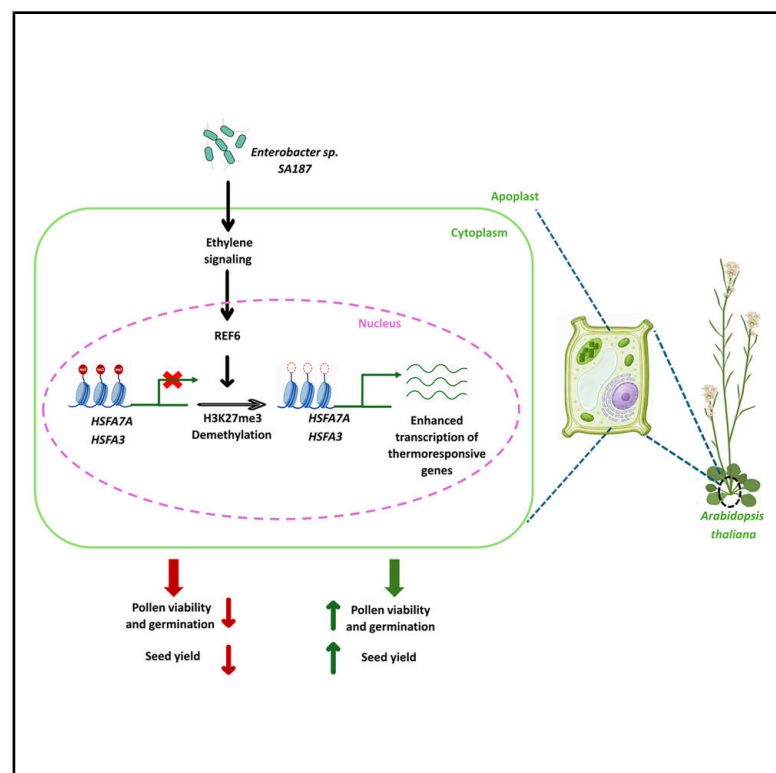


Ethylene-REF6 regulatory module enables endophyte-mediated thermotolerance of plant growth and reproduction

Graphical abstract



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In brief

Fatima et al. show that the ethylene signaling-REF6 module activates thermoresponsive gene expression via histone demethylation thereby protecting plant growth and reproduction. The beneficial endophyte *Enterobacter sp. SA187* enhances plant growth and reproductive thermotolerance through ethylene-REF6 regulation resulting in improved pollen viability and seed production under elevated temperature (eT).

Highlights

- Ethylene activates thermoresponsive genes via REF6-dependent H3K27me3 demethylation
- The ethylene-REF6 pathway sustains plant growth and reproduction under eT
- *Enterobacter sp. SA187* protects plant reproduction via the ethylene-REF6 module under eT



Article

Ethylene-REF6 regulatory module enables endophyte-mediated thermotolerance of plant growth and reproduction

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SUMMARY

Global warming affects plant growth and yield, posing a major threat to global food security. Male sexual reproduction is the most sensitive stage for plant yield under elevated temperature (eT). We show here that under eT conditions, plant growth and male reproduction depend on the ethylene-mediated activity of the histone demethylase REF6. Although eT-compromised plant performance of wild-type plants can be fortified by treatment with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), *ref6* mutant plants are insensitive to ACC, showing that ethylene-dependent eT-induced genes depend on REF6 histone demethylase activity. Sexual reproduction and yield, including pollen viability and germination, are strongly compromised in ethylene and *ref6* mutants under eT conditions. Intriguingly, we demonstrate that the bacterial endophyte *Enterobacter* sp. SA187 deploys ethylene-mediated REF6 to protect plant growth and reproduction to eT. These results show that microbiome-assisted approaches can help to ensure food safety under changing climate conditions.

INTRODUCTION

Climate change has significantly increased global temperatures, adversely affecting plant growth and overall agricultural productivity.^{1–3} Exposure to high temperatures leads to extensive cellular damage in plants, disrupting membrane stability, denaturing proteins, and impairing enzymatic activities, which collectively hinder growth and development. Prolonged heat stress can result in oxidative damage, metabolic imbalance, and even cell death if protective mechanisms are not activated.^{4,5} Even moderate rises in temperature can cause severe stress.⁶ Elevated temperatures (eT) not only negatively impact plant growth but also strongly impact reproduction and yield. Heat stress disrupts pollen development and viability, hindering fertilization, and affecting fruit and seed formation, leading to significant yield reduction, posing a major threat to global food security.⁷

Plants use multiple molecular systems to sense elevated ambient temperatures, often exhibiting morphological responses collectively termed thermomorphogenesis. Sensing eT occurs via the red light-sensitive photoreceptor phytochrome B (phyB), a temperature-responsive RNA structural motif located in the 5' untranslated region (5'-UTR) of the *PHYTOCHROME INTERACTING FACTOR 7* (*PIF7*) transcript, and a prion-like domain in the protein EARLY FLOWERING 3 (ELF3). Acting

downstream of these thermosensory components, PIF4 functions as a key transcriptional regulator that orchestrates the hypocotyl elongation and other eT-induced morphological changes.^{8,9} Plants also undergo epigenetic adjustments, including histone modification-mediated transcriptional changes. Specifically, eT causes H2A.Z-containing nucleosomes to evict H2A.Z, regulating the global transcriptome via the INOSITOL AUXOTROPHY 80 (INO80) chromatin-remodeling complex. In this context, ethylene signaling also emerges as a positive regulator in eT responses, as the key ethylene signaling factors EIN2 and EIN3 collaborate with LHP1 and INO80 to switch chromatin bivalency,¹⁰ depositing H3K4me3 and depleting H3K27me3, thereby enabling rapid transcriptional activation in response to eT.^{10–12} Recent investigations demonstrate that RELATIVE OF EARLY FLOWERING 6 (REF6/JMJ12) plays a key role in heat adaptation by actively removing H3K27me3 from heat-associated gene loci, forming part of a regulatory feedback circuit with HSFA2 that governs thermomorphogenesis and transgenerational heat memory.^{12,13} Genome-wide chromatin immunoprecipitation analyses have revealed that REF6 demethylates critical thermoresponsive genes, including *GIBBERELLIN 20-OXIDASE 2* (*GA20ox2*), *BASIC-HELIX-LOOP-HELIX 87*, thereby enabling their active expression in response to eT.¹² *ref6* mutants display compromised hypocotyl elongation at eT, confirming the importance of its enzymatic



activity. Furthermore, REF6 operates synergistically with PIF4, a major thermomorphogenic transcription factor, co-targeting downstream genes to orchestrate growth adaptations to eT.¹² Beyond its role in immediate temperature responses, REF6 also contributes to transgenerational adaptation through epigenetic memory. The heat shock transcription factor HSFA2 and REF6 were shown to establish a positive feedback loop that maintains the active transcriptional state of HSFA2 across generations. This epigenetic memory mechanism involves REF6-mediated removal of H3K27me3 marks, allowing sustained *HSFA2* expression, which in turn activates SGIP1, an F-box E3 ubiquitin ligase. SGIP1 promotes transgenerational stress adaptation by targeting SGS3 for degradation, thereby reinforcing the inherited heat stress responses.¹³ While the vegetative and molecular responses to eT have been extensively studied in several plant species, considerably less attention has been directed toward understanding its impact on the sexual reproductive system,¹⁴ which is particularly sensitive to heat. Despite the fact that male and female gametophyte development are vulnerable to heat stress, the male reproductive system has been identified as the most heat-sensitive component.¹⁵

While plants employ a range of physiological and molecular strategies to cope with eT stress,^{1,16,17} these innate defense mechanisms are often insufficient under extreme environmental conditions, thereby prompting the need for external interventions. Among these, plant growth-promoting bacteria (PGPB) have emerged as promising allies, enhancing stress resilience through improved nutrient uptake, phytohormone production, and protection against oxidative damage.^{18–20} Notably, *Enterobacter* sp. SA187, isolated from the desert legume *Indigofera argentea*, has demonstrated the ability to promote growth and stress tolerance in *Arabidopsis thaliana* and other crops under various abiotic stresses, including salinity, acute heat and high-light stress.^{21–24} Previous research has indicated that SA187 enhances biomass accumulation, increases tolerance, and regulates gene expression involved in stress responses. While its benefits under acute heat stress are well documented, its potential in enhancing plant resilience to sustained elevated ambient temperatures remains unexplored. The objective of this study was to establish whether *Enterobacter* sp. SA187 can confer thermotolerance and protect reproductive fitness in *Arabidopsis* exposed to prolonged eT, with a focus on the underlying molecular mechanisms. We identify the ethylene signaling pathway to target the histone demethylase REF6 for priming eT-induced gene expression. Moreover, our data highlight that SA187 uses the ethylene signaling regulation of REF6 for mediating protection of plant growth, sexual reproduction, and yield to eT conditions in *Arabidopsis*.

RESULTS

Ethylene-dependent activation of histone demethylase REF6 facilitates resilience to eT

To assess the role of ethylene in protecting *Arabidopsis* growth under eT, 5-day-old seedlings were initially grown at 22°C before transfer to either 22°C (control) or 30°C (eT) for an additional 16 days. Although the ethylene signaling mutants *ein2-1* and *ein3-1* did not show major growth differences at 22°C, the higher

order *ein3 ein1 ein3* mutant displayed a stronger growth-compromised phenotype even at 22°C (Figures 1A and 1B). Under eT conditions, growth was markedly reduced in all ethylene signaling mutants, with *ein3 ein1 ein3* showing the most severe reduction in fresh weight (FW) compared with Col-0 (Figures 1A and 1B). These results indicate that ethylene signaling not only contributes to plant growth maintenance under optimal temperature but also plays a critical role in sustaining growth under eT stress. To explore the molecular mechanism behind eT-driven growth reduction, we conducted RT-qPCR on key thermoresponsive marker genes after exposing plants to 30°C for 6 h. These genes included *HSFA7A* (Heat Shock Factor A7A), *HSFA3* (Heat Shock Factor A3), and *REF6* (Relative of Early Flowering 6) (Figure 1C) as well as *GA20ox2* (Gibberellin 20-oxidase 2), *IAA29* (Auxin-responsive protein IAA29), *SGIP1* (Small GTPase-interacting protein 1), and *BRM* (Brahma) (Figure S1A). We observed that the transcript levels of these genes were enhanced in response to eT, including the histone demethylase REF6, which plays a pivotal role in resilience to eT.^{12,13} REF6 regulates the expression of genes involved in thermomorphogenesis by demethylating histone H3 lysine27me3 (H3K27me3), thereby reshaping the chromatin landscape to activate temperature responsive genes. REF6 activity influences various aspects of plant development and stress responses, including the elongation of hypocotyls and other morphological adaptations triggered by eT.¹² Therefore, we investigated whether *REF6* plays a role in eT stress tolerance by analyzing the *REF6* mutant lines. Our phenotypic analysis revealed that *ref6-1* and *ref6-5* mutant plants showed significant growth defects under eT conditions (Figures 1A and 1B). Importantly, both ethylene *ein2-1* and *ref6-1* mutant plants were compromised in eT-induced gene expression of the thermoresponsive marker genes (Figures 1C and S1A).

To further examine whether the REF6 homologs ELF6 and JMJ13 also contribute to plant growth regulation, we analyzed the phenotype and FW of *elf6-C*, *jmj13-1*, *ref6-5 elf6-C*, and *ref6-5 elf6-C jmj13-1* mutants under control and eT conditions (Figure S2). Under control conditions, both *elf6-C* and *jmj13-1* showed slightly reduced FW compared with wild-type (WT) Col-0, whereas the *ref6-5 elf6-C* double mutant and *ref6-5 elf6-C jmj13-1* triple mutant displayed a stronger growth-compromised phenotype. Under eT, *elf6-C*, *ref6-5 elf6-C*, and *ref6-5 elf6-C jmj13-1* showed reduced growth compared with Col-0, with the double and triple mutants showing the most severe reduction. By contrast, *jmj13-1* did not show a growth-compromised phenotype under eT. These results indicate that ELF6 contributes to plant growth maintenance under eT, whereas JMJ13 appears to be more important for basal growth under control conditions. The stronger phenotype of the *ref6-5 elf6-C* double and *ref6-5 elf6-C jmj13-1* triple mutants further suggests that combined loss of H3K27me3 demethylase activity compromises overall plant growth capacity, both under control and eT conditions.

