

Microbial Metabolic Configuration Reweights Plant Growth–Defense Regulatory Networks in *Nicotiana benthamiana*

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Abstract

Plant growth-promoting rhizobacteria (PGPR) enhance host fitness through phytohormone modulation, induced systemic resistance, and pathogen suppression, yet whether variation in bacterial metabolic state independently shapes plant transcriptional architecture remains unresolved. Here, we compare genome-wide transcriptional responses of *Nicotiana benthamiana* to a characterized PGPR strain (JS) and a UV-induced mutant (MT) harboring frameshift disruptions in *nadB* and *sacX*, genes encoding enzymes involved in NAD⁺ biosynthesis and carbon catabolite regulation. MT-treated plants retained the directional framework of JS-induced transcriptional responses but exhibited attenuated amplitude and redistributed module weighting, consistent with transcriptional buffering rather than qualitative regulatory reprogramming. JS exposure coordinated pathways associated with energy metabolism, terpenoid and phenylpropanoid biosynthesis, and plant-pathogen interaction, whereas MT redistributed metabolic emphasis toward carbon and lipid metabolism categories. Hormone-associated analyses revealed that JS promoted coordinated auxin-, gibberellin-, and jasmonate/ethylene-centered signaling, while MT exhibited differential salicylic acid- versus jasmonate-associated weighting, implicating bacterial metabolic configuration as a key determinant of the growth-defense hormonal balance. Transcription factor profiling identified reconfigured AP2/ERF, GRAS, Dof, and MADS-box family representation under MT, indicating that metabolic perturbation propagates through upstream regulatory hierarchies. Phenotypically, MT reduced shoot growth promotion while preserving root architecture and antifungal efficacy, revealing a functional decoupling between metabolically tunable growth-promoting outputs and resilient biocontrol traits. These findings establish microbial metabolic architecture as a quantitative modulatory parameter that reshapes the amplitude and coordination of plant growth- and defense-associated regulatory networks without altering their directional polarity, and support incorporating bacterial metabolic profiling alongside strain identity as a rational selection criterion for next-generation bioinoculant development in sustainable agriculture.

Introduction

Plant growth regulation emerges from the coordinated integration of developmental, metabolic, and defense-associated signaling networks that dynamically balance biomass accumulation with environmental responsiveness. In agricultural soils, beneficial rhizobacteria represent one of the most influential biotic factors capable of reshaping this regulatory equilibrium. Through direct rhizosphere colonization and volatile organic compound (VOC)-mediated signaling, plant growth-promoting rhizobacteria (PGPR) modulate hormonal homeostasis, transcriptional reprogramming, and metabolic allocation underlying shoot elongation, root system architecture, and systemic resistance (Bais et al., 2006; de Andrade et al., 2023; Jung et al., 2012). Because plant developmental outputs are tightly coupled to hormone-driven transcriptional coordination, the metabolic activity and physiological state of beneficial soil bacteria are critical determinants of plant growth outcomes.

PGPR-mediated growth promotion has predominantly been characterized as a strain-specific property evaluated through phenotypic comparisons. However, plant growth regulation does not arise simply from microbial presence; rather, it emerges from the reorganization of interconnected hormonal networks that integrate auxin, gibberellin (GA), cytokinin (CK), brassinosteroid (BR), jasmonate (JA), ethylene (ET), and

abscisic acid (ABA) signaling. These pathways converge on transcription factor hierarchies and metabolic nodes that govern carbon allocation, secondary metabolite production, and stress responsiveness. Despite extensive documentation of PGPR-associated phenotypes, whether variation in bacterial metabolic state within a shared genetic background can reshape plant growth-regulatory transcriptional architecture remains largely unresolved—a gap that limits mechanistic understanding of how plant regulatory systems interpret metabolically distinct microbial cues.

Members of the genus *Bacillus* are widely employed as bioinoculants because of their environmental persistence and metabolic versatility (Bressuire-Isoard et al., 2018; Rai et al., 2020). The strain *Bacillus subtilis* JS has previously been shown to promote plant growth and modulate defense signaling through VOC-mediated interactions in tobacco and related plant species (J. S. Kim et al., 2015, 2017), eliciting coordinated activation of hormone-associated growth programs alongside defense-related pathways. Nevertheless, whether such host responses depend intrinsically on the metabolic configuration of the interacting bacterium—rather than solely on strain identity—remains unaddressed. Microbial central metabolism governs redox balance, carbon flux, and metabolite emission, including VOC composition. Perturbation of core metabolic pathways may therefore alter the chemical signals perceived by the plant and modify the resulting transcriptional and metabolic integration within the host.

Plant regulatory networks governing growth are closely interconnected with defense-associated pathways. Activation of JA and ET signaling frequently coincides with modulation of auxin-, GA-, CK-, and BR-dependent programs, reflecting integrated coordination of growth and stress responses. Central carbon metabolism and secondary metabolite biosynthesis further mediate resource allocation between biomass accumulation and protective functions. If microbial metabolic perturbation alters redox status or carbon-associated metabolite output, corresponding shifts in plant transcriptional and metabolic coordination may be elicited. In this framework, plant growth regulation may be functionally linked to bacterial central metabolism through signal-dependent redistribution of transcription factor networks and hormone-associated regulatory modules.

To test this hypothesis, we generated a metabolically altered derivative of *Bacillus subtilis* JS through UV-C mutagenesis (M. Guo et al., 2019; Kiriukhin et al., 2014; Vahed et al., 2013), a strategy that preserves the parental genomic framework while introducing heritable mutations. Among the selected variants, frameshift disruptions were identified in *nadB*, which encodes L-aspartate oxidase involved in NAD⁺ biosynthesis and redox homeostasis (Gerdes et al., 2002), and *sacX*, which is associated with sucrose-related carbon catabolite regulation (Soussan et al., 2022). Because these loci occupy central metabolic nodes rather than canonical secondary metabolite biosynthetic clusters, this isogenic system enables direct evaluation of how modification of bacterial redox and carbon flux influences plant transcriptional coordination, independent of strain-level divergence.

Using *Nicotiana benthamiana* as a model host, we compared physiological and genome-wide transcriptomic responses following exposure to the wild-type JS strain and its metabolically altered derivative (MT). Growth parameters including shoot biomass and leaf development were quantified, and differential gene expression profiles were determined by transcriptome sequencing. Functional enrichment analyses were

conducted to evaluate hormone-associated and defense-related pathway organization, followed by transcription factor family redistribution analysis. Integration of these datasets enabled construction of a plant-centered regulatory model describing transcriptional network reorganization in response to metabolically distinct bacterial inputs.

By comparing host responses to a growth-promoting wild-type strain with those elicited by a metabolically reconfigured isogenic derivative, this study addresses the question of whether plant growth-regulatory networks are sensitive to bacterial central metabolic state, independent of genetic identity. Demonstrating correspondence between bacterial metabolic perturbation and the redistribution of plant hormonal and transcriptional modules would establish microbial metabolic architecture as a modulatory dimension of plant–microbe interaction—a dimension that has been largely overlooked in PGPR research. Elucidating this functional linkage provides a mechanistic framework for future genetic and metabolic engineering strategies aimed at enhancing crop productivity and disease resistance through the rational design of microbial metabolic configurations in sustainable agricultural systems.

Materials and methods

Preparation of the bacterial inoculum

The PGPR *B. subtilis* JS (JS) was used as an experimental strain. The reference PGPR strains *Pseudomonas putida* (Israr et al., 2016) (PP) and *B. megaterium* (Ortíz-Castro et al., 2008) (BM) were included, whereas *Escherichia coli* DH5α (EC) was used as a negative control. For each strain, 2 μL of a glycerol stock (approximately 1×10^8 CFU mL⁻¹) stored at -80°C was inoculated into 2 mL of Tryptic Soy Broth (TSB; KisanBio, Korea) and cultured at 26°C with shaking at 180 rpm for 18 h. When the culture reached an OD₆₀₀ of 0.5 (approximately 1×10^8 CFU mL⁻¹), 100 μL of the suspension was spread onto Tryptic Soy Agar (TSA; KisanBio, Korea) plates.

Inducing random mutations in *B. subtilis* JS using UV light

- UV-C mutagenesis and mutant isolation

Mutagenesis was induced with a UV lamp (253.7 nm; Sanyo Denki, Japan). TSA plates spread with JS were placed 40 cm from the light source and exposed to UV-C irradiation in 10-s increments from 0 to 300 s. Survival rates were calculated for each exposure, and the irradiation time yielding approximately 1% survival (50 s) was selected as the mutagenesis condition. Plates were incubated at 26°C for 18 h, and surviving colonies were considered UV-induced mutants. The resulting strains were suspended in 17.5% glycerol, rapidly frozen in liquid nitrogen, and stored long-term at -80°C in a deep freezer.

- Whole genome sequencing-based variant discovery

Bacterial genomic DNA was extracted using the Takara MiniBest Bacterial Genomic DNA Extraction Kit v3.0 (Takara, Japan). Whole-genome variant analysis of the mutant strain was performed against the previously

reported JS genome (GenBank accession no. CP003492.1). Genomic DNA was sequenced by a commercial service provider (DNA Link, Korea) using PacBio long-read sequencing. Raw reads were aligned to the reference genome with BWA, and variants were called DeepVariant. The resulting variant call format (VCF) files were parsed and processed with SAMtools and BCFtools. Variants were further filtered with VCFcons and BCFtools, retaining only positions meeting the following criteria: minimum base coverage ≥ 4 , minimum variant allele frequency ≥ 0.5 , and minimum QUAL ≥ 0 . Whole-genome variant statistics were obtained with BCFtools.

- Quantification of extracellular total aspartate and total NAD(H)

To determine the metabolic changes following the *nadB* defect, total L-aspartate and total NAD(H) were quantified in bacterial culture supernatants. Aspartate was quantified using the Aspartate Assay Kit (Abcam, USA), and NAD(H) was quantified using the NAD/NADH Assay Kit (Abcam, USA). The culture supernatant was measured directly, without acid-alkali fractionation, to quantify the total NAD(H) pool available to participate in the reaction. All measurements were performed with sterile TSB medium included as a control, and each condition was analyzed in at least three independent biological replicates.

- In vitro antifungal activity assay against phytopathogenic fungi

The antifungal activity of JS and its UV-induced mutant (MT) was evaluated against the plant-pathogenic fungi *Alternaria alternata*, *Colletotrichum scovillei*, *Fusarium oxysporum*, *F. solani*, and *F. verticillioides*. Each fungus was inoculated at the center of a Petri dish containing potato dextrose agar (PDA; MBcell, Korea) and incubated to allow mycelial growth. Subsequently, four sterile filter-paper discs (4 mm diameter) were placed at the cardinal positions relative to the central colony, and 10 μL of bacterial culture (Mock, JS, MT, and EC), adjusted to OD600 = 0.5 was spotted onto each disc. Plates were incubated for 3 days, after which antifungal activity was assessed by visual inspection of mycelial growth inhibition around the discs.

- Headspace SPME-GC-MS profiling

For VOC analysis, 3 mL of bacterial culture (TSB broth) adjusted to OD600 = 1.0 was collected and filtered through a 0.22 μm membrane filter to remove cells completely, yielding a cell-free supernatant. The clarified supernatant was transferred to a 20 mL headspace vial, and NaCl was added to a final concentration of 35% (w/v) to promote salting-out of volatile constituents. VOCs were extracted by solid-phase microextraction (SPME) with a DVB/CAR/PDMS fiber (50/30 μm coating). Samples were equilibrated at 40°C for 10 min, followed by adsorption of volatiles onto the SPME fiber at the same temperature for 30 min. GC-MS analysis was performed on an Agilent GC-MS system. A non-polar HP-5MS UI capillary column (30 m $\times$ 0.25 mm, film thickness 0.25 μm) was used for separation, with helium as the carrier gas at a constant flow rate of 1 mL min⁻¹. The oven temperature program was set to hold at 40°C for 2 min, ramp to 245°C at 8°C min⁻¹, and hold at 245°C for 3 min. The mass spectrometer was operated in electron impact (EI) ionization mode at 70 eV, with the ion source temperature set to 230°C. Mass spectra were acquired over an m/z range of 25–530 to detect volatile compounds. Compound identification was performed by matching

spectra against the National Institute of Standards and Technology (NIST) mass spectral library, and only compounds with a similarity index $\geq 80\%$ were retained as final identifications. The relative abundance of each compound was expressed as the percentage of the individual peak area relative to the total ion chromatogram.

