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Alternative splicing of *PIF4* regulates plant development under heat stress

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eLife Assessment

This manuscript identifies temperature-dependent alternative splicing of PIF4 in Arabidopsis thaliana and shows that heat stress promotes the accumulation of a short exon 5-skipping isoform that is predicted to encode a non-functional protein. This finding is **important**, and it provides an intriguing new layer of regulation for PIF4; however, the strength of the mechanistic conclusions is limited, and several key conclusions rely on indirect evidence. As a result, while the data robustly demonstrate heat-regulated alternative splicing of PIF4, the causal role of PIF4 isoforms' balance in shaping heat-induced developmental responses remains only partially supported and the strength of the evidence presented is **incomplete**. This work will be of interest to biologists working on alternative splicing.

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Abstract

The Phytochrome-Interacting Factor 4 (PIF4) is a key player in the integration of multiple internal and external stimuli to optimize different aspects of plant development. While both the DNA encoding this transcription factor and its protein are known to be under tight control, no regulation at the RNA level has been previously reported. Our genomic analysis revealed that the exon/intron structure of the basic Helix-Loop-Helix (bHLH) DNA binding domain of PIF4 is conserved and pointed to skipping of an exon in this region specifically in response to heat stress. We then showed that this alternative splicing event downregulates PIF4 function under heat, which in etiolated seedlings induces photomorphogenic-related traits. Our results disclose a role for PIFs in plant responses to heat and reveal a new regulatory layer for the control of PIF4 function, underscoring the critical role of posttranscriptional regulatory processes in the molecular integration of environmental cues.

Introduction

Phytochrome Interacting Factors (PIFs) belong to the basic Helix-Loop-Helix (bHLH) family of transcription factors. The bHLH protein domain consists of two segments: the basic region, required for DNA binding, and the helix-loop-helix, responsible for hetero and homodimerization (Toledo-Ortiz et al., 2003). PIFs are also characterized by the presence of a protein domain that interacts with photoactivated phytochromes (Favero, 2020). This interaction promotes the degradation of PIFs in the light and is crucial for their role as major regulators of light-regulated biological processes (Leivar and Monte, 2014). In etiolated seedlings, which germinate and develop in subterranean darkness, PIFs are active and repress photomorphogenic features, such as chlorophyll biosynthesis, cotyledon expansion, and repression of hypocotyl elongation (Leivar et al., 2008; Shin et al., 2009).

Besides their well-known function in adjusting seedling development to light, PIFs, particularly PIF4, have over the past years also been implicated in the regulation of different biological processes such as immunity (Gangappa et al., 2017), morphological adaptations to high ambient temperatures (Koini et al., 2009), stomatal development (Casson et al., 2009), leaf senescence (Sakuraba et al., 2014), freezing tolerance (Lee and Thomashow, 2012), salt tolerance (Wang et al., 2025), anthocyanin biosynthesis (Liu et al., 2015), or fatty acid biosynthesis (Liao et al., 2025). PIF4 has thus emerged as a key integrator of multiple external and internal signals to optimize plant development (Choi and Oh, 2016; Lucyshyn and Wigge, 2009).

Several studies have described the molecular mechanisms that control *PIF4* gene expression, protein levels, and activity (Leivar and Quail, 2011; Paik et al., 2017; Pham et al., 2018), but its posttranscriptional regulation has never been characterized. Here we show that alternative splicing, a posttranscriptional process generating multiple mRNAs from the same gene, produces two different *PIF4* transcripts specifically in response to heat stress.

Temperature deviations from the optimal range significantly impact plant development and survival. Increases in temperature are classified as either high ambient temperature or excessively hot temperatures. High ambient temperature is typically 5-6ºC above the optimum temperature (22ºC for *Arabidopsis thaliana*), while excessively hot temperatures exceed this range (Li et al., 2018). These distinct temperature ranges activate independent signaling pathways, leading to different physiological outcomes. Warm temperatures induce thermomorphogenesis, which generally promotes growth and development in a PIF4-dependent manner (Quint et al., 2016). Conversely, excessively hot temperatures trigger stress-responsive pathways aimed at adjusting growth and physiology to mitigate the negative effects of heat (Kan et al., 2023). To date, the role of PIFs in temperature signaling has centered on thermomorphogenesis, with only a few studies having explored the role of PIF proteins in heat stress responses (Li et al., 2021; Yang et al., 2022). Intriguingly, our results reveal that heat stress induces photomorphogenic features in etiolated seedlings and that this developmental response is mediated by an alternative splice form of *PIF4*.

Results

PIF4 is alternatively spliced in response to heat stress

Our analysis of the exon and intron positioning in all 15 members of the XV subfamily of *Arabidopsis thaliana* bHLH transcription factors that includes PIFs (Toledo-Ortiz et al., 2003) showed that, with the sole exception of the less conserved member HFR1, the intron/exon distribution of the bHLH domain is maintained. Its 150-bp long sequence is distributed among three exons, following an invariable proportion: 22%, 44% and 34% (Figure 1A). In addition, the middle exon containing the largest section of the bHLH domain is always 66-bp long and surrounded by phase 0 introns, which preserve codon identities and therefore the reading frame (Figure 1A). Hence, alternative splicing of this exon will produce protein isoforms differing only in the bHLH region (Supplemental Figure 1). Given the genomic particularities of the bHLH middle exon in these genes, we investigated its splicing regulation by quantifying its PSI (Percent Spliced In; percentage of transcripts that include the exon) in publicly available *Arabidopsis thaliana* RNA-seq samples covering several environmental conditions and tissues at different developmental stages (Supplemental Table 1). This analysis revealed skipping of the exon exclusively in two genes: *PIF4* and *PIF6* (Figure 1B and Supplemental Table 1). In the case of *PIF6*, although exon skipping occurs in nearly all tissues and conditions tested, this alternative splicing event has only been shown to be functional in seeds and embryos (Penfield et al., 2010), where *PIF6* is highly expressed (Supplemental Figure 2). Strikingly, we found that the *PIF4* gene undergoes alternative splicing in this particular exon, exon 5, exclusively when plants are under heat stress (Figure 1B and Supplemental Figure 3). Given that the protein arising from this exon skipping event will lack a portion of the bHLH domain (Supplemental Figure 1), we expect heat-induced alternative splicing to reduce the amounts of functional PIF4.

