


# PIF4-induced BR synthesis is critical to diurnal and thermomorphogenic growth

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## Abstract

The *Arabidopsis* PIF4 and BES1/BZR1 transcription factors antagonize light signaling by facilitating co-activated expression of a large number of cell wall-loosening and auxin-related genes. While PIF4 directly activates expression of these targets, BES1 and BZR1 activity switch from a repressive to an activator function, depending on interaction with TOPLESS and other families of regulators including PIFs. However, the complexity of this regulation and its role in diurnal control of plant growth and brassinosteroid (BR) levels is little understood. We show by using a protein array that BES1, PIF4, and the BES1-PIF4 complex recognize different DNA elements, thus revealing a distinctive cis-regulatory code beneath BES1-repressive and PIF4 co-activation function. BES1 homodimers bind to conserved BRRE- and G-box elements in the BR biosynthetic promoters and inhibit their expression during the day, while elevated PIF4 competes for BES1 homodimer formation, resulting in de-repressed BR biosynthesis at dawn and in response to warmth. Our findings demonstrate a central role of PIF4 in BR synthesis activation, increased BR levels being essential to thermomorphogenic hypocotyl growth.

**Keywords** *Arabidopsis*; BES1; BR biosynthesis; PIF4; thermomorphogenesis

**Subject Categories** Plant Biology; Signal Transduction; Transcription

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## Introduction

The plant steroid hormones brassinosteroids (BRs) act antagonistically to light in the regulation of hypocotyl elongation (Li *et al*, 1996; Szekeres *et al*, 1996). Defects in BR synthesis or BR signaling result in de-etiolated growth in the dark, severe dwarfism, delayed senescence, and increased stress tolerance, highlighting a central role of these hormones in the control of plant growth and development (Chory *et al*, 1991; Clouse *et al*, 1996; Li *et al*, 1996; Szekeres *et al*, 1996). In a screen for mutants impaired in thermomorphogenic

growth, independent mutations in BR biosynthetic genes have also been identified, indicating that enhanced hypocotyl growth in response to elevated temperatures relies on increased BR levels (Gray *et al*, 1998; Ibanez *et al*, 2018). The mechanism by which external light and temperature signals are integrated into BR signaling is, however, not fully understood. BRs are perceived by the transmembrane receptor kinase BRASSINOSTEROID INSENSITIVE 1 (BRI1), which binds these hormones via a conserved island region in the LRR extracellular domain (Kinoshita *et al*, 2005). Binding of BRs releases BRI1 from inhibition by BRI1 KINASE INHIBITOR 1 (BKI1) and enables BRI1 to associate with the BAK1 (BRI1 ASSOCIATED PROTEIN KINASE 1) co-receptor (Li & Nam, 2002). Trans-phosphorylation of these receptor kinases activates the BRI1 cytosolic kinase domain and triggers a phosphorylation cascade that leads to activation of the BRI1 SUPPRESSOR 1 (BSU1) phosphatase (Mora-Garcia *et al*, 2004; Kim *et al*, 2011). Activated BSU1 is then responsible of dephosphorylating Y200 of BR-INSENSITIVE 2 (BIN2) and inactivates this negative signaling GSK3 kinase (Kim *et al*, 2011), which allows nuclear accumulation of the non-phosphorylated BES1 (BRI1-EMS-SUPPRESSOR 1) and BZR1 (BRASSINAZOLE RESISTANT 1) factors, acting as key regulators of BR-regulated gene expression (He *et al*, 2005; Yin *et al*, 2005; Sun *et al*, 2010; Yu *et al*, 2011).

BES1 and BZR1 are transcriptional repressors, but they activate gene expression in concert with other transcriptional regulators (Yin *et al*, 2005; Sun *et al*, 2010). Genome-wide analyses showed that BES1/BZR1 up- or down-regulate thousands of BR-responsive genes, although the exact mechanism for this dual regulatory function is not fully understood. In the context of down-regulated genes (such as those for BR biosynthetic enzymes), it has been proposed that BES1 and BZR1 bind as monomers to conserved BRRE-elements (Sun *et al*, 2010; Wang *et al*, 2012) and recruit the co-repressor TPL to suppress their expression (Oh *et al*, 2014; Ryu *et al*, 2014; Espinosa-Ruiz *et al*, 2017). In contrast, BZR1 and BES1 are thought to induce gene expression through their binding to G- and E-box cis-elements. Recognition of these elements is accepted to be mediated through heterodimerization with PIF4, to co-activate together multiple elongation-related targets (Sun *et al*, 2010; Oh *et al*, 2012). DNA binding specificity of the monomeric BES1 and BZR1 proteins, and

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the BES1-PIF4 complex is however not well established, as it is largely unknown how interaction with PIF4 is dynamically regulated.

Here, we analyzed DNA recognition by BES1, PIF4, and the BES1-PIF4 complex and show that these proteins bind different *cis*-elements. We show that BES1 recognizes both BRRE- and G-box elements as homodimer but not in a monomeric form. Moreover, interaction with PIF4 changes BES1 DNA binding specificity to recognition of a CATGTG element (PBE or PIF-binding element), which is enriched in the PIF + BES-UP target promoters. Notably, BR-biosynthesis genes and PIF + BES-activated targets display similar peak expression levels at dawn, coinciding with maximal activity of the PIF4 factor (Nozue *et al*, 2007). This suggests a role of PIF4 in modulating BES1-repressive activity, by competing for BES1 homodimer formation. Consistent with this function, we observed that expression of BR biosynthetic genes is strongly induced in PIF4 over-expressor lines, while it is suppressed in *pifq* mutants. PIF4 and PIF5 accumulate under warm temperatures (Koini *et al*, 2009; Foreman *et al*, 2011), and we show that the *DWF4*, *CPD*, and *BR6ox2* genes are up-regulated at 28°C in wild-type plants but not in *pifq* mutants. Together, our findings reveal a critical role of PIF4 in increasing BR levels in response to warm temperatures, and we demonstrate that this regulation is essential for thermomorphogenic growth.

## Results

### Association with PIF4 modifies BES1 DNA binding specificity

To investigate the molecular mechanisms that dictate the switch of BES1 activity from a repressive to co-activator function, we performed protein binding microarray (PBM) studies (Godoy *et al*, 2011) to define the DNA recognition motifs of BES1, PIF4, and PIF4-BES1 proteins. To this end, we expressed the PIF4-His and BES1-MBP-tagged constructs in *Escherichia coli* and used the affinity-purified proteins for PBM hybridization followed by detection with anti-His or anti-MBP antibodies. To obtain the PIF4-BES1 complex, both PIF4-His and BES1-MBP proteins were co-expressed from the same vector and purified through a His-affinity matrix. This protein fraction was then used for PBM hybridization and

detection with an anti-MBP antibody, to secure that obtained signals did correspond to the BES1-MBP/PIF4-His heterodimer. BES1-MBP was found in these studies to bind with high affinities a 5'-CACGTG-3' (G-box) DNA motif and the 5'-CGTGTG-3' and 5'-CGTGCG-3' (BRRE-box) elements, whereas PIF4-His recognized both a G-box and 5'-CATGTG-3' (PBE-box) motif (Fig 1A and B). More interestingly, the PIF4-BES1 complex did not recognize the BRRE-*cis*-elements, but displayed preferential binding to the PBE-box variant (Fig 1A and B), previously described as a PIF-binding element (Zhang *et al*, 2013; Pfeiffer *et al*, 2014). These results highlight that interaction with PIF4 alters BES1 DNA recognition and prevents this factor from binding to the BRRE-repressive motifs, while it recruits the BES1 protein to PBE-box target elements.

To confirm these results, we performed electrophoretic mobility shift assays (EMSA) using DNA probes for the *pPIL1*, *pPRE5*, and *pDWF4* promoters. These genes have been reported to be directly activated by PIF4 (*PIL1*) and the PIF4 + BES1 complex (*PRES*) (Leivar & Quail, 2011; Hornitschek *et al*, 2012; Oh *et al*, 2012) or repressed by BES1/BZR1 (*DWF4*) (Kim *et al*, 2006) and show conserved G-box (*PIL1*), PBE- (*PRES*) and BRRE- (*DWF4*) motifs in their promoter regions (Fig 1C). Consistent with the PBM results, the PIF4, BES1, and PIF4 + BES1 proteins bound with comparable affinities to the G-box element in the *pPIL1* promoter. The PBE-*cis*-element in *pPRE5* was bound by PIF4 and PIF4 + BES1, but it was not recognized by the BES1 factor. On the other side, the BRRE-binding site in the *pDWF4* promoter was only recognized by BES1 (Fig 1C). Overall, these results confirm those obtained by PBM hybridization and demonstrate that DNA binding specificities of the PIF4 and BES1 factors differ from those of the PIF4 + BES1 complex, suggesting that they regulate a different set of genomic targets. Moreover, DNA affinity purification sequencing (DAP-seq) studies with the BZR1 or BEH2-BEH4 proteins identified an identical recognition motif as we report here for BES1 (O'Malley *et al*, 2016). Genomic regions bound by these factors are significantly enriched in the BRRE- and G-box elements (Appendix Fig S1B and C), indicating that all these proteins recognize identical DNA motifs and likely have a redundant function. BES1 and BZR1 were observed in phylogenetic tree analyses to be more closely related than the rest of family members (Appendix Fig S1A). These factors share almost

### Figure 1. Complex formation with PIF4 modifies BES1 DNA binding specificity and its transcriptional function.

Protein binding microarrays (PBM) were probed with PIF4-His, MBP-BES1, and the PIF4-His/MBP-BES1 complex. For PIF4-BES1 complex purification, both proteins were co-expressed in *Escherichia coli* using the double expression pCOLADuet-1 vector. An anti-MBP antibody was used for BES1 and BES1-PIF4 signal detection, while PIF4 was detected with an anti-His antibody.

