TCP Transcription Factors Associate with PHYTOCHROME INTERACTING FACTOR 4 and CRYPTOCHROME 1 to Regulate Thermomorphogenesis in Arabidopsis thaliana

Yu Zhou, Qingqing Xun, Dongzhi Zhang, Minghui Lv, Yang Ou, Jia Li

ljia@lzu.edu.cn

HIGHLIGHTS
TCP transcription factors promote PIF4-mediated thermoresponsive hypocotyl growth
TCP17 interacts with PIF4 to promote the transcriptional activity of PIF4
CRY1 interacts with TCP17 and represses the binding affinity of TCP17 with PIF4
Higher temperature releases TCP17 from the repression of CRY1

Zhou et al., iScience 15, 600–610
May 31, 2019 © 2019 The Authors.
https://doi.org/10.1016/j.isci.2019.04.002
TCP Transcription Factors Associate with PHYTOCHROME INTERACTING FACTOR 4 and CRYPTOCHROME 1 to Regulate Thermomorphogenesis in Arabidopsis thaliana

Yu Zhou, Qingqing Xun, Dongzhi Zhang, Minghui Lv, Yang Ou, and Jia Li

SUMMARY

Temperature, one of the most critical environmental cues, greatly affects plant growth, development, and reproduction. PHYTOCHROME-INTERACTING FACTOR 4 (PIF4), a key transcription factor in light signaling pathway, plays a central role in temperature-mediated growth responses. How higher temperature regulates the function of PIF4, however, is not well understood. Here we demonstrate that three phylogenetically related TEOSINTE BRANCHED 1/CYCLOIDEA/PCF (TCP) transcription factors, TCP5, TCP13, and TCP17, play fundamental roles in promoting thermoresponsive hypocotyl growth by positively regulating the activity of PIF4. TCP17 was found to interact with a blue light receptor, CRYPTOCHROME 1 (CRY1), at lower temperature, leading to reduced activity of TCP17. Higher temperature can increase the stability of TCP17, and release TCP17 from the CRY1-TCP17 complex, allowing it to upregulate the expression of PIF4 and enhance the transcriptional activity of PIF4. This study revealed the important roles of TCPs in regulating the activity of PIF4 in thermomorphogenesis.

INTRODUCTION

Plants are sessile in nature, growth and development of which have to coordinate with their ever-changing living environments for better survival and reproduction (Lau and Deng, 2010; McClung et al., 2016). In addition to light, water, and nutrients, ambient temperature is another key environmental factor regulating multiple physiological processes in the life cycle of a plant (McClung et al., 2016; Vert and Chory, 2011; Wigge, 2013). Elevated temperature can cause a series of morphological changes of a plant, including elongated hypocotyls, early flowering, and reduced reproduction (McClung et al., 2016; Vert et al., 2012; Wigge, 2013).

PHYTOCHROME-INTERACTING FACTOR 4 (PIF4), a key regulator in light signal transduction (Castillon et al., 2007; Huq and Quail, 2002; Leivar and Monte, 2014; Leivar and Quail, 2011), also acts as a central hub in a thermoresponsive pathway (Koini et al., 2009; Wigge, 2013). PIF4 integrates with several endogenous growth-regulating phytohormones, including auxin, gibberellins, and brassinosteroids, to mediate the expression of a series of high-temperature responsive genes (Franklin et al., 2011; Koini et al., 2009; Oh et al., 2012; Stavang et al., 2009; Sun et al., 2012). Owing to the critical roles of PIF4 in connecting environmental signals to endogenous responses, its function is tightly regulated (Leivar and Monte, 2014; Leivar and Quail, 2011).

Recently, a red/far-red light photoreceptor PHYTOCHROME B (PHYB) was proposed as a temperature sensor in Arabidopsis (Jung et al., 2016; Legris et al., 2016). Elevated ambient temperature signal can be perceived by PHYB, turning PHYB from its bioactive form (Pfr) to an inactive form (Pr) (Jung et al., 2016; Legris et al., 2016). Pfr physically associates with PIF4 and blocks its transcription activity. However, Pr cannot interact with PIF4, allowing PIF4 to upregulate the expression of thermoresponsive genes, promoting hypocotyl growth at high ambient temperatures (Jung et al., 2016; Legris et al., 2016). In addition to PHYB, CRYPTOCHROME 1 (CRY1), a photolyase-like blue light receptor originally isolated from Arabidopsis (Briggs and Huala, 1999; Lin, 2002; Sancar, 2003; Sancar et al., 2000), which inhibits hypocotyl elongation in blue light by forming a complex with SUPPRESSOR OF PHYA-105 (SPA1) and CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) (Deng et al., 1991; Lian et al., 2011; Liu et al., 2011), was reported to regulate thermoresponsive hypocotyl growth by inhibiting the transcriptional level
of PIF4 and interacting with PIF4 in blue light to suppress the activity of PIF4, especially at an elevated temperature (Ma et al., 2016).

Despite the critical role of photoreceptors in temperature sensing, multiple components in photomorphogenesis and circadian rhythm were also found to regulate the activity of PIF4 in thermomorphogenesis. DETECT1/COP1 and HY5 were demonstrated to regulate high-temperature-induced growth by promoting PIF4 transcript abundance through ELONGATED HYPOCOTYL 5 (HY5) (Delker et al., 2014). Besides the DET1/COP1-HY5 cascade in regulating the expression of PIF4, there are also distinct mechanisms between DET1/COP1 and HY5 in regulating hypocotyl growth at high temperatures. DET1/COP1 complex is necessary for upregulating PIF4 expression and stability of PIF4 (Gangappa and Kumar, 2017), whereas HY5 competes with PIF4 for G-box motifs in the promoters of its target genes (Gangappa and Kumar, 2017; Toledo-Ortiz et al., 2014). EARLY FLOWERING 3 (ELF3), an important component of evening complex of circadian clock, was found to suppress the transcription levels of PIF4 and PIF5 (Nusinow et al., 2011). ELF3 interacts with PIF4 and blocks the role of PIF4 in activating the expression of thermoresponsive genes (Box et al., 2015; Nieto et al., 2015). In a recent study, TOC1/PRR5, another key component in circadian clock, was revealed to interact with PIF4, inhibiting circadian gating of PIF4 in thermomorphogenesis (Zhu et al., 2016). FLOWERING TIME CONTROL PROTEIN, an RNA-binding protein, acts as another important factor in regulating temperature-mediated flowering and hypocotyl growth by suppressing the activity of PIF4 (Blazquez et al., 2003; Lee et al., 2014; Macknight et al., 1997).

Given the fact that many factors have been proposed to interact with PIF4 and inhibit its activity, regulatory components positively regulating the activity of PIF4 in thermoresponses, however, are poorly understood. In this study, we demonstrated that TCP transcription factors positively regulate thermoresponsive hypocotyl elongation by increasing PIF4 expression and the transcriptional activity of PIF4. High temperature increases the transcriptional activity of TCP17 toward PIF4 and the interaction between TCP17 and PIF4 by relieving the repression of TCP17 from CRY1. Our studies reveal a novel molecular mechanism of TCPs in integrating the functions of CRY1 and PIF4 to regulate hypocotyl growth at high ambient temperatures.

