Enterobacter sp. SA187-induced coordinated regulation of high-affinity nitrate transporters and ethylene signaling enhances nitrogen content and plant growth under low nitrate

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Abstract

Sustainable crop production demands solutions to reduce the over use of synthetic nitrogen fertilizers, and plant-growth-promoting bacteria (PGPB) offer a promising alternative by enhancing nutrient acquisition under limiting conditions. This study shows that the endophytic bacterium *Enterobacter* sp. SA187 significantly improved the growth of *Arabidopsis thaliana* seedlings under low nitrate conditions. SA187 inoculation led to marked increases in biomass, primary root length, and lateral root density, with growth-promoting effects becoming more important with the severity of nitrate limitation. Analyses suggested enhanced nitrate uptake and improved nitrogen partitioning as indicated by reduced shoot carbon:nitrogen ratios. Transcriptomic profiling identified SA187-driven reprogramming of host gene expression, including the induction of high-affinity nitrate transporters (HATs) and hormone-responsive pathways. Functional analyses confirmed that the growth-promoting effect of SA187 required both ethylene signaling and specific HATs. The *ein2*, *nrt2.5*, and *nrt2.6* mutants failed to show

SA187-induced growth promotion, and expression analysis revealed that *NRT2.5* was regulated downstream of ethylene signaling, while *NRT2.6* was partially independent. These findings position SA187 as a conditionally beneficial endophyte that can improve plant growth through coordinated, context-dependent regulation of nitrate uptake and allocation. Such results highlight the potential of SA187 as a bioinoculant for improving plant performance under nitrate-limited conditions and thus reduce fertilizer dependency.

I. Introduction

Nitrogen (N) is essential for plant growth, development, and productivity. As a core component of nucleic acids, proteins, amino acids, chlorophyll, and secondary metabolites, N coordinates numerous physiological and metabolic processes (Krapp, 2015; O'Brien et al., 2016). Consequently, N-deficiency results in chlorosis, reduced photosynthetic capacity, stunted growth, and yield losses (Ding et al., 2005; Hawkesford et al., 2012; Ahmad Wani et al., 2022). Although atmospheric nitrogen (N_2) is abundant, most plants cannot directly use this inert form, instead they rely on bioavailable N-compounds such as nitrate (NO_3^-), ammonium (NH_4^+), and amino acids. Among these, NO_3^- generally predominates as the available N-form in well-aerated soils (Glass, 2009; Krapp et al., 2014). However, the availability of bioavailable N-compounds is often limited in agricultural systems thus making this a significant factor limiting crop yields worldwide (LeBauer and Treseder, 2008; Sinclair and Rufty, 2012).

To alleviate N-limitations and ensure global food security, agriculture has relied on synthetic N-based fertilizers. While this has significantly improved crop yields, their use remains inefficient, costly and they are a source of greenhouse gases and pollution. N-use-efficiency (NUE) remains remarkably low as less than 50% of applied N is taken up by plants, while the remainder is lost to volatilization, leaching, and denitrification. These losses not only reduce productivity and economic returns for farmers but also cause widespread environmental degradation (Liu et al., 2010; Ali et al., 2025). Adverse impacts include groundwater nitrate contamination, eutrophication, greenhouse gas emissions (such as N_2O), ozone depletion, and long-term soil degradation, with serious consequences for ecosystems and human health (FAO and UNEP, 2021; Sutton et al., 2021; Nath et al., 2023; Govindasamy et al., 2023; de Vries et al., 2024). In parallel, the production of N-based fertilizers via the Haber–Bosch process is energy-intensive

and carbon (C)-emitting, adding further environmental and economic burdens (Cherkasov et al., 2015; Rouwenhorst and Lefferts, 2020). Therefore, there is a need to improve plant N-uptake and N-utilization so as to support plant growth under low-N-input conditions in the context of an environmentally-friendly, sustainable agriculture.

Plant-growth-promoting bacteria (PGPB) have emerged as promising biological tools to reduce reliance on synthetic inputs. Historically, research has extensively focused on symbiotic N_2-fixing bacteria (e.g., *Rhizobia*, *Frankia* spp.). However, emerging studies demonstrate that even non-N_2-fixing endophytic bacteria can significantly improve plant growth directly, by enhancing nutrient availability, mobilizing soil minerals such as phosphorus, potassium, iron and N, and indirectly, by modulating hormonal signaling pathways, root architecture, defense and systemic stress responses (Bakhshandeh et al., 2020; De Zelicourt et al., 2013; Di Benedetto et al., 2017; Etesami & Adl, 2020; Glick, 2012, 2020; Pathania et al., 2020; Ramakrishna et al., 2019). Despite increasing interest, the mechanistic details underlying these beneficial plant–microbe interactions, particularly under N-limiting conditions, remain insufficiently characterized.

Among promising plant-associated endophytes, *Enterobacter* sp. SA187 (SA187) has emerged as a particularly effective beneficial endophytic bacterium. Initially isolated from the desert-adapted legume *Indigofera argentea* (Andrés-Barrao et al., 2017), SA187 effectively enhances resilience across a wide spectrum of abiotic stresses, including drought, salinity, heat, and high light. The beneficial effects of SA187 are mediated primarily through modulation of critical physiological and molecular processes such as hormonal homeostasis, particularly involving ethylene signaling pathways, maintenance of nutrient and redox balances, and activation of stress-responsive networks (de Zélicourt et al., 2018; Rahman et al., 2025; Rolli et al., 2022; Shekhawat et al., 2024; Shekhawat et al., 2021). Although previous studies have demonstrated plant growth

improvement by *Enterobacter* species under nutrient-limited conditions, through mechanisms such as N_2-fixation, improved nutrient acquisition and altered root system architecture (Shankar et al., 2011; Lin et al., 2012; Madhaiyan et al., 2013; Zhu et al., 2013; Tsegaye et al., 2022), the role of SA187 under low N conditions has not been investigated.

Nitrate uptake and allocation within higher plants are primarily regulated by specialized nitrate transporters belonging to two main families: the NRT1/PTR family (NPF) comprising mostly low-affinity transporters (LATs), and the NRT2 family of high-affinity nitrate transporters (HATs). NRT2 family members are important under low nitrate availability conditions since they transport nitrate at very low external concentrations (Krapp et al., 2014; Zhang et al., 2024). HATs regulate not only soil-derived nitrate uptake but also nitrate distribution within plant tissues and organs, significantly influencing NUE and plant adaptation under nitrate scarcity (Wang et al., 2012; Lezhneva et al., 2014; Fan et al., 2017). Moreover, hormonal signals, notably ethylene, have been implicated in modulating the expression and functional activity of nitrate transporters (Tian et al., 2009; Zhang et al., 2014; Zheng et al., 2013). Ethylene signaling, mediated through EIN2 and EIN3, plays a central role in integrating nutrient and environmental cues, but its role in regulating nitrate acquisition via plant–microbe interactions remains poorly understood (Iqbal et al., 2017; Khan et al., 2017; Leblanc et al., 2008). Although SA187 is known to modulate ethylene signaling to enhance stress tolerance (de Zélicourt et al., 2018; Shekhawat et al., 2024), to date its specific role on nitrate transporter regulation and plant N-metabolism has not been explored. Since ethylene is known to interact with several nitrate transporters (including NRT2.1) (Tian et al., 2009; Zheng et al., 2013), it is possible that SA187-mediated modulation of ethylene signaling could significantly influence nitrate uptake and nitrate acquisition under low nitrate conditions.

This study investigated the beneficial impact of SA187 on the growth of *Arabidopsis thaliana* seedlings with respect to nitrate concentration, with a focus on growth responses, nitrate transporter expression, nitrate content, C:N ratios and ethylene signaling. Using phenotypic, elemental, transcriptomic, and genetic analyses, including HATs and ethylene-insensitive mutants, this work revealed that SA187 appeared to improve nitrate uptake and aerial allocation through a combination of ethylene-dependent and -independent regulatory pathways. These findings provide mechanistic insights into how a beneficial endophyte can modulate plant nutrient physiology, offering a path forward for microbial strategies to potentially enhance NUE under N-limited conditions.

II. Materials and Methods

A. Bacterial and plant material

Arabidopsis thaliana (L.) Heynh., ecotype Columbia-0 (Col-0, wild-type), and selected mutants in a Col-0 background: *ein2-1* (Guzmán and Ecker, 1990), *nrt2.5* (GK 213H10), *nrt2.6* (SM 3.35179), and a *nrt2.5 x nrt2.6* double mutant (Kechid et al., 2013) were used in this study. The *Enterobacter* sp. SA187 strain, originally isolated from root nodules of *Indigofera argentea* in Jizan, Saudi Arabia (Andrés-Barrao et al., 2017), was used in its GFP-tagged form (de Zélicourt et al., 2018).

