PIF4–Mediated Activation of YUCCA8 Expression Integrates Temperature into the Auxin Pathway in Regulating Arabidopsis Hypocotyl Growth

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Abstract
Higher plants adapt their growth to high temperature by a dramatic change in plant architecture. It has been shown that the transcriptional regulator phytochrome-interacting factor 4 (PIF4) and the phytohormone auxin are involved in the regulation of high temperature–induced hypocotyl elongation in Arabidopsis. Here we report that PIF4 regulates high temperature–induced hypocotyl elongation through direct activation of the auxin biosynthetic gene YUCCA8 (YUC8). We show that high temperature co-upregulates the transcript abundance of PIF4 and YUC8. PIF4–dependency of high temperature–mediated induction of YUC8 expression as well as auxin biosynthesis, together with the finding that overexpression of PIF4 leads to increased expression of YUC8 and elevated free IAA levels in planta, suggests a possibility that PIF4 directly activates YUC8 expression. Indeed, gel shift and chromatin immunoprecipitation experiments demonstrate that PIF4 associates with the G-box–containing promoter region of YUC8. Transient expression assay in Nicotiana benthamiana leaves support that PIF4 directly activates YUC8 expression in vivo. Significantly, we show that the yuc8 mutation can largely suppress the long-hypocotyl phenotype of PIF4–overexpression plants and also can reduce high temperature–induced hypocotyl elongation. Genetic analyses reveal that the shy2-2 mutation, which harbors a stabilized mutant form of the IAA3 protein and therefore is defective in high temperature–induced hypocotyl elongation, largely suppresses the high-temperature phenotype of PIF4–overexpression plants. Taken together, our results illuminate a molecular framework by which the PIF4 transcriptional regulator integrates its action into the auxin pathway through activating the expression of specific auxin biosynthetic gene. These studies advance our understanding on the molecular mechanism underlying high temperature–induced adaptation in plant architecture.

Introduction
Higher plants continually sense environmental conditions to adapt their growth and development. To a large extent, this is achieved through integrating environmental cues into the growth-regulating hormonal pathways. Exposure of Arabidopsis thaliana plants to high temperature (29°C) results in dramatic plant architecture changes including rapid hypocotyl elongation, leaf hyponasty, and early flowering [1–4]. High temperature–induced hypocotyl elongation of Arabidopsis plants provides an ideal model system to investigate the regulatory mechanisms underlying adaptive growth of plants to their ever-changing environments. Among the endogenous cues involved in the regulation of high temperature–induced hypocotyl elongation is the plant hormone auxin [3]. An early observation revealed a correlation between high temperature–induced hypocotyl elongation and high temperature–induced elevation of endogenous free indole-3-acetic acid (IAA) levels [3]. Genetic analyses found that high temperature–induced hypocotyl elongation is sharply reduced in Arabidopsis mutants defective in auxin biosynthesis, transport or signaling [3]. Together, these data attribute an essential role of the auxin pathway in mediating high temperature–induced hypocotyl elongation.

It is long-recognized that auxin has profound effects on plant growth and development. A combination of physiological, biochemical, pharmacological and molecular genetic studies provide an ever-growing body of insights on our understanding of the auxin biosynthesis pathway [5,6]. It is generally believed that, IAA, the main auxin in higher plants, can be synthesized from tryptophan (Trp)-dependent and -independent pathways [5]. Among the best-characterized enzymes involved in the Trp-dependent auxin biosynthetic pathway are the YUCCA (YUC) family of flavin-containing monoxygenases [5,7–9] and the TRYPOTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1/TRYPOTOPHAN AMINOTRANSFERASE-RELATED (TAA1/TAR) family of aminotransferases [5,10,11]. A wealth of genetic evidence indicated that, while inactivating members of the TAA1 family genes causes dramatic developmental defects [8,9], overexpression of the IUC family genes leads to auxin overproduction and long hypocotyl
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Author Summary

Exposure of Arabidopsis to high temperature (29°C) results in a dramatic hypocotyl elongation. The basic helix-loop-helix transcription factor PIF4 and the phytohormone auxin play essential roles in high temperature–mediated induction of Arabidopsis hypocotyl elongation. However, the possible molecular linkage between PIF4 and the auxin pathway in regulating high temperature–induced adaptive growth remains unknown. Here, we report that high temperature–induced elevation of YUCCA8 (YUC8) transcripts and endogenous free IAA levels is dependent on the function of PIF4. In particular, we provide evidence that PIF4 directly activates the expression of YUC8 to upregulate auxin biosynthesis, as a consequence, achieves high temperature–induced hypocotyl elongation. In addition, we found that SHY2/IAA3 is an important component of the PIF4–auxin pathway in regulating high temperature–induced hypocotyl elongation. Overall, our results establish a direct connection between the PIF4 transcription factor and the auxin pathway in regulating high temperature–induced adaptation growth.

Figure 1. Loss of PIF4 Function Impairs High Temperature–Induced Elevation of YUC8 Transcripts and Endogenous Free IAA Levels.

