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Drought inhibits thermomorphogenesis via salicylic acid-mediated suppression of ELF3 phase separation

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SUMMARY

Plants are constantly exposed to environmental changes and must respond carefully to ensure survival and growth. Under high temperatures, many plants exhibit a series of morphological and developmental adjustments, including increased hypocotyl and petiole elongation. These adaptations, collectively termed thermomorphogenesis, promote transpiration and water loss, thereby enhancing evaporative cooling. However, this phenomenon has primarily been described under well-watered conditions, whereas in nature, heat often coincides with other environmental challenges, such as drought. How thermomorphogenesis integrates with water shortage conditions, where excess water loss can be detrimental, remains unclear. Here, we demonstrate that restricting water availability and mimicking drought stress with mannitol or PEG inhibit thermomorphogenesis. Mechanistically, both mannitol and PEG treatments reduce high temperature-induced transcriptional activation of *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*), a central regulator of thermomorphogenesis. This suppression is contributed to by the enhanced production of plant phytohormone salicylic acid (SA), which disrupts phase separation and prevents the deactivation of EARLY FLOWERING 3 (ELF3), a repressor of *PIF4*, at high temperatures, thereby inhibiting *PIF4* activation. Our study highlights the trade-off between cooling at high temperatures and minimizing excessive water loss under water-limited conditions, providing insights into plant responses to complex environmental challenges.

INTRODUCTION

Plants are capable of adjusting their development and growth in response to environmental signals. Understanding the mechanisms underlying these adaptive responses offers valuable opportunities for developing crops resilient to environmental challenges. Temperature is a critical environmental signal that regulates plant growth. In response to high temperatures, many plants, such as *Arabidopsis thaliana*, modify their morphology through a phytohormone-mediated process known as thermomorphogenesis. This adaptation includes increased hypocotyl and petiole elongation, resulting in an open structure that enhances plant cooling efficiency by promoting water evaporation (Casal & Balasubramanian, 2019; Crawford et al., 2012; Quint et al., 2016).

The basic helix–loop–helix (bHLH) transcription factor PHYTOCHROME INTERACTING FACTOR 4 (PIF4) is a central regulator in thermomorphogenesis (Franklin et al., 2011; Koini et al., 2009; Sun et al., 2012). *PIF4* is transcriptionally repressed at low temperatures by the evening complex (EC), which consists of EARLY FLOWERING 3 (ELF3), ELF4, and LUX ARRHYTHMO (LUX) (Box et al., 2015). In addition, ELF3 directly interacts with the PIF4 protein in an EC-independent manner and inhibits its activity by preventing PIF4 from activating its target genes (Nieto et al., 2015). Loss of ELF3 leads to the activation of PIF4 target genes in a PIF4-dependent manner (Filo et al., 2015). Elevated temperatures trigger the phase separation of the ELF3 protein, disrupting EC-mediated transcriptional repression of *PIF4* (Jung et al., 2020), and potentially weakening ELF3′s ability to inhibit PIF4 protein activity. Furthermore, the stability and activity of the PIF4 protein are regulated by several repressors, including LONG HYPOCOTYL 5 (HY5), phytochrome B (phyB), TIMING OF CAB EXPRESSION1 (TOC1), and GIGANTEA (GI) (Li et al., 2024; Lorrain et al., 2008; Nohales et al., 2019; Park et al., 2020; Toledo-Ortiz et al., 2014; Zhu et al., 2016). At high temperatures, activated PIF4 directly promotes the transcription of many auxin pathway genes, such as *YUCCA8* (*YUC8*), *INDOLE-3-ACETIC ACID INDUCIBLE 29*

(*IAA29*), *SMALL AUXIN UPREGULATED RNA 20* (*SAUR20*), and *SAUR22*. This enhances auxin synthesis and signaling, thereby inducing cellular elongation (Franklin et al., 2011; Koini et al., 2009; Sun et al., 2012).

While thermomorphogenesis enhances the plant cooling capacity, it is also associated with excessive water loss (Crawford et al., 2012). In nature, multiple environmental challenges often occur simultaneously; for instance, heat frequently coincides with drought (Kong et al., 2020; Mazdiyasni & AghaKouchak, 2015). Understanding how plants coordinate responses to combined heat and drought stress is crucial for revealing the interaction between different stress pathways and informing the development of crops resilient to increasingly complex environmental challenges. However, the integration of thermomorphogenesis with water shortage conditions remains unexplored. Several thermomorphogenesis regulators, including ELF3 and GI, are key circadian oscillator components that also influence the circadian regulation of transpiration and water use efficiency (WUE) (Siemiatkowska et al., 2022; Simon et al., 2020; Sothern et al., 2002). Loss of ELF3 results in a marked reduction in WUE (Simon et al., 2020), accompanied by disrupted circadian rhythmicity of stomatal aperture and a constitutively open-stomata phenotype (Kinoshita et al., 2011). Drought induces the accumulation of the phytohormone abscisic acid (ABA), which in turn triggers various adaptive responses in plants, such as stomatal closure and the activation of stress-responsive genes (Zhu, 2016). Interestingly, exogenous application of ABA inhibits hypocotyl elongation under high temperatures, suggesting that ABA is a negative regulator of thermomorphogenesis (Xu & Zhu, 2020). Another plant hormone, salicylic acid (SA), although best known for its crucial role in plant defense against pathogens, has also been implicated in mediating abiotic stress responses (Liu et al., 2022). SA production is suppressed at high temperatures (Huot et al., 2017; Kim et al., 2022), suggesting a trade-off between thermotolerance and plant immunity.

In this study, we aimed to investigate the impact of water deficit on thermomorphogenesis and found that mild water limitation or drought-mimicking conditions effectively suppress thermomorphogenic responses. Mechanistically, drought inhibits thermomorphogenesis primarily by suppressing the high temperature-induced transcriptional activation of *PIF4*. This suppression is mediated by the increased accumulation of salicylic acid (SA), which prevents the deactivation of ELF3 by disrupting its high temperature-induced phase separation, thereby maintaining *PIF4* repression under simultaneous drought and high temperature conditions. Our findings shed light on how plants integrate responses to multiple environmental challenges, providing insights for developing crops resilient to complex environmental conditions.

RESULTS

Drought inhibits plant thermomorphogenic growth

To investigate whether drought affects plant thermomorphogenesis, we limited water supply for *Arabidopsis* wild type (WT) Columbia (Col) plants grown at 21°C (low temperature) and 27°C (high temperature). For control plants, water was provided sufficiently to fully saturate the soil, while drought-treated plants received a reduced amount of water. We found that reducing water supply to 30% of the usual amount still maintained the plants in relatively healthy condition, without strongly impacting growth rate or causing leaf wilting (Figure 1A; Figure S1a), indicating that our mild water limitation conditions do not constrain basic life activities in the plants. However, limited water supply significantly inhibited high temperature-induced petiole elongation (Figure 1A–C), suggesting that mild drought conditions inhibit thermomorphogenesis.

To test whether limited water supply affects high temperature-induced hypocotyl elongation, another indicator of thermomorphogenesis, *Arabidopsis* WT seedlings were grown on agar plates with or without mannitol, which lowers water potential and mimics drought-induced osmotic stress (Verslues et al., 2006). Mannitol treatment strongly inhibited high temperature-induced hypocotyl elongation (Figure 1D,E) while having no effect on root elongation (Figure S1b,c). Hence, our mild mannitol treatment also effectively suppressed thermomorphogenesis without affecting basic cellular activities. The lack of suppression of hypocotyl elongation by mannitol treatment at low temperatures is likely due to the inherently low water loss under such conditions. Comparable results were obtained using PEG to induce osmotic stress (Figure S1d, e). Based on these findings, we primarily used mannitol in subsequent experiments.

