Root endophyte induced plant thermotolerance by constitutive chromatin modification at heat stress memory gene loci

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Abstract

Global warming has become a critical challenge to food security, causing severe yield losses of major crops worldwide. Conventional and transgenic breeding strategies to enhance plant thermotolerance are laborious and expensive. Therefore, the use of beneficial microbes could be an alternative approach. Here, we report that the root endophyte \textit{Enterobacter} sp. SA187 induces thermotolerance in wheat in the laboratory as well as in open-field agriculture. To unravel the molecular mechanisms, we used \textit{Arabidopsis thaliana} as model plant. SA187 reprogrammed the \textit{Arabidopsis} transcriptome via HSFA\textsubscript{2}-dependent enhancement of H3K4me3 levels at heat stress memory gene loci. Unlike thermopriming, SA187-induced thermotolerance is mediated by ethylene signaling via the transcription factor \textit{EIN3}. In contrast to the transient chromatin modification by thermopriming, SA187 induces constitutive H3K4me3 modification of heat stress memory genes, generating robust thermotolerance in plants. Importantly, microbial community composition of wheat plants in open-field agriculture is not influenced by SA187, indicating that beneficial microbes can be a powerful tool to enhance thermotolerance of crops in a sustainable manner.

Keywords \textit{EIN3}; endophyte; HSFA\textsubscript{2}; memory gene; thermotolerance

Introduction

Among other effects, global warming increases average temperatures and causes severe heat waves, challenging plant growth, and agriculture worldwide (Mittler & Blumwald, 2010; Ling \textit{et al}, 2018). Elevated temperatures cause severe cellular injury to plants resulting in a collapse of cellular organization and inhibition of plant growth (Abd El-Daim \textit{et al}, 2014). In order to cope with heat stress (HS), plants have developed several strategies such as basal heat tolerance and acquired heat tolerance. In basal heat tolerance, plants have a natural capacity to deal with heat stress, whereas in acquired thermotolerance, called heat acclimation or thermopriming, plants acquire tolerance to lethal levels via a short pre-exposure to a mild HS, a phenomenon that is known as priming (Yeh \textit{et al}, 2012). Depending on the recovery time before the secondary acute heat stress exposure, acquired thermotolerance is distinguished between “short-term acquired thermotolerance” (SAT) and “long-term acquired thermotolerance” (LAT). Acquired thermotolerance establishes a molecular stress memory state that helps to protect plants from acute heat stress damage and cell death (Sani \textit{et al}, 2013). Thermopriming is responsible for the higher expression of HS transcription factors (HSPs) regulating the expression of heat-shock proteins (HSPs) and antioxidant genes, which are responsible for responding robustly and quickly to the exposure and subsequent recovery from HS (Conrath \textit{et al}, 2006; Hilker \textit{et al}, 2016; Lin \textit{et al}, 2018). Heat-shock factor-A2 (HSFA\textsubscript{2}) is required for the active memory of HS in plants (Charng \textit{et al}, 2007; Scharf \textit{et al}, 2012). The expression of HSFA\textsubscript{2} is regulated by the four transcription factors HSFA\textsubscript{1A}, B, D, and E, which activate the expression of HSFA\textsubscript{2}. In turn, HSFA\textsubscript{2} amplifies the transcriptional induction of a subset of HS response and memory genes (Mishra \textit{et al}, 2002; Schramm \textit{et al}, 2006; Liu \textit{et al}, 2011; Yoshida \textit{et al}, 2011; Yeh \textit{et al}, 2012; Liu & Charng, 2013; Stief \textit{et al}, 2014). HSFA\textsubscript{2} binds transiently in a hit and run mode at the promoter region of HS memory genes, thereby facilitating di- and trimethylation of lysine 4 on histone H3 (H3K4me2, H3K4me3) (Lämke \textit{et al}, 2016a). Thermopriming-induced chromatin modification at the APX2 and HSP18.2 heat stress memory genes induces a transcriptional memory that leads to transcriptional
hyper-activation upon a recurring heat stress (Bruce et al., 2007; Vriet et al., 2015; Lämke et al., 2016b; Lämke & Bäurle, 2017).

Thermopriming could be applied in agriculture to make crops more heat stress resistant and productive (Li & Charng, 2012), but its application is not feasible under field conditions. In this context, the use of beneficial root endophytes and rhizobacteria (known as plant growth-promoting bacteria, PGPB) might be a more reliable method to promote plant growth under abiotic stress conditions (Márquez et al., 2007; de Zélicourt et al., 2018; Numan et al., 2018; Saad et al., 2020). Here, we report that the root endophytic bacterium Enterobacter sp. SA187, isolated from root nodules of the indigenous desert plant Indigofera argentea (Andrés-Barrao et al., 2017), significantly enhances thermotolerance in wheat under laboratory and field conditions without significantly affecting the endogenous microbiome composition. In Arabidopsis, SA187 regulates the transcription dynamics of selected heat stress memory genes by hypermethylation of histone H3K4. Thermoprimed heat tolerance depends on the transcription factors HSFA1A, B, D, and E and the downstream master regulator HSFA2 (Lämke et al., 2016b; Lämke & Bäurle, 2017). Whereas thermopriming and root endophyte-induced thermotolerance both depend on HSFA2, SA187 functions independently of HSFA1A, B, D, and E. Instead, SA187-induced thermotolerance acts on HSFA2 via the ethylene signaling pathway and the transcription factor EIN3. In summary, our study unravels a novel mechanism of root endophyte-induced thermotolerance, opening the way to use beneficial microbes to adapt agriculture to the challenges of global warming.

Results

SA187 induces thermotolerance in wheat

In order to assess the effect of SA187 on the growth of wheat under laboratory conditions, we colonized wheat plants with SA187 and exposed 6-day-old plants to 44°C HS for 2 h. In contrast to non-colonized plants which showed chlorotic leaf damage and stopped growth upon HS (Fig 1A), SA187-colonized wheat was protected against HS chlorotic leaf damage (Fig 1A) and continued growth (Fig 1B).

