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Report

High Temperature-Mediated Adaptations in Plant Architecture Require the bHLH Transcription Factor PIF4

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Summary

Exposure of *Arabidopsis* plants to high temperature (28°C) results in a dramatic change in plant development. Responses to high temperature include rapid extension of plant axes, leaf hyponasty, and early flowering [1, 2]. These phenotypes parallel plant responses to the threat of vegetational shade and have been shown to involve the hormone auxin [1, 3]. In this work, we demonstrate that high temperature-induced architectural adaptations are mediated through the bHLH transcriptional regulator PHYTOCHROME INTERACTING FACTOR 4 (PIF4). Roles for PIF4 have previously been established in both light and gibberellin (GA) signaling, through interactions with phytochromes and DELLA proteins, respectively [4–6]. Mutants deficient in PIF4 do not display elongation responses or leaf hyponasty upon transfer to high temperature. High temperature-mediated induction of the auxin-responsive gene *IAA29* is also abolished in these plants. An early flowering response to high temperature is maintained in *pif4* mutants, suggesting that architectural and flowering responses operate via separate signaling pathways. The role of PIF4 in temperature signaling does not, however, appear to operate through interaction with either phytochrome or DELLA proteins, suggesting the existence of a novel regulatory mechanism. We conclude that PIF4 is an important component of plant high temperature signaling and integrates multiple environmental cues during plant development.

Results

High Temperature-Mediated Hypocotyl Elongation Is Abolished in *pif4* Mutants

The phenotypic similarity of plant responses to high temperature ([1, 2], this study) and low red to far red ratio light (low R:FR) [3, 4] suggests the possible existence of shared regulatory mechanisms in the perception of these environmental stimuli. The latter is a component of vegetational shade and is perceived by the phytochrome family of plant photoreceptors [5]. Responses to low R:FR are regulated, in part, by stabilization of the transcriptional regulators PIF4 and PIF5 [5]. The role of PIF4 and PIF5 in mediating plant responses to elevated temperature was therefore investigated. The elongation of wild-type (WT) and *pif* mutant hypocotyls was recorded after 3 day treatment at both 22°C and 28°C. Mutants deficient in PIF4 and PIF5 [4] were analyzed alongside mutants deficient in PIF3. The latter performs a significant role in seedling de-etiolation [7] but has not been demonstrated to perform a significant role in low R:FR signaling. Striking hypocotyl elongation responses were observed in WT, *pif3*, and *pif5* mutant seedlings at the higher temperature, although a mildly attenuated response was observed in *pif5* mutants (Figure 1). No elongation response was observed in *pif4* seedlings transferred to 28°C, suggesting a fundamental role for PIF4 in regulating this process (Figure 1). Similar data were obtained with a second allele, *pif4-2* (Figure S1 available online).

Petiole Elongation and Leaf Hyponasty Responses to High Temperature Are Severely Defective in *pif4* Mutants

The role of PIF4 in mediating petiole elongation responses to high temperature was investigated after transfer of 2-week-old rosettes from 22°C to 28°C. Petiole length was recorded in leaves 4 and 5 at both temperatures when plants at 28°C displayed a 1 cm bolt. Wild-type, *pif3*, and *pif5* plants displayed clear increases in petiole length at the higher temperature, although a slightly attenuated response was observed in *pif5* mutants. As with hypocotyl elongation, petiole elongation responses to high temperature were abolished in *pif4* mutants (Figure 2). A similar response was observed in the *pif4-2* allele (Figure S2A).

Upwards elevation of leaves (leaf hyponasty) is a common response to both low R:FR and high temperature in many plants [8]. This response was analyzed in WT and *pif4* mutants transferred from 22°C to 28°C for 1 week. A dramatic hyponasty response was observed in leaves 4 and 5 of WT plants (Figures 3A and 3B). This response was severely defective in *pif4* mutants. A small hyponasty response was observed in leaf 4 of *pif4-101* mutants but no hyponasty responses were recorded in mutants containing the *pif4-2* allele (Figures 3A and 3B; Figure S2B). Together, these data suggest a major role for PIF4 in mediating petiole elongation and leaf hyponasty responses to elevated temperature.

