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The E3 ligase XBAT35 mediates thermoresponsive hypocotyl growth by targeting ELF3 for degradation in *Arabidopsis*

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Short title:

XBAT35 mediates ELF3 degradation

Summary

Plants are capable of coordination of their growth and development with ambient temperatures. EARLY FLOWERING3 (ELF3), an essential component of plant circadian clock, is also involved in ambient temperature sensing, as well as in inhibiting the expression and protein activity of the thermoresponsive regulator Phytochrome interacting factor 4 (PIF4). The ELF3 activity is subjected to attenuation in response to warm temperature,

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however, how the protein level of ELF3 is regulated at warm temperature remains less understood. Here, we report that the E3 ligase XB3 ORTHOLOG 5 IN ARABIDOPSIS THALIANA, XBAT35, mediates ELF3 degradation. XBAT35 interacts with ELF3 and ubiquitinates ELF3. Loss-of-function mutation of *XBAT35* increases the protein level of ELF3 and confers a short-hypocotyl phenotype under warm temperature conditions. Thus, our findings establish that XBAT35 mediates ELF3 degradation to lift the inhibition of ELF3 on PIF4 for promoting thermoresponsive hypocotyl growth in plants.

Keywords: ELF3, E3 ubiquitin ligase, hypocotyl growth, PIF4, thermomorphogenesis, Thermosensor, warm temperature, XBAT35

INTRODUCTION

Plants are able to sense ambient temperature signals and adjust their growth and development programmes in response to warming temperature fluctuations in a process called thermomorphogenesis (Casal and Balasubramanian 2019). In *Arabidopsis*, the bHLH family protein PIF4 plays a central role in promoting thermoresponsive hypocotyl growth (Koini et al., 2009). It recognizes G-box (CACGTG)-containing *cis*-elements and regulates downstream target genes involved in auxin biosynthesis and auxin-signaling (Franklin et al., 2011; Sun et al., 2012). PIF4 also forms a protein complex with BRI1 EMS SUPPRESSOR 1 (BES1) under warm temperature conditions to activate the expression of BR biosynthetic genes for facilitating BR-mediated hypocotyl elongation (Oh et al., 2012; Ibanez et al., 2018; Martinez et al., 2018). Other plant hormones, such as GA and ethylene, are also involved in promoting hypocotyl growth under elevated ambient temperature conditions (Stavang et al., 2009; Park et al., 2020; Hao et al., 2021; Kim et al., 2021).

The first identified warm temperature sensor is phytochrome B (phyB) (Jung et al., 2016; Legris et al., 2016). Warm temperatures accelerate the conversion of phyB from active Pfr to inactive Pr, releasing the inhibitory effects of phyB on PIF4 (Jung et al., 2016; Legris et al., 2016). A recent study showed that *PIF7* RNA perhaps is another type of thermosensor (Chung et al., 2020). Under warm temperature conditions, the secondary structure of *PIF7* undergoes conformational changes, which enhances protein translation of PIF7 (Chung et al., 2020). PIF7 promotes downstream gene expression either alone or together with PIF4 (Chung et al., 2020; Fiorucci et al., 2020). Genetic association studies showed that the Evening Complex component ELF3 is

also important for plant thermomorphogenesis (Box et al., 2015; Raschke et al., 2015), and ELF3 was recently proposed as a thermosensor (Jung et al. 2020). Under warm temperature conditions, ELF3 undergoes liquid-liquid phase separation, which presumably reduces the inhibitory effect of ELF3 on PIF4 (Vu et al., 2019; Jung et al., 2020). However, how the protein stability of ELF3 is regulated under warm temperature conditions is less understood. In the current study, we demonstrate that the ubiquitin E3 ligase XBAT35 regulates thermoresponsive hypocotyl elongation through controlling ELF3 degradation in *Arabidopsis*.