To test the effect of SA187 on wheat grown under natural desert field conditions of high temperatures, wheat was grown during three growing seasons (2014–2018) at the ICBA field station (Dubai, UAE) characterized by low rainfall and high temperatures of up to 42°C during a typical winter vegetation period (Fig EV1A). When mature plants were assessed for agronomic traits, SA187-treated wheat showed a 10–14% increase in plant height, 22% more seeds/spike, and a 12% increase in the 1,000 seeds weight (Fig EV1B). Importantly, a consistent increase of 30%, 40%, and 20% in grain yield for SA187-treated plants was observed in the three seasons, giving 2.6 t/ha of seeds (Appendix Fig S1). Similarly, consistent increases in plant biomass of SA187-treated wheat were observed in the three growing seasons (Fig 1C).

SA187 induces thermotolerance in Arabidopsis thaliana

To investigate the molecular mechanism of SA187-induced plant thermotolerance, we examined whether SA187 can enhance long-term acquired thermotolerance (LAT) in Arabidopsis. We evaluated fresh weight, % survival, and % bleaching and green leaves by comparing heat-stressed (HS) to thermoprimed/heat-acclimated (LAT) plants following an acute HS of 44°C (HS) with and without SA187 (+/- 187). For LAT treatment, 9-day-old plants that were grown at 22°C were treated at 37°C for 3 h. After 2 days of recovery at 22°C, a 44°C HS was applied. After a further incubation of 4 days at 22°C, 15-day-old plants were analyzed for phenotypes and transcriptomes. For HS treatment, 11-day-old plants that were grown at 22°C were directly exposed to 44°C before further incubation for 4 days at 22°C. Non-heat-stressed (NHS) plants were grown at 22°C for 15 days in parallel as control (Fig 1D).

Compared to NHS plants, HS resulted in a major reduction of plant fresh weight, survival, and number of green leaves, while LAT significantly protected plants from HS (Fig 1D–G). Comparing SA187-colonized (HS + 187) to non-colonized plants (HS), HS + 187 exhibited 57% higher fresh weight, 26% better survival, and 34% more green leaves (Fig 1E–G). This effect is HS-specific, as under control conditions of 22°C, SA187-colonized (NHS + 187) and non-colonized plants (NHS) displayed comparable growth, fresh weight, and survival levels (Fig 1E–G). These data show that SA187 protects Arabidopsis from HS to a similar degree as thermopriming.

Differential transcripional responses of SA187-colonized Arabidopsis to heat stress

To determine the genome-wide extent of HS-induced changes, we performed transcriptome profiling of SA187-colonized and non-colonized 15-day-old plants under non-HS (NHS), LAT, and direct HS (HS) conditions (Fig 1D). In comparison to NHS plants, and considering a log2 FC ≥ 2 or ≤ −2 (P < 0.05), HS displayed 4609, LAT 1704, HS + 187 2130, while LAT + 187 1740 DEGs (Fig 2A and B, Dataset EV1). Among all treatments, HS showed a maximum number of 2,627 DEGs that were unique to 44°C. Gene enrichment analysis (AgriGO platform, FDR ≤ 0.05) for the common genes in all HS treatments is presented in Fig EV2A and B. Gene ontology (GO) analysis of the 1,436 up-regulated HS-specific DEGs indicated enrichment in protein phosphorylation and signal transduction, and responses to abscisic acid, temperature, water, and cell death, while the 1,191 down-regulated DEGs showed enrichment for processes of oxidation–reduction, photosynthesis, ion transport, growth, and responses to auxin and gibberellin (Fig 2A and B). In summary, the HS-induced gene sets are typical for plants exposed to severe stress halting growth and inducing stress responses and survival.

Next, we investigated how SA187 affects the transcriptome of A. thaliana upon HS. Under NHS, 303 genes were differentially expressed in SA187-colonized plants when compared to non-colonized control plants (Dataset EV2). In contrast, 2,130 DEGs were found in SA187-colonized (HS + 187) compared to non-colonized HS-treated plants (HS) (Dataset EV2). The transcriptome data were organized into 10 groups by hierarchical clustering and analyzed for gene ontology enrichment (Fig 2C). Cluster 3 contains genes that are down-regulated under HS but not in SA187-colonized plants (HS + 187) showing enrichment in the regulation of gene expression, RNA biosynthesis, and response to auxin. Cluster 4 genes, which are significantly induced by SA187 under HS conditions, are involved in processes of defense, ion homeostasis, oxidation–reduction, and response to ethylene. Cluster 7, representing genes that
Figure 1. SA187 induces thermotolerance in wheat and Arabidopsis.

A The phenotype of non-colonized (Mock) and SA187-colonized (187) 5-day-old wheat seedlings after HS treatment at 44°C for 2 h.

B Fresh weight quantification of 9-day-old wheat seedlings upon 44°C heat stress (HS) and 22°C normal (NHS) conditions (without and with SA187) after 3 days of recovery at 22°C.

C Plant biomass of wheat obtained upon cultivation with and without SA187 in three consecutive growing seasons of open-field agriculture.

D Experimental scheme of heat experiments and phenotype of Arabidopsis seedlings with and without SA187. Top, plants with long-term acquired thermotolerance (LAT) treatment: 9-day-old plants that were grown at 22°C were treated at 37°C for 3 h and then returned to 22°C for 2 days. At day 11, plants were heat-stressed at 44°C for 30 min and incubated for recovery at 22°C. Middle, plants with HS treatment: 11-day-old plants that were grown at 22°C were treated at 44°C for 30 min and incubated for recovery at 22°C. Bottom, plants with NHS treatment: control plants were grown in parallel at 22°C for 15 days.

E Fresh weight of 15-day-old plants with and without SA187 under NHS, HS, and LAT temperature regimes.

F Percent survival of 15-day-old plants with and without SA187 under NHS, HS, and LAT conditions.

G Percentage of bleached and green leaves of 15-day-old plants with and without SA187 under NHS, HS, LAT conditions.