An Early Flowering Response to High Temperature Is Retained in *pif4* Mutants

The potent induction of *Arabidopsis* flowering by high temperature is well established and has been demonstrated to involve the transcriptional regulators *FLOWERING LOCUS C* and *FLOWERING LOCUS M* [2]. Mutants deficient in PIF4 displayed a similarly early flowering phenotype to WT plants upon transfer to 28°C (Figure 3C; Figure S2C). Such data suggest that the role of PIF4 is confined to plant architectural adaptations to high temperature. The genetic separation of signaling pathways regulating elongation growth and early flowering responses to environmental stress is consistent with studies of low R:FR-treated plants [9, 10].

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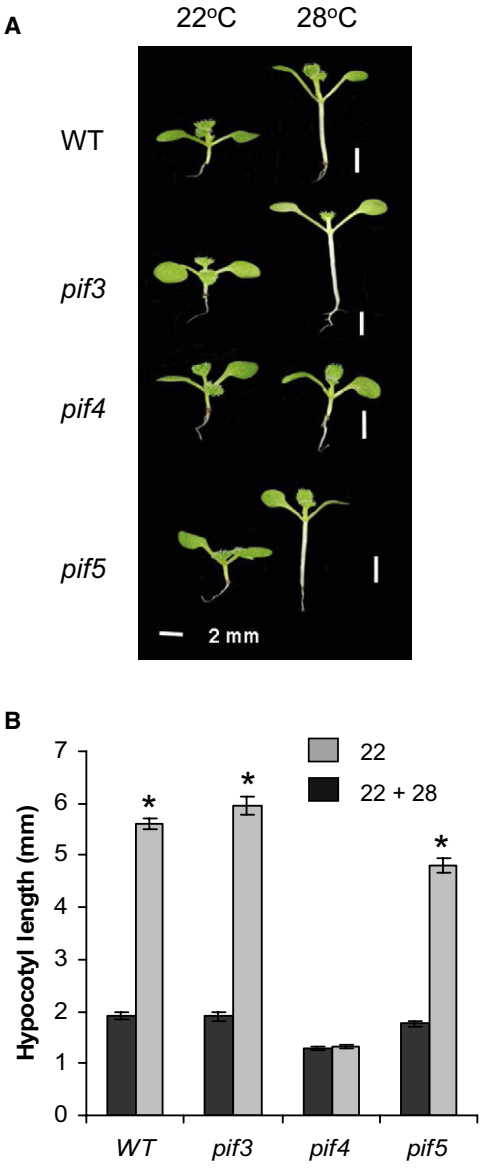

Figure 1. High Temperature-Mediated Hypocotyl Elongation in *pif* Mutants

Photographs (A) and hypocotyl lengths (B) of WT and *pif* mutant seedlings grown in continuous irradiation at different temperatures. Plants were grown at 22°C for 4 days before transfer to 28°C for 3 days. Control plants were maintained at 22°C. Error bars represent SE. Asterisks represent a significant difference from the 22°C sample with Student's t test ($p \leq 0.05$).