Since we found that ethylene signaling and *REF6* are both important for plant growth at eT (Figures 1A and 1B), we hypothesized that H3K27me3 levels of thermoresponsive genes might be regulated in an ethylene and/or REF6-dependent manner. To test this hypothesis, we performed ChIP-qPCR analysis of

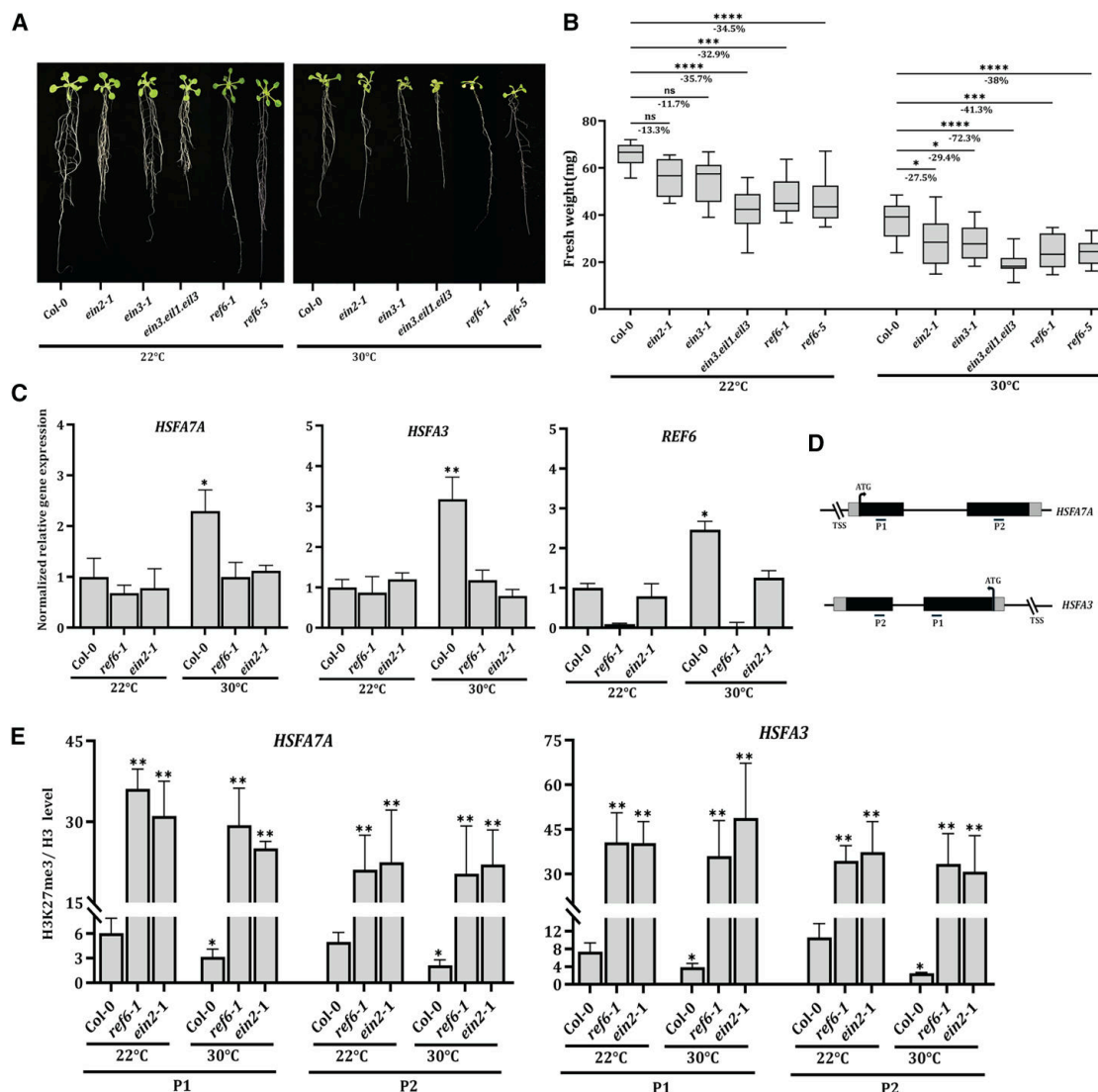


Figure 1. Ethylene and histone demethylase *ref6* mutants are hypersensitive to eT

(A and B) Phenotypes (A) and fresh weight data (B) of wild-type Col-0, ethylene signaling mutant (*ein2-1*, *ein3-1*, and *ein3 eil1 eil3*) and REF6 mutant (*ref6-1* and *ref6-5*) plants under normal (22°C) and eT (30°C) conditions. Boxplots show data from three independent biological replicates (total no. of plants $n = 36$). For each line, asterisks indicate the statistical differences based on the one-way ANOVA analyses followed by Šidák's multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). n.s., not significant.

(C) Transcript levels of *HEAT SHOCK FACTOR A7A* (*HSFA7A*), *HEAT SHOCK FACTOR A3* (*HSFA3*) and *RELATIVE OF EARLY FLOWERING 6* (*REF6*) genes determined by RT-qPCR in Col-0, *ein2-1* and *ref6-1* plants. Gene expression was first normalized to β -tubulin, and then values were recalculated relative to non-colonized Col-0 plants at 22°C. Data correspond to the mean $\pm$ SEM of three biological replicates, each comprising three technical replicates.

(D) Schematic representation of *HSFA7A* and *HSFA3* gene structures with regions targeted for ChIP-qPCR. Gene models show exon-intron organization. The gray boxes represent the 5' untranslated regions (UTRs), black boxes indicate exons, and angled arrows mark the transcription start sites. The numbered regions below each gene model (indicated by black lines) denote the genomic segments analyzed by ChIP-qPCR.

(E) Normalized relative enrichment of the H3K27me3 mark at the indicated gene loci of *HSFA7A* and *HSFA3* in Col-0, *ref6-1* and *ein2-1* mutant plants grown at 22°C and 30°C, determined by chromatin immunoprecipitation followed by qPCR (ChIP-qPCR). P1 and P2 regions indicate the sites targeted for ChIP-qPCR. Amplification values were normalized to total input and H3. The plots represent the mean of three independent biological replicates. Data correspond to the mean $\pm$ SEM.

In (C) and (E), statistical significance was determined using Student's *t* test, with asterisks denoting significant differences (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

the heat shock factor *HSFA7A* and *HSFA3* gene loci in WT Col-0. The regions targeted for amplification are labeled as P1 and P2 in the gene model (Figure 1D). For both *HSFA7A* and *HSFA3*, P1 is located within the 5' region of exon 1, whereas P2 is positioned in

a downstream exon region of the respective gene locus (Figure 1D). ChIP-qPCR analysis revealed a significant depletion of the H3K27me3 mark in WT Col-0 under eT conditions suggesting that the increased expression of *HSFA7A* and *HSFA3*

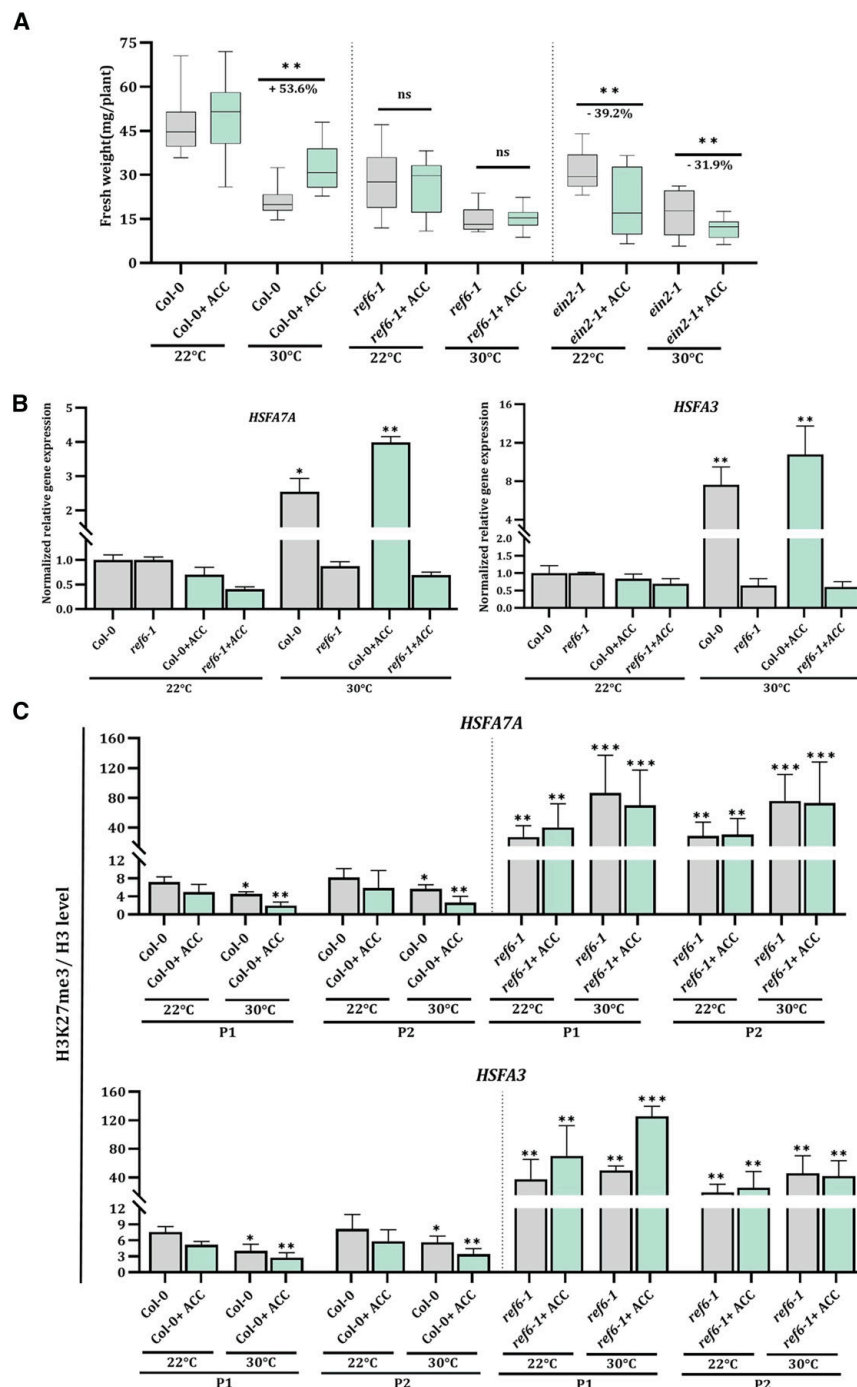


Figure 2. ACC confers thermotolerance via REF6-dependent manner

(A) Fresh weight of 16-day-old Col-0, *ref6-1*, and *ein2-1* plants grown under normal (22°C) and eT (30°C) conditions, with or without exogenous 1 μ M 1-aminocyclopropane-1-carboxylic acid (ACC) treatment. Boxplots show data from three independent biological replicates (total no. of plants $n = 36$). Asterisks indicate the statistical differences based on one-way ANOVA analyses followed by Šidák's multiple comparisons test (* $p < 0.05$, ** $p < 0.01$). n.s., not significant. (B) Transcript levels of HEAT SHOCK FACTOR 7A (*HSF7A*) and HEAT SHOCK FACTOR 3 (*HSF3*) were determined by RT-qPCR in Col-0 and *ref6-1* plants under 22°C and eT (after 6 h of 30°C), with or without ACC treatment. Expression was normalized to β -tubulin and recalculated relative to control grown at 22°C without ACC treatment. Each plot represents the mean $\pm$ SEM of three biological and three technical replicates (total no. of plants $n = 36$). (C) Relative enrichment of H3K27me3 at *HSF7A* and *HSF3* loci in Col-0 and *ref6-1* plants grown at 22°C and 30°C with or without ACC treatment, determined

(legend continued on next page)

in eT plants is a consequence of reduced H3K27me3 levels (Figure 1E). We also performed ChIP-qPCR analysis on ethylene *ein2-1* and *ref6-1* mutant plants. We observed an increase in the enrichment of H3K27me3 levels in the *HSFA7A* and *HSFA3* genes in the ethylene and histone demethylase *ref6-1* mutant compared to WT Col-0 plants (Figure 1E). This finding aligns with our RT-qPCR data, which revealed significantly lower transcript levels of thermoresponsive genes in *ein2-1* and *ref6-1* compared to Col-0 (Figures 1C and S1A).

The ethylene precursor ACC enhances plant tolerance to eT in a REF6-dependent manner

To validate the role of ethylene in REF6-dependent tolerance to eT, we applied exogenous 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, to evaluate whether ACC can confer protection at eT. FW analysis showed that ACC treatment had no significant effect on plant growth under control conditions (22°C). However, under eT (30°C), ACC markedly enhanced thermotolerance in WT plants, resulting in an increase in FW to 53% (Figure 2A). In contrast, ACC application was unable to rescue thermosensitivity in the ethylene signaling mutant *ein2-1*, further confirming that ethylene perception is essential for this response (Figure 2A). Similarly, ACC also failed to enhance thermotolerance in the *ref6-1* mutant background, indicating that REF6 is indispensable for ACC-mediated heat resilience. Furthermore, transcript analysis revealed that ACC treatment markedly enhanced the expression of *HSFA7A* and *HSFA3* (Figure 2B) and of other thermoresponsive genes (Figure S3) in WT Col-0, whereas this induction was absent in *ref6-1* mutant plants (Figures 2B and S3). Consistently, ChIP-qPCR assays demonstrated that ACC application significantly reduced the enrichment of the H3K27me3 repressive mark at *HSFA7A* and *HSFA3* gene loci at eT in WT Col-0 (Figure 2C), but not in the *ref6-1* mutant background (Figure 2C). These results confirm that REF6 enzymatic activity is essential for ACC-induced chromatin remodeling at heat-responsive gene loci, establishing that ethylene functions upstream of REF6 and requires REF6-mediated H3K27me3 demethylation to activate the transcriptional networks necessary for sustained thermotolerance.

Role of ethylene signaling and the histone demethylase REF6 in *Arabidopsis* sexual reproduction and yield under eT conditions

eT strongly affects the sexual reproduction of plants, with pollen viability being the most sensitive factor.^{7,25,26} To investigate the role of ethylene signaling and histone demethylation by REF6 in male sexual reproduction, we performed pollen viability analysis in the ethylene signaling mutants (*ein2-1* and *ein3-1*) and *ref6* mutants (*ref6-1* and *ref6-5*) and observed that, even under normal temperature conditions, ethylene and *ref6* mutant pollen exhibited significantly lower viability compared with WT Col-0. Upon exposure to eT, pollen viability was further compromised

in these mutant lines (Figures 3A and 3B). A similar trend was observed for the pollen germination and reproductive performance, where *ein2-1* and *ref6-1* mutants showed compromised pollen germination and produced markedly fewer seeds per silique even under control conditions, with the reduction becoming more severe under eT conditions compared with Col-0 (Figures 3C and 3D). These results demonstrate that both functional ethylene signaling and the coordinated activity of the histone demethylase REF6 are essential for reproduction and thermotolerance.

