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ARTICLE

AFP2 Coordinates the Activity of PIF7 for Thermomorphogenesis in Arabidopsis Seedlings

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ABSTRACT

Ambient temperature induces the hypocotyl elongation of seedling, called as thermomorphogenesis. It has been reported that the bHLH transcriptional factor PIF7 acts as the critical component to modulate plant thermomorphogenesis, but the underlying mechanism remains elusive. The phytohormone abscisic acid (ABA) suppresses the hypocotyl elongation under high temperature (HT) stress. As the ABI5 binding protein, AFP2 acts as the negative factor to control ABA signaling. In this study, we first identified AFP2 as the interaction protein of PIF7 *in vitro* and *in vivo*. Phenotype analysis revealed that overexpressing *AFP2* reduced the hypocotyl elongation, while loss-of-function *afp2* mutant showed longer hypocotyl under HT. Consistently, overexpressing *AFP2* impaired the transactivation effect of PIF7 on auxin biosynthesis related genes *YUC8* and *IAA19*, which possibly resulted into the shorter hypocotyl in the transgenic line overexpressing *AFP2* or co-overexpressing *AFP2* and *PIF7*. Thus, these data suggest that AFP2 suppressed PIF7 activity to suppress hypocotyl elongation. Furthermore, we found that HT gradually induced the degradation of AFP2 that possibly released the inhibitory effect of AFP2 on PIF7, thus induced hypocotyl elongation under HT. Taken together, our result reveals the novel function of AFP2 in coordinating thermomorphogenesis through sophistically modulating PIF7 activity.

KEYWORDS

Thermomorphogenesis; AFP2; PIF7; hypocotyl elongation

1 Introduction

Plant has evolved an elaborate mechanism to adapt the environment fluctuation. Ambient temperature over the optimal range dramatically affects the plant architecture, for example, longer petioles, up-ward leave and elongated hypocotyl. These architecture changes, also termed as thermomorphogenesis, promote plant to cool down their leaf surface temperature [1–3]. Accumulated evidence demonstrates that plant photoreceptor phytochromes B (phyB) is functional as the thermo-sensor to perceive the environment change and thus coordinate plant thermomorphogenesis [4]. PIF4 belongs to the bHLH transcriptional factor, phyB interacts with PIF4 to repress its activity, even trigger its degradation under light condition [5,6]. However, ambient high temperature inactivates phyB activity, thus release PIF4 activity, which directly activates the expression of several auxin biosynthesis or signaling related genes, subsequently induce cell elongation [1,3]. Besides PIF4 as the key positive thermomorphogenesis, there exist other seven PIFs, such as PIF3 and PIF5 that also modulate hypocotyl elongation for plant response to environmental stress

[7–11]. Recently PIF7 is also identified as the necessary factor for thermomorphogenesis under warm cycling daytime temperature, PIF7 directly activates the transcription of key gene such as auxin biosynthesis genes such as YUC8 [12,13]. Warm environment also indued the quick accumulation of PIF7, suggesting the feedback regulation network underlying PIF7 during thermomorphogenesis, though detail mechanism needs more investigated.

Apart from auxin biosynthesis, another phytohormone including ABA also regulates plant thermomorphogenesis, directly treatment with ABA strongly suppresses hypocotyl elongation under high temperature stress, the hypocotyl elongation in ABA biosynthesis deficiency mutant is longer after heat stress, suggesting the negative role of ABA signal during thermomorphogenesis [14–16]. In Arabidopsis, the bZIP transcriptional factor ABI5 acts as the important component for ABA signal transduction [17,18], AFPs as the interaction protein of ABI5 affects the protein level of ABI5, thus control seed germination after ABA treatment [19–21]. There are four members of *AFPs* in the Arabidopsis genome, including *AFP1* to *AFP4*. AFP1 is reported to negatively regulate ABI5 protein stability, and seed germination of *afp1* mutant contains higher ABI5 protein level and show more sensitive to ABA stimulation [19]. Similar to *AFP1*, *AFP2* and *AFP3* are also functional epistatically to *ABI5*, therefore affect the seed germination in response to ABA signal [21]. Seed germination of *afp2* or *afp3* mutant was lower after saline or drought treatment. Our previous study also showed that AFP2 recruits its corepressor TPR2 to suppress CONSTANS activity to delay flowering time [22]. What is more, we found that overexpressing *AFP2* presents high germination percentage under high temperature condition while the seed germination of *afp2* was lower, we also found that AFP2 dependents on ABI5 to control seed germination under high temperature [23]. However, whether or how AFP2 control seedling thermomorphogenesis remains elusive so far.

In this study, we first confirm that *PIF7* indeed acted as the necessary factor to control seedling hypocotyl elongation in response to ambient high temperature. Further biochemical analysis showed that PIF7 interacts with AFP2 in planta, and AFP2 suppressed PIF7 activity, subsequently suppressing *PIF7*-mediated thermomorphogenesis. In agreement with it, we found that overexpressing *AFP2* suppressed seedling hypocotyl elongation, while *afp2* mutant showed longer hypocotyl. Therefore our data reveal the novel function of *AFP2* in controlling seedling thermomorphogenesis through *PIF7*.

2 Method and Material

2.1 Arabidopsis Materials and Growth

Arabidopsis thaliana ecotype Columbia (Col-0) plants were used as the wild type. The mutant of *pif7-1* (Sail_622_G02) were obtained from the Arabidopsis Biological Resource Center. Seeds of each genotype were surface sterilized and sown on Murashige and Skoog medium consisting of 0.8% agar, stratified at 4°C in the dark for 3 days, and transferred to a growth chamber at 23°C under constant light. After 10 days, the seedlings were transferred to soil (3:1 [v/v] potting soil:vermiculite) and grown in a greenhouse under a 16-h white light (100–120 μmol m^{-2} s^{-1})/8-h dark cycle. Seeds used for measurement of hypocotyl length analysis were simultaneously harvested from plants grown in the same batches at 22°C under long-day (16 h light/8 h dark) conditions. Dry seeds were stored at room temperature for 2–5 months before germination analysis.