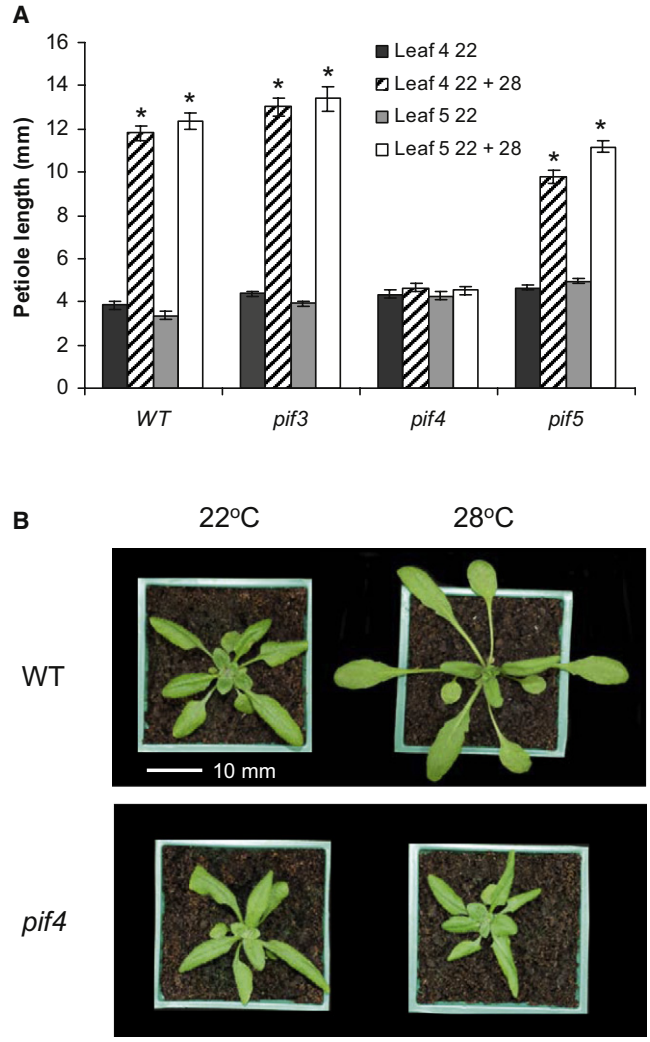

Figure 2. High Temperature-Mediated Petiole Elongation in *pif* Mutants

(A) Petiole lengths of WT and *pif* mutants grown in continuous irradiation at different temperatures. Plants were grown at 22°C for 2 weeks before transfer to 28°C. Control plants were maintained at 22°C. Measurements were performed when plants grown at 28°C displayed a 1 cm bolt. Error bars represent SE. Asterisks represent a significant difference from the 22°C sample with Student's t test ($p \leq 0.05$).

(B) Photographs of WT and *pif4* mutants at 5 weeks.

High Temperature-Mediated Alterations in Plant Architecture Can Operate Independently of Phytochromes and DELLA Proteins

Two molecular mechanisms have been documented to regulate PIF4 function. Abundance of PIF4 has been shown to be modulated via physical interaction with the phytochromes. The interaction of PIF4 with active phyB results in the phosphorylation and degradation of PIF4, thereby suppressing elongation growth in high R:FR conditions [4, 5]. By contrast, binding of phyA to PIF4 has been observed to be relatively weak [4]. The activities of both PIF3 and PIF4 have been shown to be modulated through physical interaction with DELLA proteins [6, 11]. The interaction of PIF4 with DELLAs restrains elongation growth by preventing PIF4 binding to target promoters. Degradation of DELLAs by the plant hormone GA relieves growth restraint, in part through enhancing PIF4 activity [6].

It is well established that phytochrome photoequilibrium is temperature sensitive [12]. The possibility that high temperature may inactivate phytochrome activity was investigated through analysis of high temperature-mediated hypocotyl elongation in phytochrome-deficient mutants. Triple mutants, deficient in phytochromes B, D, and E, displayed significant hypocotyl elongation after transfer to 28°C (Figure 4A). These phytochromes are the major phytochromes repressing elongation growth in high R:FR conditions [13]. The elongation observed in *phyBDE* mutants transferred to 28°C confirms that high temperature responses can operate independently of these phytochromes. The weak interaction of phyA and PIF4 [4] and limited activity of phyC in the absence of phyB, D, and E [13] suggests that high temperature-mediated