RESULTS
TCPs Positively Regulate Thermomorphogenesis

High-temperature-induced morphological changes are reminiscent of what is seen in a shade condition, suggesting a possibly common molecular mechanism between these two signaling pathways (Legris et al., 2017; Quint et al., 2016). Our previous studies revealed that three phylogenetically related TCP transcription factors, TCP5, TCP13, and TCP17, play a crucial role in promoting hypocotyl elongation in shade (Zhou et al., 2018). To investigate whether these TCPs are also required for thermoresponsive hypocotyl growth, we analyzed the hypocotyl responses of loss- or gain-of-function mutants of these three TCPs to elevated temperature. We found that the hypocotyl growth responses of tcp5, tcp13, or tcp17 single mutant to higher temperature are similar to those of Col-0 (Figures S1A and S1B). However, a tcp5 tcp17 double mutant showed a significantly impaired hypocotyl response to elevated temperature (Figures S1A and S1B) and the thermoresponsive defect of the tcp5 tcp13 tcp17 triple mutant, 3tcp, is more significant than that of the tcp5 tcp17 double mutant (Figures 1A, 1B, S1A, and S1B). In contrast, transgenic seedlings overexpressing TCP5, TCP13, or TCP17 showed greatly elongated hypocotyls even at 22°C, the optimal Arabidopsis growth temperature in a laboratory condition, indicating constitutive thermomorphogenesis (Figures S1A and S1B). These results suggested a redundant role of TCP5, TCP13, and TCP17 in promoting thermomorphogenesis.

To understand whether the expressions of TCPs are regulated by temperature, we investigated the transcriptional responses of TCP5, TCP13, and TCP17 to a higher-temperature treatment in wild-type seedlings. Seven-day-old Col-0 seedlings grown at 22°C and a long-day (LD, 16-h light/8-h dark) condition were transferred to 28°C or kept at 22°C for 4 h before collected for RNA extraction. We examined the mRNA levels of TCP5, TCP13, and TCP17 by a real-time PCR assay and observed that the transcriptional levels of these TCPs were slightly increased after a higher temperature treatment compared with those under 22°C (Figure 1C). Our previous studies demonstrated that TCP17 is an unstable protein in light, and its stability can be dramatically increased by shade treatment (Zhou et al., 2018). We therefore tested whether higher temperature can also affect the protein stability of TCP17. Seedlings of a representative homozygous proTCP17::TCP17-GFP transgenic line were grown under LD at 22°C condition for 7 days, and half
of them were transferred to and kept at 28°C (zeitgeber time 12) to ZT-24 (dark) before being collected for protein extraction. Our immunoblotting results showed that TCP17-GFP was greatly accumulated in the dark, and that higher temperature has no obvious effect on the additional accumulation of TCP17 (Figure 1D). However, higher temperature can significantly elevate the protein level of TCP17 in the light (Figure 1C). To exclude the impact of the transcription of TCP17, protein analyses were conducted by using transgenic plants from a representative transgenic line constitutively expressing TCP17-FLAG (35S::TCP17-FLAG). Our immunoblotting results showed that the TCP17-FLAG level was greatly increased after transferring 22°C-grown 35S::TCP17-FLAG seedlings to 28°C for additional 3 or 6 h (Figure 1E). In contrast, the accumulation of TCP17-FLAG was significantly decreased after transferring 28°C-pretreated seedlings to 22°C for indicated time periods (Figure S1C).

We also found that the degradation of TCP17 at 22°C was significantly suppressed by the treatment of MG132 (Figure S1D), suggesting the contribution of a 26S proteasome pathway to the instability of TCP17 at 22°C. Our results indicated that higher temperature increases the protein stability of TCP17, allowing it to be accumulated in the nucleus and promote thermomorphogenesis.

PIF4 Is Essential for TCP17 to Promote Hypocotyl Growth at Higher Temperature

Previous studies demonstrated that PIF4 acts as a key factor in regulating thermoresponsive hypocotyl growth (Wigge, 2013). To reveal whether TCP17 promotes thermoresponsive hypocotyl growth by regulating the function of PIF4, genetic and biochemical analyses were carried out to investigate the interaction between PIF4 and TCPs. We generated pif4/35S::TCP17-FLAG (pif4/TCP17-OX) plants by crossing pif4 with the representative transgenic line of 35S::TCP17-FLAG (TCP17-OX). The obtained pif4/TCP17-OX seedlings displayed significantly reduced hypocotyl elongation at 22°C compared with the TCP17-OX transgenic seedlings (Figures 2A and 2B). In addition, the thermoresponse of pif4/TCP17-OX seedlings was
greatly impaired, showing a response similar to that of the pif4 mutant (Figures 2A and 2B). Consistently, the results of real-time RT-PCR analyses showed that the expression levels of several known PIF4 target genes, YUC8, IAA19, and IAA29 (Ma et al., 2016), were significantly increased in TCP17-OX transgenic seedlings, whereas they were decreased in 3tcp (Figures S2A–S2C). Furthermore, the responses of YUC8, IAA19, and IAA29 to elevated temperature were greatly impaired in 3tcp (Figures S2A–S2C). As a central regulator of thermomorphogenesis, PIF4 promotes hypocotyl elongation at higher temperature by increasing the expression of YUC8 and TAA1 (Franklin et al., 2011; Sun et al., 2012), whose encoded proteins are key enzymes catalyzing free indole-3-acetic acid biosynthesis (Tao et al., 2008; Zhao, 2010; Zhao et al., 2001). Consistently, pif4 showed diminished higher-temperature-induced auxin accumulation and hypocotyl growth (Franklin et al., 2011; Koini et al., 2009; Sun et al., 2012). Our hypocotyl analyses showed that exogenous treatment of picloram, an analog of auxin, can significantly rescue hypocotyl growth of 3tcp (Figures S2D and S2E), indicating that the PIF4-auxin cascade is required for TCP17-promoted thermoresponsive hypocotyl elongation. These results demonstrated that TCP17-induced thermoresponsive hypocotyl growth is largely dependent on the function of PIF4.

TCPs Regulate Thermomorphogenesis by Promoting PIF4 Expression and the Transcriptional Activity of PIF4

Our previous studies demonstrated that TCP17 can directly bind to the promoter of PIF4 to elevate its expression during shade avoidance (Zhou et al., 2018). To determine whether TCPs can promote the
expression of PIF4 in response to high temperature, we analyzed the mRNA levels of PIF4 in Col-0, 3tcp, and TCP17-OX seedlings after treatment with higher temperature for different time periods. Our real-time RT-PCR analyses showed that at 28°C, the expression of PIF4 was significantly increased in TCP17-OX transgenic plants and decreased in 3tcp, and the response of PIF4 expression to high temperature was greatly impaired in 3tcp (Figure 2C). These results indicated that high-temperature-mediated upregulation of PIF4 is partially via TCP transcription factors. We also investigated whether PIF4 regulates the expression of TCPs in response to elevated temperature. The results from real-time RT-PCR showed that, compared with wild-type, the expression levels of TCP5 and TCP17 from pif4 were significantly reduced at 28°C (Figure S2F). In addition, the expression of these three TCPs were greatly increased in PIF4-OX (35S::PIF4-FLAG) transgenic plants (Figure S2F). These results indicated that the expression of TCPs can also be regulated by PIF4 in thermoresponses.