2.2 Hypocotyl Length Analysis

The seed was cold stratified at 4°C for 3 days and then sowed on 1/2MS medium for germination in the grow camber under a 16-h white light (100–120 μmol m^{-2} s^{-1})/8-h dark cycle. For hypocotyl elongation measurements, seeds were sown on 1/2MS on the growth media. Seedlings were grown on vertical plates in an incubator for 3 d at 22°C. High temperature treatment (28°C) started on day 4 for 3 days.

2.3 Transgenic Plants

To generate transgenic plants overexpressing *PIF7*, the full-length coding sequence of this gene was amplified using the primers listed in Supplemental Table 1. The amplified fragments were cloned into the Nde/EcoRI (New England Biolabs) site of the *pRI101-6Flag* vector [6 × Flag tag inserted in the EcoRI/SacI site of the pRI101-AN vector (3262; Takara)] to generate the *PIF7-Flash* construct, using an In-fusion HD Cloning Kit (638911, Clontech). These constructs were transformed into Arabidopsis plants using the *Agrobacterium tumefaciens*-mediated floral dip method (Clough and Bent, 1998).

2.4 Yeast Two-Hybrid Analysis

To check the interaction of AFP2 and PIF7 in yeast, sequences encoding AFP2 and PIF7 were amplified using the primers listed in Supplemental Table 1. The PCR products were cloned into the prey vector pGADT7 (630442, Clontech) and bait vector pGBKT7 (630443, Clontech) to generate pGBKT7-AFP2 (BD-AFP2) and pGADT7-PIF7 (AD-PIF7), respectively, using In-fusion Cloning Technology (Clontech). Yeast strains Y187 and AH109 were transformed with the prey and bait vectors, respectively, by polyethylene glycol-mediated yeast transformation as described by the manufacturer. After screening on minus Trp medium (630413, Clontech) or minus Leu medium (630414, Clontech), three independent clones were mated and grown on minus Trp/Leu medium (630417, Clontech) for 3 d to confirm mating success, and the corresponding clones were transferred to minus TPR/Leu/His/Ade medium (630428, Clontech) to measure their growth status.

2.5 Co-immunoprecipitation Analysis

The transgenic plants *AFP2-FLAG* and *PIF7-Flash/AFP2-FLAG* were used in Co-IP assays to detect the interaction between *AFP2* and *PIF7* in planta. Seedlings were grown in a grown cabinet under long-day (16 h light/8 h dark) conditions at 22°C for 7 days. The samples were harvested, ground to a fine powder in liquid nitrogen, and homogenized in IP buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA [pH 8.0], 1% Triton X-100, 0.6 mM PMSF, 20 μM MG132, Roche protease inhibitor cocktail). The extracts were centrifuged at 14,000 g for 20 min. The supernatant was mixed with 20 μl anti-MYC resin (ChromoTek) for 4 h at 4°C. The beads were washed three times with washing buffer (50 mM sodium phosphate buffer, pH 7.0, 100 mM NaCl, 5 mM EDTA, 0.1% [v/v] Triton X-100, 20 μM MG132, and a protease inhibitor tablet). After brief centrifugation, the immunoprecipitates were separated on a 12% (w/v) SDS-polyacrylamide gel and detected by immunoblot analysis with anti-Myc (Clontech) or anti-Flag (Sigma-Aldrich) antibodies, and the immunoblot signals were detected using an ECL Kit (Invitrogen).

2.6 BiFC Analysis

BiFC experiments were performed using the leaves of *Nicotiana benthamiana* plants grown in a greenhouse (16 h light/8 h dark) for 2 weeks as described previously [22]. In brief, full-length or truncated *AFP2* and *PIF7* were recombined into the binary BiFC vector pSPYNE and pSPYCE, respectively. The constructs were transformed into competent *A. tumefaciens* strain LBA4404 cells (9115, Clontech), and the cultures were incubated in a rotator at 250 rpm for 12 h at 28°C. After culture, Agrobacteria harboring nYFP or cYFP were mixed together and centrifuged at 12000 *g* for 5 min at 4°C, and the pellets were dissolved in injection solution (10 mM Tri-HCl buffer, 25 mM $MgCl_2$, pH 5.6) to an OD_{600} of 0.1. The Agrobacterium solution was injected into *N. benthamiana* leaves, and YFP fluorescence was observed 3 d after injection under a confocal microscope (LSM710; Zeiss).

2.7 RT-qPCR

Total RNA was extracted from the seedling samples using a Plant Total RNA extraction kit (NEB) and reverse-transcribed to cDNA using the reverse transcriptase kit (Takara Bio) according to the manufacturer's instructions. RT-qPCR was performed using SYBR Premix Ex Taq (Perfect Real Time; Takara Bio) on a

LightCycler 96 (Roche). The gene expression results were normalized to the expression of *PP2A* as the internal control. All experiments were performed three biological replicates and technical repeats [23,24]. Primers are listed in Supplemental Tab. 1.

2.8 Transient Transactivation Assay

The transient transactivation assay was performed in Arabidopsis protoplasts as described previously. For transient transcription activity assays using specific promoters, the *YUC8* and *IAA19* promoter sequence was amplified from Col-0 genomic DNA and cloned into the pGreenII-0800-LUC vector as a reporter and the *REN* gene under the control of the cauliflower mosaic virus **(**CaMV**)** 35S promoter in the pGreenII 0800-LUC vector as the internal control. The coding sequences of *AFP2* and *PIF7* were cloned into pGREEN-62-SK under the control of the 35S promoter and used as effectors. Reporters and effectors were transformed into Arabidopsis protoplasts in different combinations and incubated in the dark for 18 h. LUC and REN activities were separately determined at 18 h post-transformation using the Dual-Luciferase Reporter Assay System (Promega, E1910).

