ELF3 polyQ variation in *Arabidopsis thaliana* reveals a PIF4-independent role in thermoresponsive flowering.

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Abstract

Plants have elaborate genetic mechanisms controlling developmental responses to environmental stimuli. A particularly important environmental parameter is temperature. Previous work has identified ELF3 and PIF4 as key players in the *Arabidopsis thaliana* temperature response, and the ELF3 polyglutamine (polyQ) domain as a source of functional variability in ELF3. We used transgenic analysis to test the hypothesis that ELF3 polyQ variation modulates temperature sensing as an input to temperature-mediated morphological changes. We found little evidence that the polyQ domain has a specific role in temperature sensing beyond mediating overall ELF3 function. Instead, we made the serendipitous discovery that ELF3 plays a role in thermoresponsive flowering at elevated temperatures, a response previously shown to require PIF4. Unexpectedly, ELF3's role in thermoresponsive flowering was independent of PIF4. Here, we present evidence that ELF3 acts through the photoperiodic pathway, indicating a previously unknown symmetry between low and high ambient temperature responses. These findings tie together disparate observations into a coherent framework in which multiple pathways converge in accelerating flowering in response to temperature, with some such pathways modulated by photoperiod.

Introduction

The responses of plants to temperature variation are of central importance to food security in a changing world (Battisti and Naylor, 2009). The elucidation of genetic pathways underlying these responses is therefore a central mission of plant science (Quint et al., 2016). Many studies have made impressive strides in understanding the phenomena of circadian temperature compensation (Gould et al., 2006; Edwards et al., 2006; Thines and Harmon, 2010), thermoresponsive flowering (Blázquez et al., 2003; Balasubramanian et al., 2006; Strasser et al., 2009; Kumar et al., 2012; Song et al., 2013), and temperature effects on plant morphology (Koini et al., 2009; Johansson et al., 2014; Lee et al., 2014; Box et al., 2014; Mizuno et al., 2014b; Raschke et al., 2015). Various studies have converged on PIF4 as a master regulator of temperature responses, and ELF3 as an input to PIF4 integration, among many other genes and

pathways. Given previously-described regulatory interactions between ELF3 and PIF4 (Nusinow et al., 2011; Nieto et al., 2014; Nozue et al., 2007), it is reasonable to predict that these components of thermal response operate in the same pathway for various thermal response phenotypes (Lorenzo et al., 2016). Recent reports support this expectation, focusing largely on hypocotyl elongation as a model for understanding this regulatory relationship (Box et al., 2014; Mizuno et al., 2014b; Raschke et al., 2015).

ELF3 serves to repress hypocotyl elongation by reducing PIF4 levels. This repression of PIF4 occurs at both the transcriptional level, through the role of ELF3 in the Evening Complex (EC) (Nusinow et al., 2011; Nozue et al., 2007), and at the post-translational level, through PIF4 destabilization by the phytochrome phyB in cooperation with ELF3 (Lorrain et al., 2008). Light sensing enforces circadian oscillations of the EC and other components, leading to calibration of the circadian clock (McWatters et al., 2000; Covington et al., 2001), which in turn results in diurnal repression of hypocotyl elongation through repression of PIF4 and its semi-redundant paralog PIF5 (Nusinow et al., 2011; Nozue et al., 2007). ELF3 also plays a crucial role as a flowering repressor (Zagotta et al., 1996). Consequently, *elf3* null mutants are skotomorphogenic, with elongated hypocotyls under light conditions, and flower early.

PIF4 is one of a family of basic helix-loop-helix (bHLH) “phytochrome-interacting factors” (PIFs), transcription factors with overlapping functions promoting skotomorphogenesis. Under dark conditions, the PIFs act to target phyB for ubiquitin-mediated degradation by the E3 ubiquitin ligase COP1, thereby repressing photomorphogenesis (Leivar and Quail, 2011). Under light conditions, degradation of PIFs is mediated by direct interactions with photoactivated phyB (Lorrain et al., 2008). PIF4 is distinct from the other PIFs in having specific roles in temperature sensing and flowering (Proveniers and van Zanten, 2013). *pif4* null mutants show short hypocotyls with photomorphogenic attributes even in the dark (Bai et al., 2012).

At elevated ambient temperatures (27º-29º), the wiring of these various signaling pathways and the role of these proteins change. Several independent studies have recently found that warm temperatures, specifically during dark periods (Thines et al., 2014), inhibit the activity of the EC by an unknown mechanism (Mizuno et al., 2014b; Box et al., 2014; Raschke et al., 2015), leading to increased expression of *PIF4* and its

targets (Koini et al., 2009; Proveniers and van Zanten, 2013). This increased PIF4 activity leads to several morphological temperature responses through various signaling pathways. For instance, hypocotyls are elongated following PIF4 induction of auxin signaling via an auxin biosynthesis pathway (Proveniers and van Zanten, 2013; Lee et al., 2014). *PIF4* is required for the acceleration of flowering at 27ºC under short photoperiods (Kumar et al., 2012; Thines et al., 2014). Chromatin accessibility at the *FT* promoter may also be a limiting factor for PIF4 binding (Kumar et al., 2012), though these observations have been disputed (Galvão et al., 2015). In contrast, under continuous light, *pif4* null mutants have an intact temperature-dependent acceleration of flowering (Koini et al., 2009). *pif1*, *pif3*, *pif4*, and *pif5* null mutants show no aberrant flowering phenotypes under standard growth conditions either singly or in combination (Shin et al., 2009). Lastly, *pif4* null mutants lose the normal elongation of petioles under high temperatures (Koini et al., 2009). It is unclear why PIF4 does not affect thermoresponsive flowering under continuous light; yet, this phenomenon may reflect low PIF4 levels under these conditions due to inhibition by phyB. Regardless of light conditions, however, PIF4 is always necessary for petiole elongation under increased temperatures. Moreover, under longer photoperiods and higher temperature a flowering acceleration still exists (Balasubramanian et al., 2006; Koini et al., 2009), requiring that some PIF4-independent thermoresponsive flowering pathway must be in effect at higher ambient temperatures. Recent reviews of the literature tend to emphasize the primacy of PIF4 in this response (Song et al., 2013; Wigge, 2013; Liu et al., 2015), although the condition of high ambient temperature with short photoperiods is probably rare in the field.

Modeling has suggested the existence of a “Y” component of temperature sensing upstream of PIF4 (Johansson et al., 2014). Other recent studies have identified ELF3 as a plausible candidate for Y, given its function upstream of PIF4 at room temperature (Nusinow et al., 2011), its role in temperature response (Mizuno et al., 2014b; Box et al., 2014; Raschke et al., 2015), its physical interaction with PIF4, and its post-translational inhibition of PIF4 (Nieto et al., 2014). However, further studies have implicated other candidates, such as FCA (Lee et al., 2014), and mathematical modeling has suggested that ELF3/EC complex regulation alone is insufficient to

explain PIF4 thermal regulation (Seaton et al., 2015; Box et al., 2014). The exact mechanism of this response has yet to be unraveled.