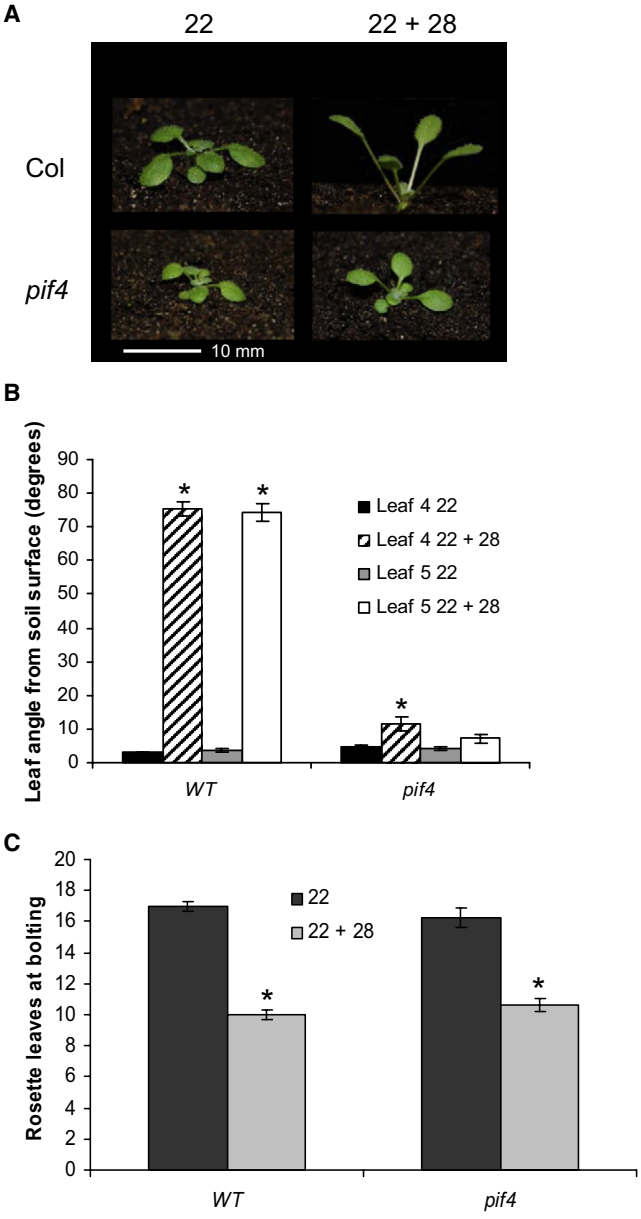

Figure 3. High Temperature-Mediated Leaf Hyponasty and Flowering Responses in *pif4* Mutants

(A and B) Photographs (A) and leaf angle measurements (B) of plants grown in continuous irradiation at different temperatures. Plants were grown at 22°C for 2 weeks before transfer to 28°C. Control plants were maintained at 22°C. Measurements were recorded of the leaf angle from the soil surface after 1 week of high temperature treatment.

(C) Flowering time of *pif4* mutants grown at high temperature. Flowering time was recorded as the number of rosette leaves when plants displayed a 1 cm bolt. Error bars represent SE. Asterisks represent a significant difference from the 22°C sample with Student's t test ($p \leq 0.05$).