2.9 Total Protein Extract and Western Blotting

For immunoblot analysis, freshly harvested or after-ripened seeds were rapidly frozen in liquid nitrogen and ground in extraction buffer (50 mM sodium phosphate, pH 7.0, 100 mM NaCl, 5 mM EDTA, 0.1% [v/v] Triton X-100, 0.1% [w/v] sodium deoxycholate, and protease inhibitor tablet; Roche). Following centrifugation at 14,000 g for 5 min at 4°C, the supernatant was collected. Approximately 10 mg of total protein was separated on a 12% (w/v) SDS–PAGE gel and transferred to a nitrocellulose membrane, which was then probed with the appropriate primary antibody (anti-Myc, anti-GST, 1:3000, Clontech; anti-Flag, 1:3000, Sigma-Aldrich) and horseradish-conjugated goat anti-mouse secondary antibody (1:3000, Promega). Signals were detected using an ECL Kit (Invitrogen).

2.10 Subcellular Localization Analysis

The coding sequence of *PIF7* or *AFP2* was cloned into pRI101-NGFP, pRI101-m Cherry, or pRI101-CFP. The constructs were transformed into Agrobacterium strain GV3101. The Agrobacterium cultures were infiltrated into *N. benthamiana* leaves, and fluorescent signals from GFP, CFP, or mCherry fluorescent protein (YFP) were observed at 72 h post-infiltration.

3 Results

3.1 The Thermomorphogenic Response Requires PIF7 in Arabidopsis

It is known that *PIF4* plays the vital role in hypocotyl elongation during thermomorphogenesis [13]. Here we accessed the function of its homology, PIF7, during hypocotyl elongation. At first we generated the transgenic line expressing GUS marker under the control of the native promoter of *PIF7* (*pPIF7:GUS*). GUS staining showed the expression of PIF7 in the cotyledon and the hypocotyl of the young seedling. High temperature treatment partially increased the GUS staining in cotyledon and hypocotyl (Fig. 1A) suggesting the potential function of PIF7 during heat stress. We then obtained the loss-of-function mutant, SAIL_622_G02, from ABRC, in which a T-DNA was inserted into its third and fourth exon through flanking primers analysis, such insertion also abolished the functional transcripts of *PIF7* (Fig. 1B), thus such mutant was named as *pif7-1*. We also generated the transgenic line overexpressing *PIF7-Flash* fusion under the control of the 35S promoter (*35S:PIF7-Flash*, abbreviated as *PIF7-Flash*), the expression of *PIF7-Flash* was also confirmed by western blotting analysis using anti-MYC antibody (Fig. 1C). Under the normal condition at 22°C, the hypocotyl length of *PIF7-Flash* was longer than wild-type Col and *pif7-1* mutant, ambient HTs treatment promoted the hypocotyl elongation, but HT did not obviously promote the hypocotyl elongation in *pif7-1* mutant, and *PIF7-Flash* seedling still showed longer hypocotyl independent of ambient environment temperature (Figs. 1C and 1D). Thus, these data suggest that *PIF7* is associated with the hypocotyl elongation during thermomorphogenesis.

