Title: Phytochromes Function as Thermosensors in Arabidopsis

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Abstract: Plants are responsive to temperature, and can distinguish differences of 1ºC. In Arabidopsis, warmer temperature accelerates flowering and increases elongation growth (thermomorphogenesis). The mechanisms of temperature perception are however largely unknown. We describe a major thermosensory role for the phytochromes (red light receptors) during the night. Phytochrome null plants display a constitutive warm temperature response, and consistent with this, we show in this background that the warm temperature transcriptome

becomes de-repressed at low temperatures. We have discovered phytochrome B (phyB) directly associates with the promoters of key target genes in a temperature dependent manner. The rate of phyB inactivation is proportional to temperature in the dark, enabling phytochromes to function as thermal timers, integrating temperature information over the course of the night.

One Sentence Summary: The plant temperature transcriptome is controlled at night by phytochromes, acting as thermoresponsive transcriptional repressors.

Main Text:

Plant development is responsive to temperature, and the phenology and distribution of crops and wild plants have already altered in response to climate change (*1*, *2*). In *Arabidopsis thaliana,* warm temperature-mediated elongation growth and flowering is dependent on the bHLH transcription factors PHYTOCHROME INTERACTING FACTOR4 and 5 (PIF4 and 5) (*3*–*6*). Growth at 27ºC reduces the activity of the Evening Complex (EC) resulting in greater *PIF4* transcription. The EC is a transcriptional repressor made up of the proteins EARLY FLOWERING3 (ELF3), ELF4 and LUX ARRHYTHMO (LUX) (*7*–*9*). To test if the EC is also required for hypocotyl elongation responses below 22ºC, we examined the behavior of *elf3-1* and *lux-4* at 12 and 17ºC. Hypocotyl elongation in *elf3-1* and *lux-4* is largely suppressed at lower temperatures (Fig. 1A, B), which is consistent with cold temperatures being able to suppress *PIF4* overexpression phenotypes (*10*). Since *PHYTOCHROME B (PHYB)* was identified as a QTL for thermal responsiveness and PIF4 activity is regulated by phytochromes (*8*, *11*), we investigated whether these red light receptors control hypocotyl elongation in the range 12 to 22ºC. Plants lacking phytochrome activity (*12*) show constitutively long hypocotyls at 12ºC and 17ºC. Thus phytochromes are essential for responding to temperature (Fig. 1C, D and Fig. S1).

We used transcriptome analysis to determine whether disrupted thermomorphogenesis in *phyABCDE* is specific for temperature signaling or is a consequence of misregulated growth pathways. To capture diurnal variation in thermoresponsiveness, we sampled seedlings over 24 hours at 22 and 27ºC. Clustering analysis reveals 20 groups of transcripts (Fig. 2A and Fig. S3; described in supplement). Thermomorphogenesis occurs predominantly at night and is driven by *PIF4*. Consistent with this, we observe *PIF4* is present in cluster 20, which is more highly expressed at 27ºC during darkness. Clusters 15 and 16 represent the other major groups of

nighttime thermally responsive genes, and these are strongly induced in *phyABCDE.* These clusters are enriched for genes involved in hormone signaling and elongation growth (*YUCCA8, YUCCA9, BRASSINOSTEROID INSENSITIVE2, LIKE AUXIN RESISTANT1, AUXIN RESPONSE FACTOR7* and *9, BIG and TRANSPORT INHIBITOR1*) (Tables S3-S5). These results indicate that the nighttime warm temperature transcriptome is specifically affected when phytochrome activity is altered. We used Principal Component (PC) analysis to investigate the overall responses of the transcriptome to temperature and phytochromes. For PC1, which explains 40.8% of the expression variance, *phyABCDE* at 22ºC occupies a similar position to wild type at 27ºC (Fig. 2B). At nighttime there is a positive correlation between gene expression changes in response to temperature and to *phyABCDE;* this relationship is lost during the day (Fig. 2C). Phytochromes are interconvertible photoreceptors (*14–16*): phyB switches between an inactive Pr state and an active Pfr state upon absorbing red- and far-red light respectively. In a thermal relaxation reaction, Pfr in the dark spontaneously reverts to the Pr state (*17–19*). We hypothesized that the reversion of Pfr to the inactive Pr state may contribute to the subsequent increase in expression of the warm temperature transcriptome during the night. We therefore examined plants containing a constitutively active version of phyB. The Y276H point mutation in phyB (YHB) prevents the dark reversion reaction (*20*, *21*), locking phyB in the active Pfr state. This results in constitutive repression of the warm temperature transcriptome throughout the night (Fig. 2A and Fig. S3). *YHB* plants also phenocopy cool grown plants, showing little thermoresponsive elongation growth even at 27ºC (Fig. S2). Across the time-course, 79% of the temperature transcriptome is misregulated in *phyABCDE*, *YHB* or both backgrounds (Fig. 2D).