- Box plot showing the enrichment scores (E-scores) of all possible 8-mers containing the G-box (CACGTG), PBE-box (CATGTG), and BRRE-elements (CGTGC/TG). Boxes represent the 25–75% quartiles and the black line the median of distribution. Bars indicate the 1–25% (above) and 75–100% (below) quartiles. E-scores above 0.4 denote that binding of the proteins toward the indicated DNA element is statistically significant. Dashed blue line indicates the 0.4 threshold.
- Sequence logo representation of the top scoring 8-mers obtained by hybridization with the PIF4, BES1, and PIF4-BES1 proteins.
- Electrophoretic mobility shift assays (EMSA) showing interaction of the PIF4, BES1, and PIF4-BES1 proteins with the conserved G-box, PBE-, and BRRE-elements in the *PIL1* (At2g46970), *PRES* (At3g28857), and *DWF4* (At3g50660) promoters. Increasing amounts of protein were used for the assay.
- BES1 binds both BRRE- and G-box elements as a homodimer. A deletion of BES1 (delN) fused to MBP (MBP-delN) and the complete protein (MBP-BES1) was co-expressed in *E. coli*. Formation of intermediate mobility bands, indicative of DNA recognition by a dimeric form of the protein, was tested in EMSA assays. A signal corresponding to the dimeric full-length MBP-BES1 and MBP-delN complex was detected with both DNA probes. Increasing amounts of the proteins were used in the assay.
- Co-expression of the PIF4 and BES1 effector constructs leads to synergistic *pPIL1* activation and reverses BES1-dependent inhibition of the *pDWF4* reporter. The *pPIL1* and *pDWF4* promoters including three G-boxes (green boxes) and two BRRE-elements (orange boxes) were fused to the firefly luciferase reporter gene (*LUC*) and co-transfected with *35S::PIF4*, *35S::BES1*, and *35S::bes1-D* effector constructs into *Nicotiana benthamiana* leaves. Leaf disks were collected 48 h after infiltration, and luciferase activity was measured in a microplate luminometer. Error bars represent SD ( $n = 24$ ).

Source data are available online for this figure.

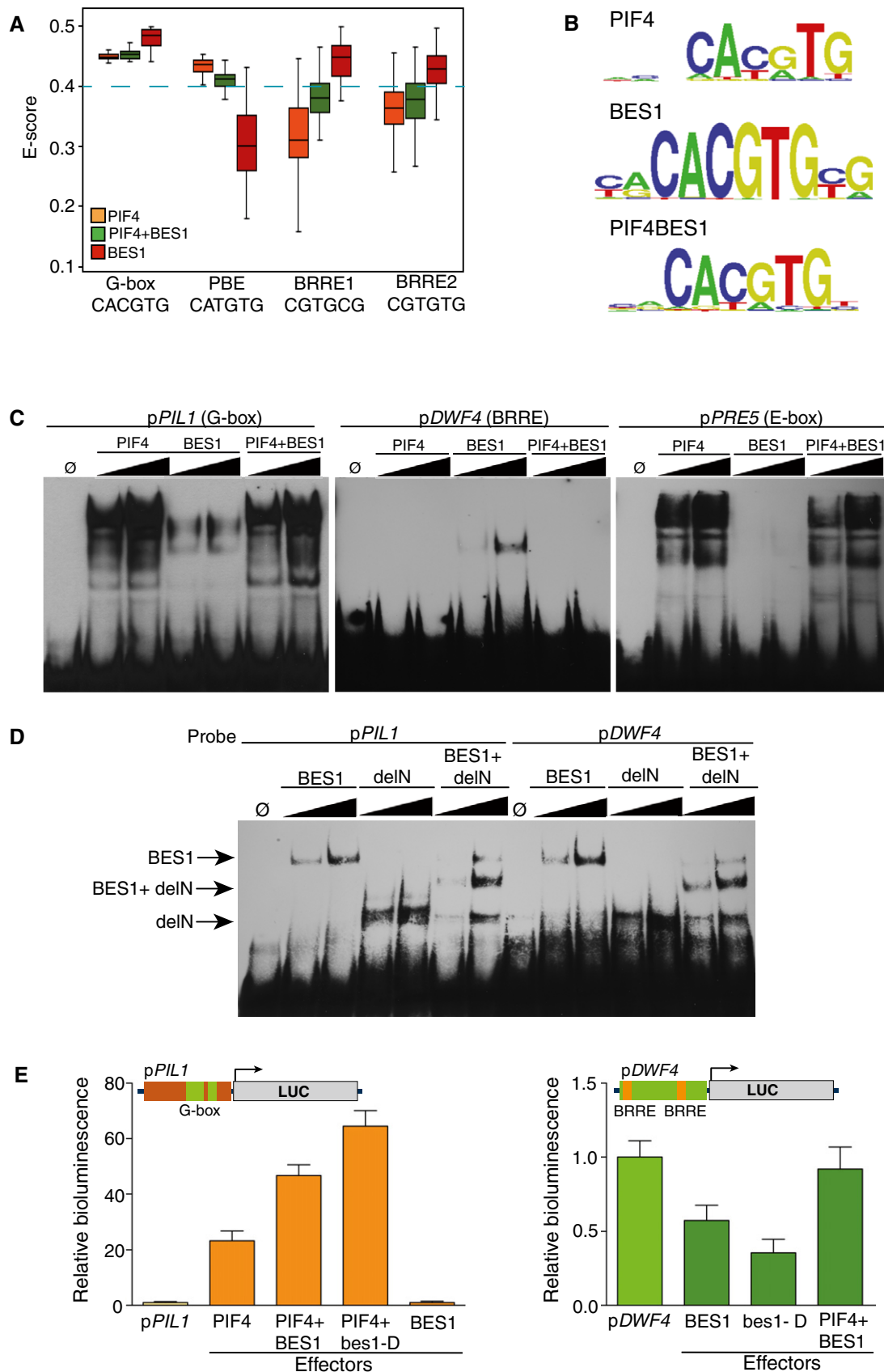


Figure 1.

identical bHLH domains, and PIF4-BZR1 is thereby predicted to bind the same PBE- motif as identified here for PIF4-BES1.

BES1 and BZR1 were proposed to bind the BRRE-binding motif as monomers, while recognizing the E-box elements on heterodimerization with other TFs (Wang *et al*, 2012). To assess this possibility, a truncated form of BES1 (delN in Fig 1D) lacking the C-terminal end was fused to the MBP-tag and employed for EMSA studies. Both delN and full-length BES1 proteins were co-expressed in *E. coli* and subsequently analyzed with the pDWF4 (BRRE-box) and pPIL1 (G-box) probes for formation of intermediate mobility DNA complexes. As shown in Fig 1D, an intermediate band corresponding to the delN + BES1 dimeric protein was observed with both DNA probes. This indicates that BES1 binds both the BRRE- and G-box as a dimer, instead of a monomeric form. This finding has important functional implications, as it suggests that complex formation with other factors will hamper BRRE- recognition, by interfering with BES1 homodimerization.

### PIF4 interaction changes transcriptional activity of the BES1 factor

To examine how PIF4-BES1 complex formation affects transcriptional outputs by these factors, the pPIL1::Luc and pDWF4::Luc reporter constructs were used in transient transactivation assays (Fig 1E). The two reporters were infiltrated in *Nicotiana benthamiana* leaves together with the 35S::PIF4, 35S::BES1, and the constitutively active 35S::bes1-D (Yin *et al*, 2002) effector constructs, either singly or in combination. As expected, PIF4 activated the pPIL1::Luc reporter, and a synergistic activation of this construct was observed on co-expression of PIF4 with the BES1 or bes1-d proteins (Fig 1D). BES1 alone, however, did not activate this promoter, showing that BES1 is unable to up-regulate the PIL1 gene by itself. The expression of the pDWF4::Luc reporter was suppressed by BES1, and more so by the constitutively active bes1-d protein (Fig 1E). Remarkably, co-expression of BES1 and PIF4 restored pDWF4::Luc expression, hence indicating that increased PIF4 levels reverse transcriptional repression by BES1. This suggests that PIF4 disrupts BES1 dimerization, thus competing for BES1 binding to its cognate BRRE- sites in the pDWF4 promoter.

Notably, in yeast two-hybrid and bimolecular fluorescence complementation (BiFC) assays, we observed that interaction with PIF4 not only implicates the reported BES1 N-terminal DNA-binding domain (Oh *et al*, 2012), but also requires of the BES1 C-terminal region (Appendix Fig S2A). This region includes the ETHYLENE RESPONSE FACTOR (ERF)-associated amphiphilic repression (EAR) domain for TOPLESS (TPL) protein–protein interaction (Ryu *et al*,

2014) and a BIN2-docking motif (Peng *et al*, 2010), with mutations within the EAR domain being established to inactivate BES1 function (Espinosa-Ruiz *et al*, 2017). Thus, it is formally possible that mutations in the C-terminal EAR lessen PIF4 binding affinity, while PIF4 complex formation hinders interaction with the co-repressor TPL. As such, PIF4 physical contact with the BES1 C-terminal region may be critical to reverse BES1-repressive activity and to recruitment of this factor to the promoters of co-activated loci.