As a key factor mediating ambient temperature response, PIF4 can be regulated at multiple levels (Quint et al., 2016; Wigge, 2013). In addition to increasing the expression of PIF4, high temperature also can impact the transcriptional activity of PIF4 (Quint et al., 2016; Wigge, 2013). To examine whether TCPs are involved in promoting the transcriptional activity of PIF4, we analyzed the hypocotyl responses of Col-0, 3tcp, PIF4-OX, and 3tcp/PIF4-OX to higher-temperature treatment. PIF4-OX transgenic plants showed extremely elongated hypocotyls compared with Col-0, whereas the hypocotyls of 3tcp/PIF4-OX seedlings were much shorter than those of PIF4-OX seedlings (Figure 2D). Consistently, our real-time PCR analyses showed that the expression levels of PIF4 target genes, YUC8, IAA19, and IAA29, were dramatically elevated in PIF4-OX plants compared with Col-0 plants (Figures 2E–2G). The expression of these three genes in 3tcp/PIF4-OX plants, however, was significantly impaired, especially at higher temperature, compared with that in PIF4-OX plants (Figures 2E–2G). Our genetic and molecular data indicated that TCPs are required for PIF4-promoted thermoresponses.

High Temperature Enhances the Interaction between TCP17 and PIF4 to Increase the Transcriptional Activity of PIF4

To determine whether TCPs promote the transcriptional activity of PIF4 via direct interaction with PIF4, we investigated the physical interaction between TCPs and PIF4 in vitro and in vivo. We detected the interaction between TCP17 and PIF4 in planta by a bimolecular fluorescence complementation (BIFC) assay. As shown in Figure 3A, strong fluorescence was observed in the nuclei of Nicotiana benthamiana leaf cells co-infiltrated with Agrobacterium harboring TCP17-cYFP and PIF4-nYFP plasmids. Interaction between TCP5 and PIF4 was also observed (Figure 3A). We further verified the interaction between TCP17 and PIF4 using a yeast two-hybrid system. Because of high auto-activation of PIF4, the activation domain (AD) at the N terminus of PIF4 was deleted (PIF4-dAD) before being cloned into a bait vector (Figure 3B). Our results indicated that TCP17 physically interacts with PIF4 in yeast (Figure 3C). The in vivo interaction between PIF4 and TCP17 was confirmed by a co-immunoprecipitation (colP) assay. We found that PIF4 was co-immunoprecipitated with TCP17 from plants at 22°C (Figure 3D). Such colP was significantly increased upon higher-temperature treatment (Figure 3D).

Consistent with the results that higher temperature can promote the direct interaction between TCP17 and PIF4, our chromatin immunoprecipitation (ChIP) followed by real-time RT-PCR using the aforementioned 35S::TCP17-FLAG (TCP17-OX) transgenic plants showed that TCP17 can associate with the G-box-motif-containing regions in the promoters of YUC8, and IAA19 that PIF4 binds to (Figures 3E and 3F). The association between TCP17 and the promoter regions of YUC8 and IAA19 was greatly enhanced by higher temperature (Figure 3F). To further investigate whether TCP17 affects the transcription activity of PIF4, a transient transcription assay was carried out to analyze the effects of TCPs on PIF4 transcription activities, by using a firefly luciferase (LUC) gene driven by the promoter of YUC8 (pYUC8::LUC) as a reporter system. Co-infiltration analysis in N. benthamiana leaves indicated that co-expression of PIF4 and TCP5, or PIF4 and TCP17, can drastically increase the expression of LUC, when compared with the one only expressing PIF4 (Figure 3G). These results proved that TCP17 forms a complex with PIF4 in vitro and in vivo, and the interaction was significantly increased by higher temperature, leading to significantly enhanced transcription activity of PIF4.

TCPS Are Involved in CRY1-Mediated Thermomorphogenesis

Previous studies demonstrated that a blue light receptor CRY1 can interact with PIF4 in a blue-light-dependent manner to repress the transcription activity of PIF4 and growth responses to elevated temperature...
However, mechanisms by which CRY1 regulates the activity of PIF4 in response to temperature changes are not well understood. In addition, transcription factors interacting with CRY1 to regulate the expression of PIF4 in thermoresponse remain elusive.

To reveal whether TCPs are required for CRY1-mediated thermoresponsive hypocotyl growth, we examined the genetic interaction between TCPs and CRY1. The hypocotyl responses of Col-0, 3tcp, cry1, 3tcp cry1, 35S::CRY1-HA, 35S::TCP17-GFP, and 35S::CRY1-HA/35S::TCP17-GFP to elevated temperature were analyzed. Consistent with the results from a previous study (Ma et al., 2016), cry1 showed dramatically elongated hypocotyls at both 22°C and 28°C; the hypocotyl length of 3tcp cry1, however, was greatly reduced compared with cry1 (Figures 4A and 4B). In addition, the hypocotyls of 35S::CRY1-HA transgenic seedlings showed a greatly reduced response to higher temperature, and TCP17-induced hypocotyl elongation at high temperature was significantly impaired in the 35S::CRY1-HA background (Figures 4A and 4B). Consistently, our real-time RT-PCR analyses showed that the expressions of PIF4-targeted genes, YUC8, IAA19, and IAA29 from cry1 are much higher than that from Col-0, whereas the transcriptional levels of these genes are greatly impaired in the 3tcp cry1 quadruple mutant compared with cry1 (Figures 4C–4E). Our genetic and molecular data strongly demonstrated that CRY1 inhibits thermomorphogenesis partially via repressing the functions of TCPs.
CRY1 Physically Interacts with TCP17 in a Temperature-Dependent Manner

To determine whether these TCPs are involved in CRY1-regulated thermomorphogenesis by directly interacting with CRY1, we investigated the physical interaction between CRY1 and TCPs in vitro and in vivo. In a BIFC assay, strong fluorescence was observed in the nucleus of *N. benthamiana* leaf cells after co-infiltration with *Agrobacterium* mixtures harboring CRY1-cYFP and TCP17-nYFP or CRY1-cYFP and TCP5-nYFP plasmids (Figure 5A). Also, we tested the interaction between TCP17 and CRY1 in a yeast two-hybrid system. *Arabidopsis* CRY1 is a photolyase-like blue light receptor (Briggs and Huala, 1999; Lin, 2002). CRY1 contains two functional domains, an N-terminal photolyase-related (PHR) domain for chromophore binding and a C-terminal extension (CCE) domain for protein-protein interactions (Figure 5B) (Yu et al., 2010). Because of strong autoactivation of full-length CRY1 protein, we tested the interaction between TCP17 and PHR or CCE domain and found that TCP17 interacts strongly with PHR, but not with CCE domain (Figure 5C). Consistently, *E. coli*-purified TCP17 and CRY1 also showed interaction in an in vitro pull-down assay (Figure 5D). More remarkably, the interaction between CRY1 and TCP17 in *Arabidopsis* showed temperature dependence. CRY1 was co-immunoprecipitated with TCP17 from plants grown at 22°C, but the interaction was greatly reduced at 28°C (Figure 5E).