(A–B) High temperature-induced expression patterns of PIF4 and YUC8 in wild type (Col-0) or the pif4 mutant. Six-d-old Col-0 and pif4 seedlings grown at 22°C in continuous light were transferred to 29°C in continuous light or were continually placed at 22°C for a 24 h time course, respectively. The 22°C-grown and 29°C-grown seedlings for each time point were harvested at the same time for RNA extraction and qRT-PCR analyses. Transcript levels of target genes were normalized to the ACTIN7 expression and were relative to those of untreated Col-0 seedlings (0 h). Data shown are average ± SD of triplicate reactions. Shown are representative data from one biological replicate; three biological replicates were conducted, yielding similar results. (C) High temperature-induced elevation of free IAA levels in hypocotyls of Col-0 and pif4. The hypocotyls of 6-d-old wild-type and pif4 mutant seedlings grown at 22°C and 29°C in continuous light, respectively, were harvested for free IAA measurement. Data shown are average ± SD. Student’s t-test between 22°C and 29°C grown plants for each genotype was performed (**, P < 0.01). Shown are representative data from one biological replicate; this experiment was conducted for three biological replicates, yielding similar results.

doi:10.1371/journal.pgen.1002594.g001

that high temperature induced a rapid elevation of PIF4 transcript levels and that the pif4 mutant largely lost the robust enhancement of hypocotyl elongation induced by high temperature [1].

In the context that both the transcription factor PIF4 and the phytohormone auxin are required for high temperature-induced hypocotyl elongation, a fascinating hypothesis is that PIF4 may directly link the auxin pathway in regulating plant adaptation growth to high temperature. We provide here evidence that, in response to high temperature, PIF4 directly activates YUC8 expression and thus elevates endogenous free IAA levels. We also show that the SHY2/IAA3 protein is a downstream component of the PIF4–auxin signaling pathway in regulating high temperature–induced hypocotyl elongation. Our results exemplify how a transcriptional regulator integrates environmental cues with endogenous hormonal signaling to mediate specialized developmental changes in regulating plant adaptive growth.

Results

Loss of PIF4 Function Impairs High Temperature–Induced Elevation of YUC8 Transcripts and Endogenous Free IAA Levels

It has been shown that high temperature activates the expression of the transcription factor PIF4 [1, 16] and elevates endogenous free IAA levels [3] in Arabidopsis. To explore the possible molecular linkage between PIF4 and the auxin pathway in regulating high temperature–mediated adaptation growth, we examined high temperature–induced expression of PIF4 and the YUCCA1 (YUC) family of auxin biosynthetic genes [5]. Consistent with previous reports [1, 16], when wild type (WT) seedlings grown at 22°C for 6 days were transferred to 29°C in continuous light over a 24 h time course, PIF4 transcript abundance was transiently elevated to a peak level at 3 h after transfer (Figure 1A). Correlating with an increased expression of PIF4, high temperature also markedly increased transcript abundance of YUC8 with a peak at 3 h in WT seedlings (Figure 1B). Closer observation with a narrower range of time points revealed that high temperature–mediated induction of YUC8...
expression occurred generally later than that of PIF4 (Figure S1). Parallel experiments indicated that high temperature did not upregulate the expression of other YUC family genes tested (Figure S2). We then compared high temperature-induced YUC8 expression between WT and the pif4 mutant, which has been shown to be defective in high temperature-induced adaptations in plant architecture (Figure 1B). As shown in Figure 1B, the basal expression levels of YUC8 were already low in pif4 seedlings and, significantly, high temperature-induced upregulation of YUC8 expression was largely abolished in this mutant, indicating that the function of PIF4 is important for the basal- and high temperature-induced expression of YUC8.

The pif4 mutation impairs high temperature-induced upregulation of YUC8 expression suggests that this mutation may also affect high temperature-induced elevation of free IAA levels. To test this, we compared high temperature-induced elevation of free IAA levels in WT and pif4 seedlings. For these experiments, we grew seedlings at 22°C or 29°C in continuous light for 6 days and collected hypocotyls for IAA measurement. Consistent with a previous report [3], high temperature increased free IAA levels of WT seedlings by around 50% (Figure 1C). As expected, high temperature-induced elevation of free IAA levels was abolished in the pif4 mutant (Figure 1C), indicating that PIF4 is also required for high temperature-induced elevation of auxin biosynthesis. Together, these results suggest that PIF4 and YUC8 may function in linking temperature and auxin pathway in regulating hypocotyl elongation.

Overexpression of PIF4 Upregulates YUC8 Expression and Leads to Elevated Endogenous Free IAA Levels

As a first step to test the possibility that PIF4 may directly regulate YUC8 expression during high temperature-induced adaptation growth, we examined YUC8 expression in transgenic plants overexpressing PIF4 (35S-PIF4). Like the reported yucca mutants which contain increased endogenous auxin levels [7], 35S-PIF4 plants show a long hypocotyl phenotype that resembles high temperature-grown WT seedlings (Figure S3). As revealed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays, the expression of YUC8 (Figure 2A), but not that of TAA1 (Figure S4), was substantially increased in 35S-PIF4 seedlings as compared to WT. We also generated PIF4-overexpression plants (pMDC7-PIF4) using the chemical inducible vector pMDC7 [17]. In the presence of the chemical inducer estradiol, pMDC7-PIF4 seedlings show increased expression of PIF4 (Figure 2B) and display a long hypocotyl phenotype like 35S-PIF4 seedlings (Figure S5). As expected, YUC8 expression was considerably elevated following estradiol induction (Figure 2C). Consistently, measurement of auxin revealed that the free IAA levels in 35S-PIF4 plants were increased by 50% as compared to those in WT plants (Figure 2D). In line with increased free IAA levels in 35S-PIF4 plants, the expression of the auxin responsive DR5:GUS, a widely used reporter of auxin response, was clearly enhanced in the basal region of 35S-PIF4 hypocotyls (Figure S6). These data together indicate that overexpression of PIF4 leads to increased expression of the auxin biosynthetic gene YUC8 and, as a result, elevated endogenous free IAA levels in planta.