Data information: Dashed lines indicate the heat acclimation on day 9 at 37°C for 3 h; HS treatment on day 11 at 44°C; sampling time for targeted transcriptome after recovery at 22°C for 1, 24, 48, 72 h, and 4 days: RNA-seq analysis, fresh weight, percent survival, and bleached/green leaves measurements after recovery at 22°C for 4 days. For percent survival, plants with green leaves were scored as live plant out of 12 plants for each biological repeat in different temperature treatments. For bleached and green leaves, a total no. of green and bleached leaves were scored per 12 plants and were divided by 12 to score number of green and bleached leaves/plant. All treatments are compared with direct 44°C heat-stressed plants. Plots represent the mean of 3 biological replicates (n = 36, 12 plants/biological repeat for Arabidopsis and n = 27, 9 plants/biological repeat for wheat seedlings). Error bars represent SE (± SE at C, F), SD (± SE at E, G). Asterisks indicate a statistical difference based on the Mann–Whitney test (*P < 0.05) for the field data and Student’s t-test (**P < 0.01; ***P < 0.001) for the Arabidopsis data. Scale bars correspond to 1 cm. Source data are available online for this figure.
are up-regulated by SA187 independently of the growth conditions, is enriched in cell wall organization, oxidation–reduction, and response to auxin. Cluster 5 displays commonly down-regulated genes in HS and HS + 187, which are mainly involved in oxidation–reduction, photosynthesis, amino acid export, secondary metabolism, and hormone responses. Finally, cluster 10 comprises the largest set of differentially expressed genes that are strongly up-regulated by HS but show only moderate induction by HS + 187. These genes mainly consist of HS-regulated genes responding to abiotic stimulus, hormone, water deprivation, oxidative stress, temperature, glucosinolate, and sulfur metabolism, as well as flavonoid biosynthesis.

Comparing LAT with HS (Dataset EV2), the 1,157 up-regulated genes were enriched for biosynthetic processes, responses to auxin and gibberellin, and photosynthesis, while the GO enrichment for the 1,907 down-regulated gene set was enriched for genes involved in response to stress, defense, heat, hormone, and water (Fig EV2C and D).

Since SA187 and LAT modulate the A. thaliana HS transcriptome, we searched for common features between the two treatments. This analysis revealed that the large majority of HS + 187 DEGs overlap with those upon LAT treatment 69% (382) of up- and 80% (736) of down-regulated DEGs (Dataset EV3). The commonly up-regulated DEGs of HS + SA187 and LAT were enriched for cell wall organization and responses to auxin and gibberellin, while the down-regulated DEGs showed GO enrichment for responses to temperature, water, ABA, SA, and JA biosynthesis (Fig 2D and E). The vast overlap in the HS DEG transcriptomes suggests that SA187 and LAT use a common mechanism to protect Arabidopsis from extreme temperatures.

**SA187 enhances the expression of heat-responsive and memory genes upon heat stress**

We next investigated the expression pattern of 8 heat-responsive genes (HSP101, HSP70, HSP70b, GA3OX1, ATERDJ3A, HSP90, XTR6, and MIPS2), HSFA2, and 2 memory genes HSP18.2 and APX2 (Liu et al, 2018). We performed qRT-PCR analysis of heat-responsive genes at 1 h after exposure to 44°C for 30 min (Fig EV3) and at 1, 24, 48, 72, and 96 h for HSFA2, HSP101, APX2, and HSP18.2 (Fig 3A–D). Under ambient conditions with no HS treatment (NHS), SA187 (NHS + 187) did not change the expression of these heat-responsive and memory genes (Figs 3A–D and EV3). Compared to either non-colonized or non-thermoprimed plants, HS + 187 and LAT plants showed higher transcript levels of HSFA2, HSP101, APX2, and HSP18.2 genes in the time range of 1–48 h upon 44°C HS treatment (Fig 3A–D). At later time points, expression of the thermo- and 187-primed genes returned to normal or even lower transcript levels than in unprimed plants (Fig 3A–D). This pattern of gene expression correlates with the improved survival of thermoprimed and SA187-colonized plants under severe HS conditions.

**SA187 induces sustained H3K4me3 levels at Arabidopsis APX2 and HSP18.2 gene loci**

Recently, chromatin modifications, in particular histone H3K4me3, were shown to be involved in maintaining the prolonged expression of heat-responsive memory genes, such as APX2 and HSP18.2, to protect plants from subsequent severe HS (Liu et al, 2015; Lämke et al, 2016a; Lämke et al, 2016b; Lämke & Bäurle, 2017). To test whether H3K4me3 is also involved in providing elevated and prolonged expression of the HS memory genes in SA187-colonized plants, we evaluated H3K4me3 levels at the APX2 and HSP18.2 gene loci as representatives of the HS memory-related genes in 9-day-old plants (Fig 3E and F). We performed ChIP-qPCR of plants that were primed at 37°C for 3 h (P, P + 187) and their respective non-primed controls (NP, NP + 187) before recovery for 24 and 72 h at 22°C (Fig 3E and F). In accordance with previous studies (Lämke et al, 2016a; Liu et al, 2018 17,28), the 37°C thermoprimed plants showed enrichment of H3K4me3 at regions 2 and 3 of APX2 and region 2 of HSP18.2 gene (Fig 3G). The ChIP assays of SA187-colonized plants (NHS + 187) also showed significant enrichment for H3K4me3 at the APX2 and HSP18.2 loci (Fig 3G). Importantly, although expression levels had been reset to normal levels for APX2 and HSP18.2 at 72 h after HS treatment (Fig 3C and D), H3K4me3 levels remained enriched in both thermoprimed and 187-colonized plants (Fig 3G), indicating that SA187 colonization enhances H3K4me3 accumulation at HS memory gene loci.

**SA187-induced heat stress tolerance is mediated by HSFA2 and ethylene signaling**

Since HSFA2 regulates HS-responsive genes (Liu & Charng, 2013), we examined whether SA187-induced thermotolerance might also be mediated via HSFA2. hsfA2 mutant plants were strongly compromised in the beneficial effect of both SA187 and LAT, indicating that HSFA2 is important for SA187- and thermopriming-induced heat tolerance (Fig 4A–C). The beneficial effect of SA187 was further recovered in HSFA2 complementation line pHSFA2::HSFA2-GFP (Appendix Fig S2A–C).

Thermopriming regulates HSFA2 via the set of four upstream transcription factors HSFA1A, B, D, and E. To test whether SA187-induced thermotolerance is also dependent on HSFA1s, we analyzed a quadruple HSFA1A, B, D, and E (hsfa1q) mutant for LAT- and SA187-induced thermotolerance. As shown in Fig 4A–C, thermopriming was completely compromised in hsfA1q mutant plants, whereas SA187 could still enhance the survival of HS-treated hsfA1q (Fig EV4A for control plants and EV4B–D for green leaves).