**CRY1 Inhibits the Activity of TCP17 in Promoting PIF4 Expression and the Interaction between TCP17 and PIF4**

The temperature-dependent interaction between TCP17 and CRY1 suggests a fundamental role of CRY1 in regulating the function of TCP17 in thermomorphogenesis. Our results showed that the stability of TCP17 protein is regulated by temperature. To investigate whether the degradation of TCP17 at low temperature is mediated by CRY1, we tested the response of TCP17 protein in cry1 or 3SS::CRY1-HA background to different temperatures. Our immunoblotting analysis showed that the level of TCP17 protein from 3SS::CRY-HA or cry1 in response to temperature changes was not significantly altered compared with that from Col-0 (Figure S3A). This result suggested that the degradation of TCP17 at lower temperature is not caused by the interaction between CRY1 and TCP17. The detailed mechanism by which temperature regulates the stability of TCP17 needs to be clarified in the future.
Previous studies demonstrated that a CRY1 loss-of-function mutant can greatly elevate the expression of PIF4 in response to high temperature (Ma et al., 2016). To reveal whether CRY1 is involved in TCP-mediated regulation of PIF4 expression, we examined the responses of PIF4 from cry1 and 3tcp cry1 to elevated...
temperature. Our data showed that the expression of PIF4 from cry1 was much higher than that from Col-0, whereas the PIF4 expression from 3tcp cry1 in response to high temperature was significantly reduced compared with that from cry1 (Figure S3B). Our further transient transcription assay in N. benthamiana by using a pPIF4::LUC reporter system showed that CRY1 can significantly reduce the transcriptional activity of TCPs toward PIF4 (Figure S3C). These data indicated that CRY1 negatively regulates PIF4 expression partially by repressing the activity of TCPs.

Considering the results that high temperature releases TCP17 from the TCP17-CRY1 complex (Figure 5E), and increases the interaction between TCP17 and PIF4 (Figure 3D), we hypothesized that CRY1 forms a complex with TCP17 to suppress the interaction between TCP17 and PIF4. To investigate whether CRY1 affects the interaction between TCP17 and PIF4, we transiently expressed TCP17-nYFP and PIF4-cYFP with or without CRY1 in N. benthamiana leaves. Consistently, strong fluorescence was observed in the cells co-expressing TCP17-nYFP and PIF4-cYFP. When CRY1 was co-expressed with TCP17-nYFP and PIF4-cYFP, the fluorescence signals were significantly reduced and faded (Figure 5F). Consistently, the results from a ChIP experiment followed by PCR showed that under 22°C, loss of function of CRY1 (cry1) can significantly increase the binding affinity of TCP17 to the G-boxes in the promoters of PIF4 target genes, similar to that from Col-0 grown under 28°C (Figure S4), indicating enhanced binding activity of TCP17 to PIF4. In summary, our data demonstrated that CRY1 represses the interaction between TCP17 and PIF4, leading to lower PIF4 activity in regulating the expression of its target genes.

**DISCUSSION**

In this study, we illustrate a molecular framework that TCP transcription factors act as positive regulators in thermomorphogenesis by promoting the function of PIF4 at both transcriptional and post-transcriptional levels (Figure 5G). The regulation of TCP17 by temperature is at multiple different levels. TCP17 protein shows a very low abundance at 22°C. Elevated ambient temperature can increase the stability of TCP17, resulting in the accumulation of TCP17. The activity of TCP17 is also regulated by temperature. At a lower temperature, CRY1 physically interacts with TCP17 and inhibits not only the transcriptional activity of TCP17 but also the interaction between TCP17 and PIF4, leading to greatly reduced mRNA abundance and transcriptional activity of PIF4. The elevated ambient temperature can suppress the interaction between TCP17 and CRY1. Subsequently, TCP17 promotes the expression of PIF4, and the interaction between PIF4 and TCP17 is enhanced, leading to increased transcription activity of PIF4 toward its downstream thermo-responsive genes. Our results not only demonstrated novel roles of TCPs in regulating thermomorphogenesis but also proved that CRY1 can negatively regulate thermoresponse not only by directly inhibiting the transcription activity of PIF4 but also by repressing the activities of its positive regulators, like TCPs. These results contribute to our better understanding of the regulatory mechanisms of higher temperature on plant growth and development. The knowledge can be used for future crop improvements via molecular breeding or genetic engineering for higher productivity under a wide range of temperatures.

**Limitations of the Study**

In this study, we demonstrated the important role of TCP transcription factors in regulating thermo-responsive hypocotyl growth. The stability of TCP17 protein is greatly increased at high temperature, the detailed mechanism of which is still unknown. In addition, as a temperature sensor, PHYB regulates thermomorphogenesis by repressing the activity of PIF4. Whether TCPs are involved in PHYB-mediated thermoresponses will be a very interesting research direction.

**METHODS**

All methods can be found in the accompanying Transparent Methods supplemental file.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.04.002.

**ACKNOWLEDGMENTS**

We are grateful to Dr. Yuval Eshed from Weizmann Institute of Science, Israel, for kindly providing the 3tcp mutant and to Dr. Hongquan Yang from Fudan University, China, for kindly sharing the CRY1 antibody. This study was supported by the National Natural Science Foundation of China (grant numbers 90917019, 31470380, and 31530005 to J. L.).
AUTHOR CONTRIBUTIONS

Conceptualization, Y.Z. and J.L.; Investigation, Y.Z., Q.X., D.Z., Y.O., and M.L.; Formal Analysis, Y.Z.; Writing – Original Draft, Y.Z. and J.L.; Writing – Review & Editing, Y.Z., and J.L.; Supervision, J.L.; Funding Acquisition, J.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Blazquez, M.A., Ahn, J.H., and Weigel, D. (2003). A thermosensory pathway controlling flowering time in Arabidopsis thaliana. Nat. Genet. 33, 168–171.

Box, M.S., Huang, B.E., Domijan, M., Jaeger, K.E., Khattak, A.K., Yoo, S.J., Sedivy, E.L., Jones, D.M., Hearn, T.J., Webb, A.A.R., et al. (2015). ELF3 controls thermoresponsive growth in Arabidopsis. Curr. Biol. 25, 194–199.

Briggs, W.R., and Huala, E. (1999). Blue-light photoreceptors in higher plants. Annu. Rev. Cell Dev. Biol. 15, 33–62.

Castillon, A., Shen, H., and Huq, E. (2007). Phytochrome Interacting Factors: central players in phytochrome-mediated light signaling networks. Trends Plant Sci. 12, 514–521.

Delker, C., Sonntag, L., James, G.V., Janitzka, P., Ibanez, C., Ziemann, H., Peterson, T., Denk, K., Mull, S., Ziegler, J., et al. (2014). The DET1-COP1-HYS pathway constitutes a multipurpose signaling module regulating plant photomorphogenesis and thermomorphogenesis. Cell Rep. 9, 1983–1989.

Deng, X.W., Caspar, T., and Quail, P.H. (1991). COP1, a regulatory locus involved in light-controlled development and gene expression in Arabidopsis. Genes Dev. 5, 1172–1182.