PIF4 Directly Binds to the Promoter Region of YUC8

Three lines of evidence support a scenario that the PIF4 transcription factor may directly regulate YUC8 expression during high temperature-induced adaptation growth. First, underlying high temperature-induced hypocotyl elongation, high temperature upregulates the expression of PIF4 in a similar fashion to that of YUC8. Second, high temperature-induced upregulation of YUC8 expression requires the function of PIF4. Third, overexpression of PIF4 leads to increased expression of YUC8 and elevated free IAA levels in planta. Given that PIF4 specifically binds to a core DNA G-box motif (CACGTG) of its target gene promoters [18], we searched for the presence of G-box motifs in the promoter regions of the 11 YUC family genes present in the Arabidopsis genome. As shown in Figure 3A, G-box motifs were found not only in the promoter of YUC8, whose expression was significantly induced by high temperature (Figure 1), but also in the promoters of YUC5, YUC9 and YUC16, whose expression was not or slightly induced by high temperature (Figure S2). To test the idea that PIF4 may actually bind to the G-box-containing regions of these YUC genes, we performed chromatin immuno-precipitation (ChIP) assays using a previously reported

![Figure 2. Overexpression of PIF4 Increases the Expression of YUC8 and Elevates Endogenous Free IAA Levels.](image-url)

(A) YUC8 expression in wild type (Col-0) and 35S-PIF4 plants. Six-d-old Col-0 and 35S-PIF4 seedlings grown in normal growth conditions (22°C) were harvested at the same time for RNA extraction and qRT-PCR analyses. Transcript levels of YUC8 were normalized to the ACTIN7 expression and then were relative to those of Col-0 seedlings. Data shown are average and SD of triplicate reactions. Student’s t-test between Col-0 and 35S-PIF4 seedlings was performed (**, P < 0.01). Shown are representative data from one biological replicate; three biological replicates were conducted, yielding similar results. (B–C) PIF4 and YUC8 expression in transgenic plants containing a chemical-inducible construct pMDC7-PIF4. Eight-d-old pMDC7-PIF4 seedlings were untreated or treated with 10 μM estradiol for 3 h before harvest for RNA extraction and qRT-PCR analyses. Transcript levels of target genes were normalized to the ACTIN7 expression and then were relative to those of untreated seedlings (0 h). Data shown are average and SD of triplicate reactions. Student’s t-test between estradiol-treated and untreated plants was performed (**, P < 0.01). Shown are representative data from one biological replicate; three biological replicates were conducted, yielding similar results. (D) Overexpression of PIF4 leads to increased free IAA levels. Eight-d-old seedlings of wild type and 35S-PIF4 seedlings grown in normal growth conditions (22°C) were harvested at the same time for free IAA measurement. Data shown are average ± SD. Student’s t-test between wild-type and 35S-PIF4 plants was performed (**, P < 0.01). Shown are representative data from one biological replicate; this experiment was conducted for three biological replicates, yielding similar results.

doi:10.1371/journal.pgen.1002594.g002
transgenic line expressing a fusion of PIF4 to the haemagglutinin (HA) antigen (PIF4-HA) [19] and anti-HA antibody (Abcam). PCR amplification of the promoter regions of the four YUC genes showed that PIF4-HA specifically bound to the G-box-containing promoter region of YUC8, but not to the G-box-containing promoter regions of YUC5, YUC9, and YUC10 (Figure 3B). These results suggest that PIF4 associates with the G-box DNA motifs in the promoter region of YUC8 in vivo. Further evidence supporting this conclusion came from electrophoretic mobility-shift assays (EMSA) using PIF4 protein expressed in vitro. As shown in Figure 3C, PIF4 bound to the G-box-containing DNA fragments present in the promoter region of YUC8 and, this binding could be effectively competed by the addition of excess amount of unlabeled G-box-containing DNA probes (Figure 3C). As a control, we showed that DNA probes containing a mutated G-box motif (CACGGG) failed to compete the binding of PIF4 to the G-box-containing DNA fragments (Figure 3C). Together, these results support that the PIF4 transcription factor regulates YUC8 expression by directly binding to its promoter region.

PIF4 Activates YUC8 Expression in the Transient Expression Assay
Next, using the well-established transient expression assay of Nicotiana benthamiana leaves, we verified the activation effect of PIF4 on the expression of a reporter containing the YUC8 promoter fused with the firefly luciferase (LUC) gene. When the pYUC8:LUC reporter was infiltrated into N. benthamiana, the LUC activity could be detected at lower level (Figure 4A, B). Coexpression of pYUC8:LUC with the 35S:PIF4 construct led to an obvious induction in luminescence.
intensity (Figure 4A, 4B), suggesting that ectopic expression of PIF4 can activate pYUC8:LUC expression in this transient expression assay. In a parallel experiment, pYUC8(mut):LUC, in which the two G-boxes of the YUC8 promoter were deleted and fused with LUC, together with 35S:PIF4 were co-infiltrated into N. benthamiana leaves. As shown in Figure 4, the activation effect of PIF4 on pYUC8(mut):LUC expression was abolished. Together, our transient expression assays in N. benthamiana leaves confirmed that PIF4 directly activates YUC8 expression in vivo.