Enterobacter sp. SA187 restores *Arabidopsis* growth under prolonged eT

Enterobacter sp. SA187 was found to protect plants from acute short-term heat stress,²³ however, these conditions do not resemble natural environments. To test whether SA187 can also protect plants under constant eT at 30°C, SA187-colonized and non-colonized *Arabidopsis* plants were grown for five days at 22°C before shifting them for 16 days to eT at 30°C and control at 22°C, following the scheme in Figure S1B. For the plant growth assessment, we examined phenotype, total FW, dry weight (DW), lateral root density (LRD), ion-leakage, chlorophyll levels and hypocotyl elongation (Figures 4A–4F and S4). The results show that while SA187 colonization had no significant effect on plant growth at 22°C, SA187 conferred a strong protective effect to eT stress at 30°C. Compared to non-colonized plants, which exhibited a biomass reduction of approximately 51% in both FW and DW to eT, SA187-colonized plants retained 40% and 25% higher FW and DW, respectively (Figures 4A–4C). Furthermore, SA187 colonization enhanced LRD under both control and eT conditions. Notably, SA187 mitigated the eT-induced suppression of root branching observed in non-colonized plants (Figure 4D). eT also caused a reduction in chlorophyll content in non-colonized plants, whereas SA187-colonized plants maintained significantly higher total chlorophyll levels (Figure 4E). Moreover, SA187 colonization significantly reduced electrolyte leakage compared to non-colonized plants at 30°C, indicating enhanced cell membrane stability under eT stress (Figure 4F). To further investigate the morphological responses, we also analyzed hypocotyl elongation, a well-known thermomorphogenesis trait.^{8,27,28} Hypocotyl elongation was measured in seedlings grown continuously at 22°C and 30°C for four consecutive days. At 30°C, Col-0 plants exhibited a marked increase in hypocotyl length, which was further enhanced in SA187-colonized plants, whereas no significant differences were observed between treatments at 22°C (Figures S4A and S4B). Microscopic analysis also revealed an increase in hypocotyl thickness under eT, consistent with previous observations.⁸ SA187-colonized plants exhibited a slight, though statistically non-significant, increase of 6% and 9% in thickness at 22°C and 30°C, respectively, compared to non-colonized controls (Figures S4C and S4D). Overall, SA187 improved biomass, root development, and stress resilience, effectively mitigating heat-induced

by ChIP-qPCR. P1 and P2 represent distinct coding regions within each gene. Values were normalized to total input and H3 and represent mean ± SEM of three independent biological and three technical replicates ($n = 200$ – 300 plants).

In (B) and (C), statistical significance was assessed using Student's *t* test ($p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$), calculated relative to wild-type Col-0 plants grown at 22°C.

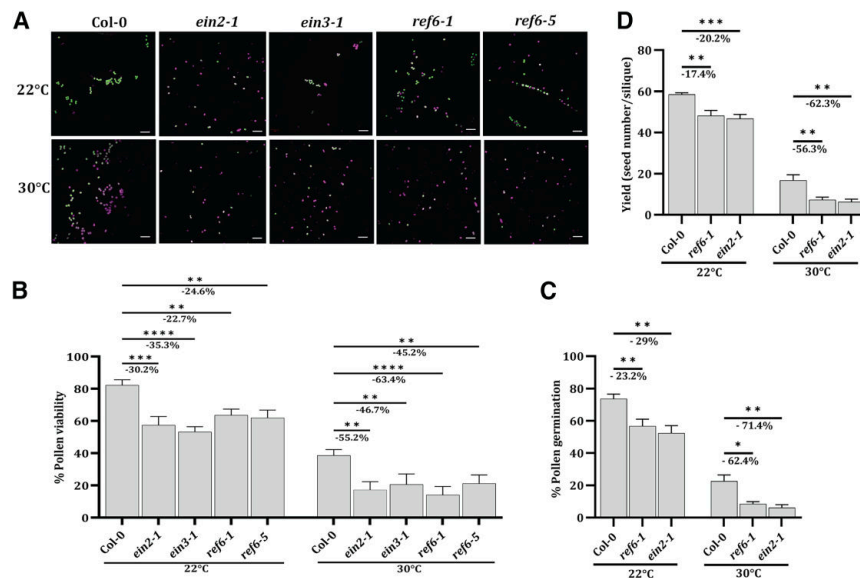


Figure 3. Pollen viability and germination are compromised in ethylene and histone demethylase *ref6-1* mutants under eT conditions

(A) Confocal image showing pollen viability in wild-type Col-0, ethylene signaling mutant (*ein2-1*, *ein3-1*, and *ein3 eil1 eil3*) and REF6 mutant (*ref6-1* and *ref6-5*) plants under normal (22°C) and eT (30°C) conditions. Pollen grains were stained with fluorescein diacetate (FDA; viable, green) and propidium iodide (PI; non-viable, magenta). Scale bars, 100 μ m.

(B) Percent viability was determined by counting 500–700 pollen grains across three independent biological replicates.

(C) Pollen germination percentage was calculated from 200 to 300 pollen grains across three biological replicates.

(D) Quantification of average seed number per silique across three independent biological replicates ($n = 36$ siliques/plant).

In (B)–(D), statistical significance was determined by one-way ANOVA followed by Šidák's multiple comparisons test ($p < 0.05$; $**p < 0.01$; $***p < 0.001$); ns, not significant.

damage in *Arabidopsis*. Additionally, successful colonization of *Arabidopsis* roots by GFP-labeled *Enterobacter* sp. SA187 was confirmed using confocal laser scanning microscopy (Figure S5A–S5C).

SA187 enhances plant resilience to eT by regulating the expression of thermoresponsive genes

To explore the molecular mechanism underlying the SA187-induced thermomorphogenesis and growth enhancement, we conducted RT-qPCR on key thermoresponsive marker genes. We observed that the transcript levels of most of these thermoresponsive marker genes, including *GA20ox2*, *HSFA7A*, *IAA29*, *HSFA3*, *SGIP1*, *BRM*, *PIF4*, and *REF6*, were already elevated at 22°C in SA187-colonized plants compared to non-colonized plants. Upon eT, their expression showed a further significant increase (Figure 4G). These results suggest that SA187 colonization enhanced the expression of thermoresponsive genes, potentially further enhancing the ability of the colonized plants to cope with and respond to eT at the molecular level.

Ethylene signaling and the histone H3K27 demethylase REF6 play key roles in SA187-mediated plant growth rescue to eT stress

Given the importance of the ethylene signaling pathway that regulates the histone demethylase REF6-dependent thermoresponsive gene expression, we investigated whether SA187 uses the same or a different mechanism to protect plants to eT. First, we

compared the phenotypes between SA187-colonized and non-colonized WT versus ethylene signaling mutants (*ein2-1*, *ein3-1* and *ein3 eil1 eil3*) and *ref6* mutant (*ref6-1* and *ref6-5*) plants. Unlike SA187-colonized WT Col-0, SA187-colonized ethylene signaling and *ref6* mutant plants were compromised in the SA187 mediated beneficial effect concerning eT stress (Figures 5A and 5B; Figures S6A and S6B). Hypocotyl measurements showed that *ein2-1* and *ref6-1* mutants displayed significantly reduced hypocotyl length compared with Col-0 at eT. Interestingly, SA187 colonization failed to increase hypocotyl elongation in the mutant background under eT (Figures S4E and S4F). These results highlight the critical role of ethylene signaling and REF6 in mediating plant protection to eT stress by SA187.

We also analyzed the role of ELF6 and JMJ13 in SA187-mediated thermotolerance (Figures S6C and S6D). We found that, similar to *ref6* mutants, the *elf6-C* mutant also failed to exhibit SA187-mediated growth promotion under eT, indicating that ELF6 is required for this response. In contrast, the *jmj13-1* mutant retained the beneficial effect of SA187, suggesting that JMJ13 is not involved in this pathway. To further assess potential redundancy, we analyzed *ref6-5 elf6-C* double mutants and *ref6-5 elf6-C jmj13-1* triple mutants. Interestingly, both double and triple mutants displayed complete loss of SA187-mediated growth rescue unlike WT under eT, consistent with the REF6 regulatory pathway (Figures S6C and S6D). Therefore, the impaired SA187 effect in these mutants likely reflects a combination of defective chromatin-mediated stress regulation and reduced growth capacity.

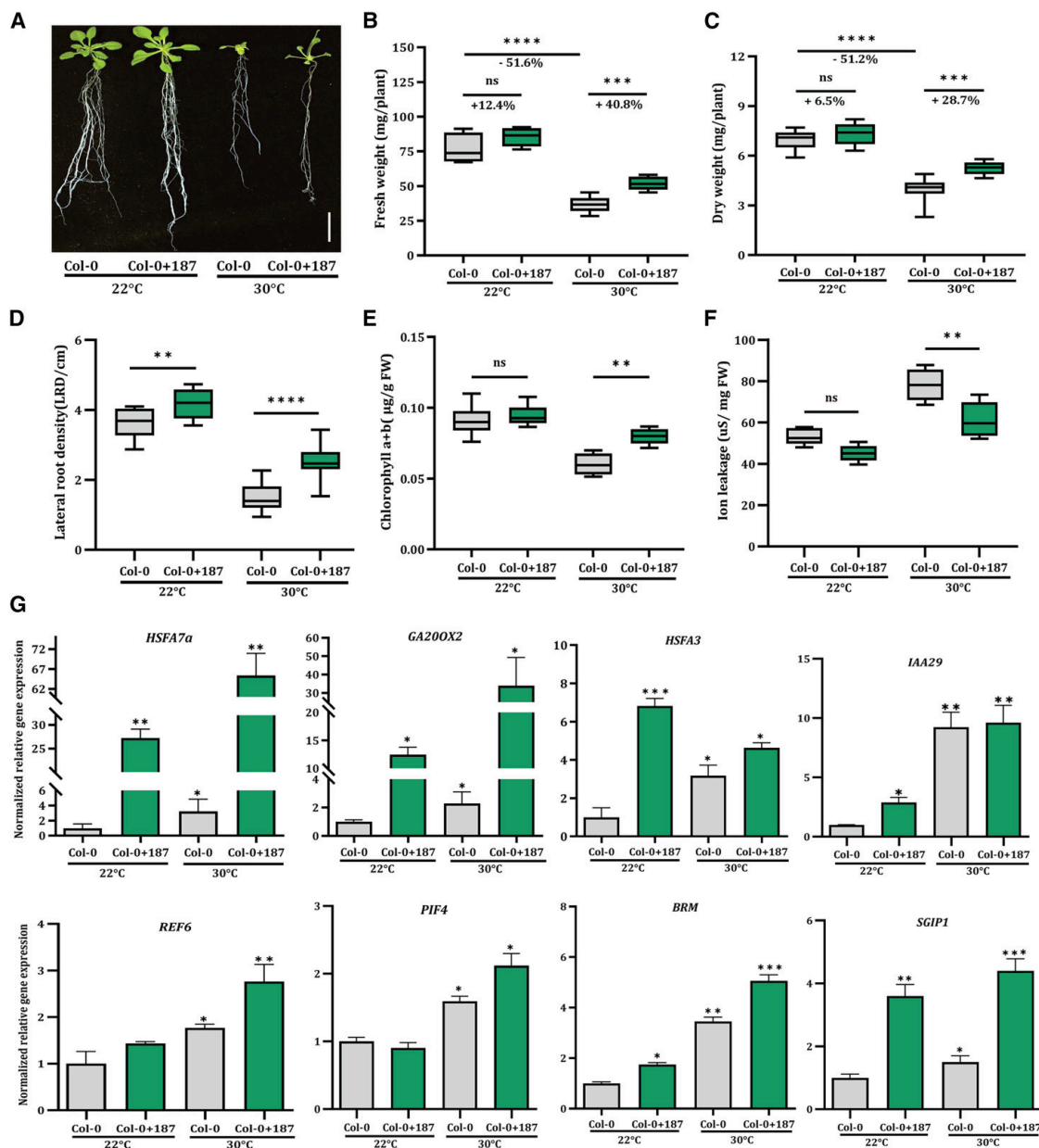


Figure 4. *Enterobacter* sp. SA187 protects plant growth by enhancing thermoresponsive gene expression under eT conditions

(A) Representative phenotype of 16-day-old non-colonized and SA187-colonized *Arabidopsis* seedlings grown at 22°C or 30°C.

(B–D) Quantification of total fresh weight (B), total dry weight (C), and lateral root density (D) of 16-day-old non-colonized and SA187-colonized plants grown under 22°C and 30°C. Boxplots show data from three independent biological replicates (total no. of plants $n = 36$).

(E and F) Total chlorophyll content (E) and ion leakage (F) represented as electrolyte conductivity in 16-day-old seedlings grown at 22°C or 30°C, comparing SA187-colonized and non-colonized plants. Boxplots show data from three independent biological replicates (total no. of plants $n = 9$).

(G) Quantitative RT-PCR analysis of *HEAT SHOCK FACTOR A7A* (*HSFA7A*), *GIBBERELLIN 20-OXIDASE 2* (*GA20ox2*), *HEAT SHOCK FACTOR A3* (*HSFA3*), *INDOLE-3-ACETIC ACID INDUCIBLE 29* (*IAA29*), *RELATIVE OF EARLY FLOWERING 6* (*REF6*), *PHYTOCHROME-INTERACTING FACTOR 4* (*PIF4*), *BRADMA* (*BRM*), and *SENSITIVE TO GIBBERELLIN INSENSITIVE 1 SUPPRESSOR 1 PROTEIN 1* (*SGIP1*) under control (22°C) and eT (after 6 h of 30°C) conditions in colonized and non-colonized plants. Gene expression was first normalized to β -tubulin, and then values were recalculated relative to the control (non-colonized plants at 22°C). Data correspond to the mean $\pm$ SEM of three biological replicates and three technical replicates (total no. of plants $n = 36$). Statistical significance was determined using Student's t test, with asterisks denoting significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$); ns, not significant.

In (B)–(F), asterisks indicate statistically significant differences determined by one-way ANOVA followed by Šidák's multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). n.s., not significant.