### The PBE-cis-element is overrepresented in the promoters of PIF4 and BES1 co-activated targets

To further explore whether PBE-elements are enriched in the promoters of BES1- and PIF4-regulated loci, we conducted microarray studies on dark grown *bes1-D* and *bes1-D;pif4pif5* plants. Differentially expressed genes (DEGs) obtained from these studies were combined with published datasets for BR-regulated gene expression (Goda *et al*, 2004, 2008; Nemhauser *et al*, 2004, 2006; Vert *et al*, 2005) and with genes reported to be differentially expressed in *bzr1-1D* and *bri1-116* mutants (Sun *et al*, 2010; Oh *et al*, 2012). This led to a total of 5,679 BR-responsive genes equally distributed between UP- and DOWN-regulated transcripts (Fig 2A; Appendix Table S1). A list of 5,324 PIF-regulated genes was also obtained by merging our *pif4pif5* data, with previous gene expression profiles of PIF4-OX lines and *pifq* mutants (Leivar *et al*, 2009; Lorrain *et al*, 2009; Shin *et al*, 2009; Nozue *et al*, 2011; Hornitschek *et al*, 2012; Oh *et al*, 2012; Zhang *et al*, 2013; Appendix Table S2). As for BR-responsive, these genes were evenly distributed among activated and repressed genes, and as expected, they showed a significant overlap with the BR- dataset, with 40% of the PIF-activated genes being also induced by BRs, while 33% of the PIF-repressed genes are BR-repressed, versus to 9.4% or 8.9% expected randomly (Fig 2A).

We next gathered all genes that in ChIP-chip or ChIP-seq studies had been identified as directly targeted by PIFs or BES1/BZR1 (Sun *et al*, 2010; Yu *et al*, 2011; Hornitschek *et al*, 2012; Oh *et al*, 2012; Zhang *et al*, 2013), yielding a list of 4,301 BES1- and 4,794 PIF-target loci (Appendix Tables S3 and S4). Overall, around 40% of the PIF- targets overlapped with genes directly regulated by BES1 or BZR1, in agreement with a coordinated function of these factors in gene regulation (Fig 2A). Moreover, by contrasting each the DEG and directly regulated datasets, we observed that PIF-activated genes are significantly enriched among the PIF-bound targets (19.1% of genes, versus 9.3% expected by chance,  $P$ -value < 0.01), while a comparable enrichment was not observed for PIF-repressed

**Figure 2. The PBE-cis-element is overrepresented in the promoters of PIF + BES1 co-activated targets.**

- A Venn diagrams representing the genes up-regulated (yellow) and down-regulated (purple) by PIFs or BES1/BZR1 (BES), and the direct target loci (green) of these transcription factors. Gene sets derive from our *bes1-D* and *bes1-D;pif4pif5* microarray analyses and from published RNA-seq studies of *pifq*, *bzr1-1D*, and *bri1-116* mutants (Appendix Tables S1 and S2). The direct target PIF and BES datasets were obtained from published ChIP-chip or ChIP-seq experiments with these factors (Appendix Tables S3 and S4). Histogram bars represent the percentage of PIF-, BES-, and PIF + BES-regulated genes that are directly bound by these transcription factors, as compared to all the *Arabidopsis* genes (total genes, in gray) (chi-squared test, \*\* $P$ -value < 0.01, ns indicates no significant differences).
- B Heat map and fold-change graph representation of the genes identified in (A). Expression data were obtained from Genevestigator, and heat map and clustering analyses were performed using the MeV software. PIF + BES-UP indicates genes up-regulated by PIF and BES and identified as targets of both the PIF and BES factors. BES-DOWN are BES-repressed targets, while BES-UP corresponds to BES-induced targets. The PIF-UP cluster includes PIF-induced genes directly bound by the PIF factors.
- C Percentages of occurrence of the indicated motifs in the PIF, BES, or PIF + BES target loci, as compared with all *Arabidopsis* genes (genome, in gray) (chi-squared test, \*\* $P$ -value < 0.01, ns indicates no significant differences).

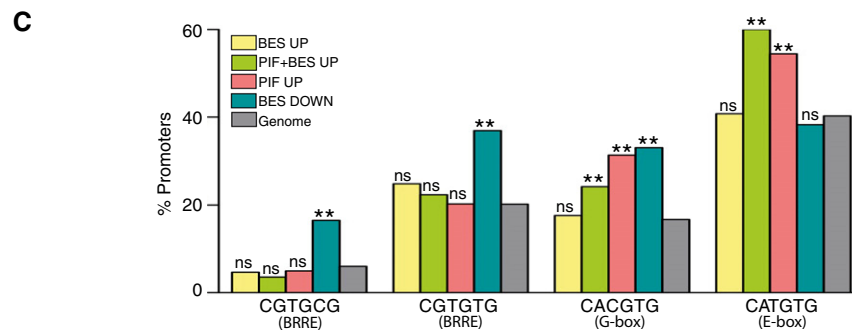
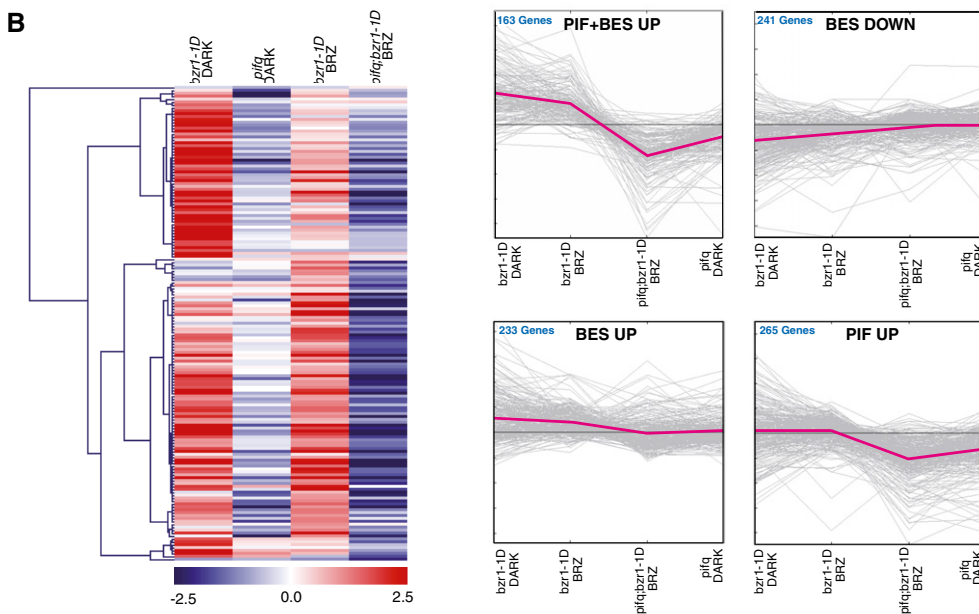
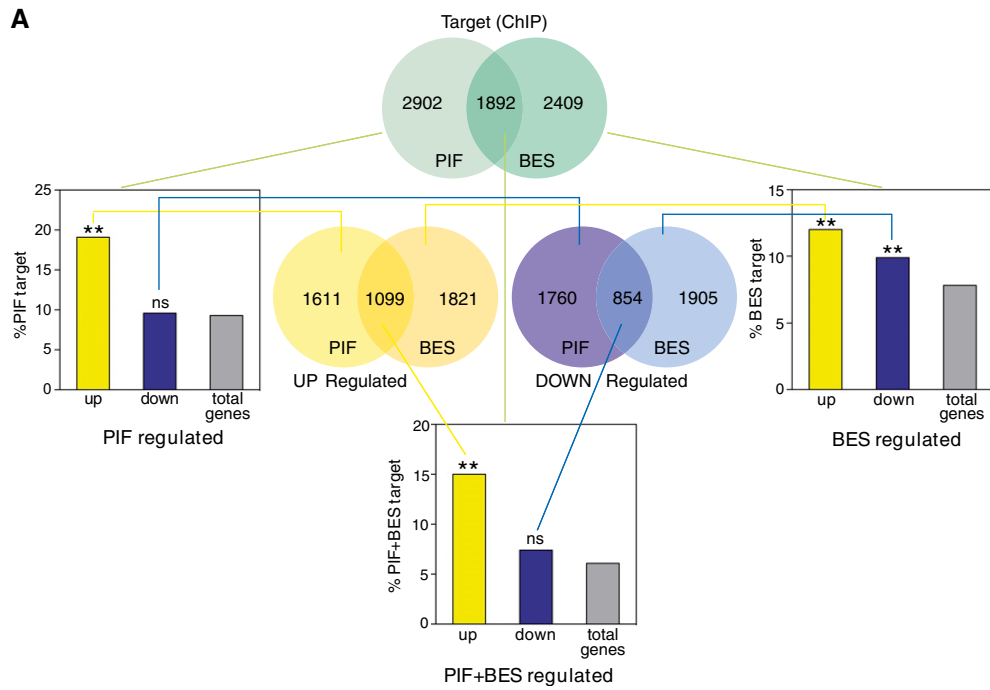


Figure 2.



genes (9.6% of genes, in a similar range as expected randomly). This indicates that PIFs act mainly as transcriptional activators and that down-regulated genes correspond to indirect targets of these factors (Fig 2A). As for BES1/BZR1 function, both induced and repressed genes were similarly overrepresented amid the BES1-specific targets (12% of the induced and 9.9% of the repressed genes, versus 7.8% expected randomly), thus reflecting a dual role of these factors as transcriptional activators and repressors (Fig 2A). Finally, BES and PIF shared loci were exclusively enriched in genes with an up-regulated pattern of expression (14.9% induced and 6% repressed genes, versus 5.8% randomly expected), consistent with the reported role of BES1/BZR1 in co-activated expression of a subset of PIF targets (Oh *et al*, 2012).

These targets showed in a heat map to be differently misregulated in *bzr1-1d* and *pifq* mutants (Fig 2B), hence identifying four main gene clusters that we designated as PIF + BES-UP, PIF-UP, BES-UP, and BES-DOWN. The PIF + BES-UP cluster includes 163 genes, which are up-regulated in *bzr1-1d* seedlings, but whose expression is suppressed in *bzr1-1d;pifq* and *pifq* mutants (Fig 2B). Moreover, *bzr1-1d* activation of these genes is reduced by the BR biosynthesis inhibitor brassinazole (BRZ), which is shown to promote BIN2-mediated phosphorylation and destabilization of the PIF4 factor (Bernardo-Garcia *et al*, 2014). The PIF-UP cluster includes 265 genes whose transcription is mostly induced by PIFs, since their expression is not affected in *bzr1-1D* seedlings. Conversely, the BES-UP and BES-DOWN clusters contain 233 and 241 genes, which are respectively activated or repressed in *bzr1-1D* plants, but whose expression is not affected in *pifq* mutants (Fig 2B).