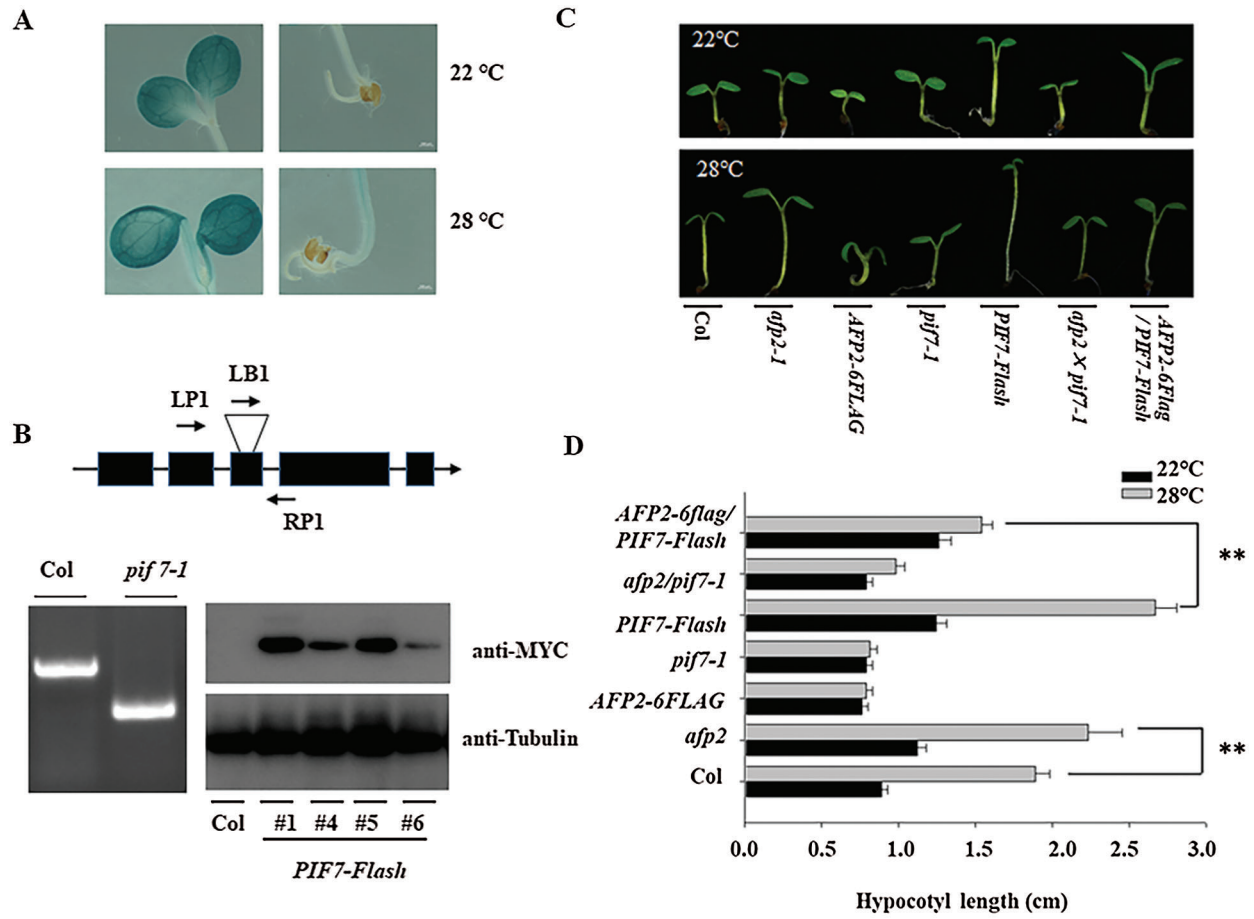

Figure 1: HT induced the hypocotyl length through *PIF7*. (A) HT slightly induced the expression of GUS in the transgenic *pPIF7: GUS*. Upper panel: the transgenic line under 22°C; Lower panel: the transgenic line under 28°C. (B) Validating the genotype of *pif7-1* and *PIF7-Flash* overexpression lines by anti-MYC antibody. The T-DNA insertion position was confirmed by corresponding primers in *pif7-1* mutant. Upper panel: the genomic structure of *PIF7* and the T-DNA insertion position. The exon was shown as a black box, and the T-DNA insertion position was shown as a triangle. Bottom panel (Left): confirming the T-DNA insertion position by corresponding primers. Bottom panel (Right): The strong immunoblotting signal could be detected in the transgenic *PIF7-Flash* line by anti-MYC antibody. The top diagram indicates the genomic structure of *PIF7* and the T-DNA insertion position. (C&D) HT induced the hypocotyl elongation. The seedling of Col, *pif7-1*, *afp2-1*, *PIF7*-Flash, *AFP2*-FLAG, *afp2/pif7* and *AFP2*-FALG/*pif7* seedling was placed under 22°C or 28°C for 3 days, and the hypocotyl length was measured. The values are shown as means SD of triplicate experiments. Asterisks indicate significant difference by Student's *t* test (**$p < 0.01$)

3.2 PIF7 Activates the Expression of YUC8 and IAA19 for Hypocotyl Elongation

The auxin-biosynthesis related genes including *YUC8* and *IAA19* is dramatically induced by ambient HT, and mediates PIF4-dependent hypocotyl elongation during thermomorphogenesis [1]. Here we also compared their expression in *pif7-1* mutant or *PIF7-Flash* seedlings, and found HT indeed induced the expression of *YUC8* and *IAA19*, such induction was weak in the *pfi7-1* seedlings, but stronger in the *PIF7-Flash* seedlings (Fig. 2). Similarly, *PIF7* also regulated the expression of *IAA19* and *SAUR24*,

Thus, this genetic analysis suggests that *PIF7* promote hypocotyl elongation through auxin-biosynthesis related gene such as *YUC8*, etc.

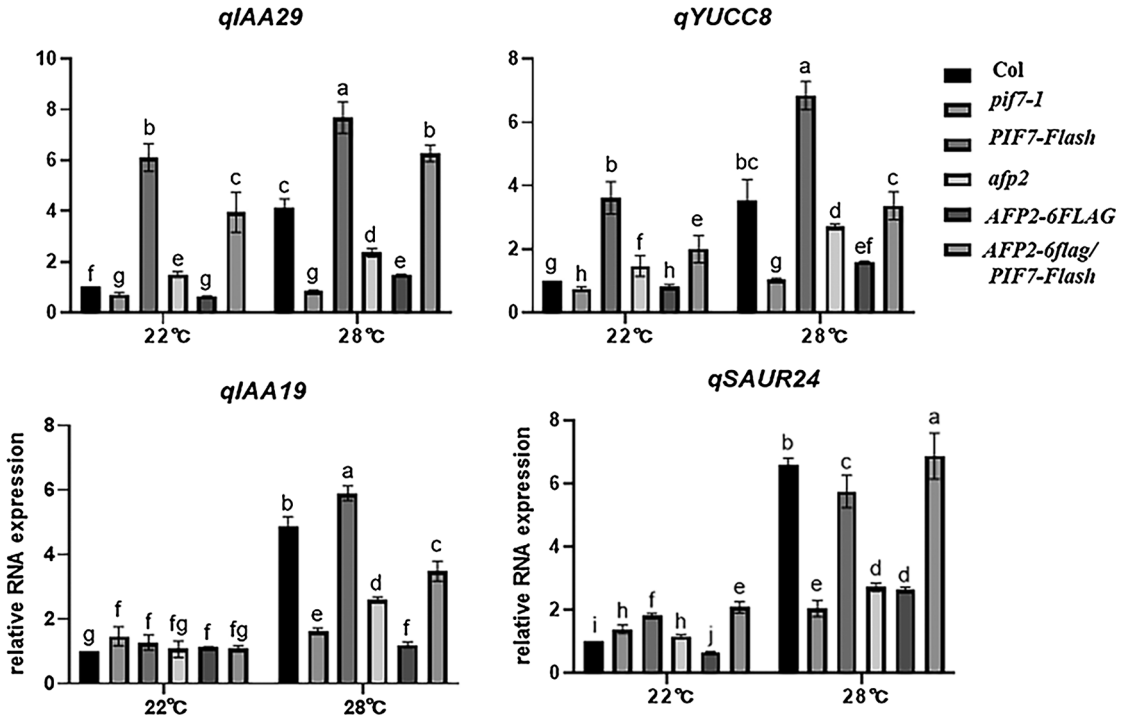

Figure 2: HT induced the expression of auxin-biosynthesis related genes

The wild-type Col seedling was treated with 28°C for indicated time, and the total RNA was extracted for RT-qPCR analysis. The *PP2A* was used as the internal control. The values are shown as means SD of triplicate experiments. Bars with different letters are significantly different at $p < 0.05$ (Tukey's test).