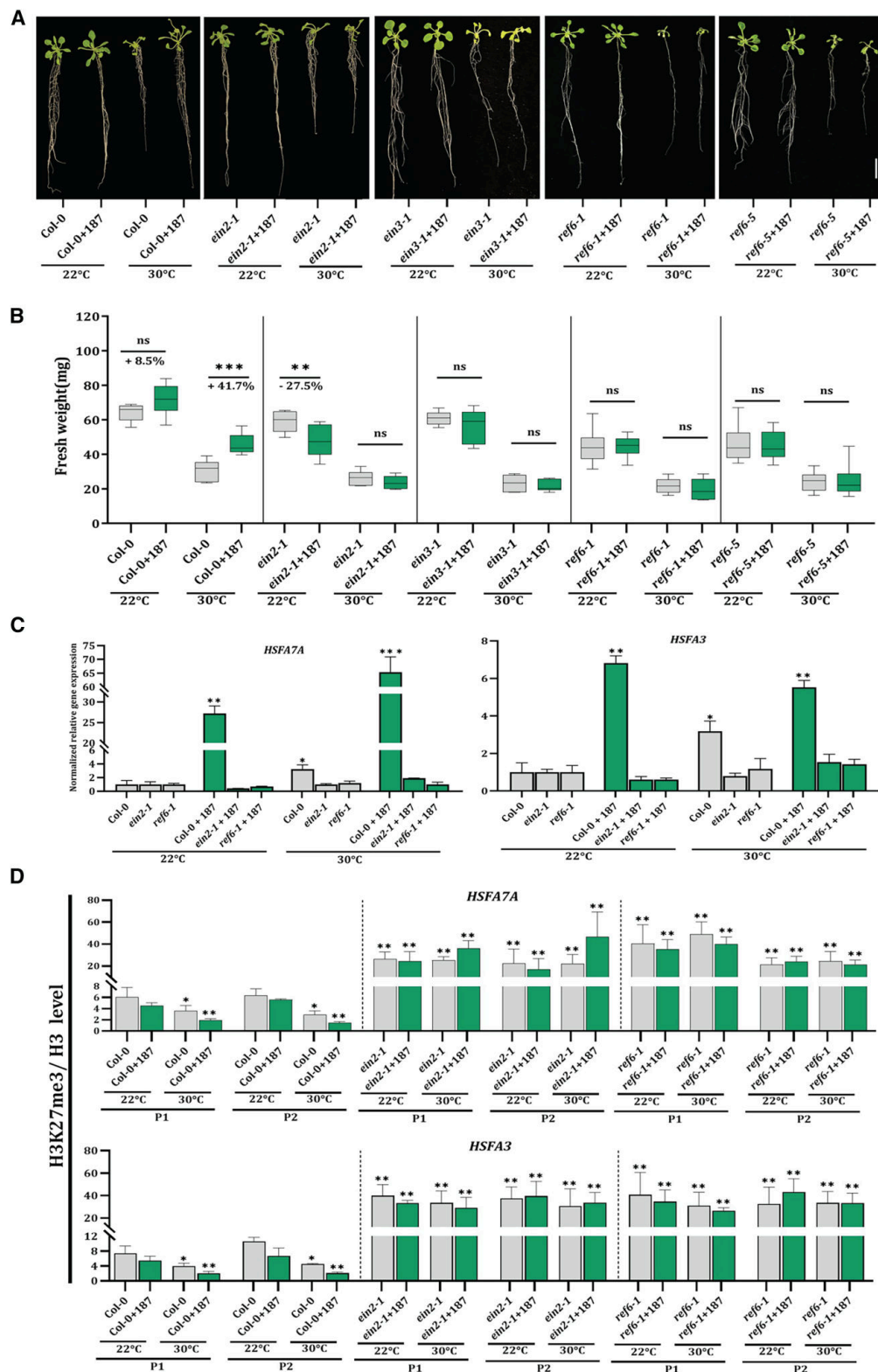


Figure 5. SA187 enhanced plant performance under eT is compromised in ethylene and histone demethylase mutants

(A and B) Phenotypes and total fresh weight (B) of wild-type and ethylene signaling (*ein2-1* and *ein3-1*) and REF6 mutants (*ref6-1* and *ref6-5*) plants upon SA187 colonization under normal (22°C) or eT (30°C) conditions. Boxplots show data from three independent biological replicates (total no. of plants $n = 36$). For each

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REF6-dependent reduction of H3K27me3 levels regulates SA187-induced expression of thermoresponsive genes

Next, we performed gene expression analysis in the *ein2-1* and *ref6-1* mutants, focusing on the thermoresponsive marker genes *GA20ox2*, *HSFA7A*, *IAA29*, *HSFA3*, *SGIP1*, and *BRM*. The upregulation of these genes by SA187 under eT stress (Figure 4G) was completely abolished in the *ein2-1* and *ref6-1* mutant plants (Figures 5C and S7). These results highlight the pivotal role of *REF6* in orchestrating the SA187-induced gene expression of *Arabidopsis* to eT stress.

Since we found that *REF6* is essential for SA187-mediated plant growth rescue (Figures 5A and 5B) and that *REF6* transcript levels were upregulated at eT (Figure 4G), we hypothesized that SA187 might reduce H3K27me3 levels on heat shock factor loci. To test this hypothesis, we performed ChIP-qPCR analysis of heat shock factors *HSFA7A* and *HSFA3* in SA187-colonized and non-colonized Col-0 plants. The analysis revealed a significant depletion of the H3K27me3 mark in these genes in SA187-colonized plants under normal and eT conditions (Figure 5D). These results suggest that the increased expression of *HSFA7A* and *HSFA3* genes in SA187-colonized plants is a consequence of reduced H3K27me3 levels. We also performed ChIP-qPCR analysis on *ein2-1* and *ref6-1* mutants to examine the effect of SA187 and eT. We observed an increase in the enrichment of H3K27me3 levels in the *HSFA7A* and *HSFA3* gene loci in the *ein2-1* and *ref6-1* mutants compared to WT Col-0 plants (Figure 5D).

To ensure consistent bacterial colonization across different *Arabidopsis* genotypes, we assessed the impact of *ref6-1* and *ein2-1* mutations, as well as eT stress, on SA187 colonization. Quantitative analysis of colony-forming units (CFUs) at day 16 revealed no significant differences in SA187 colonization levels between WT Col-0 and the mutant lines (Figure S5D). These results indicate that mutations in *REF6* and *EIN2* do not impair the ability of SA187 to colonize *Arabidopsis*, confirming that the observed phenotypic differences are attributable to host signaling pathways rather than differences in bacterial establishment.

SA187 protects pollen viability and germination to eT via ethylene and REF6

The enhanced growth observed in SA187-colonized plants under eT conditions prompted us to evaluate their reproductive performance by assessing pollen viability, germination, as well as seed set per silique. Three-week-old *Arabidopsis thaliana* plants were exposed for 4 days to eT (30°C) and then pollen sam-

ples were collected at the onset of flowering for further analysis. Pollen viability was determined by using fluorescein diacetate (FDA) and propidium iodide (PI) staining, followed by microscopic analysis to distinguish viable from non-viable pollen grains. In viable pollen grains, FDA is converted to fluorescein by cytoplasmic esterase activity and PI labels pectin in cell walls. Dead pollen were characterized by an absence of esterase activity and thus lack fluorescein-derived fluorescence and become permeable to PI, therefore displaying PI fluorescence inside the pollen.²⁹ Viable pollen were identified by green fluorescence, while magenta-stained pollen grains were considered non-viable (Figure 6A). As shown in Figure 6B, at 22°C, pollen viability was robust, showing approximately 85% viability, with a slight enhancement in SA187-colonized plants. However, under eT stress (30°C), pollen viability declined to 45%. Compared to pollen of control plants, pollen of SA187-colonized plants subjected to eT stress showed 48.5% enhanced pollen viability (Figure 6B). Since not all pollen germinated, we examined pollen germination after 18 h by bright-field microscopy (Figure 6C). Under eT at 30°C, a marked decline in pollen germination was observed, while SA187-colonized plants showed substantially higher germination rates, reaching 167% (Figure 6D). Moreover, pollen tubes from SA187-colonized plants were significantly longer than those of non-colonized plants at eT (Figure S8). To assess the physiological relevance of these findings with respect to reproductive success, we quantified seed set and silique length following exposure to eT. At 22°C, both SA187-colonized and non-colonized plants produced similar seed numbers (Figures 6E and 6F). In contrast, non-colonized plants exposed to eT exhibited a pronounced reduction, with many siliques failing to form proper seeds and displaying several empty locules (white arrowhead in Figure 6E). Remarkably, SA187-colonized plants subjected to the same stress conditions exhibited substantial protection of reproductive performance, showing a higher proportion of filled locules (green arrowhead, Figure 6E), up to a 241% increase in seed number and significantly longer siliques compared with non-colonized plants (Figures 6E and 6F).

To further investigate the role of ethylene signaling and *REF6* in SA187-mediated pollen protection at eT, we performed pollen viability and germination analyses in SA187-colonized *ein2-1* and *ref6-1* mutants. As shown before (Figures 3A and 3B), *ein2-1* and *ref6-1* mutants already exhibited significantly lower pollen viability and germination at normal temperatures of 22°C as well as at 30°C compared to WT. Notably, SA187 colonization failed to alleviate the eT-induced loss of pollen viability and

line, asterisks indicate the statistical differences based on the one-way ANOVA analyses followed by Šidák's multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). n.s., not significant.

(C) Transcript levels of *HEAT SHOCK FACTOR A7A* (*HSFA7A*) and *HEAT SHOCK FACTOR A3* (*HSFA3*) were determined by RT-qPCR in Col-0, *ein2-1* and *ref6-1* under control (22°C) and eT (after 6 h of 30°C) conditions in colonized and non-colonized plants. Gene expression was first normalized to β -tubulin, and then values were recalculated relative to the control (non-colonized plants at 22°C). Data correspond to the mean $\pm$ SEM of three biological replicates, each comprising three technical replicates.

(D) Normalized relative enrichment of the H3K27me3 mark at the indicated gene loci of *HSFA7A* and *HSFA3* in non-colonized and SA187-colonized in Col-0, *ein2-1* and *ref6-1* mutants grown at 22°C and 30°C, determined by chromatin immunoprecipitation followed by qPCR (ChIP-qPCR). P1 and P2 regions indicate the sites targeted for ChIP-qPCR. Amplification values were normalized to total input and H3. The plots represent the means of 3 biological and 3 technical replicates ($n = 200$ –300 plants). Error bars indicate standard error (SEM).

In (C) and (D), statistical significance was assessed using Student's *t* test (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$), calculated relative to wild-type Col-0 plants grown at 22°C.

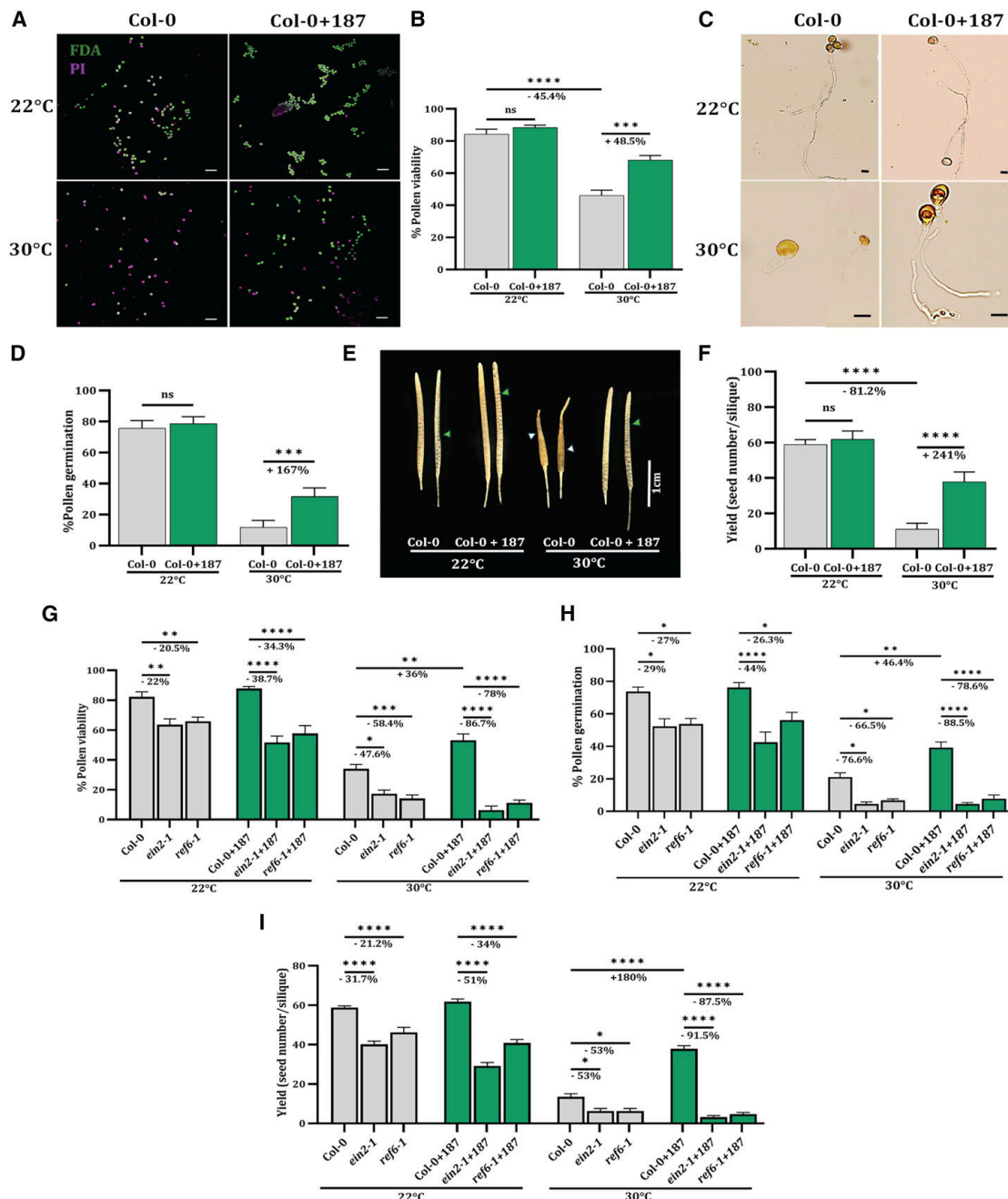


Figure 6. Ethylene dependent protection of pollen viability and germination under eT by SA187

(A) Confocal image showing pollen viability in SA187-colonized and non-colonized wild-type plants under normal (22°C) and eT (30°C) conditions. Pollen grains were stained with fluorescein diacetate (FDA; viable, green) and propidium iodide (PI; non-viable, magenta). Scale bars, 100 µm.