PhyA is able to bind promoters (*22*), and PIF3 can recruit phyB to G-boxes in vitro (*23*), so we tested if phyB controls temperature responsive genes directly. Chromatin immunopurification of phyB coupled with sequencing (ChIP-seq) revealed phyB to bind >100 sites (~95 promoters), with more targets bound at 17ºC than 27ºC (Fig. 3A). Phytochrome signaling is reduced at warmer temperatures, and even for the 33 sites bound by phyB at both temperatures, less binding occurs at 27ºC (Fig. 3B). Most phyB target genes are expressed at night in response to temperature (Fig S4, Table S1). It has been suggested that phyB may modulate transcription (*23*), and our results indicate it acts as a transcriptional repressor, since most targets are upregulated under conditions that reduce phytochrome activity during night-time, while during daytime this relationship is lost (Fig. 3C,D and Fig. S6, S9). We do not observe a change in *PHYB* expression or phyB protein levels in response to temperature, consistent with the effect of temperature on phyB being direct (Fig. S11).

Most of the phyB bound peaks contain G-boxes (Fig. 3E and Fig. S7), motifs bound by PIFs. We found overlap between phyB peaks and reported binding sites for PIF1, 3, 4, and 5 (*24*–*26*) (Fig. 3E, F). PhyB preferring sites that are bound by all 4 PIFs were enriched at both 17 and 27ºC (Only 7% of the identified peaks are shared between all 4 PIFs in (*24*)). Of the loci that are bound by phyB at both 17ºC and 27ºC, only three are not bound by PIFs. Compared to other sites, phyB at PIF binding sites shows a stronger enrichment (Fig. S5). These loci likely represent sites for facilitated PIF binding and co-enrichment for phyB. The *ATHB2* promoter illustrates how phyB binding correlates to the binding sites of PIFs (Fig. 3G; Fig. S8). To further resolve the phyB peaks we used X-ChIP (*27*). Verified multiple PIF bound sites corresponded with phyB bound sites (Fig. S10). Although light induces degradation of PIFs, there are significant levels of natively expressed PIF4 during the daytime (*28*). Thus phyB at promoters may recruit or modulate action of other regulators, providing a mechanism to increase the dynamic range of the transcriptional regulation of target genes. Precedent exists for a transcriptional activator being converted into a repressor by a ligand, for example FD is an activator when bound to FT and a repressor when bound to TFL1 (*29*).

Changes in the activity of a transcriptional repressor, R, can explain the thermoresponsive expression of *PIF4* and *LUX,* and thermal responsiveness of R can be accounted for by *ELF3* (*8*). However transcripts of PIF4 target genes such as *ATHB2*, cluster separately from *PIF4* and show greater responsiveness to phytochrome signaling (Fig. 2A). We concluded that phytochromes provide additional temperature dependent regulation of PIF4. Thus, active phytochromes (Pfr) repress activity of PIF4 and its ability to activate *ATHB2* (Fig. 4A). In the dark, the amount of Pfr is determined by the dark reversion rate, *b*. We used *ATHB2* expression data from L*er*, *phyABCDE* and *YHB* to calibrate this model (Fig. S12). Using parameters from the previous model (*8*), we allowed the model to determine which values for *b* are consistent with the thermoresponsive gene expression profiles (Table S5). The model picks values of *b* that vary as a function of temperature: the half-life of Pfr is 2.09 h at 22ºC and 1.53 h at 27ºC. If *b* is not allowed to vary with temperature, the model does not fit the data as well (Fig. S13). Our model recapitulates *ATHB2* expression in response to temperature, suggesting that dual control of *PIF4* at transcriptional and post-translational levels explains regulation of gene expression to control expansion growth (Fig. 4B).