The YUC8 Gene Is Required for PIF4–Mediated Hypocotyl Elongation

To determine the genetic relationship between PIF4 and YUC8, we identified a yuc8 mutant (SALK_096110) which harbors a T-DNA insertion that markedly reduced the expression levels of the YUC8 gene (Figure S7). We show that the yuc8 mutant is defective in high temperature-induced hypocotyl growth (Figure 5A). We then introduced the above-described 35S:PIF4 construct into the genetic background of the yuc8 mutant through genetic crossing. As shown in Figure 5B, the yuc8 mutation substantially suppressed the long-hypocotyl phenotype of 35S:PIF4 plants, supporting that YUC8 acts genetically downstream of PIF4 in regulating high temperature-induced hypocotyl elongation.

SHY2/IAA3 Is an Important Component of the PIF4–Auxin Pathway in Regulating High Temperature–Induced Hypocotyl Elongation

Several elegant observations have demonstrated the involvement of PIF4 and auxin in regulating adaptive growth of plants to

Figure 4. PIF4 Activates YUC8 Expression, as Revealed by Transient Assays of N. benthamiana Leaves. (A) Transient expression assays showing that PIF4 activates the expression of YUC8. Representative images of N. benthamiana leaves 72 h after infiltration are shown. The right panel indicates the infiltrated constructs. (B) Quantitative analysis of luminescence intensity in (A). Five independent determinations were assessed. Error bars represent SD. Asterisks denote Student’s t-test significance compared with control plants: ***, P<0.001. (C) qRT-PCR analysis of PIF4 expression in the infiltrated leaf areas shown in (A). Total RNAs were extracted from leaves of N. benthamiana infiltrated with the constructs. Five independent determinations were assessed. Error bars represent SD.

doi:10.1371/journal.pgen.1002594.g004

Figure 5. The yuc8 Mutation Reduces the Induction of Hypocotyl Elongation by High Temperature and PIF4 Overexpression. (A) Hypocotyl length showing that the yuc8 mutation reduces high temperature-induced hypocotyl elongation. Four-d-old seedlings grown at 22 °C were transferred to 29 °C in continuous light for additional 2 d before hypocotyl length measurement. Data shown are average±SD. Asterisks represent Student’s t-test significance between 29 °C and 22 °C grown plants for each genotype or between pairs indicated with brackets (**, P<0.01). Shown are representative data from one biological replicate; three biological replicates were conducted, yielding similar results. (B) Hypocotyl length showing that the yuc8 mutation partially suppresses the long-hypocotyl phenotype of 35S:PIF4 plants. The hypocotyl length of 6-d-old seedlings of the indicated genotypes grown at 22 °C was measured. Data shown are average±SD. Asterisks represent Student’s t-test significance between transgenic/mutant and wild-type plants or between pairs indicated with brackets (*, P<0.05; **, P<0.01). Shown are representative data from one biological replicate; three biological replicates were conducted, yielding similar results.

doi:10.1371/journal.pgen.1002594.g005
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Figure 6. The shy2-2 Mutation Suppresses the Long-Hypocotyl Phenotype of 35S-PIF4 Seedlings. (A) Representative images showing that shy2-2 suppresses the long-hypocotyl phenotype of 35S-PIF4. Shown are 6-d-old seedlings of Col-0, Ler, shy2-2, 35S-PIF4 and 35S-PIF4/shy2-2 grown at 22°C. (B) Hypocotyl length showing that shy2-2 suppresses the long-hypocotyl phenotype of 35S-PIF4. Hypocotyl length of six-d-old seedlings of the indicated genotypes grown at 22°C was measured. Data shown are average ± SD. Student’s t-test between mutant/transgenic and wild-type seedlings was performed (**, P < 0.01). Shown are representative data from one biological replicate; three biological replicates were conducted, yielding similar results. doi:10.1371/journal.pgen.1002594.g006

Discussion

As sessile organisms, plants have evolved remarkable ability to adapt their development to the ever-changing environmental conditions. Exposure of plants to high temperature results in dramatic changes in plant architecture, including elongation responses and leaf hyponasty. High temperatures can also considerably reduce plant biomass, raising concerns over future crop productivity and food security. Therefore, the modulation of plant architecture by high temperature is a subject of considerable agricultural significance, particularly with regard to global climate change. An ever-growing body of evidence in *Arabidopsis* has implicated that high temperature-induced plant architecture remodeling relies on the interplays between multiple external and internal cues including light, circadian clock, auxin, gibberellin and others [25,26]. Particularly, recent studies reveal that a group of bHLH transcription factors play a central role in modulating developmental responses to both light and temperature [1,14,16,27–29].