In our previous study on SA187 under salt stress (de Zélicourt et al, 2018), we found that SA187 rescues plants from salt stress via the ethylene signaling pathway. To test whether ethylene plays a role in SA187-induced thermotolerance, we analyzed the ethylene insensitive ein3-1 and ein2-1 mutants. As shown in Fig 4A–C, ein3-1 mutants were strongly compromised in SA187-induced thermotolerance but not by thermopriming (LAT), indicating that EIN3 mediates SA187-induced thermotolerance but not thermopriming in A. thaliana. Since ein2-1 mutants were similarly unresponsive to SA187 but fully responsive to LAT thermopriming in thermotolerance tests (Appendix Fig S3A–D for ein2-1), we conclude that ethylene signaling mediates SA187-induced plant thermotolerance via the EIN3 transcription factor. Importantly, thermopriming was not compromised in ein2-1 or in ein3-1 mutants (Fig 4A–C), indicating that thermopriming functions independently of ethylene signaling. To corroborate that ethylene induces thermotolerance in plants, we
also treated Arabidopsis with a low dose of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). Plant thermotolerance could be similarly improved by 1 μM ACC as by SA187 colonization (Fig 4D and E), further supporting the notion that SA187 induced HS tolerance is mediated by plant ethylene signaling.

To clarify whether ethylene acts upstream or in parallel to HSFA2 signaling, we next grew hasfa2 mutant plants in the presence or absence of 1 μM ACC before HS treatment at 44°C. Fresh weight (Fig 4F) and survival measurements (Appendix Fig S4) clearly showed that HSFA2 is necessary for mediating ethylene signaling.

Figure 2.
thereby placing ethylene signaling upstream of HSFA2-mediated thermitolerance.

To confirm that ethylene mediates thermitolerance by SA187, we performed qPCR and ChiP-qPCR of ein3-1 mutant plants for APX2 and HSPI18.2. SA187-colonized (HS + 187) ein3-1 plants did not display higher and prolonged transcripts levels in the two HS memory genes HSPI18.2 and APX2 than HS plants (Fig 5A and B), confirming EIN3-dependent induction of thermitolerance by SA187. However, 37°C thermodprimed ein3-1 plants exhibited higher expression levels for these memory genes compared to non-primed HS-induced levels, showing that thermodpriming functions independently of EIN3. ChiP-qPCR of ein3-1 that were primed at 37°C for 3 h (P) after 24 and 72 h of recovery at 22°C (Fig 5C) showed that the 37°C thermodprimed plants had enriched H3K4me3 levels at regions 2 and 3 of APX2 and region 2 of HSPI18.2 compared to the non-primed ein3-1 control (NP) (Fig 5C). In contrast, ChiP assays of SA187-colonized plants (HS + 187) did not show H3K4me3 enrichment at the APX2 and HSPI18.2 loci (Fig 5C), confirming that EIN3 mediates H3K4me3 priming by SA187.

To confirm that hsfa2 mediates thermodpriming and SA187-induced thermitolerance, we also performed qPCR and ChiP-qPCR of ein3-1 and hsfa2 mutants for APX2 and HSPI18.2, and the qPCR data for APX2 and HSPI18.2 did not show higher expression upon LAT thermo- or SA187-priming than in naïve non-treated NHS hsfa2 mutants (Fig 5D and E). Likewise, at 24 and 72 h of recovery at 22°C from HS, ChiP-qPCR of both 37°C thermodprimed and SA187-colonized hsfa2 mutants showed no significant enrichment of H3K4me3 on regions 2 and 3 of APX2 and region 2 of HSPI18.2 gene loci (Fig 5F). Taken together, these results indicate HSFA2 mediates thermitolerance of both SA187 and thermodpriming.

To eliminate the possible discrepancies in bacterial colonization, we evaluated the effect of hsfa2, ein3-1 mutants, and 44°C heat stress on SA187 colonization. The results at 0, 1, 24, and 48 h of heat stress in Col-0, hsfa2, and ein3-1 mutants revealed no significant differences in colony forming unit (CFU) levels between wild type and mutants, indicating that the mutants do not affect the overall colonization in A. thaliana under NHS or HS conditions (Fig EV5).

**SA187 does not alter wheat microbiome composition**

To explore whether the inoculation of SA187 changes the natural microbiome of the wheat root endosphere, we determined the bacterial composition of the soil and root endosphere of wheat plants. A principal component analysis of the relative abundance of operational taxonomic units (OTUs) showed a clear separation between soil and the SA187-treated or non-treated root endosphere samples (Fig 6A). Microbiome analysis of the V5–V7 region of the 16S rRNA gene demonstrated that the three samples exhibited a different composition of bacterial phyla with a high abundance of Cyanobacteria, Firmicutes in soil, whereas Proteobacteria and Actinobacteria were highly abundant in the wheat root endosphere. This observation was made for a number of crop plants, such as maize, barley, rice, and grapevine (Lundberg et al, 2012; Bulgarelli et al, 2015 30–31). PERMNOVA showed that the bacterial communities between non-treated and SA187-treated root endosphere samples were not significantly different (pairwise t = 1.23, P > 0.23), while ANOISIM (R: 0.8107, significance: 0.0039) indicated significant differences. These results reveal that non-treated and SA187-treated root samples did not differ in overall species composition but they differ in overall heterogeneity of species composition (beta diversity), indicating that the application of SA187 did not change the composition of the wheat microbiome (Fig 6B). SA187-treated or non-treated root endosphere samples exhibited twenty different families at > 1% abundance (Fig 6C) and compared to soil, wheat root endosphere samples were dominated by Xanthomonadaceae, Rhizobiaceae, and Enterobacteriaceae. Streptomycesaceae, Actinosynnemataceae, and Staphylococcaceae were less abundant in root samples in comparison to soil (Fig 5C). SA187 changed the distribution of bacterial families in roots by a decrease in Xanthomonadaceae, Rhizobiaceae, and Enterobacteriaceae but an increase in Microonosporaceae and Microbacteriaceae. In summary, although SA187 did not significantly change the microbial community itself, the percentage of microbes varied after SA187 treatment.