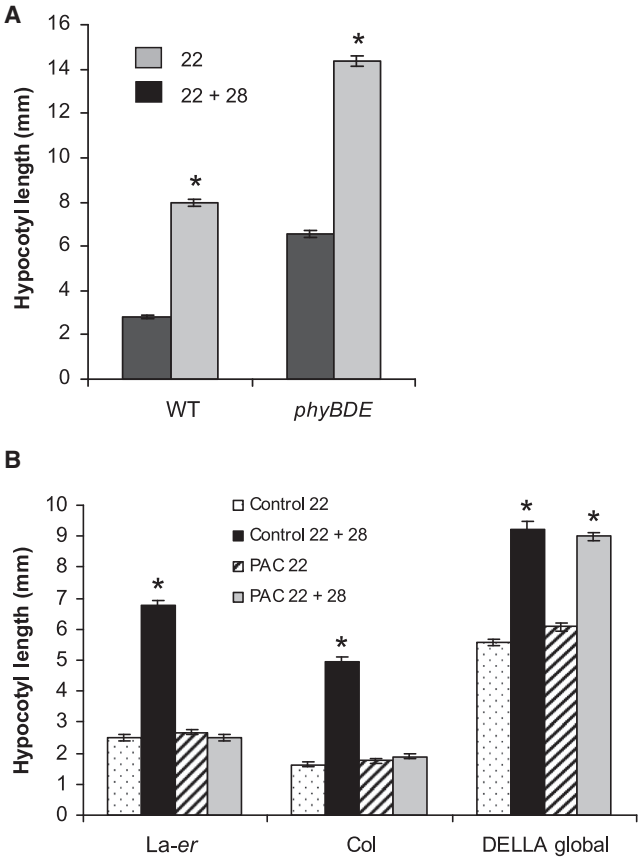

Figure 4. High Temperature-Mediated Hypocotyl Elongation in Phytochrome and DELLA-Deficient Mutants

WT and *phyBDE* mutants (A) and WT and DELLA global deficient mutants (B) grown in the presence and absence of the GA biosynthesis inhibitor Paclobutrazol (PAC). Plants were grown at 22°C for 4 days before transfer to 28°C for 3 days. Control plants were maintained at 22°C. Error bars represent SE. Asterisks represent a significant difference from the 22°C sample with Student's t test ($p \leq 0.05$).

elongation growth is not regulated through inactivation of the phytochromes. Low R:FR ratio-mediated gene expression responses were additionally retained at 28°C (Figure 5B, next section), confirming significant phytochrome activity at elevated temperatures.

The possibility that PIF4/DELLA interaction may be altered at 28°C was investigated through analysis of a DELLA global mutant, deficient in all five DELLA proteins. Mutant plants displayed significant hypocotyl and petiole elongation upon transfer to high temperature (Figure 4B; Figure S3). These data demonstrate that high temperature-mediated elongation growth can occur independently of DELLA action, although a possible contribution of DELLA proteins to WT responses cannot be completely eliminated. The infusion of soil with Paclobutrazol (PAC), a GA biosynthesis inhibitor, abolished high temperature-mediated hypocotyl elongation in WT plants, but not DELLA global mutants (Figure 4B). These data suggest GA biosynthesis to be permissive, rather than regulatory, for this response. The GA-deficient *ga4-1* mutant has been shown to display a mildly attenuated hypocotyl elongation growth response to high temperature [1]. It is therefore likely that the paclobutrazol treatment used in these experiments is more effective in reducing GA levels than is the *ga4-1* mutation. The absence of PAC-mediated inhibition of elongation responses to high temperature in DELLA global mutants suggests that the inhibition observed in WT seedlings results from unnaturally high DELLA accumulation in these plants. It is likely that these accumulated DELLAs act, in part, to prevent high temperature-mediated elongation growth through inhibition of PIF4 function. The possibility does, however, exist that unnaturally high DELLA levels may inhibit