Our model infers a temperature responsive half-life for Pfr. Dark reversion of phytochromes from various species is a thermal relaxation reaction (*15*). We observed that the *in vivo* dark

reversion rate of phyB: $phyB_{Pfr}$ $t_{1/2}$ showed an exponential relationship to temperature over one order of magnitude between 4 and 27ºC ($t_{1/2} = 10^{(-0.0496T + 1.315)}$; (T, temperature, ºC; $t_{1/2}$ half life, h), $R^2 = 0.99$) (Fig. 4C, Fig. S14). The half-lives at 22ºC and 27ºC ($t_{1/2}$ 1.79 h and 0.86 h respectively) are comparable to those seen in the model.

Since phytochromes are central regulators of plant responses to the environment, their acquisition of a thermally responsive behavior suggests an evolutionary route to integrate temperature information into development. Single amino acid changes as well as post-translational modifications can alter the dark reversion rate, suggesting sequence diversification among phytochromes affords diversity in thermal responsiveness for different climates (*19*, *21*, *30*–*32*). The Pfr dark reversion rate represents a sensitive mechanism to integrate small differences in temperature over the night in order to make developmental decisions.

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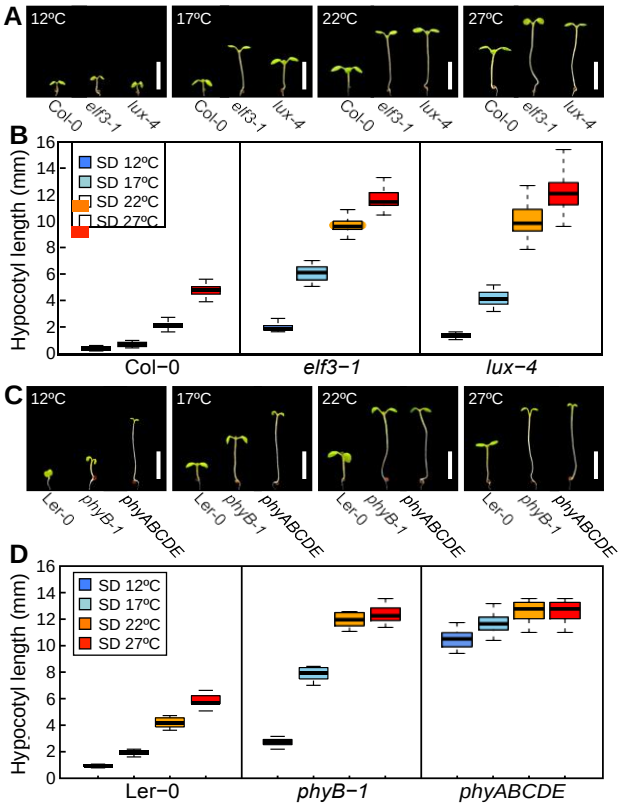

Fig. 1 Phytochromes are essential for correct thermomorphogenesis at lower temperatures. (A) Seedlings for Col-0, *elf3-1* and *lux-4* respectively grown at indicated temperatures for 8 days under short photoperiods. Scale bars, 5 mm. (B) Hypocotyl length boxplots for the indicated genotypes grown at different temperatures as in (A). (C) Seedlings for L*er*, *phyB-1* and *phyABCDE* respectively grown at indicated temperatures for 8 days under short photoperiods. Scale bars, 5 mm. (D) Hypocotyl length boxplots for the indicated genotypes grown at different temperatures as in (C).