Specifically, the mechanism by which EC/ELF3 activity is reduced under elevated temperatures ("temperature sensing") is not known. We recently used transgenic experiments to demonstrate that ELF3 function is dependent on the unit copy number of its C-terminal polyglutamine (polyQ) tract (Undurraga et al., 2012). This protein region is likely disordered, and disordered proteins can evince structural changes in response to physical parameters such as temperature (Uversky, 2009). Thermal remodeling of this polyQ tract is a plausible mechanism by which ELF3 activity could be modulated through temperature. Moreover, this polyQ tract shows substantial natural variation, with most variants showing strong interactions with the genetic background, such that different backgrounds prefer their endogenous ELF3-polyQ variant (Undurraga et al., 2012). Furthermore, in flies, variable repeats are associated with local temperature compensation adaptations (Sawyer, 1997). In short, the ELF3-polyQ is an attractive candidate for adaptive variation in the ecologically relevant trait of temperature response (Gemayel et al., 2010).

In this study, we used previously described transgenic polyQ variants of ELF3 in two *A. thaliana* genetic backgrounds to dissect the contribution of the polyQ tract to temperature response, and to better understand ELF3 function in temperature sensing. We show that polyQ repeat copy number modulates temperature sensing by affecting ELF3 function. However, this effect appears to be largely the result of overall effects on ELF3 function rather than being specific to temperature. Surprisingly, we found that ELF3's role in thermoresponsive flowering appears to be entirely independent of PIF4 in the reference genetic background, Columbia. We postulate that ELF3's primary role in thermoresponsive flowering is PIF4-independent and occurs through the photoperiodic pathway, and that this role is in turn dependent on the genetic background.

Results

The hypocotyl elongation temperature response is modulated by the ELF3 polyQ tract affecting overall gene function.

Many recent studies have noted the involvement of ELF3 in temperature-dependent hypocotyl elongation (Mizuno et al., 2014a, 2014b; Box et al., 2014; Raschke et al., 2015), concluding that ELF3 protein activity is reduced under elevated temperatures, thereby relieving ELF3 repression of *PIF4* expression. *PIF4* up-regulation then leads to the observed hypocotyl elongation. We examined whether polyQ tract variation in ELF3 in two backgrounds affects hypocotyl elongation at 27º (Figure 1, Table S1). In the Ws background (Figure 1A), the endogenous ELF3 (16Q) partially rescues *elf3*, and another variant (9Q) fully rescues the hypocotyl temperature response. Other variants with more or fewer Qs rescue inefficiently. In the Col background, we observed a somewhat different pattern, though the same lines, in addition to the 20Q variant, showed partial rescue (Figure 1B, Table S1). However, the endogenous 7Q variant, among other variants, failed to rescue the response, consistent with previous observations that these transgenic lines are hypomorphic in the Col background (Undurraga et al., 2012). Overall, patterns of hypocotyl response to elevated temperature were not linear, though it is unclear in the Col background whether this might be due to poor complementation. Thus, the response to elevated temperature was similar to other, previously tested ELF3-dependent traits (Undurraga et al., 2012). Robust thermal responses were strongly correlated with the overall functionality of each ELF3 variant (Figure 1C), consistent with recent reports suggesting that the mechanism of ELF3 thermal response in hypocotyl elongation is the de-repression of PIF4 (Raschke et al., 2015; Nomoto et al., 2012; Mizuno et al., 2014b, 2014a, 2015; Box et al., 2014). Among Ws lines, which do show robust rescues with the endogenous variant, we cannot reject the hypothesis that the different transgenic lines respond to elevated temperature in proportion to ELF3 functionality. From our data, conclusions about the effects of polyQ variation on thermoresponsive hypocotyl elongation in the Col background would be premature. Nonetheless, our various transgenic lines remain informative as an allelic series of ELF3 function. In summary, one means by which ELF3 polyQ variation mediates thermally-responsive hypocotyl elongation is through affecting overall ELF3 functionality.

Expression of PIF4 and PIF4 targets as a function of temperature and ELF3. To evaluate the hypothesis that the temperature response defects in the transgenic lines is due to up-regulation of PIF4 and PIF4 targets, we measured transcript levels of *PIF4* and its target *AtHB2* in seedlings of selected lines from both backgrounds at 22ºC and 27ºC (Figure 1D). Like other groups (Raschke et al., 2015; Mizuno et al., 2014b), we observed an inverse relationship between ELF3 functionality and transcript levels of *PIF4* and *AtHB2*, and also that ELF3 function has a larger effect on expression of *PIF4* than of *AtHB2*. Similar to previous observations, there appeared to be an effect of elevated temperature on *PIF4* transcript levels independent of *ELF3* genotype. Col transgenic lines complement *PIF4* repression poorly, consistent with their poor complementation of hypocotyl phenotypes. We observed a small effect of elevated temperature on *AtHB2* expression in WT lines, but *AtHB2* responses in transgenic lines (even those with intact thermal responses) were less clear. We conclude that de-repression by ELF3 is one factor potentially controlling temperature-dependent PIF4 activation. Notably, the ELF3 lines with the strongest temperature response (for example 16Q in the Ws background) showed the most robust repression and de-repression of *PIF4* expression. However, it was not clear whether de-repression of PIF4 and its targets is sufficient to explain temperature response defects of *elf3* null mutants.

ELF3-polyQ variation affects thermoresponsive adult morphology and flowering time. Following the expectation that ELF3's temperature response acts through PIF4, we reasoned that ELF3 should also play a role in other PIF4-dependent thermal responses. One well-known response to elevated temperature involves adult morphologies, wherein leaf angle from the ground increases and petioles elongate. *pif4* mutants do not have these responses when grown at elevated temperatures (Koini et al., 2009). We therefore measured petiole length in our ELF3-polyQ transgenic lines, expecting that, due to general PIF4 de-repression, poorly-functioning ELF3-polyQ lines would show no response (perhaps due to constitutively elongated petioles, similar to hypocotyls; Figure 2). In stark contrast to these expectations, we found that all lines had a robust petiole response to temperature (Figure 2 A, B, Table S2). This effect was apparent in both Ws (Figure 2A) and Col backgrounds (Figure 2B). Moreover, this response is actually

accentuated in *elf3* null mutants and in poorly-functioning ELF3 polyQ variants (Figures 2A, B).

Further, we measured flowering time in transgenic lines as the number of rosette leaves at flowering (Figures 2C, D, Table S3). Accelerated flowering is a well-known temperature response for which *PIF4* is not required under longer photoperiods (Koini et al., 2009). Hence, we expected that loss of *ELF3* function should also not affect thermoresponsive flowering. In contrast to our expectations, in the Col background, *elf3* mutants had an abrogated flowering response to elevated temperature (Figure 2D). Moreover, most variants in the Col background entirely failed to rescue this phenotype. Previous work indicated that many of these lines fail to rescue other *elf3* null phenotypes (Undurraga et al., 2012), but the involvement of ELF3 in thermoresponsive flowering is unexpected. In contrast to Col, Ws is known to lack a robust flowering response to elevated temperature under these conditions (Ibañez et al., 2015), and indeed, variants in the Ws background generally showed no thermoresponsive flowering (Figure 2C). Thus, ELF3 polyQ variation does not suffice to enhance the negligible thermoresponsive flowering in the Ws background under these conditions. In light of this data, the roles of ELF3 and PIF4 in the elevated temperature response appear to be independent of one another under these experimental conditions. This result is intriguing, given that the PIF4 pathway is the best-recognized mechanism for thermoresponsive flowering at high temperatures (Song et al., 2013; Wigge, 2013; Kumar et al., 2012; Liu et al., 2015). Therefore, we suggest that ELF3 acts in a PIF4-independent pathway for thermoresponsive flowering at high temperatures, similar to the acknowledged complexity of thermoresponsive flowering at lower temperatures (Strasser et al., 2009; Blázquez et al., 2003; Song et al., 2013).