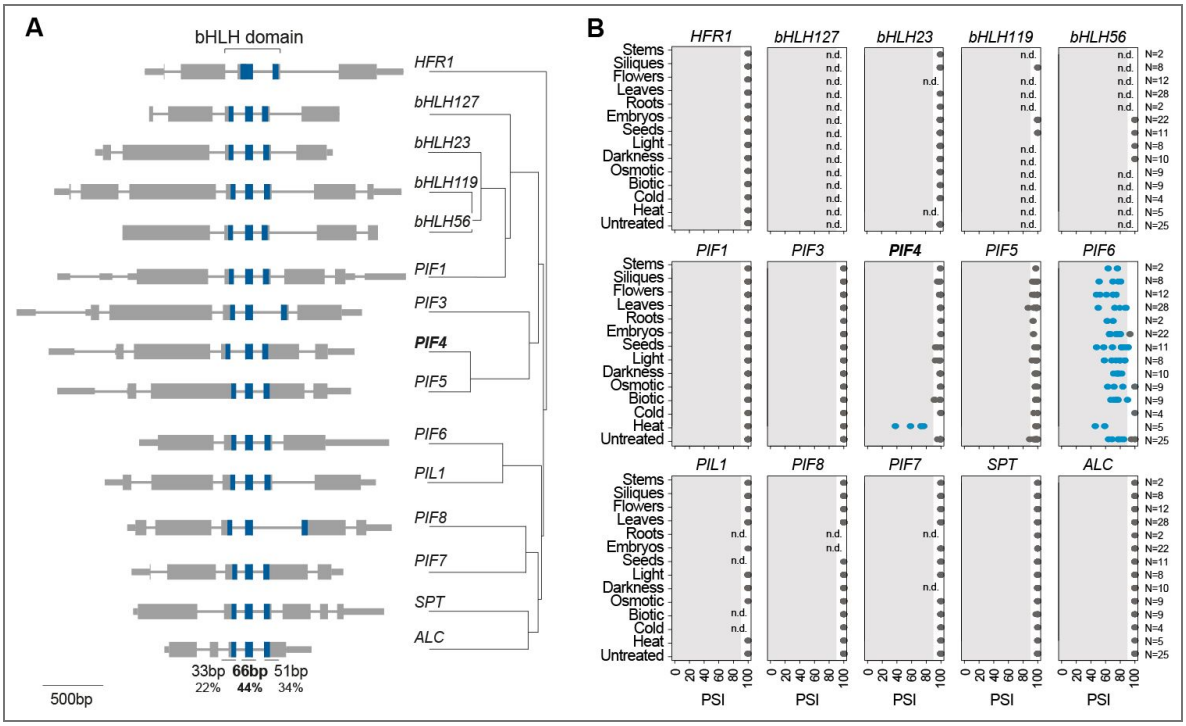

Figure 1. Alternative splicing regulation of the bHLH central exon in the XV subfamily of Arabidopsis *thaliana* bHLH transcription factors.

(A) Exon and intron gene distribution (left) and phylogeny (right; adapted from Leivar and Quail, 2011) of all members of the XV subfamily of Arabidopsis bHLH transcription factors. The bHLH subdomains are shown in blue. For all genes with the exception of *HFR1*, percentages indicate the proportion of the bHLH domain encoded in each exon. **(B)** PSI (Percent Spliced In) of the bHLH central exon using publicly available RNA-seq data covering several *Arabidopsis thaliana* tissues and environmental conditions (Supplemental Table 1). Blue dots indicate PSI<90, the generally considered cutoff for exon skipping. n.d., not detected.

Heat stress induces photomorphogenesis in the dark

To study the impact of the exon skipping event in the bHLH domain of *PIF4*, we applied heat stress (37 ºC) to 3-day-old etiolated seedlings, a developmental stage at which PIFs are known to be functional in repressing photomorphogenesis (Leivar et al., 2008; Shin et al., 2009). First, we confirmed that the heat-induced *PIF4* alternative splicing event occurs in etiolated seedlings as well (Figure 2A), also showing that it does not occur in response to light and is sustained along time (Figure 2A and 2B). Remarkably, heat treatment of dark-grown seedlings partially induces photomorphogenesis — cotyledons open and hypocotyl elongation is repressed (Figure 2C and 2D). These morphological changes, although less pronounced, are characteristic of seedlings lacking PIF activity, as is the case with etiolated seedlings transferred to light or dark-grown quadruple *pif1pif3pif4pif5* (*pifq*) mutants (Figure 2B and Supplemental Figure 4) (Leivar et al., 2009, Leivar et al., 2008; Shin et al., 2009). Moreover, we quantified protochlorophyllide (Pchlide), the phototoxic chlorophyll precursor that, when overaccumulated in etiolated seedings, leads to photobleaching upon light exposure. This analysis revealed higher levels of Pchlide and increased photobleaching in wild-type (WT) etiolated seedlings exposed to heat for 24 hours prior to light exposure, a trend also partially phenocopying *pifq* mutants (Figure 2E, 2F and Supplemental Figure 5) (Leivar et al., 2009). These phenotypes are consistent with heat-induced alternative splicing reducing the amounts of functional PIF4. Analysis of *PIF1*, *PIF3*, *PIF4* and *PIF5* expression levels under 37ºC demonstrated that none of these genes were transcriptionally downregulated by heat stress (Figure 2G and Supplemental Figure 6), discarding a strong reduction in *PIF* transcript levels as the cause of the observed phenotypes. Heat-stressed etiolated seedlings are phenotypically more similar to higher order *pif* mutants than to *pif4* single mutants (Supplemental Figure 7 and 8) (Leivar et al., 2008; Shin et al., 2009), suggesting that the *PIF4-S* splice form generated by exon skipping may act as a dominant negative rather than being merely inactive. Previous studies have reported that different PIF4 protein isoforms can exert dominant negative effects, inhibiting the activity of other PIFs, and that alternative splicing can produce dominant negative transcription factor isoforms (Gangappa et al., 2017; Kim et al., 2020; Nicolas et al., 2015; Seo et al., 2011). However, direct evidence is needed to confirm that the *PIF4* short isoform functions as a dominant negative, reducing the activity of the long isoform and other PIFs. Nevertheless, because heat-induced phenotypes are milder than those observed in *pifq* seedlings (Figure 2C, 2E and 2F), a substantial fraction of PIFs likely remains functional at 37 ºC. This is consistent with heat-induced alternative splicing affecting only around 50% of transcribed *PIF4* mRNAs (Figure 2A and 2B).

Heat-induced photomorphogenesis depends on *PIF4* alternative splicing

To confirm the implication of *PIF4* alternative splicing in the physiological changes undergone by heat-stressed etiolated seedlings, we quantified the morphological and chlorophyll-related phenotypes of transgenic plants expressing predominantly the long *PIF4* splice form in the *pif4-101* mutant background, (*PIF4p::PIF4-L*; Figure 3A and Supplemental Figure 9). Importantly, both the heat-induced Pchlide accumulation and cotyledon opening were strongly reduced in these lines, while the repression of hypocotyl elongation was maintained (Figure 3B, 3C and Supplemental Figure 10). In agreement with the reduction of Pchlide in *PIF4-L* plants, a non-significant but correlating trend in their photobleaching phenotypes was also observed (Figure 3B). Importantly, *PIF4-L.1* expresses the long isoform at levels similar to those of WT plants (Supplemental Figure 9A), ruling out the possibility that the suppression of heat-induced phenotypes (cotyledon opening and Pchlide accumulation) is due to elevated *PIF4* expression levels. In addition, consistent with the comparable alternative splicing levels observed in heat-stressed WT and *pif4* seedlings (Figure 3A and Supplemental Figure 9B), the skipped exon is located upstream of the *pif4-101* mutation (Supplemental Figure 9C), and the phenotypes are also comparable (Figure 3). Similar results were obtained with the other commonly used *pif4* mutant (*pif4-2*; Leivar et al., 2008), which harbors a similar insertion site (Supplemental Figure 9D). Overall, our results substantiate a role for *PIF4* alternative splicing in controlling heat-induced