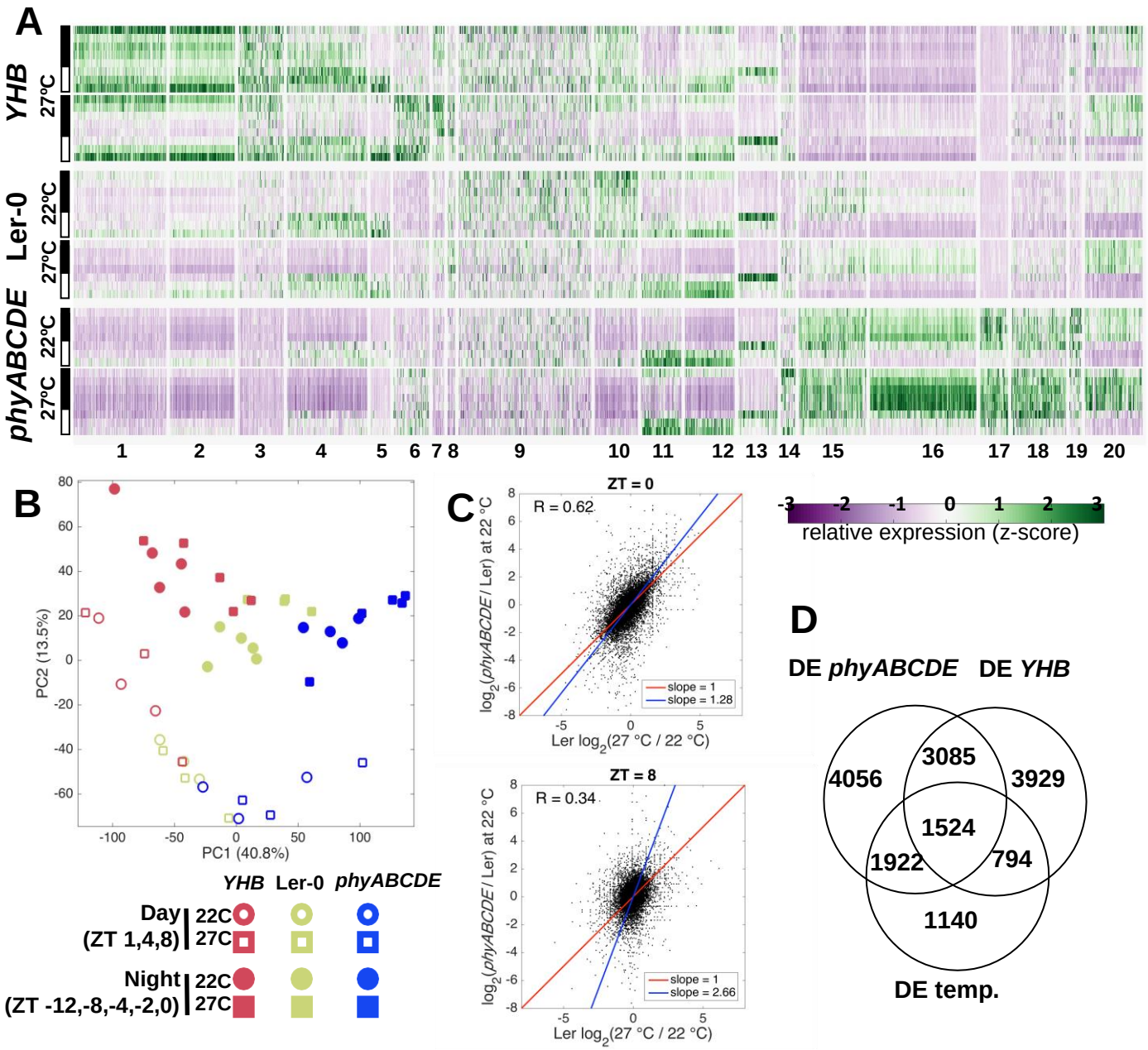

Fig. 2 Phytochromes control the temperature transcriptome at night. (A) Clustering of all RNA-seq time course samples for *phyABCDE*, L*er*, and *YHB* at 22ºC and 27ºC. Samples were collected every 4 h from ZT=0 with additional timepoints at ZT=1 and ZT=22. Black bars indicate night, white bars day. Clustering was performed on the expression-filtered dataset using a Gaussian mixture model; number of clusters was assumed to be a random variable. The number of clusters was automatically learned using an empirical Bayes approach (variational bayesian inference). (B) Principal components analysis on the expression-filtered dataset using genes as features. (C) Transcripts up-regulated in the *phyABCDE* background correlated positively with genes induced by temperature at dawn (ZT=0, top) but not at dusk (ZT=8, bottom). (D) Sets of differentially expressed genes from phytochrome and temperature transcriptomes overlap.