In vitro VOC-mediated plant growth promotion assay

To evaluate the growth-promoting effects of JS and MT, *N. benthamiana* was used as the host plant. Seeds were surface-sterilized in 70% (v/v) ethanol for 30 s, then treated with 2.5% (v/v) sodium hypochlorite (NaOCl) for 90 s and rinsed at least five times with sterile distilled water. Sterilized seeds were sown on half-strength Murashige and Skoog medium (1/2 MS; with vitamins, 2.205 g L⁻¹; sucrose, 10 g L⁻¹; pH 5.75). Partitioned Petri dishes (SPL, Korea) were used, with 1/2 MS medium poured into one compartment and TSA medium into the other. Six days after sowing, five uniformly grown seedlings were transplanted onto the 1/2 MS side of each dish. Seedlings were maintained in a growth chamber at 26°C under a 16/8 h light/dark photoperiod. Three days after transplantation, 10 μ L of bacterial culture ($OD_{600} = 0.5$; approximately 1×10^8 CFU mL⁻¹) was spotted onto the center of the TSA compartment. Treatments included TSB medium (Mock), JS, MT, PP, BM, and EC as an inoculated negative control. Negative controls were used to confirm whether JS and MT function as PGPRs. These included the PGPRs PP and BM, and the non-PGPR EC.

In addition, to assess root development phenotypes, seedlings were arranged (eight per plate) in square dishes (SPL, Korea). Bacterial cultures (10 μ L; $OD_{600} = 0.5$) were applied 5 cm from the primary root tip. Treatments included Mock, JS, MT, and EC.

Plant growth conditions, bacterial treatment, and total RNA isolation

Three treatment groups were analyzed: (1) Mock-treated *N. benthamiana*, (2) JS-treated *N. benthamiana*, and (3) MT-treated *N. benthamiana*. Each treatment was performed in three biological replicates for both RNA-seq and qRT-PCR validation. *N. benthamiana* seeds were kindly provided by the Korea Research Institute of Bioscience and Biotechnology (KRIBB). All plants were grown in a greenhouse under a 12/12 h light/dark photoperiod at 30°C. Bacterial cultures (approximately 1×10^8 CFU mL⁻¹) were diluted in 1 L of distilled water, and 15 mL of the suspension was applied to each plant as a soil drench. Leaf tissues were harvested 18 h after rhizosphere inoculation. Samples were immediately frozen in liquid nitrogen and ground to a fine powder. Total RNA was extracted using a modified CTAB method (S. H. Kim & Hamada, 2005).

RNA-seq-based transcriptome profiling and functional enrichment analysis

RNA libraries were prepared with the TruSeq Stranded mRNA Library Prep Kit (Illumina, USA) and sequenced on an Illumina NovaSeq 6000 platform in a 101 bp paired-end format. Raw paired-end reads

were aligned to the *N. benthamiana* reference genome using HISAT v2.2.1 (D. Kim et al., 2015). The reference genome was downloaded from NCBI GenBank (assembly accession GCA_034376525.1; Ko et al., 2024). Genome indices were built with hisat2-build, and alignments were converted to sorted BAM files using SAMtools. Transcript abundance was quantified with StringTie v2.2.2 (Pertea et al., 2015) using the reference annotation file as a guide (-G), and restricting quantification to known transcripts (-e). Ballgown-compatible outputs were generated (-B), and a gene-level count matrix was produced by prepDE.py3. Differential expression analysis was performed in R using DESeq2 v1.44.0 (Love et al., 2014). Read counts were normalized by the DESeq2 size factor, and significance was assessed using the Wald test under a negative binomial model. Multiple testing correction was performed using the Benjamini-Hochberg procedure, and genes with $FDR < 0.05$ and $|\log_2FC| \geq 3$ were defined as significantly differentially expressed genes (DEGs). Given the high variability observed in the dataset, we applied these conservation thresholds to prioritize the most robust and reliable biological signals.

Functional enrichment analysis was performed using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Functional annotation was performed using EggNOG-mapper v2 (Cantalapiedra et al., 2021; Huerta-Cepas et al., 2019). KEGG pathway visualization was implemented as bubble plots using TBtools-II v2.152 (Chen et al., 2023) and SRplot (Tang et al., 2023). Candidate transcription factor (TF) genes were identified by annotating conserved protein domains with InterProScan against the Pfam database, and genes containing hallmark domains of major plant TF families (e.g., AP2/ERF, MYB, WRKY) were selected.

Gene symbols were assigned based on BLASTP searches against the NCBI RefSeq protein database, and each gene was annotated as the putative homolog of the top hit ($E\text{-value} < 1e\text{-}20$; identity $\geq 40\%$).

Quantitative real-time PCR (qRT-PCR) analysis

First-strand cDNA was synthesized from 1 μg of total RNA using the PrimeScript™ II 1st strand cDNA Synthesis Kit (Takara, Japan) with oligo(dT) primers, following the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed with TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Japan) on a MIC real-time PCR system (Bio Molecular Systems, Australia). Six genes with $|\log_2FC| \geq 2$ in MT relative to JS in the RNA-seq dataset were selected for validation as representative markers of the principal regulatory axes identified in the preceding analyses, and their relative expression levels were compared. Primers were designed using the GenScript online PCR primer design tool and NCBI Primer-BLAST with default parameters: $T_m = 59 - 6^\circ\text{C}$; GC = 45–55%; amplicon size = 130–200 bp (Table S1).

Statistical analysis

Plant growth-promotion data were tested for normality with the Shapiro-Wilk test, followed by one-way ANOVA. Post hoc comparisons were performed using Tukey's HSD test ($p < 0.05$). qRT-PCR data were analyzed with a t-test to assess significance relative to the mock-treated control ($0.01 < p < 0.05$, *; $p < 0.01$, **). For GO and KEGG enrichment analyses, FDR values were calculated using the Benjamini-Hochberg method. All statistical analyses and visualizations were performed in R v4.3.1.

Results

Metabolic perturbation framework targeting central redox and carbon nodes in *Bacillus subtilis* JS

To establish an experimental framework for examining how alteration of bacterial central metabolism influences plant growth–regulatory responses, UV-C–induced mutants were generated from *B. subtilis* JS, and whole-genome sequencing was performed on a representative surviving colony (Fig. S1). This approach enabled identification of stable genomic alterations within an otherwise shared genetic background, thereby allowing subsequent plant responses to be interpreted in relation to defined metabolic perturbations rather than broad strain divergence.

Whole-genome sequencing identified four single-base insertions in the mutant strain (Table 1), each predicted to introduce frameshifts resulting in loss-of-function effects. A cytosine insertion in *nadB* (L-aspartate oxidase; GCCCCC→GCCCCC) disrupted an enzyme catalyzing an early step in de novo NAD biosynthesis. The predicted frameshift is expected to impair enzymatic activity. Consistent with disruption of NAD-associated metabolism, L-aspartate accumulated, and total NAD(H) production decreased in the mutant strain (Fig. 1), supporting perturbation of NAD-linked metabolic processes.

An adenosine insertion in *sacX* (CA→CAA), encoding a regulator within the phosphotransferase system (PTS)-based sucrose transport pathway, was also identified. Frameshift occurred within a conserved region and may affect sucrose sensing or phosphotransfer regulation. Although no significant differences in bulk sucrose utilization were detected under the tested conditions (data not shown), disruption of *sacX* may influence coordination of carbon flux and intracellular energy balance rather than overall substrate consumption. Such alteration of central carbon regulation could affect metabolic output relevant to plant-associated interactions, although this was not directly quantified in the present study.

Two additional insertions were detected, including a guanine insertion (CGG→CGGG) within a permease-encoding gene and a cytosine insertion (TCCCC→TCCCCC) in a non-coding region at position 2,644,385. The functional consequences of these variants remain unresolved and were not further prioritized.

Among the identified mutations, *nadB* and *sacX* reside within core metabolic pathways governing redox homeostasis and carbon allocation. Because these loci represent central metabolic nodes rather than secondary metabolite biosynthetic genes, the resulting strain provides a defined system in which bacterial metabolic configuration is altered while the parental genetic background is largely preserved. Subsequent analyses therefore focus on determining how perturbation of these metabolic axes influences bacterial physiological state and associated plant responses.

Metabolic alteration of *Bacillus subtilis* JS differentially modulates volatile-mediated plant growth and antifungal responses

To evaluate how alteration of bacterial central metabolism influences volatile-mediated plant growth regulation, the physiological responses of *N. benthamiana* were examined following exposure to VOCs emitted by JS and MT.

Exposure to JS-derived VOCs significantly enhanced shoot growth relative to the mock control, resulting in an approximately twofold increase in fresh weight (0.12 g vs. 0.06 g) together with increased leaf number.

These observations are consistent with previous reports describing volatile-mediated growth promotion by *Bacillus* species (Ryu et al., 2003; Tahir et al., 2017). Exposure to VOCs from the MT strain also promoted plant growth compared with the mock treatment; however, both leaf number and biomass were consistently lower than those observed under JS treatment. Thus, while growth-promoting activity was maintained, the magnitude of shoot growth stimulation was quantitatively reduced under MT exposure. This attenuation is compatible with the possibility that disruption of *nadB* and *sacX*, predicted to affect redox balance and carbon-associated metabolism, influences the intensity or composition of volatile-associated signaling without eliminating growth-regulatory capacity (Marinoni et al., 2008).

Root architectural analysis further demonstrated pronounced stimulation of lateral root formation in both JS- and MT-exposed plants compared with mock and EC controls, including increased lateral root density, branching frequency, and primary root elongation (Fig. S2). Notably, despite reduced shoot biomass accumulation under MT treatment, root system architecture was largely comparable between MT and JS exposures. These findings indicate that alteration of bacterial central metabolism exerts differential quantitative effects on shoot and root growth outputs, while preserving core root developmental responses.

Antifungal assays revealed strong inhibition of mycelial growth of *C. scovillei*, *A. alternata*, *F. oxysporum*, and *F. solani* under exposure to both JS and MT (Fig. S3), with no substantial differences in inhibition patterns between strains. These results indicate that disruption of central metabolic loci did not abolish broad-spectrum antifungal activity and suggest that the reduced shoot growth observed under MT exposure does not reflect a general loss of biological functionality.

Qualitative profiling of culture supernatants identified 2,3-butanediol, acetoin, and methyl salicylate as major emitted compounds (Fig. S4). Although these metabolites have been previously associated with plant growth promotion and defense activation, quantitative analyses were not conducted in the present study, limiting inference regarding strain-specific emission levels. Nevertheless, the combined phenotypic observations indicate that perturbation of bacterial central metabolism is associated with modification of the magnitude of volatile-mediated plant growth responses, while preserving fundamental plant-modulatory capacity. Direct linkage between specific metabolic lesions and VOC emission profiles will require future quantitative GC–MS analyses.

Plant transcriptional architecture is quantitatively reconfigured in response to metabolically altered bacterial exposure

To determine how alteration of bacterial central metabolism influences plant growth-associated transcriptional regulation, RNA-seq analysis was performed on *N. benthamiana* leaf tissues following direct root-zone application of Mock, JS, and MT (three biological replicates per treatment; Table S2; Table S3). This comparative framework enabled evaluation of how plant transcriptional programs respond to differences in bacterial metabolic configuration within a shared genetic background.

