Mediator Subunit MED25 Physically Interacts with PHYTOCHROME INTERACTING FACTOR4 to Regulate Shade-Induced Hypocotyl Elongation in Tomato

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Shade triggers important adaptive responses such as the shade-avoidance syndrome, which enable plants to respond to the depletion of photosynthetically active light. The basic helix-loop-helix transcription factors PHYTOCHROME INTERACTING FACTORS (PIFs) play a key role in the shade-avoidance syndrome network by regulating the biosynthesis of multiple phytohormones and the expression of cell expansion-related genes. Although much has been learned about the regulation of PIFs in response to shade at the protein level, relatively little is known about the PIF-dependent transcriptional regulation of shade-responsive genes. Mediator is an evolutionarily conserved transcriptional coactivator complex that bridges gene-specific transcription factors with the RNA polymerase II (Pol II) machinery to regulate gene transcription. Here, we report that tomato (Solanum lycopersicum) PIF4 plays an important role in shade-induced hypocotyl elongation by regulating the expression of genes that encode auxin biosynthesis and auxin signaling proteins. During this process, Mediator subunit 25 (MED25) physically interacts with PIF4 at the promoter regions of PIF4 target genes and also recruits Pol II to induce gene transcription. Thus, MED25 directly bridges the communication between PIF4 and Pol II general transcriptional machinery to regulate shade-induced hypocotyl elongation. Overall, our results reveal a novel role of MED25 in PIF4-mediated transcriptional regulation under shade.

Plant growth is strongly influenced by the presence of neighboring plants that compete for light and other resources. When plants are under canopy-induced shade, phytochromes detect a reduction in the ratio of red light to far-red light (R/FR; Ballaré et al., 1990). This allows shade-intolerant plants to sense overcrowding by competitors. Consequently, plants initiate escape mechanisms, collectively known as the shade-avoidance syndrome (SAS), to ensure survival. Typical shade-avoidance responses of plants include repressed seed germination, promoted hypocotyl and petiole growth, altered leaf angle, arrested leaf and root development, acceleration of flowering, and reduced branching (Cerdán and Chory, 2003; Casal, 2012; González-Grandío et al., 2013).

In Arabidopsis (Arabidopsis thaliana), SAS is primarily mediated by the photoreceptor phytochrome B (phyB; Halliday et al., 1994; Schepens et al., 2004; Keller et al., 2011). The phytochromes exist in two interconvertible forms: the R-absorbing state (Pr), or inactive form, and the FR-absorbing state (Pfr), or active form. The Pr and Pfr forms of phyB are maintained in an R/FR ratio-dependent balance. Under white light (WL; high R/FR ratio), the photoequilibrium is displaced toward the active Pfr form, which interacts with a group of PHYTOCHROME INTERACTING FACTORS (PIFs) in the nucleus and triggers their rapid phosphorylation...
and degradation. However, under shade (low R/FR ratio), the photoequilibrium is displaced toward the inactive Pr form, leading to the accumulation of PIFs in the nucleus (Cifuentes-Esquivel et al., 2013), which promotes the expression of shade-responsive genes, thus enabling plants to elongate and escape shade (Lorrain et al., 2008; Li et al., 2012).

PIF proteins, a subfamily of basic helix-loop-helix (bHLH) transcription factors, play a critical role in mediating shade-induced rapid transcriptome reprogramming (Lorrain et al., 2008; Horntschek et al., 2012; Leivar et al., 2012; Li et al., 2012). The Arabidopsis genome encodes eight PIF or PIF-like (PIL) proteins (PIF1, PIF3–PIF8, and PIL1), all of which contain a conserved active phyB-binding domain that is required for their interaction with the Pr form of phyB (Lee and Choi, 2017). PIF proteins directly regulate the expression of both positive and negative regulators of SAS (Roig-Villanova and Martinez-García, 2016). Among the positive regulators are several auxin biosynthesis \textit{YUCCA} (\textit{YUCC} \textit{YUCC}) genes and auxin-responsive genes. It has been shown that PIF4, PIF5, and PIF7 directly regulate the expression of auxin-biosynthesis genes \textit{YUC}2, \textit{YUC}5, \textit{YUC}8, and \textit{YUC}9 and auxin-responsive genes \textit{IAA}19 and \textit{IAA}29, thus linking the perception of shade with rapid plant growth (Horntschek et al., 2012; Li et al., 2012).

Physiological and molecular studies have been conducted to investigate the effects of the shade-avoidance response in tomato (\textit{Solanum lycopersicum}; Chitwood et al., 2012, 2015; Bush et al., 2015). Tomato plants exhibit elongated internodes and petioles as well as changes in overall leaf area and leaf mass per unit area in response to shade (Chitwood et al., 2015). Expression analyses have identified a group of auxin-related genes whose expression correlates with the strength of the shade-avoidance response (Bush et al., 2015), implicating the role of auxin in regulating SAS in tomato. Recently, a few studies identified and characterized tomato \textit{PIF} genes (Llorente et al., 2016; Rosado et al., 2016). Tomato PIF1a modulates carotenoid biosynthesis during fruit ripening via a mechanism virtually identical to that used by its closest Arabidopsis homolog, PIF1 (Llorente et al., 2016). Amino acid sequence alignments indicated that Arabidopsis and tomato PIF homologs share 27% to 51% sequence identity. Despite the low identity score, all tomato PIF amino acid sequences contain active phytochrome B binding (APB) motifs and bHLH domains, thus reinforcing their identity as PIF proteins (Rosado et al., 2016). However, whether tomato PIF proteins effectively regulate SAS remains poorly understood.

Although the role of PIF proteins in regulating SAS is well-established in Arabidopsis, the precise mechanism underlying PIF-mediated transcriptional regulation remains enigmatic. In particular, it remains unclear how PIF transcription factors relay regulatory signals to the RNA polymerase II (Pol II) transcriptional machinery to transcribe specific genes during SAS.