PIF4 Is an Integrator between High Temperature and Auxin Pathway in Regulating Adaptive Hypocotyl Growth

In this study, we discovered that, as a molecular integrator, the PIF4 transcription factor links high temperature to the auxin pathway in regulating high temperature-induced hypocotyl elongation. Several lines of evidence support this finding: First, underlying the long-standing observation that high temperature induces a dramatic elongation of the hypocotyl, we showed that high temperature triggers an elevation of the transcript abundance of both *PIF4* and *YUC8* (Figure 1). Second, high temperature-induced upregulation of *YUC8* expression largely depends on the function of PIF4 (Figure 1). Third, overexpression of *PIF4* leads to increased expression of *YUC8* and elevated endogenous free IAA levels (Figure 2). Fourth, as revealed by ChIP and EMSA assays, PIF4 specifically binds to a core DNA G-box motif (CACGTTG) present in the promoter of the *YUC8* gene (Figure 3). Fifth, transactivation assays in *N. benthamiana* leaves support that PIF4 stimulates the activity of the *YUC8* promoter fused with a reporter (Figure 4). Finally, the *yuc8* mutation, which is defective in high temperature-induced hypocotyl elongation, is able to partially suppress the long-hypocotyl phenotype of the 35S-PIF4 plants (Figure 5). Together, these data support that, PIF4 selectively activates the expression of the auxin biosynthetic gene *YUC8*, thus integrates high temperature to the auxin pathway in regulating adaptive hypocotyl growth.

It is worthy of note that the *yuc8* mutant still retains some response to high temperature in hypocotyl elongation and that this mutation fails to completely suppress the long-hypocotyl phenotype of 35S-PIF4 plants (Figure 5). A plausible explanation for this is that the *yuc8* mutant used in this study shows reduced, but not loss of, *YUC8* expression (Figure S7). Alternatively, we could not rule out the possibility that PIF4 may activate auxin biosynthetic genes other than *YUC8*, which act weakly in PIF4-mediated hypocotyl growth in response to high temperature. A very recent report hints that the PIF4 transcription factor could target *TAA1* [29], which acts genetically upstream of the *YUC* family genes in IAA production [12,13]. Considering that overexpression of *TAA1* does not lead to any obvious developmental phenotype [11,12] and that *TAA1* and *YUCs* act in a common linear biosynthetic pathway for auxin production [6,12,13], it is reasonable to propose that *TAA1* acts together with other auxin biosynthesis genes such as *YUC8* to mediate high temperature-induced and PIF4-mediated hypocotyl elongation. However, our gene expression analyses reveal that overexpression of *PIF4* alone fails to elevate *TAA1* transcription (Figure S4).

PIF4-mediated activation of *YUC8* expression in response to high temperature exemplifies a mechanism by which environmental cues manipulate auxin, the key endogenous modulator of plant architecture. Another known physiological process in which both PIF4 and auxin are involved is shade avoidance syndrome (SAS), plant adaptive growth responses to the light signal [14,30,31]. PIF4 is therefore emerging as a molecular “hub” to integrate both temperature and light signals to regulate plant architecture remodeling [14]. Accumulating evidence reveals that,
unlike shade avoidance, where PIF4 acts redundantly with its homolog, PIF3, to regulate elongation growth, PIF4 appears to perform a dominant role in driving high temperature-induced adaptive growth [1, 14, 16, 32–34]. These studies suggest that, PIF4, and possibly other PIF family members, have specialized and overlapping functions in regulating plant adaptive growth to different environmental stimuli.

SHY2/IAA3 Is Specifically Involved in PIF4–Mediated Hypocotyl Elongation in Response to High Temperature

Our results support a scenario in which the auxin pathway acts downstream of the PIF4 transcriptional regulator in regulating high temperature-induced hypocotyl elongation. Supporting evidence for this hypothesis came from our genetic analysis showing that the axr1-12 mutation, which contains a mutation in a subunit of the heterodimeric RUB-E1 enzyme required for auxin signaling [35], completely suppressed the long-hypocotyl phenotype of 35S-PIF4 seedlings (Figure S8). Based on our current knowledge of the auxin signaling pathway, auxin mediates the expression of auxin responsive genes through the inactivation of AUX/IAA transcriptional repressors that negatively control the activity of AUXIN REPONSE FACTOR (ARF) transcription factors [36]. In the context that many gain-of-function aux/iaa mutations are associated with reduced response to exogenous auxin, but developmental defects among these mutants are frequently more specific [36], it is reasonable to speculate that specific Aux/IAA-ARF pair(s) may function in the PIF4-auxin pathway to mediate the specialized hypocotyl elongation process triggered by high temperature. In our genetic efforts to identify new components involved in the PIF4-auxin pathway in regulating high temperature-mediated hypocotyl elongation, we determined that SHY2/IAA3, but not other IAA proteins tested, has a specialized function in mediating high temperature-induced hypocotyl elongation. It is of interest in future studies to identify the ARF transcription factor(s) interacting with SHY2/IAA3 in regulating high temperature-induced hypocotyl elongation.

Materials and Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotypes Columbia (Col-0), Ler and WS were used as wild types. The pif4 mutant used in this study was the reported null allele pif4-2 [1]. Other plant materials used in this study were previously described: DR5:GUS [37], 35S-PIF4 [19], 35S:PIF4-HA [19], yucca [7], axr1-12 [38], shy2-2 [2019], slr1 [21], axr2-1 [22], axr5-1 [23] and iaa28-1 [24]. yucca (SALK_096110) was identified from the SIGnAL T-DNA collection [39].

All molecular manipulations were performed according to standard methods [40]. The PIF4 coding fragment was amplified by PCR and cloned into the AxR1/Plc1 sites of the binary vector pHMC7 [17], resulting in a chemical-inducible PIF4 expression construct. The construct was then transformed into Agrobacterium tumefaciens strain GV3101 (pMP90), which was used for transformation of Arabidopsis plants by vacuum infiltration [41].