RESULTS AND DISCUSSION

XBAT35 is involved in plant thermomorphogenesis

XBAT35 belongs to a small E3 ubiquitin ligase gene family that is involved in abiotic stress responses, apical hook curvature and lateral root development in *Arabidopsis* (Nodzon et al., 2004; Carvalho et al., 2012; Liu et al., 2017; Li et al., 2020; Yu et al., 2020). We obtained several gene-edited loss-of-function mutant alleles of *XBAT35* (Figure S1), and checked their thermoresponsive hypocotyl phenotypes. These mutants (*xbat35-2* and *xbat35-3*) grew normally with a similar hypocotyl length to that of wild-type (WT) plants under normal growth temperature conditions (22°C) (Figure 1A, B). In contrast, the hypocotyl length of *XBAT35* mutant plants was significantly shorter than that of WT plants under warm temperature conditions (29°C) (Figure 1A,B). It is known that PIF4 regulates downstream genes to promote cell expansion and hypocotyl elongation at warm temperature (Franklin et al., 2011). We then checked the expression of *PIF4* and three PIF4-regulated genes, *YUC8*, *IAA19*, and *AT1G73120*, in WT and *xbat35-2* mutant plants. Under normal temperature conditions, the expression level of *YUC8*, *IAA19* and *AT1G73120* in WT plants was similar to that in *xbat35-2* mutant plants (Figure 1C-E). Under warm temperature conditions, the expression level of *PIF4* and these three *PIF4* downstream genes was lower in *xbat35-2* mutant plants than that in WT plants, especially at ZT 24 hr (Figure 1C-F). We then checked the genetic relationship between *XBAT35* and *PIF4* by analysing the thermoresponsive phenotypes in *xbat35-3* mutant, *pif4-101* mutant, and their double mutant plants. The hypocotyl length of *xbat35-3 pif4-101* double mutant plants was similar to that of *pif4-101* mutant plants (Figure S2), suggesting that *PIF4* is epistatic to *XBAT35* in the regulation of thermoresponsive hypocotyl growth.

Together, these results demonstrate that *XBAT35* promotes thermomorphogenesis, which functions upstream of *PIF4* in *Arabidopsis*.

XBAT35 interacts with ELF3 both *in vitro* and *in vivo*

Previous study has revealed that ELF3 regulates the protein activity of PIF4 (Nieto et al. 2015). To check whether XBAT35 interacts with ELF3, *in vitro* pull-down assays were conducted. The results showed that GST-ELF3 could pull-down MBP-XBAT35 (Figure 2A). The *in vivo* interaction between XBAT35 and ELF3 was further confirmed in the split-luciferase assay, split-YFP assay, as well as in co-immunoprecipitation (Co-IP) assay in *Arabidopsis* (Figure 2B-D). Therefore, XBAT35 interacts with ELF3 both *in vitro* and *in vivo*. We also analysed the genetic relationship between *XBAT35* and *ELF3*. Phenotypic analysis showed that the hypocotyl length of *elf3-101 xbat35-2* double mutant plants was similar to that of *elf3-101* mutant plants (Figure 2E-F). We concluded that *ELF3* is epistatic to *XBAT35*, and XBAT35 may function together with ELF3 during plant thermomorphogenesis.

XBAT35 ubiquitinates ELF3 and regulates ELF3 accumulation in *Arabidopsis*

To know whether XBAT35 could ubiquitinate ELF3, *in vitro* ubiquitination assays were carried out. The native form MBP-XBAT35, but not the mutated form MBP-XBAT35M (H428A), was auto-ubiquitinated when detected in the western blot analysis with anti-Ub antibody (Figure 2G). In the presence of E1, E2, Ub, GST-ELF3 and MBP-XBAT35 in the reaction, higher molecular weight bands of GST-ELF3 were detected using *anti*-GST and *anti*-ELF3 antibody, respectively (Figure 2G). In contrast, no shifted bands were detected when a mutated form of XBAT35 (MBP-XBAT35M) was incubated in the reaction (Figure 2G). The expression level of *ELF3* was higher at 29°C than that at 22°C at ZT 24 hr, however, there was no significant difference in the expression level of *ELF3* between WT and *xbat35-2* (Figure 2H). To examine whether XBAT35 regulates ELF3 accumulation *in vivo*, we performed western blotting analysis with *anti*-ELF3 antibody in WT and *xbat35-2* mutant plants under both normal and warm temperature conditions. As recently reported (Ding et al. 2018; Zhang et al. 2021), the endogenous protein level of ELF3 was decreased in the daytime (ZT 8 hr) but increased during night time (ZT 24 hr) in WT plants in response to warm temperatures (Figure 2I-L). Under warm temperature conditions, the protein level of ELF3 was higher in *xbat35-2*