(B) Percent viability was determined by counting 500–700 pollen grains across three independent biological replicates.

(C) Representative bright-field images showing *in vitro* pollen germination after 18 h incubation on germination medium at 22°C or 30°C. The Col-0 pollen image at 30°C (lower-left image) is a composite assembled from two representative microscopic fields acquired under identical imaging conditions. Brightness and contrast adjustments were applied uniformly across the entire image and equally to all samples to improve visualization of pollen tubes (scale bars, 20 µm).

(D) Pollen germination percentage was calculated from 200 to 300 pollen grains across three independent biological replicates.

(E) Representative siliques of *Arabidopsis* from SA187-colonized and non-colonized plants grown at 22°C or 30°C. Green arrowheads indicate fully developed siliques containing visible seeds, whereas white arrowheads denote eT-stressed siliques with reduced/empty seed set. Scale bars, 1 cm.

(F) Quantification of average seed number per silique ($n = 36$ siliques/plant).

(G) Percentage pollen viability of *ein2-1* and *ref6-1* was determined by counting 350–500 pollen grains across three independent biological and three technical replicates.

(legend continued on next page)

germination in *ein2-1* and *ref6-1* mutants, in contrast to the strong protective effect observed in SA187-colonized WT plants (Figures 6G and 6H). This lack of protection extended to reproductive performance, as *ein2-1* and *ref6-1* mutants exhibited markedly fewer seeds per silique even at 22°C, with the reduction becoming more severe under eT conditions (Figure 6I). Together, these results demonstrate that both functional ethylene signaling and the coordinated activity of the histone demethylase REF6 are essential for SA187-mediated thermotolerance of *Arabidopsis* at the reproductive stage.

DISCUSSION

Global climate change poses a major threat to crop productivity, particularly by impairing plant reproductive development under prolonged heat stress. However, existing studies are largely confined to investigating vegetative traits and survival under acute high-temperature stress, leaving a critical gap in our understanding to protect reproductive health under the more ecologically relevant scenario of continuous eT.¹⁴ In this study, we show that ethylene signaling plays an important role in plant tolerance to eT. Previous research reports have shown that ethylene protects plants against heat stress-induced oxidative damage in *Arabidopsis*.³⁰ Later in another report, it was shown that ethylene targets HSFA2, the master regulator of heat stress responses, via EIN3-dependent activation of ERF95 and ERF97.³¹ These responses were, however, always carried out in response to short-term lethal doses of heat and not to longer periods of eT, as shown in our present work. We show here that under eT conditions, ethylene is required for coordinating the epigenetic regulation of thermoresponsive genes via REF6 (RELATIVE OF EARLY FLOWERING 6). REF6 is a Jumoni-domain-containing histone demethylase that specifically removes the repressive histone mark H3K27me3 from thermoresponsive gene loci, thereby promoting transcription of heat stress-related genes.^{12,13} The loss of function in the *ref6-1* mutant correlates with a significant reduction in the expression of thermoresponsive genes, including key regulators such as *HSFA7A* and *HSFA3*. Our data propose that ethylene acts upstream of REF6, as REF6 transcript levels are markedly reduced in the *ein2-1* mutant (Figure 1C). Moreover, exogenous ACC confers strong protection to WT *Arabidopsis* plants under eT conditions, but ACC fails to confer eT stress protection in the *ref6-1* mutant (Figure 2). Although ACC conversion to ethylene can produce cyanide as a coproduct,^{32,33} the low ACC concentration used in this study, together with the observed rescue of WT plants and the lack of rescue in ethylene and REF6-defective mutants (Figure 2A), supports the interpretation that the observed phenotypes are primarily linked to ethylene signaling rather than nonspecific ACC-derived toxicity. This is further supported by previous studies showing that ACC can promote beneficial

plant responses when applied at low concentrations. For example, Liu et al.³⁴ showed that ACC application at minimal concentrations improved plant growth under salinity stress. While gaseous ethylene would represent a more direct approach to activate ethylene signaling, its long-term application under controlled growth conditions is technically challenging. Therefore, ACC was used as a practical and widely accepted approach to modulate ethylene signaling.

High temperatures not only affect plant vegetative growth negatively but also both female and male reproductive processes. Although eT can lead to reduced ovule number, cause ovule abortion, and decrease ovary size, pollen viability and pollen tube growth are considered the main bottleneck in plant reproduction under heat stress.⁷ High temperature perturbs multiple layers of pollen development, including the precise timing of meiosis,^{7,35,36} the integrity of supportive tissues like the tapetum,³⁷ and the synchronization of developmental cues. These disruptions often manifest as early or abnormal pollen maturation, reduced cellular energy reserves,^{38,39} and viability,⁴⁰ ultimately compromising reproduction and yield.¹⁵ In this work, we show that ethylene signaling also plays an important role in sexual reproduction, seed set and yield of *Arabidopsis* plants under eT conditions. Importantly, we also show that *ein2-1* and *ref6-1* mutant plants show similar defects in sexual reproduction and yield, suggesting that this signaling module is equally important for vegetative growth as well as for the reproductive stages of *Arabidopsis* under eT conditions. Overall, these observations suggest that REF6 is not only required for thermomorphogenesis responses but is also essential for the transcriptional reprogramming driven by ethylene signaling under eT conditions.

Previously, we established that the beneficial endophyte *Enterobacter* sp. SA187 protects *Arabidopsis* from acute heat stress via EIN3 regulation of HSFA2,^{22,23} but so far, it was not clear whether these studies are also relevant for eT stress. Here, we show that SA187 also protects *Arabidopsis* plant growth and reproductive performance from eT (Figures 4 and 6). SA187 enhanced pollen viability, pollen germination and increased the number of seeds per silique, suggesting that SA187 supports reproductive resilience under eT (Figure 6). SA187-induced ethylene signaling enhances REF6 expression, thereby exerting control over the thermoresponsive transcription factors HSFA7A and HSFA3 and their downstream genes such as GA20ox2, IAA29, SGIP1, and BRM (Figures 5C and S5). Moreover, the beneficial effects of SA187 were lost in both ethylene signaling as well as in histone demethylase *ref6* mutants (Figures 5 and 6). These results indicate that SA187-mediated plant thermotolerance is based on fine-tuning the expression of thermoresponsive genes via the host ethylene-REF6-dependent epigenetic mechanism. While the underlying molecular mechanism was investigated mainly in vegetative tissues, the dependence of reproductive thermotolerance on ethylene

(H) Percentage of *in vitro* pollen germination in *ein2-1* and *ref6-1* mutant plants, either SA187-colonized or non-colonized, grown under normal (22°C) and eT (30°C) conditions. Values represent mean $\pm$ SEM from three independent biological replicates. Pollen germination percentages were calculated by scoring 200–300 pollen grains per replicate.

(I) Quantification of average seed number per silique in SA187-colonized and non-colonized *ein2-1* and *ref6-1* mutant plants, grown at 22°C or 30°C. Each dataset represents three independent biological replicates ($n = 36$ siliques/plant). For all analyses, statistical significance was determined by one-way ANOVA followed by Sidák's multiple comparisons test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$); ns, not significant.

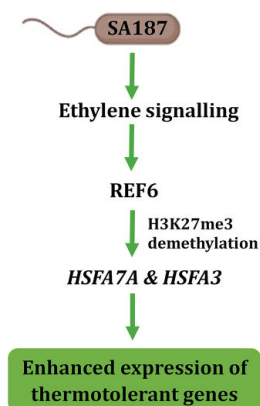


Figure 7. Model of SA187-induced thermotolerance genes via ethylene-mediated REF6 demethylase

The model illustrates that the beneficial endophytic bacterium SA187 enhances ethylene signaling, which in turn regulates the histone demethylase *RELATIVE OF EARLY FLOWERING 6* (REF6). REF6 demethylates the transcription repressive histone mark H3K27me3 at *HEAT SHOCK FACTOR A7A* (HSFA7A) and *HEAT SHOCK FACTOR A3* (HSFA3). The removal of H3K27me3 increases chromatin accessibility and promotes transcriptional activation of HSFA7A and HSFA3, which further induces the expression of thermotolerance-associated genes, thereby enhancing plant resilience, growth, and survival under eT conditions.

signaling and REF6, as well as the absence of SA187-mediated protection in the respective mutants, indicates that a similar pathway may operate in reproductive tissues. Nevertheless, its direct involvement remains to be experimentally validated in reproductive tissues.

Previous work by de Zélicourt et al.⁴¹ showed that plant colonization by SA187 induces the bacterial methionine salvage pathway, leading to the production of 2-keto-4-methylthiobutyric acid (KMBA), a sulfur-containing ethylene precursor that can be converted into ethylene in planta.⁴¹ Importantly, this study demonstrated that SA187-mediated stress tolerance requires plant ethylene signaling, but not the plant ACC-dependent ethylene biosynthetic pathway, indicating that SA187 enhances ethylene responses mainly by providing bacterial KMBA rather than by directly stimulating endogenous plant ethylene production. Given that plant ethylene biosynthesis originates from S-adenosyl methionine (SAM), a key intermediate in sulfur metabolism, this highlights a close connection between host-induced microbial sulfur metabolism and plant ethylene signaling.²² In addition, de Zélicourt et al. reported that SA187 is not restricted to the root surface but can colonize internal plant tissues, including aerial organs, and was detected in the apoplast of hypocotyls, cotyledons, first true leaves and stomata of these organs. This endophytic colonization provides a plausible basis for systemic communication between SA187 and distal plant tissues.⁴¹

Based on these findings, we propose a model in which SA187 enhances ethylene signaling, leading to the activation of the histone demethylase REF6 (Figure 7). Activated REF6 removes H3K27me3 marks at *HSFA7A* and *HSFA3* gene loci, thereby increasing chromatin accessibility and promoting their transcription. The upregulation of these heat shock factors subsequently

induces the expression of thermotolerance-associated genes, collectively enhancing plant growth, resilience, and survival under eT conditions.

Limitations of the study

While this study reveals a role for SA187-mediated thermotolerance in *Arabidopsis* through the ethylene-REF6 pathway, the relevance of this mechanism in agriculturally important crop species remains to be validated under field-like conditions. Additionally, the study primarily focuses on the protective effects of SA187 on male reproductive success, particularly pollen viability and germination. The potential roles of SA187 in female reproductive processes, such as ovule development and stigma receptivity, as well as the direct involvement of the ethylene-REF6 pathway in reproductive tissues, warrant further exploration.

RESOURCE AVAILABILITY

Lead contact

Any inquiries regarding this study or requests for materials and resources should be directed to the lead contact, Heribert Hirt (heribert.hirt@kaust.edu.sa).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the [lead contact](#) upon request.
- Seeds, microbes, and any additional information required to reanalyze the data reported in this paper are available from the [lead contact](#) upon request.
- This paper does not report original code.

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AUTHOR CONTRIBUTIONS

K.S., H.H., and A.F. designed the experiments and wrote the manuscript. A.F. performed all experiments and conducted the data analysis. L.A., W.A., H.U.F., and A.N. assisted with the experimental work. J.G.M. provided the resources. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- Leaf chlorophyll measurement
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- RNA extraction and real-time quantitative PCR analysis
- Chromatin immunoprecipitation (ChIP-qPCR)
- Assessment of SA187 colonization and root visualization via confocal microscopy
- Pollen viability, in vitro germination, and silique seed set analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Histone H3	abcam	Cat# ab1791; RRID: AB_302613
Anti-Histone H3 (tri methyl K27)	abcam	Cat# mAbcam 6002; RRID: AB_305237
Bacterial strains		
<i>Enterobacter</i> sp. SA187	C. Andrés-Barrao et al. ²²	N/A
Critical commercial assays		
NucleoSpin RNA Plant Kit	Macherey-Nagel	Cat#18080044
SuperScript III	Invitrogen™	Cat# 18080-400
SSOAdvanced Universal SYBR Green	Bio-Rad	Cat#1725271
Experimental models: Organisms/strains		
<i>Arabidopsis thaliana</i>	https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=3702	NCBI:txid3702
Oligonucleotides		
q-PCR sequences- Table S1 in Supplementary	This paper	N/A
ChIP q-PCR sequences- Table S1 in Supplementary	This paper	N/A
Software and algorithms		
ImageJ (version 1.54)	Schindelin et al. ⁴²	https://imagej.net/ij/
GraphPad Prism version 10	N/A	https://www.graphpad.com/
ZEN 3.4 (blue edition)	Carl Zeiss system	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Plant material and growth conditions

A. thaliana wild-type Col-0 seeds and mutants (*ein2-1* and *ein3-1*) were obtained from publicly available collections. Seeds of *ein3. eil1. eil3* triple mutant were kindly provided by Prof. Stanislav Kopriva. The *ref6-1* mutant seeds were kindly provided by Prof. Moussa Benhamed, while the *elf6-C*, *jmj13-1*, *ref6-5 elf6-C*, and *ref6-5 elf6-C jmj13-1* mutant lines were kindly obtained from Prof. Jose Gutierrez-Marcos laboratory. All plants were grown in controlled growth chambers at 22°C and 30°C under a 16-h light and 8-h dark photoperiod conditions.