Franklin, K.A., Lee, S.H., Patel, D., Kumar, S.V., Sarpaz, A.K., Gu, C., Ye, S., Yu, P., Breen, G., Cohen, J.D., et al. (2011). Phytochrome-interacting factor 4 (PIF4) regulates auxin biosynthesis at high temperature. Proc. Natl. Acad. Sci. U S A 108, 20231–20235.

Gangappa, S.N., and Kumar, S.V. (2017). DET1 and HYS control PIF4-mediated thermosensory elongation growth through distinct mechanisms. Cell Rep. 18, 346–351.

Huq, E., and Quail, P.H. (2002). PIF4, a phytochrome-interacting bHLH factor, functions as a negative regulator of phytochrome B signaling in Arabidopsis. EMBO J. 21, 2441–2450.

Jung, J.H., Domijan, M., Klose, C., Biswas, S., Ezer, D., Gao, M., Khattak, A.K., Box, M.S., Charoenwatan, V., Cortijo, S., et al. (2016). Phytochromes function as thermosensors in Arabidopsis. Science 354, 886–889.

Koiti, M.A., Alvey, L., Allen, T., Tilley, C.A., Harberd, N.P., Whitelam, G.C., and Franklin, K.A. (2009). High temperature-mediated adaptations in plant architecture require the bHLH transcription factor PIF4. Curr. Biol. 19, 408–413.

Lau, O.S., and Deng, X.W. (2010). Plant hormone signaling lightens up: integrators of light and hormones. Curr. Opin. Plant Biol. 13, 571–577.

Lee, H.J., Jung, J.H., Cortes Llorca, L., Kim, S.G., Lee, S., Baldwin, J.T., and Park, C.M. (2014). FCA mediates thermal adaptation of stem growth by attenuating hypocotyl growth in Arabidopsis. Nat. Commun. 5, 5473.

Legris, M., Klose, C., Burgie, E.S., Rojas, C.C., Neme, M., Hildbrunner, A., Wigge, P.A., Schäfer, E., Vierstra, R.D., and Casal, J.J. (2016). Phytochrome B integrates light and temperature signals in Arabidopsis. Science 354, 897–900.

Legris, M., Nieto, C., Sellaro, R., Prat, S., and Casal, J.J. (2017). Perception and signalling of light and temperature cues in plants. Plant J. 90, 683–697.

Leivar, P., and Monte, E. (2014). PIFs: systems integrators in plant development. Plant Cell 26, 56–78.

Leivar, P., and Quail, P.H. (2011). PIFs: pivotal components in a cellular signaling hub. Trends Plant Sci. 16, 19–28.

Lian, H.L., He, S.B., Zhang, Y.C., Zhu, D.M., Zhang, J.Y., Jia, K.P., Sun, S.X., Li, L., and Yang, H.Q. (2011). Blue-light-dependent interaction of cryptochrome 1 with SPA1 defines a dynamic signaling mechanism. Genes Dev. 25, 1023–1028.

Lin, C. (2002). Blue light receptors and signal transduction. Plant Cell 14 (Suppl.), S207–S225.

Liu, B., Zuo, Z., Liu, H., Liu, X., and Lin, C. (2011). Arabidopsis cryptochrome 1 interacts with SPA1 to suppress COP1 activity in response to blue light. Genes Dev. 25, 1029–1034.

Ma, D., Li, X., Guo, Y., Chu, J., Fang, S., Yan, C., Noel, J.P., and Liu, H. (2016). Cryptochrome 1 interacts with PIF4 to regulate high temperature-mediated hypocotyl elongation in response to blue light. Proc. Natl. Acad. Sci. U S A 113, 224–229.

Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C., et al. (1997). FCA, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding domains. Cell 89, 737–745.

McClung, C.R., Lou, P., Hermamd, V., and Kim, J.A. (2016). The importance of ambient temperature to growth and the induction of flowering. Front. Plant Sci. 7, 1266.

Nieto, C., Lopez-Salmeron, V., Daviere, J.M., and Prat, S. (2015). ELF3-PIF4 interaction regulates plant growth independently of the Evening Complex. Curr. Biol. 25, 187–193.

Nusinow, D.A., Helfer, A., Hamilton, E.E., King, J.J., Imai, M., Schultz, T.F., Farre, E.M., and Kay, S.A. (2011). The ELF4-ELF3-LUX complex links the circadian clock to diurnal control of hypocotyl growth. Nature 475, 398–402.

Oh, E., Zhu, J.Y., and Wang, Z.Y. (2012). Interaction between BZR1 and PIF4 integrates brassinosteroid and environmental responses. Nat. Cell Biol. 14, 802–809.

Quint, M., Delker, C., Franklin, K.A., Wigge, P.A., Halliday, K.J., and van Zanten, M. (2016). Molecular and genetic control of plant thermomorphogenesis. Nat. Plants 2, 15190.

Sancar, A. (2003). Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors. Chem. Rev. 103, 2203–2237.

Sancar, A., Thompson, C., Thresher, R.J., Araujo, F., Mo, J., Oszu, S., Vagas, E., Dawut, L., and Selby, C.P. (2000). Photolyase/cryptochrome family blue-light photoreceptors use light energy to repair DNA or set the circadian clock. Cold Spring Harb. Symp. Quant. Biol. 65, 157–171.

Stavang, J.A., Gallego-Bartolome, J., Gomez, M.D., Yoshida, S., Asami, T., Olsen, J.E., Garcia-Martinez, J.L., Alabadi, D., and Blazquez, M.A. (2009). Hormonal regulation of temperature-induced growth in arabidopsis. Plant J. 60, 589–601.
Sun, J., Qi, L., Li, Y., Chu, J., and Li, C. (2012). PIF4-mediated activation of YUCCA8 expression integrates temperature into the auxin pathway in regulating Arabidopsis hypocotyl growth. PLoS Genet. 8, e1002594.

Tao, Y., Ferrer, J.L., Ljung, K., Pojer, F., Hong, F., Long, J.A., Li, L., Moreno, J.E., Bowman, M.E., Ivans, L.J., et al. (2008). Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. Cell 133, 164–176.

Toledo-Ortiz, G., Johansson, H., Lee, K.P., Bou-Torrent, J., Stewart, K., Steel, G., Rodriguez-Concepcion, M., and Halliday, K.J. (2014). The HY5-PIF regulatory module coordinates light and temperature control of photosynthetic gene transcription. PLoS Genet. 10, e1004416.

Vert, G., and Chory, J. (2011). Crosstalk in cellular signaling: background noise or the real thing? Dev. Cell 21, 985–991.

Wigge, P.A. (2013). Ambient temperature signalling in plants. Curr. Opin. Plant Biol. 16, 661–666.

Yu, X., Liu, H., Klejnot, J., and Lin, C. (2010). The cryptochrome blue light receptors. Arabidopsis Book 8, e0135.

Zhao, Y. (2010). Auxin biosynthesis and its role in plant development. Annu. Rev. Plant Biol. 61, 49–64.

Zhao, Y., Christensen, S.K., Fankhauser, C., Cashman, J.R., Cohen, J.D., Weigel, D., and Chory, J. (2001). A role for flavin monooxygenase-like enzymes in auxin biosynthesis. Science 291, 306–309.