We next interrogated the upstream regulatory regions of these target loci for the elements identified in the protein array. Remarkably, the 5'-CGTGCG-3' and 5'-CGGTGTG-3' (BRRE) motifs were found to be overrepresented only in the promoters of genes in the BES-DOWN cluster, consistent with the reported role of BES1 and BZR1 in negative BR feedback regulation, by binding to conserved BRRE-*cis*-elements in the BR biosynthetic promoters (Sun *et al*, 2010). The PBE 5'-CATGTG-3' element was enriched in the PIF4-UP and PIF + BES-UP promoters, but not in the promoters of BES-UP or BES-DOWN genes, in agreement with our PBM and EMSA studies showing that BES1 does not recognize this *cis*-element. Moreover, in line with the finding that PIF4, BES1, and PIF4-BES1, bind all of them a 5'-CACGTG-3' G-box motif, this element was overrepresented in the promoters of PIF-UP, PIF + BES-UP, and BES-DOWN genes (Fig 2C), while genes in the BES1-UP cluster were not enriched in any of these elements. This suggests that BES1 activates these targets through physical interaction with other factors than PIFs and is recruited to a different DNA element.

Taken together, these data indicate that BES1/BZR1 repress BES-DOWN targets by binding to G-box and BRRE-elements in their promoters, while PIF4 recruits these factors to a new *cis*-element, the PBE (5'-CATGTG-3') motif, to co-activated expression of PIF + BES targets. Notably, PIF4 binds both G-box and PBE-elements in the absence of BES1, but interaction with BES1/BZR1 synergistically activates these genes. Also, PIF4 competes for BES1/BZR1 binding to the BRRE-elements, which contributes to de-repression of the BES-DOWN targets, including BR biosynthetic genes. We propose that BES1/BZR1 inhibition of BR levels creates a negative feedback loop that restrains excessive PIFs activity. This way, dynamic changes in BES1 and PIF4 nuclear levels tightly

control plant growth, combinatorial function of these factors providing a highly sensitive mechanism for rapid modulation of cell elongation in response to environmental cues.

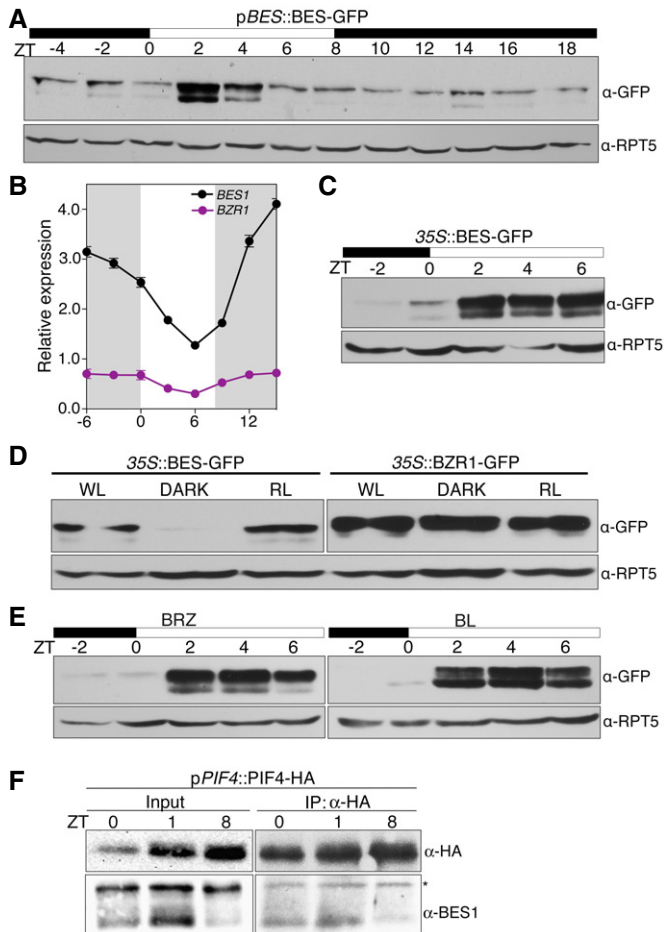
### Light stabilizes the BES1 protein

Constitutively active *bess1-d* and *bzr1-1d* proteins display significantly increased stability, which suggests that regulation of BES1 and BZR1 nuclear accumulation is critical to transcriptional control by these factors (Wang *et al*, 2002; Yin *et al*, 2002). The GSK3 kinase BRASSINOSTEROID INSENSITIVE 2 (BIN2) phosphorylates and inactivates BES1 and BZR1, in addition to promote proteasomal degradation of these factors (He *et al*, 2002; Vert & Chory, 2006; Wang *et al*, 2013a). Although it is largely accepted that BIN2-dependent phosphorylation is the principal mechanism that controls BES1/BZR1 stability, other studies demonstrated that these factors are regulated in response to cues other than BR signaling (Wang *et al*, 2013b; Kim *et al*, 2014). Here, we used *pBES1::BES1-GFP* lines to analyze the pattern of BES1 protein accumulation in short days (Fig 3A). Time course studies revealed that levels of the BES1 protein increase directly after dawn and remain high for the first 2–4 light hours, to be later reduced to lower levels as observed at night (Fig 3A). A band corresponding to the phosphorylated BES1 protein was visible across the whole 24-h interval, whereas non-phosphorylated BES1 was observed primarily after dawn (Fig 3A). To assess whether BES1 protein accumulation mirrored its expression profile, we performed qRT-PCR analyses of the *BES1* and *BZR1* transcripts in wild-type plants. As seen in Fig 3B, elevated levels of these transcripts were detected at night, whereas their expression was reduced during the day, reaching basal levels by ZT6. *BES1* was also expressed to considerably higher levels than *BZR1*, inconsistency between protein accumulation and transcript levels suggesting that BES1 is under post-translational control (Fig 3A and B). To further test this hypothesis, we analyzed levels of the protein in *35S::BES1-GFP* lines. Notably, a higher accumulation of the BES1-GFP protein is still observed in these plants during the day (Fig 3C), although transcript levels were slightly elevated at night (Appendix Fig S5D).

To further assess whether light affects BES1 protein stability, we analyzed protein levels in plants grown in continuous white light (WL), red light (RL), or in darkness (DARK). Remarkably, BES1 was detected in WL and RL, but not in darkness, in opposite to BZR1 which accumulated to constant levels (Fig 3D). Hence, in our conditions, light stabilized BES1, but did not affect the BZR1 protein. Lastly, we investigated whether light effects are mediated through changes in BR levels. As shown in Fig 3E, application of the BR biosynthesis inhibitor BRZ increased the ratio of phosphorylated to non-phosphorylated BES1, while a preferential accumulation of the non-phosphorylated protein was observed on treatment with epibrassinolide (eBL). However, neither BRZ nor eBL had an effect on BES1 levels at night, which demonstrates that BES1 stabilization during early morning responds to the presence of light.

### PIF4 mediates de-repressed expression of BR biosynthetic genes

PIF4 control of cell elongation is defined by a coincidence mechanism, whereby the circadian clock regulates *PIF4* gene expression while light inactivates the PIF4 protein, via phyB-dependent phosphorylation and proteasomal degradation (Nozue *et al*, 2007;