Mediator is a multisubunit complex conserved across eukaryotes, which plays essential roles in Pol II-dependent gene transcription (Kornberg, 2005; Malik and Roeder, 2005; Sourtourina et al., 2011; Poss et al., 2013; Allen and Taatjes, 2015). Mediator has been extensively investigated in yeast and animals for its ability to orchestrate transcription factor-dependent assembly of the Pol II preinitiation complex (PIC) via discrete interactions with signal-dependent transcription factors and Pol II (Kornberg, 2005; Malik and Roeder, 2005, 2010; Sourtourina et al., 2011). Biochemical purification of the Arabidopsis Mediator complex identified 21 conserved and six plant-specific subunits (Bäckström et al., 2007). Mediator subunit25 (MED25) is one of the best-characterized plant Mediator subunits. Studies show that MED25 performs functions in diverse areas including hormone signaling, biotic and abiotic stress responses, and plant development (Kazan, 2017; Zhai and Li, 2019).

Recently, our studies revealed that MED25 interacts with MYC2, a master transcription factor of jasmonate (JA) signaling, to regulate nearly every step of the JA-mediated transcriptional output (Chen et al., 2012; An et al., 2017; Liu et al., 2019a; You et al., 2019; Wu et al., 2020).

Although MED25 was first identified as a SAS regulator acting downstream of phyB (Cerdán and Chory, 2003), the mechanism of action of MED25 remains largely unclear. Here, we show that tomato MED25 and PIF4 promote shade-induced hypocotyl elongation. MED25 physically interacts with PIF4 and recruits Pol II general transcriptional machinery to the promoters of PIF4 target genes, which is important for PIF4-mediated gene transcription. Our results characterize the function of MED25 in linking the PIF4 transcription factor to Pol II general transcriptional machinery, thus broadening our understanding of the regulatory mechanism of MED25 in SAS.

**RESULTS**

**Tomato PIF4 Positively Regulates Shade-Induced Hypocotyl Elongation**

Arabidopsis PIF4 and PIF5 positively regulate low R/FR ratio-mediated shade avoidance, and the abundance of PIF4 and PIF5 proteins increases rapidly in WL-grown seedlings upon their transfer to simulated shade (SH), consistent with their function in promoting hypocotyl elongation (Lorrain et al., 2008). Phylogenetic analysis shows that tomato PIF4 is a homolog of Arabidopsis PIF4 and PIF5 proteins (Supplemental Fig, S1A; Llorente et al., 2016; Oh et al., 2020). To investigate whether tomato PIF4 plays a role in SAS similar to that observed for Arabidopsis PIF4 and PIF5 proteins, we used the CRISPR/Cas9 gene editing system (Deng et al., 2018) to generate \textit{pif4-}\textit{c} mutant plants (Supplemental Fig, S2, A–D). Sequence analyses revealed a 4- and a 1-bp deletion in the \textit{PIF4} open reading frame (ORF) in \textit{pif4-}\textit{c}-1 and \textit{pif4-}\textit{c}-9 mutant lines, respectively, leading to a frame-shift mutation and generation of a premature stop codon (TAA or TAG) in both lines (Supplemental Fig, S2, A–D). Despite the prediction of four potential off-target sites
using the Cas-Offinder (http://www.rgenome.net/cas-offinder/), off-target mutation was not detected at any of these POTs in pif4-c-1 and pif4-c-9 (Supplemental Fig. S3). We examined the hypocotyl length of wild-type, pif4-c-1, and pif4-c-9 seedlings in WL and SH conditions. Compared with wild-type seedlings, pif4-c-1 and pif4-c-9 mutant seedlings showed a significant reduction in hypocotyl length under WL, suggesting that PIF4 is required for hypocotyl elongation in WL conditions. (Supplemental Fig. S4, D and E).

To investigate whether tomato PIF4 regulates hypocotyl elongation under SH conditions, (Supplemental Fig. S4, D and E). Moreover, the expression of exogenous PIF4-GFP was specifically detected in the PIF4-OE-4 and PIF4-OE-14 plants (Supplemental Fig. S4B). In addition, the protein abundance of tomato PIF4 increased in response to SH (Supplemental Fig. S4C). Further, we found that the hypocotyl length of PIF4-OE seedlings was comparable to that of wild-type seedlings under WL conditions; however, under SH, the PIF4-OE seedlings exhibited significantly longer hypocotyls than wild-type seedlings, further confirming that PIF4 promotes hypocotyl elongation under SH conditions. (Supplemental Fig. S4, D and E).

It has been shown that Arabidopsis PIF4 and PIF5 regulate the expression of several auxin-biosynthetic and auxin-responsive genes to promote hypocotyl elongation (Tao et al., 2008; Horvitschek et al., 2012). To investigate whether tomato PIF4 regulates the expression of auxin-biosynthetic genes in response to shade, we first identified the tomato homologs of Arabidopsis YUC genes using Arabidopsis YUC8 and YUC9 proteins as query sequences. Two YUCCA-like flavin monooxygenases (Solyc09g064160 and Solyc06g083700), which showed the highest similarity to Arabidopsis YUC8 and YUC9, respectively, were identified and named tomato YUC8 and YUC9.

Both these proteins contained the conserved FAD- and NADPH-binding motifs (Supplemental Fig. S5). We detected the expression levels of tomato YUC8 and YUC9 genes in wild-type, pif4-c-1, and pif4-c-9 seedlings under WL and SH conditions by reverse transcription quantitative PCR (RT-qPCR). Under WL, expression levels of YUC8 and YUC9 were slightly lower in pif4-c mutant seedlings than in wild-type seedlings (Fig. 1C). In response to SH, expression levels of YUC8 and YUC9 were significantly increased in wild-type seedlings (Fig. 1C). However, shade-induced expression of YUC8 and YUC9 was decreased in pif4-c mutant seedlings compared with wild-type seedlings (Fig. 1C).