3.3 PIF7 Interacts with AFP2

To better understand the mechanism of which PIF7 control hypocotyl elongation during HTs, we used PIF7 as the bait and perform yeast two-hybrid approach to screen the Arabidopsis normalized cDNA library to look for PIF7-interaction protein. After two-round of screening, we obtained several positive clones. One clone encoding *APF2* was selected for further study as *AFP2* is also reported to particulate plant response to HTs stress. We first confirmed the interaction of PIF7 and AFP2 in yeast cell as the yeast co-expressing BD-*PIF7* and AD-*AFP2* grow well on the selective medium, but not for the BD-*PIF7* or AD-*AFP2* with their corresponding empty vector (Fig. 3A). We then generated the construct containing *PIF7-Flash* fusion under the control of *35S* promoter (*35S:PIF7-Flash*, abbreviated as *PIF7-Flash*), or the *AFP2-FLAG* fusion under the control of *35S* promoter (*35S:AFP2-FLAG*, abbreviated as *AFP2-FLAG*), and co-expressed them in tobacco leaves, and then checked their interaction between AFP2 and PIF7 (Figs. 3C and 3D). As shown in Fig. 3B, we found AFP2-FLAG could be co-immunoreacted by PIF7-Flash, but not by anti-MYC alone, suggesting the interaction between PIF7 and AFP2 in planta.

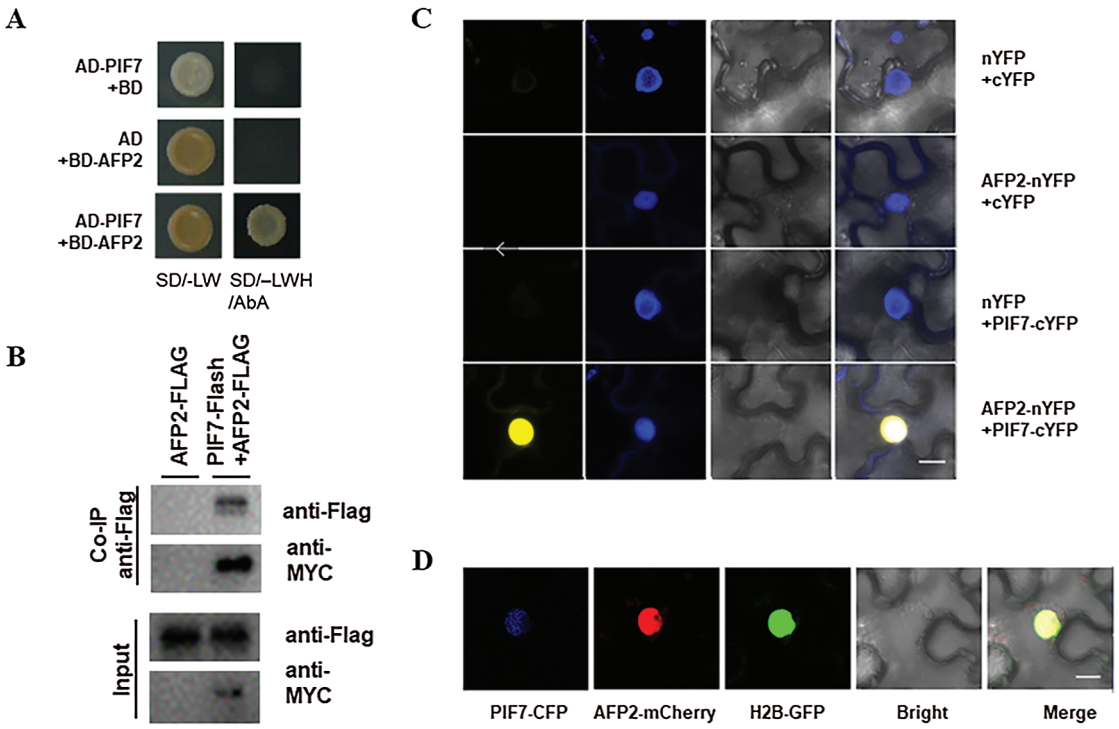

Figure 3: PIF7 interacts with AFP2 *in vivo* and *in vitro* (A) The interaction between PIF7 and APF2 by Y2H analysis. Yeast cells co-transformed with the indicated construct combinations were grown on SD medium lacking Trp/Leu (-LW) or Trp/Leu/His (-LWH) with 100 ng mL-1 Aureobasidin A (AbA). AD: DNA-activation domain of GAL4; BD: DNA-binding domain of GAL4. (B) The *in-vivo* interaction between PIF7 and AFP2 by Co-iP analysis Co-IP showed the interaction of PIF7 with AFP2 in Arabidopsis. Proteins extracted from hydrated seeds from *AFP2-FLAG* or *PIF7-Flash/AFP2-FLAG* plants were immunoprecipitated by MYC-Trap beads. Coimmunoprecipitated proteins were detected by anti-FLAG or anti-MYC antibody. Immunoblots show the presence of proteins in total protein extracts from plants (input) and fractions after immunoprecipitation by anti-FLAG or anti-MYC antibodies. (C) The interaction between PIF7 and AFP2 by BiFC analysis BiFC assay showed that PIF7-cYFP interacts with AFP2-nYFP in the nuclei of *N. benthamiana* epidermal leaf cells. PIF7 was fused to the C-terminal fragment of YFP (cYFP) to form PIF7-cYFP. Full-length AFP2 was fused with the N-terminal fragment of YFP (nYFP) to generate AFP2-nYFP. YFP fluorescence was detected in *N. benthamiana* leaves co-infiltrated with the indicated constructs. Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI). Bar = 10 μm. (D) The co-localization of PIF7 and AFP2 in the Nucleus Analysis of the colocalization of PIF7 and AFP2 in *N. benthamiana* leaves. PIF7-CFP and AFP2-mCherry colocalize in the nuclei of *N. benthamiana* epidermal leaf cells. *H2B-GFP*: a Nuclei marker. Bar = 10 μm.