**Discussion**

The increases in global temperatures have a significant negative impact on agriculture worldwide and pose a serious threat to global food security. To cope with this challenge, we propose to consider the use of beneficial microbes. Several studies reported that endophytes can enhance plant growth under abiotic stress conditions (Marquez et al, 2007; Avramova, 2015; de Zelicourt et al, 2018; Saad et al, 2020). So far, however, the application in open-field agriculture of thermitolerance by beneficial microbes was not investigated. Our work shows that the beneficial root endophyte SA187...
SA187-induced thermotolerance is associated with higher expression of heat-responsive genes and chromatin modification.

A, B Dynamics of HSFA2 and HSP101 transcript levels in control (NHS, NHS + 187), 44°C heat-stressed non-colonized and SA187-colonized (HS, HS + 187), thermoprimed (LAT), and thermoprimed and SA187-colonized plants (LAT + 187) at 1, 24, 48, 72, and 96 h of recovery at 22°C. SA187-colonized (HS + 187) and thermoprimed (LAT) plants showed higher transcript levels in comparison to plants exposed at 44°C HS for 30 min (HS) after 1 h of recovery at 22°C.

C, D Transcript levels of heat stress memory genes HSP18.1 and APX2 in control plants (NHS, NHS + 187), 44°C heat-stressed non-colonized and SA187-colonized (HS, HS + 187), thermoprimed (LAT), and thermoprimed SA187-colonized plants (LAT + 187) at 1, 24, 48, 72, and 96 h of recovery at 22°C. Transcript levels were normalized to tubulin as reference gene, and the respective 22°C NHS plants were harvested at the same time points. All treatments are compared with direct 44°C HS treatment for statistical significance.

E Schematic representation of the experimental set-up and sampling times for ChIP-PCR. 9-day-old plants were thermoprimed at 37°C for 3 h before incubation at 22°C for 24 h or 72 h.

F APX2 and HSP18.2 gene models drawn to scale (gray boxes, 5' untranslated region; orange boxes, exons; angled arrow, transcription start site). The underneath numbers with gray bar indicate the positioning of regions analyzed for ChIP-PCR, three regions of APX2 and 2 regions of HSP18.2.

G Relative enrichment of H3K4me3 at APX2 and HSP18.2 in control non-primed (NP), SA187-colonized non-primed plants (NP + 187), 37°C-primed (P), and 37°C-primed SA187-colonized plants (P + 187) at 24 and 72 h after priming as determined by chromatin immunoprecipitation-qPCR for the indicated regions of APX2 and HSP18.2. Amplification values were normalized to input and H3 and region 1 of non-primed (NP) plants.

Data information: All plots represent the means of 3 biological replicates. Error bars represent SE. Asterisks indicate a statistical difference based on Student’s t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 for differences between NP in comparison to NP + 187, P and P + 187 treatments).
enhances thermotolerance of wheat and Arabidopsis under controlled laboratory conditions. Importantly, we also show that in three independent vegetation periods, SA187 enhances thermotolerance and yield of wheat in open-field agriculture without significantly affecting the microbiome composition of the crop. These results suggest that beneficial microbes such as SA187 might be efficient means to improve global crop productivity under extreme environmental conditions. To investigate the molecular mechanism of endophyte-induced thermotolerance, we investigated SA187-induced thermotolerance in Arabidopsis.

Figure 4. Ethylene and HSFA2 signaling mediates SA187-induced thermotolerance in Arabidopsis.

A Phenotypes of SA187-colonized or non-colonized wild-type Col-0, hsfA2, hsfA1q, and ein3-1 mutant plants upon long-term acquired thermotolerance treatment (LAT). 9-day-old plants without and with SA187 (LAT, LAT + 187) were treated at 37°C for 3 h and then returned to 22°C for 2 days. At day 11, plants were heat-stressed at 44°C for 30 min and incubated for recovery at 22°C, or direct heat stress treatment (HS): 11-day-old plants without and with SA187 (HS, HS + 187) were treated at 44°C for 30 min and incubated for recovery at 22°C.

B, C Fresh weight and percent survival of Col-0, hsfA2, hsfA1q, and ein3-1 plants in HS, HS + 187, LAT, and LAT + 187 treatments. Due to the dwarf size of hsfA1q mutants, LAT treatment was performed on day 18 and HS at day 20. All treatments are compared with plants upon 44°C HS.

D Fresh weight of 1 µM 1-aminocyclopropane-1-carboxylic acid (ACC)-treated wild-type plants. 5-day-old plants were transferred to 1 µM ACC containing plates, and 11-day-old plants were HS-treated at 44°C for 30 min before recovery at 22°C for 3 days (HS, HS + ACC). LAT: 9-day-old plants without and with ACC treatment (LAT, LAT + ACC) were primed for 3 h at 37°C before incubation for 2 days at 22°C, and further heat stress of 44°C was performed at day 11 for 30 min.

E Percent survival of Col-0 plants with and without ACC under HS, HS + ACC, LAT, and LAT + ACC conditions.

F Fresh weight of hsfA2 and control Col-0 plants treated with and without ACC under non-heat stress condition (NHS, NHS + ACC) of 22°C and heat stress (HS, HS + ACC) condition of 44°C for 30 min. Percent survival was scored at day 4 of recovery from 44°C heat stress. All treatments were compared with plants upon HS.

Data information: All plots represent the means of 3 biological replicates (n = 36, 12 plants per biological repeat). Error bars represent SD of three biological repeats. Asterisks indicate a statistical difference based on Student’s t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).
Figure 5. SA187-induced sustained H3K4me3 levels at APX2 and HSP18.2 memory genes depend on ethylene and HSFA2 signaling.

A, B Transcript levels of HS memory genes HSP18.2 and APX2 in ein3-1 non-heat-stressed (NHS, NHS + 187), 44°C heat-stressed SA187-colonized (HS + 187), thermoprime (LAT), and thermoprime SA187-colonized plants (LAT + 187) at 1, 24, 48, 72, and 96 h of recovery at 22°C.

C Relative enrichment of H3K4me3 at APX2 and HSP18.2 in control non-primed (NP), SA187-colonized non-primed plants (NP + 187), 37°C-primed (P), and 37°C-primed SA187-colonized plants (P + 187) at 24 and 72 h after priming as determined by chromatin immunoprecipitation-qPCR for the indicated regions of APX2 and HSP18.2 (Fig 3F).

D, E Transcript levels of HS memory genes HSP18.2 and APX2 in hsfa2 mutant.