Sequencing on an Illumina NovaSeq 6000 platform generated 72.6–80.3 million raw reads per sample in the Mock group, 87.9–88.9 million in the JS group, and 86.0–89.0 million in the MT group. After quality filtering (Phred score ≥ 20), 90–95% of reads were retained. Mean Q30 scores exceeded 95%, and GC content ranged from 43.8% to 44.5% across treatments. Alignment to the *N. benthamiana* KLAB v2.0 reference genome (Ko et al., 2024) using HISAT v2.2.1 achieved 99.7–99.8% total mapping efficiency. Uniquely mapped reads accounted for approximately 93% in JS and MT samples and 71–91% in Mock samples, indicating overall high sequencing quality suitable for comparative transcriptomic analysis (Table 2).

Differential expression analysis using a stringent threshold ($|\log_2FC| \geq 3$, FDR < 0.05) revealed substantial transcriptional divergence among treatments (Fig. 3). Hierarchical clustering of normalized expression values (Z-score transformation; Euclidean distance) demonstrated clear separation of Mock, JS, and MT groups (Fig. 3A). Plants exposed to JS formed a distinct cluster characterized by coordinated induction and repression of extensive gene sets relative to Mock, with strong concordance among biological replicates, indicating reproducible transcriptional reprogramming in response to rhizosphere-mediated bacterial stimulation.

In contrast, plants exposed to MT occupied an intermediate transcriptional state between JS and Mock. Many gene clusters regulated under JS exposure were regulated in the same direction under MT exposure; however, both the magnitude and extent of induction or repression were consistently reduced. Gene modules strongly activated in JS-treated plants were diminished in size and expression intensity in MT-treated plants. These patterns indicate that modification of bacterial central metabolism does not reverse the directionality of plant transcriptional responses but modulates their amplitude and degree of regulatory coupling.

Pairwise DEG comparisons further supported this interpretation (Fig. 3B; Table S3). JS vs. Mock identified 460 DEGs (238 up-regulated, 222 down-regulated), whereas MT vs. Mock yielded 383 DEGs (197 up-regulated, 186 down-regulated), indicating a quantitatively attenuated transcriptional response under MT exposure. Direct comparison between MT and JS identified 72 DEGs, suggesting modulation of response strength and network configuration rather than extensive pathway replacement. Notably, 286 DEGs were shared between JS and MT relative to Mock, while 77 and 31 DEGs were unique to JS and MT, respectively.

Collectively, these results demonstrate that plants exposed to bacteria with altered central metabolic capacity engage a largely overlapping transcriptional framework compared with wild-type exposure, but exhibit reduced breadth, intensity, and combinatorial organization of growth- and defense-associated regulatory programs. Thus, variation in bacterial metabolic state is reflected in quantitative reconfiguration of plant transcriptional architecture rather than qualitative redefinition of signaling polarity.

Functional enrichment analyses reveal quantitative reorganization of plant regulatory networks under exposure to metabolically distinct bacteria

To characterize how plant functional transcriptional organization responds to bacterial metabolic alteration, GO and KEGG enrichment analyses were performed on differentially expressed genes identified across

Mock, JS, and MT treatments (Table S4; Table S5). These analyses were conducted to determine whether plant growth- and metabolism-associated regulatory modules are differentially coordinated when plants are exposed to bacteria with altered central metabolic capacity.

GO profiling revealed treatment-dependent redistribution of enriched terms across Biological Process (BP), Molecular Function (MF), and Cellular Component (CC) categories. Although both JS and MT exposures induced extensive transcriptional remodeling relative to Mock, the relative weighting and composition of enriched functional categories differed between treatments. This pattern indicates that plant regulatory coordination is modulated in a quantitative manner rather than through simple presence–absence activation of specific pathways (Fig. 4; Fig. 5; Fig. S5).

In MT-exposed plants, up-regulated genes were enriched for stress- and stimulus-responsive processes, including response to stimulus, together with DNA- and nucleic acid–binding functions in the MF category (Fig. 5A). These enrichments are consistent with increased representation of transcriptional regulatory components, including AP2/ERF and GRAS-associated modules described below. Plastid- and chloroplast-associated components were overrepresented within the CC domain, and enrichment of oxidoreductase activity and metal ion binding suggests engagement of redox-related enzymatic processes. Conversely, down-regulated genes in MT-exposed plants were enriched for sequence-specific DNA binding, nucleic acid binding, and transferase activity, while BP categories associated with biosynthetic processes, primary metabolism, and broader cellular metabolic processes were significantly depleted (Fig. 5B). These patterns indicate redistribution of transcriptional regulatory capacity and primary metabolic activity under MT exposure. Because *nadB* and *sacX* mutations alter bacterial NAD-associated metabolism and carbon flux regulation, the observed plant transcriptional shifts are consistent with exposure to a metabolically modified bacterial signal environment; however, direct mechanistic linkage remains to be experimentally validated.

KEGG pathway analysis further supported treatment-associated reorganization of plant regulatory networks. In JS vs. Mock, MT vs. Mock, and MT vs. JS comparisons, 189, 180, and 79 pathways were mapped, respectively, with 37, 36, and 1 pathways reaching statistical significance (adjusted $p < 0.05$). Plants exposed to JS were characterized by coordinated enrichment of pathways related to Metabolism (A09100), Environmental adaptation (B09159), and Energy metabolism (B09102), together with photosynthesis-antenna proteins (ko00196) and defense- and secondary metabolism–associated pathways including sesquiterpenoid and triterpenoid biosynthesis (ko00909), diterpenoid biosynthesis (ko00904), and plant–pathogen interaction (ko04626) (Fig. 6B). These enrichments reflect integrated engagement of growth- and defense-associated metabolic modules.

In contrast, MT vs. Mock analysis revealed enrichment of phenylpropanoid biosynthesis (ko00940), lipid and carbohydrate metabolism categories (B09103, B09101), and photosynthesis-related classes among down-regulated DEGs (Fig. 6C), whereas photosynthesis-antenna proteins (ko00196) and terpenoid-associated pathways were predominantly enriched among up-regulated DEGs (Fig. 6D). This distribution indicates altered weighting of primary and secondary metabolic pathways in plants exposed to bacteria with perturbed central metabolism.

Direct comparison between MT and JS identified limited statistically significant enrichment, with only Chaperones and folding catalysts reaching significance among up-regulated DEGs (enrichment factor = 7.70) (Fig. S6). Additional pathways related to hormone signaling and defense regulation—including BR biosynthesis (ko00905), phenylpropanoid biosynthesis (ko00940), terpenoid backbone biosynthesis (ko00900), flavonoid biosynthesis (ko00941), monoterpenoid biosynthesis (ko00902), and MAPK signaling (ko04016)—were detected but did not reach statistical thresholds (Fig. S6). These observations suggest subtle quantitative differences in regulatory integration rather than extensive pathway replacement.

These enrichment analyses demonstrate that plants exposed to bacteria with altered central metabolic configuration retain activation of core metabolic and defense-associated pathways; however, the coordination, relative contribution, and integration of growth-, metabolism-, and stress-related transcriptional modules are quantitatively reorganized. This pattern reflects redistribution of plant regulatory network architecture rather than qualitative loss of pathway activation.

Hormone-associated and secondary metabolic modules underpin treatment-dependent regulatory redistribution

The functional redistribution identified by GO and KEGG analyses prompted further examination of hormone-associated regulatory modules, which represent central integrators of plant growth and stress coordination. Because plant growth regulation is orchestrated through interconnected hormone signaling networks, evaluation of hormone-responsive gene expression provides a mechanistic layer linking global pathway enrichment patterns (Fig. 6) to transcriptional control of developmental and defense outputs.

A heatmap of genes associated with plant growth, development, and stress responses (Fig. S7) revealed distinct hormone- and defense-related transcriptional configurations between JS- and MT-exposed plants. Gene labels correspond to locus IDs used in this study, and gene symbols represent putative homologs assigned based on top BLASTP hits against the NCBI RefSeq database. Treatment-dependent differences in hormone-associated gene expression were evident (Table 3), indicating divergence in regulatory coordination rather than reversal of signaling polarity.

In the auxin pathway, plants exposed to JS exhibited induction of *GH3.6*, *IAA29*, *IAA15*, and *SAUR50*, whereas *AUX1* and *ARF18* were slightly reduced. Under MT exposure, induction of *GH3.6* and *IAA29* was maintained and expression levels were elevated relative to JS, while *SAUR21* and *SAUR68* were uniquely induced. These SAUR genes are associated with cell elongation and stimulus-responsive growth modulation (Ren & Gray, 2015; Sun et al., 2016). These patterns show that auxin-related growth signaling is maintained in plants under both JS and MT treatments and suggest that distinct auxin-associated signaling states may drive a reconfiguration of downstream signaling.

Within the gibberellin pathway, both treatments reduced *GASA1* and *GASA6* expression and increased *PIF4* and *GA2ox* transcripts, consistent with modulation of active GA homeostasis (Biemelt et al., 2004; Sakamoto et al., 2001). ET-associated genes, including ACO isoforms and *ERF1B*, were elevated under both exposures, reflecting activation of ET/JA-integrated transcriptional programs (Lorenzo et al., 2003). ABA signaling components displayed coordinated downregulation of PP2C genes in both treatments, whereas

MPK12 was specifically induced under MT exposure, indicating treatment-dependent variation in stress-related signaling integration (Jammes et al., 2011).

In SA- and JA-associated pathways, MT-exposed plants showed stronger induction of *SAMT1* but comparatively reduced expression of *TGA9* and *PR1* relative to JS. Although exogenous NAD⁺ has been reported to enhance SA-associated defense signaling (X. Zhang & Mou, 2009), the present transcriptomic results do not establish a direct mechanistic linkage between bacterial NAD biosynthesis and host SA output. Instead, the data indicates differential weighting of SA-associated regulatory components. JA-associated genes *LOX5* and *LOX6* were induced in both treatments, though induction magnitude was reduced in MT. BR- and CK-related genes exhibited shared reductions across treatments, while *ARR6* was specifically induced in JS.

These hormone-associated expression patterns demonstrate that plants exposed to bacteria with altered central metabolic capacity retain core growth–defense signaling polarity but exhibit quantitative redistribution in coupling strength among hormonal modules. Such rebalancing is consistent with the attenuated shoot growth observed under MT exposure.

Secondary metabolism–associated transcriptional programs further distinguished the treatments (Table 4). Terpenoid biosynthesis genes, including multiple EAS isoforms involved in capsidiol production, were induced under both treatments but more strongly in JS, with one isoform significantly elevated only under JS exposure. In the phenylpropanoid pathway, JS prominently induced *MYB61* and *RAX2*, whereas MT was characterized by broader activation of transcriptional regulators including *MYB46*, *MYB61*, *bHLH110*, and *RAX2*, together with pronounced downregulation of the phenylpropanoid repressor *MYB4* (Geng et al., 2019; Yao et al., 2021). Enzymatic components also differed, with MT preferentially inducing *CCR1* and *VS*, while JS more strongly induced BEBT/BEAT-related genes (Hou et al., 2024; Liu et al., 2018). Multiple cytochrome P450 genes associated with alkaloid metabolism and oxidative processes were differentially expressed across treatments (Ali et al., 2019; Z. Guo et al., 2025), including coordinated downregulation of *CYP90B3* and JA-Ile oxidation genes (Bancoş et al., 2002; Heitz et al., 2012; Ohnishi et al., 2006). Glycosylation-related genes involved in secondary metabolite conjugation were induced under both exposures, although with reduced magnitude in MT (Louveau & Osbourn, 2019; Tiwari et al., 2016).