Microbial strains and culture conditions

The beneficial endophyte *Enterobacter* sp. SA187 was obtained from root nodules of the desert-adapted legume *Indigofera argentea*, native to the Jizan region of Saudi Arabia.²² Cryopreserved SA187 cultures were streaked onto LB agar plates, incubated at 28°C for 24 h, and a single colony was used to initiate all subsequent experiments. For preparation of SA187-colonized seed plates, 50 mL of half-strength Murashige and Skoog (½ MS) medium containing 0.9% agar (pH 5.8) was supplemented with 0.1 mL of a freshly prepared bacterial suspension (OD₆₀₀ = 0.2), yielding a final concentration of approximately 10⁵ CFU/mL. Control plates were prepared by adding 0.1 mL of sterile LB broth to ½ MS medium instead of the bacterial suspension.

METHOD DETAILS

Plant assay and growth measurements

For all the plant growth assay, the seeds were surface sterilized with 70% ethanol and 0.05% Triton X-100 for 10 min vortex. The seeds were then rinsed three times with 100% ethanol and dried on sterilized filter paper in a laminar flow hood. Sterilized seeds were distributed on a ½MS medium (50 mL) that was supplemented with either 100 µL of Luria Broth (LB) medium (for mock) or a fresh culture of SA187 that was cultured in LB to an optical density of 0.21 (for colonized). The seeds were subjected to stratification in darkness at 4°C for a duration of 2 days. Subsequently, the plates were moved to a growth chamber where the temperature was set

at 22°C, and the photo period consisted of 16 h of light followed by 8 h of darkness. This condition was maintained for a period of 5 days. The 5 days old seedlings, with root lengths of around 1.0–1.5 cm, were transferred to a new ½MS medium plate under normal conditions 22°C and 30°C to simulate eT stress, follow the scheme in Figure S1B. Total FW was measured after 16 days of stress using a METTLER TOLEDO sensitive balance, while DW was determined after drying the biomass at –80°C for two days. For ethylene precursor treatments, 1 μM of ACC (1-aminocyclopropane-1-carboxylic acid) was added to the ½ MS medium. All experiments were conducted with three biological replicates and technical replicates.

Lateral root count and hypocotyl length measurement

For hypocotyl length measurement, 20–35 seedlings per treatment were imaged daily from day two to day five, using an HP Scanjet 8270 scanner. Hypocotyl lengths were quantified using ImageJ software (version 1.54).⁴² For lateral root analysis, seedlings were assessed on the twelfth day following stress treatment. Lateral roots were counted under a stereomicroscope, and primary root lengths were measured using ImageJ. Lateral root density (LRD) was calculated as the number of lateral roots divided by the primary root length (in cm).

Leaf chlorophyll measurement

Chlorophyll was extracted from 50 to 80 mg of frozen, ground leaf tissue using 1 mL of 80% (v/v) acetone.^{43,44} The homogenate was centrifuged at 13,000 rpm for 10 min at room temperature, and the resulting supernatant was used to measure absorbance at 537, 647, and 663 nm using a TECAN plate reader. Chlorophyll concentrations were calculated using the following equations: Chl b (μmol mL^{–1}) = 0.02405 A₆₄₇–0.004305 A₅₃₇–0.005507 A₆₆₃. Chl a (μmol mL^{–1}) = 0.01373 A₆₆₃–0.000897 A₅₃₇–0.003046 A₆₄₇. Total Chlorophyll = Chl.a + Chl.b.²⁴

Ion leakage measurement

Shoot tissues from both non-stressed and heat-stressed plants were harvested on day 16, and their FW were recorded. Samples were placed into 6-well plates containing 3 mL of a solution composed of 2.5 mM MES buffer (monohydrate free acid, pH 5.8) supplemented with 0.05% (v/v) Tween 20, following the method of Jameel et al.,⁴⁵ with a slight modification: whole shoots were used instead of leaf discs. The plates were incubated in the dark at 28°C for 24 h. After incubation, the MES solutions containing the shoot tissues were transferred into individually labeled test tubes. Membrane ion leakage was determined by measuring the electrical conductivity of the solution using a conductivity meter (Mettler Toledo AG 8603, Mettler-Toledo Group), and values were recorded in mS/m. Conductivity readings were then normalized to the fresh weight of each sample and expressed as mS/m per mg FW, allowing for accurate comparisons between treatments.

RNA extraction and real-time quantitative PCR analysis

Five-day-old Arabidopsis seedlings grown on ½ MS plates under long-day conditions were treated at 22°C or 30°C for 6 h, with or without SA187. Total RNA was isolated using the NucleoSpin RNA Plant Kit (Macherey-Nagel, Cat. 740949) and quantified with a NanoDrop-6000. cDNA was synthesized from 1 μg RNA using SuperScript III (Invitrogen, Cat. 18080044). RT-qPCR was performed on a CFX384 real-time PCR Detection System (Bio-Rad) with the program: 50°C 2 min, 95°C 10 min, 40 cycles of 95°C 10 s and 60°C 40 s. β-tubulin was used as the reference gene, and relative expression levels were normalized to the corresponding mock, non-colonized samples. Each reaction was run in three biological replicates, each with three technical. Primer sequences are provided in Table S1.

Chromatin immunoprecipitation (ChIP-qPCR)

For ChIP-qPCR, chromatin immunoprecipitation was performed as described by Shekhawat et al.²³ and Lämke et al.⁴⁶ Briefly, ~200–300 mg of 5-day-old seedlings treated with 22°C or 30°C for 6 h, with or without SA187, were cross-linked with 1% formaldehyde under vacuum for 15 min and quenched with 2 M glycine for 5 min. Tissues were flash-frozen in liquid nitrogen and stored at –80°C. Ground tissue was used for nuclei isolation, and chromatin was sheared using a Diagenode Bioruptor (10 cycles, 30 s on/off, medium setting) to ~250 bp fragments. Immunoprecipitation was performed overnight at 4°C using antibodies against H3K27me3 (Abcam, ab6002) and H3 (Abcam, ab1791), followed by capture with Dynabeads Protein A (Thermo Fisher). After washing and reverse cross-linking at 65°C overnight, DNA was extracted via phenol-chloroform, ethanol-precipitated with glycogen, and re-suspended in 20 μL of water. DNA concentrations were measured using a Nanodrop-6000. ChIP-qPCR was conducted for two regions, each of the *HSFA7a* and *HSFA3* loci. Amplification values were normalized to total input and H3. Values represent mean ± SEM of three biological replicates, each with three technical replicates.

Assessment of SA187 colonization and root visualization via confocal microscopy

Arabidopsis seedlings were germinated on ½ MS agar inoculated with SA187 (OD₆₀₀ = 0.2) and transferred to fresh ½ MS plates after 5 days, then grown under control or eT conditions for the next 15 days. On day sixteen, seedlings were collected, homogenized in 1 mL extraction buffer (10 mM MgCl₂, 0.01% Silwet L-77), serially diluted, and plated on LB agar. Following incubation at 28°C overnight, bacterial colonies were counted. The number of colony-forming units (CFUs) was normalized to fresh tissue weight (CFU mg^{–1} FW).⁴⁷ Visualization of GFP-labeled Enterobacter sp. SA187 on Arabidopsis roots was performed using a Zeiss LSM 880 inverted

confocal microscope. Five-day-old seedlings grown vertically on ½ MS plates inoculated with SA187: GFP were gently rinsed with sterile distilled water. Root segments were excised and mounted in sterile water. GFP fluorescence was excited at 488 nm and detected at 493–537 nm in single-track mode.⁴¹

Pollen viability, in vitro germination, and silique seed set analysis

Arabidopsis thaliana plants were cultivated on Jiffy trays under optimal conditions (22°C). To induce eT stress, plants were transferred to 30°C for 4 days following the bolting stage. Mature pollen was harvested from fully open flowers of both control and heat-treated plants. For viability assays, pollen from six flowers was suspended in staining buffer (290 mM sucrose, 1.27 mM Ca(NO₃)₂, 0.16 mM H₃BO₃, 1 mM KNO₃) containing 0.001% FDA and 10 μM PI, incubated for 15 min at 28°C, and imaged using a Zeiss LSM 880. FDA fluorescence was excited at 488 nm and detected at 493–584 nm, while PI was excited at 561 nm and detected at 584–718 nm. Viable pollen was identified by FDA positivity, and non-viable pollen by PI staining. For each sample, 200–300 pollen grains were scored. Pollen viability was calculated as the percentage of FDA-positive grains relative to the total number of grains.

For germination analysis, pollen grains were collected and resuspended in a pollen germination medium (PGM) prepared according to the established protocol by Muhlemann et al.⁴⁰ with slight optimization. The PGM comprised 10% (w/v) PEG 4000, 15% (w/v) sucrose, 30 mM Ca(NO₃)₂, 0.01% (w/v) boric acid, 0.02% (w/v) MgSO₄, and 0.01% (w/v) KNO₃, adjusted to pH 7.25. Samples were incubated for 18 h before mounting on microscopic slides. Pollen germination was observed using bright-field microscopy, and tube length measurements were performed using Fiji (ImageJ) software.⁴² Brightness and contrast adjustments were applied uniformly to the entire image and equally across all samples to improve visualization of pollen tubes.

For seed set analysis, siliques were harvested from plants grown under control and heat stress conditions, with or without microbial colonization. Collections were made for 2–3 weeks post-fertilization, and siliques were fixed in a 3:1 (v/v) solution of glacial acetic acid and ethanol for a minimum of 6 h. After fixation, samples were transferred to 100% ethanol and stored overnight. Images were captured the following day using an iPhone 13 Pro. Seed counts were manually performed by zooming in on the fixed silique images within ImageJ.

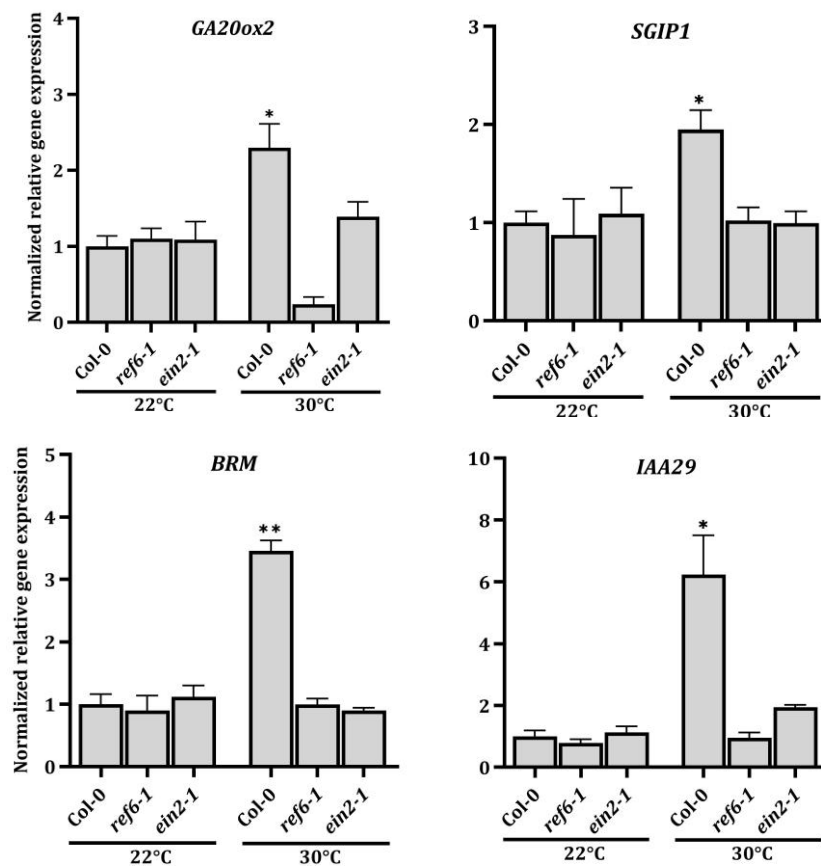
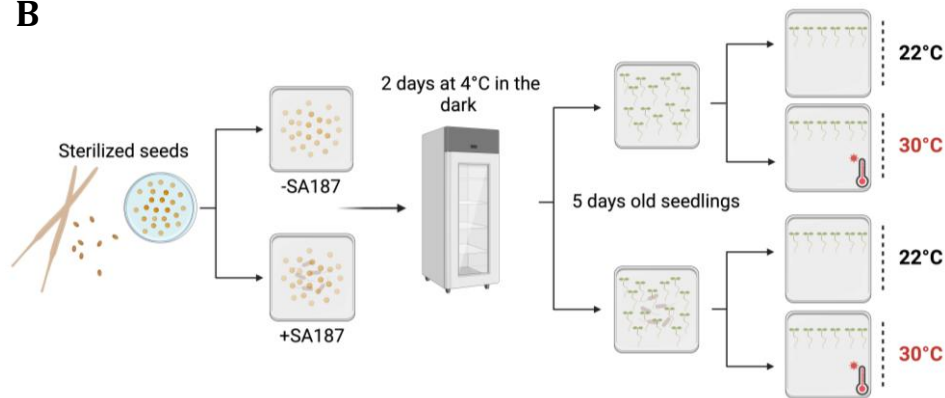
QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were performed using GraphPad Prism version 10 and Microsoft Excel 2019. Differences in qPCR and ChIP-qPCR data between Col-0 and mutant lines within each treatment were assessed using a two-tailed Student's *t* test. For physiological parameters, including FW, DW, pollen viability, and others, one-way ANOVA followed by Šidák's multiple comparisons test was used to evaluate differences between Col-0 and mutant lines under eT and/or microbial colonization treatments. Statistical details, including sample size (*n*) and significance levels, are provided in the corresponding figure legends. Boxplots show the median and interquartile range, with whiskers indicating the minimum and maximum values. Statistical significance was considered at *p* < 0.05.