ELF3 regulates thermoresponsive flowering under long days, and is not required for PIF4-dependent thermoresponsive adult morphologies. To directly address the relationship of ELF3 and PIF4 in adult thermoresponsive phenotypes, we grew *pif4* and *elf3* mutants with various thermal treatments. Previous experiments showing *pif4* thermoresponsive adult phenotypes were grown in continuous light and transferred to 27ºC at 14 days (Koini et al., 2009), whereas we transferred from 22ºC to 27ºC at day

one. Hence, it was possible that the observed inconsistencies between *elf3* and *pif4* effects on adult thermoresponsive phenotypes were a trivial consequence of experimental conditions. For instance, exposure of early seedlings to elevated temperature may have unexpected consequences in later ontogeny. Consequently, we tested both transfer conditions under long days to ensure that our results were comparable to previous experiments (Figure 3). We found that the effect of different experimental treatments is negligible, though the longer 27ºC treatment (transfer at day one) showed a slightly stronger morphological response than the shorter 27ºC treatment (transfer at 14 days, Figure 3A, B). Further, our results under long days replicated previous observations under continuous light (Koini et al., 2009), showing that PIF4 is essential for petiole elongation (Figure 3B), but dispensable for thermoresponsive flowering (Figure 3C). Our PIF4 results were in direct contrast to ELF3, which was dispensable for petiole elongation (Figure 3B), but essential for thermoresponsive flowering (Figure 3C). Indeed, petiole elongation is potentially hyper-responsive in *elf3* (Figure 3A, Figure 3B). These results confirm the apparent independence of ELF3 and PIF4 in these specific responses, and the independence adult thermal responses from early seedling temperature stimuli.

One open question was whether the dispensability of ELF3 for petiole elongation reflected increased importance of other inputs to PIF4, such as FCA, which is involved in PIF4-dependent thermoresponsive petiole elongation in seedlings (Lee et al., 2014). We therefore measured adult thermoresponsive petiole elongation in *fca* mutants (Figure 3D), and unexpectedly found no substantial difference between *fca* mutants and WT Col. We suggest that while PIF4 influences both adult petiole and hypocotyl elongation in response to elevated temperatures, regulatory rewiring across development occurs by removing FCA and ELF3 as inputs to PIF4-dependent thermomorphogenesis.

A second question was whether *elf3* still affects thermoresponsive flowering in the Ws strain under other conditions; for instance, the closely related Ws-2 strain evinces a robust thermoresponsive flowering acceleration at 22ºC relative to 16ºC but not at 28ºC relative to 22ºC (Ibañez et al., 2015). We therefore assayed flowering in Ws and the Ws-derived null mutant *elf3-4* at these temperatures (Figure 3E). Under these

conditions, Ws robustly accelerated flowering at 22ºC, whereas *elf3-4* showed no perceptible difference in flowering between the two temperatures. Thus, ELF3's role in thermoresponsive flowering is not restricted to the Col strain or a certain temperature, but rather is necessary for whatever thermoresponsive reaction norm a strain may have for flowering.

ELF3 and PIF4 regulate adult thermoresponsive phenotypes independently.

If ELF3 and PIF4 were truly independent in controlling thermal responses of adult phenotypes under long days, then *elf3 pif4* double mutants would show approximately additive phenotypes. We generated *elf3 pif4* double mutants and subjected them to the same experiments as above (omitting again the 14 day transfer treatment). Our results indicated that flowering and petiole elongation appear to constitute independent temperature responses, with PIF4 controlling the former and ELF3 controlling the latter in additive fashions (Figure 4). That is, *elf3 pif4* double mutants showed negligible thermoresponsive flowering like *elf3*, and a negligible petiole response like *pif4*. Additionally, *elf3 pif4* flowered slightly later than *elf3* at 22º, while maintaining a negligible thermal response in flowering, indicating that *elf3* mutants are not simply restricted by a physiological limit of early flowering. The additivity of these phenotypes establishes that, under these conditions, ELF3 and PIF4 must operate in separate thermal response pathways.

Previous studies have indicated that other members of the PIF family, such as PIF1, PIF3, and PIF5, have minimal roles in these same thermal response phenotypes (Koini et al., 2009; Stavang et al., 2009; Proveniers and van Zanten, 2013), and *pif4 pif5* double mutants retain substantial thermoresponsive flowering even under shorter 12 hour light : 12 hour dark photoperiods (Thines et al., 2014). These previous findings suggest that our results are not explained by redundancy between PIFs. However, to exclude this possibility, we evaluated thermoresponsive flowering in *pif4 pif5* mutants (Figure 4D), because PIF5 is most often considered to act redundantly with PIF4 (Filo et al., 2015; Thines et al., 2014; Sun et al., 2013). As expected, both *pif5* single mutants and *pif4 pif5* double mutants have intact thermoresponsive flowering. These

observations indicate that redundancy with other PIFs is not responsible for the apparent independence of PIF4 and ELF3.

Overall, the strong photoperiod-dependence of PIF4-related thermoresponsive flowering necessitates the existence of some pathway or pathways independent of PIF4 under long days, given the persistence of the phenomenon under these conditions. Based on our data, ELF3 acts in one such pathway.

Thermoresponsive flowering under long days can operate through the photoperiodic pathway. ELF3's role in thermoresponsive flowering at low ambient temperatures has previously been shown to operate in the photoperiodic pathway, through repressing *GI* expression, which in turn represses GI's direct activation of FT (Sawa and Kay, 2011; Jang et al., 2015). To evaluate whether this pathway might explain our results (Figure 5), we measured transcript levels of *GI* and *CO* in wild-type and *elf3* mutants under 22ºC and 27ºC (Figure 5A). We found that *GI* is strongly up-regulated in *elf3* null mutants of Col and Ws backgrounds, confirming previous reports in Col (Jang et al., 2015; Mizuno et al., 2014a). Further, wild-type Ws shows approximately five-fold higher basal *GI* levels compared to Col, which do not increase at higher temperatures. In contrast, Col shows very low basal *GI* levels that increase at higher temperatures to approximately the same levels as Ws. *CO* levels, however, are not substantially affected by either *elf3* mutation or increased temperature, consistent with previous reports (Strasser et al., 2009; Jang et al., 2015), and in fact may decrease at 27ºC. Thus, robust thermoresponsive flowering is correlated with low basal levels of *GI,* and with temperature-dependent *GI* up-regulation, as observed in Col. High basal *GI* levels in Ws may be associated with this strain's lack of thermoresponsive flowering and deficient circadian temperature compensation at higher ambient temperatures (Ibañez et al., 2015; Edwards et al., 2005, 2015). These observations support the model under which ELF3 acts in the photoperiodic pathway to engender thermoresponsive flowering, just as it does in response to lower ambient temperatures (Strasser et al., 2009; Jang et al., 2015).