These results indicate that plants maintain activation of core hormonal and secondary metabolic programs under both bacterial exposures; however, alteration of bacterial central metabolism is associated with redistribution and differential coordination of regulatory modules governing growth and defense integration. The observed quantitative rebalancing of hormone-associated and metabolic pathways provides a transcriptional framework consistent with reduced growth magnitude under MT treatment and establishes the basis for examining upstream transcription factor network reconfiguration.

Transcription factor network reconfiguration underlies treatment-dependent redistribution of plant regulatory modules

Because hormone-associated and secondary metabolic pathways are coordinated through hierarchical transcriptional regulation, we next examined transcription factor (TF) networks identified in the RNA-seq dataset to determine whether exposure to metabolically distinct bacterial strains is reflected at the level of upstream regulatory control. This analysis was based directly on differentially expressed genes derived from the transcriptome dataset described above.

Among MT and JS treatments, eight transcription factors met the differential expression criteria ($|\log_2FC| \geq 2$, FDR < 0.05; Table 5). Domain architectures were annotated using InterProScan and PFAM, and TF families were assigned through BLASTP searches against PlantTFDB v5.0 (E-value < 1e-06) (Jin et al., 2017), followed by additional functional curation using NCBI BLASTP.

Transcription factors upregulated under MT exposure were predominantly distributed among the AP2/ERF, GRAS, Dof, and MADS-box families. Within the AP2/ERF family, *ERF017*, *ERF017-like*, and *ERF003-like* genes showed significant induction in MT-treated plants. AP2/ERF factors are established regulators of JA/ET-associated transcriptional programs (Kong et al., 2023; Ma et al., 2024), and their increased representation under MT exposure is consistent with redistribution of JA/ET-linked regulatory modules observed in hormone-associated analyses. The GRAS family member SCARECROW-like was also strongly induced under MT exposure, indicating altered engagement of developmental and hormone-responsive transcriptional circuits. Similarly, *DOF3.4-like*, belonging to the Dof family, was significantly upregulated. Dof transcription factors are implicated in sugar-responsive and carbon-associated transcriptional regulation, aligning with the broader metabolic redistribution identified in plants exposed to bacteria with perturbed central metabolism. The MADS-box gene *AGL11* was likewise induced, suggesting modulation of developmental regulatory components under metabolically altered bacterial exposure.

In contrast, transcription factors downregulated in MT relative to JS included members of the NF-YA family. NF-YA proteins, components of the CCAAT-binding complex, integrate hormone signaling and stress-associated responses (J. E. Kim et al., 2020; Laloum et al., 2013; Siriwardana, 2025). Reduced expression of *NF-YA-2-like* and related loci is compatible with altered coordination of auxin- and growth-associated transcriptional outputs (M. Zhang et al., 2017), although functional consequences were not directly assessed in the present study.

The observed redistribution of transcription factor families indicates that exposure to bacteria with altered central metabolic capacity is reflected at the level of upstream regulatory architecture. Increased representation of AP2/ERF-, GRAS-, Dof-, and MADS-box-associated modules alongside relative reduction of NF-YA-linked regulation supports a model in which plant growth-defense integration is quantitatively reweighted rather than qualitatively redefined. This transcription factor reconfiguration provides a regulatory context for the hormone-associated and secondary metabolic redistribution described above.

qRT-PCR validation confirms robustness of treatment-dependent transcriptional reorganization

To assess the reproducibility and structural robustness of transcriptional patterns identified by RNA-seq-based differential expression analysis, qRT-PCR validation was performed on a representative subset of

genes (Fig. 7). Rather than attempting exhaustive validation of all DEGs, six genes were selected to represent principal regulatory axes identified in the preceding analyses, including hormone-associated redistribution, redox and photosynthesis-related metabolic shifts, stress-responsive signaling, and transcription factor network reconfiguration (Fig. S7).

The selected genes included a photosynthesis-related transcript (g45715.t1), redox- and defense-associated genes (g33357.t1, g17759.t1), a stress-responsive gene (g11405.t1), a transcription factor differentially expressed between MT and JS (g39000.t2), and an MT-specific DEG (g18298.t1). This panel was designed to capture distinct functional modules contributing to transcriptional separation among treatments, including metabolic reallocation, hormone-associated stress integration, and upstream regulatory control, rather than to validate individual pathways in isolation. Relative expression levels were calculated using ribosomal protein L23 and F-box genes as internal controls.

For g33357.t1, g45715.t1, and g17759.t1, qRT-PCR reproduced both the directionality and relative magnitude of expression changes observed in RNA-seq, with decreased transcript abundance detected in both JS- and MT-exposed plants. For g39000.t2, g11405.t1, and g18298.t1 under MT exposure, absolute fold-change values differed between RNA-seq and qRT-PCR measurements; however, directional trends and relative comparisons across treatments were maintained. Such quantitative discrepancies are consistent with known methodological differences between sequencing-based and amplification-based platforms, including dynamic range variation, normalization approaches, transcript isoform detection sensitivity, and primer-specific amplification efficiency.

Importantly, the preserved polarity of expression changes across validated genes supports the broader observation that plants exposed to metabolically altered bacteria retain the directional framework of JS-induced transcriptional responses while exhibiting quantitative differences in magnitude and regulatory weighting. Although the validation panel was limited in scope, the representation of genes spanning metabolic, stress-associated, and transcription factor modules strengthens confidence that the treatment-dependent differences observed in global transcriptomic analysis reflect structured regulatory reorganization rather than stochastic expression variability.

Discussion

Plant growth regulation is governed by coordinated hormonal, metabolic, and transcriptional networks that dynamically integrate environmental and biotic inputs. Among biotic modulators, PGPR have been extensively studied for their capacity to enhance host productivity through phytohormone modulation, ISR, and suppression of soil-borne pathogens (Lugtenberg & Kamilova, 2009). However, whether variation in bacterial metabolic configuration, rather than strain identity per se, shapes plant transcriptional responses has remained largely unexplored. Here we demonstrate that plant regulatory systems respond not only to the presence of a beneficial bacterium but to the specific metabolic state it presents, by comparing the characterized PGPR strain JS (J. S. Kim et al., 2015, 2017) with a UV-induced mutant carrying frameshift disruptions in *nadB* and *sacX* (Table 1) and examining how alteration of bacterial redox- and carbon-associated metabolism influences genome-wide transcriptional organization in *N. benthamiana*.

Comparative transcriptome analysis revealed that MT-treated plants retained the directional framework of JS-induced gene regulation while exhibiting reduced response amplitude and altered module composition. Hierarchical clustering positioned JS-treated samples as a distinct transcriptional state relative to Mock, whereas MT-treated samples occupied an intermediate configuration characterized by attenuated induction or repression intensity (Fig. 3A). The substantial overlap of 286 shared DEGs between JS- and MT-treated samples, together with the limited set of treatment-specific genes (Fig. 3B), indicates that bacterial metabolic perturbation modulates the quantitative deployment of regulatory programs rather than generating a qualitatively distinct transcriptional identity. This pattern is consistent with transcriptional buffering, wherein the plant immune and growth regulatory systems maintain directional coherence while adjusting response magnitude in proportion to signal intensity, as has been observed in contexts of partial PGPR colonization or attenuated bacterial metabolite production (Stringlis et al., 2018).

Functional enrichment analyses (Fig. 6; Fig. S6) further supported this interpretation. JS exposure coordinated pathways associated with energy metabolism, photosynthesis-antenna proteins (ko00196), and defense-related secondary metabolism, including sesquiterpenoid and diterpenoid biosynthesis (ko00909; ko00904) and plant-pathogen interaction (ko04626). MT exposure preserved engagement of many of these pathways but redistributed metabolic emphasis toward carbohydrate and lipid metabolism, with altered representation of phenylpropanoid biosynthesis (ko00940). As this pathway serves as a metabolic hub connecting lignin deposition, flavonoid accumulation, and salicylate biosynthesis integral to plant immunity and root-microbe communication (Vogt, 2010), the quantitative rather than qualitative shift in phenylpropanoid flux under MT likely reflects the altered carbon status of the bacterial partner following *sacX* disruption. Collectively, these results establish that bacterial metabolic state reshapes amplitude, coordination, and module weighting without reversing the directional polarity of plant regulatory responses.

Hormone-associated transcriptional programs provided mechanistic resolution of this redistribution (Table 3; Fig. S7). JS exposure promoted coordinated modulation of auxin-, GA-, CK-, and BR-related pathways alongside JA/ET-centered defense modules, consistent with the well-documented capacity of PGPR to simultaneously prime growth-associated phytohormone signaling and activate jasmonate cascades (Pozo et al., 2008). MT exposure retained induction of several auxin- and GA-associated transcripts but exhibited differential weighting of SA- and JA-related components, including altered expression of PR1, TGA-related genes, and LOX isoforms. This differential SA- versus JA-associated representation is consistent with known antagonistic and synergistic crosstalk between these pathways (Koornneef & Pieterse, 2008), and raises the possibility that bacterial metabolic configuration influences the hormonal pivot point at which growth-defense crosstalk is balanced. Although NAD⁺ has been implicated in SA-associated signaling through its role as a substrate for poly(ADP-ribose) polymerase (X. Zhang & Mou, 2009), the present data indicate redistribution of hormone-associated regulatory emphasis rather than direct causal linkage, suggesting that the growth-defense balance is plastically adjustable in response to microbial metabolic state.

Transcription factor analysis (Table 5) positioned this redistribution within an upstream regulatory framework. Increased representation of AP2/ERF-, GRAS-, Dof-, and MADS-box-associated families under MT, together with reduced NF-YA expression, reflects reconfiguration of transcriptional hierarchies rather

than isolated pathway shifts (Gutterson & Reuber, 2004; Müller & Munné-Bosch, 2015). GRAS-family proteins serve as nodes integrating gibberellin status, carbon availability, and symbiotic signaling (Hirsch et al., 2009; Torres-Galea et al., 2006), and their increased representation under MT may reflect altered hormonal balance consequent to changes in bacterial volatile profiles, consistent with qualitative VOC differences observed between JS and MT (Fig. S4). Dof transcription factors, recognized regulators of carbon-nitrogen metabolism and light-responsive gene expression (Skirycz et al., 2006; Yanagisawa, 2002), and MADS-box proteins, which coordinate developmental timing in integration with environmental cues (Theißen et al., 2016), further illustrate the breadth of upstream regulatory reorganization. Reduced NF-YA expression under MT is consistent with a dampened capacity for coordinating nitrogen-responsive and growth-associated transcriptional programs (Laloum et al., 2013). Because these TF families integrate hormone signaling, carbon-responsive regulation, and developmental coordination (Kong et al., 2023; Ma et al., 2024; M. Zhang et al., 2017), their redistribution provides a regulatory basis for the altered coupling among growth- and defense-associated modules observed under metabolically perturbed bacterial conditions.

Phenotypic outcomes were consistent with transcriptomic restructuring. Reduced shoot growth under MT paralleled attenuated transcriptional amplitude across growth-associated modules, while root architectural responses were largely preserved under both treatments (Fig. S2), suggesting that root-associated regulatory programs are less sensitive to bacterial metabolic reconfiguration than shoot growth pathways, as has been observed where PGPR volatile-mediated shoot promotion is dissociated from root colonization effects (Ryu et al., 2003; Zamioudis et al., 2013). Despite pronounced transcriptional redistribution, antifungal activity remained robust and comparable to JS (Fig. S3). This preservation demonstrates that perturbation of bacterial central metabolism through *nadB* and *sacX* disruption does not abolish core biocontrol functionality, and supports a model in which antifungal efficacy represents a constitutive or threshold-stable functional module buffered against fluctuations in bacterial redox and carbon flux, consistent with the regulatory robustness conferred by GacS/GacA, Spo0A, and quorum-sensing networks governing lipopeptide and polyketide biosynthesis in PGPR (Bais et al., 2006; Chowdhury et al., 2013; Raaijmakers & Mazzola, 2012). The decoupling between reduced growth-promoting amplitude and preserved antifungal performance indicates that microbial metabolic architecture influences regulatory amplitude without redefining categorical functional identity, and that resilience in antifungal output may further reflect redundancy in secondary metabolite production pathways or maintenance of ISR-associated signaling capacity through jasmonate-dependent pathways despite SA-module redistribution (Niu et al., 2011; Van Der Ent et al., 2008).