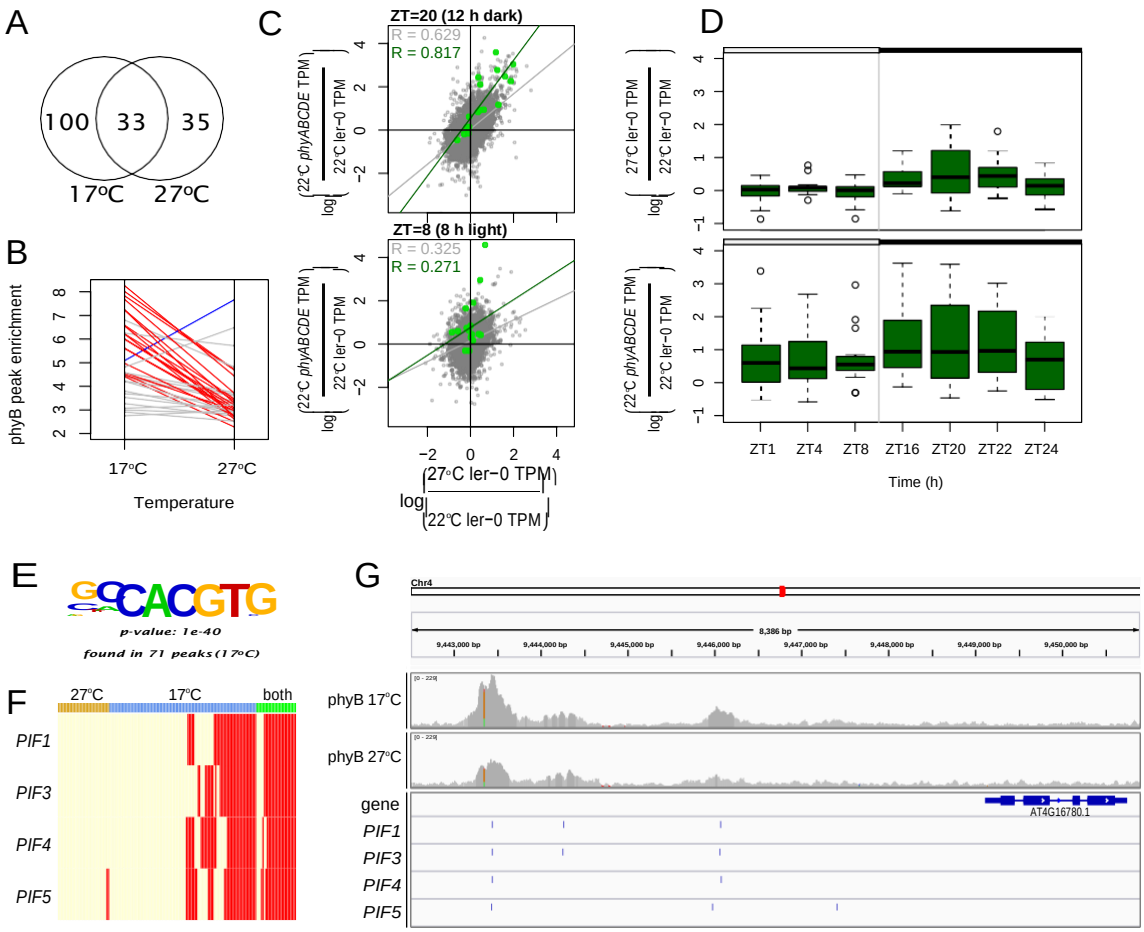

Fig. 3 PhyB binds to promoters of temperature responsive genes. (A) Comparison of phyB-myc ChIP-seq peaks identified at 17ºC and 27ºC using MACS2. In 6 instances, a single broad 17ºC peak was identified as two smaller peaks at 27ºC, each of these are considered as two separate peaks in the subsequent analysis. (B) Among the 33 peaks that are found in both 17ºC and 27ºC, a comparison was made of the phyB-myc peak enrichment (calculated as the log fold change compared to Input, by MACS2) at 17ºC and 27ºC. Values that decreased by over 50% are colored red and those that increase by over 50% are colored blue, the remaining are grey. (C) Log-fold change in expression in *phyABCDE* compared to log-fold change in expression caused by elevated temperature (27ºC vs 22ºC), at two time-points (ZT=8 and 20) in the RNA-seq time course from Fig. 2. The green points depict the transcripts of the 15 genes that are adjacent to the 33 peaks that are found in both 17ºC and 27ºC. Pearson's R is calculated for all genes (grey) and the phyB target genes (green). (D) Distributions of log-fold change in expression caused by *phyABCDE* and temperature elevation for 15 phyB target genes. (E) From the 17ºC peaks (127, considering the 6 broad peaks as a single peak), a G-box was the strongest de novo motif identified by Homer2, using shuffled sequence to form the background distribution. (F) Each column represents a different phyB peak, sorted by whether it was found at 27ºC, 17ºC or both- a red bar indicates that this peak overlaps with the center of the reported PIF binding site (23). All but three of the peaks found in both 17ºC and 27ºC are bound by multiple PIFs, almost none of the peaks found only at 27ºC are bound by PIFs. (G) IGV browser view of the *ATHB2* promoter, showing overlap between phyB-myc peaks at 17ºC and 27ºC and PIF1,3,4,5 peaks previously described (24).