If the photoperiodic pathway contributes to thermoresponsive flowering at elevated ambient temperatures in long days (LD), we would expect mutants in this

pathway to show abrogated temperature responses. Under short days (SD), members of both the autonomous and photoperiodic pathways are essential to the high temperature flowering response (Balasubramanian et al., 2006). These two pathways also contribute independently to thermoresponsive flowering at low temperatures (16ºC vs. 23ºC) (Blázquez et al., 2003; Strasser et al., 2009). Altogether, we would expect that a photoperiodic thermoresponsive flowering pathway would operate independently of both PIF4 and the autonomous pathways in long days. It is not clear whether the autonomous pathway would be independent of PIF4, given known interactions between FCA and PIF4 (Lee et al., 2014).

To evaluate whether these past results under other conditions also apply to LD and elevated temperatures, we measured flowering time at 22ºC and 27ºC in mutants in the photoperiodic pathway (*gi*, *co,* Figure 5B). We also tested mutants of the gibberellin pathway (*spy*), and a terminal floral integrator (*soc1*), which are not expected to be necessary for thermoresponsive flowering. We found robust thermal responses in all mutants except *elf3* and *gi*, closely agreeing with previous results under other conditions (Balasubramanian et al., 2006; Strasser et al., 2009; Sawa and Kay, 2011; Jang et al., 2015), and implicating GI (but not CO) as an actor in thermoresponsive flowering at elevated temperatures. Collectively, these experiments suggest that the photoperiod pathway is necessary in promoting thermoresponsive flowering in long days, and expression data in this and other studies suggests that ELF3 is likely to act within this pathway.

Discussion

ELF3 and PIF4 are both crucial integrators of temperature and light signaling in controlling *A. thaliana* development. Recent literature has emphasized the centrality of PIF4-dependent thermoresponsive regulation in a variety of phenotypes, including in flowering (Kumar et al., 2012; Song et al., 2013; Wigge, 2013). Here, we extend the previous observation that PIF4 is dispensable for thermoresponsive flowering under long photoperiod conditions (Koini et al., 2009), and identify ELF3 as essential for thermoresponsive flowering under conditions in which PIF4 is dispensable. Our results integrate previous knowledge about thermoresponsive flowering under other conditions,

and identify at least one pathway for this response that do not involve PIF4. Moreover, we show that while polyQ variation in ELF3 affects ELF3 function, the polyQ tract may not constitute a simple temperature-responsive component in itself (in Ws at least). Our results incorporate current findings into classic models of thermal responses in *A. thaliana*, allowing a comprehensive view of the genetic underpinnings of this agronomically crucial plant trait.

ELF3 polyglutamine variation affects thermoresponsive traits in part by modulating overall ELF3 activity. In previous work, we demonstrated that polyQ variation in ELF3 is (1) common, (2) affects many known ELF3-dependent phenotypes, and (3) is dependent on the genetic background (Undurraga et al., 2012). Following the recent discoveries that ELF3 is involved with temperature response (Mizuno et al., 2014b; Box et al., 2014; Raschke et al., 2015), we confirmed that ELF3 polyQ variation also affects temperature response phenotypes in a background-dependent fashion. However, we found little support for the hypothesis that the polyQ tract has a special role in temperature sensing. Instead, as was the case for other ELF3-dependent phenotypes, ELF3 polyQ variation appears to affect overall ELF3 functionality, with less functional ELF3 variants lacking robust temperature responses. The molecular mechanism by which polyQ variation affects ELF3 functionality remains unknown, in spite of substantial phenotypic characterization (Undurraga et al., 2012). Targeted mechanistic study will be required to answer this question.

ELF3-PIF4 relationship in thermoresponsive morphologies. One question that remains unanswered is to what extent ELF3 participates in PIF4-dependent thermoresponsive morphologies. While our study and previous work (Mizuno et al., 2014a; Box et al., 2014; Raschke et al., 2015) support a PIF4-ELF3 link in temperature-dependent hypocotyl elongation, this relationship disappears in the analogous case of temperature-dependent petiole elongation. These results can be explained by many hypotheses. First, it is possible that ELF3 regulation of PIF4 is only relevant at the early seedling stage. Alternatively, the model that elevated temperature down-regulates ELF3 could be inadequate, and rather PIF4 up-regulation at elevated temperatures could be due to

other inputs. One candidate that modulates PIF4 responsiveness is FCA; however, thermoresponsive hypocotyl elongation is actually intensified in *fca* mutants (Lee et al., 2014), as is petiole elongation in seedlings. More work will be needed to understand the relationship of ELF3 and PIF4 in thermomorphogenesis.

Natural variation in temperature response. Several studies have indicated that different *A. thaliana* strains respond to temperatures differently, either shifting or inverting the reaction norm of the phenotype in question (Edwards et al., 2005; Ibañez et al., 2015; Edwards et al., 2015). Ws has a shifted reaction norm with respect to temperature compared to Col for photoperiod-related phenotypes, including flowering. For instance, Ws displays accelerated flowering at 23ºC vs. 16ºC (Ibañez et al., 2015), but accelerates flowering no further at 27ºC. Thus, if thermoresponsive flowering involves ELF3 in Ws, *elf3* Ws mutants would lack flowering acceleration at 22ºC relative to 16ºC, and indeed we show this to be the case. Another example of differential mutational effects among strains is that *gi* mutants in the L*er* background display robust thermoresponsive flowering (Blázquez et al., 2003; Balasubramanian et al., 2006). It is unclear whether this finding is due to altered wiring of pathways between these backgrounds.

Thermoresponsive flowering can require PIF4 or ELF3, depending on photoperiod. Under various conditions, both ELF3 and PIF4 have been found to be crucial for thermoresponsive flowering; ELF3 is involved under long days comparing 16ºC/23ºC temperature conditions (Strasser et al., 2009; Jang et al., 2015), and PIF4 is involved under short days comparing 22ºC/27ºC temperature conditions (Kumar et al., 2012). Other members of the autonomous and the photoperiodic pathways have also been implicated in thermoresponsive flowering (Blázquez et al., 2003; Balasubramanian et al., 2006; Strasser et al., 2009) (besides other pathways, (Lee et al., 2007)). Consequently, some combination of these pathways, modulated by experimental conditions, must require ELF3 and/or PIF4. We and others (Koini et al., 2009; Thines et al., 2014) have observed that PIF4 and its paralogs are not required or minimally required for proper thermoresponsive flowering under longer photoperiods and elevated

temperatures. Hence, some other pathway or pathways must be responsible for such a temperature response. Furthermore, we and others (Strasser et al., 2009; Jang et al., 2015) have shown that ELF3 and the photoperiod pathway (excluding CO) are essential for proper thermoresponsive flowering under long days. It has been previously shown that PIF4 and the photoperiodic pathway contribute to thermoresponsive flowering via independent pathways (Kumar et al., 2012), suggesting that under longer photoperiods PIF4 activity is inhibited, allowing other mechanisms to dominate thermoresponsive flowering.