Several limitations merit consideration. Additional mutations present in the MT genome beyond *nadB* and *sacX* (Table 1) may contribute to the observed phenotypes, and their individual contributions remain to be resolved through targeted mutagenesis and complementation analysis. VOC profiling was qualitative (Fig. S4), and quantitative GC-MS analysis will be necessary to directly associate bacterial metabolic perturbation with altered volatile emission dynamics, given that even small quantitative shifts in bacterial volatile blends can produce qualitatively distinct plant transcriptional responses (Blom et al., 2011; Ryu et al., 2003). Moreover, transcript abundance does not necessarily correspond to protein activity or metabolic

flux, underscoring the need for integrative multi-omics validation including proteomics and metabolomics in future studies.

These findings carry significant practical implications that extend PGPR research beyond a strain-centric framework. In agricultural environments characterized by fluctuating nutrient availability or abiotic constraints that impose metabolic stress on soil bacteria, strains retaining disease-suppressive capacity under metabolic perturbation may provide more consistent protective effects than strains whose biocontrol activity depends on metabolically demanding biosynthetic programs. The observation that beneficial microbial functions are metabolically tunable outputs, rather than binary traits, underscores the importance of incorporating microbial metabolic profiling, including redox balance, NAD pool status, and carbon catabolite repression state, as selection criteria alongside strain identity in next-generation bioinoculant development (Backer et al., 2018; Compant et al., 2019). Collectively, the present findings establish microbial metabolic architecture as a quantitative modulatory parameter that reshapes the amplitude and coordination of plant regulatory networks without altering their directional polarity, conceptually analogous to the way in which plant nutritional and hormonal status gates the amplitude rather than the direction of immune and developmental responses (Huot et al., 2014). Recognition of this metabolic dimension positions microbial metabolic optimization as an actionable and rational principle for engineering resilient plant-microbe partnerships in sustainable agriculture.

Table 1
Single-nucleotide insertion mutations identified in the genome of *Bacillus subtilis* JS following UV-C irradiation

Position	Gene	Coding/ noncoding	Description	Change
2644385	-	unknown	hypothetical protein	TCCCC -> TCCCCC
2667843	-	Coding	permease	CGG > CGGG
2710888	nadB	Coding	L-aspartate oxidase	GCCCCC > GCCCCCC
3800380	sacX	Coding	PTS system sucrose transporter subunit IIBC	CA > CAA

Table 2
RNA-seq read statistics and mapping efficiency for Mock, JS, and MT treatments.

Group	Sample Name	Raw Reads	Clean Reads	Mapped Reads (%)	Uniquely Mapped (%)
1	Mock (Rep 1)	76,277,278	53,975,530	53,837,761 (99.74)	38,302,216 (70.96)
	Mock (Rep 2)	80,270,914	48,159,666	48,093,938 (99.86)	44,117,511 (91.61)
	Mock (Rep 3)	72,557,046	56,314,520	56,204,605 (99.80)	51,419,501 (91.31)
2	JS (Rep 1)	88,422,092	64,259,902	64,120,683 (99.78)	60,198,646 (93.68)
	JS (Rep 2)	87,937,026	64,459,866	64,327,978 (99.80)	60,192,953 (93.38)
	JS (Rep 3)	88,859,304	65,561,478	65,417,181 (99.78)	61,202,688 (93.35)
3	MT (Rep 1)	85,979,758	63,650,394	63,538,384 (99.82)	59,489,597 (93.46)
	MT (Rep 2)	89,032,848	66,685,892	66,573,611 (99.83)	62,107,285 (93.13)
	MT (Rep 3)	88,944,496	66,953,546	66,830,862 (99.82)	62,457,532 (93.28)

Table 3
Hormone-related genes differentially expressed in *Nicotiana benthamiana* in response to JS and MT treatments.

Gene ID	Accession No.	Gene symbol	Description	log ₂ FC	
				JS	MT
g1921.t1	XP_016485842.1	<i>GH3.6</i>	GRETCHEN HAGEN 3.6	7.31	7.54
g39842.t1	XP_059294911.1	<i>IAA29</i>	auxin-responsive protein IAA29	6.01	6.16
g43253.t1	XP_009599501	<i>SAUR68</i>	auxin-responsive protein SAUR68-like	-	5.98
g5199.t1	XP_009760922.1	<i>SAUR21</i>	auxin-responsive protein SAUR21-like	-	5.27
g29224.t1	XP_009758646.1	<i>SAUR50</i>	auxin-responsive protein SAUR50-like	5.66	-
g96.t1	XP_009759020.1	<i>SAUR50</i>	auxin-responsive protein SAUR50-like	5.62	-
g5192.t1	XP_015058931.1	<i>IAA15</i>	auxin-induced protein 15A-like	2.04	1.68
g4102.t1	XP_009592396.1	<i>SAUR36</i>	auxin-responsive protein SAUR36-like	2.71	-
g60.t1	XP_016479066.2	<i>AUX1</i>	auxin transporter-like protein 4	-1.88	-2.17
g28276.t1	NP_001312726.1	<i>MPK12</i>	mitogen-activated protein kinase 9-like	-	7.46
g5319.t1	XP_016452988.1	<i>PP2C24</i>	putative protein phosphatase 2C 24	-1.52	-2.62
g130.t1	NP_001334589.1	<i>CP15A</i>	cysteine proteinase 15A-like precursor	-1.74	-2.33
g31745.t1	XP_016445091.1	<i>PP2C51</i>	protein phosphatase 2C 51	-1.07	-2.09
g29874.t2	NP_001307367.1	<i>DRM1</i>	auxin repressed/dormancy associated protein	2.80	2.96
g2966.t1	XP_009598799.1	<i>ARF18</i>	auxin response factor 18-like	-2.14	-1.78
g29874.t1	NP_001307367.1	<i>DRM1</i>	auxin repressed/dormancy associated protein	2.16	2.00
g43847.t1	XP_009594361.1	<i>GASA1</i>	gibberellin-regulated protein 1-like	-2.02	-1.97
g44752.t1	PHU24083.1	<i>GASA6</i>	Gibberellin-regulated protein 6	-2.38	-2.92
g25128.t1	XP_009602219.1	<i>PIF4</i>	transcription factor PIF4	2.04	1.90
g7924.t1	XP_009793839.1	<i>ACO5</i>	1-aminocyclopropane-1-carboxylate oxidase 5-like	8.42	7.75
g33212.t1	XP_009593567.1	<i>ACO1/ACO2</i>	1-aminocyclopropane-1-carboxylate oxidase-like	8.78	6.92

Gene ID	Accession No.	Gene symbol	Description	log ₂ FC	
				JS	MT
g36454.t1	XP_070037365.1	<i>H6H</i>	hyoscyamine 6-dioxygenase-like	-7.20	-7.17
g22515.t1	XP_009593567.1	<i>ACO1/ACO2</i>	1-aminocyclopropane-1-carboxylate oxidase-like	2.15	1.56
g7190.t1	NP_001312570.2	<i>ERF1B</i>	ethylene-responsive transcription factor 1B-like	3.05	2.30
g23630.t1	NP_001312416.1	<i>CAB13</i>	chlorophyll a-b binding protein 13, chloroplastic	1.93	2.04
g15250.t1	NP_001312259.1	<i>GA2ox1</i>	gibberellin 2-beta-dioxygenase 1-like	-	6.74
g29666.t1	NP_001312509.1	<i>GA2ox</i>	gibberellin 2-beta-dioxygenase-like	6.82	6.50
g5456.t1	XP_009781853	<i>GA2ox8</i>	gibberellin 2-beta-dioxygenase 8	2.30	2.14
g23374.t1	XP_009589948.1	<i>ABR1</i>	ethylene-responsive transcription factor ABR1-like	-3.94	-3.65
g34192.t1	XP_009606951.1	-	ethylene-responsive transcription factor ABR1-like isoform X1	-8.10	-8.06
g29839.t1	XP_016487471.1	<i>PTI5</i>	pathogenesis-related genes transcriptional activator PTI5-like	2.87	2.37
g13968.t1	NP_001312457.1	<i>SAMT1</i>	salicylate carboxymethyltransferase-like	5.98	6.83
g21044.t2	XP_016485811.1	<i>TGA9</i>	transcription factor TGA9-like isoform X2	7.48	7.17
g31.t1	XP_009787159.1	<i>PR1</i>	basic form of pathogenesis-related protein 1-like	2.42	-
g38527.t1	XP_009774215.1	<i>LOX5</i>	probable linoleate 9S-lipoxygenase 5	7.46	-
g993.t1	XP_075086986.1	<i>LOX6</i>	lipoxygenase 6, chloroplastic isoform X1	6.65	6.88
g29598.t1	NP_001306251.1	<i>SSR2</i>	sterol side chain reductase 2	-7.20	-
g19400.t1	XP_016474761.2	<i>PMT5</i>	putative methyltransferase PMT5	-	-11.38
g20433.t1	XP_009623473.1	<i>ARR17</i>	two-component response regulator ARR17	2.11	1.50
g31214.t2	XP_009796867.1	<i>ARR6</i>	two-component response regulator ARR6-like	6.63	-

Table 4

Differentially expressed genes associated with secondary metabolism in *Nicotiana benthamiana* following JS and MT treatments.

Gene ID	Accession No.	Gene symbol	Description	log ₂ FC	
				JS	MT
g15511.t1	XP_009798564.2	GGPPS SSU2	heterodimeric geranylgeranyl pyrophosphate synthase small subunit 2, chloroplastic-like	-7.02	-
g29598.t1	NP_001306251.1	SSR2	sterol side chain reductase 2	-7.20	-
g23578.t1	NP_001413014.1	EAS	5-epi-aristolochene synthase 3	4.37	3.24
g10274.t1	NP_001413014.1	EAS	5-epi-aristolochene synthase 3	3.08	2.53
g4524.t1	NP_001413014.1	EAS	5-epi-aristolochene synthase 3	7.99	7.53
g22734.t1	NP_001413014.1	EAS	5-epi-aristolochene synthase 3	3.70	2.84
g10258.t1	XP_019253144.1	EAS	epi-aristolochene synthase 110	2.16	1.86
g22535.t1	XP_009791335.1	TPS3	(-)-camphene/tricyclene synthase, chloroplastic-like isoform X1	6.68	-
g32275.t1	XP_075082056.1	At3g50280	putative acetyltransferase At3g50280	2.66	2.55
g6357.t1	XP_009762834.1	MYB61	transcription factor MYB61-like	6.62	6.98
g29267.t1	XP_009798858.1	bHLH110	transcription factor bHLH110-like	-	6.90
g21330.t1	XP_009609673.1	MYP46	transcription factor MYB46-like	-	6.99
g43785.t1	XP_016507036.1	VS	vinorine synthase-like	-	6.56
g33830.t2	XP_009782740.1	CCR1	cinnamoyl-CoA reductase 1-like	-	3.88
g21737.t1	XP_009762834.1	MYB61	transcription factor MYB61-like	-	6.58
g25128.t1	XP_009602219.1	PIF4	transcription factor PIF4	2.04	1.90
g24953.t1	NP_001312867.1	BEBT	benzyl alcohol O-benzoyltransferase	2.41	1.47
g16783.t1	NP_001312833.1	RAX2	transcription factor RAX2-like	7.33	7.10
g45623.t1	XP_070033425.1	BEAT	acetyl-CoA-benzylalcohol acetyltransferase-like	6.51	-
g3753.t1	XP_075082056.1	At3g50280	putative acetyltransferase At3g50280	6.93	-
g42383.t1	NP_001312656.1	KCS20	3-ketoacyl-CoA synthase 20-like	-2.02	-2.44
g23950.t1	NP_001312656.1	KCS20	3-ketoacyl-CoA synthase 20-like	-2.00	-2.43
g22038.t1	NP_001312700.1	PPOE	polyphenol oxidase E, chloroplastic-like	-2.28	-