B. Plant growth and bacterial colonization

Arabidopsis thaliana seeds were surface-sterilized and inoculated with SA187 as described previously (Saad et al., 2018). Briefly, sterilized seeds were sown on modified half-strength Murashige and Skoog (½MS) medium (Apollo Scientific Catalogue No: PMM531B), containing basal salts only (0.5 g/L MES, pH 5.8, 9 g/L agar), under different nitrate regimes ranging from extremely-low to high-nitrate *i.e.* 0.2, 1, 2, 10, 20 mM KNO_3. KCl was added to adjust the

potassium concentration in the growth media so that their composition was identical, except for the variation in nitrate levels. For SA187 treatments (+SA187), $2x10^5$ bacteria/mL were added directly to the growth media before pouring into square petri plates. For mock controls (Mock), an equal volume of sterile LB medium was added instead. After stratification for 24 h at 4°C in the dark, plates were placed vertically in a growth chamber (16-h photoperiod, 18–20°C night/day, 120 $\mu E/m^2/s$ light intensity, 50 ± 10% relative humidity) to allow seed germination.

At 6 days post-germination (dpg), seedlings were transferred to fresh modified ½MS agar plates containing the same nitrate concentrations as in the germination plates and harvested at 16 days for fresh weight (FW) measurements, nitrate quantification and, molecular and elemental analyses. Plates were scanned at 16dpg to obtain the images for primary root length (PRL) and lateral root density (LRD) measurements.

C. Growth phenotyping

FW of individual plants was measured at harvest. PRL and LRD were measured from plate scans using Fiji/ImageJ software (Schindelin et al., 2012).

D. Elemental analyses

Dried root and shoot tissues (~1 mg per sample) were transferred to tin capsules, weighed and combusted in a Pyrocube elemental analyzer (Elementar, Lyon, France). C and N contents were determined via thermal conductivity detection (TCD) after gas separation on selective columns. Quantification was performed against certified standards: ammonium sulfate (21.2% N), sucrose (42.11% C), glutamic acid (9.51% N, 40.82% C), and glutamine (19.17% N, 41.09% C). Data are presented as % dry weight (DW).

E. Nitrate content assay

Flash-frozen tissues (≥10 mg FW) were ground under liquid N. Each sample was extracted with 500 µL preheated Milli-Q water (100°C), vortexed, and boiled for 15 min with intermittent mixing. After cooling and centrifugation (15,000×g, 15 min), 450 µL of the supernatant was collected. Nitrate content was measured colorimetrically using the Nitrite/Nitrate Assay Kit (Sigma-Aldrich, #23479), following the manufacturers protocol. Technical triplicates were performed per sample.

F. Transcriptomics

i. RNA-sequencing and data analyses

Shoot and root samples were collected from 16-day-old mock and SA187-treated plants grown at 20 mM and 1 mM nitrate, with three biological replicates per condition. Each replicate comprised pooled shoot or root tissues from 4–6 plants. Total RNA was extracted using the NucleoSpin RNA kit following the manufacturer's recommendations (Macherey-Nagel). RNA-seq libraries were prepared from 500 ng total RNA using the QuantSeq 3′ mRNA-Seq Library Prep Kit (Lexogen) and sequenced (75 bp single-end) on an Illumina NextSeq500.

Unique molecular identifiers (UMIs) were removed and appended to the read identifier with the extract command of UMI-tools (v1.0.1) (Smith et al., 2017). Reads where any UMI base quality score fell below 10 were removed. To remove adapter sequences, poly(A), poly(G) sequences, and low quality nucleotides, reads were trimmed with BBduk from the BBmap suite (v38.84) (Bushnell, 2014) with the options k=13 ktrim=r useshortkmers=t mink=5 qtrim=r trimq=10 minlength=30. Trimmed reads were then mapped onto the reference genome of *Arabidopsis thaliana* (TAIR10+ARAPORT11+MtCol0) and counted using STAR (v2.7.3a) (Dobin et al., 2013), with the following parameters --alignIntronMin 5 --alignIntronMax 60000 --alignMatesGapMax 6000 –alignEndsType Local--outFilterMultimapNmax 20 --

outFilterMultimapScoreRange 0 –outSAMprimaryFlag AllBestScore –mismatchNoverLmax 0,6. Reads with identical mapping coordinates and UMI sequences were collapsed to remove PCR duplicates using the dedup command of UMI-tools with the default directional method parameter. Deduplicated reads were counted using HTSeq version v0.12.4 (Anders et al., 2015) (htseq-count mode intersection-nonempty) based on the *A. thaliana* TAIR10-ARAPORT11 annotation. Between 2.9 (86.6% of raw reads) and 2.5 (95% of raw reads) millions of reads were associated to annotated genes.

Statistical analyses were conducted with R v3.6.2 (R Core Team, 2020) using DiCoExpress V1 (Lambert et al., 2020; Baudry et al., 2022) based on the Bioconductor package edgeR (v 3.28.0) (Robinson et al., 2010; McCarthy et al., 2012). Two separate statistical analyses were conducted on data from roots and leaves, starting from the raw count tables.

For each analysis, genes with low counts were filtered using the “filterByExpr” function where the group argument specifies the biological conditions, the min.count value was set to 3 and the min.total.count, large.n and min.prop arguments were set to their default values. Libraries were normalized using the TMM method.

For both analyses, the differential analysis was based on a negative binomial generalized linear model in which the logarithm of the average gene expression is an additive function of a nitrate effect (1mM or 20mM), an inoculation effect (SA187 or mock), their interaction and a replicate effect (3 modalities). Four comparisons were performed (i) the difference between 1 mM and 20 mM nitrate in the mock condition, (ii) the difference between 1 mM and 20 mM nitrate in the SA187 inoculated condition, (iii) the difference between mock and SA187 inoculated at 1 mM nitrate, and (iv) the difference between mock and SA187 inoculated at 20 mM nitrate. For each contrast a likelihood ratio test was applied and raw p-values were adjusted with the Benjamini–

Hochberg procedure to control the false discovery rate. The distribution of the resulting p-values followed the quality criterion described by Rigaill et al. (2016). A gene was declared differentially expressed if its adjusted p-value was lower than 0.05.

Hierarchical clustering of differentially expressed genes (DEGs) was performed using the MeV software (v4.9) with Pearson correlation and average linkage to identify SA187- and nitrate-responsive gene expression clusters. GO term enrichment analysis of DEG clusters was conducted using the R package clusterProfiler (Yu et al., 2012). Terms with adjusted p-values (FDR) < 0.05 were considered significant.

ii. qRT-PCR

cDNA synthesis was performed using the ImProm-II™ Reverse Transcription System (Promega) with oligo-dT primers. Quantitative real-time PCR was performed using SYBR Green I Master Mix (Roche) on a Bio-Rad CFX384 Real-Time PCR Detection System. Each reaction was run with technical triplicates. Gene expression was normalized to *ACTIN2* and *YSL8*, and relative expression levels were calculated using the ΔΔCt method (Livak and Schmittgen, 2001).

G. Statistical analyses and visualization

qRT-PCR data were analyzed using CFX Manager software version 2.1 (Bio-Rad). All other statistical analyses were performed in R (v4.2.1). Growth parameters (FW, PRL, and LRD) were compared using two-tailed unpaired Student's *t*-tests. Elemental composition data (C, N, and C:N ratio) and nitrate contents were analyzed using the non-parametric Mann–Whitney U test. Data visualization, including boxplots and bar graphs with error bars, was carried out using ggplot2, ggpubr, and rstatix packages in R. A threshold of $p < 0.05$ was used to determine statistical significance, with significance levels indicated directly on plots.

III. Results

A. Effect of SA187 on plant growth under varying nitrate conditions

To evaluate the impact of SA187 inoculation on seedling growth with respect to nitrate concentration, *Arabidopsis thaliana* seedlings were grown on solid media containing various concentrations of nitrate (20, 10, 2, 1, and 0.2 mM NO_3^-), with or without SA187 inoculation. Growth responses were quantified by measuring total FW (TFW), PRL, and LRD (Figure 1a–c). At high nitrate availability (20 mM), SA187 inoculation had no significant effect on TFW and PRL compared to mock controls (Figure 1a, b), however a significant increase in LRD (+27.2%) was observed (Figure 1c). As external nitrate concentration declined, SA187-mediated growth enhancements became progressively more pronounced. At 10 mM, SA187 treatment significantly increased TFW by 83.1%, PRL by 18.1%, and LRD by 32.9% (Figure 1a–c) and similar responses were seen at 2 mM nitrate, with SA187 increasing TFW by 90%, PRL by 13.4%, and LRD by 30.8%. At 1 mM nitrate, the beneficial effects of SA187 further intensified for TFW (+108.5%) and PRL (+29.3%), but the LRD increase (+32%) remained comparable to that observed at 2 and 10 mM (Figure 1a–c). Under a severe nitrate limitation (0.2 mM), the effect of SA187 inoculation was exceptionally pronounced, leading to increases of 295.5% (TFW), 121.6% (PRL), and 274.9% (LRD) compared to mock controls (Figure 1a–c). Taken together, these results indicate that SA187 substantially improves both shoot and root development, specifically when nitrate availability is low, with the magnitude of the beneficial effects inversely correlated with external nitrate levels.