Seeds were surface-sterilized for 15 min in 10% bleach, washed four times with sterile water, and plated on half-strength Murashige and Skoog (MS) medium. Plants were stratified at 4°C for 2 d in darkness and then transferred to a phytotron set at 22°C with a 16-h light/8-h dark photoperiod or in continuous light for specific experiments. For high temperature treatment, plants were directly grown at 29°C in continuous light or young seedlings were transferred to 29°C in continuous light for different times.

Gene Expression Analysis

For qRT-PCR analysis, seedlings were harvested and frozen in liquid nitrogen for RNA extraction. RNA extraction and qRT-PCR analysis were performed as previously described [37]. Primers used to quantify gene expression levels are listed in Table S1. The GUS activity assays were performed as previously described [37].

ChIP–PCR Assay

One gram of 6-d-old seedlings of 35S:PIF4-HA transgenic plants [19] and the anti-HA antibody (Abcam) were used in ChIP experiments. Chromatin immunoprecipitation (ChIP) assays were performed as previously described [42]. The enrichment of DNA fragments was determined by semi-quantitative PCR analysis. Three independent biological repeats were performed.

DNA Gel-Shift Assay

PIF4 and Luciferase (Luc) were synthesized by using the Rabbit Reticulocyte TNT system (Promega) [18, 43]. The 60-bp YUC8 promoter probes containing G-box motifs were synthesized and labeled with biotin at the 3’ end (Invitrogen). Cold competitor probes were generated from dimerized oligos of the YUC8 promoter region containing the wt-G-box (CACGGT) or mut-G-box (CAGGGG) motifs, respectively. DNA gel-shift assays were performed as described [18, 43]. Probe sequences are shown in Table S1.

Transactivation of YUC8 Promoter Activity by PIF4 in N. benthamiana Leaves

The transient expression assays were performed in N. benthamiana leaves as previously described [44]. The YUC8 promoter was amplified with the primer pairs 5-CACCATCCGATATACTGATGATGATTTGACAAGAT-3 and cloned into pENTR using the pENTR Directional TOPO cloning kit (Invitrogen). To generate YUC8 promoter with mutations, site-directed mutagenesis was used to delete the two G-boxes in the YUC8 promoter (Figure 3) using the TaKaRa MutanBEST kit. Then, the two YUC8 promoter versions were fused with the luciferase reporter gene LUC through the Gateway reactions into the plant binary vector pGW325 [45] to generate the reporter constructs pYUC8:LUC and pYUC8(mut):LUC. The PIF4 effector construct was the 35S:PIF4. For this construct, the PIF4 coding fragment was amplified by PCR with the primer pairs 5-CACCATCCGATATACTGATGATGATTTGACAAGAT-3 and 5-GTGGTC-CAAACGAGAACCGT-3. Five independent determinations were assessed. Error bars represent SD. The experiments were repeated at least five times with similar results.

Free IAA Measurement

For measurement of free IAA levels in wild-type and pif4 mutant hypocotyls in response to high temperature treatment, the hypocotyls of 6-d-old wild-type and pif4 mutant seedlings grown at 22°C and 29°C in continuous light, respectively, were harvested for free IAA measurement. For the wild-type seedlings grown at 29°C, the 2 mm length parts for each hypocotyl (above the junction between hypocotyl and root) were harvested for free IAA measurement. Eight-d-old seedlings of wild-type and 35S-PIF4 grown at 22°C in continuous light were harvested for free IAA measurement. Approximately 200 mg (fresh weight) of tissues were used for IAA extraction and measurement as previously described [46].

Supporting Information

Figure S1 Comparison of PIF4 and YUC8 Expression in Response to High Temperature Treatment. (A–B) qRT-PCR
analyses of the expression of PIF4 and YUC8 genes in wild type (Col-0) upon high temperature treatment. Six-d-old Col-0 seedlings grown at 22 °C were transferred to 29 °C in continuous light or were continually placed at 22 °C for a time course, respectively. The 22 °C and 29 °C grown seedlings for each time point were harvested at the same time for RNA extraction and qRT-PCR analyses. The transcript levels of target genes were normalized to the ACTIN7 expression and were relative to those of untreated seedlings (0 h). Data shown are average and SD of triplicate reactions. Shown are representative data from one biological replicate; three biological replicates were conducted, yielding similar results.

Figure S2 High Temperature–Induced Expression Patterns of YUCs in Wild Type (Col-0) and the pif4 Mutant. (A–E) qRT-PCR analyses of the expression of YUC genes in wild type (Col-0) and the pif4 mutant. Six-d-old Col-0 and pif4 seedlings grown at 22 °C were transferred to 29 °C in continuous light or were continually placed at 22 °C for a 24 h time course, respectively. The 22 °C and 29 °C grown seedlings for each time point were harvested at the same time for RNA extraction and qRT-PCR analyses. The transcript levels of target genes were normalized to the ACTIN7 expression and were relative to those of untreated seedlings (0 h) for each genotype. Data shown are average and SD of triplicate reactions. Shown are representative data from one biological replicate; three biological replicates were conducted, yielding similar results.

