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Research Article

Keywords: *Pseudomonas ceruminis*, complete genome, Plant growth-promoting rhizobacteria (PGPR), Transcriptome analysis

Posted Date: October 16th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10989577/v1>

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Additional Declarations: No competing interests reported.

Complete genome of ‘*Pseudomonas ceruminis*’ PGEL-SA, a *Nicotiana benthamiana* rhizosphere isolate, and its effects on plant growth

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Abstract

Plant growth-promoting rhizobacteria (PGPR) modulate plant growth and defense through diverse pathways. ‘*Pseudomonas ceruminis*’ is a lineage recently proposed based on genome-based analyses, but no complete genome-based identification or functional characterization has yet been reported. Here we sequenced the complete genome of strain PGEL-SA, isolated from rhizosphere soil of *Nicotiana benthamiana*, and analyzed its genome-based taxonomy, comparative genomic analysis, genome-encoded plant growth-promoting traits and culture-supernatant volatile profile, while describing plant responses under physically separated coculture and under direct rhizosphere inoculation. Hybrid assembly yielded a single circular genome of 5,988,567 bp (G+C content 61.9%, 5,551 genes). PGEL-SA showed an ANI of 99.18% and a dDDH of 93.6% against ‘*P. ceruminis*’ BML-PP028^T and was assigned to this lineage. In I-plate coculture with a central partition, seedlings exposed to PGEL-SA showed significantly increased shoot fresh weight and leaf number, under an unpartitioned condition, lateral root number increased and primary root elongation was inhibited. SPME-GC-MS of the culture supernatant tentatively identified dimethyl disulfide (DMDS), methyl salicylate (MeSA) and 3-methyl-1-butanol. DMDS and MeSA did not reproduce the coculture phenotype and 3-methyl-1-butanol was only partially effective, so they are presented as candidate mediators. Transcriptome analysis under rhizosphere inoculations showed upregulation of translation- and protein-biosynthesis-related genes, downregulation of sulfate transport genes and upregulation of glutathione and methionine biosynthesis genes. This

study provides a complete genomic resource for the '*P. ceruminis*' lineage, expands the known habitat range of this lineage beyond clinical settings, and establishes its function as a PGPR.

Keywords: '*Pseudomonas ceruminis*'; complete genome; Plant growth-promoting rhizobacteria (PGPR); Transcriptome analysis

Abbreviations

PGPR: Plant Growth-Promoting Rhizobacteria

WGS: Whole Genome Sequencing

ANI: Average Nucleotide Identity

dDDH: digital DNA-DNA Hybridization

VOCs: Volatile Organic Compounds

SPME-GC-MS: Solid-Phase MicroExtraction-Gas Chromatography-Mass Spectrometry

DMDS: Dimethyl Disulfide

MeSA: Methyl Salicylate

Introduction

Plant growth-promoting rhizobacteria (PGPR) are widely used as environmentally friendly biological control agents. Diverse bacteria are known, including *Bacillus*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, and *Pseudomonas* spp., which enhance plant health through complex actions in the soil (Irfan et al., 2026). PGPR fix nitrogen and convert it into plant-available forms (Kuan et al., 2016; Malik et al., 1997; Yadegari et al., 2010), and produce siderophores, iron-chelating compounds that supply plant-available iron even in iron-deficient soils (Kloepper