Mannitol treatment suppresses high temperature-induced transcriptional activation of *PIF4*

Thermomorphogenesis is accompanied by extensive changes in gene expression (Quint et al., 2016). To explore the molecular mechanism underlying drought-induced inhibition of thermomorphogenesis, we first examined the basic thermo-response of high temperature and mannitol-treated WT seedlings by assessing the expression of rapidly responsive heat shock genes such as *Heat Shock Protein 70* (*HSP70*) and *HSP101* (Kan et al., 2023). After 2 h of high-temperature treatment, these genes were activated normally (Figure 2A), suggesting that mannitol treatment does not affect the plant's basal heat response. We then compared the transcriptome of WT seedlings shifted from low to high temperatures for 12 h using RNA sequencing (RNA-seq) (Figure 2B). At high temperatures, a greater number of genes were repressed by mannitol treatment compared to those at low temperatures (Figure 2C),

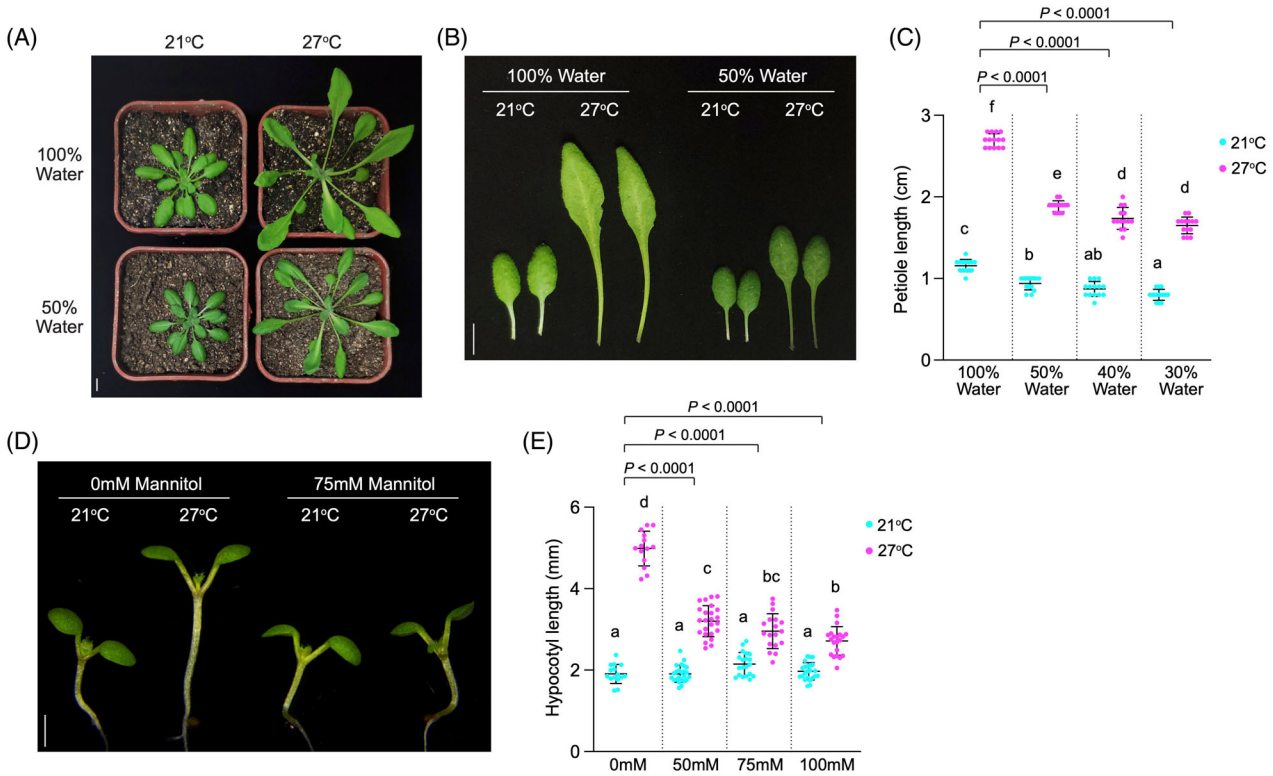

Figure 1. Drought and mannitol treatments inhibit thermomorphogenesis.
(A) Phenotypes of Col plants grown at 21°C and 27°C under well-watered and water-limited conditions. Images were taken 3 weeks after transferring 7-day-old seedlings to well-watered or water-limited soil. Scale bar: 1 cm.
(B) Petiole elongation phenotypes of the 9th and 10th true leaves of Col plants grown at 21°C and 27°C under well-watered and water-limited conditions. Images were taken 3 weeks after transferring 7-day-old seedlings to well-watered or water-limited soil. Scale bar: 1 cm.
(C) Petiole lengths of the 9th and 10th true leaves of Col plants grown at 21°C and 27°C under well-watered and water-limited conditions. For each condition, 14 leaves were measured 3 weeks after transferring 7-day-old seedlings to well-watered or water-limited soil. Values represent means ± SD. Significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences. Significance of interaction (water × temperature) was tested using two-way ANOVA, and the multiple *P*-values shown represent pairwise comparisons between different water conditions under each temperature condition.
(D) Hypocotyl elongation phenotypes of Col seedlings grown at 21°C and 27°C with or without mannitol treatment. Plants were germinated and grown at 21°C without mannitol under short-day conditions for 4 days, then shifted to 27°C or kept at 21°C with or without mannitol treatment for an additional 4 days. Scale bar: 1 mm.
(E) Hypocotyl length of Col seedlings grown at 21°C and 27°C with or without mannitol treatment. For each condition, 14–24 seedlings were measured. Values represent means ± SD. Significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences. Significance of interaction (mannitol × temperature) was tested using two-way ANOVA, and the multiple *P*-values shown represent pairwise comparisons between different mannitol conditions under each temperature condition.

indicating that mannitol has a stronger repressive effect on gene expression at high temperatures. Gene ontology (GO) analysis of mannitol-repressed genes under high temperature conditions revealed an enrichment of auxin-responsive genes (Figure 2D). Upon closer examination of these genes, we found that many are known targets of PIF4. Furthermore, mannitol treatment inhibited the high temperature-induced transcriptional activation of *PIF4* itself (Figure 2E). These findings were further verified by an independent RT-qPCR analysis (Figure 2F).

To test whether mannitol inhibits thermomorphogenesis by suppressing *PIF4* activation, we assessed the effect of mannitol on hypocotyl elongation in a *PIF4* mutant. The results showed that the loss of *PIF4* reduced the inhibitory effect of mannitol on hypocotyl elongation under high temperature conditions. In addition, the further loss of *PIF4* homologous genes completely abolished thermomorphogenic growth and eliminated sensitivity to mannitol (Figure 3A,B) (Leivar & Quail, 2011). We further employed a *35S::PIF4* line, in which *PIF4* is constitutively expressed (Figure S2a,b). Mannitol treatment exhibited a much weaker inhibitory effect on hypocotyl elongation in *35S::PIF4* compared with WT (Figure 3C,D), and a similar trend was observed with PEG treatment (Figure S2c,d). Furthermore, limiting water supply had minimal effect on suppressing petiole elongation in *35S::PIF4* plants grown in