Supplemental information

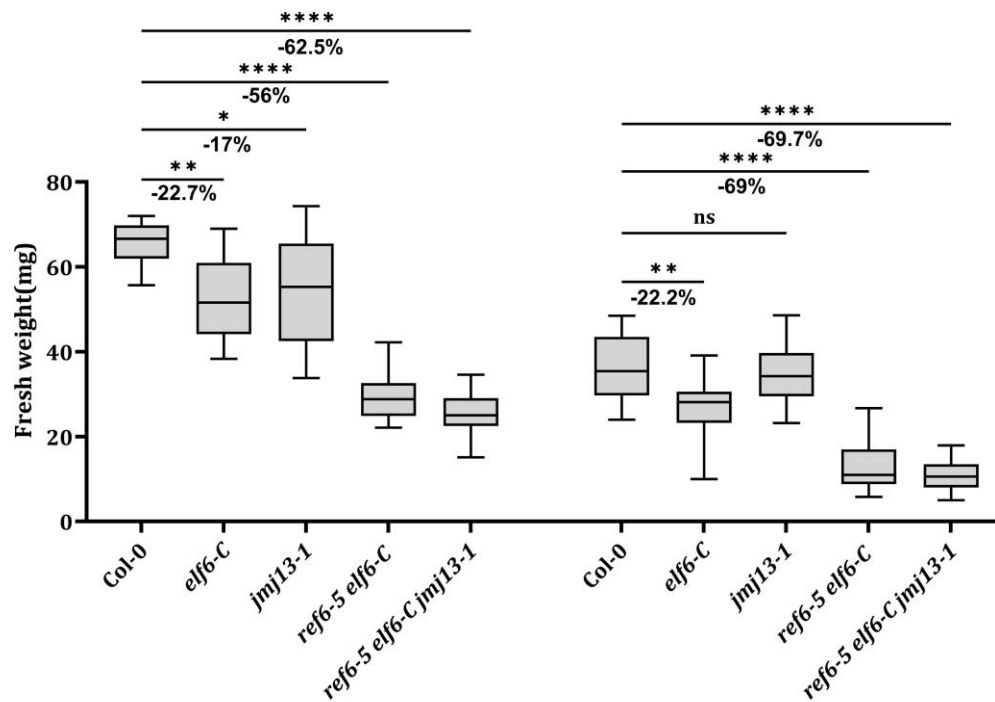
**Ethylene-REF6 regulatory module enables
endophyte-mediated thermotolerance of plant
growth and reproduction**

Anam Fatima, Kirti Shekhawat, Louai Alidrissi, Waad Alzayed, H.U. Farooq, Arun P. Nagarajan, Jose Gutierrez-Marcos, and Heribert Hirt

A**B**

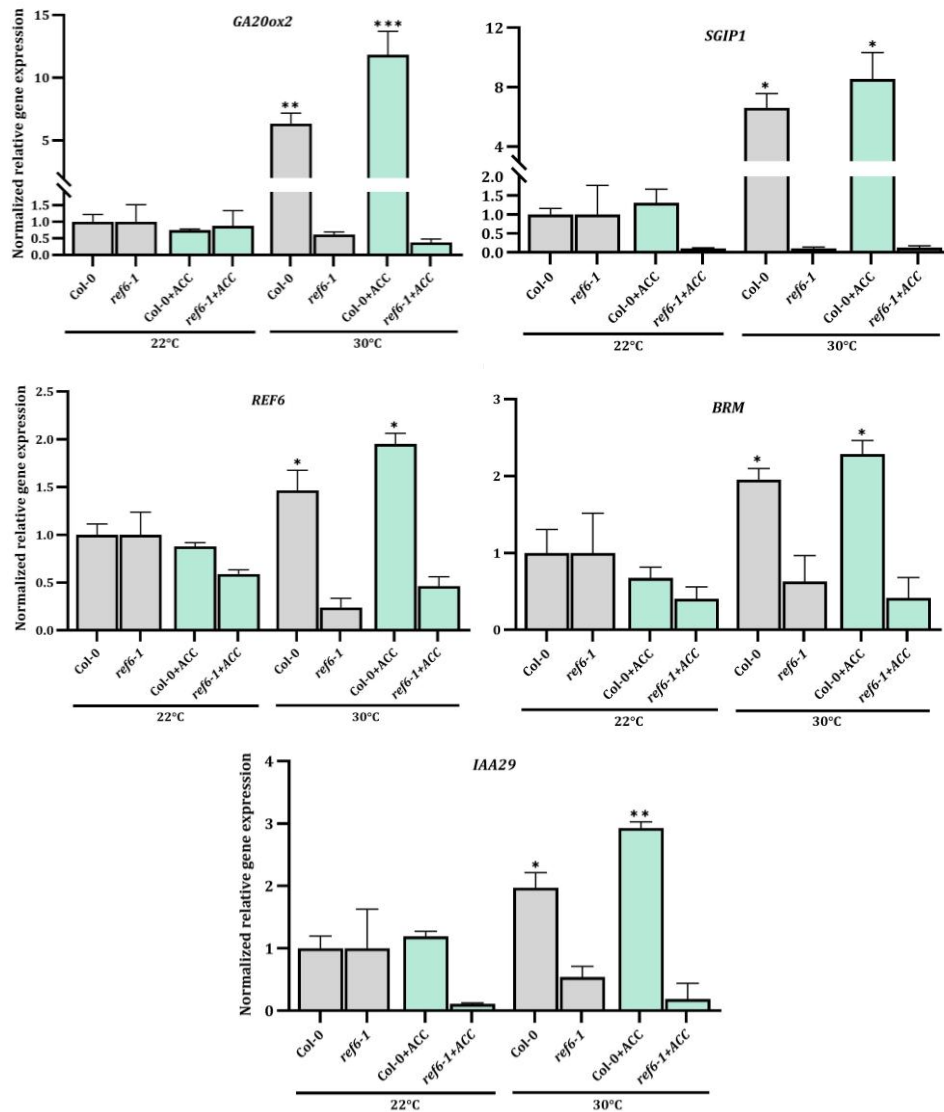
Supplementary Figure 1. Normalized relative gene expression analysis of thermoresponsive genes in wild type Col-0, *ref6-1* and *ein2-1* mutant seedlings under eT.

(A) qRT-PCR analysis of *GA20ox2*, *IAA29*, *BRM*, and *SGIP1* expression in 6-day-old seedlings grown under control (22°C) and eT (after 6h of 30°C) conditions, with or without SA187 colonization. Expression levels were normalized to β -tubulin and are shown relative to Col-0 at 22°C. Error bars represent standard error (SEM). Statistical significance was determined using Student's t-test, with asterisks denoting significant differences (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$). **(B)** Graphical illustration of the experimental workflow for plant assays conducted under normal (22°C) and eT (30°C) conditions, with or without SA187 colonization.



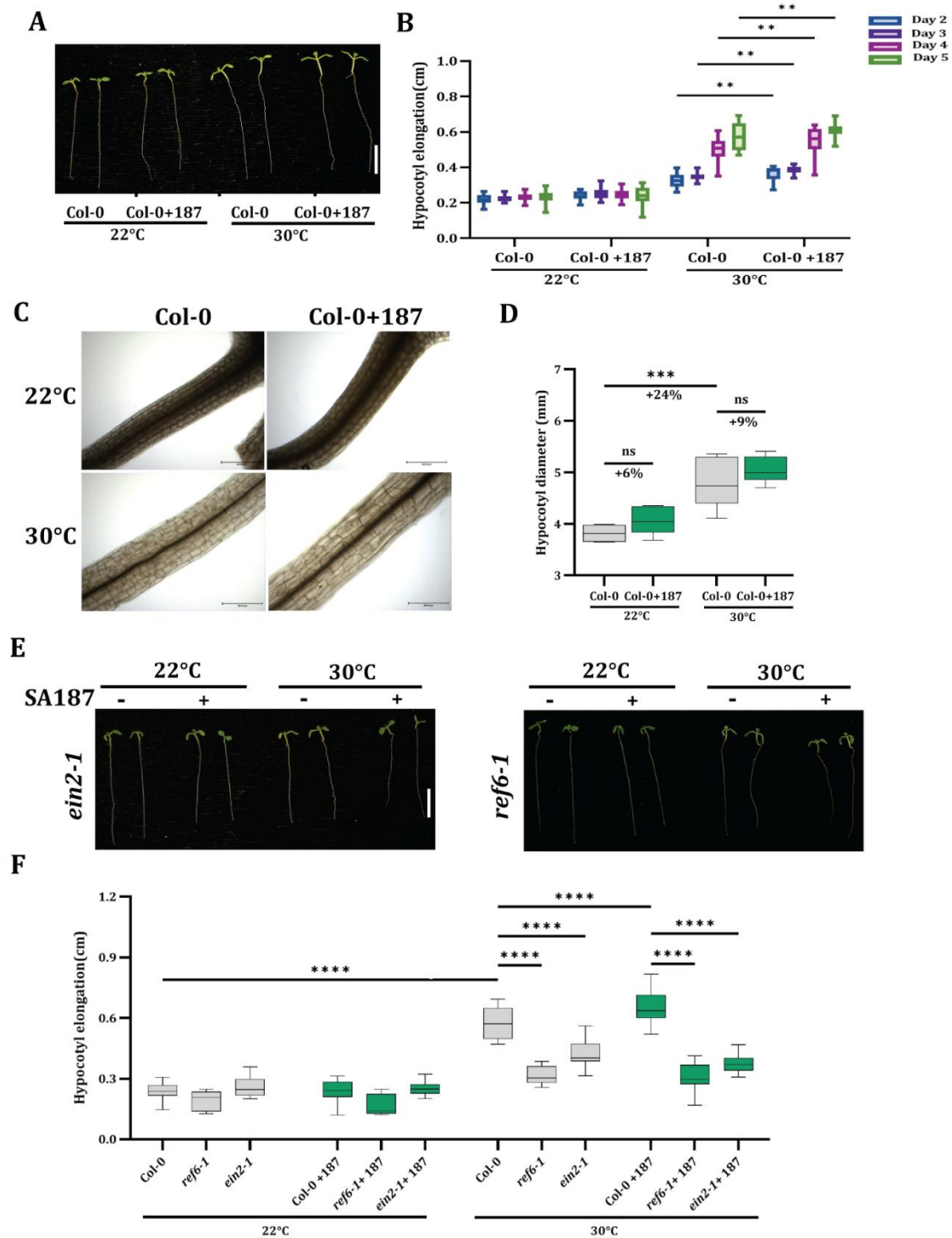
Supplementary Fig. 2. Role of REF6 homolog ELF6 and JMJ13 under normal and eT conditions.

Fresh weight data of REF6 homolog *elf6-C*, *jmj13-1* and its double mutant (*ref6-5 elf6-C*), and triple mutant (*ref6-5 elf6-C jmj13-1*) plants under normal (22°C) and eT (30°C) conditions. Boxplots show data from three independent biological replicates (Total No. of plants $n = 36$). For each line, asterisks indicate the statistical differences based on the one-way ANOVA analyses followed by Šidák's multiple comparisons test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). n.s. stands for not significant.



Supplementary Figure 3. Exogenous ACC enhances thermotolerance in a REF6-dependent manner.

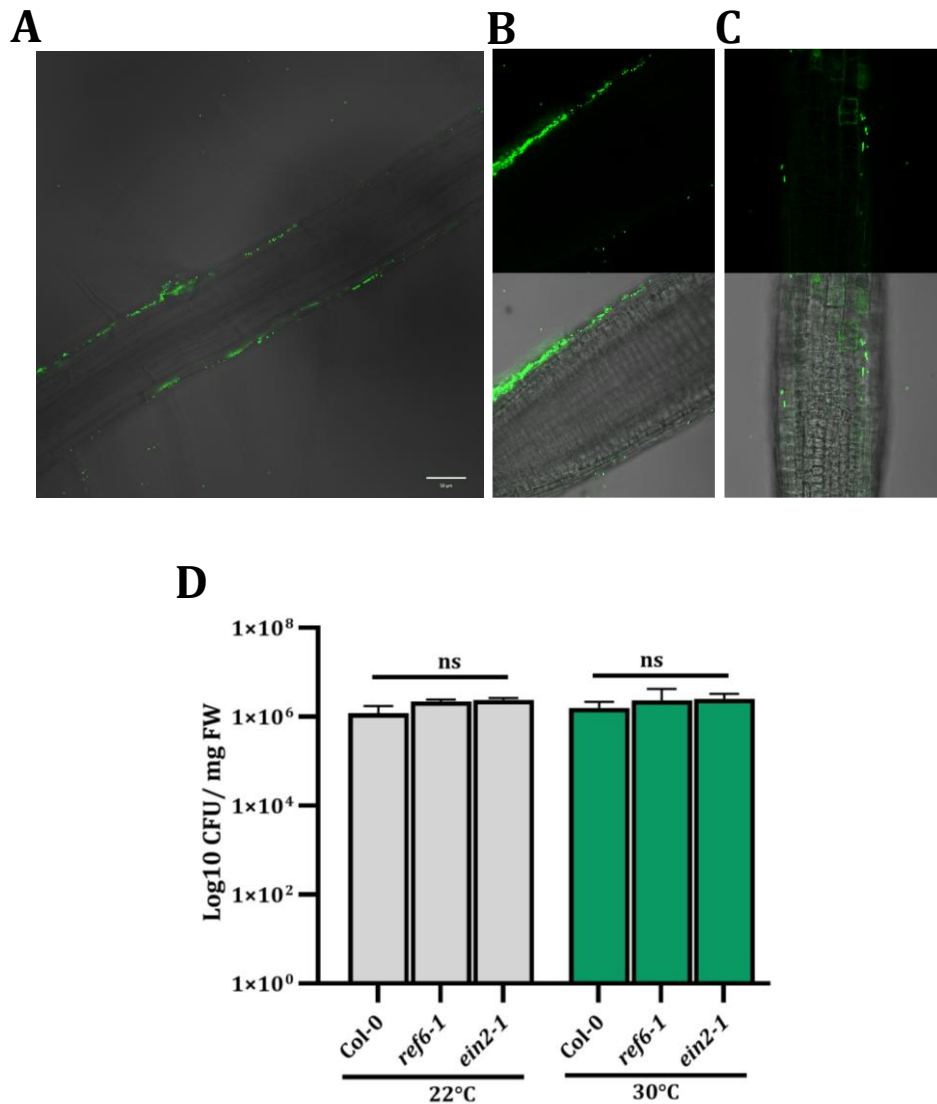
Transcript levels of *GA20ox2*, *SGIP1*, *IAA29*, *REF6*, and *BRM* were determined by qRT-PCR in wild-type *Col-0* and *ref6-1* mutant plants grown under normal (22°C) and eT (after 6h of 30°C) conditions, with or without exogenous ACC treatment. Expression values were normalized to β -tubulin and calculated relative to *Col-0* plants grown at 22°C without ACC treatment. Each plot represents the mean $\pm$ SEM of three independent biological replicates. Statistical significance was determined using Student's *t*-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).