These collected observations lead us to propose a model of thermoresponsive flowering, in which PIF4, ELF3, the photoperiodic pathway, and other pathways interact depending upon condition and genetic background (Figure 5D). Under short days or other short photoperiods, phyB activity is down-regulated, leading to up-regulation of PIF4 (Duek and Fankhauser, 2005; Huq and Quail, 2002; Luo et al., 2014; Lorrain et al., 2008), which at high levels occupies the promoter of the flowering integrator *FT* and induces flowering (Kumar et al., 2012). Constitutive overexpression of PIF4, PIF5, and PIF3 under long day conditions induces early flowering (Galvão et al., 2015), supporting the hypothesis that differences in PIF levels underlie the difference in PIF4 importance between long and short photoperiods. PIF4 activation thus appears to be the dominant mechanism of thermoresponsive flowering under short photoperiods, although *pif4* null mutants still show some thermal response. However, under longer photoperiods, phyB up-regulation leads to an attenuation of PIF4 activity, and consequently the role of PIF4 and other PIFs becomes negligible (Koini et al., 2009). This allows canonical ambient temperature responses (such as the photoperiodic pathway, including ELF3, (Strasser et al., 2009; Jang et al., 2015)) to take a dominant role in thermoresponsive flowering. Several reports have indicated that GI and COP1, but not CO, are involved in thermoresponsive flowering (Balasubramanian et al., 2006; Strasser et al., 2009; Jang et al., 2015), with GI directly binding the *FT* promoter (Jang et al., 2015). Under each of these conditions, *FT*-induced flowering is activated by a different signaling cascade initiated by elevated temperature. This interpretation leads to a holistic and coherent view of how light and temperature responses are integrated in this important plant trait.

To summarize, at least three independent mechanisms have been described that promote thermoresponsive flowering in any context. These include the photoperiodic pathway (PHYB/ELF3/GI/COP1), the autonomous pathway (PHYA/FCA/FVE/TFL1/FLC), and the PIF4-dependent pathway (PIF4/H2A.Z/gibberellin), all of which converge by regulating *FT* (although the last pathway may also act through other integrators (Thines et al., 2014; Galvão et al., 2015). The collective results of our experiments and previous work suggest that the first two pathways are necessary but not sufficient for thermoresponsive flowering, and that the third (PIF4) is sufficient but not necessary for thermoresponsive flowering. The truth of this characterization deserves further study in understanding the interdependencies of the three pathways. For instance, it has been suggested that PIF4 binding to the *FT* promoter is dependent on cooperativity with a second photoperiod-controlled actor (Seaton et al., 2015). Notably, in our interpretation the photoperiodic pathway omits CO, which shows neither a thermoresponsive flowering defect nor temperature-dependent expression responses in any study we are familiar with (Balasubramanian et al., 2006; Sawa and Kay, 2011; Jang et al., 2015).

In conclusion, we observe that ELF3 is involved in the hypocotyl response to elevated temperature as reported previously, and that this response can be abrogated by poorly-functioning ELF3-polyQ variants. We further demonstrate that ELF3 has little effect on the petiole temperature response, and is necessary for the flowering temperature response, suggesting that it functions independently of PIF4, potentially in the photoperiodic pathway. These results reiterate the complexity of these crucial environmental responses in plants, and will serve as a basis for further development of our understanding of how plants respond to elevated temperatures. In the context of climatic changes, this understanding will serve those attempting to secure the global food supply.

Methods

Plant materials and growth conditions. All mutant lines (except *pif4-2 elf3-200*) were either described previously or obtained as T-DNA insertions from the Arabidopsis Biological Resources Center at Ohio State University (Alonso et al., 2003; Kleinboelting

et al., 2012), and are described in Table S11. *pif4-2 elf3-200* was obtained via crossing and genotyping. T-DNA insertions were confirmed with primers described in Table S10. For hypocotyl assays, seedlings were grown for 15d in incubators set to SD on vertical plates as described previously (Undurraga et al., 2012). All plates were incubated at 22º for one day, after which one replicate arm was transferred to an incubator set to 27º, with another replicate arm maintained at 22º. For flowering time assays, plants were stratified 3-5d at 4º in 0.1% agarose and seeded into Sunshine #4 soil in 36-pot or 72-pot flats to germinate at 22º under LD. Replicate arms were subsequently transferred to 27º LD conditions as indicated, with others remaining at 22º. Different temperature treatments of the same experiment were identical with respect to randomization, setup, and format. At 25d, petiole length and whole leaf length (including petiole) of the third leaf were measured, and the ratio of these values was further analyzed. Flowering was defined as an inflorescence ≥1cm tall; at this point, date and rosette leaf number were recorded.

Trait data analysis. All data analysis was performed using R v3.2.1 (R Core Team, 2015). Where indicated, temperature responses were modeled using multiple regression in the form *Phenotype* ~ $\boldsymbol{\mu}$ + $\boldsymbol{\beta_G}$*Genotype* + $\boldsymbol{\beta_T}$*Temperature* + $\boldsymbol{\beta_{GxT}}$**(***Genotype* x *Temperature***)** + $\boldsymbol{\beta_E}$*Experiment* + *Error*. All experiments were included in models for transgenic experiments, and thus the $\boldsymbol{\beta_E}$ term describes systematic variation between experiments, whereas line-specific effects among transgenics should be modeled in the error term. Where temperature responses are reported, they consist of the $\boldsymbol{\beta_T}$ + $\boldsymbol{\beta_{GxT}}$ terms and associated errors ($\sqrt{\sigma_T^2 + \sigma_{GxT}^2}$ where σ_T is the standard error for $\boldsymbol{\beta_T}$ and σ_{GxT} is the standard error for $\boldsymbol{\beta_{GxT}}$), and thus are corrected for systematic experimental variation and temperature-independent genotype effects. Analysis scripts and data are provided at https://figshare.com/s/129525f02ef6e66f7bed and as File S1.

Gene expression analyses. Seedlings were grown for 1d under LD at 22º, after which one replicate arm was transferred to LD at 27º, with another replicate arm remaining at 22º, and all seedlings were harvested 6d later at indicated times. At harvest, ~30mg aerial tissue of pooled seedlings was frozen immediately in liquid nitrogen and stored at

-80º. RNA extraction, cDNA synthesis, and real-time quantitative PCR were performed as described previously (Undurraga et al., 2012), using primers in Table S10. Transcript levels were quantified using the **ΔΔ**C_t method (Pfaffl, 2001).

Acknowledgments

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Author contributions. Designed research: MOP AL CQ. Performed experiments: MOP AL. Analyzed data: MOP. Wrote the paper: MOP CQ.

Supplemental Data.

Table S1. Regression analysis of hypocotyl elongation temperature response among Col and Ws transgenic lines.

Table S2. Regression analysis of petiole : leaf length ratio temperature response among Col and Ws transgenic lines.

Table S3. Regression analysis of rosette leaf number at flowering temperature response among Col and Ws transgenic lines.

Table S4. Regression analysis of rosette leaf number at flowering and petiole : leaf length ratio temperature responses in *elf3* and *pif4*.

Table S5. Regression analysis of rosette leaf number at flowering temperature response in Ws and *elf3-4*.

Table S6. Regression analysis of petiole : leaf length ratio temperature response in Col and *fca* mutants.

Table S7. Regression analysis of rosette leaf number at flowering temperature response in *elf3 pif4* double mutants.

Table S8. Regression analysis of rosette leaf number at flowering temperature response in *pif4 pif5* double mutants.

Table S9. Regression analysis of rosette leaf number at flowering and petiole : leaf length ratio temperature responses in flowering pathway mutants.

Table S10. Primers used in this study.

Table S11. Mutant lines used in this study.

File S1. Data and analysis code.