F Relative enrichment of H3K4me3 levels at APX2 and HSP18.2 memory genes in hsfa2 mutant.

Data information: For transcript level analysis, the data were normalized to tubulin as reference gene and the respective 22°C NHS, NHS + 187 control plants, that were harvested at the same time points. For H3K4me3 enrichment analysis, amplification values were normalized to input and H3 region 1 of non-primed (NP) plants. All treatments are compared with direct 44°C HS treatment for statistical significance. All plots represent the means of 3 biological replicates. Error bars indicate SE. Asterisks indicate a statistical difference based on Student’s t-test: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 for differences between NP in comparison to NP + 187, P, and P + 187 treatments.
the model plant *Arabidopsis thaliana*. Our experimental evidences suggest that SA187 and thermopriming/heat acclimation use a partially overlapping signaling network for inducing thermotolerance. We found that SA187 induces thermotolerance to a similar degree as heat acclimation by hyper-induction of HS-responsive and HS memory genes. Interestingly, the majority of the genes induced by HS was not induced in thermoprimed or SA187-colonized plants. A comparison of the DEGs of SA187-colonized and thermoprimed plants revealed a large overlap (Fig 2D and E), suggesting that thermotolerance is associated with a transcriptional memory resulting in faster and stronger expression of heat-responsive genes upon a repeated stress signal (Stief et al., 2014; Sedaghatmehr et al., 2016; D’Urso & Brickner, 2017). Moreover, in both SA187-colonized and thermoprimed plants, gene expression of the HS memory genes APX2 and HSP18.2 was up-regulated and higher transcript levels were maintained up to 48 h after HS. Importantly, the persistent expression of these genes is known to be associated with improved plant growth under extreme temperatures (Lämke et al., 2016a; Lämke et al., 2016b; Lämke & Bäurle, 2017). The transcriptional regulation of heat-responsive genes is controlled by epigenetic factors that help maintain priming memory (Brzezinka et al., 2016), and elevated histone H3K4 methylation levels and enhanced chromatin accessibility are involved in the thermoprimed memory process (Lâmke et al., 2016a; Liu et al., 2018). This thermopriming memory is HSFA2 dependent as plants require active HSFA2 in order to elevate histone H3K4 methylation levels for memory genes. Recently, the involvement of HSFA2 was also shown for transgenerational thermomemory in *A. thaliana* (Liu et al., 2019). HSFA2 and H3K27me3 demethylase RELATIVE OF EARLY FLOWERING 6 (REF6) form a positive feedback loop to transmit long-term epigenetic memory of heat. HSFA2 is activated by heat-induced demethylation and then HSFA2 further up-regulates the expression of the H3K27me3 demethylase REF6, establishing a positive feedback loop between H3K27me3 demethylation and HSFA2 up-regulation that is critical to the thermomemory establishment and transmission to progeny (Liu et al., 2019). However, an involvement of chromatin modifications in providing HS tolerance by beneficial microorganisms is hitherto unknown. Here we show that, similar to thermopriming, SA187 induces sustained accumulation of H3K4me3 at APX2 and HSP18.2 gene loci (Fig 3G).

HSFA2 is required for the maintenance of HS-induced memory by activating the expression of HS genes (Schramm et al., 2006; Lämke et al., 2016a; Lämke et al., 2016b). Our phenotype and chromatin data show that SA187- and thermopriming-induced thermotolerance depend on HSFA2 as mutants in *hsfa2* are compromised in both processes. During HS, HSFA2 is strongly induced and this process depends on the set of the four transcription factors HSFA1A, B, D, and E. Importantly, quadruple *hsfa1q* mutant plants are compromised in thermopriming, but not SA187-induced thermotolerance. These data show that SA187 regulates HSFA2 by a different mechanism than heat acclimation. We show here that SA187-induced thermotolerance is compromised in *ein3-1* and *ein2-1* mutant plants, but not upon thermopriming. In summary, our analysis shows that thermopriming- and SA187-induced thermotolerance are both mediated by HSFA2, but thermopriming uses HSFA1 transcription factors, whereas SA187 uses the ethylene pathway and the EIN3 transcription factor. EIN3 likely does not act directly on the HSFA2 promoter, but by its downstream abiotic stress-inducible ERF transcription factors (Solano et al., 1998; Xing et al., 2017). Overexpression of ERF1 in *Arabidopsis* by the 35S CaMV promoter results in enhanced heat tolerance and up-regulation of heat tolerance genes (Cheng et al., 2013). Depending on the stress conditions, ERFs activate a specific set of stress response genes by binding to the cis-acting GCC box or its substitute TCC box and DRE elements which are also present in the HSFA2 promoter (Yang et al., 2015; Prajapati et al., 2019; Xie et al., 2019). In summary, our data suggest that...
SA187-induced thermotolerance could be mediated via EIN3-regulated expression of ERFs that subsequently act on HSFA2 (Fig 7). In terms of applying thermotolerance in agriculture, thermoprimering is a transient mechanism and is hard to apply on field-grown crops. In contrast, SA187 permanently colonizes plants, rendering them constitutively thermotolerant without any further treatment, making root endophytes a powerful tool to maintain crop production under the adverse conditions of global warming.

Materials and Methods

Bacterial inoculum and media preparation

Enterobacter sp. SA187 was isolated from root nodules of Indigofera argentea in the Jizan region of Saudi Arabia (Andrés-Barrao et al., 2017). Cryogenically maintained Enterobacter sp. SA187 were streaked out on LB agar media and incubated at 28°C for 24 h. A single colony was used for further experiments. For bacterial seed plates, 50 ml of half-Murashige and Skoog medium (MS) with 0.9% agar and a pH of 5.8 was mixed with 0.1 ml of fresh bacterial suspension with an OD of 0.2 to obtain a final number of 10^7 CFU/ml. For control plates, 0.1 ml of liquid LB was mixed with ½ MS media.