B. Effect of SA187 on C, N, and nitrate contents

To investigate how SA187 affected nitrate acquisition and internal nitrate partitioning, total C, total N, the C:N ratio, and nitrate content were measured in both shoots and roots of *Arabidopsis thaliana* grown under high (20 mM) and low (1 mM) nitrate conditions, with or without SA187

inoculation (Figure 2a–d). SA187 inoculation did not significantly alter shoot C content under either nitrate condition, but slightly reduced root C content at low nitrate (Figure 2a). N content exhibited tissue-specific responses: SA187 significantly increased shoot N content but decreased root N content under low nitrate conditions (Figure 2b). However, no significant changes in N content were observed under high nitrate conditions (20 mM) (Figure 2b). These variations in C and N content led to a significantly lower C:N ratio upon SA187 inoculation at 1 mM nitrate, both in shoots and in roots (Figure 2c). Most notably, SA187 induced a significant increase in nitrate content under low nitrate (1 mM) in both shoots and roots when compared to controls (Figure 2d). At high nitrate conditions (20 mM), on the other hand, SA187 had no significant effect on nitrate content. Collectively, these findings emphasize the role of SA187 inoculation in improving nitrate uptake, and shifting N-allocation and nitrate translocation towards aerial tissues under conditions of low nitrate. Such SA187-dependent changes could contribute to the observed increase in seedling growth.

C. SA187 mediated transcriptomic reprogramming of Arabidopsis shoots and roots

To gain insights into the molecular basis of SA187-mediated growth enhancement in the presence of low nitrate, transcriptome analyses (RNA-seq) were performed on Arabidopsis shoots and roots under high (20 mM) and low (1 mM) nitrate conditions with and without SA187 inoculation. Differential gene expression analysis identified 2,280 and 1,005 DEGs in shoots and roots, respectively (Supplementary Figure S1, Supplementary Table S2 and Table S3). Hierarchical clustering and Gene Ontology (GO) enrichment analyses were conducted to categorize the DEGs based on their expression patterns and functional annotations.

In shoots, hierarchical clustering revealed distinct expression patterns. Cluster 4 (517 genes), the largest shoot cluster, showed significant gene expression induction by SA187 in both nitrate

conditions, although predominantly under high nitrate, and included genes associated with defense, immune response, oxidative stress, ethylene, salicylic acid (SA) signaling and metabolic processes, jasmonic acid (JA), cell wall thickening and response to N-compounds. Cluster 10 (239 genes) exhibited a nitrate-dependent regulation, being upregulated under low nitrate alone but downregulated by SA187 treatment under low nitrate conditions. This cluster was enriched in genes linked to JA response, oxidative stress, amino acid metabolism, flavonoid biosynthesis, and glutathione metabolism. Genes in cluster 3 (81 genes) and cluster 9 (284 genes) demonstrated SA187-specific induction regardless of nitrate concentration. These clusters included genes involved in systemic resistance, response to N-compounds, lateral root development, auxin and ethylene signaling, and camalexin biosynthesis (Fig. 3a).

Root transcriptome profiling also revealed distinct clusters of gene expression. Cluster 3 (417 genes), the largest root cluster, exhibited down-regulation under low nitrate but pronounced induction by SA187 across both nitrate conditions. Genes in this cluster were primarily related to defense, ethylene, JA, SA, auxin, cytokinin signaling, and oxidative stress responses. Cluster 5 (234 genes) showed an inverse expression pattern to cluster 3, being upregulated under low nitrate but strongly downregulated by SA187 at both nitrate concentrations. It included genes involved in amino acid transport and metabolism, N-signaling, and oxidative stress responses. Notably, cluster 11 (151 genes) was selectively induced by SA187 under low nitrate and enriched for genes involved in root morphogenesis, nitrate import, and responses to reactive N-species (Figure 3b), suggesting a condition-specific program optimized for nutrient acquisition under stress. Targeted examination of HATs highlighted a strong SA187-induced expression of *NRT2.5* (At1g12940) in shoots, and *NRT2.4* (At5g60770) and *NRT2.6* (At3g45060) in roots, under low nitrate conditions (Fig. 3c).

Together, these findings suggested that SA187 orchestrated a transcriptional network integrating nutrient transport, hormone signaling, stress buffering, and developmental remodeling to promote plant adaptation under a low nitrate condition.

D. Role of high-affinity nitrate transporters NRT2.5, NRT2.6 and ethylene signaling in SA187-mediated growth promotion

To evaluate the functional requirement of ethylene signaling and specific HATs in SA187-induced plant growth benefits under low nitrate conditions, phenotypic analyses were performed using ethylene-insensitive (*ein2-1*), HAT single (*nrt2.5* , *nrt2.6*) and double (*nrt2.5* × *nrt2.6*) mutants under low nitrate (1 mM). Wild-type plants (Col-0) exhibited substantial growth enhancement upon SA187 inoculation, as expected. However, none of the tested mutants displayed significant growth improvements upon SA187 inoculation, indicating that both nitrate transporters and a functional ethylene signaling were indispensable for the growth-promoting effects of SA187 under low nitrate (Figure 4a).

To further dissect the regulatory link between ethylene signaling and nitrate transporter expression, transcript levels of six HAT genes (*NRT2.1–NRT2.6*) were quantified by qRT-PCR in shoots and roots of Col-0 and *ein2-1* plants at 1mM nitrate, with and without SA187 inoculation (Figure 4b). NRT2.7 was excluded due to a consistently undetectable expression. In mock-inoculated *ein2-1* plants, baseline expression of *NRT2.3*, *NRT2.4*, and *NRT2.6* was higher in shoots compared to Col-0, while in roots, *NRT2.3* and *NRT2.6* were slightly downregulated, and *NRT2.2* was slightly upregulated. To examine how SA187 differentially modulated HATs expression in Col-0 and *ein2-1*, qRT-PCR analyses were compared following SA187 inoculation of both genotypes. In shoots, *NRT2.2*, *NRT2.3*, *NRT2.4*, and *NRT2.5* were upregulated by SA187

in Col-0. However, in *ein2-1*, SA187-mediated upregulation was only observed for *NRT2.3* in both organs and for *NRT2.4* in roots, suggesting a partial disruption of the regulatory response. Notably, *NRT2.6* was downregulated by SA187 in Col-0 shoots but showed no significant regulation in *ein2-1*. In roots, SA187 induced the expression of all tested HATs in Col-0. In contrast, SA187 failed to upregulate *NRT2.1*, *NRT2.2*, and *NRT2.5* in *ein2-1* roots, despite a normal induction of the other HATs. Amongst the examined HATs, *NRT2.5* exhibited the highest fold-change in response to SA187 in both organs when compared to mock controls, highlighting it as a potential major component in SA187-mediated growth enhancement under low nitrate conditions. Taken together, these results revealed that ethylene signaling was not essential for the basal expression of most HATs but it was critical for their SA187-induced activation. A major part of the SA187-induced modulation of HAT expression was observed predominantly in roots, although some notable effects were also detected in shoots. The *ein2-1* mutation specifically disrupted SA187-mediated induction of *NRT2.2* and *NRT2.5* in both shoots and roots, *NRT2.4* and *NRT2.6* in shoots, and *NRT2.1* in roots. These findings support a model in which SA187 leverages ethylene signaling to fine-tune the expression of a subset of nitrate transporters that improve seedling growth and nitrate content under low nitrate conditions.

E. Importance of EIN2, NRT2.5, and NRT2.6 in SA187-induced nitrogen accumulation

To determine whether the SA187-induced upregulation of HATs and growth promotion is associated with changes in N-allocation, total N content (% DW) was measured in shoots and roots of Col-0, *ein2-1*, and the *nrt2.5*×*nrt2.6* double mutant under low nitrate conditions (1 mM NO_3^-) with and without SA187 inoculation (Figure 4c). In wild-type (Col-0) plants, SA187 inoculation significantly increased shoot N-content compared to mock controls. However, this

beneficial effect was abolished in both *ein2-1* and *nrt2.5×nrt2.6* shoots, with no significant differences detected between inoculated and mock-treated plants (Fig. 4c). In roots, SA187 did not significantly alter N-content in wild-type and mutant lines, suggesting that SA187 impacted N-allocation to the shoot under these experimental conditions (Fig. 4c). These results further confirmed the involvement of ethylene signaling, *NRT2.5*, and *NRT2.6* in SA187-induced promotion of shoot N content.