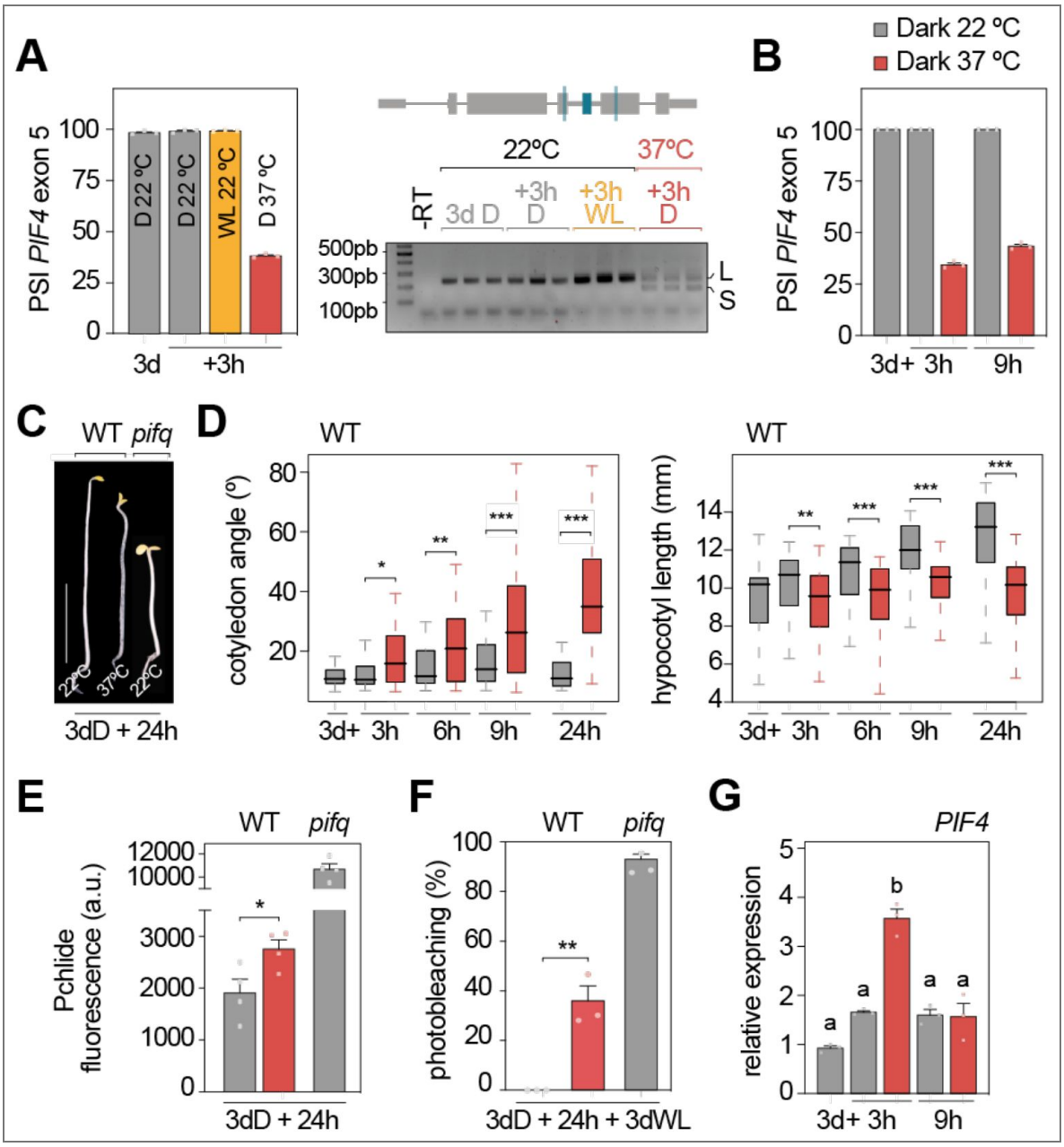

Figure 2. **Impact of heat treatment in etiolated seedlings.**

(A-B) PSI (Percent Spliced In) quantification (left) from RT-PCR (right) of the *PIF4* alternatively-spliced exon in seedlings grown in continuous dark for 3 days (d) and then transferred to 37 °C (red) or white light (WL; yellow), or kept at 22 °C for 3 (A) or 9 (B) additional hours (h) in darkness (D; gray). Data represent means ± SEM of biological triplicates. -RT, no reverse transcriptase. **(C)** Representative image of 3-day-old wild-type (WT) and *pifq* seedlings subjected or not to heat stress. Scale bar, 5 mm. **(D)** Boxplot representations of the cotyledon aperture (left) and hypocotyl length (right) of at least 35 WT seedlings grown in darkness for 3 days and then transferred to 37 °C in darkness (red) or kept in the dark at 22 °C (gray) for the indicated time in hours. Asterisks indicate statistically differences between medians (Mann Whitney test). Fluorescence of protochlorophyllide (Pchlide; 635 nm) **(E**; *n*=4) and percentage of photobleaching **(F**; *n*=3) in heat-stressed (red) or unstressed (gray) etiolated WT and *pifq* seedlings. Asterisks indicate statistically differences between averages (*t*-test). **(G)** RT-qPCR analysis of *PIF4* transcript levels in WT seedlings grown as in (*B*). Values were normalized to *PP2A* and expression levels are expressed relative to the initial time point set at one. Data represent means ± SEM of biological triplicates, and different letters denote statistically significant differences under each condition (Tukey test; $P<0.05$). n.d., not detected. (A, D, E, F) *$P<0.05$, **$P<0.01$ and ***$P<0.001$.

developmental responses in etiolated seedlings. Next, we quantified the transcriptional response to heat stress in etiolated seedlings with different levels of the long *PIF4* splice form (Figure 3A). Interestingly, we found a strong enrichment of heat-regulated genes among those reported as PIFq-regulated or PIFq-bound (Pfeiffer et al., 2014) (Figure 3D). Furthermore, heat-induced transcriptional changes in *pif4* mutants, the genetic background of *PIF4-L* seedlings, were significantly attenuated in these transgenic lines, yet the response remained far from abolished (Figure 3E). This result could be explained by some heat-induced transcriptional changes being fully PIF-independent, as shown in Figure 3D, and others being PIF-dependent but unaffected due to the considerable fraction of PIFs still functional under heat stress. Either scenario would also explain the partial reversion of the heat-induced phenotypic responses observed in *PIF4-L* lines.

To confirm the role of *PIF4* alternative splicing in regulating heat-induced responses, we generated transgenic plants expressing the short isoform of *PIF4* under the control of its endogenous promoter (*PIF4p::PIF4-S*) and evaluated their morphology in the dark under control temperature conditions. These transgenic lines (*PIF4-S*) showed higher *PIF4* expression levels than the corresponding WT control (Figure 4A), and in all three lines, the short isoform was the predominantly expressed variant (Figure 4B). We then conducted a phenotypic analysis of seedlings grown in the dark for 3 and 4 days at 21ºC. Interestingly, our results showed that enhanced production of the short isoform consistently promoted cotyledon opening, while changes in the hypocotyl length were not always detectable (Figure 4C). Thus, the cotyledon phenotype of these plants resembles that of WT plants exposed to heat stress (Figure 2C and 2D), linking the production of this isoform with heat-induced morphological adaptations. Notably, cotyledon opening in these transgenic plants at 21ºC is less pronounced than in heat-stressed plants (37ºC), indicating that the production of this isoform is not the unique mechanism underlying heat-induced cotyledon opening.

Discussion

Our study reveals, for the first time, that cotyledon opening is a developmental response of etiolated seedlings exposed to heat stress. Heat stress also exerts a repressive effect on hypocotyl elongation in etiolated seedlings, a phenomenon previously reported but not extensively studied (Hong and Vierling, 2000; Karayekov et al., 2013; Larkindale et al., 2005; Martín and Duque, 2022). Karayekov et al. linked this inhibitory effect on hypocotyls to altered functioning of light signaling components such as CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), ELONGATED HYPOCOTYL5 (HY5) and circadian clock components. Notably, they also proposed a physiological rationale for this response: dark-germinated seedlings approaching the soil surface may become more susceptible to heat shock episodes due to their proximity to sunlight. They hypothesized that the molecular mechanisms triggered by these episodes would prime seedlings for imminent light exposure. Given that the first exposure to sunlight represents a critical phase for etiolated seedlings, requiring rapid adaptation to ensure survival, we agree that the early activation of photomorphogenesis-associated traits could be advantageous. Our finding that heat promotes cotyledon opening, another hallmark of photomorphogenesis (Arsovski et al., 2012), supports the hypothesis that, in etiolated seedlings, heat may function as a signal to induce photomorphogenesis. In addition, we show that heat enhances Pchlide accumulation in dark-grown seedlings. Although the conversion of Pchlide to chlorophyllide is light-dependent, Arabidopsis seedlings accumulate Pchlide in the dark to expedite this process upon light exposure (Sperling et al., 1997). However, the amount of Pchlide must be tightly balanced with the availability of the enzyme that catalyzes its conversion to prevent the generation of reactive oxygen species and subsequent cellular damage upon illumination (Mochizuki et al., 2010; Reinbothe et al., 1996). This raises the question of whether heat-induced Pchlide accumulation in etiolated seedlings is an adaptative mechanism to accelerate chlorophyll production and optimize the transition to autotrophic development, or whether it is a side effect of prematurely activating light signaling in the dark, an outcome that, as suggested by increased photobleaching, would negatively impact seedling survival.