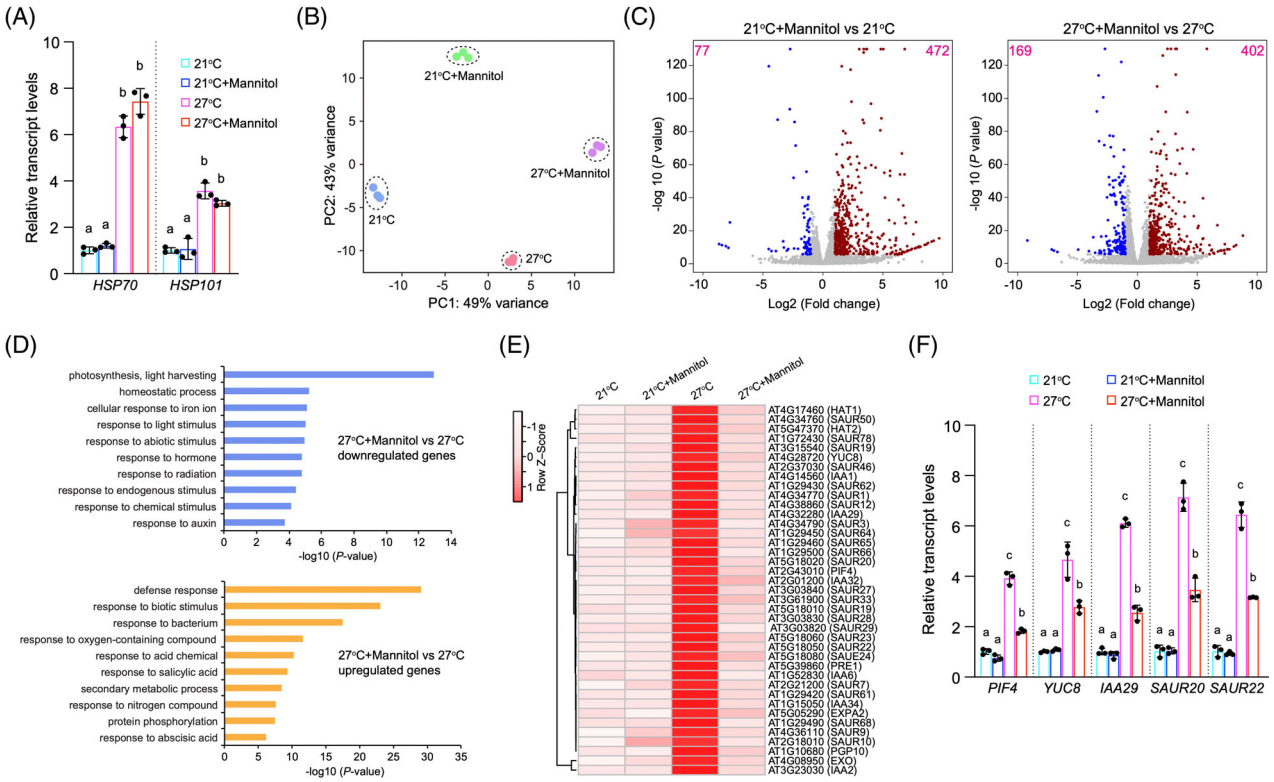

Figure 2. Mannitol treatment suppresses high temperature-induced *PIF4* activation.
(A) Relative transcript levels of *HSP70* and *HSP101* in Col seedlings grown at 21°C and 27°C with or without mannitol treatment, as determined by RT-qPCR. Seven-day-old seedlings grown at 21°C under short-day conditions were shifted to 27°C or kept at 21°C with or without 100 mM mannitol treatment at ZT1, and seedlings were collected at ZT3 for RNA extraction. Values represent means ± SD of three biological replicates. The significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences.
(B) PCA plot of Col seedlings grown under different conditions showing transcriptome differences determined by RNA-Seq. Seven-day-old seedlings grown at 21°C under short-day conditions were shifted to 27°C or kept at 21°C with or without 100 mM mannitol treatment at ZT1, and seedlings were collected at ZT13 for RNA-Seq. PC1 covers the highest amount of variance between samples; PC2 covers most of the remaining variance. Three biological replicates were performed for each condition.
(C) Volcano plots of differentially expressed genes (DEGs). The *y*-axis values correspond to -$\log_{10}$ (*P*-adjust), and the *x*-axis values correspond to $\log_2$ (fold change). Genes with at least twofold expression changes and *P*-adjust less than 0.05 are considered misexpressed. The numbers of transcript level increased and decreased genes are indicated at the top right and left corners, respectively.
(D) Gene ontology (GO) analysis of significantly upregulated and downregulated genes in response to mannitol treatment at 27°C. The top 10 representative terms are listed, ranked by *P*-value.
(E) Heatmap showing transcript levels of auxin-related genes downregulated by mannitol, as determined by RNA-Seq. Data represent the mean of three biological replicates.
(F) Relative transcript levels of *PIF4*, *YUC8*, *IAA29*, *SAUR20*, and *SAUR22* in Col seedlings grown at 21°C and 27°C with or without mannitol treatment, as determined by RT-qPCR. Seven-day-old seedlings grown at 21°C under short-day conditions were shifted to 27°C or kept at 21°C with or without 100 mM mannitol treatment at ZT1, and seedlings were collected at ZT13 for RNA extraction. Values represent means ± SD of three biological replicates. The significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences.

soil (Figure S2e,f). These results confirm that drought represses thermomorphogenesis primarily by suppressing the transcriptional activation of *PIF4*. Notably, hypocotyl elongation and petiole elongation in *35S::PIF4* were still slightly repressed by mannitol/PEG treatment and water-limitation conditions, respectively (Figure 3C,D; Figure S2c–f). Moreover, although mannitol/PEG did not reduce *PIF4* transcript levels in *35S::PIF4* (Figure S2a,b), it mildly decreased the expression of PIF4 target genes (Figure 3E,F; Figure S2g,h). This suggests that drought may also impact PIF4 protein abundance or activity.

Mannitol treatment-induced salicylic acid inhibits thermomorphogenesis by suppressing *PIF4* activation

To further investigate how mannitol treatment leads to the suppression of *PIF4* transcriptional activation at high temperatures, we examined the gene expression profile of mannitol-treated seedlings. We found that genes induced by mannitol at high temperatures were highly enriched in defense response, response to bacteria, and response to salicylic acid (SA) pathways. In addition, as expected, abscisic acid (ABA)-responsive genes were also enriched

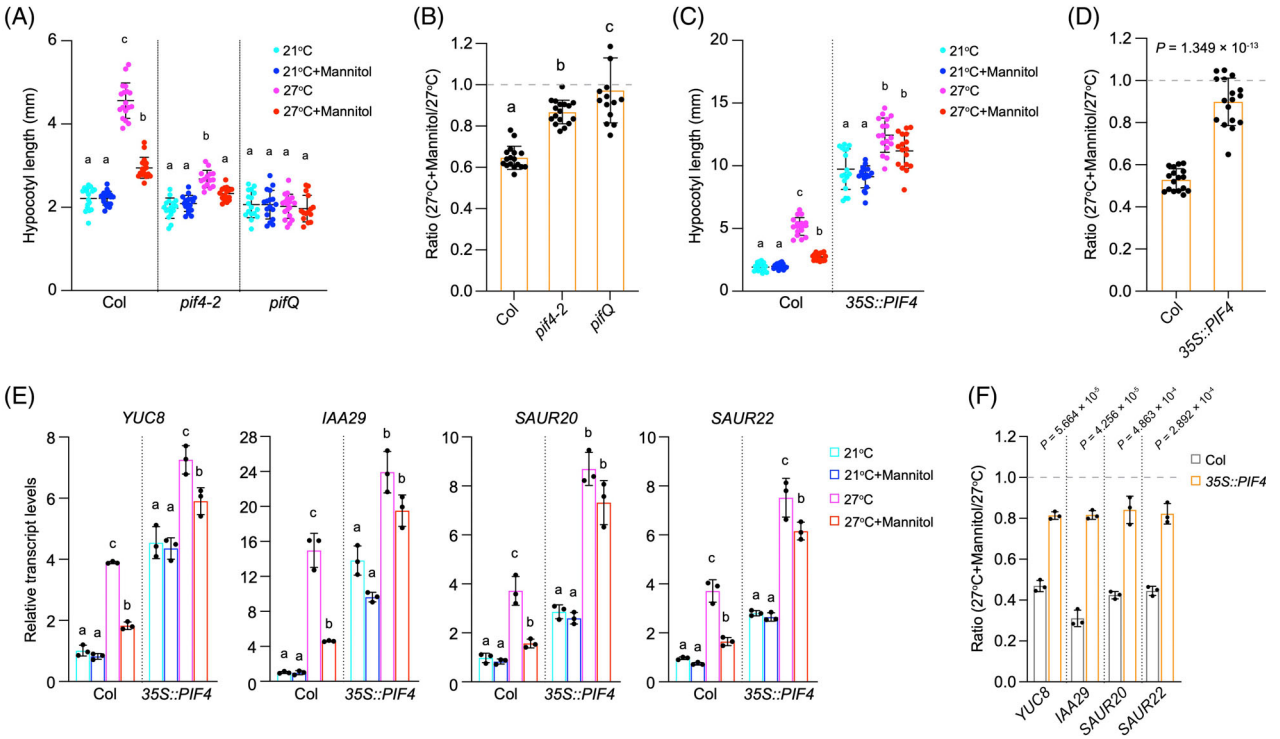

Figure 3. Mannitol suppresses thermomorphogenesis via PIF4.
(A) Hypocotyl length of Col, *pif4-2*, and *pifQ* (*pif1;pif3;pif4;pif5* quadruple mutant) seedlings grown at 21°C and 27°C with or without mannitol treatment. Plants were germinated and grown at 21°C under short-day conditions for 4 days and were then shifted to 27°C or kept at 21°C with or without 100 mM mannitol treatment for an additional 4 days. 15–19 seedlings were measured for each line in each condition. Values represent means ± SD. Significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences.
(B) The ratio of hypocotyl length in Col, *pif4-2*, and *pifQ* seedlings grown at 27°C with mannitol treatment compared to those grown without treatment. 15–17 seedlings were scored for each line. Values represent means ± SD. The significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences.
(C) Hypocotyl length of Col and *35S::PIF4* seedlings grown at 21°C and 27°C with or without mannitol treatment. Plants were germinated and grown at 21°C under short-day conditions for 4 days and were then shifted to 27°C or kept at 21°C with or without 100 mM mannitol treatment for an additional 4 days. Seventeen seedlings were measured for each line in each condition. Values represent means ± SD. Significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences.
(D) The ratio of hypocotyl length in Col and *35S::PIF4* seedlings grown at 27°C with mannitol treatment compared to those grown without treatment. Seventeen seedlings were scored for each line. Values represent means ± SD. Statistical significance was determined by two-tailed Student's *t*-test.
(E) Relative transcript levels of *YUC8, IAA29, SAUR20,* and *SAUR22* in Col and *35S::PIF4* seedlings grown at 21°C and 27°C with or without mannitol treatment, as determined by RT-qPCR. Seven-day-old seedlings grown at 21°C under short-day conditions were shifted to 27°C or kept at 21°C with or without 100 mM mannitol treatment at ZT1, and seedlings were collected at ZT13 for RNA extraction. Values represent means ± SD of three biological replicates. The significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences.
(F) The ratio of transcript levels of *YUC8, IAA29, SAUR20,* and *SAUR22* in Col and *35S::PIF4* seedlings at 27°C with mannitol treatment compared to those grown without treatment. Values represent means ± SD of three biological replicates. Statistical significance was determined by two-tailed Student's *t* test.