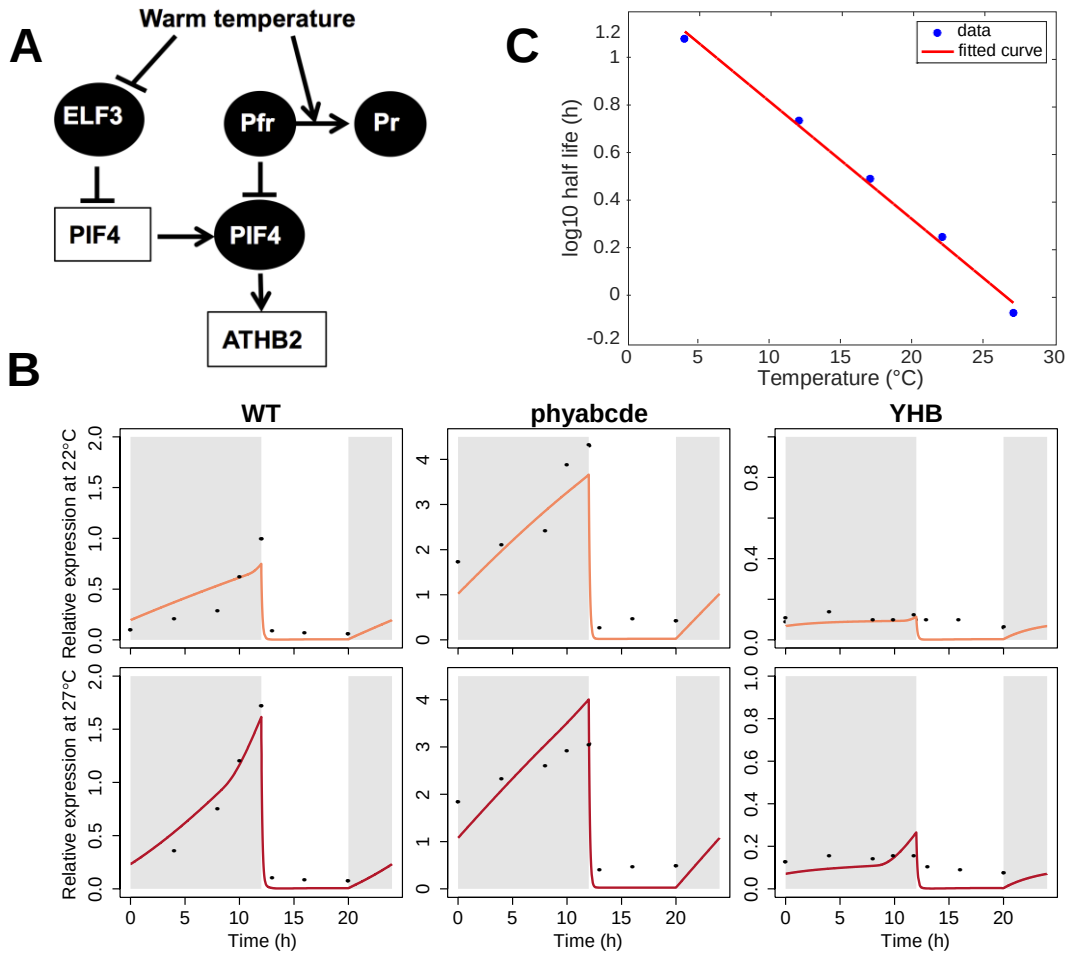

Fig. 4. A thermoresponsive gene expression network. (A) Regulatory network integrating temperature information through ELF3 activity and Pfr dark reversion. Temperature information is integrated through activity of ELF3, which acts as a repressor of *PIF4*, and deactivation of Phytochrome (Phy). Circles denote proteins and squares genes. (B) Modeled gene expression (orange and red lines) agrees with measured values (black dots). (C) The phyB Pfr half-life varies exponentially with temperature (assayed in etiolated seedlings). At lower temperatures, active Pfr persists at the end of the night, while at higher temperatures most Pfr is depleted within a few hours.