Field experiments

Experiments were carried out at the SCADA field station of the International Center for Biosaline Agriculture (ICBA), Dubai, UAE (25°05′42.0″N 55°23′23.3″E), during three growing seasons 2014/15, 2016/17, and 2017/18. ICBA field area has Hyperthermic Typic Torripsamment soil type which characterized with very low nutrient contents (Shahid et al., 2009). Soil physical and chemical properties (Table EV1) were determined according to Alzubaidy et al (2016). The compost (manure) was applied with the rate of 40 tons per hectare (t/ha) in the area selected for experiment 15 days before sowing. Seeds of Karim (Triticum durum) cultivar were coated with a mixture containing broth culture, sugar solution (10%) with a rate of 2 × 10^5 cell/kg seed. The inoculated seeds were kept for 1 h, and thereafter, the seeds were air-dried over 3MM filter paper (Whatman) and sown immediately. Control (mock-inoculated) seeds were coated with a mixture containing all components without SA187. The wheat seeds were sown manually during the first week of November from each growing seasons and harvest by end of April beginning of May. Meteorology data including temperature and rainfall were recorded (Appendix Fig S1A). The experiment was conducted using a randomized complete block design; each plot is 5 m² with three replications. All the plots were irrigated with water EC = 0.3 dS/m, the average value for the soil pH was 7.45, and the salinity of soil was EC = 1.9 dS/m. Agronomic data were collected upon wheat harvest, including plant height, biomass, tiller number, height, number and seeds per spike, 1,000 seed weight, total grain yield, and total biomass yield.

DNA extraction, 16S rRNA gene amplicon sequencing, and data processing

Total DNA extraction of endophytic bacterial community was performed as described by (Eida et al., 2018). Total DNA was extracted from 300 mg of soil or plant root material (duplicates) using the PowerSoil DNA Isolation Kit (MO BIO Laboratories). Sequencing libraries of bacterial communities of soil, rhizosphere, and root endosphere were prepared according to the Illumina 16S Metagenomic Sequencing Library Preparation guide with using the V5–V7 region (Beckers et al., 2016). The V5–V7 region was amplified using a two-step PCR protocol with V5–V7 primers (799F: 5′-TCGTCGAGAAGACGTGTGATTAAGAGACAG AACMGAGATT AGATACCCCGG-3′; 1193R: 5′-GTCTCTGCGGGGCTCGGAATGATGTAT AAGAGACAG AGCTATCACCACCTTTCC-3′, overhang adapter sequences are underlined) for the first PCR step and Illumina Nextera XT Index kit (Illumina Inc., San Diego, CA, USA) for the second PCR step. The first PCR step (amplicon PCR) was performed using the above primer set with NEBNextQ5Hot Start HiFi PCR Master Mix (New England Biolabs) in a total volume of 50 μl. The cycle conditions were as follows: initial cycle at 98°C for 1.5 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 30 s, and 65°C for 45 s with a final extension 65°C for 5 min. The duplicate PCR amplicons were then pooled and run on 2% agarose gel (140 V for 45 min, 25°C) in order to clear the samples from residual primers, primer dimers, and mitochondrial/chloroplast DNA. The lower bands corresponding to bacterial amplicons (~500–550 bp) were excised from the gels and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). Subsequently, 5 ng of purified PCR product was used for a second round of PCR amplification using Illumina Nextera XT Indexes and same cycle conditions as first step PCR for eight cycles instead of 30 cycles. Amplicons were cleaned using Agencourt AMPure XP (Beckman Coulter Inc., Brea, CA, USA) magnetic beads. Libraries were validated with Qubit dsDNA HS assay kit (Thermo Fisher Scientific) and Agilent 2100 bioanalyzer with the DNA 7500 kit (Agilent Technologies) and quantified with qPCR using KAPA.
library quantification kit (KAPA Biosystems). The library was normalized and sequenced at the KAUST Bioscience Core Labs on an Illumina MiSeq (Illumina Inc.) with 2 × 300 bp paired-end reads and V3 chemistry. MiSeq sequencing of the V5–V7 region of the 16S rRNA gene yielded 1,396,927 sequences with a mean length of 301 bp. Microbiome data were analyzed according to Eida et al. (2018) using the software MOTHUR (version 1.42.3) (Schloss et al., 2009). After quality filtering, and exclusion of chimeras amplified, sequences were annotated to bacteria. Sequences were classified against Greengenes database using bootstrapping of 60 (McDonald et al., 2012). For further analysis, sequences were clustered into operational taxonomic units (OTUs) using a 97% similarity cut-off. Alpha diversity index, beta diversity, and principal coordinate analysis (PCoA) were performed as implemented in mother (Schloss et al., 2009).

### Plant material and growth conditions for wheat and Arabidopsis thaliana

**Arabidopsis thaliana**

Col-0 seeds were obtained from publicly available collections. *hsfa1-q* and *hsfa2* seeds were obtained from Yee-yung Charrng (ABRC, Taipei, Taiwan). The HSFA2 complemented lines (pHSFA2::HSFA2-GFP) were generated by agrobacterium mediated floral dip of *hsfa2* mutant. The genomic locus of HSFA2 containing the promoter and gene body was cloned into pMDC107. HSFA2 complemented floral dip of hsfa2 mutant. The genomic locus of HSFA2 containing the promoter and gene body was cloned into pMDC107 vector generating a c-terminal GFP tag. Seeds were surface-sterilized with 0.05% SDS solution prepared in 70% ethanol, followed by three times washing with absolute ethanol. The sterilized seeds were plated on ½ MS medium agar plates seeded with 10⁶ CFU/ml SA187. Seeds were stratified at 4°C for 2 days and then plates were transferred to a growth chambers (Model CU36-L5, Percival Scientific, Perry, IA, USA) under a 16-h photoperiod and 8-h dark conditions at 22°C for germination and seedling growth.

**Heat priming and heat-shock experiment**

We developed a heat-priming platform by modifying a previous method (Larkindale & Vierling, 2008). For *A. thaliana*, 5-day-old SA187-inoculated and non-inoculated seedlings of near equal lengths were transferred to new ½ MS plates, while for wheat, 2-day-old seedlings were transferred on 50-ml falcon tubes. For HS treatments in *Arabidopsis*, plants with bacteria and without bacteria were divided into three sets of plates. In set 1, plants were given acclimation HS treatment, where 9-day-old SA187-colonized and non-colonized plants were exposed to 37°C of heat acclimation for 3 h followed by 2 days recovery at 22°C and a further 44°C HS for 30 min at day 11 (LAT). In set 2, SA187-inoculated and non-inoculated plants were exposed directly to 44°C HS from 30 min on day 11 (HS). For the 44°C treatment, we used a pre-heated water bath. In set 3, SA187-inoculated and non-inoculated plants were grown under normal conditions at 22°C (NHS). We performed the same HS procedure for all experiments and the arrows indicate the sampling points for the respective collection and data analysis of the plants (Fig 1D). For wheat experiment under laboratory conditions, 6-day-old colonized and non-colonized plants were exposed to 44°C for 2 h and fresh weight was measured for 9-day-old plants.