Figure S3 High Temperature–Induced Adaptation Growth of Wild Type and Mutants. (A) Representative images showing high temperature-induced hypocotyl elongation of the indicated genotypes. Four-d-old seedlings grown at 22 °C were transferred to 29 °C for additional 2 d before photographs were taken. (B) Measurements of hypocotyl length of seedlings shown in (A). Four-d-old seedlings grown at 22 °C were transferred to 29 °C for additional 2 d before hypocotyl lengths were measured. Data shown are average ± SD. Asterisks represent Student’s t-test significance between 29 °C and 22 °C grown plants or between transgenic/mutant lines and their wild types (**, P<0.01). Shown are representative data from one biological replicate; three biological replicates were conducted, yielding similar results. (C) Photographs of the indicated genotypes grown in soil at different temperatures. Plants were grown at 22 °C for 10 d before transfer to 29 °C for additional 12 d. Control plants were maintained at 22 °C.

Figure S4 Expression Analysis of TAA1 in Wild Type (Col-0) and 35S-PIF4 Plants. Six-d-old Col-0 and 35S-PIF4 seedlings grown in normal growth conditions (22 °C) were harvested at the same time for RNA extraction and qRT-PCR analyses. Transcript levels of YUC8 were normalized to the ACTIN7 expression and then were relative to those of Col-0 seedlings. Data shown are average and SD of triplicate reactions. Shown are representative data from one biological replicate; three biological replicates were conducted, yielding similar results.

Figure S5 Hypocotyl Growth Phenotype of pMDC7:PIF4 Transgenic Line. Six-d-old seedlings of pMDC7:PIF4 grown at 22 °C on medium without or with inducer (10 μM estradiol).

Figure S6 Tissue-Specific Expression of DR5:GUS in WT and 35S-PIF4 Plants. The 6-day-old DR5:GUS and DR5:GUS/35S-PIF4 seedlings grown at 22 °C were used for GUS activity assays. Shown are representative photographs for basal region of hypocotyl from one biological replicate; three biological replicates were conducted, yielding similar results.

Figure S7 Molecular Analysis of the yuc8 Mutant. (A) Diagram showing the T-DNA insertion site in the YUC8 gene. (B) RT-PCR analysis showing reduced expression of YUC8 in the yuc8 mutant. (C) qRT-PCR analysis showing reduced expression of YUC8 in the yuc8 mutant. The transcript levels of YUC8 were normalized to the ACTIN7 expression and were relative to those of Col-0 seedlings. Data shown are average and SD of triplicate reactions. Student’s t-test between Col-0 and yuc8 plants was performed (**, P<0.01). Shown are representative data from one biological replicate; three biological replicates were conducted, yielding similar results.

Table S1 List of the primers used in this study.

Acknowledgments

The authors thank Drs. Salome Prat, Keara A. Franklin, and Yunde Zhao for providing seeds used in this study and Dr. Philip Benfey for critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: CL, JS. Performed the experiments: JS, LQ,YL,JC. Analyzed the data: JS, CL. Wrote the paper: JS, CL.
References