IV. Discussion

A. SA187 enhances plant growth under low nitrate conditions

This study demonstrates that SA187 significantly promoted Arabidopsis growth under low nitrate conditions through a coordinated enhancement of seedling growth and root development, thus highlighting a potential role in limiting N-based fertilizer use. The observed growth-promoting effect was tightly linked to external nitrate availability. While only a negligible SA187 effect was seen under high nitrate (20 mM), SA187 significantly increased total FW, PRL, and LRD under low nitrate regimes (between 0.2–2 mM nitrate) (Figure 1). These findings position SA187 as a context-dependent growth promoting inoculant that becomes functionally more advantageous under low nitrate conditions, a hallmark of beneficial plant–microbe interactions to support host fitness under suboptimal environmental conditions. Indeed, previous studies have shown that SA187 does not promote plant growth under non-stress conditions but significantly enhances biomass and root system development under abiotic stresses such as salinity, heat, drought, and high light stress (Andrés-Barrao et al., 2017; Andres-Barrao et al., 2021; de Zélicourt et al., 2018; Rolli et al., 2022; Shekhawat et al., 2024).

Interestingly, SA187 consistently increased LRD across all tested nitrate concentrations including 20 mM when shoot biomass and PRL were unaffected. This constitutive increase in

lateral root formation, regardless of nitrate status, suggests that SA187 may activate a basal root developmental response. This may improve the plant's capacity to explore heterogeneous soil environments and exploit transient nutrient patches, an adaptive advantage even when nitrate supply is sufficient (Garnett et al., 2009). Enhancement of root branching has been observed previously in other PGPB interactions, where changes in root architecture were found to be driven by microbial modulation of plant hormone pathways, including auxins, cytokinins, and ethylene (Spaepen et al., 2008; Vacheron et al., 2013; Chen et al., 2024). The SA187 genome contains *tnaA*, encoding a tryptophanase that converts tryptophan into indole, a known auxin precursor, as well as an indole efflux pump (*acrEF*) (Andrés-Barrao et al., 2017; Synek et al., 2021). Indole and its derivatives contribute to the biosynthesis of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), key auxins involved in lateral root initiation and elongation (Trujillo-Hernandez et al., 2020; Sun et al., 2023). It is therefore possible that SA187 influences host auxin dynamics either by directly producing auxin-like metabolites or by stimulation of host endogenous auxin.

The observed root remodeling may be underpinned by an additional layer of mutualism. It has been proposed that SA187 preferentially enters plant tissues via natural openings at lateral root emergence sites (Synek et al., 2021). From this perspective, increased LRD not only benefits the plant by expanding its nutrient absorption capacity but it will also facilitate bacterial entry and endophytic establishment. Such a dual-purpose modulation, benefiting both microbe and host, exemplifies the mutualistic sophistication of beneficial endophytes. Collectively, these results suggest that SA187 promotes root system remodeling and overall growth to enhance nutrient acquisition under low N, while also potentially optimizing its own endophytic lifestyle within the plant host.

B. SA187 impacts C and N content and improves nitrate accumulation under low nitrate conditions

SA187 inoculation enhanced Arabidopsis nitrate content and elemental analyses revealed an altered internal N-distribution under low nitrate conditions. This was seen especially under low-nitrate where SA187 increased shoot total N-content while either reducing (Fig. 2b) or maintaining (Fig. 4c) it in the root system. This was accompanied by elevated nitrate levels in both organs in SA187 inoculated seedlings compared to wild-type Col-0. This suggested that SA187 not only facilitated root nitrate uptake but also enhanced its translocation to aerial tissues. Therefore, it appeared that SA187 mediated an altered N-allocation that favored N investment in aerial parts compared to roots. This was only observed under low nitrate, when sustaining photosynthetic efficiency and shoot biomass depends heavily on optimal N-partitioning. The observed shift in N-content from roots to shoots could reflect a strategy to redirect resources to maximize growth under suboptimal N-conditions. The role of PGPB in enhancing both nitrate uptake and root-to-shoot transport has been demonstrated previously in multiple plant-microbe systems. For example, *Achromobacter* sp. inoculation significantly improved biomass production and doubled nitrate uptake efficiency in *Brassica napus* (Bertrand et al., 2000). Likewise, volatile organic compounds from *Bacillus velezensis* SQR9 promoted nitrate accumulation and growth in both Arabidopsis and rice (Chen et al., 2024). Another *Enterobacter* species, *E. cloacae* subsp. *dissolvens*, was shown to improve N-content and yield of teff varieties under field conditions, indicating the broader potential of this genus in improving plant N-nutrition (Tsegaye et al., 2022).

Beyond uptake, the effective transport and assimilation of nitrate into amino acids and proteins is a key determinant of NUE and overall plant growth and productivity. By enhancing both nitrate uptake and its allocation to shoots, PGPB may help plants optimize N-use under stress conditions. Upregulated expression of nitrate transporters involved in xylem loading, such as AtNRT1.5, has been reported in response to beneficial microbes, facilitating enhanced nitrate flow to shoot tissues (Calvo et al., 2019). Such enhanced translocation may not only support vegetative growth but also influence seed development and nutrient remobilization during reproductive stages. Indeed, microbial inoculation has been shown to improve N-remobilization efficiency in crop plants, benefiting grain protein content and yield (Adesemoye et al., 2008). Although the current study focused on vegetative development, it is plausible that SA187-mediated enhancements in nitrate uptake and N-allocation may also support reproductive success and seed filling, contributing to overall NUE, a key trait for achieving sustainable crop productivity under N-limited conditions (Congreves et al., 2021).

The C/N ratio in plant tissues is an indicator of metabolic balance and nutritional status. Efficient N-assimilation requires the incorporation of C-skeletons into amino acids and structural components, thereby limiting the buildup of unused C-rich metabolites when N is scarce (Elser et al., 2010; Baslam et al., 2020; Li et al., 2022). In line with this, C:N ratios were elevated under low nitrate availability. However, SA187 inoculation significantly reduced C:N ratios under these conditions, suggesting improved N acquisition and assimilation. Notably, SA187-inoculated plants under low nitrate exhibited shoot C:N ratios comparable to those observed in plants grown under a high nitrate supply. This suggests that SA187 enhanced nitrate uptake sufficiently to restore metabolic balance even in low-nitrate environments. From an agronomic perspective, plants with low C:N ratios often have high protein contents, improved nutritional

quality, and produce residues that decompose more readily, enhancing soil fertility and nutrient recycling (Hertzler et al., 2020; De Silva et al., 2023).

Collectively, these results point to a dual contribution of SA187 in facilitating nitrate uptake and optimizing its internal use and allocation. Such a capacity highlights the potential of SA187 as a bioinoculant for improving plant N-status, especially in the context of reducing the use of synthetic fertilizers while maintaining the productivity of low-input agricultural systems.

C. Transcriptomic analysis reveals SA187-induced reprogramming of nitrogen and hormone signaling pathways under low nitrogen

Transcriptomic profiling revealed that SA187 induced a coordinated and condition-specific reprogramming of *Arabidopsis thaliana* gene expression (Figure 3). This reprogramming encompassed multiple layers of regulatory networks including nutrient acquisition, hormone-mediated development, immune modulation, and cellular stress responses. The observed expression patterns reflected a potential dynamic microbial modulation of host physiology to improve growth and resilience in low nitrate environments.