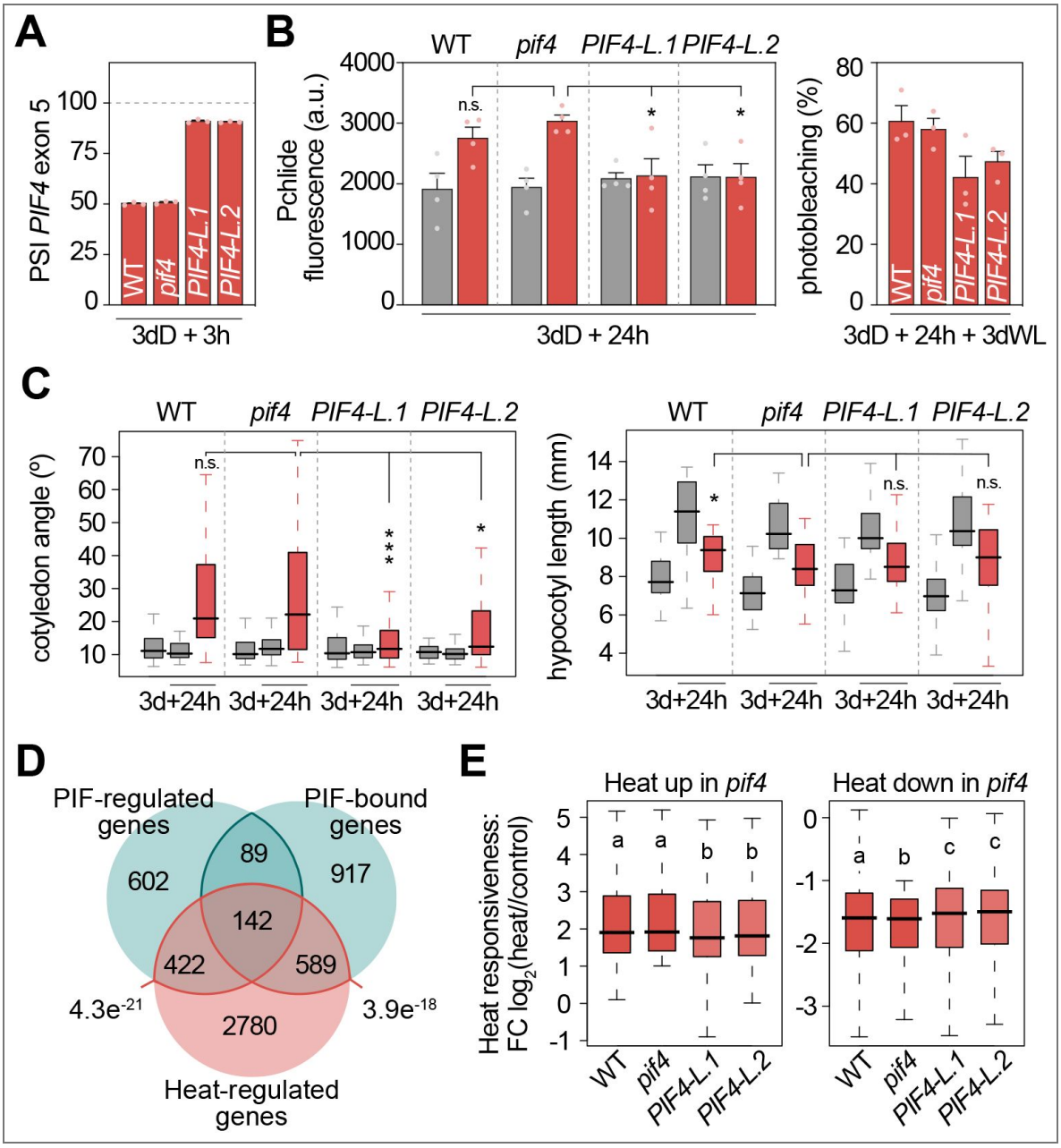

Figure 3. Enhancing expression of the *PIF4* long isoform at 37°C reduces the impact of heat stress in etiolated seedlings.

(A) PSI (Percent Spliced In) quantification of the *PIF4* alternatively-spliced exon in wild-type (WT), *pif4* and *PIF4-L* seedlings grown in continuous dark for 3 days (d) and then transferred to 37 °C for 3 additional hours (h) in darkness. Data represent means ± SEM of biological triplicates. **(B)** Protochlorophyllide (Pchlide; 635 nm) fluorescence (left; *n*=4) and percentage of photobleaching (right; *n*=3) in 3-day-old etiolated WT, *pif4* and *PIF4-L* seedlings transferred to 37 °C (red) or kept at 22 °C (gray) for 24 additional hours (h) in the dark. For photobleaching quantification, seedlings were subsequently exposed to white light (WL) for 3 days. Data represent means ± SEM of biological replicates (t-test). a.u., arbitrary units. **(C)** Boxplot representations of the cotyledon aperture (left) and hypocotyl length (right) of at least 35 WT, *pif4* and *PIF4-L* seedlings grown as in (*B*). Mann Whitney test was used to define statistically differences. **(D)** Venn diagram showing overlap among heat-regulated genes in WT seedlings defined in this study and PIF-regulated and PIF-bound genes defined previously (Pfeiffer et al., 2014) (two-sided Fisher's exact test). **(E)** Heat responsiveness (fold change; FC) in WT, *pif4* and *PIF4-L* for heat-regulated genes in *pif4* seedlings (*n*=3). Different letters denote statistically significant differences between genotypes by Dunn's test (*P*<0.05). (A-C) Asterisks indicate statistically significant differences from *pif4* (**P*<0.05, ***P*<0.01 and ****P*<0.001; n.s., not significant), and *n* the number of biological replicates.