et al., 1980). They can also directly produce phytohormones, such as indole-3-acetic acid (IAA), promoting root elongation and lateral root formation (Spaepen et al., 2007). They may directly suppress the activity of neighbouring plant pathogenic microorganisms by producing antimicrobial compounds or lytic enzymes (Thomashow et al., 1990; Whipps, 2001), and even without direct antimicrobial action they can confer resistance against pathogenic microorganisms by inducing induced systemic resistance (ISR) and systemic acquired resistance (SAR) (Pieterse et al., 2014; Van Loon et al., 1998). PGPR can also communicate with plants without direct contact by producing volatile organic compounds (VOCs). Ryu et al. (2003) first demonstrated that 2,3-butanediol and acetoin synthesized by *Bacillus subtilis* GB03 promote plant growth, and subsequent studies have shown that PGPR-produced VOCs benefit plant health, including suppression of plant pathogens and induction of ISR (Frag et al., 2013; J. S. Kim et al., 2015, 2017; Ryu et al., 2003; Wu et al., 2019). The genus *Pseudomonas* belongs to the family Pseudomonadaceae and is one of the largest genera of Gram-negative bacteria as well as one of the best-studied bacterial genera (Lalucat et al., 2022). According to the List of Prokaryotic names with Standing in Nomenclature (LPSN), more than 650 species have been described, making it one of the groups with the greatest species diversity among currently known bacterial genera (Peix et al., 2018). In particular, species such as *P. fluorescens* and *P. aeruginosa* are well known as PGPR that enhance root development, nutrient utilization, stress tolerance, and disease resistance (Chopra et al., 2020; Santoyo et al., 2025). However, many closely related species within the genus *Pseudomonas* are often not clearly distinguished by 16S rRNA sequence similarity alone, and identification based on traditional molecular markers therefore has limitations (Yarza et al., 2014). Accordingly, whole genome sequencing (WGS)-based average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) analyses have become the standard methods for precise species-level identification (Goris et al., 2007; Yoon et al., 2017). Indeed, based on WGS-derived ANI and dDDH analyses, a strain previously misidentified as *P. putida* was renamed as '*P. ceruminis*' sp. nov. (BML-PP028^T) within the *P. putida* group (Tohya et al., 2022, 2023). However, this species name does not meet the requirements for valid publication and has not been validly published under the ICNP to date; it is listed in NCBI Taxonomy as effectively published but not validly published. In this paper, the species name is therefore written in single quotation marks and BML-PP028^T is described as the proposed type strain. Although recent genome-based approaches have clarified the taxonomic position of '*P. ceruminis*', little is known about the ecological role and functional potential of this species in plant-associated environments. In particular, for plant-derived or rhizosphere-associated '*P. ceruminis*' isolates, there are few, if any, cases in which genome structure, volatile organic compound (VOC) production, and plant growth-

promoting activity have been characterized. Additional whole-genome sequences and functional descriptions are therefore needed to better understand the biological potential of this species. In this study, we investigated strain PGEL-SA, isolated from rhizosphere soil of *N. benthamiana* grown in a plant growth facility at the University of Seoul, Republic of Korea.

Accordingly, in this study we sequenced the complete genome of PGEL-SA, analyzed its genome-based taxonomy, comparative genomic analysis, and VOC profile, and separately evaluated plant growth responses under PGPR inoculated condition. These results not only expand the genome database of the under-studied '*P. ceruminis*' but also provide an important basis for investigating their mechanisms of action.

Materials and Methods

Bacteria Isolation, 16S rRNA Analysis, and Whole genome De novo Sequencing of PGEL-SA

The bacterial strain was isolated from rhizosphere soil of *N. benthamiana* collected at a plant growth facility of the University of Seoul, Republic of Korea (37°35'00.47" N and 127°03'18.20" E). The isolate was cultured in Tryptic Soy Broth (TSB, MCell, Korea) at 26 °C with shaking at 160 rpm for 18 h; for long-term storage, cells were suspended to OD₆₀₀ = 0.5, supplemented with sterile glycerol (17.5%, v/v), transferred to cryogenic vials, and stored at -80 °C.

The 16S rDNA gene was amplified with PCR using universal primers 27F and 1492R. Genomic DNA extraction and sequencing were performed at Bionics (Seoul, Republic of Korea). The 16S rRNA gene sequences were aligned against the EzBioCloud database (<https://www.ezbiocloud.net>), and phylogenetic analysis was performed to further examine genotypic characteristics. Fifty strains showing high homology in a 16S rRNA BLASTN search were used for comparison. The tree was rooted using *Azotobacter chroococcum* LMG 8756 (NR_116305.1) as an outgroup to ensure accurate topological orientation. Sequences were aligned using ClustalW. A maximum likelihood tree was inferred with IQ-TREE 3 (v3.0.1; Wong et al., 2026), with the best-fit model (TPM3u+F+I+G4) selected by ModelFinder (Kalyaanamoorthy et al., 2017), and node support was assessed with 1,000 ultrafast bootstrap replicates (Hoang et al., 2018). To confirm consistency, neighbour-joining and maximum parsimony analyses were performed in parallel using MEGA12 (Kumar et al., 2024). The resulting phylogenetic tree was visualized using FigTree (v1.4.4;

<https://tree.bio.ed.ac.uk/software/figtree/>).