3.4 AFP2 Influences the Transcriptional Activation of PIF7 to Suppress Hypocotyl Elongation

As we mentioned above, PIF7 modulates the hypocotyl elongation under HTs, we then analyzed the function of AFP2 during thermomorphogenesis. We used the *afp2-1* mutant and the transgenic line overexpressing *AFP2-FLAG* that is used before, to compare the hypocotyl length before or after HT stress. Under normal condition, all of the wild-type Col, *afp2-1* and *AFP2-FLAG* transgenic line presented the similar length, however, HTs treatment increased the hypocotyl length, but HTs-induced hypocotyl elongation was attenuated in the *AFP2-FLAG* seedlings, but more obvious for *afp2-1* mutant seedling (Figs. 1C and 1D), suggesting that AFP2 as the negative regulator controls hypocotyl elongation

during seedling thermomorphogenesis process. We then checked the *YUC8* and *IAA19* expressing among Col, *afp2-1* and *AFP2-FLAG* seedlings. As shown in Fig. 2, expressions of *YUC8* and *IAA19* were lower in the *AFP2-FLAG* lines, but higher in the *afp2-1* line, compared with Col seedlings. These data suggest that AFP2 control hypocotyl elongation through auxin biosynthesis pathway.

We further dissect the genetic relationship between *AFP2* and *PIF7* during thermomorphogenesis by reciprocally crossing *AFP2-FLAG* or *afp2* with *PIF7-Flash* or *pif7* mutant, and then checked their hypocotyl elongation after HTs stress. As shown in Figs. 1C and 1D, HTs promoted the hypocotyl elongation for wild-type Col and *PIF7-Flash* seedling, but such induction was not effective in *AFP2-FLAG* and *pif7-1* mutant seedlings. However, overexpressing *AFP2-FALG* in *PIF7-Flash* seedlings (*AFP2-FLAG/PIF7-Flash*) attenuated the hypocotyl elongation in contrast to *PIF7-Flash* seedling. In agreement with it, the expressions of *YUC8* and *IAA19* in *AFP2-FLAG/PIF7-Flash* was lower than that in *PIF7-Flash* background alone (Fig. 2). These data suggest that AFP2 antagonizes the function of PIF7 through influencing the auxin biosynthesis.

Furthermore, we checked the effects of AFP2 and PIF7 on the transcriptional level of *YUC8* or *IAA19* by transient protoplast analysis. As shown in Figs. 4A and 4B, overexpressing *PIF7* alone obviously activated the transcriptional level of *YUC8* or *IAA19*, however, coexpressing *AFP2* with *PIF7* attenuated the activation effect of *PIF7* on the transcriptional level of *YUC8* or *IAA19*, confirming the antagonistic effect between *AFP2* or *PIF7* on *YUC8* or *IAA19* expressions.

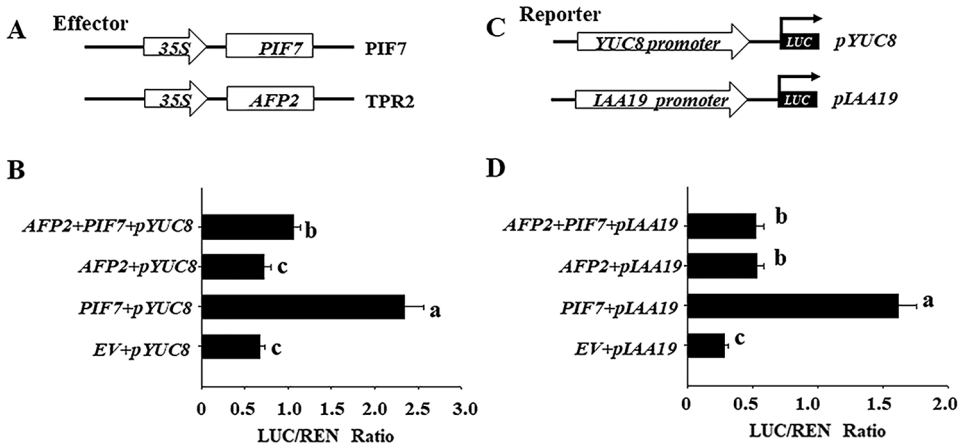

Figure 4: *PIF7* and *AFP2* antagonistically regulate the expressions of *YUC8* and *IAA19* by transient protoplast analysis. A) Schematic diagram of the effector (*PIF7* or *AFP2*) and reporter constructs (*YUC8* and *IAA19*) used in the transient transactivation assay. B) *AFP2* and *PIF7* antagonistically modulate the expressions of *YUC8* and *IAA19*. The *YUC8pro:LUC* or *IAA19pro:LUC* reporter were coexpressed with *PIF7* or *AFP2* effectors for 24 h; the firefly luciferase and Renilla luciferase (LUC/REN) ratio represents *YUC8pro:LUC* or *IAA19pro:LUC* activity relative to the internal control (35Spro:REN). Data are means ± SD of three biological replicates. Bars labelled with different letters are significantly different at $p < 0.05$ (Tukey's test).