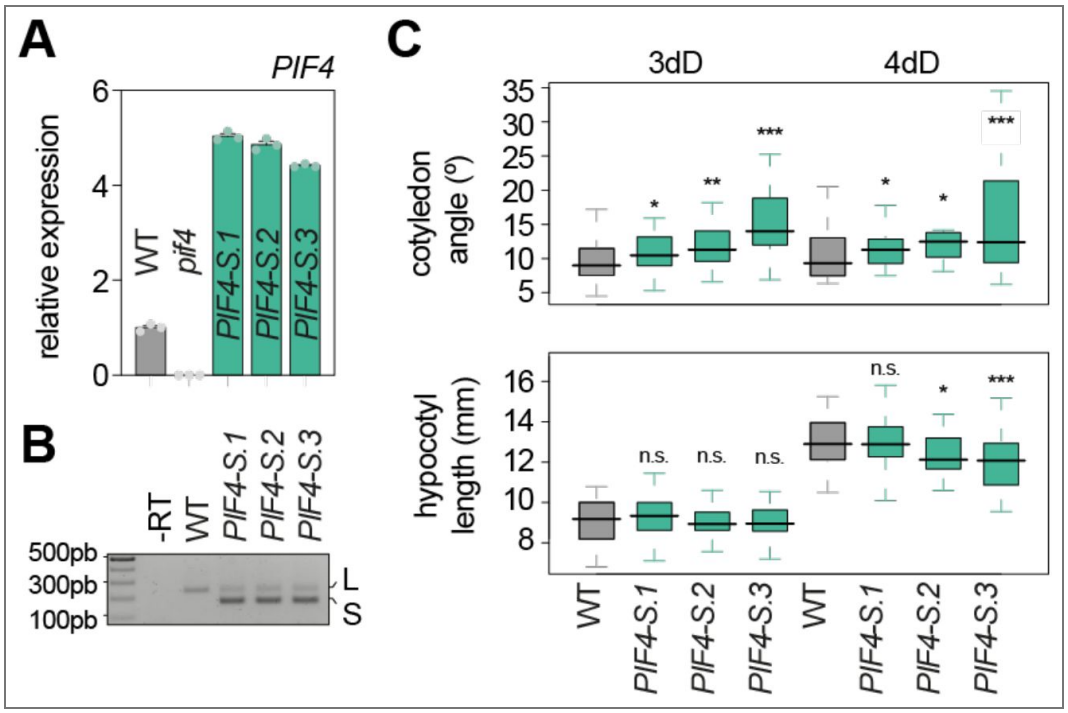

Figure 4. Enhancing expression of the *PIF4* short isoform promotes cotyledon opening in the dark.

(A) RT-qPCR analysis of *PIF4* transcript levels in wild-type (WT), *pif4* and *PIF4-S* seedlings grown for 4 days in darkness. Values were normalized to *PP2A* and expression levels are expressed relative to the WT. Data represent means ± SEM of technical triplicates. **(B)** RT-PCR of the alternatively-spliced exon of *PIF4* in seedlings grown as in (*A*). -RT, no reverse transcriptase. **(C)** Boxplot representations of the cotyledon aperture (top) and hypocotyl length (bottom) of at least 28 WT, *pif4* and *PIF4-S* seedlings grown for 3 or 4 days (d) in the dark (D). Asterisks indicate statistically differences from WT at each day (Mann Whitney test; **P*<0.05, ***P*<0.01 and ****P*<0.001; n.s., not significant).

Our data also indicate that the induction of these photomorphogenic traits in etiolated seedlings depends, at least in part, on a heat-specific regulatory event: the alternative splicing of *PIF4*. This represents a novel finding, as PIF4, despite being one of the most studied proteins in Arabidopsis, has been reported to be regulated only at the transcriptional and post-translational levels (Balcerowicz, 2020; Favero, 2020). Notably, our phenotypic analyses of seedlings with altered patterns of *PIF4* alternative splicing (*PIF4-L* and *PIF4-S*) suggest that the heat-induced isoform plays a role specifically in controlling cotyledon-related phenotypes. This implies that the mechanisms reported by Karayekov to control hypocotyl elongation under heat stress may operate in parallel with the alternative isoform of *PIF4*. Because our transcriptomic experiment was conducted using whole seedlings, we were unable to assess organ-specific effects in detail. We hypothesize that this alternative splicing event may be organ-specific or, alternatively, that the protein encoded by the heat-induced *PIF4* isoform may be preferentially active in cotyledons due to specific protein interactors and/or molecular targets. Further research into organ-specific dynamics is needed to elucidate why this alternative splicing event appears to predominantly affect cotyledon development. These findings would provide valuable insights into the organ-specific roles of PIF proteins, an emerging area of research (Dong et al., 2019; Sun et al., 2016; Zhang et al., 2021).

Our study focuses on the role of PIF4 under heat stress, a condition in which its function remains poorly understood. In fact, the few previous reports linking PIF function to heat stress have yielded contrasting results, with PIFs acting as either positive (Li et al., 2021) or negative (Yang et al., 2022) regulators of the response. Here, we significantly expand the current understanding of this transcription factor by demonstrating its involvement in heat stress and and further reinforcing its role as a key integrator of diverse environmental signals in the regulation of plant development (Paik et al., 2017). Importantly, modulating alternative splicing to alter isoform abundance is emerging as a promising strategy for developing stress-resilient plants (Alhabsi et al., 2025). In this context, investigating the role of the *PIF4* alternative isoform in heat-stressed adult plants, along with molecular strategies that specifically target the splicing sites involved in its regulation, could reveal a novel molecular target and offer an alternative genetic approach to enhance plant stress tolerance.

Methods

Plant materials

The *Arabidopsis thaliana pif1-1pif3-3pif4-2pif5-3* (*pifq*) and *pif4-2* mutant was obtained from the Nottingham Arabidopsis Stock Centre (NASC). *PIF4-L* transgenic plants expressing the *PIF4* coding region driven by the endogenous promoter, together with its respective *pif4* mutant control (*pif4-101*; Lorrain et al., 2008) were kindly provided by U. Pedmale (Cold Spring Harbor Laboratory, USA). *PIF4-S* transgenic plants were generated by PCR amplification and cloning a 1227-bp fragment containing the coding sequence region of the short splice variant under the control of a 2505-bp fragment upstream the PIF4 start codon corresponding to the PIF4 promoter, in the eGFP-tagged version of the binary pBA002 vector using the XbaI/AatI restriction sites (5'-GACGTTTCTAGAATGGAACACCAAGGTTGGAG-3'and 5'-GTGACGTCCGAGTGGTCCAAACGAGAAC-3'). The pPIF4 promoter was insertved into the promoterless pBA002 via HindIII/XbaI restriction sites (5'-TGTGAAGCTTCCAAAGTAATAAAAGTTGCCACAAC-3'and 5'-GACGTTTCTAGAGTCAGATCTCTGGAGA CATTTC-3'). The resulting constructs were introduced into *Agrobacterium tumefaciens* strain EHA105 and subsequently used for agroinfiltration-mediated transformation of Col-0 seedlings (Clough and Bent, 1998).

Phenotypical and photobleaching analysis

Sterile seeds were sown on MS medium containing 1X Murashige and Skoog (MS) salts (Duchefa Biochemie), 2.5 mM MES (pH 5.7), 0.5 mM myo-inositol, and 0.8% agar (w/v). After stratification for 4 days at 4 ºC in darkness, seeds were subjected to a 3-hour light pulse to induce germination and then transferred to continuous darkness for 69 hours at 22 ºC. Maintaining the absence of light,

seedlings were then either kept at 22 ºC for control conditions or transferred to 37 ºC for heat stress. Hypocotyl and cotyledon measurements of at least 30 seedlings and two biological replicates were carried out using the National Institutes of Health ImageJ software as described before (Sentandreu et al., 2011). Pictures were taken before and after exposure to stress as indicated in each figure. Photobleaching experiments were adapted from previous studies (Leivar et al., 2009), with 3-day-old etiolated seedlings being grown under control conditions or at 37 ºC for 24 hours and then transferred to continuous white light for 3 additional days (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$). At this point, the percentage of seedlings that failed to become green were scored in three biological replicates.

Protochlorophyllide quantification

Approximately 30 sterile seeds, sown on MS medium and stratified for 4 days at 4 ºC in the dark, were subjected to a 3-hour light pulse before being transferred to continuous darkness for 69 hours at 22 ºC. Seedlings were then either kept under these conditions (control) or transferred to 37 ºC in the dark to induce heat stress. Whole seedlings were collected in the dark 24 hours later, flash-frozen in liquid N_2,and ground before extraction with 0.75 mL ice-cold 9:1 acetone:0.1 M NH4OH, as described previously (Terry and Kacprzak, 2019). The resulting mixture was vortexed for 1 minute and then centrifuged at 14,000 rpm at 4 ºC for 5 minutes. After supernatant recovery, the protochlorophyllide (Pchlide) content was determined as the peak value (635 nm) of the fluorescence emission spectrum between 600-700 nm, measured with a bandwidth of 5 nm after excitation at 440 nm and using a Synergy Neo2 microplate reader (Biotek). Pchlide data is shown as the average of Pchlide per seedling of four biological replicates.