mutant plants than that in WT plants at both ZT 8 hr and ZT 24 hr (Figure 2I-L). Similar to the observations in *bbx18-2 bbx23-2* mutant plants (Ding et al. 2018), the ELF3 protein level was slightly higher in *xbat35-2* mutant than that in WT plants at ZT 24 hr at 22°C (Figure K-L), suggesting that XBAT35 probably also controls ELF3 accumulation under normal ambient temperature conditions. Nonetheless, these results demonstrate that XBAT35 ubiquitinates ELF3 *in vitro* and reduces the accumulation of ELF3 *in vivo* in *Arabidopsis.*

XBAT35 and XBAT31 function redundantly to regulate ELF3 protein stability during thermomorphogenesis

Recently we reported that XBAT31 regulates ELF3 stability under warm temperature conditions (Zhang et al. 2021). To investigate whether these two proteins have redundant functions in thermomorphogenesis, we produced *xbat31-3 xbat35-2* double mutant plants by crossing the respective single mutant plants and checked their thermoresponsive hypocotyl phenotypes. Under normal growth temperature conditions, the hypocotyl length of *xbat31-3 xbat35-2* double mutant plants was similar to that of WT plants (Figure 3A, B). In contrast, under warm temperature conditions, the hypocotyl length of *xbat31-3 xbat35-2* double mutant plants was shorter than that of either *xbat31-3* or *xbat35-2* single mutant plants (Figure 3A, B). We also performed cell free degradation assays with total protein extracts from WT and *xbat31-1 xbat35-3* double mutant plants. Using the *anti*-ELF3 antibody, we found that ELF3 degraded faster in samples from WT plants than that from *xbat31-1 xbat35-3* double mutant plants (Figure 3C, D), which could be inhibited by adding the proteasome inhibitor MG132 to the assays (Figure 3E, F). Therefore, *XBAT35* is functionally redundant to *XBAT31* in regulating ELF3 stability during thermomorphogenesis.

Warm temperatures enhance the accumulation of BBX18, a BBX domain-containing protein that regulates ELF3 accumulation via XBAT31 in *Arabidopsis* (Ding et al., 2018; Zhang et al., 2021). Interestingly, XBAT35 interacted with BBX18 in pull-down assays, as well as in split-luciferase and split-YFP assays (Figure S3). Therefore, it is possible that under warm temperature conditions, BBX18 also recruits XBAT35 to target ELF3 for degradation in *Arabidopsis* (Figure 3G). Although XBAT35 regulated ELF3 accumulation at both ZT 8 hr and ZT24 hr in *Arabidopsis* (Figure 2I-L), the expression of warm temperature-induced genes in *xbat35-2* mutant plants was more affected at ZT 24 hr (Figure 1C-F), while the expression of those genes

in *xbat31-1* mutant plants was more affected at ZT 8 hr (Zhang et al., 2021). These results suggest that XBAT31 and XBAT35 are timely different in terms of their dominant roles in mediating ELF3 degradation and thermoresponsive gene expression. ELF3 is an essential component of the Evening Complex (EC) that regulates circadian clock in plants (McWatters et al., 2000). EC is recruited to the promoter of *PIF4* to suppress *PIF4* expression (Nomoto et al., 2012). In addition, ELF3 physically interacts with PIF4 and prevents PIF4 from activating its transcriptional targets in an EC-independent manner (Nieto et al., 2015). Thus, ELF3 is a negative regulator of PIF4 and inhibits thermoresponsive hypocotyl growth. Based on our previous results on XBAT31 (Zhang et al., 2021) and the current results on XBAT35, we concluded that XBAT35 and XBAT31 ubiquitinate and promote ELF3 degradation under warm temperature conditions, which releases the inhibitory effects of ELF3 on PIF4 and integrates ambient temperature signals with plant growth and development.