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Figure Legends

Figure 1. Response to elevated temperature (27º, relative to 22º) among transgenic lines expressing ELF3-polyQ variants. Mean response and error were estimated by regression, based on two independently-generated transgenic lines for each genotype, with n >= 30 seedlings of each genotype in each condition (Table S1). WT = Ws, *elf3* = *elf3* mutant+vector control, 0Q = *elf3* mutant+*ELF3* transgene lacking polyQ, etc. Error bars indicate standard error of the mean. (A): Ws (Wassilewskija) strain background. Lines are generated in an *elf3-4* background. (B): Response in the Col (Columbia) strain background, lines were generated in an *elf3-200* background. (C): Temperature response is a function of ELF3 functionality (repression of hypocotyl elongation at 22º). Simple means of 22º hypocotyl length, regression estimates of temperature response. PCC = Pearson correlation coefficient; p-value is from a Pearson correlation test. (D): Effect of background, temperature, and polyQ on *PIF4 and AtHB2* expression. Error bars represent the standard error of the mean across 3 technical replicates. White bars represent 22º expression, red bars 27º expression for each line. Tissue was collected from 7d seedlings at ZT0. This experiment was repeated with similar results.

Figure 2. Adult plant responses to elevated temperature (27º, relative to 22º) in long days among transgenic lines expressing different ELF3-polyQ variants. (A) and (C): Response in the Ws (Wassilewskija) strain background. Lines are in an *elf3-4* background. (B) and (D): Response in the Col (Columbia) strain background, lines are in an *elf3-200* background. (A) and (B) display PL:LL temperature response, (C) and (D) display RLN temperature response. Average responses and errors were estimated in a regression model accounting for variation between experiments (Tables S2, S3), based on two to three independently-generated transgenic lines for each genotype. n >= 24 plants overall for each genotype in each condition. PL:LL = petiole to leaf length ratio at 25 days post germination, RLN = rosette leaf number at flowering, WT = wild type, *elf3* = *elf3* mutant+vector control, 0Q = *elf3* mutant+*ELF3* transgene with entire polyglutamine removed, etc. Error bars indicate standard error.

Figure 3. *elf3* and *pif4* null mutant phenotypes are independent under LD treatments and robust to conditions. (A), (B), and (C): 22º: constant 22º LD growth; 27º 14d: transfer from 22º to 27º at 14 days post-germination; 27º 1d: transfer from 22º to 27º at 1 day post-germination. (A): Col (WT), *elf3-200*, and *pif4-2* plants grown under long days with three different temperature regimes were photographed at 20 days post germination. Experiment was repeated with similar results. (B and D): Petiole elongation responses of the indicated genotypes, measured by ratio of petiole to whole leaf length at 25 days post germination. (C) and (E): Flowering temperature response of indicated genotypes under indicated conditions, measured by rosette leaf number (RLN) at flowering. For each experiment, n > 10 plants for each genotype in each treatment. Regression analysis of data in Tables S4, S5, S6.

Figure 4. Double mutant analysis confirms PIF4 and ELF3 independence in adult temperature responses and non-redundancy of PIF4 with PIF5. (A): Col, *elf3-200*, *pif4-2,* and *elf3-200 pif4-2* plants grown under long days with two different temperature regimes were photographed at 25 days post germination. (B): Petiole elongation

responses of the indicated genotypes, measured by ratio of petiole to whole leaf length at 25 days post germination. (C): Flowering temperature response of indicated genotypes, measured by rosette leaf number (RLN) at flowering. (B) and (C): n > 8 plants for each genotype in each treatment. All “27º” plants were seeded and incubated one day at 22º before transfer to 27º. Experiments were repeated with similar results. Regression analysis of data reported in Tables S7 and S8.

Figure 5. ELF3 and GI regulate thermoresponsive flowering. (A): Temperature-responsive expression of photoperiodic pathway components. Expression of each gene is quantified relative to levels in Ws at 22º (Ws 22 = 1.0). This experiment was repeated with similar results. *elf3-4*: *elf3* null in Ws background; *elf3-200*: *elf3* null in Col background. (B): Thermoresponsive flowering in various flowering mutants. LD RLN = rosette leaf number at flowering under long days. * : interaction term for genotype by environment at $p < 0.01$; details of regression model in Table S9. (C) Thermoresponsive petiole elongation in various flowering mutants. For (B) and (C), n >= 8 plants of each genotype in each condition; white boxes indicate measurements at 22º, red boxes indicate measurements at 27º. *gi: gi-2*, *co*: *co-101*, *spy*: *spy-3*, *soc1*: *soc1* T-DNA insertion, *elf3: elf3-200*. This experiment was repeated with similar results. (D): Models of thermoresponsive flowering under long and short photoperiods. Dashed edges indicate speculated temperature sensing mechanisms. Edges with increased weight indicate relative increases of influence between conditions. Pathways are indicated, along with other important actors reported elsewhere.