Gene ID	Accession No.	Gene symbol	Description	log ₂ FC	
				JS	MT
g28871.t1	XP_0097777704.1	KCS6	3-ketoacyl-CoA synthase 6	-2.10	-2.30
g24880.t1	XP_019238731.1	MYB15	transcription factor MYB15-like	-2.47	-
g27577.t1	XP_0097777704.1	KCS6	3-ketoacyl-CoA synthase 6-like	-2.13	-2.61
g5219.t2	XP_019246582.1	bHLH63	PREDICTED: transcription factor bHLH63-like	-2.77	-
g7134.t1	XP_049407119.1	KCS20	3-ketoacyl-CoA synthase 20-like	-2.16	-3.11
g20246.t1	XP_075076252.1	HCT	shikimate O-hydroxycinnamoyltransferase-like	-6.83	-6.79
g2306.t1	XP_049407119.1	KCS20	3-ketoacyl-CoA synthase 20-like	-1.66	-2.34
g23099.t1	XP_055821788.1	ADH4	alcohol dehydrogenase-like 4	-	-2.14
g44634.t1	NP_001312657.1	MYB4	myb-related protein Myb4-like	-	-6.94
g6075.t1	NP_001312606.1	CYP74D3	divinyl ether synthase CYP74D3	9.36	8.66
g23862.t1	XP_070016465.1	CYP749A22	cytochrome P450 CYP749A22-like	-	6.92
g1567.t1	XP_016456046.1	KA02	ent-kaurenoic acid oxidase 2-like isoform X1	6.78	7.04
g41113.t1	XP_016483719.2	CYP707A2	abscisic acid 8'-hydroxylase CYP707A2-like	7.53	6.51
g31372.t1	KAJ8553992.1	-	hypothetical protein K7X08_024670	-2.16	-1.14
g31373.t1	KAJ8553992.1	-	hypothetical protein K7X08_024670	-2.24	-1.13
g26300.t1	XP_016466139.1	CYP77A1	cytochrome P450 77A1-like	-2.75	-2.66
g22324.t1	NP_001275219.1	CYP86A33	CYP86A33 fatty acid omega-hydroxylase	-2.27	-2.74
g10979.t1	XP_009762653.1	CYP94B3	cytochrome P450 94B3-like	-2.83	-1.74
g30036.t1	XP_016466139.1	CYP77A2	LOW QUALITY PROTEIN: cytochrome P450 77A2-like	-2.29	-2.48
g29316.t1	NP_001266259.2	CYP90B3	cytochrome P450 90B3	-2.06	-1.58
g44728.t1	NP_001313000.1	CYP73A5	cytochrome P450 CYP73A100-like	-2.12	-2.01
g43562.t1	NP_001312976.1	CYP82E4	nicotine N-demethylase CYP82E4	-6.19	-10.89
g6803.t1	XP_016502493.2	CYP78A6	cytochrome P450 78A6-like	-7.16	-
g260.t1	XP_016483719.2	CYP707A2	abscisic acid 8'-hydroxylase CYP707A2-like	-6.85	-

Gene ID	Accession No.	Gene symbol	Description	log ₂ FC	
				JS	MT
g4651.t1	NP_001275219.1	<i>CYP86A33</i>	CYP86A33 fatty acid omega-hydroxylase	-1.66	-2.11
g658.t1	NP_001275219.1	<i>CYP86A33</i>	CYP86A33 fatty acid omega-hydroxylase	-1.80	-2.07
g45163.t1	XP_016462768.2	<i>CYP71D20</i>	premnaspirodien oxygenase	-1.86	-2.14
g826.t1	NP_001311496.1	<i>CYP98A2</i>	cytochrome P450 98A2-like	-1.40	-2.20
g44516.t1	XP_016444031.1	<i>CYP94C1</i>	cytochrome P450 94C1	-2.49	-1.53
g37612.t1	XP_009765329.1	<i>UGT74E2</i>	UDP-glycosyltransferase 74E2-like	2.64	2.15
g6375.t1	WIW42767.1	<i>UGT family</i>	UDP-glycosyltransferase	7.56	7.43
g7341.t1	UHH90483.1	<i>UGT family</i>	UDP-glycosyltransferase	2.67	1.68
g24111.t1	WIW42654.1	<i>UGT family</i>	UDP-glycosyltransferase	3.03	1.28
g9469.t1	XP_009777953.1	<i>GT7</i>	putative glycosyltransferase 7 isoform X1	-2.04	-1.70
g19281.t1	WIW42715.1	<i>UGT family</i>	UDP-glycosyltransferase	-2.54	-2.21

Table 5
Differentially expressed transcription factors distinguishing MT from JS treatments in *Nicotiana benthamiana*.

Gene ID	Description	Accession No.	TF Family	Domain	InterPro IDs	Log ₂ FC
g2895.t1	ethylene-responsive transcription factor ERF017	XP_016443748.1	AP2/ERF	AP2/ERF	IPR001471 (PFAM)	2.32
g3221.t1	ethylene-responsive transcription factor ERF017-like	XP_009613570.1	AP2/ERF	AP2/ERF	IPR001471 (PFAM)	2.13
g13483.t1	protein SCARECROW-like	XP_009615228.1	GRAS	GRAS	IPR005202 (PFAM); PF16635 (PFAM)	7.72
g36702.t1	PREDICTED: dof zinc finger protein DOF3.4-like isoform X2	XP_019243796.1	Dof	Zf-Dof-type	IPR003851 (PFAM); PF14354 (PFAM)	6.54
g31416.t1	agamous-like MADS-box protein AGL11 isoform X2	XP_019267507.1	MIKC_MADS	MADS-box domain, K-box domain	IPR002100 (PFAM); IPR002487 (PFAM); IPR021279 (PFAM); IPR009579 (PFAM)	6.45
g45870.t1	ethylene-responsive transcription factor ERF003-like	XP_009771954.1	AP2/ERF	AP2/ERF	IPR001471 (PFAM)	6.42
g39000.t2	nuclear transcription factor Y subunit A-2-like isoform X4	XP_070011954.1	NF-YA	-	IPR001289 (PFAM)	-8.48
g13430.t2	uncharacterized protein LOC107768790 isoform X1	XP_016443427.1	NF-YA	-	IPR001289 (PFAM)	-2.49

Abbreviations

JS

Bacillus subtilis JS

MT
Bacillus subtilis JS mutant
PGPR
Plant growth-promoting rhizobacteria
UV
Ultraviolet
NAD
Nicotinamide adenine dinucleotide
VOC
Volatile Organic Compound
GC-MS
Gas chromatography-mass spectrometry
ISR
Induced systemic resistance
IAA
Indole-3-acetic acid
GA
Gibberellin
CK
Cytokinin
ET
Ethylene
BR
Brassinosteroid
SA
Salicylic acid
JA
Jasmonic acid
ABA
abscisic acid
TF
Transcription factor

Declarations

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Author Contribution

Seung-A Kim conceived and designed the study, performed the experiments, analyzed and interpreted the data, and drafted the manuscript. Jihyun F. Kim contributed to data analysis and interpretation and assisted with manuscript editing. Soohyun Oh assisted with experiments and data acquisition. Beom Seok Kim and Eui-Hwan Chung provided resources and contributed to technical support and interpretation of results. Seungill Kim contributed to study design and supervised parts of the work. Sun-Hyung Kim supervised the study, acquired funding, and critically revised the manuscript. All authors reviewed and approved the final manuscript. Sun-Hyung Kim is the corresponding author.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

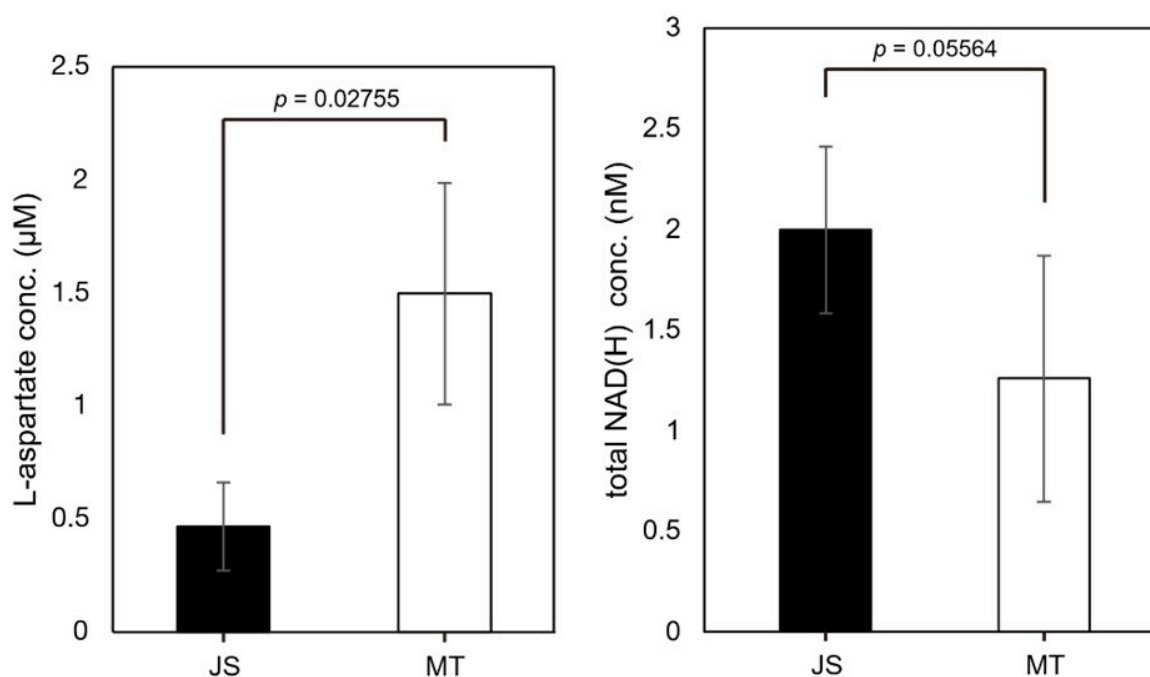


Fig. 1.

Figure 1

Quantification of L-aspartate and NAD(H) in *Bacillus subtilis* JS and UV-induced mutant cultures.

Supernatant levels of L-aspartate and total NAD(H) were quantified in *B. subtilis* JS (JS) and the UV-induced mutant strain (MT). Data are presented as mean $\pm$ standard deviation (SD) from three independent biological replicates per strain ($n = 3$). Statistical significance between strains was assessed using a two-sided Welch's two-sample *t*-test.