In the RT-qPCR assays, ACTIN2 was used as a control for normalization since its expression is not affected by the mutations and different conditions according to our RNA-Seq results (Supplemental Fig. S6). These results suggest that PIF4 positively regulates shade-induced expression of YUC8, YUC9, and IAA19.

To test whether PIF4 directly regulates YUC8, YUC9, and IAA19 expression by binding to their promoters, we performed chromatin immunoprecipitation (ChIP) assays using PIF4-GFP OE lines. Given that Arabidopsis PIF4 specifically binds to G-box or G-box-like motifs in its target gene promoters (Sun et al., 2012), we searched for the promoter regions of YUC8, YUC9, and IAA19 genes in the tomato genome. A G-box motif (CACGTG) was identified in the YUC9 promoter, and G-box-like motifs, specifically CATGTG and CACATG, were identified in YUC8 and IAA19 gene promoters, respectively. We performed ChIP assays using 6-d-old PIF4-GFP seedlings, and the ChIP-qPCR results revealed that PIF4 was significantly enriched at the G-box or G-box-like motifs in YUC8, YUC9, and IAA19 gene promoters under SH conditions (Fig. 1, D and E). To detect whether the ectopic expression of PIF4-GFP driven by the 3SS promoter could affect the ChIP results, we also performed ChIP assays using hypocotyls of 6-d-old PIF4-GFP seedlings, since PIF4 was expressed in hypocotyls in the wild-type seedlings (Supplemental Fig. S7A). Moreover, RT-qPCR analysis showed that the expression of YUC8, YUC9, and IAA19 could be induced in hypocotyls under SH (Supplemental Fig. S7B). As shown in Supplemental Figure S7C, PIF4 was specifically enriched in the G-box or G-box-like motifs of the target promoters in response to SH, which is consistent with the result using the whole seedling of PIF4-GFP plants (Fig. 1E). These results suggest that PIF4 binds to the G-box or G-box-like motifs of YUC8, YUC9, and IAA19 promoters under SH in vivo. Furthermore, electrophoretic mobility shift assays (EMSA) indicated that a glutathione S-transferase (GST)-PIF4 fusion protein binds to the promoter of YUC9 in a G-box-dependent manner (Fig. 1F). These results demonstrate that tomato PIF4 promotes the expression of YUC8, YUC9, and IAA19 under shade by directly binding to their promoters.

Collectively, our results indicate that tomato PIF4 promotes shade-induced hypocotyl elongation by directly regulating the expression of several auxin-biosynthetic and -responsive genes.

**MED25 Physically Interacts with PIF4**

Since the Mediator complex functions as a bridge between transcription factors and Pol II to modulate gene transcription, we set out to determine the specific Mediator subunit(s) needed for relaying the transcriptional activation function of PIF4. Previously, the Arabidopsis Mediator complex subunit MED25 was shown to regulate hypocotyl elongation in response to shade (Cerdán and Chory, 2003). This observation led us to hypothesize that
Figure 1. PIF4 positively regulates hypocotyl elongation under simulated shade. A and B, Images (A) and quantification (B) of the hypocotyl length of wild-type (WT) and pif4-c mutant tomato seedlings grown under WL or exposed to SH. Three-day-old seedlings were either kept under WL or transferred to SH for 3 d. Data represent the mean ± SE of at least 20 plants. Scale bar = 1 cm. The percentage on the columns represents the hypocotyl length relative to the wild type under WL or SH, respectively. Different lowercase letters indicate
MED25 directly interacts with PIF4. To test this hypothesis, we performed in vitro pull-down experiments using purified maltose-binding protein (MBP)-tagged MED25 (MBP-MED25) and GST-PIF4. The GST-PIF4 recombinant fusion protein, but not GST, was able to pull-down MBP-MED25 (Fig. 2A), indicating that PIF4 interacts with MED25 in vitro. To determine whether PIF4 interacts with MED25 in vivo, we performed communoprecipitation experiments by coexpressing MED25-myc and PIF4-GFP in Nicotiana benthamiana leaf cells. Cell extracts were immunopurified using anti-GFP antibody, and the resultant immunoprecipitates were fractionated via SDS-PAGE and detected using antimyc antibody. The MED25-myc fusion protein was detected in cell extracts when coexpressed with PIF4-GFP but not when coexpressed with GFP (negative control), suggesting that PIF4 interacts with MED25 in plant cells (Fig. 2B).

To identify the protein domain of PIF4 necessary for its interaction with MED25, we conducted yeast two-hybrid (Y2H) assays. Full-length or truncated versions of PIF4 (Fig. 2C) were fused to the GAL4 activation domain to generate prey vectors, and full-length MED25 was fused to the GAL4 DNA-binding domain to generate the bait vector. The results of Y2H assays showed that the N-terminal domain (NTD) of PIF4 was sufficient for PIF4-MED25 interaction (Fig. 2C). Deletion of the NTD of PIF4 eliminated the interaction between PIF4 and MED25, whereas deletion of the bHLH and C-terminal domains (CTDs) did not affect this interaction (Fig. 2C). These results suggest that the NTD of PIF4 is involved in its interaction with MED25. Similarly, to identify the protein domain of MED25 responsible for its interaction with PIF4, we divided the MED25 protein into a von Willebrand factor A domain, a nonconserved middle domain, an ACID domain, and a Gln-rich domain (Fig. 2D). Y2H assays indicated that the ACID domain of MED25 is essential for its interaction with PIF4 (Fig. 2D; Supplemental Fig. S8). Together, our results indicate that PIF4 interacts with MED25 both in vitro and in vivo.