**Figure 3. The BES1 protein is stabilized in the light.**

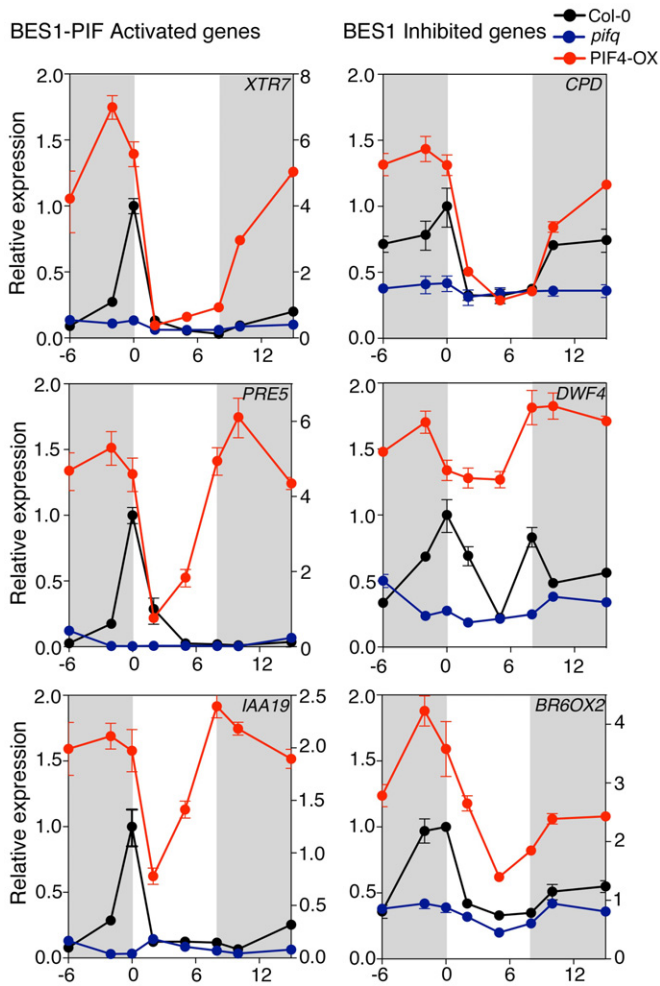
- A** Time course analysis of BES1 protein accumulation levels. *pBES1::BES1-GFP* seedlings were grown under short days for 6 days, and samples were harvested every 2 h for a 24-h interval. Total protein extracts were used for Western blot analysis with an anti-GFP antibody to detect the BES1-GFP protein. Hybridization with anti-RPT5 antibody is included as a loading control. Dark and light periods are represented as black and white bars, respectively. The experiment was repeated twice with similar results.
- B** Diurnal oscillation of *BES1* and *BZR1* transcripts. Col-0 seedlings were grown for 6 days under short days, and samples were harvested every 3 h. *BES1* and *BZR1* expression levels were determined by qRT-PCR analyses and normalized to *PP2A*. Relative levels are the mean of three technical replicates, and error bars represent standard deviation. The experiment was performed three times with similar results. Dark periods are indicated by shading.
- C** Western blot analyses of *35S::BES-GFP* seedlings grown for 6 days under short days. Samples were collected every 2 h, starting 2 h before lights on (ZT-2) to ZT6. Western blots were hybridized with an anti-GFP antibody to detect the BES1-GFP protein and anti-RPT5 as a loading control.
- D** BES1 but not BZR1 is stabilized in the light. *35S::BES1-GFP* and *35S::BZR1-GFP* seedlings were grown for 6 days in continuous white light (WL), red light (RL), and continuous dark (DARK), and the BES1 and BZR1 proteins analyzed by Western blot using an anti-GFP antibody. Hybridization with an anti-RPT5 antibody was used as a loading control.
- E** BES1 accumulation is independent of brassinosteroid levels. Six-day-old *35S::BES-GFP* seedlings were treated overnight with 100 nM epi-brassinolide (BL) or 0.5 μM brassinazole (BRZ). The BES1 and BZR1 proteins analyzed by Western blot hybridization with anti-GFP and anti-RPT5 antibodies.
- F** *In vivo* analysis of PIF4-BES1 complex formation. Six-day-old *pPIF4::PIF4-HA* seedlings grown under short-day conditions were collected at ZT0, ZT1, and ZT8. PIF4-HA was immunoprecipitated with an anti-HA antibody and the pulled-down BES1 protein immunodetected with an anti-BES1 antibody. \*indicates a non-specific band by the primary antibody used to immunoprecipitation.

Source data are available online for this figure.

Lorrain *et al*, 2008; Niwa *et al*, 2009). Therefore, to gain additional understanding on the functional significance of PIF4-BES1 interaction in diurnal regulation of hypocotyl growth, we investigated expression levels of the *XTR7*, *PRES5*, and *IAA19* genes in detail. Wild-type, *PIF4-OX*, *pifq*, and *bes1-d* plants were grown in short-day (SD) conditions, and levels of these transcripts analyzed over a 24-h time course. These genes belong to the PIF + BES-UP cluster, and so we reasoned that their peak expression levels should reflect PIF4 and BES1 temporal association. The BES-DOWN *CPD*, *DWF4*, and *BR6ox2* (*CYP85A2*) BR biosynthetic genes were similarly analyzed as readout of BES1 homodimer formation.

In wild-type plants, *XTR7*, *PRES5*, and *IAA19* displayed a narrow peak of expression at dawn (Fig 4), in accordance with their previously reported expression patterns (Nozue *et al*, 2007; Nomoto *et al*, 2012). This peak is however lost in *pifq* mutants (Fig 4), which corroborates an essential role of PIFs in transcriptional activation and recruitment of the BES1 factor to these promoters. In line with this function, transcripts of *XTR7*, *PRES5*, and *IAA19* were strongly up-regulated in *PIF4-OX* lines at night, while their expression levels were reduced upon illumination (Fig 4), consistent with the light-mediated inhibition of PIF4 by phyB (Lorrain *et al*, 2008). Furthermore, in constitutive *bes1-D* and *bzr1-1D* mutants, expression of these genes was elevated only at dawn, supporting that BES1/BZR1-mediated co-activation of these targets requires of

transcriptionally active PIFs (Appendix Figs S3 and S4), while loss-of-function *bes1* and *bzr1* mutants showed normal levels of expression of these transcripts (Appendix Fig S5A), in support of a redundant function of BES1 and BZR1 in their regulation. These results suggest that PIF4 is limiting to PIF4-BES1 complex formation and that interaction between these factors mostly takes place at dawn, when increased *PIF4* transcription and the absence of light allow PIF4 accumulation in its active form. Western blot detection of BES1 in *pPIF4::PIF4-HA* extracts immunoprecipitated with an anti-HA antibody actually showed that endogenous BES1 is more efficiently pulled-down at ZT0–ZT1 than ZT8, although levels of the PIF4-HA protein are still elevated by the end of the day (Fig 3F). Also, the nuclear non-phosphorylated form of BES1 is detected in these fractions, consistent with an interaction of these proteins in the nucleus. On the other side, an analogous dawn-phased peak of expression as for PIF + BES-UP targets was observed for the *CPD*, *DWF4*, and *BR6ox2* genes (Fig 4). Transcript levels of these BES-DOWN targets later decreased during the day, to slowly rise at night. More remarkably, their peak expression was suppressed in *pifq* mutants, whereas they were strongly up-regulated at night in *PIF4-OX* lines. Reduced levels of *CPD*, *DWF4*, and *BR6ox2* transcripts were also detected in the constitutive *bes1-d* and *bzr1-1D* mutants (Appendix Figs S3 and S4), in agreement with a function of BES1 in negative regulation of these targets. Overall, these findings indicate that PIF4 de-represses BR biosynthetic gene transcription, presumably by preventing dimeric BES1 accumulation, which negatively regulates these genes.



**Figure 4. PIF4 mediates de-repressed expression of BR biosynthetic genes.**

Col-0 (black), *PIF4-OX* (red), and *pif4* (blue) seedlings were grown for 6 days under short days, and samples were harvested at the indicated time points. Total RNA was used for qRT-PCR quantification of PIF + BES-UP and the BR biosynthetic BES-DOWN transcripts, using *PP2A* as endogenous control. Error bars represent SD of three technical replicates. Dark periods are shown in gray. Relative expression of the *XTR7*, *PRE5*, *IAA19*, and *BR6ox2* genes in *PIF4-OX* lines is referred to the right y axis of the graph. The experiment was repeated three times with similar results.

Notably, *BES1* was transcribed in our growth conditions to higher levels at night and the protein showed a preferential stabilization in the light (Fig 3), while *PIF4* transcriptional activity is highest at dawn, when rise in *PIF4* transcription coincides with absence of light (Nozue *et al*, 2007; Niwa *et al*, 2009). This suggests that during late night, *BES1* accumulates mainly in a complex with *PIF4*, which explains the observed rise in PIF + *BES1*-UP transcript levels and up-regulated expression of *CPD*, *DWF4*, and *BR6ox2* genes. This temporal rise in BR levels may also play a critical role in mediating *BIN2* inactivation during late night, thus favoring nuclear accumulation of both the transcriptionally active *BES1* and *PIF4* proteins. Immediately after dawn, however, opposite regulation of these factors by light is anticipated to modify their nuclear balance

toward an excess of *BES1*. This allows *BES1* dimerization, with *BES1* repressive action then leading to BR biosynthetic gene inhibition as observed during the day. At the same time, a drop in BR levels is predicted to result in *BES1* inactivation and thus would account for the gradual up-regulation of BR biosynthetic genes observed during early night. Overall, these findings underscore that the opposite effects of light on *PIF4* and *BES1* protein stability have a relevant role in orchestrating combinatorial activity of these factors and in diurnal regulation of BR levels.

#### PIF4 promotes thermomorphogenic hypocotyl elongation by activating BR synthesis

*PIF4*, and to a lesser extent *PIF5*, are key activators of thermosensory growth (Koini *et al*, 2009; Stavang *et al*, 2009; Kumar *et al*, 2012). Warmer temperatures were shown to reduce binding of the evening complex (EC), *ELF3*, *ELF4*, and *LUX* proteins to its target promoters, which leads to increased *PIF4* transcription during the night (Box *et al*, 2015; Raschke *et al*, 2015) and activated expression of auxin-related genes, such as *YUCCA8* and several *SAURs* (Stavang *et al*, 2009; Franklin *et al*, 2011). As we had found that *PIF4* positively regulates *CPD*, *DWF4*, and *BR6ox2* gene expression, we asked whether this regulation plays a relevant role in thermomorphogenesis. In wild-type plants, we observed that *CPD*, *DWF4*, and *BR6ox2* transcripts were elevated during the night in seedlings grown at 28°C compared to those grown at 22°C. The greatest temperature-induced increase in these genes occurred in the late night, with temperature showing much smaller effects during the day (Fig 5A). A similar up-regulation of these genes was not observed in *pif4* mutants, demonstrating that PIFs play an essential role in temperature-dependent activation of BR synthesis (Fig 5A). Analyses of *XTR7* transcript levels (used as a positive control) showed that this *PIF4* target is strongly up-regulated at 28°C during late night. By contrast, in the *pif4* mutant, it is expressed to basal levels and does not respond to temperature (Fig 5B).