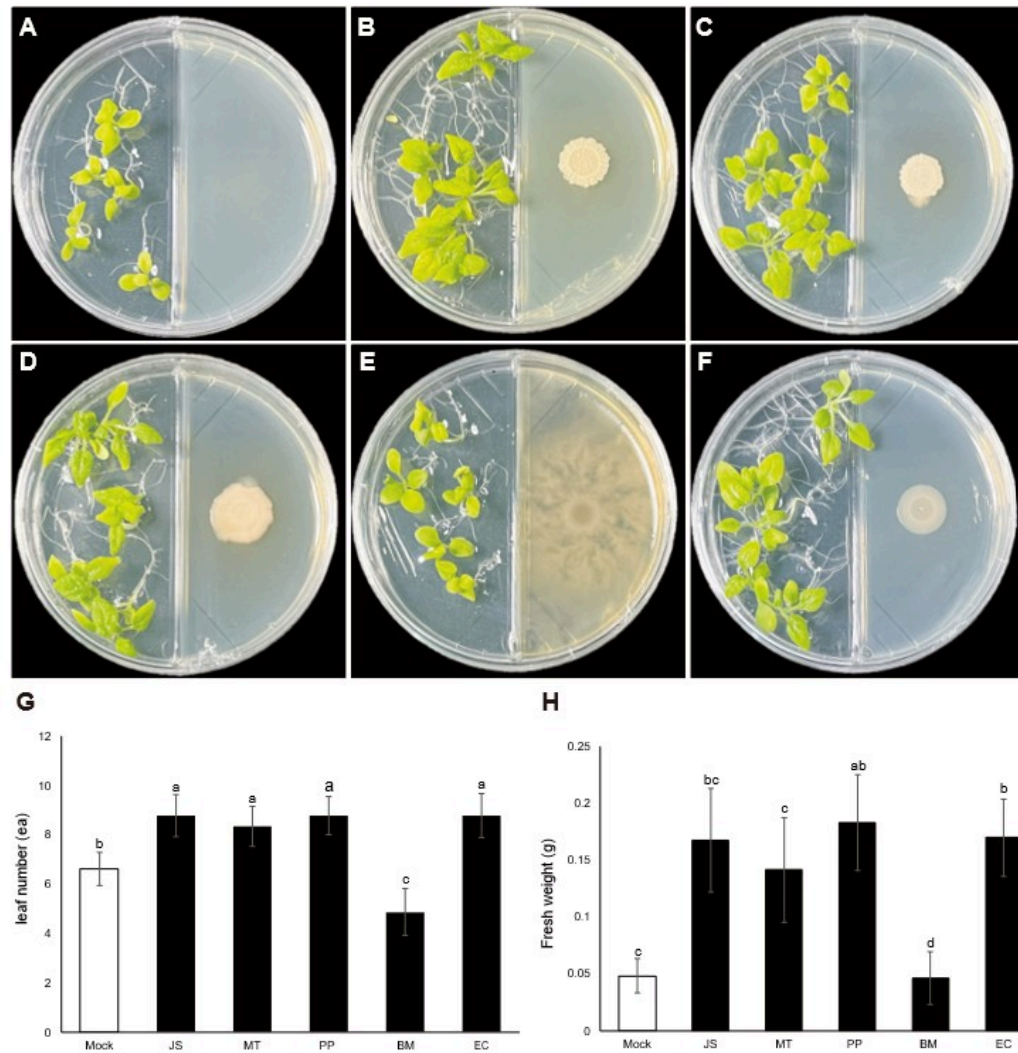


Fig. 2.

Figure 2

Volatile-mediated growth promotion of *Nicotiana benthamiana* by *Bacillus subtilis* JS and related bacterial strains. Representative images of *N. benthamiana* seedlings grown in a split-plate under different bacterial treatments: (A) Mock control, (B) *B. subtilis* JS (JS), (C) UV-induced *B. subtilis* JS mutant (MT), (D) *Pseudomonas putida* (PP), (E) *B. megaterium* (BM), and (F) *Escherichia coli* (EC). Quantitative analyses of (G) leaf number and (H) fresh biomass are shown for each treatment. Data represent the mean $\pm$ standard error (SE) from 40 biological replicates per treatment ($n = 40$). Different letters (a–d) indicate statistically significant differences among treatments, whereas groups sharing the same letter are not significantly different, as determined by Tukey's honestly significant difference (HSD) test ($p < 0.05$).

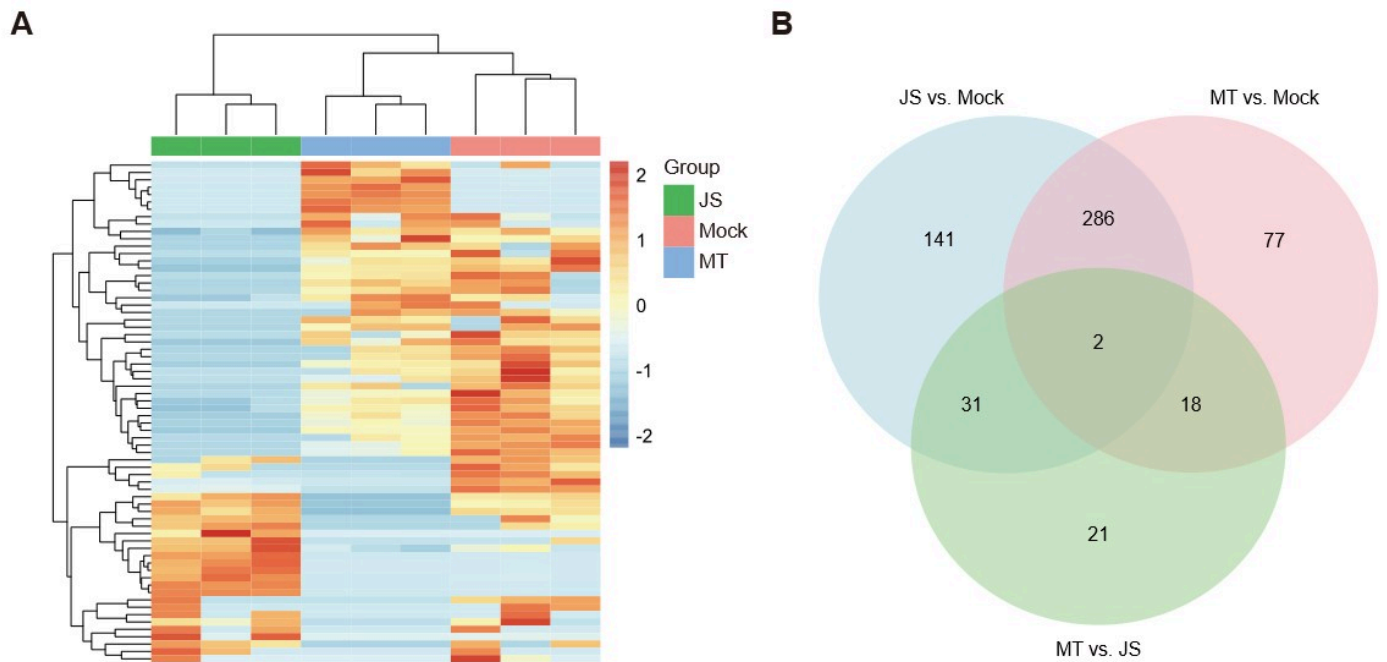


Fig. 3.

Figure 3

Differential gene expression patterns induced by Mock, JS, and MT treatments in *Nicotiana benthamiana*. (A) Heatmap showing hierarchical clustering of differentially expressed genes (DEGs) identified among Mock-, *B. subtilis*JS- (JS), and UV-induced JS mutant (MT)-treated plants. Genes with an absolute $\log_2$ fold change ($|\log_2\text{FC}| \geq 3$) and adjusted p-value (padj) < 0.05 were selected, and normalized expression values were Z-score-transformed prior to clustering. The color scale represents relative expression levels across samples. (B) Venn diagram illustrates the overlap and uniqueness of DEGs identified in pairwise comparisons among Mock, JS, and MT treatments, highlighting both shared and treatment-specific transcriptional responses.

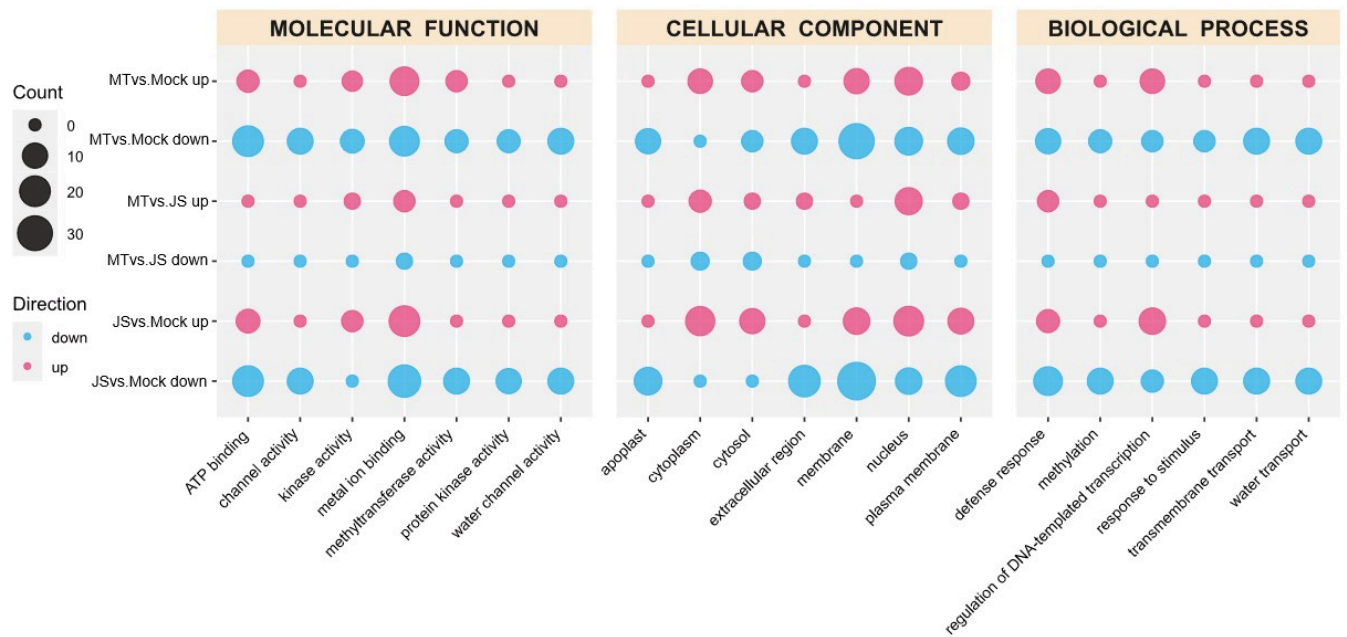


Fig. 4.

Figure 4

Gene Ontology (GO) enrichment analysis of differentially expressed genes in response to bacterial treatments. Bubble plots showing the top 20 significantly enriched Gene Ontology (GO) terms across the three major GO categories: Molecular Function, Cellular Component, and Biological Process. Enrichment analysis was performed using differentially expressed genes identified under Mock, *Bacillus subtilis* JS (JS), and UV-induced JS mutant (MT) treatments. Bubble size represents the number of genes associated with each GO term, while color indicates the direction of regulation (up- or down-regulated). Only GO terms meeting the statistical significance threshold (adjusted p-value < 0.05) are shown.