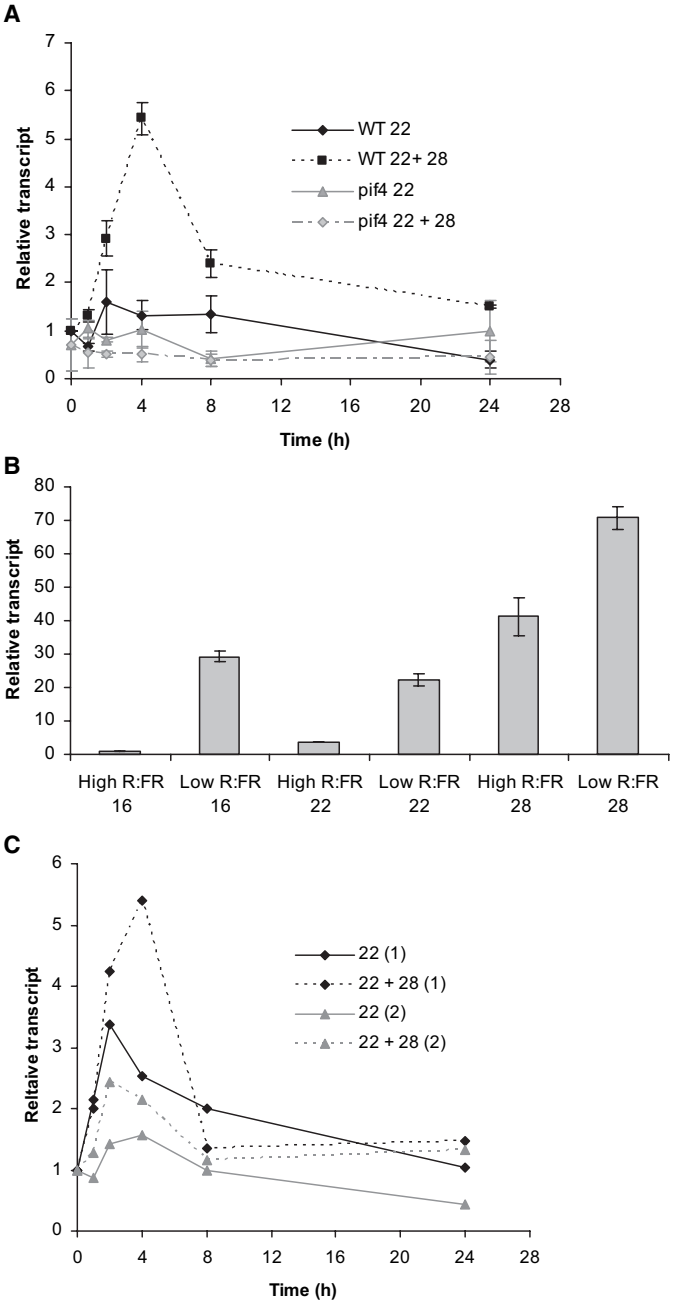

Figure 5. *IAA29* and *PIF4* Transcript Abundance after High Temperature and Low R:FR Treatments

(A) *IAA29* transcript abundance measured by qPCR in WT and *pif4* mutants grown in continuous light for 7 days at 22°C and transferred to a water bath at either 22°C or 28°C for 24 hr. Samples were harvested at 0, 1, 2, 4, 8, and 24 hr. Mean data from two biological repeats are shown with range bars.

(B) *IAA29* expression in 3-week-old rosettes grown in continuous light at 16°C, transferred to the same light conditions at different temperatures for 24 hr, and treated with low R:FR at each temperature for a further 24 hr. Error bars represent the SE from 3 determinations.

(C) *PIF4* transcript abundance in WT plants measured as in (A). Data from two biological repeats are shown (1 and 2).

high temperature-mediated elongation growth through additional PIF4-independent mechanisms.

High Temperature-Mediated Increases in Transcript Abundance of the Auxin-Responsive Gene *IAA29* Are Abolished *pif4* Mutants