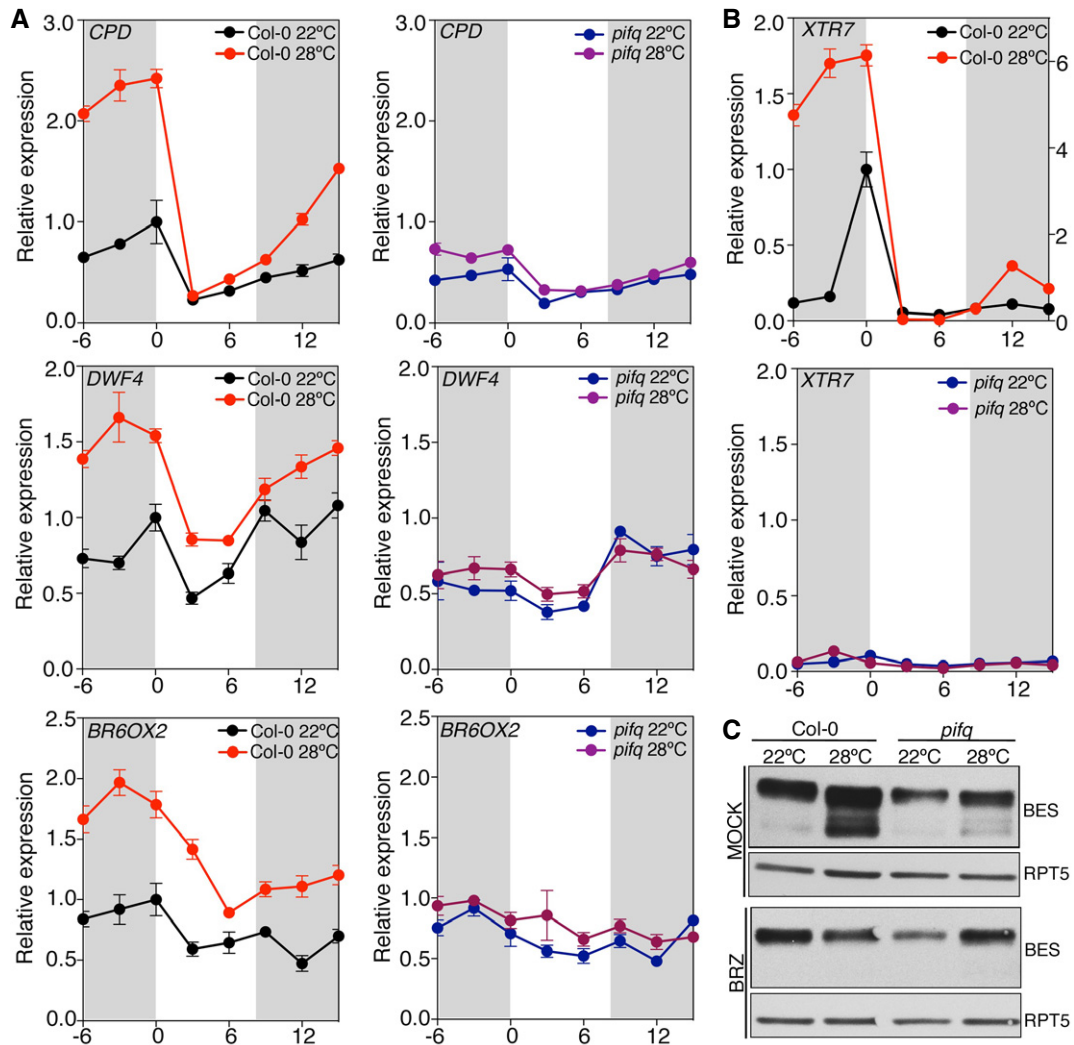
As previously reported, *pif4pif5* mutants showed reduced temperature-mediated hypocotyl elongation (Koini *et al*, 2009; Stavang *et al*, 2009) and we observed an even more temperature-unresponsive phenotype in *pif4* mutants (Appendix Fig S6). Moreover, eBL restituted thermomorphogenic growth of both mutants, whereas the inhibitor BRZ compromised temperature-induced growth of wild-type plants, demonstrating that PIF-mediated control of BR synthesis is essential to this response. Consistent with this regulation, an increased proportion of non-phosphorylated *BES1* was observed in wild-type seedlings grown at 28°C (Fig 5C). By opposite, levels of non-phosphorylated *BES1* were significantly reduced in the *pif4* mutant, despite these plants accumulated comparable levels of phosphorylated *BES1* when grown in the presence of BRZ (Fig 5C).

Collectively, these data illustrate that *PIF4*-dependent control of BR synthesis not only contributes to coordinate hypocotyl elongation during late night, but is critical to the promotion of hypocotyl growth in warmth.

## Discussion

In this study, we showed that *BES1* binds to DNA as a dimeric and not as a monomeric protein and that complex formation with *PIF4* modifies the DNA *cis*-elements recognized by this factor (Fig 1).





**Figure 5. PIF4 activates expression of BR-synthesis genes in response to elevated temperatures.**

**A** Col-0 and *pifq* seedlings were grown under short days at 22 and 28°C, and samples were collected at the indicated time points. Expression of BR biosynthetic genes was analyzed by qRT-PCR, using the constitutive *PP2A* gene as internal control. Error bars represent SD of three technical replicates. The experiment was repeated twice with similar results.

**B** Activation of the *XTR7* PIF + BES-UP target was studied as a positive control of the experiment. Error bars represent SD of three technical replicates. Dark periods are shown in gray. The experiment was performed three times with similar results.

**C** Increased levels of dephosphorylated BES1 at elevated temperatures depend on PIFs activation of BR synthesis. Col-0 and *pifq* seedlings were grown for 6 days under short days and 22°C or 28°C, on half strength MS media (mock) or media supplemented with 0.5 μM BRZ. BES1 was analyzed by Western blot hybridization with an anti-BES1 antibody. RPT5 was used as a loading control.

Source data are available online for this figure.

Dimeric BES1 acts as a transcriptional repressor by binding to G-box and BRRE-element which are found to be enriched in the promoters of BR biosynthetic genes and BES1-DOWN targets, and recruits the co-repressor TPL to these loci (Ryu *et al*, 2014; Espinosa-Ruiz *et al*, 2017). Interaction with TPL relies on an EAR domain located at the C-terminal end of the BES1 and BZR1 proteins. We observed that complex formation with PIF4 (Appendix Fig S2A and B) requires of the BES1 C-terminal region, in addition to the N-terminal DNA-binding HLH domain (Appendix Fig S2A and C). Therefore, physical contact with PIF4 presumably hinders TPL interaction and mediates the switch of BES1-repressive activity into a co-activator function. Interestingly, mutations in the EAR domain result in BES1

inactivation (Ryu *et al*, 2014; Espinosa-Ruiz *et al*, 2017), and it is possible that this effect is caused in part by weakened PIF4 interaction. Our findings showed that PIF4 modifies the DNA binding specificity of BES1, to recognition of a 5'-CATGTG-3' motif, previously identified as a PIF-binding element (PBE) (Zhang *et al*, 2013; Pfeiffer *et al*, 2014). Notably, this motif is overrepresented in the promoters of genes that are up-regulated by PIF4 or the PIF4-BES1 complex (Fig 2), underscoring that combinatorial regulation by PIF4 and BES1 is responsible of co-activated expression of multiple target loci. Moreover, these elements are not enriched in genes activated by BES1 independent of PIFs, raising the possibility that BES1 binds other families of TFs to co-activate these targets.

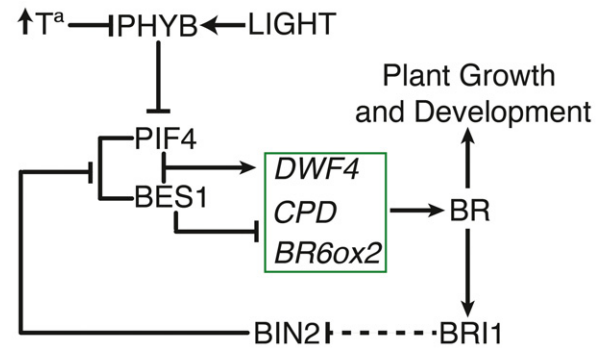
Our results reveal that PIF4-BES1 interaction plays an important role in diurnal regulation of BR synthesis, most probably by competing for BES1 dimerization. In our growth conditions, *BES1* transcript levels were elevated at night while decreased during the day, as opposed to the BES1 protein that is stabilized during early day (Fig 3). We showed that the pattern of BES1 protein accumulation is not regulated by BR signaling, but is dependent on light, as application of eBL or the inhibitor BRZ changed the ratio of phosphorylated to non-phosphorylated protein, but did not increase BES1 protein levels during late night (Fig 3). Although COP1 has been reported to degrade phosphorylated BZR1 in darkness (Kim *et al.*, 2014), and SINAT E3 ligases to degrade the dephosphorylated BES1 protein in the light (Nolan *et al.*, 2017; Yang *et al.*, 2017), a similar light-dependent degradation of these proteins was not observed in our study, highlighting that growth conditions strongly affect BES1/BZR1 stability. These results underscore that regulation of BES1/BZR1 protein levels is far more complex than previously anticipated and that future studies will be required to understand light-dependent control of BES1 accumulation.

The *BES1* gene was shown in a recent report to encode two different isoforms, designated as BES1-Long (BES1-L) and BES1-Short (BES1-S), with BES1-L having a stronger activity in promoting BR signaling (Jiang *et al.*, 2015). All experiments in this work were carried out with the regular BES1-S isoform as we observed that BES1-L does not display the same light-dependent stabilization as BES1-S (Appendix Fig S5B). Transcript levels for the BES1-L isoform were also less abundant than for the shorter protein (Appendix Fig S5C), which suggests that BES1-S is the preponderant form in our growth conditions, and has a role in diurnal BR levels regulation as it is stabilized by light.

We could observe that transcription of BR biosynthetic genes was elevated at dawn, coinciding with the peak expression of PIF + BES-UP targets (Fig 4). This expression profile suggests that increased levels of the PIF4 protein during late night de-repress these BES-DOWN targets. Light, on the other hand, inactivates PIF4 while stabilizes BES1, enabling BES1 dimerization to suppress BR biosynthetic gene expression during daytime. Consistent with this model of regulation, *CPD*, *DWF4*, and *BR6ox2* were induced in *PIF4-OX* lines, whereas their transcript levels were notably reduced in *pifq* and *bes1-d/bzr1-1D* mutants (Fig 4, Appendix Figs S3 and S4).