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AUTHOR CONTRIBUTIONS

J.X.L. and L.L.Z. designed the experiments; L.L.Z., W.L., and Y.Y.T., performed the experiments; J.X.L. and L.L.Z. analysed the data; J.X.L. S.J.D., and L.L.Z. wrote the paper. All authors read and approved of the content.

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Figures

Figure 1. XBAT35 positively regulates hypocotyl growth under warm temperature conditions in *Arabidopsis*. (**A**-**B**) Phenotypic analysis of the *XBAT35* loss-of-function mutants during thermomorphogenesis. Error bars represent *SD* (n=24). The *pif4-101* mutant was included as a control. Letters above the bars indicate significant differences as determined by HSD test ($P < 0.05$). (**C**-**F**) The expression of *PIF4* or *PIF4*-regulated thermoresponsive genes. Relative gene expression level is the expression in each sample normalized to that in WT at ZT 8 hr at 22°C, both of which were normalized to that of *PP2A*. Error bars depict *SE* (n=3).

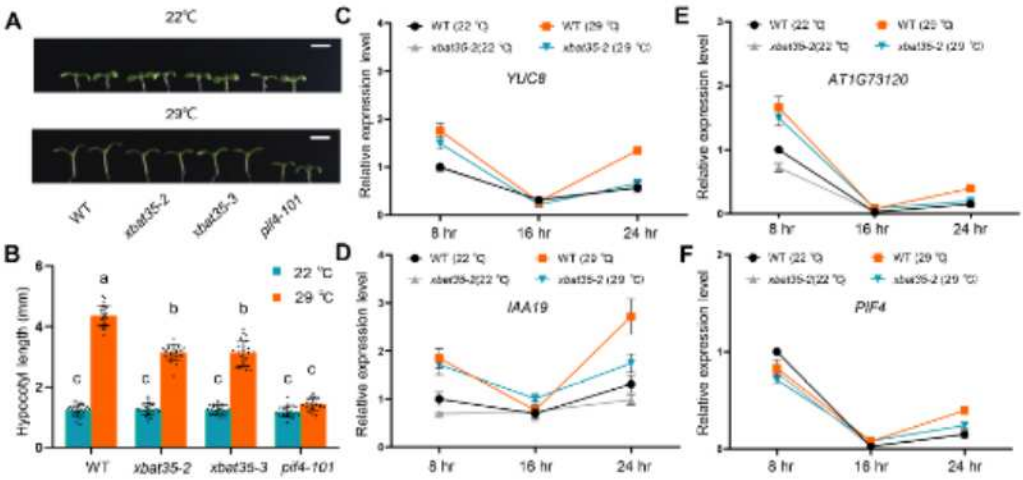

Figure 2. XBAT35 interacts and ubiquitinates ELF3 to regulate ELF3 accumulation in *Arabidopsis*. (**A**-**D**) Protein-protein interactions between XBAT35 and ELF3 in

pull-down assays (**A**), split-luciferase assays (**B**), split-YFP assays (**C**) and Co-IP assays (**D**). Bar = 50 μm. (**E-F**) Genetic analysis between *XBAT35* and *ELF3*. The *xbat35-2* mutant was crossed to the *elf3-101* mutant to generate the double mutant *elf3-101 xbat35-2*. Error bars represent *SD* (n=24). The *pif4-101* mutant was included as a control. (**G**) Ubiquitination assays *in vitro*. GST-ELF3 was incubated with MBP-XBAT35 plus E1, E2 and ubiquitin (Ub) in the reaction. MBP-XBAT35M has a mutation in the RING domain. Brackets highlight the ubiquitinated bands. (**H**) *ELF3* expression in WT and *xbat35-2* mutant plants. Relative gene expression level is the expression in each sample normalized to that in WT at ZT 8 hr at 22°C, both of which were normalized to that of *PP2A*. (**I-L**) ELF3 accumulation in WT and *xbat35-2* mutant plants. Tubulin was a loading control. Protein band intensities from three independent experiments were quantified (J and L) and represented gels from one replicate were shown (**I** and **K**). Error bars represent *SE* (n=3). Letters above the bars indicate significant differences as determined by HSD test in **F**, **J** and **L** ($P < 0.05$).