The similarities between *Arabidopsis* responses to high temperature [1, 2] and low R:FR [3] were investigated at the molecular level through expression analysis of marker genes associated with low R:FR signaling. Plates of seedlings grown in continuous irradiation at 22°C were transferred to water baths at either 22°C or 28°C in identical light conditions. This treatment ensured uniform heating of plants and standardized gene expression kinetics between biological repeats. Plant temperature was monitored throughout the experiment with a thermal imaging camera to ensure that only plants displaying uniform heating were harvested. The transcript abundance of multiple genes reported to respond significantly and robustly to low R:FR treatment was analyzed throughout a 24 hr time course ([14, 15], data not shown). One gene (*IAA29*) was observed to repeatedly display increased transcript abundance after transfer to high temperature. This increase peaked at 4 hr after transfer and decreased again by 24 hr (Figure 5A). No high temperature-mediated increase in *IAA29* transcript abundance was observed in *pif4* mutants (Figure 5A). Similar data were obtained in adult rosettes, although differences between 22°C and 28°C were not observed until the 24 hr time point (Figure S4A). Rapid and sustained increases in *IAA29* transcript abundance have been reported after low R:FR treatment of *Arabidopsis* [3, 14–16]. The possible additivity of increased temperature and low R:FR signals on *IAA29* levels was investigated through analysis of plants transferred to elevated temperatures and low R:FR. Plants were initially grown at a cool temperature (16°C), acclimated to a higher temperature (either 22°C or 28°C) for 24 hr, and then subjected to a 24 hr low R:FR treatment. This temperature shift alone resulted in considerably increased *IAA29* transcript abundance at 28°C. A smaller increase was observed at 22°C (Figure 5B). Responsivity to low R:FR was retained at all temperatures, providing further evidence that phytochrome function is not inactivated at higher temperatures.

Transfer to High Temperature Transiently Elevates *PIF4* Levels

The increased PIF4 function observed at higher temperatures was investigated through analysis of *PIF4* transcript abundance. Seedlings grown in continuous irradiation at 22°C were transferred to water baths at either 22°C or 28°C, as described above. Tissue was harvested at multiple time points over a 24 hr time course. A small transient increase in *PIF4* transcript abundance was recorded after transfer to both water baths in two separate biological repeats and was greater at the higher temperature (1 and 2, Figure 5C). The elevated transcript abundance observed after transfer to the 22°C water bath likely represents the small temperature increase experienced by these plants (see Experimental Procedures). A similar response to both treatments was recorded in adult rosettes (Figure S4B).

Conclusions

In this work, we have shown that PIF4 is essential for high temperature-mediated adaptations in plant architecture.

Given the functional redundancy between PIF family members in regulating light-mediated elongation growth [5, 18, 19], the dominance of PIF4 in this response is highly unusual. We have demonstrated that high temperature-mediated elongation growth can occur in the absence of phytochromes and DELLA proteins, suggesting the existence of a novel molecular mechanism. Hypocotyl elongation responses to high temperature involve the plant hormone auxin [1]. We observed the auxin-responsive gene *IAA29* to display increased transcript abundance upon transfer to high temperature, a response that was abolished in *pif4* mutants. This gene has previously been shown to be a component of auxin-mediated elongation growth in shade-avoidance responses to low R:FR [3]. It is therefore possible that PIF4 functions as a key regulator of an auxin-mediated signaling pathway controlling architectural adaptations to high temperature. This pathway may additionally function as a component of shade avoidance, suggesting a prominent role for PIF4 as a node of crosstalk between light and temperature signaling [1, 3, 5]. Light and cold signals have previously been shown to intersect through a bHLH protein, SPATULA, in the control of *Arabidopsis* seed germination [17]. Together, these data suggest that bHLH proteins may function as integrators of multiple environmental signals. Mildly attenuated elongation responses to high temperature were observed in *pif5* mutants. bHLH proteins can heterodimerize [18], so it is possible that an absence of PIF5 may perturb the activity of PIF4 signaling complexes.

Transiently increased *PIF4* transcript abundance was observed after transfer of plants to high temperature. Analysis of protein abundance under similar conditions would therefore be of interest. Low R:FR ratio-mediated elongation responses have been shown to operate, in part, through stabilization of PIF4 [5]. The possibility exists that high temperature acts to stabilize PIF4 protein in addition to transiently elevating *PIF4* transcript levels. This would provide a mechanism for the role of PIF4 in longer- and shorter-term responses to high temperature, respectively. Alternatively, high temperature may act to enhance PIF4 function to mediate the dramatic architectural responses observed. The identification of such a key regulator provides an important advance in understanding how plants respond to changes in ambient temperature. Furthermore, the future molecular dissection of PIF4 signaling pathways may provide valuable insight into the response of plants to temperature elevations associated with global climate change.