Whether PIF4 positively regulates BR biosynthetic genes by directly binding to these targets remains an open question. In fact, ChIP-PCR studies showed that PIF4 binds the *DWF4* and *BR6ox2* promoters (Oh *et al.*, 2012; Wei *et al.*, 2017), and in our EMSA studies, both the G-box and PBE-elements were equally recognized by the PIF4 and PIF4-BES1 proteins (Fig 1). Hence, it is possible that PIF4 and PIF4-BES1 directly bind the conserved G-box elements in the BR biosynthetic promoters. Otherwise, PIF4 may also facilitate BR biosynthetic gene expression by interfering with BES1 homodimer formation, hence precluding dimeric BES1 of binding to the G-box and BRRE-elements in these promoters, and inhibition of their expression through recruitment of the co-repressor TPL (Oh *et al.*, 2014; Ryu *et al.*, 2014).

Furthermore, we provide evidence showing that this regulation is critical to thermomorphogenic hypocotyl elongation. We showed that warm temperatures cause up-regulated expression of BR biosynthetic genes, and that this response is compromised in the *pifq* mutant, which fails to show temperature-mediated hypocotyl



**Figure 6. Model for BR-dependent diurnal and ambient temperature control of hypocotyl elongation.**

In this model, an increase in PIF4 protein levels as a consequence of elevated temperatures promotes formation of the PIF4-BES1 complex, and activated expression of the *DWF4*, *CPD*, and *BR6ox2* BR biosynthetic genes. Increased BR levels support plant growth by inactivating BIN2 and promoting accumulation of the active PIF4 and BES1 proteins. In response to light, phyB destabilizes PIF4, and the BES1 dimeric protein is dominant over the PIF4-BES1 complex. Dimeric BES1 represses expression of BR biosynthetic genes and leads to decreased BRs levels. As a consequence, BIN2 is reactivated and phosphorylates PIF4 and BES1, inactivating these factors, which leads to increased transcription of BR biosynthetic genes during the night. Light and temperature cues thus control PIF4 and BES1 levels, the nuclear balance of both factors providing a highly sensitive mechanism for rapid modulation of plant growth and development in response to environmental cues.

elongation at 28°C (Fig 5 and Appendix Fig S6). Notably, BL restores thermomorphogenic growth of *pif4pif5* and *pifq* mutants, while the inhibitor BRZ blocks enhanced elongation of wild-type plants (Appendix Fig S6), hence demonstrating that the elongation defects of *pifq* mutants are caused by impaired thermo-responsive activation of BR biosynthetic genes. In agreement with a prevalent role of this regulation in temperature-induced hypocotyl growth, mutations affecting the *DWF7* and *ROT3* genes were recently identified in a screen for *okapi* mutants that reversed the hypersensitive thermo-response of *Arabidopsis Rrs-7* accessions (Ibanez *et al.*, 2018). Defects in these BR biosynthetic genes also block response to temperature in the Col-0 background, whereas BL rescues thermomorphogenic defects of these mutants (Ibanez *et al.*, 2018), evidencing that temperature-induced hypocotyl elongation is strictly dependent on BRs.

We propose that increased *PIF4* transcription as a result of elevated temperatures allows PIF4 protein accumulation during late night and alters PIF4/BES1 nuclear balance toward an overabundance of PIF4. Most BES1 is then bound to PIF4, and the PIF4-BES1 complex is therefore dominant over the BES1 dimeric protein. This leads to activation of the auxin-related and cell wall-modifying PIF-UP and PIF + BES-UP targets supporting cell growth, in addition to de-repressed transcription of BR biosynthetic genes. Increased BR levels in turn, positively feedback on this regulation, by signaling inactivation of BIN2, which phosphorylates and destabilizes the PIF3 and PIF4 factors (Bernardo-Garcia *et al.*, 2014; Ling *et al.*, 2017), in addition to modulate BES1 and BZR1 subcellular localization by phosphorylation of these proteins (Ryu *et al.*, 2010, 2007; Fig 6). Indeed, BZR1 was recently reported to translocate into the nucleus at elevated temperatures (Ibanez *et al.*, 2018), and we showed that

dephosphorylated BES1 levels increase in the wild type at 28°C (Fig 5). Hence, the switch in BES1 transcriptional activity from a repressive into a co-activator function, coupled with recognition of distinct promoter *cis*-elements by the dimeric BES1 and heterodimeric PIF4-BES1 proteins, provides mechanistic detail on how concerted function of these factors coordinates thermomorphogenic growth.

## Materials and Methods

### Plant material and growth conditions

*Arabidopsis thaliana* Col-0 wild-type plants were used in this study. The *bes1-D* (Yin *et al*, 2002; introgressed into Col-0), *pif4pif5* (de Lucas *et al*, 2008), and *pifq* (Shin *et al*, 2009) mutants, as well as *pBES1-BES1-GFP* (Yin *et al*, 2002) and *35S::BZR1-GFP* (Wang *et al*, 2002) lines were in the Col-0 background. *35S::BES1-GFP* plants were generated by cloning the *BES1* CDS (*BES1topof-BES1-R*) without stop codon into the pENTR™/D-TOPO vector and subsequent mobilization by LR clonase (Invitrogen) reaction into the pGWB5 vector. This construct was introduced into *Agrobacterium tumefaciens* (GV3101) and used for Col-0 plants transformation by floral dipping. The double-mutant *bes1-D;pif4pif5* was generated by crossing *pif4pif5* and *bes1-D* mutants. Seedlings were grown for 6 days under short-day conditions (8-h light/16-h dark) at 22°C, or under continuous white light, red light, or dark conditions. For thermomorphogenic studies, seedlings were grown under short-day conditions at 22°C for 24 h and then transferred to 28°C or kept at 22°C for five additional days.

Seeds were surface-sterilized for 15 min in 70% (v/v) ethanol and 0.01% (v/v) Triton X-100, followed by two washes of 2 min in 96% (v/v) ethanol, and let dry in a sterile bench. Seeds were then sown on half strength Murashige and Skoog (MS) media with Gamborg's vitamins (Duchefa) and 1% sucrose (w/v), supplemented with 0.8% agar (w/v), and stratified at 4°C for 4 days in the dark. Germination was synchronized by 4 h illumination, before transferring plates to darkness for 20 additional hours. Brassinazole (TCI America) and epi-brassinolide (SIGMA) treatments were performed at 0.5 and 0.1 μM, respectively. After germination, seedlings were moved to the plates containing the different treatments and grown in the conditions indicated. Treatments in liquid medium were performed by moving seedlings to multiwell plates containing 1.5 ml half strength MS liquid media supplemented with 0.5% Suc/well and the corresponding treatments and incubation for 16 h with continuous shaking.

For hypocotyl growth measurements, plants were grown for 6 days on vertical plates. Plates were photographed, and the length of the hypocotyl was determined with the ImageJ software.

### Yeast two-hybrid assay

Yeast two-hybrid interaction assays of the PIF4 and BES1 proteins were performed with the GAL4 Two-Hybrid System (Clontech). The complete CDS of PIF4 and BES1 was amplified with primers *PIF4YFPf/PIF4YFPr* and *BES1topof/BES1-R*, cloned into the pENTRY™/D-TOPO vector, and subsequently mobilized into the pGADT7 and pGBKT7 (Clontech) Gateway-compatible vectors. PIF4 deletions were generated by using the following primer

combinations: *PIF4YFPf/APB\_PIF4r* for PIF4del1, *PIF4sinAPBf/PIF4YFPr* for PIF4del2, *AtPIF4del3f/PIF4YFPr* for PIF4del3, and *AtPIF4del4f/PIF4YFPr* for PIF4del4. For BES1 deletions, the following primer combinations were used: *BES1del2f/BES1-R* for BES1del2, *BES1topof/BES1del3r* for BES1del3, and *BES1del4f/BES1-R* for BES1del4. BES1del1 was obtained by HindIII/SacI digestion of the BES1del2 construct in the TOPO vector. Constructs were transformed into the AH109 yeast strain, by the lithium acetate method, and reporter gene activation was analyzed by selection on SD-LW and SD-LWHA plates.

### Bimolecular fluorescence complementation assay

Full-length coding regions for the *Arabidopsis* PIF4 and BES1 proteins and the BES1del2 deletion in the pENTR™/D-TOPO vector (Invitrogen) were mobilized by LR-reaction (Invitrogen) into the binary pBiFC vectors containing the N- and C-terminal YFP fragments (YFP<sup>N43</sup> and YFP<sup>C43</sup>). The BES1delN deletion was obtained by PCR amplification with primers *BES1topof/BES1delNr*. Generated constructs were transformed into the *A. tumefaciens* GV3101 strain and co-infiltrated into *N. benthamiana* leaves, using the indicated combinations. The p19 protein was used to suppress gene silencing (Voinnet *et al*, 2003). *Agrobacteria* strains bearing the BiFC constructs and the p19 silencing suppressor were suspended in 10 mM MES pH 5.5, 10 mM MgSO<sub>4</sub>, and 150 μM acetosyringone at a OD<sub>600</sub> ratio of 0.7:0.7:1.0, respectively, for infiltration. One day after infiltration, leaves were treated with 10 μM MG132 (VWR Calbiochem) and 24 h later they were observed under a Leica TCS SP5 laser scanning confocal microscope, with an excitation beam splitter TD 488/561/633 and an emission band width between 495 and 556 nm.