**MED25 Acts as a Coactivator of PIF4 to Mediate Shade-Induced Hypocotyl Elongation**

To elucidate the biological significance of PIF4-MED25 interaction, we analyzed the shade-induced, hypocotyl-elongation phenotype of MED25 antisense (MED25-AS) lines; in these lines, the expression level of endogenous MED25 is substantially reduced compared with the wild type (Liu et al., 2019a). Under WL conditions, the hypocotyl lengths of two MED25-AS lines were similar to those of wild-type plants; however, under SH conditions, both MED25-AS lines showed significantly shorter hypocotyl lengths than wild-type plants (Fig. 3, A and B), suggesting that MED25 positively regulates shade-induced hypocotyl elongation. To confirm the role of MED25 in regulating hypocotyl elongation under shade, we also analyzed the hypocotyl lengths of MED25 overexpression (MED25-OE) lines, which expressed MED25 to significantly higher levels compared to wild-type plants (Liu et al., 2019a). The hypocotyl lengths of two MED25-OE lines were similar to those of wild-type plants under WL conditions but significantly enhanced under SH conditions (Supplemental Fig. S9). Consistent with the morphological phenotypes, shade-induced expression levels of YUC8, YUC9, and IAA19 were dramatically reduced in MED25-AS plants compared with wild-type plants (Fig. 3C; Supplemental Fig. S6). These results prompted us to investigate whether MED25 acts as a coactivator of PIF4 during the transcriptional regulation of PIF4 target genes. To test this possibility, we cloned the 1,987-bp YUC9 promoter sequence into the dual-LUC reporter system to generate the ProYUC9:LUC reporter construct (Fig. 3, D and E). Coexpression of PIF4 with ProYUC9:LUC in N. benthamiana leaves led to significantly increased LUC activity (Fig. 3, D and E), suggesting that PIF4 activates the expression of ProYUC9:LUC. When MED25 was coexpressed with PIF4 and the ProYUC9:LUC reporter construct, the PIF4-dependent activation of LUC activity was further enhanced (Fig. 3, D and E). In parallel control experiments, GFP had an insignificant effect on PIF4-dependent activation of LUC activity. Together, these results substantiate that MED25 acts as a coactivator of PIF4 in regulating shade-induced hypocotyl elongation.

**MED25 and PIF4 Coregulate the Expression of Shade-Responsive Genes**

To evaluate the impact of PIF4 and MED25 on shade-responsive gene expression on a genome-wide scale, we performed RNA-sequencing (RNA-seq) experiments using wild-type, pif4-c-9, and MED25-AS-3 seedlings grown under WL (WT_WL, pif4_WL, and med25_WL, respectively) or SH (WT_SH, pif4_SH, and med25_SH, respectively) or SH (WT_SH, pif4_SH, and med25_SH, respectively).
Genes with 1.5-fold change in expression ($P < 0.05$) were considered to be differentially expressed. Under SH, 2,074 genes were differentially expressed in pif4-c-9 plants compared with wild-type plants; these genes were designated as PIF4-regulated, shade-responsive genes (fold-change > 1.5, FDR-adjusted $P < 0.05$; Fig. 4A; Supplemental Dataset 1). Similarly, 1,312 genes showed differential expression between MED25-AS-3 and the wild type; these genes were designated as MED25-regulated, shade-responsive genes (fold-change > 1.5; FDR-adjusted $P < 0.05$; Fig. 4A; Supplemental Dataset 2). Comparison of these two datasets revealed 742 overlapping genes, which were defined as PIF4- and MED25-coregulated, shade-responsive genes (fold-change > 1.5, FDR-adjusted $P < 0.05$; Fig. 4A; Supplemental Dataset 3). Among these, 306 genes were upregulated and 317 genes were downregulated by both PIF4 and MED25 (Fig. 4B). These results indicate that ~57% (742 of 1,312) of MED25-regulated, shade-responsive genes and 36% (742 of 2,074) of PIF4-regulated, shade-responsive genes are coregulated by MED25 and PIF4. Gene ontology (GO) analysis indicated that these genes are enriched in pathways related to auxin response, cell wall biogenesis process, organonitrogen compound catabolic process, and cell wall macromolecule metabolic process (Fig. 4C). Many well-characterized, growth-associated genes were identified as MED25- and PIF4-coregulated genes, including auxin-biosynthetic genes ($YUC8$ and $YUC9$), auxin-responsive genes ($IAA11$, $IAA19$, $IAA26$, and $IAA29$; Wu et al., 2012; Huai et al., 2018), small auxin upregulated RNA genes ($SAUR$, $SAUR3$, $SAUR4$, and $SAUR61$; Liu et al., 2019b), and a cell wall-modifying expansion gene ($EXP2$; Català et al., 2000). Remarkably, shade-mediated upregulation of these genes was suppressed in MED25-AS and pif4-c plants (Fig. 4D), indicating that PIF4 and MED25 play critical roles in shade-mediated activation of these genes. Together, these data suggest that MED25 and PIF4 coregulate the expression of a subset of shade-responsive genes.

**MED25 Recruits Pol II to the Promoters of PIF4 Target Genes**

It has been shown that transcription factors recruit MED25 to the promoter region of target genes to
activate gene transcription (Chen et al., 2012; Ito et al., 2016; Liu et al., 2016; Ren et al., 2020). To measure the enrichment of MED25 on the chromatin of the PIF4 targets, we performed ChIP-qPCR assays using transgenic plants expressing a translational fusion of MED25 with the GFP reporter (MED25-GFP; Liu et al., 2019a). Since MED25 was ubiquitously expressed in various tissues of wild-type plants (Supplemental Fig. S10), we used whole seedlings of 6-d-old MED25-GFP plants in the analyses. Results of ChIP-qPCR analyses revealed significant enrichment of MED25 at the G-box or G-box-like motifs in YUC8, YUC9, and IAA19 promoters under SH conditions (Fig. 5, A and B).