Experimental Procedures

Plant Material

All experiments were performed with the functionally null *pif4-101* and *pif4-2* alleles. Both are in the Columbia background and have been described previously [6, 17]. The *pif5* (Columbia), *pif3-1* (Columbia), and *phyBphyDphyE* (Landsberg *erecta,* La-*er*) alleles are described elsewhere [5, 7, 13, 20]. The global DELLA mutant is homozygous for mutant alleles at the five *Arabidopsis* DELLA loci (*gai-t6*, *rga-t2*, *rgl1-1*, *rgl2-1*, and *rgl3-4*) and was constructed by crossing a *gai-t6 rga-t2 rgl1-1 rgl2-1* homozygote [21] with a *rgl3-4* homozygote (a T-DNA insertion from the publicly available SAIL collection [Columbia background]) that had been backcrossed six times successively onto the La-*er* background. A line homozygous for the mutant alleles at all five loci were selected from the F2 on the basis of resistance to paclobutrazol (a GA biosynthesis inhibitor) and further screened via PCR assay.

Plant Growth

Seeds were germinated on 5 cm Petri dishes containing a 3:1 mixture of soil and horticultural sand as described previously [5]. All experiments were performed in continuous irradiation at constant temperature in matching growth cabinets (Fi-troton 600H, Sanyo Gallencamp). White light was provided by fluorescent tubes at a photon irradiance of 100 μmol m^{-2} s^{-1} and a red to far-red ratio of 2.7. For low R:FR treatments, the same photon irradiance of Photosynthetically Active Radiation (PAR) was used with a R:FR of 0.1. For mature plant analyses, uniformly sized seedlings were transferred to individual pots at 7 days. For experiments with paclobutrazol, seedlings were grown as above for 7 days and watered with 1 μM Paclobutrazol in 0.01% EtOH on days 4 and 5. Control plants were watered similarly with 0.01% EtOH.

Physiological Analyses

Measurements of hypocotyl and petiole length were recorded with IMAGE J software (http://www.rsb.info.nih.gov/ij). Leaf hyponasty was recorded as the angle between individual leaves and the soil surface with a protractor. Flowering time was recorded as the number of rosette leaves when plants displayed a 1 cm bolt. A minimum of 20 seedlings were used for hypocotyl measurement and 10 plants for petiole, hyponasty, and flowering measurements. Each experiment was repeated multiple times with similar results.

Gene Expression

Gene expression was analyzed in 7-day-old seedlings and 3-week-old rosettes. Plants were transferred to a water bath at either 22°C or 28°C in the light conditions described above. This treatment ensured rapid and uniform heating of plants. Plant temperature was monitored with a portable thermal imaging camera throughout the experiment (http://www.flirthermography.co.uk/). Plants displaying uniform heating were harvested at 0, 1, 4, 8, and 24 hr. Thermal imaging showed high temperature-treated seedlings to increase from 20°C to 28°C within 1 hr of water bath transfer. Control seedlings displayed a small increase in temperature from 20°C to 22°C (data not shown). RNA extraction and qPCR procedures have been described previously [16]. The primers used were ActinF (TCAGATGCCCAGAAGTGTTGTTCC), ActinR (CCGTACAGATCCTTCCTGATATCC), IAA29F (ATCACCATCATTGCCCGTAT), IAA29R (ATTGCCACACCATCCATCTT), PIF4F (GCCGATGGAGATGTTGAGAT), and PIF4R (CCAACCTAGTGGTCCAAACG).

Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://www.current-biology.com/supplemental/S0960-9822(09)00618-6.

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