3.5 HT Programmatically Induces the Degradation of AFP2 Protein

We previously study showed that *AFP2* present circadian expression during daytime and nighttime. Here we further explore the expressing profile of *AFP2* during seedling thermomorphogenesis. HTs treatment did not obviously affect the transcriptional level of *AFP2*. We then generated the transgenic seedling expressing the *AFP2-FLAG* fusion under the control of its native promoter (*pAFP2:AFP2-FLAG*),

and checked the endogenous AFP2 protein level after HT treatment. As show in Fig. 5A, HT gradually decreased the protein abundance of AFP2 at the first 24 h treatment and sustained the lower level of AFP2 during the following 48 h treatment, suggesting that HT induced the degradation of AFP2. To further test the effect of HT on the stability of AFP2, we expressed GST-AFP2 in bacteria and used the extracted GST-AFP2 protein to test the effect of HT on AFP2 stability by cell-free analysis. As shown in Fig. 5B upper panel, we incubated GST-AFP2 with the crude protein extraction from the seedling subjected with 48 h of HT treatment, observed the gradually degradation of GST-AFP2. As the control, the crude protein extract from the seedling after HT treatment with additional proteasome inhibitor MG132 did not affect the stability of GST-APF2 (Fig. 5C, bottom panel). These data suggest that HT induced the degradation of AFP2 through ubiquitin-dependent degradation pathway.

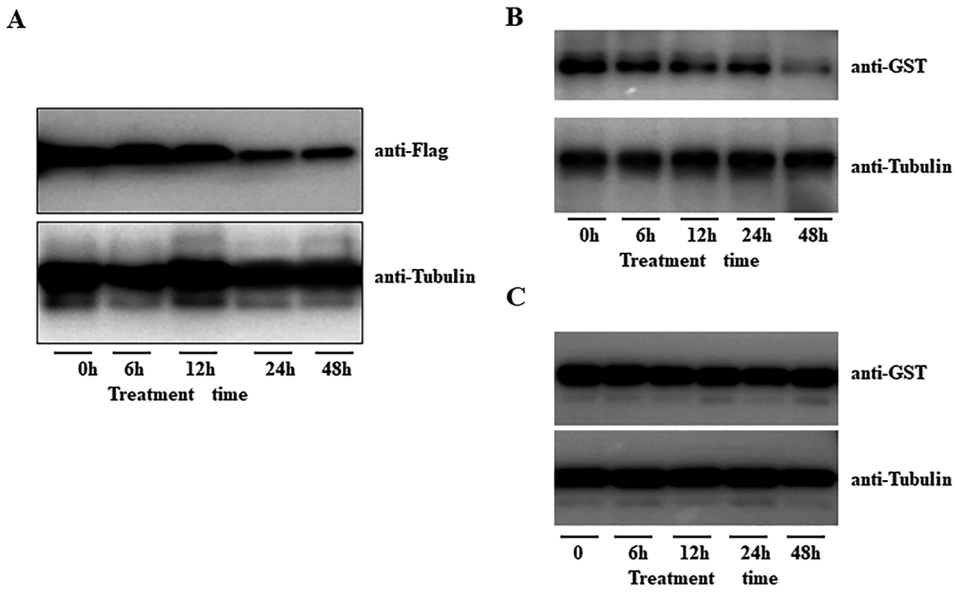

Figure 5: HT induced the degradation of AFP2. A) HT gradually induced the degradation of AFP2-FLAG in the transgenic pAFP2: *AFP2-FLAG*. One-week-old seedling of pAFP2: *AFP2-FLAG* was placed under 28°C for indicated time, and the protein abundance of AFP2-FLAG was checked by anti-FLAG. Anti-tubulin was used as the loading control. B, C) Cell-free analysis of the degradation of GST-AFP2 in planta. The purified GST-AFP2 was incubated the total protein extract from the seedling under 28°C for 3 days, and the degradation speed of GST-AFP2 could be detected by anti-GST antibody (upper panel). The anti-tubulin antibody was used as the loading control (lower panel). As for the control, the protein extract for the seedling under normal condition was incubated with GST-AFP2 for 6 h, 12 h or 24 h, and the degradation of GST-AFP2 was detected (B). For inhibitory experiment, the seedling was pretreated with the proteasome inhibitor MG132 at 10 M for 12 h following HT treatment for 3 days of HT at 28°C for cell-free analysis (C).

4 Discussion

Previous study demonstrates that PIF7 binds the promoter of CBFs, the key gene family to control plant cold acclimation. To coordinate plant freezing tolerance, different photoperiod length, for example long-day (LD) or short-day (SD) condition affect the expression and protein stability of PIF7; subsequently affect its binding to CBF for freezing tolerance [25]. During this process, PIF4 cooperates with PIF7 to control CBF expressing and freezing tolerance [1,25]. In this study, we first investigated the role of PIF7 during thermomorphogenesis, and found overexpressing PIF7 presented longer hypocotyl, whereas

the loss-of-function *pif7* mutant showed relative shorter hypocotyl compared with wild-type Col under HT stress, which confirm that PIF7 is necessary for hypocotyl elongation during thermomorphogenesis. RT-qPCR analysis revealed that *PIF7* mediated HT-dependent *YUC8* and *IAA19* expressing, since inactivating *PIF7* compromised HT-induced *YUC8* and *IAA19* expressing, and overexpressing *PIF7* continuously activated *YUC8* and *IAA19* expressing, which can explain the longer hypocotyl of *PIF7-Flash* before or after HT treatment [13]. In agreement with our result, *PIF7* is important for early response to elevated temperature for Arabidopsis seedling. Like cold stress, high temperature also induced the rapid accumulation of PIF7 protein to activate auxin biosynthesis for long hypocotyl elongation, however high temperature represses the transcriptional level of *PIF7*, suggesting the different mechanism for HT to *PIF7* transcription or protein expression. Meanwhile, both of *pif4* or *pif7* mutant show insensitive to cold stress or HT, suggesting the PIF4 and PIF7 forms heterodimers to regulate plant in response to ambient temperature stress.