### Quantification of SA187-colonization

Seedlings were germinated on ½ MS agar plates inoculated with SA187, and 5-day-old seedlings were transferred to new ½ MS plates (12 seedlings per plate). At day 11, plants were heat-stressed 44°C HS from 30 min, and samples were taken at 0 h as control, 1, 24, and 48 h after 44°C HS for CFU analysis. Plants were ground in Eppendorf tubes using homogenizer. Further, sample was re-suspended in 1 ml of extraction buffer containing 10 mM MgCl₂, 0.01% Silwet L-77, vortexed for 10 min. Serial dilutions were performed and samples were plated on LB agar plates, and colony forming units (CFUs) were counted after overnight incubation at 28°C. Calculated number of CFUs was normalized per mg of plant material. The experiment was conducted in three biological replicates, each with two technical replicates per condition.

### RNA extraction, reverse transcription, and qRT–PCR

For RNA-seq analysis, total plant RNA was extracted from non-heat-stressed (NHS), HS + 187, heat stress (HS), HS + 187, long-term acquired thermotolerance (LAT), and HS + 187 treatments after 96 h of HS treatment (from 15-day-old seedlings), while for targeted gene expression studies, plant samples were harvested from NHS, HS + 187, HS HS + 187, LAT, and HS + 187 samples after 1, 24, 48, 72, and 96 h of HS treatment using the Nucleospin RNA plant kit (Macherey-Nagel), including DNasel treatment, according to the following manufacturer’s recommendations. For qPCR analysis, the total RNA was reverse-transcribed using a using Superscript III (Invitrogen): 1 μg of total RNA and oligo-dT primer. For *Arabidopsis* gene expression analyses, tubulin was used as a reference gene. All reactions were done in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) as follows: 50°C for 2 min, 95°C for 10 min; 40× (95°C for 10 s and 60°C for 40 s). All reactions were performed in three biological replicates, and each reaction as a technical replicate. Accession numbers of the genes studied were HSFA2 (At2g26150), HSP101 (At1g74310), HSP70 (At1g15550), GAPX1 (At1g15550), TXR6 (At4g25810), MIPS2 (At2g22240), HSP90 (At5g52640), ATERDJ3A (At3g08970), HSP18.2 (At5g59720), APX2 (At3g09640), and TUB6 (At5g12250). A list of all primers used in the current study is provided in Table EV2.

### Bioinformatics analysis of RNA-seq data

We performed mRNA libraries with 1 μg of total plant RNA using a stranded mRNA Library Prep kit (Illumina). Pooled libraries were sequenced using Illumina HiSeq 4000 platform which resulted in paired-end reads of length 151 bps. Sequenced reads were checked for quality using FASTQC (Andrews, 2012). Adapter sequences and low-quality reads or base pairs were trimmed using Trimmomatic. Pooled libraries were sequenced with 24, and 48 h after 44°C HS for CFU analysis. Plants were ground in Eppendorf tubes using homogenizer. Further, sample was re-suspended in 1 ml of extraction buffer containing 10 mM MgCl₂, 0.01% Silwet L-77, vortexed for 10 min. Serial dilutions were performed and samples were plated on LB agar plates, and colony forming units (CFUs) were counted after overnight incubation at 28°C. Calculated number of CFUs was normalized per mg of plant material. The experiment was conducted in three biological replicates, each with two technical replicates per condition.
(P-value ≤ 0.05; statistical correction: Benjamini–Hochberg; FDR ≤ 0.05) in cuffdiff were used. Post-processing and visualization of differential expression were done using cummeRbund v2.0.0. A cut-off of 2-fold change and P-value less than 0.05 were set to identify the up- and down-regulated genes between two conditions. Venny (Oliveros, 2007–2015) was used to identify the genes common or unique to different conditions compared. AgrigO (Tian et al., 2017) was used to find the corresponding GO terms (FDR ≤ 0.05) and the functions of the respective genes.

**Chromatin immunoprecipitation**

We conducted ChIP as described in previous studies (Lämke et al., 2016a). In short, roughly 500 mg of 10- and 12-day-old seedlings were cross-linked by vacuum-infiltrating 1% formaldehyde for 15 min. Formaldehyde was quenched using 2 M glycine. Samples were stored at −80°C until further processing. Further, nucleic acid extraction was performed and chromatin was sonicated using a Diagenode Bioruptor (medium setting, 14 cycles each with 30 s on/30 s off with ice cooling), resulting DNA was extracted using the phenol–chloroform method and precipitated with ice chilled ethanol and glycogen (Invitrogen), and then re-suspended in 20 µl of water. ChIP-PCR was performed for three regions of APX2 and two regions of HSP18.2 gene loci. Amplification values were normalized to H3 (normalized signal modification/normalized signal H3). The given values in graphs are the means of three biological replicates, with each replicate was normalized to the respective 22°C control with no HS (NHS) sample before averaging.

**Data availability**

The data set supporting the results of this article is included within the article and its additional files. The raw data from the RNA-seq samples were submitted to the National Centre for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database, under project GSE143635, accession numbers GSM4264089-GSM4264106. The data are publicly available and accessible at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE143635. The raw microbiome data (MiSeq data) are accessible https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE147553 (Enter token wplwqqaarzkrmf into the box).

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**Author contributions**

KS, MMS, AS, and HH conceptualized and designed the experiments. KS standardized the HS protocol and phenotyping experiments. HA-M and Kmas performed the field experiments. AAE and FA did the microbiome library and data analysis. AS and KS performed qRT–PCR for targeted transcriptome at 1, 24, 48, 72, and 96 h, and AS helped with ChIP-qPCR experiment. Kmar performed bioinformatics analysis of raw RNA-seq data. Rj helped with phenotype experiments and RNA extractions. KS analyzed the data. HH supervised the experiments and analysis of the data. KS, MMS, and HH wrote the manuscript. All authors approved the final version of the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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