Whole genome sequencing of PGEL-SA, from DNA extraction to genome assembly, was performed at Macrogen (Seoul, Republic of Korea), using a hybrid strategy combining the Illumina and PacBio sequencing platforms. High-quality genomic DNA was first extracted, a TruSeq Nano DNA (350) library was constructed, and sequencing was performed on the NovaSeq X platform (Illumina, USA). In parallel, a PacBio Microbial Library was constructed and SMRT (Single Molecule, Real-Time) sequencing was performed. Assembly of the resulting PacBio long-read data was carried out using the Microbial Genome Analysis Application included in SMRT Link (v25.1.0.257715) (Chin et al., 2013). After the initial assembly step, Inspector (v1.0.1) was used to correct errors in the contig sequence and improve assembly accuracy (Chen et al., 2021). The Illumina short-read data produced above were then applied in Pilon (v1.22) for final sequence polishing (Walker et al., 2014). Genome assembly completeness was assessed by BUSCO (v5.1.3) analysis. Based on the genome map, coding gene, tRNA, rRNA, and tmRNA prediction was performed using Prokka (v1.14.6) software (Seemann, 2014). To characterize the functions of the predicted CDSs, functional annotation was performed using egg-NOG-mapper (v2.1.10) and the eggNOG database (v5.0.2) (Cantalapiedra et al., 2021; Huerta-Cepas et al., 2019), and protein domain and motif information was additionally obtained through InterProScan (v5.34-73.0) (Jones et al., 2014).

Genome-based taxonomic and comparative genomic analyses

The genomic similarity between strain PGEL-SA and eleven closely related *Pseudomonas* spp. was quantified using OrthoANI values with the OrthoANI Tool (OAT) (Yoon et al., 2017). dDDH values were calculated using the Genome-to-Genome Distance Calculator (GGDC 3.0) web server; because some of the compared strains are in draft status, formula 2, which is applicable to incompletely sequenced genomes, was used (<https://ggdc.dsmz.de/home.php>) (Meier-Kolthoff et al., 2013, 2022). The twelve strains were annotated using Prokka, and BLASTP-based gene-cluster comparative genomic analysis was performed using Roary (v3.12.0) (with the following flags: -e (create a multiFASTA alignment of core genes using PRANK), --mafft (core gene alignment with MAFFT), and -i 50 (minimum percentage identity for blastp; 50%)). Because this analysis includes multiple different *Pseudomonas* species rather than multiple isolates of a single species, a minimum identity of 50% was applied for interspecies comparison.

Genome-based prediction of plant growth-promoting traits

To search the complete genome for plant growth-promoting traits (PGPTs), the PGPT-Pred tool of the PlaBAs v1.02 web resource was used (Patz et al., 2021). The predicted protein sequences of PGEL-SA were queried against the PGPT ontology (PGPT-BASE) using the default blastp+hmmer approach (blastp v2.10.1+, HMMER v3.3). Protein sequences were aligned to the tool-specific protein databases, best hits were selected at an e-value threshold of 1e-5, and the presence of the corresponding PFAM domains was confirmed with HMMER. Annotated proteins were mapped to the hierarchical PGPT ontology, which comprises direct effects (biofertilization, phytohormone/plant-signal production, and bioremediation) and indirect effects (plant colonization, competitive exclusion, stress control/biocontrol, and plant immune response stimulation).