Several evidences propose ABA affects thermomorphogenesis process, including auxin biosynthesis or auxin-responsive gene expression [14,19]. AFP2 as the ABI5 binding protein negatively regulates ABA signal [19]. Our previous study also showed that *AFP2* enhance seed germination tolerance to HT through weakening ABI5 activity [26]. In this study we identified AFP2 as the interaction of PIF7, such interaction was validated by a series of experiment including yeast two-hybrid experiment, Co-IP experiment, colocalization experiment also revealed both of AFP2 and PIF7 localized in the nucleus (Fig. 3D). Meanwhile, the *afp2* mutant displayed longer hypocotyl, while overexpressing *AFP2* showed shorter hypocotyl after HT stress, suggesting that *AFP2* acts as the negative regulator to control thermomorphogenesis. Furthermore, genetic analysis using different crossed line showed that overexpressing *AFP2* in the *PIF7*-Flash attenuated the longer hypocotyl phenotype in contrast to *PIF7*-Flash line. Coordinately, the transcriptional level of *YUC8* and *IAA19* was also lower in the crossed *AFP2*-FLAG/*PIF7*-Flash line than that in *PIF7*-Flash line alone. These data suggest that *AFP2* represses the trans-activating of *PIF7* to its target *YUC8* and *IAA19* for hypocotyl elongation (Fig. 2). As AFP2 protein contains the EAR motif, which can recruit TPL/TPR complex and HDAC complex, and HDAC complex can epigenetically erase the histone acetylation modification in the chromatin of target gene locus, as a result, epigenetically repress these target gene expressions. Supporting this possibility, our previous study also showed that CONSNTANS recruits AFP2 and HDACs to coordinates the activation of CONSTANS to FT expression [22]. Besides the acetylation modification, *PIF7* is reported to recruits MRG1/MRG2 complex, thus brings histone-acetylases to increase histone acetylation level to promote the expression of *YUC8* and *IAA19* during shade response [27]. Thus, these evidences, including our data, suggest the complicated regulatory mechanism for PIF7 for plant response to environment stress.

In this study we also noticed that HT gradually increased the protein accumulation of AFP2 in the transgenic *pAFP2: AFP2-FLAG* lines, but the transcriptional level of AFP2 did not obviously change after HT treatment. Cell-free analysis also showed that HT retarded the degradation of AFP2, suggesting the post-transcriptional regulation mechanism for AFP2 expression, possibly suppressing proteasome-dependent ubiquitin system, as the proteasome inhibitor MG132 suppressed the degradation of AFP2 under normal condition. In consistent with the differential profile of AFP2 transcript and its protein expression, HT also differentially regulated the transcriptional level of PIF7 and its protein expression [13]. We speculate that HT induced the gradually accumulation of AFP2 as the brake to slow down the PIF7 activity during the long-term HT treatment, as AFP2 interacts with PIF7 as the AFP2/PIF7 complex, which possible recruits TPL/TPR complex and HDAC to reduce histone acetylation modification level, ultimately reduce its target gene, such as YUC8 and IAA19 expression, and guarantee the appropriate PIF7 activity to avoid excess elongation of hypocotyl under HT condition (Fig. 6).

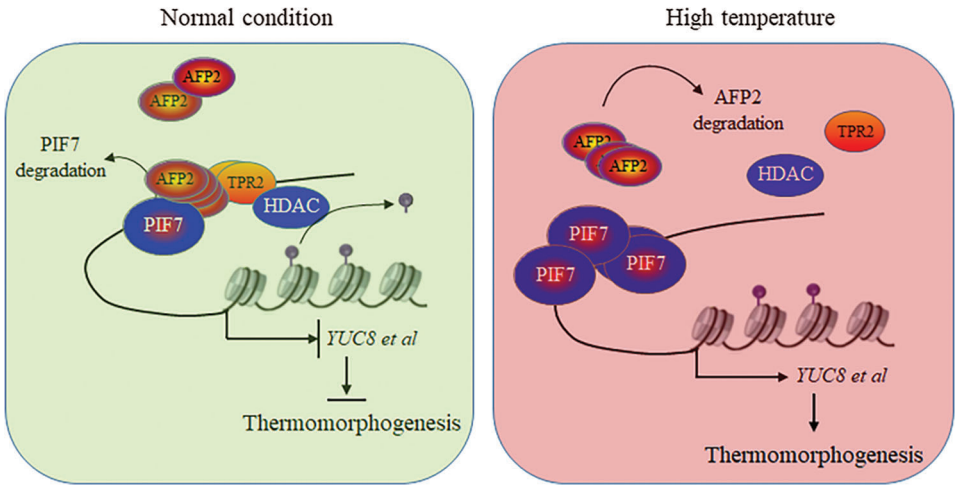

Figure 6: The propose model to illustrate the cooperation mechanism between PIF7 and AFP2 to regulate seedling thermomorphogenesis

After all, in this study we confirmed that *PIF7* activated YUC8 and IAA19 expression for longer hypocotyl elongation, or thermomorphogenesis, after HT stress. Meanwhile, we identified *AFP2* as the new regulator coordinates *PIF7* during thermomorphogenesis. We propose a model to illustrate the possible mechanism by which AFP2 cooperates with PIF7 to modulate thermomorphogenesis. As shown in Fig. 6, HT induced the rapid accumulation of PIF7 to activate YUC8 and IAA19 for longer hypocotyl, meanwhile, long-term HT treatment gradually increased the protein abundance of AFP2, which interacts with PIF7 and recruit HDAC to repress the trans-activation activity of PIF7 on YUC8 and IAA19 to avoid the excessive damage of HT on seedling growth. In a word, *AFP2* as the novel regulator interacts with *PIF7* as the PIF7/AFP2 complex to sophisticate modulate thermomorphogenesis for the young seedling.

Under normal condition, AFP2 interacts with PIF7, which then recruit the corepressor TPR2 to orchestrate the transactivation activity of PIF7 on auxin-biosynthesis related gene expressions, thus present normal hypocotyl growth. However, HT treatment induces the degradation of AFP2, which could not efficiently recruit TPR2, thus release PIF7 activity to activate the expressions of auxin-biosynthesis genes, ultimately accelerate hypocotyl elongation for thermomorphogenesis.

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