(Figure 2D). Previous studies have reported that exogenous application of ABA suppresses thermomorphogenesis (Xu & Zhu, 2020). However, the effect of SA on thermomorphogenesis remains unclear. Interestingly, the expression of key genes involved in SA biosynthesis, such as *CALMODULIN BINDING PROTEIN 60G* (*CBP60g*) *and ISOCHORISMATE SYNTHASE 1* (*ICS1*) (Peng et al., 2021), was upregulated by mannitol treatment (Figure S3a). Based on these observations, we focused on SA and first measured its content in response to mannitol treatment. Without mannitol, SA content was slightly reduced by elevated temperatures. However, mannitol treatment led to an increase in SA content (Figure 4A), consistent with the induction of SA biosynthesis genes. Similarly, plants grown under water-limited soil conditions or treated with PEG also accumulated higher levels of SA (Figure S3b,c).

We then treated seedlings with SA and found that applying SA could inhibit high temperature-induced hypocotyl elongation (Figure 4B,C). Furthermore, SA treatment repressed the activation of *PIF4* and its target genes at high temperatures (Figure 4D). The effect of SA on inhibiting hypocotyl elongation was strongly reduced when *PIF4* is constitutively expressed in the *35S::PIF4* line (Figure 4E,F; Figure S3d). This is also reflected by the reduced responsiveness of PIF4 target genes in *35S::PIF4* (Figure 4G,H). Nevertheless, similar to mannitol and PEG, SA slightly

repressed PIF4 target genes in *35S::PIF4*, suggesting that SA may influence PIF4 protein abundance or activity as well.

To determine whether mannitol represses thermomorphogenesis through SA, we applied mannitol to the *sid2-2* mutant and a *NahG* transgenic line. The *sid2-2* mutant has a mutation in ICS1, the main enzyme involved in SA synthesis (Wildermuth et al., 2001), while the *NahG* line constitutively expresses an SA hydroxylase from *Pseudomonas putida*, which converts SA to catechol, thus reducing SA levels in the plant (Delaney et al., 1994). Both *sid2-2* and the *NahG* line displayed reduced sensitivity to mannitol treatment (Figure S3e,f), indicating that the mannitol-induced repression of thermomorphogenesis depends on SA. However, *sid2-2* and the *NahG* line still showed partial responsiveness to mannitol, which could be attributed to residual SA production in these plants (Garcion et al., 2008; Vlot et al., 2009) or the involvement of ABA in the absence of SA.

Mannitol and SA repress thermomorphogenesis via ELF3

PIF4 transcription, protein abundance, or activity are suppressed by several repressors, including the evening complex (EC), LONG HYPOCOTYL 5 (HY5), phytochrome B