Zhou, Y., Zhang, D., An, J., Yin, H., Fang, S., Chu, J., Zhao, Y., and Li, J. (2018). TCP transcription factors regulate shade avoidance via directly mediating the expression of both PHYTOCHROME INTERACTING FACTORS and auxin biosynthetic genes. Plant Physiol. 176, 1850–1861.

Zhu, J.Y., Oh, E., Wang, T., and Wang, Z.Y. (2016). TOC1-PIF4 interaction mediates the circadian gating of thermoresponsive growth in Arabidopsis. Nat. Commun. 7, 13692.
Supplemental Information

TCP Transcription Factors Associate
with PHYTOCHROME INTERACTING FACTOR 4
and CRYPTOCHROME 1 to Regulate
Thermomorphogenesis in *Arabidopsis thaliana*

Yu Zhou, Qingqing Xun, Dongzhi Zhang, Minghui Lv, Yang Ou, and Jia Li
Figure S1. Related to Figure 1. The redundantly role of TCPs in regulating thermomorphogenesis positively. (A) Phenotypes of Col-0, tcp5, tcp13, tcp17, tcp5 tcp17, 3tcp, 35S::HA-TCP5 (TCP5-OX), 35S::TCP13-GFP (TCP13-OX), and 35S::TCP17-FLAG (TCP17-OX) grown at 22°C and 28°C conditions. Scan bars represent 1 cm. (B) Measurements of the hypocotyl length shown in (A). Data shown were average and SEM (n≥20). ns p>0.05, *p<0.05 and **p < 0.01; based on student’s t test, which was performed by comparing each mutant to Col-0 grown under the same temperature. (C) The protein level of TCP17 was greatly reduced at lower temperature. Seven-day-old 35S::TCP17-FLAG seedlings grown under LD, 22°C, were pretreated at 28°C for 24 hours and then transferred to 22°C or remained at 28°C for 3 or 6 hours. (D) The degradation of TCP17 protein at lower temperature was repressed by MG132. 28°C pre-treated 35S::TCP17-FLAG seedlings were transferred to 1/2 MS media with or without MG132 and incubated at 22°C for 6
hours, as control, 35S::TCP17-FLAG seedlings were treated at 28°C in 1/2 MS media without MG132 for 6 hours. In (C) and (D), immunoblot was carried out to detect the level of TCP17-FLAG and Tubulin by using an anti-FLAG or an anti-tubulin antibody, respectively.
**Figure S2.** Related to Figure 2. TCPs play important roles in regulating PIF4-mediated thermoreponses.

(A-C) The responses of PIF4 targeted genes from Col-0, TCP17-OX, or 3tcp to elevated temperature. Seven-day-old seedlings grown under 22°C, LD condition were transferred to 28°C or kept at 22°C for 2, 4, and 6 hours before being collected for RNA extraction and real time PCR analyses. Data shown are the average and SEM of three biological replicates. *p < 0.05 and **p < 0.01; based on the student’s t test, showing the comparison of TCP17-OX-28°C or 3tcp-28°C to Col-28°C. (D, E) PIC can rescue the short hypocotyl phenotype of 3tcp. Col-0 and 3tcp seedlings were grown under LD condition at 22°C with various concentration of an auxin analog, picloram (PIC), for 5 days and then transferred to 22°C (D) or 28°C (E) for additional 3 days before measurement was taken. Data shown were average and SEM (n≥20). ns p>0.05, *p< 0.05 and **p < 0.01; based on the student’s t test. (F) The expression of TCP5, TCP13, and TCP17 from Col-0, PIF4-OX, and pif4 in response to elevated temperature. Seedlings were grown under LD, 22°C for 7 days, and then were transferred to 28°C or remained at 22°C for 4 hours. Data shown are the average and SEM of three biological replicates. *p < 0.05 and **p < 0.01; based on the student’s t test.
Figure S3. Related to Figure 5. CRY1 inhibits the activity of TCP17 to repress the expression of PIF4. (A) The response of TCP17 protein to temperature changes are not altered by CRY1. Seven-day-old 35S::TCP17-FLAG, CRY1-OX/35S::TCP17-FLAG, cry1/35S::TCP17-FLAG seedlings grown under 22°C were pre-treated at 28°C for 1 day, and then transferred half of them to 22°C for 3 hours before being collected for protein extraction. Immunoblotting was taken out to detect the TCP17-FLAG protein level by using an anti-FLAG antibody. Tubulin probed by an anti-TUB antibody was used as an internal control. (B) The expression level of PIF4 from Col-0, 3tcp, cry1 and 3tcp cry1 in response to elevated temperature. Seedling were grown under LD, 22°C condition for 7 days, and then were transferred to 28°C or remained at 22°C for 4 hours before being collected for analyses. Data shown were average and SEM. *p<0.05 and **p < 0.01; based on student’s t test. (C) CRY1 inhibits the transcriptional activity of TCP5 and TCP17 in a transient assay using N. benthamiana leaves. pPIF4::LUC was co-expressed with 35S::CRY1-HA, 3S::TCP5-HA, 35S::TCP17-FLAG, 3S::TCP5-HA with 35S::CRY1-HA, or 35S::TCP17-FLAG with 35S::CRY1-HA, respectively. The luciferase activities were imaged 48 hours after co-infiltrated.
Figure S4. Related to Figure 5. CHIP assay on the promoters of *YUC8* and *IAA19*
from Col-0, *TCP17-OX (35S::TCP17-FLAG)*, and *cry1/TCP17-OX (cry1-35S::TCP17-FLAG)*.

Seedlings were grown under LD, 22°C for 10 days, and then half of them were
transferred to 28°C for 4 hours before collected for CHIP. P1, P2, and P3 are
described as in Figure 3E and 3F. Data shown are the average and SD. *p* < 0.05 and
**p** < 0.01. Student’s t tests were used for the statistical analyses.
Table S1. Related to Figure 1, Figure 2, Figure 3, and Figure 4. Primer sequences used in this study.

| Primers for Q-RT PCR analysis |  |
|-------------------------------|--|
| Actin2-F                      | TCAGATGCCAGAAGTGGTTGCTCC |
| Actin2-R                      | CCGTACAGATCCTCCTGATATCC |
| TCP17-Q-F                     | TCTGGTAACGTCACTGTGC |
| TCP17-Q-R                     | ACCACCACGGAAACGAAG |
| TCP5-Q-F                      | TCCTACTCTCCGGAATGA |
| TCP5-Q-R                      | AAGAGCTGAAGATGACCGGC |
| TCP13-Q-F                     | TTAGGGTTCACGCCTTTT |
| TCP13-Q-R                     | GCTGAATAGCCGGTGGGACT |
| PIF4-Q-F                      | TGCATCAACACGACCGTA |
| PIF4-Q-R                      | TGCTCACTCCGTGGT |
| YUC8-Q-F                      | TGATCGGTTGTTTACGAGGA |
| YUC8-Q-R                      | CTTGACGTTTCGTGGGTTT |
| IAA19-Q-F                     | GGTGACAACCTGCGATACGTTACCA |
| IAA19-Q-R                     | CCCGTCATCCGATCTTTT |
| IAA29-Q-F                     | TCCGTGGAACGCTATCCT |
| IAA29-Q-R                     | ACCGTGTCATATCAAGATGTTT |