To examine whether the depletion of PIF4 affects the enrichment level of MED25, we introduced MED25-GFP into pif4-c-1 plants by crossing. ChIP-qPCR analyses revealed that shade-induced enrichment of MED25 on YUC8, YUC9, and IAA19 promoters was substantially lower in pif4-c-1 plants than in wild-type plants (Fig. 5, A and B). Given that PIF4 did not affect MED25 protein levels (Supplemental Fig. S11), these results demonstrate that the enrichment and function of MED25 on PIF4 target-gene promoters depend on PIF4.

Previous studies showed that Arabidopsis MED25 associates with both DNA-bound MYC2 and Pol II, thus serving as an integrative hub for the transcriptional
Figure 4. Transcriptome analyses of PIF4- and MED25-regulated genes. A, Venn diagrams showing the overlap of genes between pif4-c and MED25-AS plants grown under SH. B, Heat map showing the shade-associated differentially expressed genes coregulated by PIF4 and MED25 in tomato, according to RNA-seq data. C, GO analysis of shade-associated genes coregulated by PIF4 and MED25. D, Expression of a few selected SH-responsive genes in wild-type (WT), pif4-c-9, and MED25-AS-3 plants in RNA-seq experiments.
Figure 5. Enrichment of MED25 and Pol II on PIF4 target gene promoters. A, Schematic representation of the amplicons of YUC8, YUC9, and IAA19 promoters used for ChIP-qPCR. Positions of the transcription start site (TSS) and transcription termination site (TTS) are indicated. B and C, ChIP-qPCR assays showing the enrichment of tomato MED25 (B) and Pol II (C) on the promoters of YUC8, YUC9, and IAA19 genes under WL and SH. ACTIN2 was used as a nonspecific target (control). Data represent means ± SD (n = 3). Different lowercase letters indicate significant differences by one-way ANOVA and Tukey posthoc test (P < 0.05). WT, Wild type. D, A proposed working model showing the coordinated regulation of shade signaling pathways by MED25 and PIF4. The MED25 transcriptional coactivator physically interacts with PIF4 and promotes its binding to the promoter of auxin biosynthetic and signaling genes (such as YUC9 and IAA19), leading to their activation and hypocotyl elongation.
regulation of JA signaling genes (Chen et al., 2012). In this context, our finding that the tomato MED25 physically associates with PIF4 and affects its function suggests that the mutation of MED25 could impair the recruitment of the Pol II general transcriptional machinery during shade-induced gene transcription. To test this speculation, we assessed the effect of MED25 depletion on shade-induced recruitment of the CTD of the largest subunit of Pol II to PIF4 target-gene promoters. The results of ChIP-qPCR assays revealed that shade-induced recruitment of Pol II CTD to the promoters of YUC8, YUC9, and IAA19 was markedly reduced in MED25-AS plants compared with wild-type plants (Fig. 5, A and C). Taken together, these results suggest that MED25 recruits Pol II to the promoters of PIF4 target genes and creates a direct bridge between PIF4 and Pol II general transcriptional machinery.

DISCUSSION

In response to shade, plants trigger genome-wide transcriptional reprogramming, which is largely regulated by PIF transcription factors (Lorrain et al., 2008; Li et al., 2012). Shade (low R/FR ratio) increases the stability of PIF proteins, which accumulate in the nucleus and promote the expression of shade-responsive genes (Lorrain et al., 2008; Li et al., 2012). Whereas much effort in this field has been devoted to understanding the regulatory mechanism of PIFs at the protein level, we know relatively little about the mechanism of PIF-mediated transcriptional regulation. In this study, we provide several lines of evidence showing that tomato MED25, a multifunctional subunit of the Mediator complex, plays a positive role in PIF4-mediated gene transcription during shade-induced hypocotyl elongation. First, reduction in the expression of MED25 in MED25-AS plants reduced hypocotyl elongation under shade (Fig. 3), which is similar to the phenotype of pif4-c mutant lines (Fig. 1). Second, MED25 enhanced the function of PIF4 in regulating the expression of PIF4 target genes, as shown by the dual-LUC reporter assay (Fig. 3). Third, MED25 physically interacted with PIF4 both in vivo and in vitro (Fig. 2). Fourth, PIF recruited MED25 to target promoter regions, which in turn recruited the Pol II transcriptional machinery to the same promoter regions, as shown by ChIP-qPCR assays (Fig. 5, A–C). Finally, 36% of the PIF4-regulated shade-responsive genes were coregulated by MED25 (Fig. 4). Together, these results demonstrate an important and novel biological role of MED25 in PIF4-mediated transcriptional regulation during shade-induced hypocotyl elongation (Fig. 5D).

In Arabidopsis, PIF4 and PIF5 are critical regulators of shade-induced rapid plant growth, as they directly control the expression of genes encoding auxin biosynthesis and auxin signaling components (Horvitschek et al., 2012; Li et al., 2012). Tomato PIF4 is a homolog of Arabidopsis PIF4 and PIF5 (Llortente et al., 2016). Here, we provide several lines of evidence showing that tomato PIF4 also plays important roles in regulating shade-induced hypocotyl elongation. In tomato, depletion of PIF4 decreased shade-induced hypocotyl elongation and reduced the expression of auxin biosynthetic and auxin-responsive genes (Fig. 1, A–C). RNA-seq analysis revealed that the PIF4-regulated shade-responsive gene set was enriched in auxin-related genes, including auxin-biosynthetic genes, auxin-responsive genes, and SAUR genes (Fig. 4). Furthermore, ChIP-qPCR and EMSA analyses revealed that PIF4 directly targeted the promoters of YUC8, YUC9, and IAA19 to regulate their expression (Fig. 1, D and E). These results suggest that the mechanism of shade-induced hypocotyl elongation is conserved in tomato and Arabidopsis.