The notable induction of defense- and immune-related genes by SA187, especially under sufficient nitrate conditions, suggested an underlying mechanism of induced systemic resistance (ISR). It is a common plant defense strategy triggered by beneficial microbes, allowing plants to rapidly and robustly respond to eventual biotic stress conditions (Van der Ent et al., 2009; Pieterse et al., 2014). This priming effect by SA187 aligns closely with previous reports demonstrating ISR induction by beneficial rhizobacteria (Zamioudis et al., 2013; de Zélicourt et al., 2018). The largest SA187-induced gene cluster in shoots (Cluster 4) was enriched for genes associated with defense signaling, oxidative stress responses, and hormone pathways including ethylene, SA and JA. These genes were strongly induced by SA187 under high nitrate conditions

and to a lesser extent under low nitrate, suggesting a nitrate-independent component of defense priming. Such transcriptional activation is consistent with microbe-ISR, a process where non-pathogenic rhizobacteria enhance plant immunity (Zehnder et al., 2001; Choudhary et al., 2007; Van der Ent et al., 2009; Pieterse et al., 2014). The concurrent induction of SA and JA signaling components points to a nuanced immune regulation, potentially enabling both basal defense and biotic stress tolerance. Studies have shown that an increased N-supply can lead to plant susceptibility to pathogens (Mur et al., 2016; Jeon, 2019; Maina et al., 2024). Interestingly, although SA187 did not enhance biomass under high nitrate, its activation of immune pathways suggested it could confer fitness advantages by strengthening plant immunity. Consistent with this idea, shoot Clusters 3 and 9 displayed SA187-induced expression irrespective of N-status. These clusters were enriched in genes responding to microbial symbionts, hormone signaling (SA, JA, auxin, ethylene), N-compounds, and root developmental regulators such as lateral root initiation and camalexin biosynthesis. The nitrate-independent induction of these clusters suggested a basal SA187-triggered transcriptional program integrating developmental cues with immune modulation, likely enabling dual growth-promotion and stress-buffering effects. The co-enrichment of hypoxia-responsive genes could be linked to bacterial modulation of the rhizosphere redox environment, potentially altering local oxygen tension or reactive oxygen species homeostasis, both known to influence root development and immune status (Bailey-Serres and Voesenek, 2008; Considine and Foyer, 2014).

On the other hand, shoot Cluster 10 displayed a nitrate-sensitive signature where genes were upregulated under low nitrate but repressed upon SA187 inoculation. This cluster included genes related to detoxification, glutathione metabolism enzymes, and flavonoid biosynthetic, classical metabolic markers of cellular stress responses to N-deprivation and oxidative imbalance (Lea et

al., 2007; Krapp et al., 2011; Curci et al., 2017; Liang and He, 2018; Li et al., 2023; Yang et al., 2024). SA187-mediated suppression of these stress markers under low nitrate again suggested a buffering effect, whereby SA187 reduced the perceived severity or molecular consequences of low nitrate.

Root transcriptome profiling revealed a comparable complexity. The largest cluster (Cluster 3) was strongly induced by SA187 under both nitrate regimes and contained genes involved in immune signaling, phytohormone pathways (ethylene, SA, JA, auxin, cytokinin), and oxidative stress responses. SA187 is known to colonize the root endosphere, therefore this widespread induction probably reflected direct microbe-host signaling at the root interface. Cluster 5 was characterized by a classical stress signature under low nitrate that was downregulated by SA187. This included genes related to amino acid metabolism, redox balance, and hypoxia, consistent with earlier findings that SA187 alleviates cellular stress via metabolic reprogramming (de Zélicourt et al., 2018; Andres-Barrao et al., 2021; Shekhawat et al., 2024). A strikingly distinct pattern was observed in Cluster 11, where genes were upregulated by SA187 only under low nitrate. Functional enrichment revealed root morphogenesis, root hair elongation, nitrate import, and cellular responses to reactive N species. The exclusive upregulation of this cluster under SA187–low nitrate conditions suggested a tightly controlled transcriptional module integrating nutrient signals with bacterial cues to selectively activate root developmental pathways when most needed.

Among the most functionally relevant findings was the upregulation of high-affinity nitrate transporter genes (*NRT2.5* in shoots, *NRT2.4* and *NRT2.6* in roots). These transporters are critical under low external nitrate concentrations (Lezhneva et al., 2014; Krapp et al., 2014). Their

induction under low nitrate conditions suggested that SA187 benefits observed in terms of growth and nitrate acquisition and partitioning concerned NRT2.4, NRT2.5 and NRT2.6.

Collectively, the transcriptomic data supported a model in which SA187 dynamically rewired plant gene expression to coordinate developmental and metabolic adaptations, enhance nitrate uptake and allocation, and buffer physiological stress responses. These multifaceted changes may enable SA187-inoculated plants to sustain growth and development under low nitrate conditions and position this endophyte as a potent microbial tool for improving NUE and resilience in crops.

D. SA187-mediated growth promotion requires coordinated action of ethylene signaling and high-affinity nitrate transporters

Functional analyses suggested that both ethylene signaling and specific high-affinity nitrate transporters, particularly *NRT2.5* and *NRT2.6*, were required for SA187-mediated growth promotion under low nitrate conditions. Phenotypic analyses confirmed that the *ein2-1*, *nrt2.5*, *nrt2.6*, *nrt2.5×nrt2.6* mutants failed to show significant growth improvements following SA187 inoculation, unlike wild-type plants. Indeed, previous reports have demonstrated the crucial regulatory role of ethylene in modulating nitrate transporter expression and root system architecture under varying N-conditions. Ethylene signaling, mediated through *EIN2/EIN3* pathways, has been previously shown to regulate critical nitrate transporter genes such as *NRT1.1* and *NRT2.1*, fine-tuning nitrate uptake and root developmental responses to external N-availability (Tian et al., 2009; Zheng et al., 2013). For instance, ethylene-mediated repression of lateral root growth under high nitrate conditions required intact *EIN2-1* signaling, and ethylene-insensitive mutants (*ein2-1*, *etr1-3*) exhibited dysregulated root responses to nitrate signals (Tian et al., 2009). Additionally, Zheng et al. (2013) identified a feedback regulatory loop whereby low

nitrate availability induced ethylene biosynthesis, subsequently repressing *NRT2.1* expression to avoid excessive nitrate uptake under prolonged deficiency. Under abiotic stresses, ethylene also modulated stress-induced nitrate allocation to roots via coordinated regulation of *NRT1.5* (xylem loading) and *NRT1.8* (xylem unloading), further exemplifying its central role in plant nutrient homeostasis and stress adaptation (Zhang et al., 2014).

The requirement for ethylene signaling in this context was further supported by SA187-induced expression of *NRT2.1–NRT2.6* in wild-type roots, and selective induction of *NRT2.2*, *NRT2.3*, *NRT2.4*, and *NRT2.5* in shoots. In contrast, *NRT2.6* was downregulated in shoots following SA187 treatment. Among all transporters, *NRT2.5* displayed the strongest induction, suggesting a central role in SA187-mediated enhancement of nitrate content and N-allocation. This transcriptional activation was largely abolished in *ein2-1*, which failed to upregulate key transporters including *NRT2.1* in roots, and *NRT2.2* and *NRT2.5* in both shoots and roots. These results clearly position these genes downstream of ethylene signaling in the SA187 response. Interestingly, SA187-induced *NRT2.6* downregulation in shoots was not observed in *ein2-1*, while root expression of *NRT2.6* remained upregulated in both genotypes, suggesting a partially ethylene-dependent regulatory pattern that was dependent in shoots but independent in roots.

The significance of *NRT2.5* in this context is consistent with its critical role under prolonged N-starvation. Indeed, *NRT2.5* was found to be essential not only for efficient nitrate uptake but also for phloem loading and N-remobilization, supporting plant growth under sustained N-limitation (Lezhneva et al., 2014). Similarly, *NRT2.4*, which was also significantly upregulated by SA187 in both organs, has been shown to contribute to nitrate uptake and remobilization, particularly in lateral roots under severe N-deficiency (Kiba et al., 2012). While a precise role for *NRT2.6* in nitrate transport remains poorly defined, earlier studies suggested its involvement in pathogen-

induced oxidative stress responses rather than direct nitrate acquisition (Dechorgnat et al., 2012). However, the requirement of *NRT2.6* for SA187-mediated growth enhancement aligns with observations from beneficial interactions involving other bacteria such as *Phyllobacterium brassicacearum* STM196, where mutants of *NRT2.6* (and *NRT2.5*) lose beneficial bacteria growth effects (Kechid et al., 2013).

The existence of both ethylene-dependent and ethylene-independent regulations of nitrate transporter expression by SA187 inoculation was observed when comparing wild-type and *ein2-1* seedlings. While *NRT2.2*, *NRT2.5*, *NRT2.6* (shoots) and *NRT2.1* (roots) clearly required ethylene signaling for SA187-induced expression, the regulation of *NRT2.3* in both organs and *NRT2.4* and *NRT2.6* in roots appeared to be independent of ethylene. This dual regulatory system may provide robustness to nitrate acquisition and translocation strategies under fluctuating environmental conditions where ethylene signaling may be perturbed, for example, under combined stress scenarios. Such regulatory flexibility shows the complexity of microbial modulation of plant nutrient responses and highlights the sophisticated molecular interplay microbial signals, hormone-mediated transcriptional networks, and nitrate transporter systems.