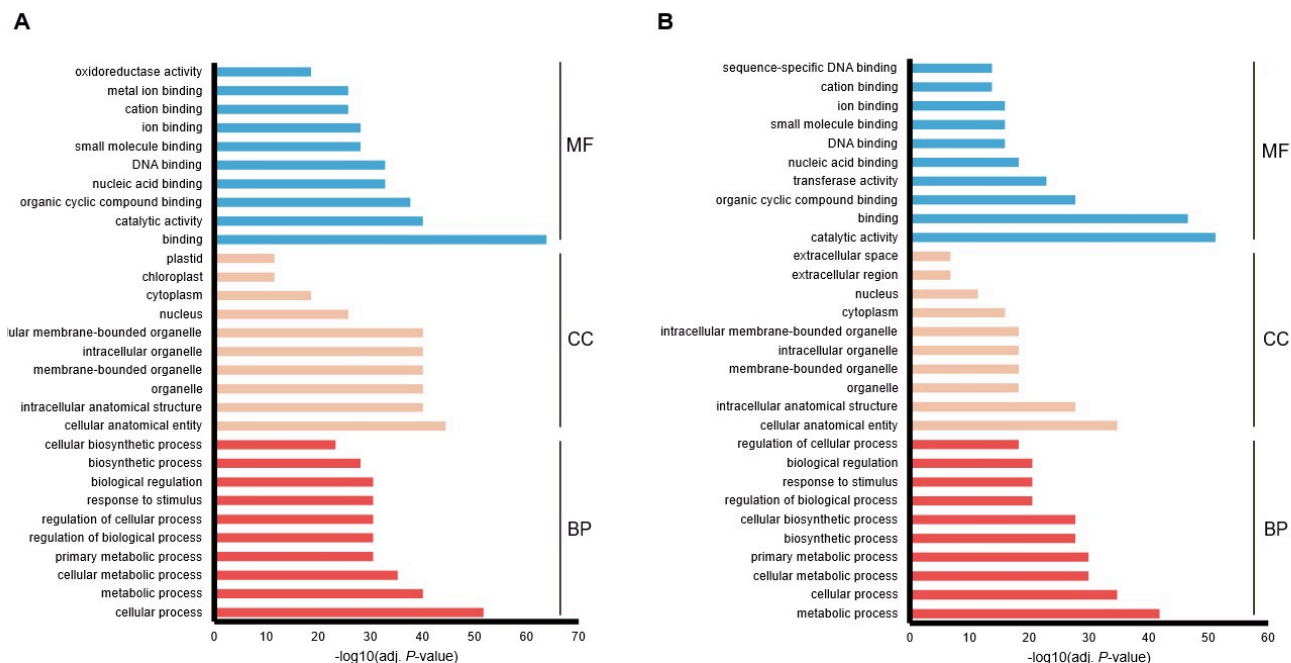


Fig. 5.

Figure 5

Gene Ontology (GO) enrichment analysis of differentially expressed genes between UV-induced *Bacillus subtilis* JS mutant (MT)– and JS-treated *Nicotiana benthamiana* plants. **A**, GO terms enriched among genes up-regulated in MT relative to JS ($\log_2$ fold change > 2, FDR < 0.05). **B**, GO terms enriched among genes down-regulated in MT relative to JS ($\log_2$ fold change < -2, FDR < 0.05). GO terms are grouped into Molecular Function (MF), Cellular Component (CC), and Biological Process (BP) categories. Bars represent enrichment scores for each GO term, highlighting functional pathways differentially modulated by MT compared with JS.

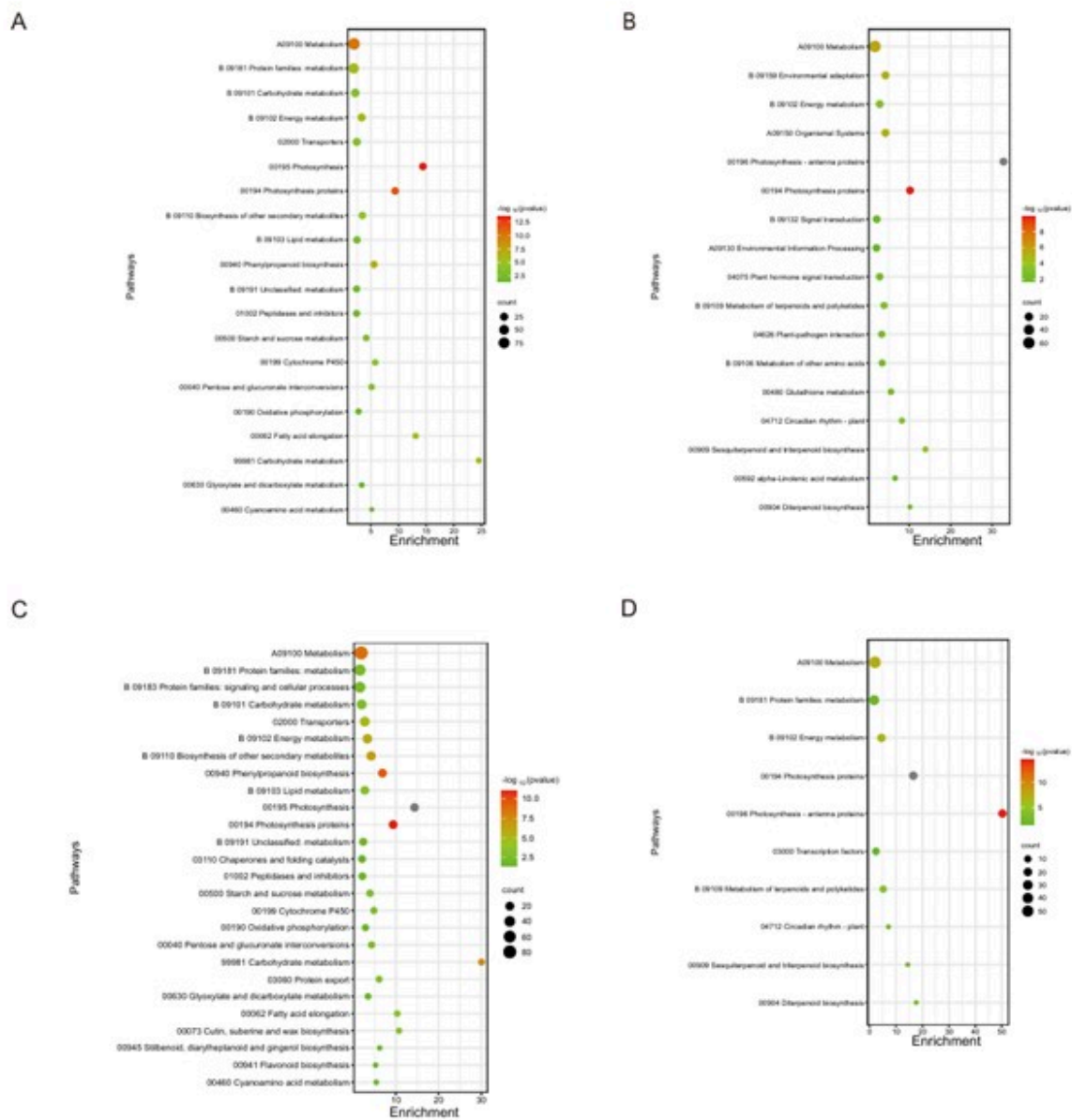


Figure 6

KEGG pathway enrichment analysis reveals distinct metabolic and signaling programs induced by JS and MT treatments. KEGG pathway enrichment analysis of differentially expressed genes (DEGs) identified in pairwise comparisons between bacterial treatments and the Mock control. **A**, Down-regulated pathways in JS vs. Mock, **B**, up-regulated pathways in JS vs. Mock, **C and D**, down-regulated pathways in MT vs. Mock, and up-regulated pathways in MT vs. Mock. Each dot represents an enriched KEGG pathway; dot size corresponds to the number of DEGs associated with the pathway, and dot color indicates statistical significance expressed as $-\log_{10}(\text{adjusted } p)$. Only pathways meeting the significance threshold (adjusted $p < 0.05$) are shown.

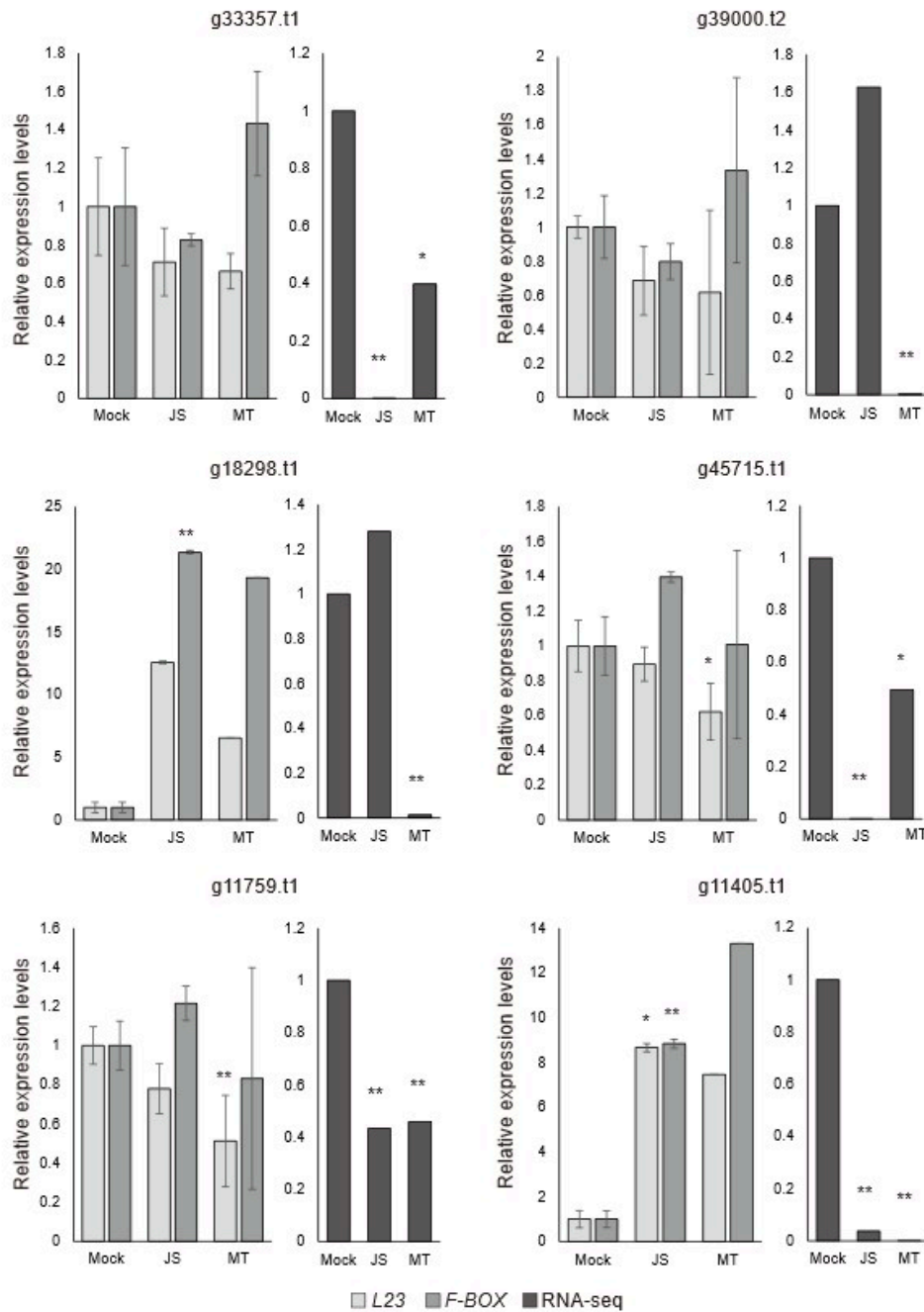


Fig. 7.

Figure 7

Validation of RNA-seq results by qRT-PCR analysis of selected genes. Comparison of relative expression levels determined by RNA-seq and quantitative real-time PCR (qRT-PCR) for six representative genes involved in photosynthesis, redox regulation, stress response, defense, and transcriptional control. Gene expression levels were measured in *Nicotiana benthamiana* leaves treated with Mock, *Bacillus subtilis* JS (JS), or the UV-induced mutant (MT). qRT-PCR analyses were performed using three independent biological

replicates, each analyzed in triplicate. Relative transcript abundance was calculated using the $2^{-\Delta\Delta C_t}$ method, with **L23** and **F-BOX** used as internal reference genes. RNA-seq expression values are shown alongside qRT-PCR results for direct comparison. Data are presented as mean $\pm$ SD. Asterisks indicate statistically significant differences relative to the Mock control. *, $P \leq 0.05$; **, $P < 0.01$.

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