In addition to PIF4 and PIF5, Arabidopsis PIF7 also has been shown to be involved in SAS (Li et al., 2012; de Wit et al., 2015; Mizuno et al., 2015). Interestingly, the action mode of PIF7 in regulating SAS is distinct from that of PIF4 and PIF5. Whereas low R/FR increased the stability and accumulation of PIF4 and PIF5 (Leivar et al., 2008), shade leads to a rapid dephosphorylation of PIF7, thereby enhancing its binding to target promoters (Li et al., 2012). Phylogenetic analysis showed that there are two PIF7 homologs in the tomato genome, PIF7a and PIF7b (Supplemental Fig. S1A; Oh et al., 2020). Our RNA-seq and RT-qPCR analyses revealed that the expression of both PIF7a and PIF7b was reduced in response to shade (Supplemental Fig. S1, B and C), suggesting that these PIF7 proteins of tomato might play distinct functions from PIF4 in regulating SAS. It will be interesting in future studies to elucidate the action mechanism of tomato PIF7 in regulating SAS.

Mediator is a eukaryotic multisubunit transcriptional coactivator complex that regulates various aspects of gene transcription. The most well-characterized function of Mediator is its ability to regulate PIC formation. Mediator is recruited to target gene promoters and enhancer regions via direct interactions with specific transcription factors, while maintaining direct physical interactions with Pol II and other PIC components (Holstege et al., 1998; Asturias et al., 1999; Myers et al., 2001; Davis et al., 2002; Bernecky et al., 2011). PIF4 is a master transcription factor that integrates versatile environmental and hormonal signals during plant growth (Lau and Deng, 2010; Leivar and Quail, 2011). However, the Mediator subunit involved in PIF4 transcriptional activity is unknown. Our study revealed that MED25 plays a critical role in the regulation of PIF4 transcriptional activity. Importantly, we showed that MED25 physically interacts with PIF4 (Fig. 2). Mutations in PIF4 and MED25 similarly affected the expression of several genes, including those involved in auxin biosynthesis and signaling (YUC8, YUC9, and IAA19; Figs. 1 and 3). MED25 was recruited to the promoters of these genes in a PIF4-dependent manner (Fig. 5, A and B). Moreover, the binding of Pol II CTD to the promoter regions of these target genes required MED25 (Fig. 5, A and C). Together, our results suggest...
that during shade-induced transcription of PIF4 target genes, MED25 serves as a bridge between PIF4 and the Pol II transcriptional machinery for PIC assembly.

Arabidopsis MED25 was originally identified as PFT1, which acts downstream of phyB to regulate SAS by modifying flowering time and hypocotyl elongation (Cerdán and Chory, 2003). Subsequent studies revealed that MED25 integrates photoperiod and age pathways to regulate flowering time (Inigo et al., 2012; Liu et al., 2017; Yao et al., 2019). CONSTANS (CO), a central component of the photoperiod pathway that promotes flowering, has been identified as an important target of MED25 (Inigo et al., 2012). A recent study showed that MED25 interacts with TEOSINTE BRANCHED/CYCLOIDEA/PCF (TCP) transcription factors and FLOWERING BHLH transcription activators to facilitate CO transcription and promote flowering (Liu et al., 2017). In parallel, another study suggested that MED25 interacts with SQUAMATA PROMOTER BINDING PROTEIN-LIKE 10 (SPL10) transcription factor to regulate flowering time through the age pathway (Yao et al., 2019). In this study, PIF4-targeted, shade-responsive genes were also regulated by MED25, demonstrating that MED25 regulates shade-induced hypocotyl elongation through the PIF4-mediated pathway. Moreover, the MED25-PIF4 interaction and MED25 enrichment on YUC8, YUC9, and IAA19 promoters elucidate how MED25 regulates shade-induced hypocotyl elongation. Thus, our results, together with those of previous studies, suggest that through interactions with different transcription factors, MED25 integrates multiple pathways to regulate SAS.

MED25 is a multifunctional regulator that not only links signal-specific transcription factors with the Pol II transcriptional machinery for PIC assembly, but also plays important roles in many other steps of gene transcription. Previously, we showed that in addition to interacting with the master transcription factor MYC2, MED25 also interacts with multiple genetic and epigenetic regulators of JA signaling, and controls almost every step of MYC2-dependent gene transcription, including nuclear hormone receptor activation, epigenetic regulation, mRNA processing, transcriptional termination, and chromatin loop formation (Chen et al., 2012; An et al., 2017; Liu et al., 2019a; You et al., 2019; Zhai and Li, 2019; Wu et al., 2020). Among these MED25-interacting regulators, LEUNIG-HOMOLOG (LUH) served as a scaffold to stabilize the MYC2–MED25 activation complex by enhancing interaction with MYC2, MED25, and other coactivators (You et al., 2019). LUH and LEUNIG (LUG) are two highly homologous members of the Groucho family of transcriptional regulators, which associate with transcription factors through SEUSS (SEU) adaptor protein (Gregis et al., 2006; Sridhar et al., 2006). Interestingly, LUH has also been reported as a coregulator of PIF1-mediated transcription during seed germination (Lee et al., 2015). Moreover, it was shown that SEU interacts with PIF4 and acts as a central regulator that integrates light and temperature signals to control plant growth (Huai et al., 2018). Further elucidation of the relationship between MED25, LUH, and SEU during PIF4-mediated gene transcription will provide deeper insight into transcriptional regulation by PIF transcription factors.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

Tomato (*Solanum lycopersicum* ‘Ailsa Craig’) was used as the wild type for generating all transgenic or mutant lines. The *MED25-AS* (Liu et al., 2019a) and *MED25-GFP* (Liu et al., 2019a) genotypes have been described previously, and the pif4-c mutant and PIF4-GFP OE lines were generated in this study. The *MED25* transgene was introduced into the pif4-c background via crossing. Homozygous plants were selected by genotyping. To analyze plant phenotype under shade, surface-sterilized tomato seeds were germinated by soaking in sterile water at 25°C for 48 h. Tomato seedlings were incubated on one-half strength Murashige and Skoog medium (1% [w/v] Suc and 0.8% [w/v] agar [pH 5.8]) in a growth chamber under continuous WL (200 μmol photons m⁻² s⁻¹) at 26°C for 2 d. Subsequently, seedlings were either left under WL or transferred to SH (R, 640–720 nm, 20 μmol m⁻² s⁻¹; FR, 720–750 nm, 31 μmol m⁻² s⁻¹; R/FR ratio = 0.65) for 4 d. Then, hypocotyl lengths of at least 20 seedlings were measured using ImageJ software (https://imagej.nih.gov/ij/).