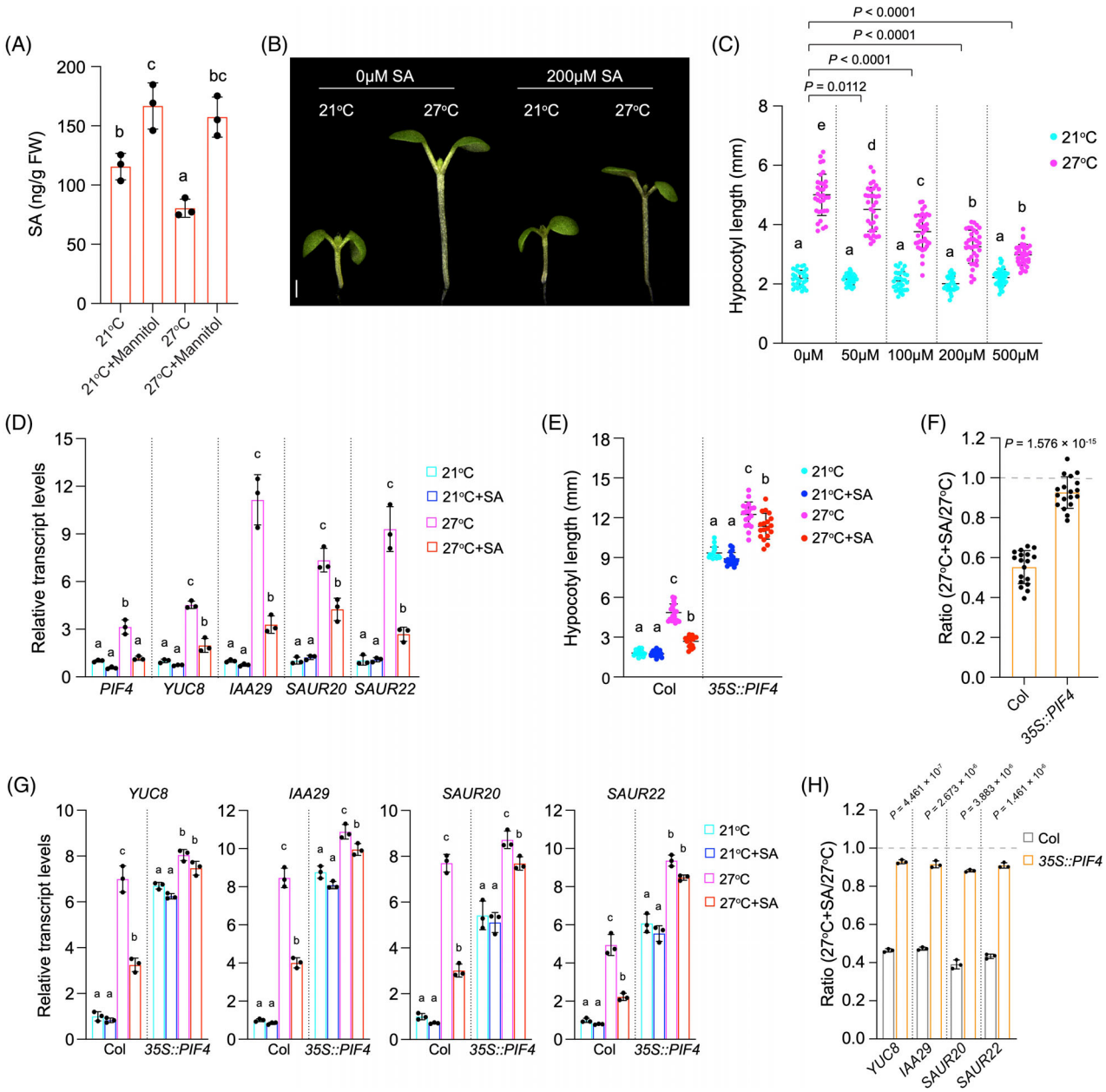

Figure 4. Mannitol-induced salicylic acid suppresses *PIF4* activation.
(A) SA levels in Col seedlings grown at 21°C and 27°C with or without mannitol treatment. Seven-day-old seedlings grown at 21°C were shifted to 27°C or kept at 21°C with or without 100 mM mannitol treatment at ZT1, and seedlings were collected at ZT13 for SA measurement. Values represent means ± SD of three biological replicates. The significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences. FW, fresh weight.
(B) Hypocotyl elongation phenotypes of Col seedlings grown at 21°C and 27°C with or without SA treatment. Plants were germinated and grown at 21°C without SA under short-day conditions for 4 days, then shifted to 27°C or kept at 21°C with or without SA treatment for an additional 4 days. Scale bar: 1 mm.
(C) Hypocotyl length of Col seedlings grown at 21°C and 27°C with or without SA treatment. For each condition, 29 to 38 seedlings were measured. Values represent means ± SD. Significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences. Significance of interaction (SA × temperature) was tested using two-way ANOVA, and the multiple *P*-values shown represent pairwise comparisons between different SA concentrations under each temperature condition.
(D) Relative transcript levels of *PIF4*, *YUC8*, *IAA29*, *SAUR20*, and *SAUR22* in Col seedlings grown at 21°C and 27°C with or without SA treatment, as determined by RT-qPCR. Seven-day-old seedlings grown at 21°C under short-day conditions were shifted to 27°C or kept at 21°C with or without 200 μM SA treatment at ZT1, and seedlings were collected at ZT13 for RNA extraction. Values represent means ± SD of three biological replicates. The significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences.
(E) Hypocotyl length of Col and *35S::PIF4* seedlings grown at 21°C and 27°C with or without SA treatment. Plants were germinated and grown at 21°C under short-day conditions for 4 days, and were then shifted to 27°C or kept at 21°C with or without 200 μM SA treatment for an additional 4 days. Eighteen seedlings were measured for each line in each condition. Values represent means ± SD. Significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences.
(F) The ratio of hypocotyl length in Col and *35S::PIF4* seedlings grown at 27°C with SA treatment compared to those grown without treatment. Eighteen seedlings were scored for each line. Values represent means ± SD. Statistical significance was determined by two-tailed Student's *t*-test.
(G) Relative transcript levels of *YUC8*, *IAA29*, *SAUR20*, and *SAUR22* in Col and *35S::PIF4* seedlings grown at 21°C and 27°C with or without SA treatment, as determined by RT-qPCR. Seven-day-old seedlings grown at 21°C under short-day conditions were shifted to 27°C or kept at 21°C with or without 200 μM SA treatment at ZT1, and seedlings were collected at ZT13 for RNA extraction. Values represent means ± SD of three biological replicates. The significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences.
(H) The ratio of transcript levels of *YUC8*, *IAA29*, *SAUR20*, and *SAUR22* in Col and *35S::PIF4* seedlings at 27°C with SA treatment compared to those grown without treatment. Values represent means ± SD of three biological replicates. Statistical significance was determined by two-tailed Student's *t*-test.

(phyB), TIMING OF CAB EXPRESSION1 (TOC1), and GIGANTEA (GI) (Delker et al., 2014; Lorrain et al., 2008; Nohales et al., 2019; Nusinow et al., 2011; Park et al., 2020; Zhu et al., 2016). To determine whether these repressors are involved in mannitol-mediated inhibition of thermomorphogenesis, we analyzed corresponding mutant lines. Our results revealed that the loss of EC components EARLY FLOWERING 3 (ELF3), ELF4, and LUX ARRHYTHMO (LUX) provided the strongest relief from the repression of hypocotyl elongation by mannitol (Figure 5A; Figure S4a). Moreover, the loss of ELF3 reduced the sensitivity of petiole elongation to water limitation and of hypocotyl elongation to PEG treatment under high temperature conditions (Figure S4b–e). Further analysis of *PIF4* transcript levels revealed that the loss of ELF3 almost completely abolished the inhibitory effect of mannitol/PEG on *PIF4* transcription at high temperatures (Figure 5B,C; Figure S4f,g). Consistently, the repression of PIF4 target genes by mannitol/PEG at high temperatures was relieved in the absence of ELF3 (Figure 5D,E; Figure S4h,i).

As mannitol represses thermomorphogenesis through SA, we then tested whether ELF3 is essential for SA-mediated suppression of thermomorphogenesis as well. Indeed, the loss of ELF3 greatly reduced the inhibitory effect of SA on hypocotyl elongation at high temperatures (Figure 5F,G). Furthermore, the suppression of *PIF4* transcription and its target genes by SA at high temperatures was strongly compromised by the loss of ELF3 (Figure 5H–K). Together, we conclude that mannitol and SA repress thermomorphogenesis through ELF3.

Mannitol and SA inhibit high temperature-induced phase separation of ELF3

To investigate the impact of mannitol and SA on ELF3, we first analyzed *ELF3* transcript levels following mannitol or SA treatments and observed no significant effect on *ELF3* transcription (Figure S5a,b). In addition, mannitol or SA treatments did not affect the ELF3 protein abundance (Figure S5c). At low temperatures, ELF3 is dispersed in the nucleus, allowing it to form EC with ELF4 and LUX to repress gene transcription. Temperature elevation induces phase separation of ELF3 protein, which inhibits ELF3 chromatin binding and EC formation, thereby enabling gene activation (Jung et al., 2020). To test whether mannitol and SA affect the phase separation of ELF3, we employed an *elf3-7;gELF3-GFP* line, which behaves identically to the WT (Figure S5d,e). Mannitol and SA treatments significantly reduced the number of high temperature-induced ELF3 speckles (Figure 6A,B). Consistently, high temperature-mediated dissociation of ELF3 from the *PIF4* promoter was compromised by mannitol and SA (Figure 6C,D), suggesting that ELF3 continuously suppresses *PIF4* transcription in the presence of mannitol and SA.

DISCUSSION

A key yet largely unexplored question in plant environmental responses is how plants adapt to multiple simultaneous challenges. In this study, we address this by examining plant responses to concurrent heat and drought. Our findings reveal that limiting water supply or mimicking

drought-induced osmotic stress with mannitol or PEG effectively suppresses thermomorphogenesis (Figure 1; Figure S1). Similarly, although heat promotes stomatal opening, drought has an antagonistic effect. When both conditions are present, the impact of drought dominates, and the heat-induced increase in stomatal opening is abolished under drought (Rizhsky et al., 2002; Sato et al., 2024; Zandalinas et al., 2016). These observations indicate that plants prioritize reducing water loss over cooling under water-limited conditions. This adaptive strategy likely