E. Ethylene signaling and nitrate transporters coordinate SA187-induced nitrogen allocation to shoots

The loss of SA187-mediated growth promotion in *ein2-1* and *nrt2.5×nrt2.6* mutants, combined with the altered expression of HATs in ethylene-insensitive *ein2-1*, led to the hypothesis that both ethylene signaling and functional NRT2.5 and NRT2.6 were critical for SA187-driven N-allocation to shoots. In wild-type plants, SA187 significantly increased shoot N-content, consistent with enhanced nitrate uptake and preferential allocation to aerial tissues. The complete loss of this effect in *ein2-1* and *nrt2.5×nrt2.6* confirmed that both ethylene signaling and (at least

one of) the HATs were required for this shift in N-allocation induced by SA187. The loss of this response in *ein2-1* reinforced the idea of ethylene as a central regulator in SA187-induced nitrate transport and N-allocation. Transcriptomic and qRT-PCR data consistently showed that *NRT2.5* expression was strongly induced by SA187 in wild-type plants but not in *ein2-1*, demonstrating that this transporter functioned downstream of ethylene signaling. These findings align with earlier studies indicating that ethylene can regulate nitrate transporter expression under N-limitation (Tian et al., 2009; Zheng et al., 2013), and support the view that ethylene functions as a key signaling hub linking microbial perception with nitrate uptake pathways. Similarly, the failure of SA187 to enhance shoot N-content in the *nrt2.5×nrt2.6* double mutant confirmed the essential contribution of these transporters to nitrate acquisition and distribution. SA187-dependent regulation of *NRT2.5* and *NRT2.6* under low nitrate, as observed in wild-type plants, indicates their roles in mediating improved N-allocation to shoots. Given that shoot N-accumulation correlated with growth improvement, this transporter-dependent redistribution is likely a prerequisite for the observed biomass gains.

These findings collectively support a model in which SA187 promotes nitrate uptake and N-allocation to the shoot through coordinated regulation of HATs expression via both ethylene-dependent and ethylene-independent pathways. *NRT2.5* functions downstream of ethylene signaling in both shoots and roots, while *NRT2.6* shows a dual regulatory pattern; ethylene-dependent in the shoot, but ethylene-independent in the root (Figure 5). This coordinated spatial and hormonal regulatory control allows SA187-mediated fine-tuning of nitrate uptake and N-allocation through multiple entry points in the signaling network, enabling improved plant N-status and internal redistribution under low nitrate conditions. These findings advance our

understanding of plant–microbe interactions and offer a framework for leveraging beneficial microbes to enhance NUE and promote sustainable low N-based fertilizer agricultural practices.

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Author Approvals

All authors have seen and approved the final version of the manuscript. The work described has not been published previously and is not accepted for publication elsewhere.

Competing Interests

The authors declare no competing interests.

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Figures

Figure 1. The beneficial effect of SA187 on *Arabidopsis thaliana* plant growth parameters under high and low nitrate. (a) Total fresh weight (mg) per seedling; (b) Primary root length (cm); (c) Lateral root density (n/cm = number/cm). Boxplots depict the median (horizontal line), interquartile range (box), and whiskers to 1.5× IQR; individual data points are overlaid as black dots, and white circles denote group means ($n \geq 40$). Percentage above each bracket gives the change in mean with +SA187 relative to Mock. Statistical significance between treatments at each nitrate level was determined by two-sided, unpaired Student's t-test: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; NS, not significant.

Figure 2. The effect of SA187 on *Arabidopsis thaliana* shoot and root C and N composition under high and low nitrate. Panels show boxplots of (a) total C content (% DW), (b) total N content (% DW), (c) C:N ratio, and (d) nitrate content (μmol g^{-1} FW) in shoots and roots for mock and SA187 treatments at each nitrate concentration. Boxplots display the median (center line), interquartile range (box), and whiskers (1.5× IQR); individual samples are plotted as black dots, white circles mark group means. Mock and +SA187 are distinguished by lighter versus darker colors. Wilcoxon rank sum test (Mann–Whitney U test) was used to compare treatments within each nitrate condition (significance: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, NS = non-significant).

Figure 3. Global transcriptomic responses to SA187 in *Arabidopsis thaliana* shoots and roots under high and low nitrate. Hierarchical clustering heat maps of normalized gene expression (genes mean-centered across all samples) in **(a)** shoots and **(b)** roots of wild-type Col-0 seedlings treated with Mock or SA187 under high (20 mM) and low (1 mM) nitrate. Clustering was performed in MeV v4 using Pearson correlation and average linkage. Key clusters showing

the most relevant induction or repression patterns are highlighted and annotated with enriched Gene Ontology (GO) biological processes obtained by G:Profiler. The color scale denotes relative expression (yellow = high, blue = low). The original mean counts were subjected to data adjustment by normalizing genes across all samples. Hierarchical clustering was performed using average linkage under Pearson correlation (MeV version 4). **(c)** Bar plots representing RNA-seq differential expression of selected HAT candidate genes (AT1G12940, *NRT2.5*; AT3G45060, *NRT2.6*; AT5G60770, *NRT2.4*) significantly regulated by SA187 treatment at 1 mM nitrate. Significance was determined by RNA-seq data analysis, with asterisks (***) denoting statistically significant differences (p-value < 0.001).

Figure 4. Effect of SA187 inoculation on *Arabidopsis thaliana* wild-type (Col-0) and mutant seedlings grown under low nitrate. (a) Total FW of seedlings grown with Mock or +SA187 treatments. Boxplots show median (center line), interquartile range (box) and whiskers to 1.5× IQR; individual plants are overlaid as black dots and white circles denote group means ($n \geq 40$). Percentage above each bracket gives the relative change in mean fresh weight with +SA187 versus Mock. Statistical significance was determined by two-sided unpaired t-tests within each genotype (**P < 0.01, ***P < 0.001). (b) qRT-PCR analysis of high-affinity nitrate transporter genes in shoots (upper panels) and roots (lower panels) of Arabidopsis Col-0 and *ein2-1* seedlings. Expression was calculated by the ΔΔCt method and is shown as $\log_2$ fold-change relative to the Col-0 Mock samples for each tissue. Black asterisks indicate significance between Mock and +SA187 treatments (*P < 0.05; **P < 0.01; ***P < 0.001); grey asterisks denote differences between Col-0 and *ein2-1* under the mock treatment. Error bars represent ± SEM of three biological replicates ($n \geq 3$). (c) Total N content (% DW) in shoots and roots under mock or SA187 treatments in Col-0, *ein2-1*, and *nrt2.5*×*nrt2.6* seedlings grown under 1 mM nitrate.

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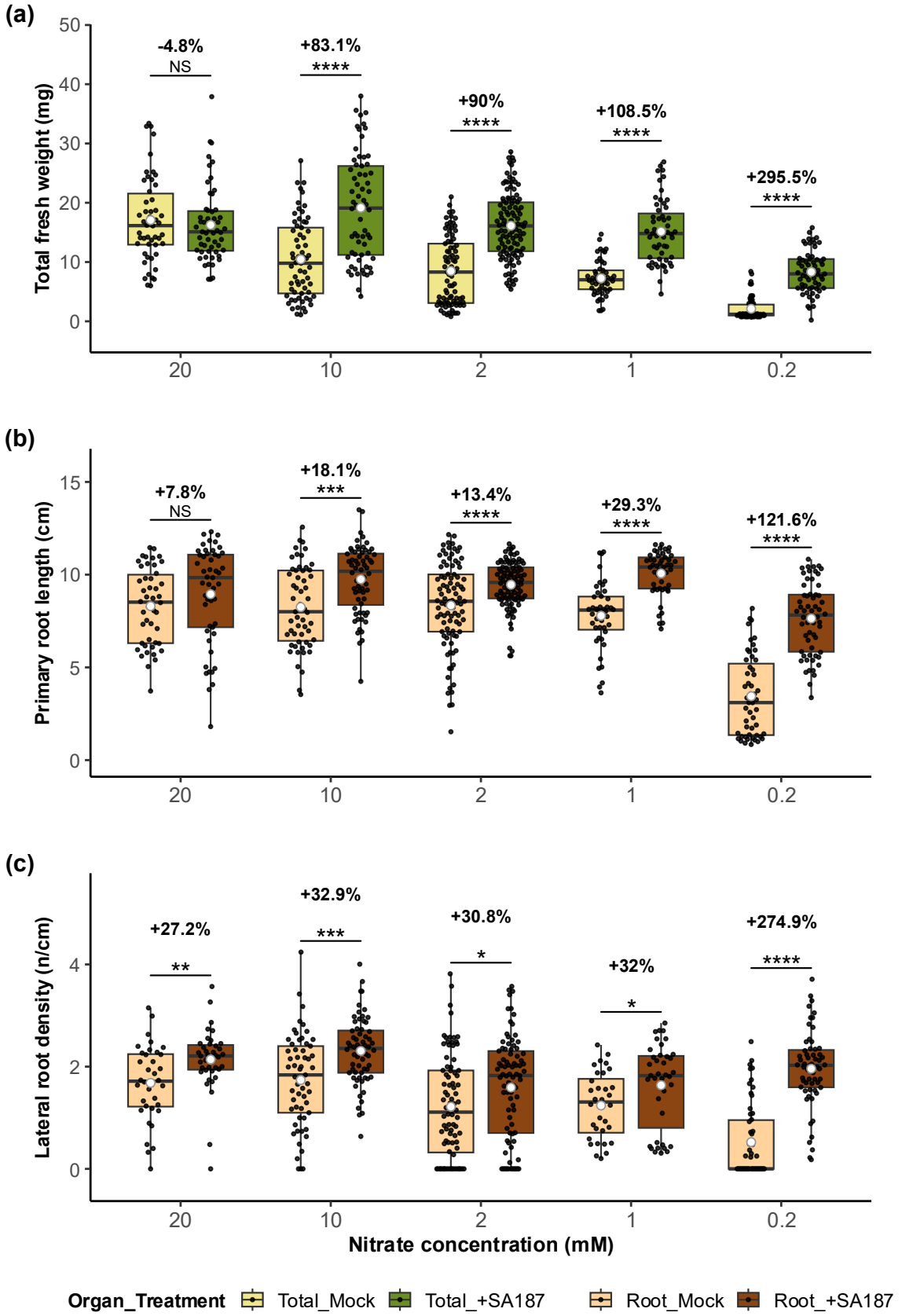
Supplementary Figure 1. Differential gene expression in *Arabidopsis thaliana* under high or low nitrate and with or without SA187 inoculation. Transcriptomic comparisons of Arabidopsis seedlings inoculated with SA187 versus mock-treated controls under low (1 mM) and high (20 mM) nitrate concentrations. (a) DEGs in shoot tissues. (b) DEGs in root tissues. Each comparison indicates the number of significantly upregulated (red) and downregulated (purple) genes identified by pairwise contrasts. Arrows represent the direction of each comparison.