**Generation of PIF4-GFP OE Tomato Lines**

The full-length *PIF4* coding sequence (CDS) was amplified by PCR and cloned into the pK7FWG2 vector downstream of the Cauliflower mosaic virus (CaMV) 355 promoter to generate an in-frame PIF4-GFP fusion. Primers used for plasmid construction are listed in Supplemental Table S1. The resulting PIF4-GFP construct was introduced into wild-type tomato plants (*Ailsa Craig*) via *Agrobacterium tumefaciens*-mediated transformation (Du et al., 2014). Transformsants were selected based on their resistance to kanamycin. Homozygous *zyg* T3 or T4 transgenic lines were used for phenotypic and molecular characterization.

**Generation of pif4-c Mutant Lines Using the CRISPR/Cas9 Technology**

Two 19-bp fragments were selected from the *PIF4* CDS (nucleotides 28–46 and 171–189) to synthesize guide RNAs (gRNAs) for editing *PIF4* using CRISPR/Cas9 technology. The gRNA sequences were amplified from the pHSE401 vector (template) by PCR using forward and reverse primers containing these 19-bp sequences (Supplemental Table S1). The tomato U6-26-PIF4-gRNA cassette and the CRISPR/Cas9 binary vector pCBC-DTT2_tomatoU6 were digested with BsaI, and the cassette was cloned into the binary vector to generate the pCBC-DTT2_tomatoU6-PIF4 vector. The final binary vector was introduced into wild-type tomato plants via *A. tumefaciens*-mediated transformation (Du et al., 2014). CRISPR/Cas9-induced mutations were genotyped by PCR, followed by DNA sequencing. Primers used for plasmid construction are listed in Supplemental Table S1. Cas9-free T2 plants carrying mutations in *PIF4* were identified for further experiments.

**Y2H Assays**

The Y2H assays were performed using the Matchmaker GAL4 Two-Hybrid System (Clontech). Full-length and partial CDSs of tomato PIF4 were cloned into the pGADT7 vector, and those of MED25 were cloned into the pGBK7T7 vector. Primers used for plasmid construction are listed in Supplemental Table S1. Constructs used to test protein–protein interactions were cotransformed into yeast (Saccharomyces cerevisiae) strain AH109. Cotransformation of empty pGBK7T7 and pGADT7 vectors served as a negative control. The transformed yeast cells were selected on synthetic defined (SD) solid medium lacking Leu and Trp (SD/-2). To assess protein–protein interactions, the transformed yeast cells were suspended in SD/-2 liquid medium (OD₅₆₂ = 1.0). Then, 5 mL of each yeast culture containing a unique combination of plasmids was plated on SD medium lacking Leu, Trp, His, and Ade (SD/-4). Plates were incubated at 30°C, and the growth of yeast cells was examined after 3 d.

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In Vitro Pull-Down Assays

To produce MBP-MED25 recombinant protein, the full-length MED25 CDS was PCR-amplified and cloned into the pMAL-c2X vector. To produce GST-tagged PIF4 protein, the full-length PIF4 CDS was amplified and cloned into the pGEX-4T-3 vector. The primers used for generating these constructs are listed in Supplemental Table S1. The recombinant vectors were transformed into E. coli BL21 (DE3) cells. Then, 0.5 mM isopropyl-β-D-thiogalactoside was added to the bacterial cultures to induce the expression of MBP-MED25 and GST-PIF4 fusion proteins, which were then purified using amyllose resin (New England Biolabs) and GST Bind Resin (Millipore), respectively.

Immunoblot Analysis

Protein extraction and immunoblotting were performed according to standard protocols. Six-day-old seedlings were ground to a fine powder in liquid nitrogen and then transferred to extraction buffer (50 mM Tris-HCl [pH 7.5], 10 mM EDTA, 50 mM NaCl, 50 mM diethiothreitol, 2% [v/v] Nonidet P-40, and Roche protease inhibitor cocktail). Protein samples were mixed with SDS protein loading buffer and boiled for 10 min. Then, these samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Immunoblots were probed with anti-GFP antibody (1:2,000; catalog no. M20004, Abmart). Ponceau S-stained membranes were used as loading controls.