Gene expression and PSI quantification from RNA extraction

Total RNA was extracted from *Arabidopsis thaliana* seedlings using the InnuPREP Plant RNA kit (Analytik Jena BioSolutions) and 1 µg treated with DNase I to remove genomic DNA. cDNA synthesis using the oligo dT primer and the enzyme SuperScript III reverse transcriptase (Invitrogen) was conducted in the presence of RNase Out (Invitrogen). The cDNA was then used to quantify either gene expression or exon skipping of *PIF4*'s fifth exon. In both cases, three biological replicates were analyzed for each condition and/or genotype tested. Gene expression was measured by Reverse Transcription-quantitative PCR (RT-qPCR) using a QuantStudioTM 7 Flex Real-Time PCR System 384-well format and the Absolute SYBR Green ROX Mix (Thermo Scientific) on 2.5 µL of cDNA (diluted 1:10) per 10 µL of reaction volume, containing 300 nM of each gene-specific primer (see below). The *PP2A* gene was used for normalization (Shin et al., 2007). Exon skipping of the fifth exon of *PIF4* was quantified from RT-PCRs using primers spanning the two adjacent exons. These primer sequences were obtained from PastDB (Plant alternative splicing and transcription Data Base; *www.pastdb.crg.eu*; Martín et al., 2021). The resulting bands were quantified using the National Institutes of Health ImageJ software.

RNA sequencing

RNA was extracted from 3-day-old WT, *pif4-101*, *PIF4-L.1* and *PIF4-L.2* etiolated seedlings grown for 3 hours at 37 ºC or 22 ºC in complete darkness. Oligo dT, non-strand specific libraries from triplicate biological replicates were built and sequenced using NextSeq500 at the Gulbenkian Institute for Molecular Medicine (GIMM). An average of 15 million 75-nucleotide single-end reads were generated per sample. Raw sequencing data was submitted to the Sequence Read Archive (accession number GSE200247).

Gene expression quantification from RNA sequencing data

Quantification of *Arabidopsis thaliana* transcript expression from our RNA-seq experiment (GSE200247) and public sequencing data (Dataset S1) was performed using vast-tools v2.5.1 and v2.2.2 (Martín et al., 2021; Tapial et al., 2017), respectively. For each Arabidopsis transcript, this tool provides the number of mapped reads per million mapped reads divided by the number of uniquely mappable positions of the transcript (cRPKM; corrected-for-mappability reads per kbp

of mappable sequence per million mapped reads) (Labbé et al., 2012). To identify genes differentially expressed between different temperatures, we used vast-tools compare_expr using the option -norm to perform a quantile normalization of cRPKM values between samples. Next, we filtered out the genes that were not expressed at cRPKM > 5 and had read counts > 50 across all the replicates of at least one of the samples compared. Finally, differentially-expressed genes were defined as those with a fold change of at least 2 between each of the individual replicates from each genotype. See *https://github.com/vastgroup/vast-tools* for details.

PSI quantification from RNA sequencing data

We employed vast-tools v2.2.2 to quantify alternative splicing from public sequencing data (Martín et al., 2021; Tapial et al., 2017). This tool quantifies exon skipping (ES), intron retention (IR) and alternative donor (ALTD) and acceptor (ALTA) site choices. For all these types of events, vast-tools estimates the Percent Spliced In (PSI) of the alternative sequence using only exon-exon (or exon-intron for IR) junction reads and provides information about the read coverage See *https://github.com/vastgroup/vast-tools* for details. Data shown in Figure 1 and Supplemental Table 1 indicate the PSI quantification of specific alternative splicing events in the subfamily XV of the bHLH transcription factors (see below) using a wide array of samples (Supplemental Table 1).

Data availability

RNA-seq data have been deposited in Gene Expression Omnibus (GEO) (GSE200247).

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Additional information

Author contributions

M.N.-G., B.A., D.S., T.L. and G.M performed the experiments and analyzed the data. All authors discussed the results. G.M. conceived the project and designed research. G.M. and P.D. wrote the manuscript.

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Additional files

Supplementary file. Supplemental Figures 1-10 and Supplemental Tables 1-2.

Supplemental Table 1. List of publicly available RNA sequencing samples used. Samples available at the Short Read Archive (SRA) were used to quantify the PSI (Percent Spliced In) and expression values shown in Figure 1, Supplemental Figure 2 and Supplemental Figure 3. Columns indicate our sample classification based on their growth conditions and tissue source, which are summarized in the sample description column, as well as the original SRA code number and name. For simplification PSI quantification was performed in samples originated from merging the biological replicates.

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Peer reviews

Reviewer #1 (Public review):

This manuscript by Niño-González and collaborators shows that PIF4 undergoes alternative splicing in response to elevated temperature, generating distinct isoforms that may contribute to early seedling responses of Arabidopsis thaliana to heat stress (37 {degree sign}C). This work provides an intriguing perspective on how PIF activity may be modulated under stress conditions.

The authors report rapid heat-induced changes in seedling morphology, with cotyledon angle and hypocotyl length altered as early as 3 hours after transfer to 37 {degree sign}C. These responses correlate with a transient increase in PIF4 transcript levels, followed by a return to control values at later time points. Notably, heat induces preferential production of an exon 5-skipping isoform of PIF4. The resulting short protein variant (PIF4-S) lacks part of the bHLH domain and is therefore unlikely to be transcriptionally active.

To explore functional consequences, the authors expressed the exon 5 inclusion (functional) isoform, PIF4-L, in the pif4-101 mutant background. Some heat-induced phenotypes, such as protochlorophyllide accumulation and subsequent photobleaching, were reduced or absent in these lines. Interestingly, pif4-101 mutants themselves largely resemble WT plants for most heat-responsive traits, with the exception of hypocotyl length. PIF4-L expression specifically attenuates the cotyledon angle response to heat, without strongly affecting hypocotyl elongation.

An important point is that PIF4 itself is not essential for the observed heat responses, as pif4 mutants respond largely like wild-type plants. This implies that the phenotypes described are likely controlled by multiple PIFs acting redundantly. In this context, the generation of the PIF4-S isoform may represent one of several mechanisms by which heat stress reduces overall functional PIF levels, rather than a PIF4-specific regulatory switch.

Other caveats should be considered when interpreting the work. The functional relevance of the PIF4-S isoform under heat stress is not tested, as heat responses of these transgenic lines were not examined. Transcriptome analysis of heat-stressed WT, pif4-101 mutant, and PIF4-L-expressing plants revealed an enrichment of PIF-regulated genes, supporting a possible role for this family of transcription factors in the heat stress response. Notably, the heat responsiveness of the mutant and of the transgenic lines differs only marginally from that of WT plants. In addition, the study relies primarily on total transcript-level analyses, without quantitative assessment of individual PIF isoforms or direct measurement of PIF protein abundance. Given that other PIFs are also expressed and may be subject to alternative RNA processing, it needs to be determined whether PIF4-S alone could exert a dominant effect, counteracting all the other functional PIFs by itself, under heat stress. Hence, the proposed model is a plausible but still incomplete framework that requires further experimental validation and analysis.

Altogether, the results presented in this manuscript could also be interpreted as follows: multiple PIFs contribute to the observed phenotypes in response to heat, with overlapping (redundant) functions. Heat stress may reduce functional PIF levels through different mechanisms, one of which is the regulation of alternative splicing, as shown here for PIF4, leading to the production of non-functional proteins or protein variants that could act as negative competitors (such as PIF4-S). Restoring PIF levels to values of control conditions could therefore reverse heat-induced phenotypes, as observed in the PIF4-L expression lines.