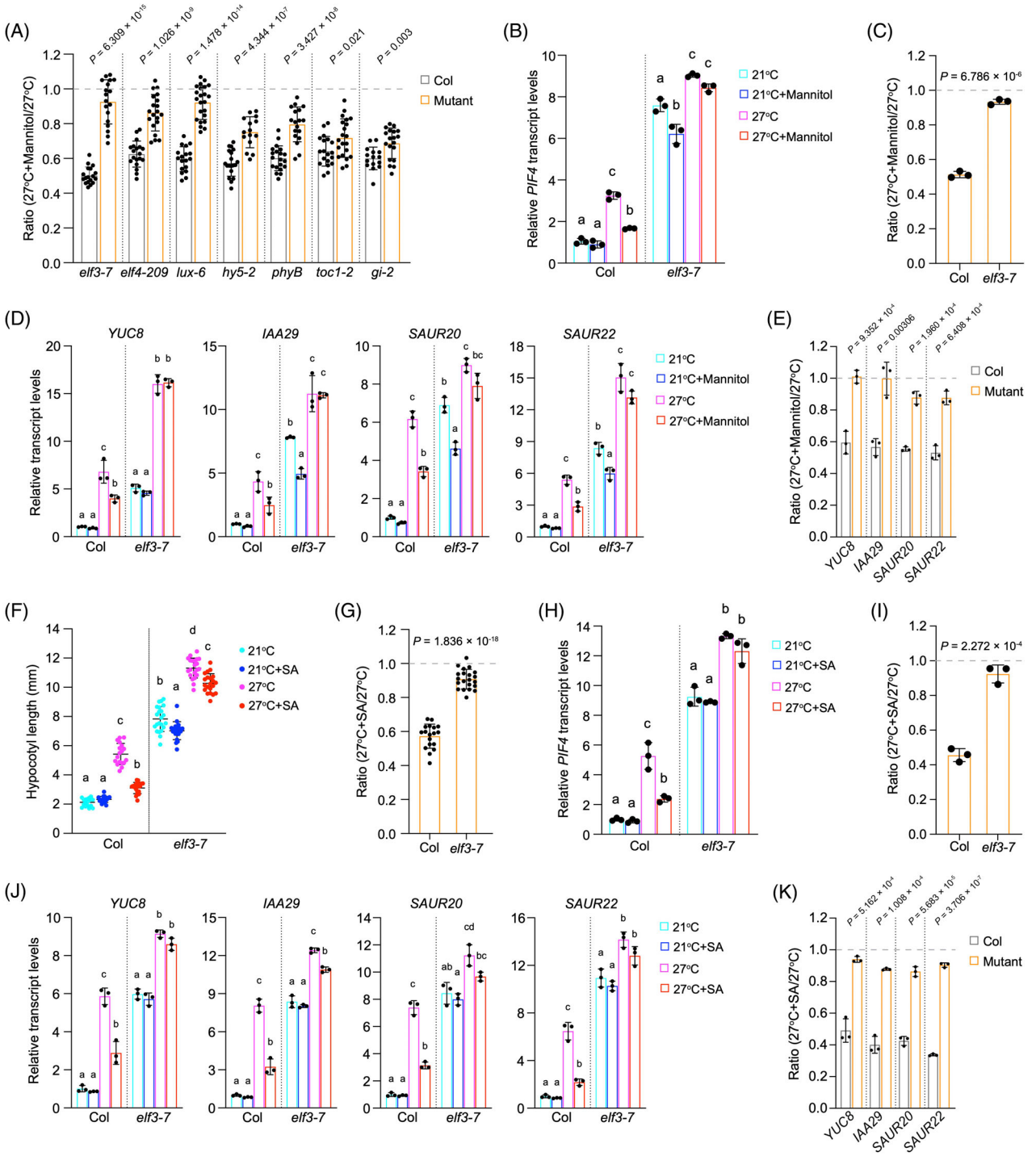

Figure 5. Mannitol and SA suppress *PIF4* activation via ELF3.
(A) The ratio of hypocotyl length in Col and tested mutants grown at 27°C with mannitol treatment compared to those grown without treatment. 15–23 seedlings were scored for each line. Values represent means ± SD. Statistical significance was determined by two-tailed Student's *t*-test.
(B, H) Relative transcript levels of *PIF4* in Col and *elf3-7* seedlings grown at 21°C and 27°C with or without mannitol (B)/SA (H) treatment, as determined by RT-qPCR. Seven-day-old seedlings grown at 21°C under short-day conditions were shifted to 27°C or kept at 21°C with or without 100 mM mannitol (B)/200 μM SA (H) treatment at ZT1, and seedlings were collected at ZT13 for RNA extraction. Values represent means ± SD of three biological replicates. The significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences.
(C, I). The ratio of transcript levels of *PIF4* in Col and *elf3-7* seedlings at 27°C with mannitol (C)/SA (I) treatment compared to those grown without treatment. Values represent means ± SD of three biological replicates. Statistical significance was determined by two-tailed Student's *t*-test.
(D, J) Relative transcript levels of *YUC8*, *IAA29*, *SAUR20*, and *SAUR22* in Col and *elf3-7* seedlings grown at 21°C and 27°C with or without mannitol (D)/SA (J) treatment, as determined by RT-qPCR. Seven-day-old seedlings grown at 21°C under short-day conditions were shifted to 27°C or kept at 21°C with or without 100 mM mannitol (D)/200 μM SA (J) treatment at ZT1, and seedlings were collected at ZT13 for RNA extraction. Values represent means ± SD of three biological replicates. The significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences.
(E, K). The ratio of transcript levels of *YUC8*, *IAA29*, *SAUR20*, and *SAUR22* in Col and *elf3-7* seedlings at 27°C with mannitol (E)/SA (K) treatment compared to those grown without treatment. Values represent means ± SD of three biological replicates. Statistical significance was determined by two-tailed Student's *t*-test.
(F) Hypocotyl length of Col and *elf3-7* seedlings grown at 21°C and 27°C with or without SA treatment. Plants were germinated and grown at 21°C under short-day conditions for 4 days, and were then shifted to 27°C or kept at 21°C with or without 200 μM SA treatment for an additional 4 days. 18–21 seedlings were measured for each line in each condition. Values represent means ± SD. Significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences.
(G) The ratio of hypocotyl length in Col and *elf3-7* seedlings grown at 27°C with SA treatment compared to those grown without treatment. 18–21 seedlings were scored for each line. Values represent means ± SD. Statistical significance was determined by two-tailed Student's *t*-test.

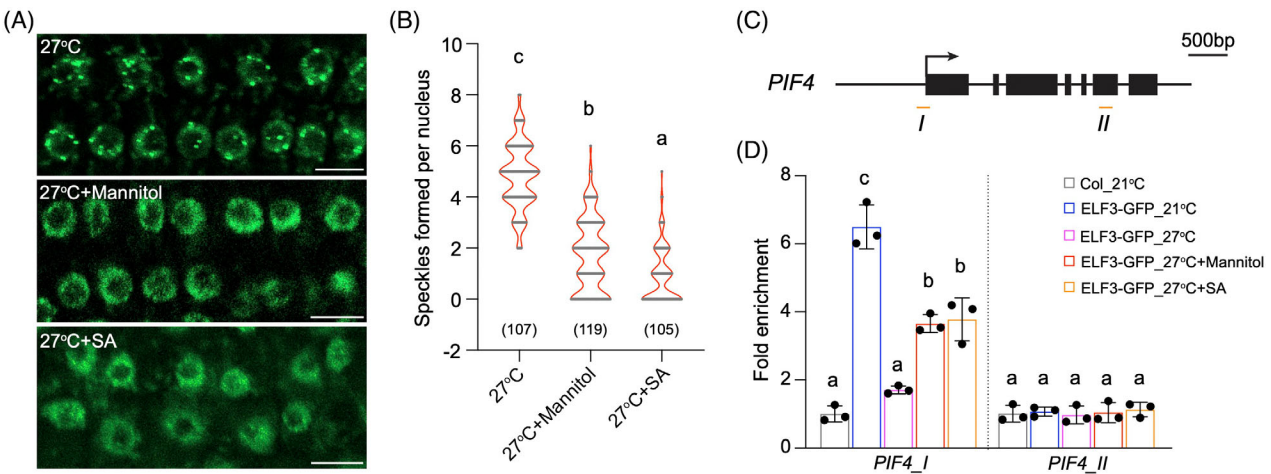

Figure 6. Mannitol and SA suppress high temperature-induced ELF3 phase separation.
(A) ELF3-GFP signals in root nuclei at 27°C with or without mannitol or SA treatment. Four-day-old *elf3-7;gELF3-GFP* seedlings grown at 21°C under short-day conditions were shifted to 27°C with or without 100 mM mannitol/200 μM SA treatment at ZT1, and ELF3-GFP signals in roots were observed at ZT13. Scale bars: 10 μm.
(B) Quantification of ELF3 speckles in root nuclei at 27°C with or without mannitol or SA treatment. Numbers in parentheses indicate the amounts of counted nuclei.
(C) Schematic structures of *PIF4*. The arrow indicates the transcription start site and boxes represent exons. Orange lines indicate regions examined by ChIP-qPCR.
(D) ELF3 enrichment levels at *PIF4* at 21°C and 27°C with or without mannitol or SA treatment, as determined by ChIP-qPCR. Seven-day-old seedlings grown at 21°C under short-day conditions were shifted to 27°C or kept at 21°C with or without 100 mM mannitol/200 μM SA treatment at ZT1, and seedlings were collected at ZT13 for ChIP assay. DNA fragments immunoprecipitated by anti-GFP were first normalized by input and then normalized to Col (control). Values represent means ± SD of three biological repeats. The significance of differences was tested using one-way ANOVA with Tukey's test ($P < 0.05$), with different letters indicating statistically significant differences.

enhances plant survival by addressing the more critical challenge of drought stress over temperature elevation.

We found that mannitol treatment strongly suppresses the high temperature-induced transcriptional activation of *PIF4* (Figure 2E,F). Moreover, constitutive expression of *PIF4* largely abolishes drought-mediated suppression of thermomorphogenesis (Figure 3; Figure S2), demonstrating that drought primarily exerts its effects by regulating *PIF4* transcription. Nevertheless, the *35S::PIF4* line remains slightly responsive to drought treatment, suggesting the involvement of additional mechanisms beyond transcriptional regulation. This is in agreement with the

partial loss of sensitivity of thermomorphogenesis to mannitol treatment observed in the absence of phyB and HY5 (Figure 5A; Figure S4a), which repress PIF4 protein stability and activity, respectively (Lorrain et al., 2008; Toledo-Ortiz et al., 2014).

SA is a plant hormone essential for plant defense against pathogens (Vlot et al., 2009). Moreover, SA has been shown to regulate plant tolerance to various abiotic stresses (Liu et al., 2022). High temperatures inhibit both basal and pathogen-induced SA production, suggesting that plants compromise their defense capabilities to adapt to heat (Huot et al., 2017; Kim et al., 2022). We found that drought treatment enhances SA biosynthesis (Figure 4A; Figure S3a–c). Exogenous application of SA resembles the inhibitory effects of mannitol on thermomorphogenesis, and SA is required for the repression induced by mannitol (Figure 4; Figure S3e,f). Thus, compared to the potential threat of pathogen infection, the more immediate and critical threat of drought stress likely drives plants to restore and increase SA production under high temperature conditions.