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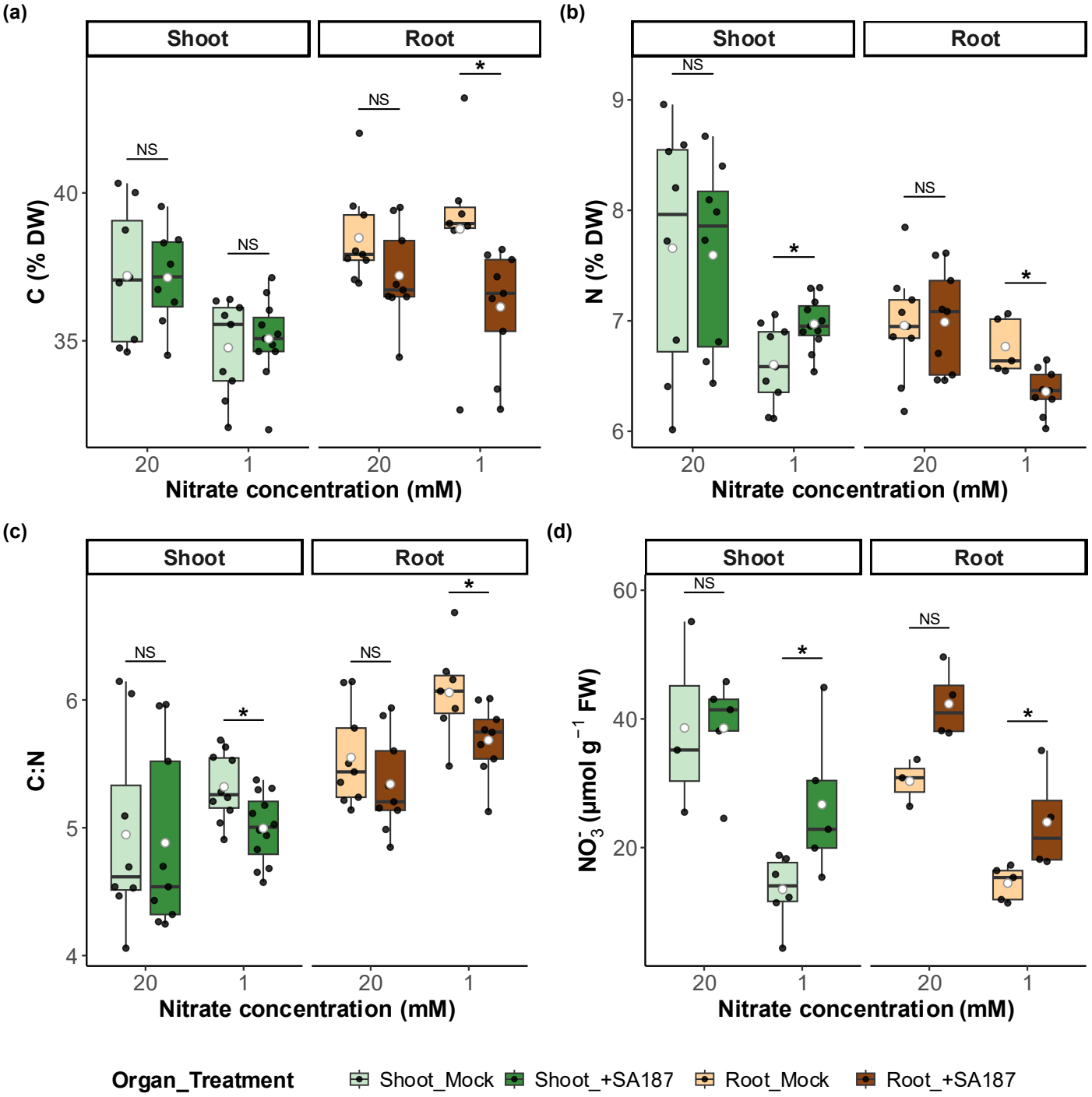

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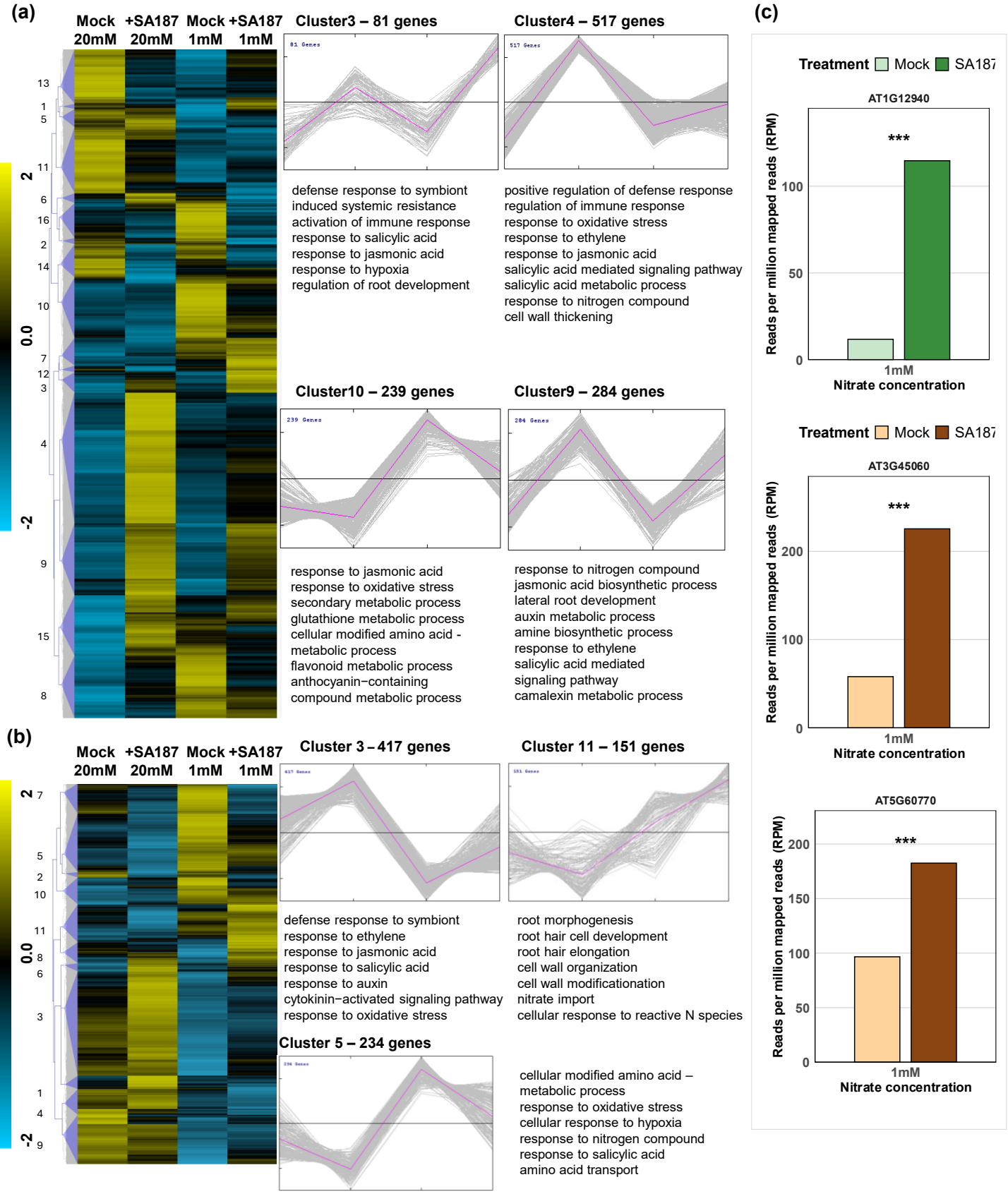