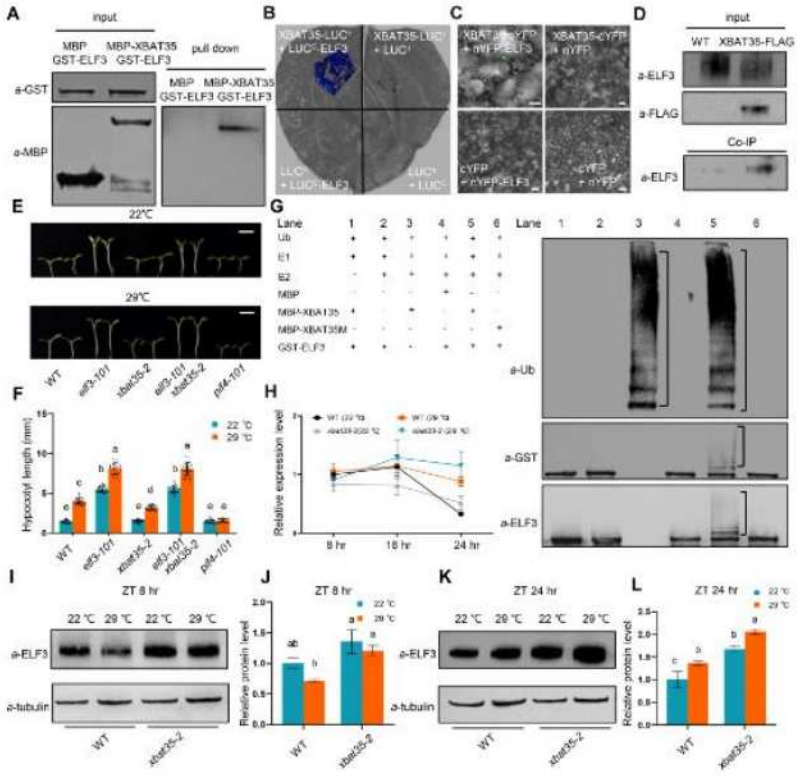

Figure 3. XBAT35 is functionally redundant to XBAT31 during plant thermomorphogenesis. (**A-B**) Phenotypic analysis of the single and double mutant plants of *XBAT31* and *XBAT35*. Error bars depict SD (n=24). The *pif4-101* mutant was used as a control. Bar = 5 mm. Letters above the bars indicate significant differences as determined by HSD test ($P < 0.05$). (**C-F**) Cell free degradation of ELF3 in

samples from WT and *xbat31-1 xbat35-3* double mutant plants. Total protein extracts were incubated with or without the proteasome inhibitor MG132 for 0-80 minutes (min), and the protein level of ELF3 was detected using the *anti*-ELF3 antibody. Tubulin was used as a loading control, and CHX was added to inhibit protein synthesis. Protein band intensities from three independent experiments were quantified (**D** and **F**), and represented gels from one replicate were shown (C and E). Error bars represent SE (n=3). **, $P < 0.01$. (**G**) A simplified working model for the role of XBAT31 and XBAT35 in regulating thermoresponsive hypocotyl growth under warm temperature conditions. In WT plants, BBX18 recruits XBAT31/XBAT35 to target ELF3 for ubiquitination-mediated protein degradation under warm temperature conditions, releasing the inhibitory effects of ELF3 on PIF4 for promoting thermoresponsive hypocotyl growth. In contrast, in *xbat31-1 xbat35-3* double mutant plants, more ELF3 is accumulated under warm temperature conditions, which inhibits both the expression of *PIF4* and protein activity of PIF4, leading to less thermoresponsive growth.