We show that drought inhibits thermomorphogenesis primarily through ELF3, a key component of the EC complex that represses *PIF4* transcription (Figure 5; Figure S4). ELF3 exhibits phase separation at elevated temperatures, resulting in dense aggregation and functional inactivation (Jung et al., 2020). This temperature-dependent phase separation is primarily driven by a prion-like domain containing polyglutamine (polyQ) repeats (Jung et al., 2020). Notably, natural variation in polyQ length has been observed among *Arabidopsis* accessions (Undurraga et al., 2012), suggesting a potential link between polyQ variation and environmental adaptation (Wilkinson & Strader, 2020). However, studies have not found compelling evidence supporting that polyQ length variation plays a role in temperature adaptation or is associated with differential temperature responsive phenotypes among *Arabidopsis* accessions (Press et al., 2016; Zhu et al., 2024). Our results show that mannitol or SA treatment suppressed high temperature-induced phase separation of ELF3, enabling sustained binding of ELF3 to the *PIF4* promoter (Figure 6). This mechanism explains the lack of full activation of *PIF4* transcription by high temperatures under mannitol or SA treatment. In addition, given that ELF3 inhibits PIF4 protein activity (Nieto et al., 2015), the prevention of ELF3 phase separation under these treatments may also affect PIF4 protein activation in response to high temperatures. It would be of interest to investigate whether natural variation in polyQ length is associated with differential responses to combined heat and drought stress.

Based on these results, we propose that drought/osmotic stress suppresses thermomorphogenesis by stimulating SA production, which in turn inhibits ELF3 phase separation at high temperatures. This inhibition prevents the high temperature-induced activation of *PIF4* transcription and may also impair the activation of PIF4 protein activity (Figure 7). This likely represents a trade-off mechanism to prevent excessive water loss caused by thermomorphogenesis under water-limited conditions. Interestingly, loss of ELF3 results in a continuously open-stomata phenotype (Kinoshita et al., 2011). Therefore, preventing high temperature-induced ELF3 deactivation, such as by disrupting its phase separation, may also help restrict stomatal opening and reduce water loss under drought. Supporting this notion, *elf3* mutant plants showed reduced survival rates under drought conditions (Figure S5f,g), highlighting the crucial role of ELF3 in drought resistance. Our findings thus reveal a novel regulatory mechanism involving ELF3 in the plant response to simultaneous heat and drought. However, how drought/osmotic stress stimulates SA biosynthesis and the mechanism by which SA inhibits ELF3 phase separation remain to be elucidated. Together, these findings enhance the understanding of plant responses to concurrent heat and drought, offering valuable insights for developing crops resilient to complex environmental challenges.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis Columbia-0 was used as the wild type, and all *Arabidopsis* materials used in this study were in Columbia (Col) background. *elf3-7* (Hicks et al., 2001), *elf4-209* (Zhang et al., 2018), *lux-6* (Zhang et al., 2018), *hy5-2* (Bou-Torrent et al., 2015), *phyB* (Salk_022035) (Ruckle et al., 2007), *gi-2* (Huq et al., 2000), *toc1-2* (Ito et al., 2008), *pif4-2* (Sun et al., 2012), *pifQ* (Leivar et al., 2008), *35S::PIF4* (Xue et al., 2021), *sid2-2* (Wildermuth et al., 2001), and *NahG* (Delaney et al., 1994) have been reported previously.

To examine hypocotyl and root elongation phenotypes, plants were germinated on 1/2 Murashige and Skoog (1/2 MS) plates and grown at 21°C under short-day conditions (8 h light and 16 h dark) for 4 days, and were then shifted to 27°C or kept at 21°C with or without mannitol/PEG/SA treatment for an additional 4 days. To examine petiole elongation phenotypes, plants were germinated on 1/2 MS plates at 21°C and grown for 7 days. Prior to transplanting, equal weights of soil were placed into plant growth containers (8 cm × 8 cm × 8 cm). For the well-watered condition, water was added to fully saturate the soil (100% water content). For water-limited conditions, 50%, 40%, or 30% of the full water volume was used. 7-day-old seedlings were then transplanted into the prepared soils, with one seedling per container, and grown at either 21 or 27°C under short-day conditions with 50% humidity. Watering was performed every 3 days by applying two drops of water directly to the soil surface at the shoot-root junction for the well-watered condition and one drop for the water-limited condition. For drought treatment, equal weights of soil were placed into plant growth containers (6 cm × 6 cm × 5.5 cm), and 7-day-old seedlings were transferred to fully saturate soil, with four seedlings per container, and grown under long-day conditions (16 h light and 8 h dark) at 25°C with 50% humidity. Watering was withheld for 18 days following transplantation, after which rewatering was performed.

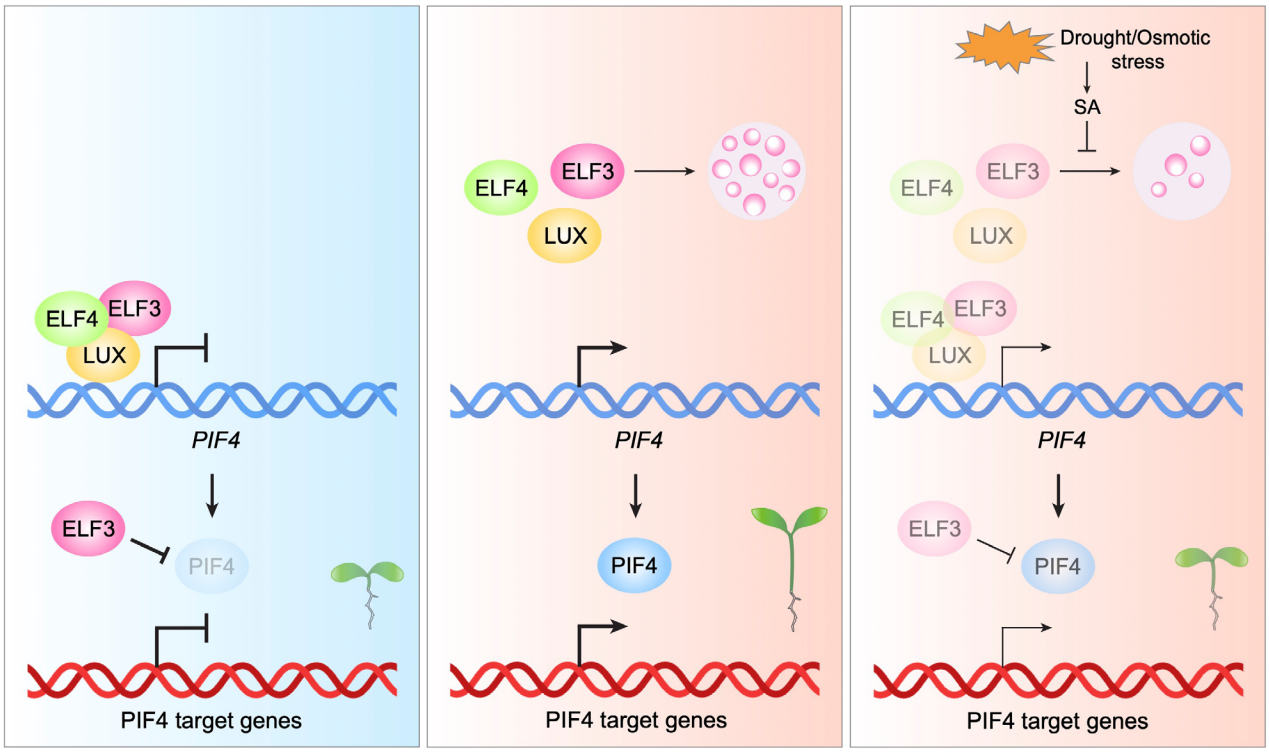

Figure 7. Proposed model of drought/osmotic stress suppressing thermomorphogenesis.
At low temperatures, ELF3, ELF4, and LUX form EC to repress *PIF4* transcription. In addition, ELF3 can inhibit PIF4 protein activity independent of ELF4 and LUX. Under well-watered conditions, high temperature triggers ELF3 phase separation, causing the dissociation of EC from the *PIF4* promoter and subsequent transcriptional activation of *PIF4*, as well as activation of PIF4 protein. However, under limited water supply, drought/osmotic stress stimulates SA biosynthesis. SA inhibits ELF3 phase separation at high temperatures, thereby suppressing both the transcriptional activation of *PIF4* and its protein activity. As a result, *PIF4* target genes are not fully activated, and thermomorphogenesis is repressed.