Figure 1

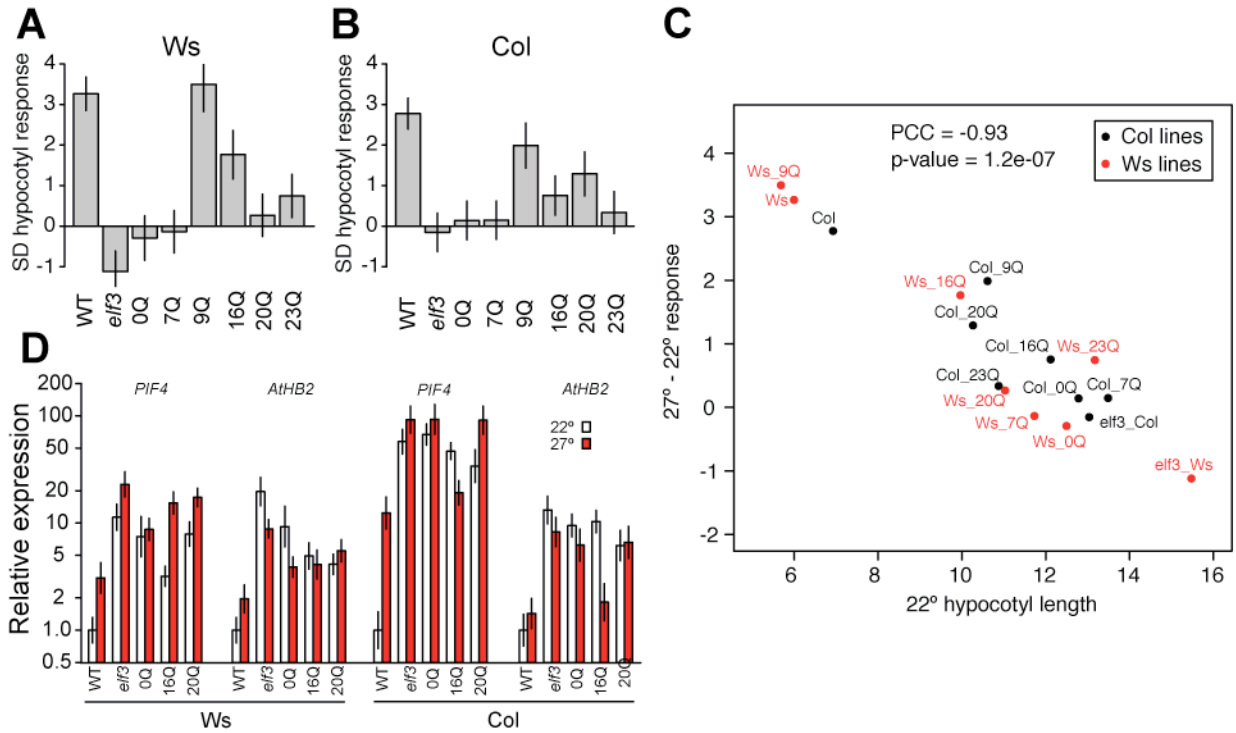

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Figure 2

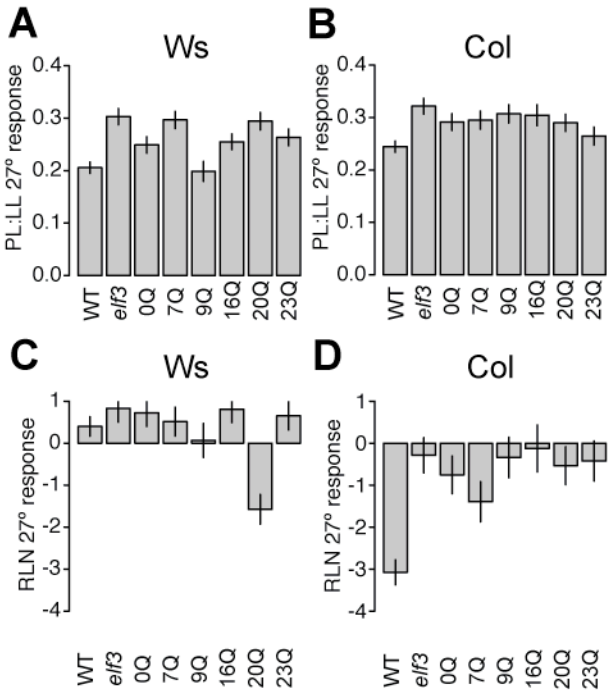

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Figure 3

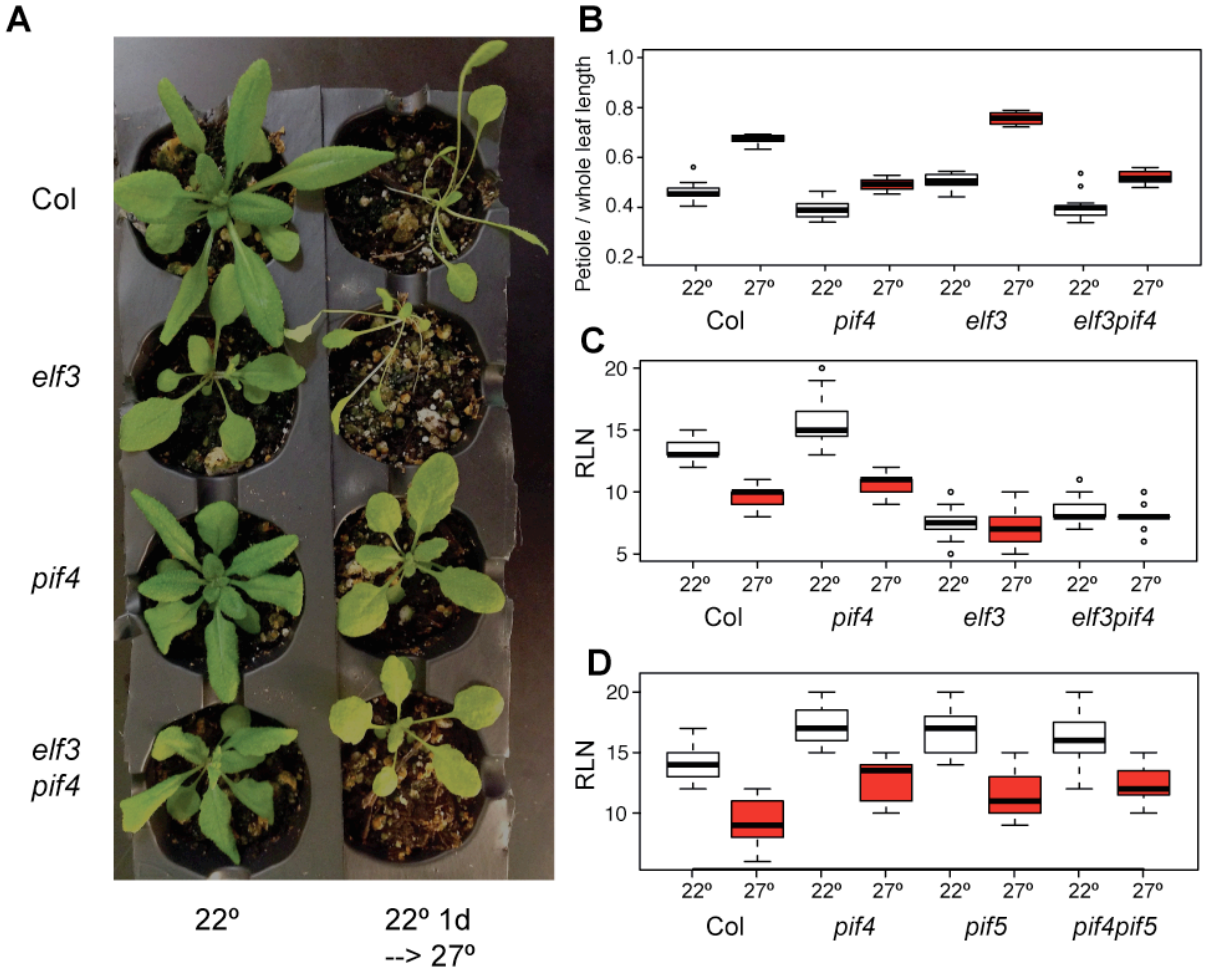

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Figure 4

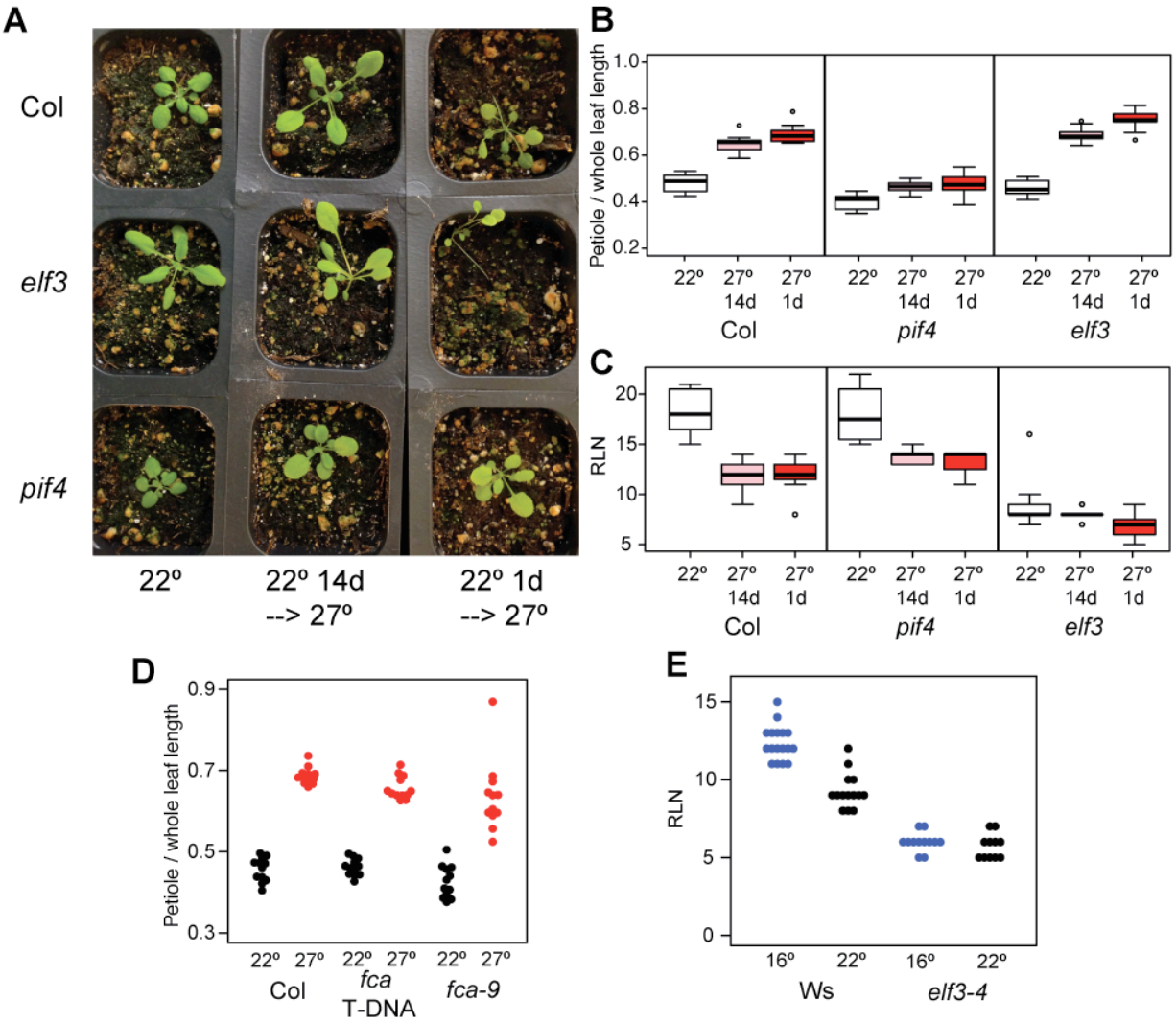

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Figure 5

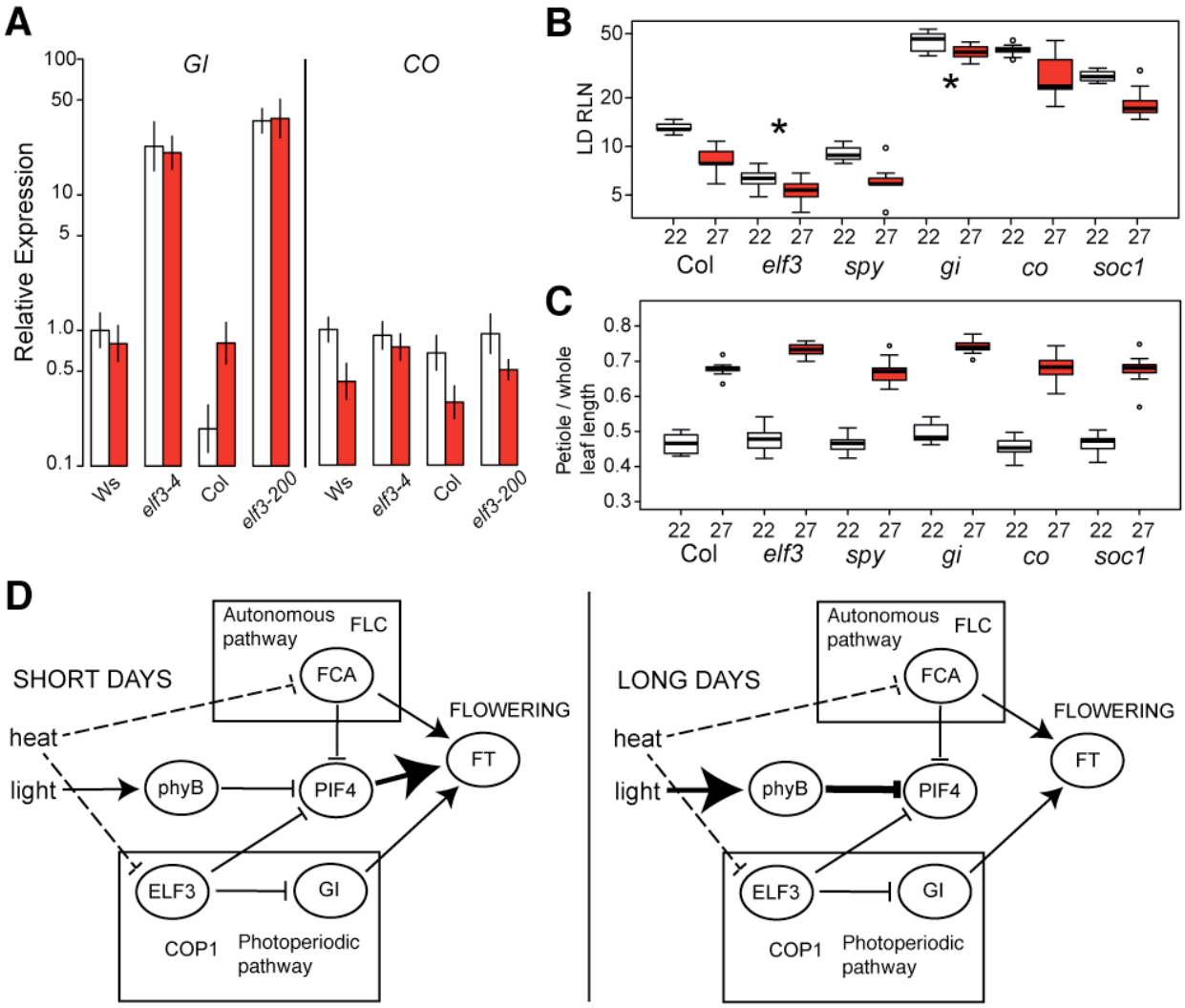

Figure 5. ELF3 and GI regulate thermoresponsive flowering. (A): Temperature-responsive expression of photoperiodic pathway components. Expression of each gene is quantified relative to levels in Ws at 22º (Ws 22 = 1.0). This experiment was repeated with similar results. *elf3-4*: *elf3* null in Ws background; *elf3-200*: *elf3* null in Col background. (B): Thermoresponsive flowering in various flowering mutants. LD RLN = rosette leaf number at flowering under long days. * : interaction term for genotype by environment at $p < 0.01$; details of regression model in Table S9. (C) Thermoresponsive petiole elongation in various flowering mutants. For (B) and (C), n >= 8 plants of each genotype in each condition; white boxes indicate measurements at 22º, red boxes indicate measurements at 27º. *gi: gi-2*, *co: co-101, spy: spy-3, soc1: soc1* T-DNA insertion, *elf3: elf3-200*. This experiment was repeated with similar results. (D): Models of thermoresponsive flowering under long and short photoperiods. Dashed edges indicate speculated temperature sensing mechanisms. Edges with increased weight indicate relative increases of influence between conditions. Pathways are indicated, along with other important actors reported elsewhere.