### Electrophoretic mobility shift assay

Electrophoretic mobility shift assay assays were performed with biotin-labeled probes. To this end, DNA fragments for the *pPIL1*, *pPRES*, and *pDWF4* promoter regions were amplified by using 5'-biotinylated oligonucleotides (Appendix Table S5). Gels were transferred after electrophoretic separation to a nitrocellulose membrane and detected with the LightShift Chemiluminescent EMSA Kit (Pierce). The PIF4-His and MBP-BES1 recombinant proteins were incubated in a 20 μl reaction mixture containing 20 mM Hepes pH 7.9, 50 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT, 50 ng/ml poly (di-dC), 10% glycerol, and 0.1% NP-40 for 5 min at room temperature. The biotin-labeled probes were then added to the mixture and incubated on ice for 20 more minutes, before separation on 4% native polyacrylamide gels in 0.5× TBE buffer. The labeled probes were detected with streptavidin, according to the instructions provided by the manufacturer (Pierce).

### Protein binding microarray

For expression of the recombinant proteins, BES1 was cloned into the pMAL-c2X vector (NEB), encoding the MBP (maltose binding protein) in front of the Gateway recombination cassette. This fusion was then inserted into the pCOLADuet-1 (Novagen) vector by amplification with primers BES-COLA-2F (includes an *RcaI* restriction site, compatible with *NcoI*) and BES-COLA-2R, and PCR product

digestion with the *RcaI* and *AscI* enzymes, before ligation into the *NcoI/AscI* sites (MCS1) of the pCOLADuet-1 vector. The PIF4 CDS was cloned into the vector pET-23a (+) (Novagen) by amplification with primers PIF4-1F and PIF4-1R, which respectively include the *NdeI* and *EcoRI* sites (Appendix Table S5), besides a linker of five glycines. The PIF4 His-tag fusion was then amplified using primers PIF4-COLA-2F and PIF4-COLA-2R (includes an *AvrII* recognition site), and cut with *NdeI* and *AvrII*, for ligation into the pCOLADuet-1 *NdeI/AvrII* sites (MCS2). *Escherichia coli* Rossetta cells were used to express the recombinant proteins. The cultures were grown until an OD<sub>600</sub> of 0.6 and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 37°C. The PIF4 6XHis protein was purified through Ni-NTA agarose beads (Qiagen), and the MBP-BES1 fusion through an amylose resin (NEB), following the specifications of the manufacturers. For the PBM, a 10 nucleotides design was selected. Generation of the double-stranded microarray, protein incubation, and immunological detection of the protein-DNA complexes was as described by Godoy *et al* (2011). Three independent hybridizations were performed with the purified protein fractions obtained from recombinant *E. coli* cultures expressing the PIF4-His, MBP-BES1, and PIF4-His + MBP-BES1 proteins.

#### RNA extraction and quantitative real-time PCR analysis

Total RNA was isolated from whole seedlings using the High Pure Isolation Kit (Roche) and DNase-treated when bound to the column, following the manufacturer instructions. One microgram of RNA was reverse-transcribed into cDNA using the Transcriptor First-Strand cDNA Synthesis Kit (Roche) and random hexamers. The cDNA reaction was diluted to 40 μl, and real-time PCR amplification performed with 1 μl of cDNA, using the Power SYBR Green PCR Master Mix (Applied Biosystems), and a 7500 Real-Time PCR System (Applied Biosystems). Expression levels were calculated relative to *PP2A* by means of the  $\Delta\Delta CT$  threshold cycle method (Applied Biosystems). Primer sequences are provided in Appendix Table S5. Quantitative RT-PCR determinations were done in at least two independent biological replicates with similar results.

#### Protein extraction and Western blot analysis

Seedlings were homogenized in an extraction buffer containing (50 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 150 mM NaCl, 2.5 mM EDTA, 10% glycerol, 25 mM β-glycerophosphate, 0.1% NP-40, 10 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 100 μM PMSF, and 5 μM β-mercaptoethanol and protease inhibitors). Extracts were centrifuged twice at 13,000 g for 10 min at 4°C, and protein concentration in the supernatant was determined by the Bradford assay (Bio-Rad). Protein samples were boiled in TMx2 loading buffer for 5 min and 30 μg of protein separated on 8% SDS-PAGE gels. Gels were transferred to 0.2 μm nitrocellulose membranes (Amersham) and homogeneous protein transfer confirmed by Ponceau red staining. Membranes were incubated in blocking buffer (1× PBS buffer including 0.1% Tween 20 and 5% dried nonfat milk) for 1 h at room temperature, before overnight incubation at 4°C with anti-GFP (Roche), anti-BES1, or anti-RPT5 (Kwok *et al*, 1999) antibodies. Finally, membranes were incubated for another hour with a peroxidase-conjugated secondary antibody. The proteins were visualized with the Supersignal West Pico and Femto substrates (Pierce).

#### Luciferase assay

For the luciferase assay, *N. benthamiana* leaves were infiltrated *Agrobacterium* strains bearing the constructs expressing the *PIL1* (*pPIL1-1F/pPIL1-1R*) or *DWF4* (*pDWF4-F/pDWF4-R*) promoters fused to the Luciferase reporter gene (pLUC-Trap3 vector), and the *35S::BES1-GFP*, *35S::PIF4-HA*, or *35S::bes1-D-GFP* effector constructs, to test for activated expression of these genes. Two days after infiltration, 0.5-cm-diameter disks were collected from the leaves and transferred to 96-well microtiter plates containing 165 μl 0.5× MS liquid media and 35 μl of 1× D-Luciferin substrate (20 μg/ml; Promega). Luminescence was measured with a LB 960 Microplate Luminometer (Berthold). One disk was used per well and at least 12 disk replicates per sample, and the average Luminescence values represented.

#### In vivo co-IP assay

Six-day-old *pPIF4::PIF4-HA* seedlings grown under short-day conditions were collected at ZT0, ZT1, and ZT8 and cross-linked with 1% formaldehyde for 15 min under vacuum. The reaction was terminated by adding glycine to a final concentration of 125 mM and samples were frozen in liquid nitrogen, before homogenization in protein extraction buffer (25 mM Tris-HCl, pH 8.0, 75 mM NaCl, 0.2% Nonidet P-40, 10 mM β-mercaptoethanol, 1 mM PMSF, protease inhibitors (Roche), and 10 μM MG132). Extracts were centrifuged twice for 15 min at 13,000 g and 4°C, and the supernatants kept for co-immunoprecipitation assays. 40 μl of anti-HA sepharose beads was added to the tissue extracts, and the mixture was incubated for 3 h at 4°C. Beads were washed four times with 500 μl extraction buffer and the retained proteins eluted by incubation for 15 min at 65°C with 50 μl of pre-heated 95°C 2× SDS protein loading buffer. 10 μl (anti-HA) and 40 μl (anti-BES1) of the Co-IP samples were used for immunodetection as described previously.

#### Microarray analysis

Microarray was performed with *bes1-D*, *pif4pif5*, *bes1-D;pif4pif5*, and Col-0 genotypes. 6-day-old seedlings grown in the dark were used for RNA extraction and Affymetrix microarray hybridization. Total RNA was extracted from whole seedlings with the High Pure RNA Isolation Kit (Roche). Three biological replicates were used for expression studies. Background correction and normalization were performed using the LIMMA package (Smyth & Speed, 2003). Genes were considered as differentially expressed if fold change was > 2 and the corrected *P*-value (Rank Products) < 0.1. Enrichment in DNA elements in the 1-kb upstream region of these genes was analyzed using the motif analysis tool from TAIR. Statistical analyses were performed using the SPSS statistical package. Hierarchical clustering of the directly regulated genes was obtained using the MEV TIGR free software (<http://mev.tm4.org/>).

#### Statistical analysis

For the analysis of the protein binding microarrays, raw signal intensities were normalized and adjusted with the Perl script “normalize\_agilent\_array.pl”, and enrichment scores (E-Scores) for all the possible 8-mers and position weight matrices for the best scoring 8-mers were obtained using the Perl script “seed\_and\_wobble.pl”.



These analyses were carried out with the PBM Analysis Suite ([http://the\\_brain.bwh.harvard.edu/PBMAAnalysisSuite/index.html](http://the_brain.bwh.harvard.edu/PBMAAnalysisSuite/index.html)). Box plots were obtained using the SPSS statistical program, and logos for the best motifs with enoLOGOS (<http://www.benoslab.pitt.edu/cgi-bin/enologos/enologos.cgi>).

To analyze the overlap of PIF and BES targets and PIF- and BES-regulated genes, lists from published arrays, ChIP-ChIP and ChIP-seq data, were constructed (Appendix Tables S1–S4). Genes were categorized as direct targets or UP-/DOWN-regulated if present in at least one list, excluding those found to be simultaneously up- and down-regulated in different datasets. Lists of the different PIF- and BES-regulated and target genes were used to construct Venn diagrams using the Venny tool (<http://bioinfogp.cnb.csic.es/tools/venny/>). A chi-squared test was performed to calculate gene enrichment of the overlapping datasets compared to total *Arabidopsis* genes (TAIR) using the SPSS statistical program. Gene enrichment was considered statistically significant with a *P*-value < 0.01.

## Data accessibility

Microarray results have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) with the accession code GSE85211.

**Expanded View** for this article is available online.

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## Author contributions

SP, AE-R, and CM designed the experiments. MdL performed initial studies, and SB-G carried out the yeast two-hybrid assays. CM generated the purified proteins and carried out the BiFC BES1-PIF4 interaction analyses. JMF-Z hybridized the protein binding microarray and analyzed the corresponding DNA recognition motifs. CM performed the EMSA, Co-IP, and Western blot studies. qRT-PCR analyses were carried out by AE-R and CM, and the transactivation assays by SP. Microarray hybridization and promoter *cis*-element studies were carried by AE-R. CM and SP wrote the manuscript. All authors revised the manuscript.

## Conflict of interest

The authors declare that they have no conflict of interest.

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