Media preparation

1/2 MS medium was prepared by dissolving 2.2 g of Murashige & Skoog Basal Salt Mixture (Phytotech, M524) and 0.5 g of MES monohydrate (Macklin, M813152) in 1 L of water, followed by adjusting the pH to 5.8. To mimic drought-induced osmotic stress, the non-metabolizable osmotic agents mannitol and PEG were used. Mannitol-containing media were prepared by directly dissolving mannitol (Sigma, M1902) into the 1/2 MS medium at the desired concentrations. Subsequently, 8 g of agar was added, and the medium was autoclaved. To prepare polyethylene glycol (PEG)-infused plates, PEG-6000 (Sigma, 81 260) was dissolved in autoclaved 1/2 MS liquid medium to make the PEG overlay solution at the desired concentrations. Subsequently, 30 mL of the PEG solution was gently added on top of 20 mL of solidified 1/2 MS agar medium, and the plates were allowed to equilibrate overnight. To prepare salicylic acid-containing media, sodium salicylic acid (Sigma, 71 945) was dissolved in distilled water to make a 100 mM stock solution. The appropriate volume of this stock was added to autoclaved 1/2 MS medium to achieve the desired final concentration before pouring the media plates.

Vector construction

The gELF3-GFP construct was generated by cloning the full-length genomic sequence of *ELF3*, including its native promoter, from Col into the pGWB540 vector (Nakagawa et al., 2007) by BP/LR reactions (Thermo Fisher Scientific), without any modifications to the sequence, to create a C-terminal fusion with GFP. For the 35S::ELF3-HA construct, the full-length coding sequence of *ELF3* from Col was cloned into a modified pGWB513 plasmid containing the CaMV 35S promoter (Zhao et al., 2023) by BP/LR reactions (Thermo Fisher Scientific), to fuse it with an HA tag. Primers used for amplification are specified in Table S1.

RNA-Seq and RT-qPCR

For both RNA-Seq and RT-qPCR, 7-day-old seedlings grown at 21°C under short-day conditions were shifted to 27°C or kept at 21°C with or without 100 mM mannitol/20% PEG/200 μM SA treatment at ZT1, and seedlings were collected at ZT13 for RNA extraction. Total RNA was extracted with Eastep Super Total RNA Extraction Kit (Promega, LS1040). Three independent biological replicates were performed for each line and condition. For RNA-seq, sequencing libraries were prepared with the NEBNext Ultra RNA library prep kit for Illumina (NEB, 7530 L) according to the manufacturer's instruction. Prepared libraries were sequenced on a NovaSeq 6000 platform and paired-end 150 bp reads were generated. Adapter trimming was performed and low quality reads were filtered with fastp version 0.20.1 (Chen et al., 2018). Reads were aligned to the *Arabidopsis* genome (TAIR10) using Hisat2 version 2.1.10 (Kim et al., 2019). Reads per gene were counted by HTseq version 0.11.2 (Anders et al., 2015). Transcripts per million (TPM) values were generated using R. Differential gene expression analysis was performed using DESeq2 version 1.26.0 (Love et al., 2014). Genes were considered as differentially

expressed in RNA-Seq if they exhibited a more than two-fold change in expression and had a *P* adjust value <0.05. Gene ontology analysis was performed with DAVID (https://david.ncifcrf.gov/) (Huang et al., 2009). For RT-qPCR, reverse transcription was performed using HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, R312-02). Real-time quantitative PCR was conducted on an Applied Biosystems QuantStudio 6 Flex Real-Time PCR System or a Bio-Rad CFX384 Touch Real-Time PCR Detection System using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Q711-02). *PP2A* was used as an endogenous control for normalization. Primers used for amplification are listed in Table S1.

ChIP-qPCR

To analyze ELF3 binding at the *PIF4* locus, 7-day-old Col and *elf3-7;gELF3-GFP* seedlings grown at 21°C under short-day conditions were shifted to 27°C or kept at 21°C with or without 100 mM mannitol/200 μM SA treatment at ZT1, and seedlings were collected at ZT13. Materials were fixed with 1% formaldehyde. Chromatin was resuspended with ChIP lysis buffer [50 mM HEPES pH7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.3% SDS, and 1 × protease inhibitor cocktail (Sigma, S8830)] and sheared by sonication. Sheared chromatin was diluted two times with ChIP dilution buffer [50 mM HEPES pH7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.01% SDS, and 1 × protease inhibitor cocktail (Sigma, S8830)] and subjected to immunoprecipitation with an anti-GFP antibody (ThermoFisher Scientific, A-11122) (dilution rate: 1:150). The amount of input and immunoprecipitated DNA was quantified by real-time PCR. Three independent biological replicates were performed for each line and condition. Primers used for amplification are specified in Table S1.

Western blot

To examine the ELF3 protein abundance upon mannitol/SA treatment, 7-day-old seedlings of *elf3-7;35S::ELF3-HA* grown at 21°C under short-day conditions were shifted to 27°C with or without 100 mM mannitol/200 μM SA treatment at ZT1, and seedlings were collected at ZT13. Total protein was extracted with the protein extraction buffer [200 mM Tris pH7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, and 1 × protease inhibitor cocktail (Sigma, S8830)]. Protein was transferred to a 0.2 μm nitrocellulose membrane (Bio-Rad) after being separated by SDS-PAGE. ELF3-HA was detected using anti-HA (CST, 3724) (dilution rate: 1:2500) as the primary antibody and Goat anti-Rabbit IgG (H + L)-HRP Conjugated (ThermoFisher Scientific, 31 460) (dilution rate: 1:5000) as the secondary antibody. Actin was detected using anti-Actin (Abclonal, AC009) (dilution rate: 1:5000) as the primary antibody and Goat anti-Mouse IgG (H + L)-HRP Conjugated (ThermoFisher Scientific, 31 430) (dilution rate: 1:5000) as the secondary antibody.

SA content quantification

SA measurement was performed as previously described (Forcat et al., 2008). To measure SA content in response to mannitol/PEG treatment, 7-day-old seedlings grown at 21°C were shifted to 27°C or kept at 21°C with or without 100 mM mannitol/20% PEG treatment at ZT1, and seedlings were collected at ZT13. For SA measurement under water-limited soil conditions, aerial parts of the plants were collected 3 weeks after transplanting. Tissues were flash-frozen in liquid nitrogen and ground to fine powder. A 100 mg aliquot of tissue powder was extracted with 400 μL of 10% methanol containing 1% acetic acid. D_4-SA was added as an internal standard. After centrifugation at 13000 ***g*** for 10 min at 4°C, the supernatant was transferred to a new tube, and the pellet was re-extracted with an additional 400 μL of 10% methanol containing 1% acetic acid. The supernatants were combined and filtered before analysis with a UPLC system (Waters) coupled to a 5500 Qtrap MS system (SCIEX).

Observation of ELF3-GFP signals

To analyze ELF3 phase separation under mannitol/SA treatment, 4-day-old *elf3-7;gELF3-GFP* seedlings grown at 21°C under short-day conditions were shifted to 27°C with or without 100 mM mannitol/200 μM SA treatment at ZT1, and ELF3-GFP signals in roots were imaged at ZT13. Images were captured using a Zeiss LSM980 confocal laser scanning microscope. Excitation was performed at 488 nm, and emission was collected between 500 and 550 nm. For each condition, five roots were imaged, and 17 to 27 nuclei per root were analyzed to count the number of speckles formed within the nuclei.

Statistical analysis

Statistical tests performed are indicated in the figure captions. The significance of differences was determined with two-tailed Student's *t*-test, one-way ANOVA with Tukey's test, or two-way ANOVA To calculate the ratios, the average values of data obtained at 27°C without treatment were first determined. Each value at 27°C with treatment was then divided by this average to obtain the ratios.

AUTHOR CONTRIBUTIONS

RS, MX, and DJ designed experiments; RS, MX, XL, HL, and DJ performed experiments; RS, MX, HZ, and DJ analyzed data; DJ wrote the manuscript with the help of RS.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Gene Expression Omnibus at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE287933, reference number GSE287933.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Plant growth phenotypes grown with or without drought/mannitol/PEG treatment.

Figure S2. Gene expression and phenotypic analysis in *35S::PIF4* plants.

Figure S3. Analysis of the involvement of SA in drought-mediated suppression of thermomorphogenesis.

Figure S4. Analysis of hypocotyl elongation and gene expression in PIF4 repressor mutants.

Figure S5. Analysis of ELF3 expression upon mannitol and SA treatments, and drought resistance in *elf3* mutant plants.

Table S1. Primers used in RT-qPCR and ChIP-qPCR tests.

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