Supplementary Figure 4. SA187 promotes hypocotyl elongation under eT in Arabidopsis.

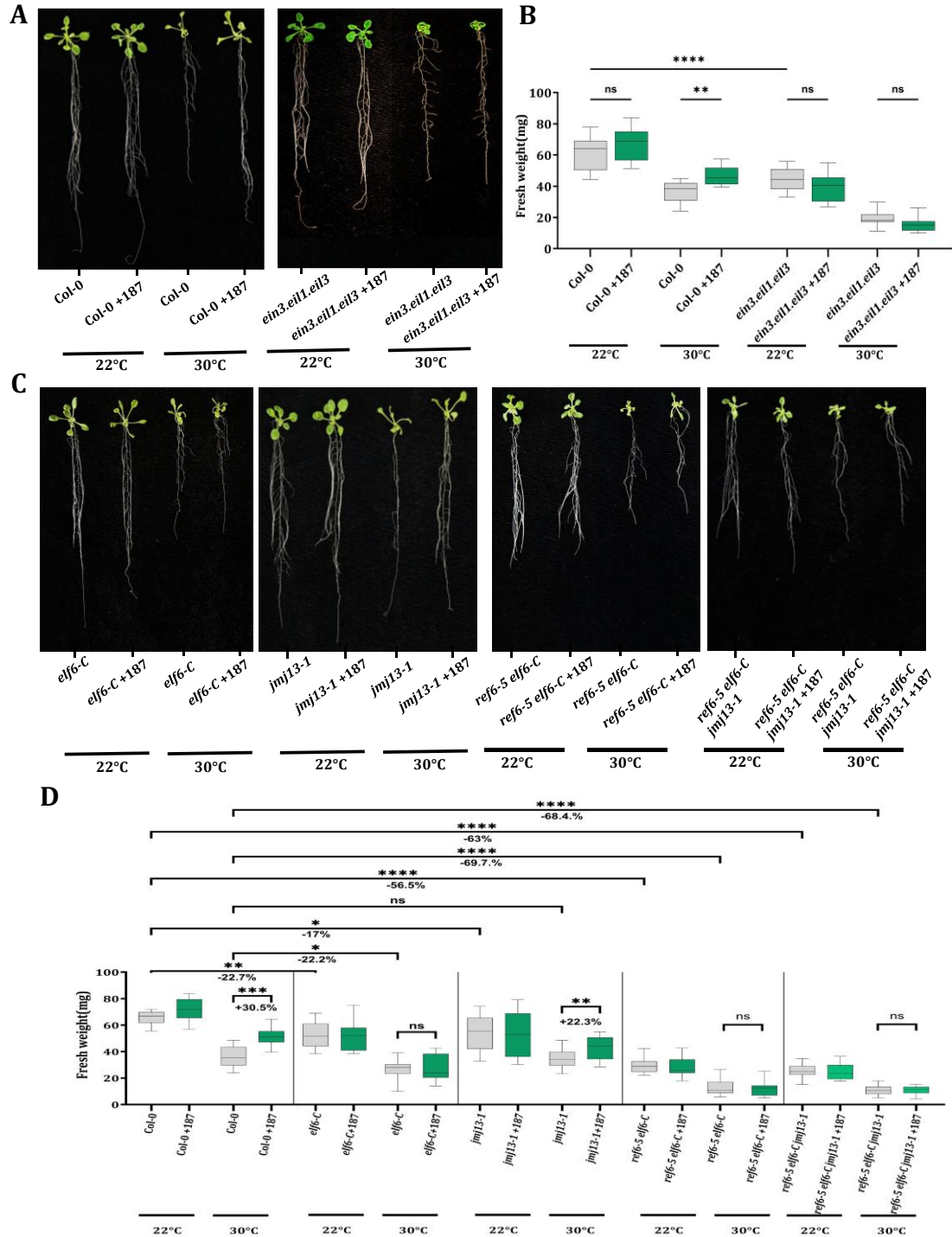
(A-D) Representative image of four-day old *A. thaliana* Col-0 seedlings grown at 22°C and 30°C, with and without SA187 colonization. Scale bar = 1 cm(A). Box plot showing hypocotyl elongation of Col-0 seedlings germinated for five days, then transferred to fresh ½ MS plates and grown at 22 °C or 30 °C from day 2 to day 5 (n = 30) (B). Bright-field microscopic images of the hypocotyl region showing radial cell elongation at 22°C and 30°C. Scale bar = 200 µm (C). Quantification of hypocotyl diameter in Col-0 seedlings under the 22°C and 30°C (n=18-36) (D). (E & F) Representative image and hypocotyl elongation data of four-day old *A. thaliana* mutant *ein2-1* and *ref6-1* mutant seedlings with and without SA187 colonization at 22°C and 30°C. Scale bar = 1 cm. For all the analysis, statistical significance was

determined by one-way ANOVA with Šídák's multiple comparisons test. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). ns: stands for not significant.



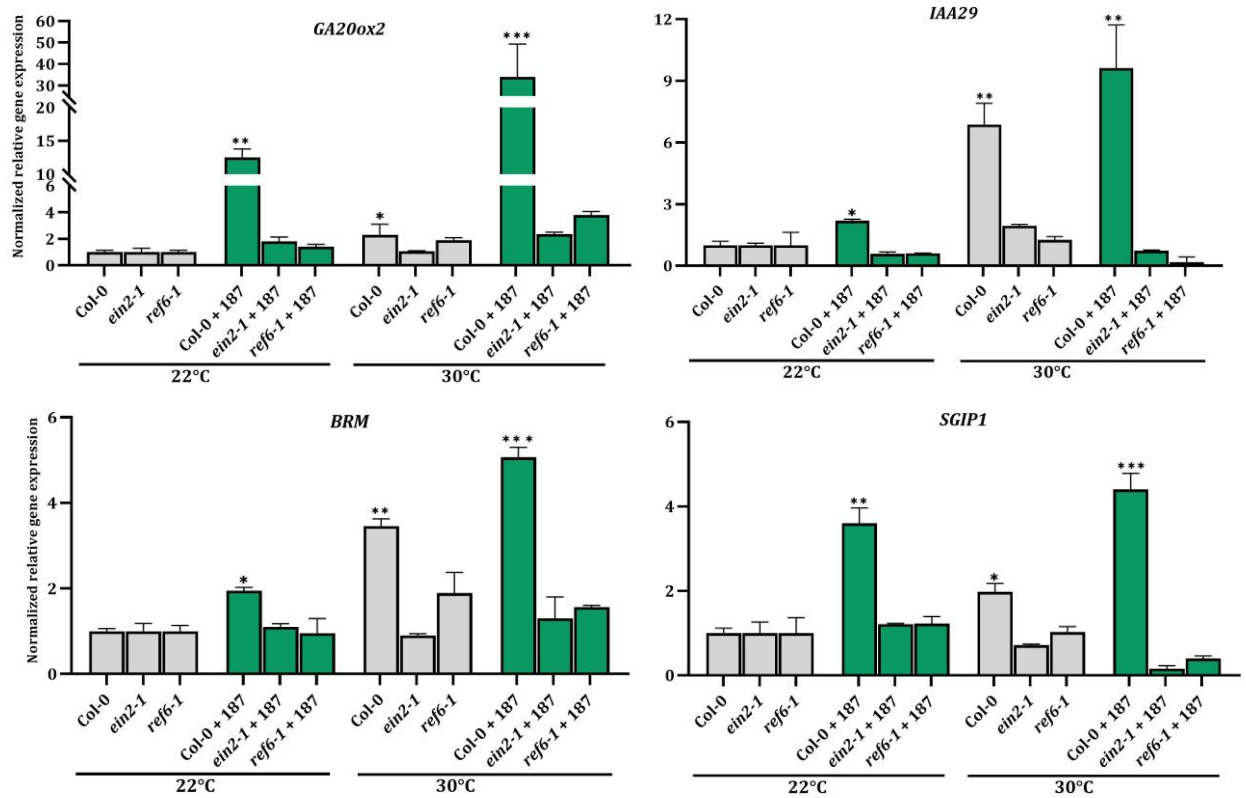
Supplementary Figure 5. GFP-tagged *Enterobacter* sp. SA187 colonization and quantitative analysis of colony-forming units (CFU) in Arabidopsis at eT.

(A-C) Confocal images of five-day-old wild-type Col-0 seedling roots imaged at 20X magnification. Merged fluorescence and brightfield image showing bacterial colonization along the root surface. Green fluorescence indicates the presence of GFP-tagged SA187 cells. Scale bar = 50 μm . (A). Upper panel: GFP fluorescence; Lower panel: corresponding brightfield overlay showing bacteria aligned along the epidermal cell layer (B). Colonization near the root tip zone, with GFP signals visible in epidermal and cortical regions (C). **(D)** Quantitative analysis of colony-forming units (CFUs) of 16-day old SA187 colonized plants at 22°C and 30°C. Statistical differences based on the one-way ANOVA analyses followed by Šídák's multiple comparisons test. n.s. stands for not significant.

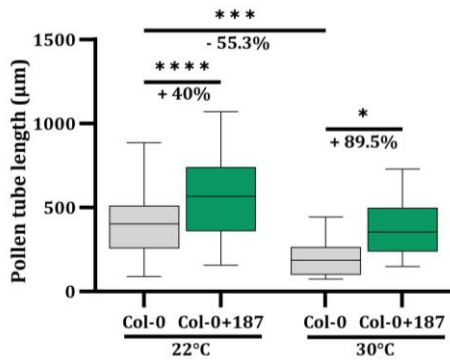


Supplementary Figure 6. Role of Ethylene signaling high order mutant and REF6 homolog ELF6 and JMJ13.

(A-B) Phenotypes (A) and fresh weight data (B) of wild type Col-0 and ethylene signaling high order mutant *ein3.eil1.eil3* plants under normal (22°C) and eT (30°C) conditions. (C-D) Phenotypes (A) and fresh weight data (B) of REF6 homolog *elf6-1*, *jmj13-1* and its double (*ref6-5 elf6-C*), and triple mutant (*ref6-5 elf6-C jmj13-1*) plants under normal and eT conditions. Boxplots show data from three independent biological replicates (Total No. of plants n = 36). For each line, asterisks indicate the statistical differences based on the one-way ANOVA analyses followed by Šidák's multiple comparisons test (*P < 0.05, **P < 0.01, ***P < 0.001). n.s. stands for not significant.



Supplementary Figure 7. Normalized relative gene expression analysis of thermoresponsive genes in wild type Col-0, *ein2-1* and *ref6-1* mutant seedlings with or without SA187 colonization. qRT-PCR analysis of *GA20ox2*, *SGIP1*, *BRM* and *IAA29* expression in 6-day-old seedlings grown under control (22°C) and eT (after 6h of 30°C) conditions, with or without SA187 colonization. Expression levels were normalized to β -tubulin and are shown relative to Col-0 at 22°C. Error bars represent standard error (SEM). Statistical significance was determined using Student's t-test, with asterisks denoting significant differences (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).



Supplementary Figure 8. Pollen tube length measurement under eT in colonized and non-colonized wild-type Col-0.

The box plot shows the quantification of pollen tube length in colonized and non-colonized wild-type Col-0 plants under 22°C and 30°C conditions. Data represents three independent biological replicates (n = 95-120 per condition). Statistical differences based on the one-way ANOVA analyses followed by Šidák's multiple comparisons test (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.001).

Table S1

Primers used in this study for qRT-PCR and ChIP qPCR	
Oligo Name	Primer sequence 5' to 3'
HSFA3-F	TTGGTTGATGACCCGACTCTTG
HSFA3-R	TTCCCACTTGTCAGTATCAATCTTTC
HSFA7A-F	CTTTCTCCACGATTCTCCTTCCT
HSFA7A-R	AAAGGGTTTCTCCTCTTGATGTTCT
REF6-F	AGGTCAACAGCCAAACGAATAGC
REF6-R	CTCTCGACGGCTTCTGATGTCTC
BRM-F	TTAGAAAGGAAAAGGATTAGGC
BRM-R	GCCGTTTCGCATAACCTCA
SGIP1-QF	CATTCTCCCCGTAGATGCTGA
SGIP1-QR	CAAATCCTCGGACGCCAAC
GA20ox2-F	CATTAGGACAAGAGTTCGAGCAG
GA20ox2-R	GGAGGATAATGATTGAGCCTCA
PIF4-F	GCCGATGGAGATGTTGAGAT
PIF4-R	CCAACCTAGTGGTCCAAACG
IAA29-F	ATCACCATCATTGCCCGTAT
IAA29-R	TTGCCACACCATCCATCTTA
β - tubulin-F	CAAGATGCTACAGCCGGAGAGGA
β - tubulin-R	AGAAGAAAGTGAATGCTGCTTGCT
ChIP-qPCR Primers	
HSFA3-P1-ChIP-QF	CCCATGCCACTAGACATCTTAC
HSFA3-P1-ChIP-QR	CGGGTCATCAACCAAATCA
HSFA3-P2-ChIP-QF	AGCACCACCAGCAGCCTCAA
HSFA3-P2-ChIP-QR	AAGTCATCCCTCCTAAACCCT
HSFA7A-P1-ChIP-QF	CCAATGGAGGGTTTACACGA

HSFA7A-P1-ChIP-QR	TGAAATGACGAGGAAGGAGAAT
HSFA7A-P2-ChIP-QF	CTTCACCAAGCCATGACGC
HSFA7A-P2-ChIP-QR	TG TTCCTTCTATCCTCTGTTCCA