ChIP Assay

Six-day-old PIF4-GFP, MED25-GFP, and MED25-GFP/pif4-c seedlings were grown under WL or SH. Two grams of whole seedlings or hypocotyls of the indicated genotypes were harvested and crosslinked with 1% [v/v] formaldehyde for 10 min at room temperature. To stop the crosslinking reaction, 0.125 M Gly was added to each sample, and vacuum and filtration was continued for 5 min at room temperature. The crosslinked samples were ground to a fine powder in liquid nitrogen. Then, the chromatin complex was isolated, resuspended in nuclei lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1% [v/v] SDS, 1% [v/v] Triton X-100, 0.1% [w/v] sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and 1× Roche protease inhibitor mixture), and sonicated to shear the chromatin into ~500 bp fragments. Then, 30 μL of the chromatin sample was removed and saved as input (control). The remaining chromatin sample was incubated with polyclonal anti-GFP antibody (AB9290, lot GR203024-L, Abcam) overnight at 4°C. The immunoprecipitated complexes were collected using protein G beads (Invitrogen) and washed with four different buffers in the following order: low-salt wash buffer (150 mM NaCl, 0.2% [w/v] SDS, 0.5% [v/v] Triton X-100, 2 mM EDTA), high-salt wash buffer (500 mM NaCl, 0.2% [w/v] SDS, 0.5% [v/v] Triton X-100, 2 mM EDTA, and 20 mM Tris-HCl [pH 8.0]), LIG wash buffer (0.25% LIG, 0.5% [w/v] Nonidet P-40, 0.5% [w/v] sodium deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl [pH 8.0]), and TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA). The washed samples were then eluted off the protein G beads using elution buffer (1% [w/v] SDS and 100 mM NaHCO3). The protein-DNA crosslinks were reversed by incubating the immunoprecipitated sample at 65°C overnight. Then, the DNA was recovered using the QIAquick PCR Purification Kit (Qiagen) and analyzed by quantitative real-time PCR (qPCR). Primers used to perform qPCR were designed to amplify regions of YUC9, YUC8, and YUC9 promoters that were conserved or did not contain G-box and/or G-box-like motifs (PIF4-binding sites; Supplemental Table S1). ACTIN2 was used as a nonspecific target gene (control). The qPCR data were normalized relative to the input, and enrichment of promoter fragments was expressed as fold enrichment.

EMSA

The full-length CDS of PIF4 were PCR-amplified and cloned into pGEX-4T-3. The recombinant MBP fusion proteins were expressed in E. coli BL21 (DE3) cells and purified to homogeneity using GST resin. Oligonucleotide probes were synthesized and labeled with biotin at the 5′ ends (Invitrogen). EMSAs were performed as described previously (Chen et al., 2011; Du et al., 2014). Briefly, biotin-labeled probes were incubated with GST fusion proteins at room temperature for 20 min, and free and bound probes were separated by PAGE. Mutated YUC9 probes in which the specific transcription factor-binding motif 5′-CACCTG-3′ was replaced by 5′-AAAAAA-3′ were used as negative controls. Probes used for EMSA are listed in Supplemental Table S1.

RNA-Seq and Data Analysis

RNA-seq analysis was performed using 6-d-old wild-type, pIF4-c, and MED25-AS-3 seedlings grown under continuous WL or SH in a growth chamber. Three biological replicates, each containing 18 independent samples, were used for RNA extraction. Total RNA was extracted from each sample using the RNeasy Plant Mini Kit (Qiagen) and treated with DNase I. The quality of total RNA was assessed using a NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer. Then, 3 μg total RNA was used to construct the Illumina sequencing libraries according to the manufacturer’s instructions. The libraries were sequenced on the Illumina HiSeq 2500 platform (Berry Genomics) to generate 6 Gb of high-quality 150-bp paired-end reads.

RNA-seq reads were aligned to the tomato genome ITAG3.2 (https://solgenomics.net). Gene expression levels in all biological replicates were calculated using Salmon. Two-way ANOVA was performed to determine whether genotype or treatment had a significant effect on the expression level of a certain gene (FDR-adjusted P < 0.05). Genes differentially expressed between samples were identified using the DESeq2 package (Love et al., 2014), with standard parameters (FDR-adjusted P < 0.05). GO enrichment analysis was performed using the AGRIgo classification system (http://systemsbiology.cau.edu.cn/ agrigoV2/index.php) with default parameters. GO term enrichment was shown by the most specific subclass in the enrichment analysis.

Transient Expression Assays

To conduct transient transcriptional activity assays, the YUC9 promoter (1,987 bp) was amplified from the genomic DNA of wild-type tomato plants and cloned into the pGreenII 0800-LUC vector (Hellens et al., 2005) to generate the reporter construct. The pGreen II 0800-LUC vector containing the Renilla luciferase (REN) gene under the control of the CaMV 35S promoter was used as an internal control. PIF4-GFP and MED25-MYC were used as effector constructs. Primers used for generating these constructs are listed in Supplemental Table S1. The constructs were transformed into A. tumefaciens cells. The transformed cells were incubated, harvested, and resuspended in infiltration buffer (10 mM MES, 0.2 mM acetoxyxymethyl, and 10 mM MgCl2) to obtain an OD600 value of 0.5. Equal volumes of transformed A. tumefaciens cells were mixed in different combinations and co-infiltrated into Nicotiana benthamiana leaves using a needleless syringe. Firefly LUC and REN activities were measured using the Dual-LUC Reporter Assay System (Promega) according to the manufacturer’s instructions, and LUC:REN ratios were calculated. Three independent biological replicates were performed for each agroinfiltration.

RT-qPCR Assay

cDNA was prepared from 2 μg of total RNA with SuperScript III reverse transcriptase (Invitrogen) and quantified on a Roche 480 cycler with the SYBR Green kit (Takara). The expression levels of target genes were normalized against ACTIN2. Each qPCR analysis was repeated three times for technical replicates, and the mean value was recorded for each biological replicate. Data from three independent biological replicates were collected, and error bars represent the sd from three biological replicates. Statistical significance was evaluated with Tukey posthoc test or Student’s t test. Primers are listed in Supplemental Table S1.

Accession Numbers

Sequence data from this article can be found in the Sol Genomics Network initiative under the following accession numbers: Solyc12g070100 (MED25), Solyc07g043580 (PIF4), Solyc05g115540 (PIF7a), Solyc06g069600 (PIF7b), Solyc03g120380 (YUC19), Solyc09g064160 (YUC8), Solyc06g083700 (YUC9), and Solyc11g005330 (ACTIN2).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Phylogenetic relationship of PIF proteins from tomato and Arabidopsis.
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