Main concerns:

(1) The existence of a shorter isoform of PIF4 and PIF6 is relevant, and PIF4 could indeed play a role in the context of heat stress, as it does in thermomorphogenesis. In this sense, the interplay between PIF4-S and PIF4-L might be linked to plant morphological responses to heat; however, the present work requires further investigation to determine whether this is indeed the case. It is important to note that pif4 mutants behave similarly to WT plants, indicating that PIF4 is not necessary for the observed responses. These phenotypes are therefore most likely related to several PIFs rather than to one specific family member. The results obtained with the transgenic lines expressing PIF4-L or PIF4-S support this interpretation, as increasing a functional PIF (PIF4-L) reduces some phenotypes, while expressing a dominant-negative version mimics heat-induced phenotypes under control conditions. Thus, it is reasonable to interpret that under heat stress, functional PIF levels are reduced through multiple mechanisms, alternative splicing and PIF4-S generation being one of them in the case of PIF4, but likely with additional effects on other family members. This clearly requires further study.

(2) RT-qPCR quantification of total PIF4 transcripts, as well as the long and short isoforms under the tested conditions, is necessary. While we agree with the authors that PIF4-S could act as a dominant-negative factor, demonstrating this requires comparison of phenotypes under heat versus control conditions using the PIF4-S transgenic lines. Importantly, for the authors' hypothesis to be valid, PIF4-S must be able to outcompete other PIFs; therefore, accurate quantification of its expression levels across conditions is crucial. Combining the results shown in Figures 2A and Figure 2G suggests that the levels of the functional PIF4-L isoform are unchanged or even reduced after 3 h of heat treatment, as the increase in total PIF4 does not fully compensate for the diversion toward PIF4-S. Additionally, it would be equally relevant to quantify the expression of other PIFs (or at least those shown in Suppl. Fig. 6) to determine whether PIF4-S could exert such a strong effect even when expressed at relatively low levels. By "proper quantification", we refer specifically to functional protein-coding variants, as in the PIF4-L case. Supplemental Figure 6 shows that PIF3 and PIF5 appear unaffected by heat, while PIF1 expression is increased. However, JBrowse data for dark-grown seedlings indicate that PIF1 is subject to alternative transcription initiation, alternative splicing, and alternative polyadenylation at its 3' end. A similar situation occurs

for PIF3, at least at the 5' end of the transcriptional unit. Therefore, alternative RNA processing mechanisms may play a key role in modulating functional PIF protein levels in response to heat. Without considering diverted isoforms of other PIFs, the interpretation becomes problematic, as PIF1 is upregulated by heat, and PIF4-S would therefore need to overcome its activity as well. This is particularly relevant given that the cotyledon angle phenotype at 37 {degree sign}C appears even stronger than in the pif1pif3pif5 triple mutant, if such a comparison is feasible.

(3) In addition, PP2A is a well-established housekeeping gene for normalization across different light regimes, as its expression is not affected by light. However, we are not convinced this holds true under heat stress conditions (see Li et al., Plant Cell 2019 Jul 29;31(10):2353-2369. doi:10.1105/tpc.19.00519).

(4) Furthermore, the mechanistic conclusions would be strengthened by directly assessing PIF protein levels, for example, by western blot analysis, to determine whether changes in transcript isoform abundance translate into corresponding changes in protein accumulation under heat stress.

(5) Importantly, the authors' interpretation that "PIF4-L.1 expresses the long isoform at levels similar to those of WT plants (Supplemental Figure 9A), ruling out the possibility that the suppression of heat-induced phenotypes (cotyledon opening and Pchlide accumulation) is due to elevated PIF4 expression levels" is not correct. The RT-qPCR assay quantifies all isoforms containing exon 6, which include both long and short variants with respect to exon 5 inclusion. Since WT plants at 37 {degree sign}C express both isoforms (L/S ≈ 60/40), the PIF4-L lines actually express 2-4-fold higher levels of the functional PIF4 isoform, based on the values shown in the figures.

(6) Figure 3B should include a statistical analysis, as it appears that PIF4-L expression does not significantly reduce photobleaching. Cotyledon angle is not affected by either the pif4 mutation or PIF4-L expression under 22 {degree sign}C conditions (Figure 3C). However, after 24 h at 37 {degree sign}C, there is a clear effect, with cotyledon angles closer to those observed in WT plants at 22 {degree sign}C. Regarding hypocotyl length, although statistical testing was not performed, it is evident that pif4-101 affects this parameter, while PIF4-L expression in this background does not substantially alter the mutant response.

Other comments:

(1) We do not believe that Figure 3E is an optimal way to demonstrate attenuation of transcriptional changes by PIF4-L expression in pif4 mutants. A heat map representation would likely be more direct and informative.

The authors should consider expressing another functional PIF in the pif4 mutant background to determine whether the observed effects are specific to PIF4, as proposed, or whether they reflect a general PIF function.

(2) It would also be informative to examine the response under Light + 37 {degree sign}C conditions. Since PIF4 mRNA accumulation is induced by light, the authors should test whether plants incubated in light show a similar response to heat or whether it is attenuated. Potential cross-regulation between light and heat responses would be worth exploring.

(3) As the authors acknowledge in the introduction, most of our knowledge regarding PIFs in temperature signalling has focused on thermomorphogenesis. Therefore, we believe it is important to place these new findings (exon 5 skipping) within that framework, as they could help explain observations made under better-characterized conditions. In addition, would be interesting to see the phenotypes of the pifq mutant under heat stress. Even though this mutant line displays a heat-stress-like phenotype under control conditions, it may still

respond to heat treatment. If so, this would indicate that PIFs are not fully determinative of this response.

(4) The authors should clearly state the genetic background of the PIF4-S expression lines, which appear to be in the pif4-101 background but are not explicitly described as such in the manuscript.

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Reviewer #2 (Public review):

The manuscript "Alternative splicing of PIF4 regulates plant development under heat stress" by Niño-González et al. describes a heat-responsive alternative splicing (AS) event in PIF4 in Arabidopsis and its potential impact on seedling development. The authors observe that etiolated ings exposed to heat respond with a more photomorphogenic developmental behaviour, as reflected, for example, by increased cotyledon opening and reduced hypocotyl elongation. They propose that the AS event in PIF4 may contribute to this response, due to reduced formation of the full-length PIF4 protein and an increase in the shorter PIF4 protein with potentially dominant negative functions.

Expressing the individual variants in a pif4 mutant background was used to further examine their function. In the case of the full-length PIF4 variant, some of the heat-induced phenotypes were suppressed. For the lines overexpressing the shorter PIF4 variant, heat responses were not examined.

The authors describe an interesting phenotype and present an appealing model of how AS of PIF4, a well-known key regulator of developmental processes including light- and temperature responses, might be involved. However, I don't think that the authors provide strong evidence for their model, and the unaltered heat response of pif4 mutants argues against a major role of this gene and its AS event under these conditions. Regarding the heat responses, it remains open how distinct those are from thermomorphogenesis.

Weaknesses:

(1) In the manuscript, it is emphasized that previous studies on PIFs' role in temperature responses have mainly focused on thermomorphogenesis under high ambient temperature and not under hot temperatures causing heat stress. How do the authors know that the effects they are looking at are specific to hot temperatures and do not also occur at more moderate temperature increases? So, what would PIF4 splicing look like upon a shift from 22{degree sign}C to 28{degree sign}C (instead of 37{degree sign}C as used in the manuscript)?