| Primers for ChIP-qPCR analysis |  |
|--------------------------------|--|
| P1-F                           | atcggtgcctcaaatcca |
| P1-R                           | TTTCTACGGACCATTTT |
| P2-F                           | GATATCAAATGACTCCACGTGTC |
| P2-R                           | TCCGTGAAACGCTCTTCTTC |
| P3-F                           | GGTGCATAATGGTGTGTTGA |
| P3-R                           | GATGGGGTTTAGAGGACGTAGG |

| Primers used for cloning       |  |
|--------------------------------|--|
| TCP5-F                         | AAAAGCAGGCTTC ATGAGATCAG GAGAATGTA |
| TCP5-R                         | AGAAAGCTGGTGC TCAAGAATCTGATTACCT |
| TCP5-NR                        | AGAAAGCAGGTTCAATCTGATTACCT |
| TCP13-F                        | AAAAGCAGGCT TCAGATATCG TCTCTGGAA |
| TCP13-R                        | AGAAAGCAGGTTCA ATGAGATCAG ACTTTTCTC |

| Primers used for cloning       |  |
|--------------------------------|--|
| TCP5-F                         | AAAAGCAGGCTTC ATGAGATCAG GAGAATGTA |
| TCP5-R                         | AGAAAGCTGGTGC TCAAGAATCTGATTACCT |
| TCP5-NR                        | AGAAAGCAGGTTCAATCTGATTACCT |
| TCP13-F                        | AAAAGCAGGCTTC ATGAAATCG TCTCTTTG |
| TCP13-R                        | AGAAAGCAGGTTCA ATGAGATCAG ACTTTTCTC |
| Sequence ID   | Primer Sequence                  |
|--------------|----------------------------------|
| TCP13-NR     | AGAAAGCTGGGTCCATATGGTGATCCTTCCTC|
| TCP17-F      | AAAAAAGCAGGCTTC ATGGGAAT AAAAAGAAGA |
| TCP17-R      | AGAAAGCTGGGTCCCTACTCGATATGGTCTGGTTGT|
| TCP17-NR     | AGAAAGCTGGGTCCCTCGATATGGTCTGGTTGT|
| PIF4-F       | AAAAAAGCAGGCTTCATGGAACACC AAGGTTGGAG|
| PIF4-R       | AGAAAGCTGGGTCCCTAGTGGTCCAAACGAGAACCCTG|
| PIF4-NR      | AGAAAGCTGGGTCCCTAGTGGTCCAAACGAGAACCCTG|
| proTCP17-F   | AAAAAAGCAGGCTTC aatatggtccggtttgcagttg |
| proYUC8-F    | AAAAAAGCAGGCTTC ATCCGATATGATAACGAT |
| proYUC8-R    | AGAAAGCTGGGTGTTGAAGTTGTATTGGAAA |
| CRY1-F       | AAAAAAGCAGGCTTC ATGTCTGGGT CTGTATCTGG |
| CRY1-R       | AGAAAGCTGGGTGTTACCCGGTTTGTAAGAAGCCGTC |
| CRY1-NR      | AGAAAGCTGGGTGTTACCCGGTTTGTAAGAAGCCGTC |
| proPIF4-F    | AAAAAAGCAGGCTTCgatatggtccattacaagtaggcac |
| proPIF4-R    | AGAAAGCTGGGTGTCGATCTCTGGAGACATTTCAG |

77
78
Transparent methods

Plant materials and growth condition

All the plants used in this study are Columbia accession. tcp5 (CS116350), tcp13 (CS313854), tcp17 (SALK_148580), pif4 (SALK_140393), and cry1 (SALK_069292) were obtained from ABRC. tcp5 tcp17 double mutant was generated by crossing tcp5 with tcp17. 3tcp triple mutant was obtained from Yuval Eshed’s lab and was previously described (Efroni et al., 2008; Zhou et al., 2018). For constitutive overexpression, full length coding sequences of TCP5, TCP13, TCP17, PIF4, CRY1, and were cloned into pEarley Gate201 (35S::HA-TCP5 and 35S::HA-TCP13), pBIB-BASTA-35S::GWR-FLAG (35S::TCP17-FLAG and 35S::PIF4-FLAG), pBIB-HYG-35S::GWR-GFP (35S::PIF4-GFP, and 35S::TCP17-GFP), and pBIB-HYG-35S::GWR-HA (35S::CRY1-HA) using a Gateway cloning approach. For proTCP17::TCP17-GFP transgenic plants, promoter region (1500 bp upstream of ATG) and coding sequence of TCP17 amplified from genomic DNA was cloned into pBIB-BASTA-GWR-GFP by the Gateway cloning approach. pif4/35S::TCP17-FLAG was obtained by crossing pif4 with 35S::TCP17-FLAG. 3tcp/35S::PIF4-FLAG plants were generated by crossing 3tcp with 35S::PIF4-FLAG. 3tcp cry1 quadruple mutant was obtained by crossing 3tcp with cry1. CRY1-OX/TCP17-OX plants were generated by crossing 35S::TCP17-GFP with 35S::CRY1-HA. 35S::CRY1-HA/35S::TCP17-FLAG plants were generated by crossing 35S::CRY1-HA with 35S::TCP17-FLAG. 35S::PIF4-GFP/35S::TCP17-FLAG plants were generated by transforming 35S::PIF4-GFP into a 35S::TCP17-FLAG background. All the plants were grown in a greenhouse set at 22°C and a long day condition (LD, 16h light/8h dark) for general growth and seed harvesting.

Hypocotyl measurements

Surface-sterilized seeds were planted on 1/2 Murashige and Skoog (MS) medium containing 1 % sucrose and 0.8 % agar. For PIC treatment, various concentrations of PIC were mixed in the 1/2 MS medium. After 2 day vernalization, the plates were
placed under LD, 22°C condition for 5 days and then transferred to LD, 28°C or remained at LD, 22°C for additional 3 days before being harvested for analyses. Hypocotyls of seedlings were scanned and Image J software was used to quantify hypocotyl lengths. At least 20 seedlings were measured for each independent experiment. At least three biological replicates were carried out for each quantitative analysis.

**RNA extraction and real time PCR**
Seedlings were grown under 22°C, LD condition for 7 days, and then were transferred to 28°C or remained at 22°C for different time periods. Total RNAs were extracted using a Plant Total RNA extraction kit (Tiangen), and 1 μg total RNA was used for the first-strand cDNA synthesis using an Invitrogen reverse transcriptase kit, according to the manufacturer’s instructions. Real time PCR was performed by using SYBR Premix Ex Taq II (TaKaRa) on an Applied Biosystems Step One Plus Real-time PCR system. The relative expression shown was the mean from 3 biological replicates after normalized against ACTIN2.

**Immunoblotting**
Protein extraction and immunoblotting were carried out as described previously (Zhou et al., 2018). proTCP17::TCP17-GFP and 35S::TCP17-FLAG were grown under LD, 22°C condition for 7 days, then were treated for various time periods under different temperature conditions, before the whole seedlings being collected for protein extraction. The protein levels of TCP17-GFP and TCP17-FLAG were detected with an anti-GFP (Roche) or an anti-FLAG antibody (Abmart) respectively.
TUBULIN probed with an anti-tubulin antibody (sigma) was used for as an internal control. The experiments were repeated three times and the similar results were obtained. One of the representative results was shown.