1. Koini MA, Abeys L, Allen T, Tilley CA, Harberd NP, et al. (2009) High temperature-mediated adaptations in plant architecture require the bHLH transcription factor PIF4. Curr Biol 19: 408–413.
2. Balasubramanian S, Sureshkumar S, Lenep J, Weigel D (2008) Potential induction of Arabidopsis thaliana flowering by elevated growth temperature. PLoS Genet 2: e106. doi:10.1371/journal.pgen.0020106.
3. Gray WM, Ostin A, Sandberg G, Romanos CP, Estelle M (1998) High temperature promotes auxin-mediated hypocotyl elongation in Arabidopsis. Proc Natl Acad Sci USA 95: 7197–7202.
4. Kumar NV, Wigge PA (2010) H2AZ-containing nucleosomes mediate the thermoregulatory response in Arabidopsis. Cell 140: 136–147.
5. Zhao Y (2010) Auxin biosynthesis and its role in plant development. Annu Rev Plant Biol 61: 49–64.
6. Mashiguchi K, Tanaka K, Sakai T, Sugawara S, Kawaide H, et al. (2011) The main auxin biosynthesis pathway in Arabidopsis. Proc Natl Acad Sci USA 108: 18512–18517.
7. Zhao Y, Christensen SK, Fankhauser C, Cashman JR, Cohen J, et al. (2001) A role for flavin monooxygenase-like enzymes in auxin biosynthesis. Science 291: 306–309.
8. Cheng Y, Dai X, Zhao Y (2006) Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in Arabidopsis. Genes Dev 20: 1790–1799.
9. Cheng Y, Dai X, Zhao Y (2005) Auxin synthesized by the YUCCA flavin monooxygenases is essential for embryogenesis and leaf formation in Arabidopsis. Plant Cell 17: 2430–2439.
10. Tao Y, Ferrer JR, Ljung K, Poger F, Hong F, et al. (2008) Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. Cell 133: 177–191.
11. Stepanova AN, Robertson-Hoyt J, Yun J, Benavente LM, Xie DY, et al. (2011) TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. Cell 133: 177–191.
12. Won C, Shen X, Mashiguchi K, Zheng Z, Dai X, et al. (2011) Conversion of tryptophan to indole-3-acetic acid by Tryptophan Aminotransferases of Arabidopsis and Yuccas in Arabidopsis. Proc Natl Acad Sci USA 108: 18510–18513.
13. Stepanova AN, Yun J, Robles LM, Novak O, He W, et al. (2011) The Arabidopsis YUCCA flavin monooxygenases functions in the indole-3-pyruvic acid branch of auxin biosynthesis. Plant Cell 23: 3961–3973.
14. Leivar P, Quail PH (2011) PIFs: pivotal components in a cellular signaling hub. Trends Plants Sci 16: 19–28.
15. Moon J, Zhu I, Shen H, Huq E (2008) PIF1 directly and indirectly regulates chlorophyll biosynthesis to optimize the greening process in Arabidopsis. Proc Natl Acad Sci USA 105: 9413–9418.
16. de Lucas M, Daviere JM, Rodriguez-Falcon M, Poutin M, Iglesias-Pedraza JM, et al. (2008) A molecular framework for light and gibberellin control of cell elongation. Nat Cell 45: 480–484.
17. Tian Q, Uhrig NJ, Reed JW (2002) Arabidopsis SHY2/IAA3 inhibits auxin-regulated gene expression. Plant Cell 14: 301–319.
18. Fukaki H, Tameda S, Masuda H, Taka S (2002) Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA13 gene of Arabidopsis. Plant J 29: 153–168.
19. Nagpal P, Walker LM, Young JC, Sonawala A, Timpte C, et al. (2000) Axr2 encodes a member of the Aux/IAA protein family. Plant Physiol 123: 563–574.
20. Yang X, Lee S, So JH, Dharmasiri S, Dharmasiri N, et al. (2004) The IAA1 protein is encoded by IAA1 and is a substrate of SCF(TIR1). Plant J 40: 772–782.
21. Rogg LE, Lauwelli J, Bartel B (2001) A gain-of-function mutation in IAA28 suppresses lateral root development. Plant Cell 13: 465–480.
22. Patel D, Franklin KA (2009) Temperature-regulation of plant architecture. Plant Signal Behav 4: 577–579.
23. Casson SA, Franklin KA, Gray JE, Grierson CS, Whitelam GC, Hetherington AM (2009) Phytochrome B and PIF4 regulate stomatal development in response to light quantity. Curr Biol 19: 229–234.
24. Lucyshyn D, Wigge PA (2009) Plant development: PIF4 integrates diverse environmental signals. Curr Biol 19: R263–266.
25. Franklin KA, Lee SH, Patel D, Kumar SV, Sparte AK, et al. (2011) PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) regulates auxin biosynthesis at high temperature. Proc Natl Acad Sci USA 108: 20231–20235.
26. Tshifely J, Martinez-Garcia JF, Josse EM (2009) Integration of light and auxin signaling. Cold Spring Harb Perspect Biol 1: a001586.
27. Franklin KA (2008) Shade-avoidance. New Phytol 179: 930–944.
28. Nunez K, Harmer SL, Maloof JN (2011) Genomic analysis of circadian clock-, light-, and growth-correlated genes reveals PHYTOCHROME-INTERACTING FACTORS as a modulator of auxin signaling in Arabidopsis. Plant Physiol 156: 357–372.
29. Lorrain S, Allen T, Duck PD, Whitelam GC, Fankhauser C (2008) Phytochrome-mediated inhibition of shade avoidance involves degradation of growth-promoting bHLH transcription factors. Plant Cell 19: 312–323.
30. Hornitschek K, Lorrain S, Zoev V, Michieli O, Fankhauser C (2009) Inhibition of the shade avoidance response by formation of non-DNA binding bHLH heterodimers. EMBO J 28: 3893–3902.
31. Pozzo JC, Timpte C, Tan S, Callis J, Estelle M (1998) The ubiquitin-related protein RUB1 and auxin response in Arabidopsis. Science 2004: 1760–1763.
32. Chapman EJ, Estelle M (2004) Mechanism of auxin-regulated gene expression in plants. Annu Rev Genet 36: 265–289.
33. Sun J, Xu Y, Ye S, Jiang H, Chen Q, et al. (2009) ABA-dependent AXR1 protein is a negative regulator of phytochrome B signaling and shade avoidance. Proc Natl Acad Sci USA 106: 18510–18513.
34. Nozue K, Harmer SL, Maloof JN (2011) Growth and development of the axr1 mutants of Arabidopsis. Plant Cell 22: 1071–1080.
35. Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, et al. (2003) Genome-wide isogenous mutagenesis of Arabidopsis thaliana. Science 301: 655–657.
36. Sambrook J, Russell D (2001) Molecular cloning, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
37. Bechtold N, Pelletier G (1998) In planta Agrobacterium-mediated transformation of adult Arabidopsis thaliana plants by vacuum infiltration. Methods Mol Biol 62: 259–266.
38. Gendrel AV, Lippman Z, Marriens R, Colon V (2005) Profiling histone modification patterns in plants using genomic tiling microarrays. Nat Methods 2: 213–218.
39. Huq E, Quail PH (2002) PIF4, a phytochrome-interacting bHLH factor, functions as a negative regulator of phytochrome B signaling in Arabidopsis. EMBO J 21: 2441–2450.
40. Chen Q, Sun J, Zhai Q, Zhou W, Qi L, et al. (2011) The basic helix-loop-helix transcription factor MYC2 directly represses PLETHORA expression during jasmonate-mediated modulation of the root stem cell niche in Arabidopsis. Plant Cell 23: 3335–3352.
41. Nakagawa T, Kirome T, Hino T, Tanaka K, Kawamukai M, et al. (2007) Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J Biosci Bioeng 104: 34–41.
42. Zhou W, Wei L, Xu J, Zhai Q, Jiang H, et al. (2010) Arabidopsis Tyrosylprotein sulfotransferase acts in the auxin/PLETHORA pathway in regulating postembryonic maintenance of the root stem cell niche. Plant Physiol 156: 3692–3703.