(2) The potential role of PIF4 and its AS event in the heat response is the key point of this manuscript, as also reflected by the title. As summarized above, I don't see direct evidence for this and a functional characterization of the AS event is lacking. First, the pif4 mutant doesn't show an altered response, which argues against its requirement under these conditions, and in particular against the proposed model that a shortened version of PIF4 acts in a dominant negative manner. Second, the impact of AS on PIF4 protein levels remains open. Antibodies against PIF4 exist and have been used before, e.g. in Lee et al. (2021), Nat Comm, and Fan et al. (2025), Nat Comm - both studies address the role of PIF4 in thermomorphogenesis and should also be discussed in this manuscript. Detecting PIF4 proteins would allow testing if indeed both PIF4 protein variants are detectable and whether, upon heat stress, the longer variant decreases while the shorter variant increases. This could be expected based on transcript data; however, due to regulation at multiple steps, a correlation between transcript and protein levels might not exist. Third, the transgenic lines expressing either the short or long PIF4 variant do not really reflect the situation in the wild type and might be/are overexpression lines. Specifically, constructs for both variants lack the UTRs according to the

description in the method section. Furthermore, is the short version expressed as GFP fusion, as I understood from the method description? The PIF4-L mutants have similar PIF levels as the WT (SFig. 9); however, this refers to total transcripts, which makes a difference in the wild type, in particular under heat stress. Comparing here only the PIF4-L levels would be more informative. Accordingly, the transgenic lines may overexpress PIF4-L compared to the wild type. All the PIF4-S lines show 4 to 5-fold overexpression (again for total transcripts) compared to WT. Including lines with lower overexpression levels would be needed for a direct comparison to the wild type. Moreover, immunoblot analysis of the PIF4 protein would be needed for a direct comparison between the wild type and the two types of mutants.

(3) Apart from the question of what level of (over)expression the transgenic lines have, several aspects of the phenotyping experiments are not in line with a simple model of PIF4 regulation or have not been addressed. Expressing the long PIF4 variant in the pif4 mutant background suppresses some of the heat-induced changes, but not the hypocotyl shortening, suggesting that the hypocotyl effect is not caused by a heat-induced lack of PIF4.

When expressing the short variant, the authors observe increased cotyledon opening in darkness, consistent with a suppression of skotomorphogenesis due to a negative function of PIF4-S, at least when it is overexpressed. For hypocotyl length, no consistent difference between wild type and PIF4-S lines was observed: seedlings grown for 3 d in darkness had identical lengths, for 4-d-old seedlings, the PIF4-S lines did not give consistent results: PIF4S.1 (which has highest transgene expression) had same length as wild type; a pronounced difference was only seen for PIF4-S.3, which is the line with lowest expression. Have the experiments been reproduced with independent seed badges? I'm also wondering why the authors haven't performed the heat stress experiments with these PIF4-S lines, as they did for the PIF4-L mutants. According to the authors' model, the PIF4-S lines might show an opposite response compared to the PIF4-L lines, i.e. an even more pronounced heat effect compared to the wild type.

(4) Why was the heat effect on AS of PIF6 not further analysed? Previous work showed the role of PIF6 in seed development and germination; in line with this, PIF6 expression is particularly high in embryos and seeds, but it is also expressed and alternatively spliced in other tissues and conditions, as shown in Figure 1 and SFigure 2. From the data in Figure 1, it looks like the AS pattern in heat might also be different from other conditions. So, it would be interesting to see how AS of PIF6 changes in the control and heat samples that the authors analysed for PIF4 AS, in particular, if this response is distinct for PIF4 versus PIF6.

(5) The presentation of the RNA-seq data is incomplete. According to the method section, WT, pif4-101, PIF4-L.1 and PIF4-L.2 seedlings upon 3 h heat/control treatment were analysed. Why are DE and DAS genes and comparisons of different genotypes not shown? The FC data displayed in Figure 2E and the overlap between heat-regulated genes (Fig. 3D; only in WT) and PIF regulation show only some aspects of the data.

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Reviewer #3 (Public review):

Summary:

PIFs play a pivotal role not only in light and temperature signaling pathways, but in many other signaling pathways regulating plant development by modulating transcription of a large number of genes both directly and indirectly. Similarly, alternative splicing (AS) plays a critical role in shaping the splice isoforms of thousands of genes under different environmental conditions to regulate plant development. In fact, AS of PIF6 has been shown to be involved in seed development. PIF4 is a central transcription factor integrating light and temperature signaling pathways. However, AS of PIF4 has not been involved in any

pathways. This story first describes how AS of PIF4 is regulated by heat stress, and this regulation is involved in heat stress signaling to regulate plant development. This is an important finding of general interest.

Strengths:

The authors first describe AS of PIF4 is regulated by heat stress, and this regulation is involved in heat stress signaling to regulate plant development.

Weaknesses:

There are many loose ends in this story that need to be tied up.

Major points:

(1) The authors are showing only the AS transcripts by PCR, but no protein data. Given that the hypothesis is that the short form of PIF4 is functioning in a dominant negative fashion, the authors need to show that this short isoform expresses a protein. In addition, they need to show that this form is functioning in a dominant negative fashion with other PIFs, either by showing that this form reduces the DNA binding and/or transcriptional responses of other PIFs.

(2) The two mutant alleles used for this study (pif4-100 and pif4-2) have T-DNA insertion after the AS exon. Do these alleles express any short version of the protein? The previous studies showed no protein production, and thus, they may not function as a dominant negative form. Usually, the T-DNA insertion alleles may express truncated transcripts, but many do not express any protein due to a lack of stop codon and/or degradation of the transcripts. But in this case, the mutants are behaving like WT. The authors need to show that these alleles are expressing a truncated version of the PIF4 protein.

(3) Figure 4 shows phenotypes of independent lines expressing the PIF4 short version. The authors analyzed only the cotyledon and hypocotyl phenotypes, but not Pchlide or bleaching assays. The authors need to do a thorough phenotype analysis, including heat-stress phenotypes of these lines, to test if the data make sense with their hypothesis.

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Author response:

We would like to thank the Editor and the three Reviewers for their detailed assessment of our manuscript and their constructive feedback. We found the suggestions valuable for refining our work. Before presenting the fully updated manuscript, we would like to clarify a few points in this initial response. This manuscript identifies a heat-induced, alternativelyspliced short isoform of *PIF4* (*PIF4-S*) that contributes to the physiological responses observed in heat-stressed etiolated seedlings. First, we agree with all Reviewers that including PIF4 protein data will strengthen our findings an more definitely demonstrate the generation of a protein-coding alternative isoform under heat stress. Therefore, this will be one of our main priorities in the revision. Evidence for the functionality of this alternative isoform is clearly demonstrated by the distinct phenotypes exhibited by transgenic lines expressing either the long or the short versions of *PIF4*. Nevertheless, we agree that a more comprehensive characterization of these lines, as well as of the *pif4* mutant lines, will further strengthen the demonstration of the functional relevance of this alternative splicing event. In addition, we will extend the phenotypic analysis of the *PIF4-S* lines to heat stress conditions. Importantly, the phenotypes observed in these lines suggest that additional molecular mechanisms may act in parallel with this alternative splicing event to regulate development in heat-stressed etiolated seedlings. As proposed by Reviewer #1, other PIFs may be involved in this response, and we will address this possibility. We will also provide new experimental

data to show that alternative splicing in this gene is specific to heat stress and does not occur in other PIFs. Finally, we would like to clarify that the main scope of this manuscript is to demonstrate the functional relevance of the alternative isoform generated by splicing in *PIF4* under heat stress. A detailed investigation of its molecular mode of action is beyond the scope of the present study. We sincerely appreciate the thoughtful feedback provided by all Reviewers. We will carefully consider their suggestions and use them to guide the inclusion of additional experiments and analyses in our revised manuscript to reinforce and clarify our conclusions.

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