**Yeast two-hybrid assay**
The full length of TCP17 cDNA was cloned into a pGADT7 vector and the various
fragments of PIF4 and CRY1 were cloned into the pGBK7 vector based on manufacturer’s instructions (Clontech). The construct pGADT7-TCP17 was transformed into Y187 yeast cells, and the constructs containing various fragments of PIF4 or CRY1 were transformed into Y2H Gold yeast cells. After selected on a synthetic dropout medium without Leu (-Leu, for pGADT7-TCP17) or Trp (-Trp, for pGBK7-PIF4-dAD, pGBK7-CCE, and pGBK7-PHR), the yeast cells harboring pGADT7-TCP17 were mated with yeast cells harboring pGBK7-PIF4-dAD, pGBK7-CCE, or pGBK7-PHR for 24 hours, and then grown on synthetic dropout medium without Leu and Trp (-Leu/Trp). Tree clones of each plate were picked up and grown on the synthetic dropout medium without Leu, Trp, His, and Adnine (-Leu/Trp/His/Adnine) containing 25 mM Aureobasidin A (ABA) to detect the interactions between TCP17 and PIF4 or CRY1.

BIFC

The full length cDNA of TCP17, and TCP5 were cloned into pEarley Gate201-nYFP, while PIF4, CRY1 was cloned into pEarley Gate202-cYFP. Agrobacterium harboring each plasmid was incubated in LB medium containing 10 mM MES (PH 5.7) and 20 mM acetylsyringone at 28°C overnight with shaking. After centrifugation, the pellets were resuspended in MS medium with 10 mM MES (PH 5.7), 10 mM MgCl2, and 150 mM acetylsyringone to make a final concentration with OD600 up to 0.6. For co-transfections, equal volume of appropriate agrobacteria was mixed and the mixtures were incubated at room temperature for 3 hours before injection. After 48 hours of infiltration, the fluorescence was observed by a Leica confocal microscope.

Pull down assay

Full length coding sequence of TCP17 was fused in frame to the C terminus of the MBP tag by cloning into a pMAL-cRI-GWR vector. To make a FLAG-CRY1 fusion protein, CRY1 was cloned into the pFLAG-MAC vector by gateway method. For pull down assay, E. coli expressed MBP-TCP17 was purified using an amylose resin (NEB) following the manufacturer’s instructions. The purified MBP-TCP17 protein was
incubated with the *E. coli*-purified FLAG-CRY1 protein under 4°C for 2 hours. After
eluted using an elution buffer (10 mM maltose, 10 mM Tris-HCl, PH 7.5), the
pull-down products were detected by immunoblotting using an anti-MBP or an
anti-FLAG antibody.

**Coimmunoprecipitation (co-IP)**
Col and 35S::TCP17-FLAG seedlings were used for detecting the interaction between
TCP17 and CRY1, and for the interaction between PIF4 and TCP17, 35S::PIF4-GFP
and 35S::PIF4-GFP/35S::TCP17-FLAG seedlings were used. Seven days old
seedlings grown in a LD, 22°C growth chamber were transferred to 28°C or kept at
22°C for 4 hours before harvested for analyses. The tissues were grounded to fine
powder in liquid nitrogen, and homogenized in IP buffer (50 mM Tris-HCl (PH 7.5), 1
mM EDTA, 75 mM NaCl, 0.5 % Triton X-100, 5 % Glycerol). After sonicated 5 times
(10 seconds each time) with power output setting at 65 W, the extracts were
centrifuged at 13,000 rpm for 15 min. The supernatant was mixed with 40 μl of
anti-FLAG Affinity Matrix (SIGMA), and incubated at 4°C for 4 hours. The beads
were washed 5 times with washing buffer (50 mM Tris-HCl (PH 8.0), 150 mM NaCl,
0.1 % Triton X-100). The bound proteins were eluted from the affinity beads with 2×
SDS loading buffer boiled at 95°C for 10 min. The immunoprecipitation products
were analyzed by immunoblot using an anti-FLAG, an anti-GFP, or an anti-CRY1
antibody.

**Chromatin immunoprecipitation**
Chromatin immunoprecipitation (ChIP) assays were performed as described
previously (Ni et al., 2009). Col-0, 35S::TCP17-FLAG and cry1/35S::TCP17-FLAG
transgenic seedlings were grown in LD condition at 22°C for indicated time, and then
transferred to 28°C or kept at 22°C for 4 hours. Two grams of plants were collected
for ChIP assay. Chromatin was isolated and sonicated to generate DNA fragments
with size ranging from 200 bp to 1000 bp. 40 μl of the anti-FLAG Affinity Matrix
(SIGMA) were used for chromatin immunoprecipitation. Precipitated DNA was
analyzed by a real-time PCR. Three independent biological repeats were performed, and similar results were obtained. Relative fold enrichment shown in the results was the mean from one biological replicate after normalized against ACTIN2, and then against the respective input DNA samples. Student’s t test was used for the statistical analyses.

**Luciferase imaging**

The transient expression assays in *N. benthamiana* leaves were carried out as previously described (Walley et al., 2007). The reporter *pPIF4::LUC* and *pYUC8::LUC* constructs were generated by inserting the promoter of PIF4 or YUC8 into the *pGWB235* binary vector by the Gateway cloning approach. *35S::TCP17-FLAG, 35S::HA-TCP5, 35S::PIF4-GFP,* and *35S::CRY1-HA* were used as effectors. The analyses were repeated three times, and similar results were obtained. Luciferase activities were imaged using a Lumazone CA 1300B camera.

**Accession Numbers**

*TCP5* (AT5G60970), *TCP13* (AT3G02150), *TCP17* (AT5G08070), *PIF4* (AT2G43010), *CRY1* (AT4G08920), *YUC8* (AT4G28720), *IAA19* (AT3G15540), *IAA29* (AT4G32280).
Supplemental References

Efroni, I., Blum, E., Goldshmidt, A., and Eshed, Y. (2008). A protracted and dynamic maturation schedule underlies Arabidopsis leaf development. The Plant Cell 20, 2293-2306.

Ni, Z., Kim, E.D., Ha, M., Lackey, E., Liu, J., Zhang, Y., Sun, Q., and Chen, Z.J. (2009). Altered circadian rhythms regulate growth vigour in hybrids and allopolyploids. Nature 457, 327-331.

Walley, J.W., Coughlan, S., Hudson, M.E., Covington, M.F., Kaspi, R., Banu, G., Harmer, S.L., and Dehesh, K. (2007). Mechanical stress induces biotic and abiotic stress responses via a novel cis-element. PLoS Genetics 3, 1800-1812.

Zhou, Y., Zhang, D., An, J., Yin, H., Fang, S., Chu, J., Zhao, Y., and Li, J. (2018). TCP Transcription Factors Regulate Shade Avoidance via Directly Mediating the Expression of Both PHYTOCHROME INTERACTING FACTORS and Auxin Biosynthetic Genes. Plant Physiology 176, 1850-1861.