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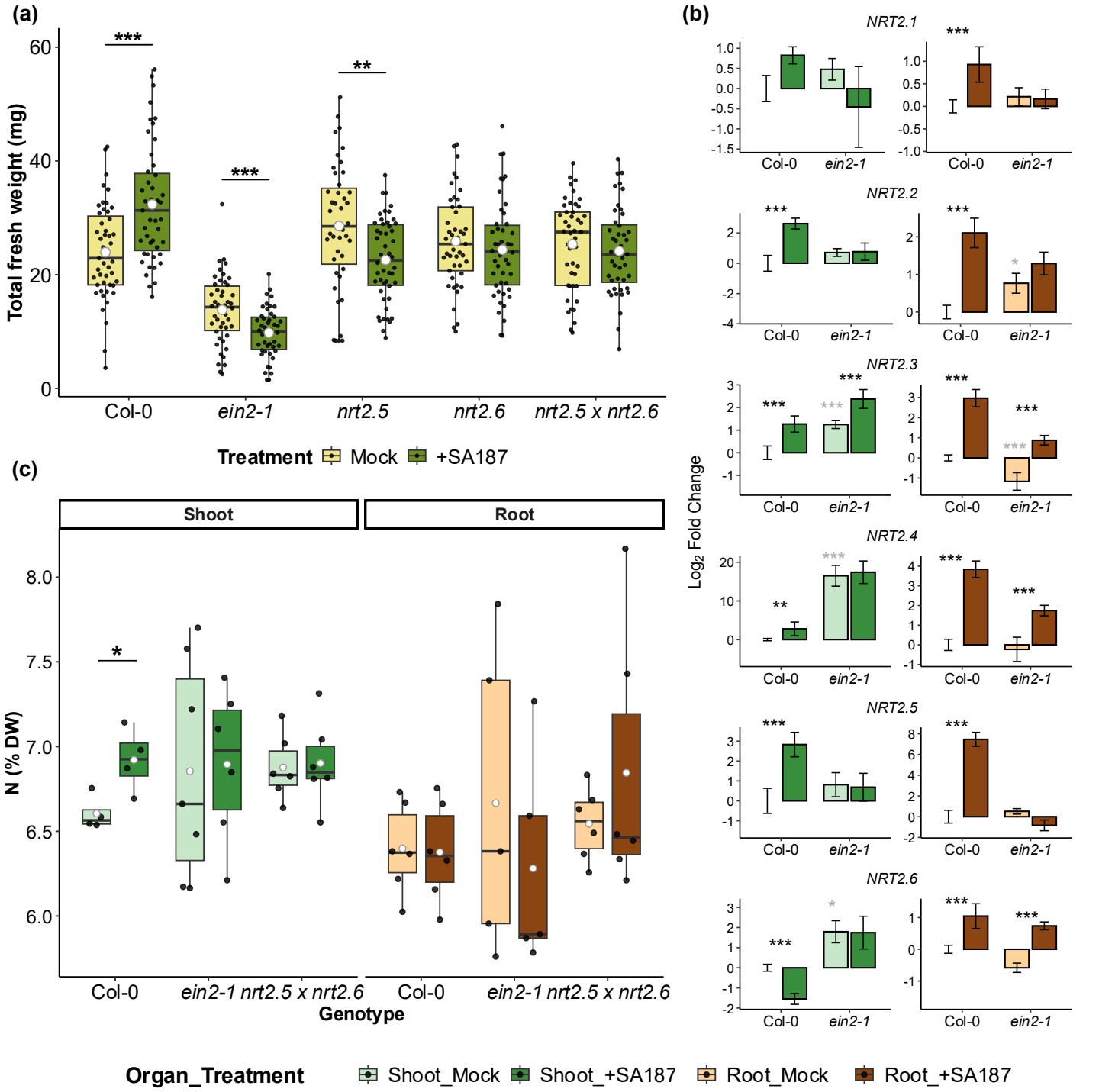

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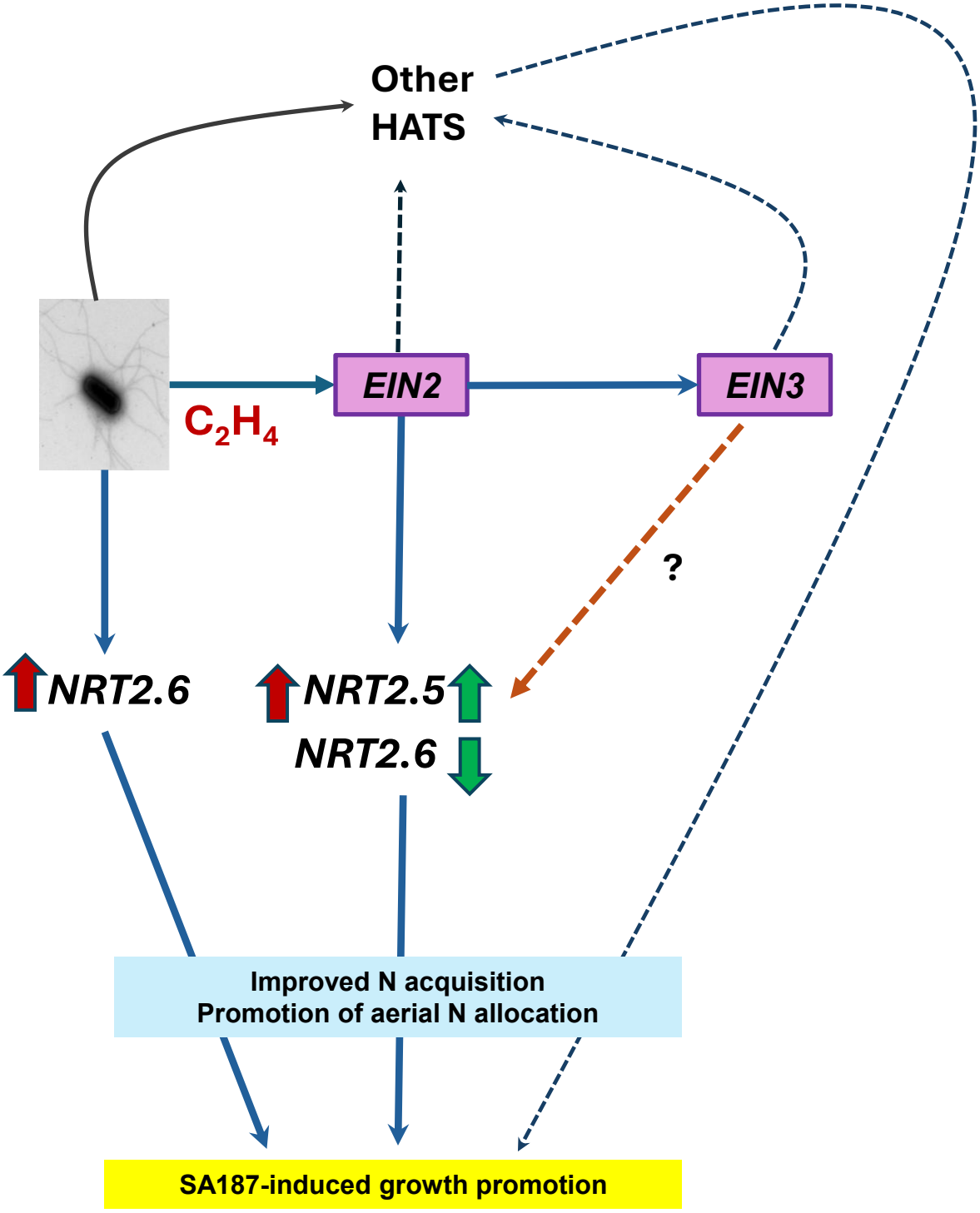

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