Qualitative screening of volatile compounds by SPME-GC-MS

To analyze the VOCs produced by PGEL-SA, gas chromatography-mass spectrometry (GC-MS) was performed. The strain was cultured in TSB medium, and the supernatant obtained after removal of bacterial cells was used as the analytical sample; the analysis was performed once on a single sample. To increase the extraction efficiency of volatile components, NaCl was added to a final concentration of 35%. Uninoculated TSB medium prepared under the same conditions was used as the control. VOCs were extracted by the solid phase microextraction (SPME) method. The sample was equilibrated at 40 °C for 10 min, and volatile components in the headspace were then adsorbed onto a DVB/CAR/PDMS fiber (50/30 µm) at 40 °C for 30 min. The extracted VOCs were analyzed using a GC-MS system (Agilent, USA). Separation was performed on an HP-5MS UI column (30 m × 0.25 mm × 0.25 µm) with a carrier gas flow rate of 1 mL/min. The GC oven temperature program was as follows: an initial hold at 40 °C for 2 min, a ramp to 245 °C at 8 °C/min, and a final hold at 245 °C for 3 min. MS analysis was performed in electron ionisation (EI) mode with an ionisation energy of 70 eV. The ion source temperature was maintained at 230 °C, and the mass scan range was set to 25-530 m/z. The detected compounds were tentatively identified by comparison with the NIST11 mass spectral library. Authentic standards and retention-index-based confirmation were not used. The relative content of each compound was expressed as the percentage (%) of the individual peak area relative to the total area of all detected peaks.

Plant growth assays under separated and unpartitioned coculture conditions

N. benthamiana Nb_KLAB seeds were provided by the Korea Research Institute of Bioscience & Biotechnology

(KRIBB), Daejeon, Korea. *N. benthamiana* seeds were sterilized in 70% (v/v) ethanol for 30 s and in 3 % (v/v) NaOCl solution for 90 s, washed five times with sterile distilled water, and plated on 1/2 MS medium (Murashige & Skoog Medium 2.205 g/L, sucrose 10 g/L, gelrite 3 g/L, pH 5.75). Seeds were germinated under a 16 h light/8 h dark cycle at 26 °C. For the I-plate assay, 1/2 MS medium was dispensed on one side and Tryptic Soy Agar (TSA, MCell, Korea) on the other, and five *N. benthamiana* seedlings were placed on the 1/2 MS side. On the TSA side, 10 µL of PGEL-SA culture was spot-inoculated. At 7 days after inoculation (DAI), leaf number and fresh weight were measured (3 plates, 15 plants in total). To examine root development, nine seedlings were placed on a square dish, and 10 µL of PGEL-SA was spot-inoculated onto filter paper. At 7 DAI, primary root growth and lateral root number in response to PGEL-SA treatment were measured (3 plates, 27 plants in total). In addition, to examine the effects of individual volatile compounds, dimethyl disulfide (DMDS), methyl salicylate (MeSA), and 3-methyl-1-butanol were diluted in ethanol and applied. Each compound was spotted onto filter paper as 10 µL at concentrations of 50 µM and 100 µM. As controls, a solvent control treated with an equal volume of ethanol (EtOH), an untreated control (Mock), and a live PGEL-SA treatment were included, and fresh weight and leaf number were measured (3 plates, 3 plants per plate; 9 plants in total).

Rhizosphere inoculation and RNA sequencing

'*P. ceruminis*' PGEL-SA was cultured in TSB medium at 26 °C to OD₆₀₀ = 0.5. The culture was centrifuged, and the pellet was resuspended in PBS buffer to obtain a final suspension of OD₆₀₀ = 0.1. 5 mL of this suspension was inoculated into the rhizosphere of *N. benthamiana* plants grown for 30 days in a growth chamber at 26 °C (16 h light/8 h dark) to allow stable establishment of the bacteria in the rhizosphere. The experimental groups were prepared as follows: (1) Mock, seedlings without any treatment; (2) PGEL-SA, seedlings with PGEL-SA inoculation. Samples from each group were collected 18 h and 96 h after treatment, and RNA-seq was performed with three replicates per group.

Total RNA was extracted from leaves of *N. benthamiana* Nb_KLAB plants using a modified CTAB method (S. H. Kim & Hamada, 2005). RNA was quantified using the Quant-iTTM RiboGreen RNA Assay Kit (Invitrogen, Carlsbad, CA, USA) and a Victor Nivo Multimode Microplate Reader (PerkinElmer, Waltham, MA, USA), and RNA quality was verified using a Bioanalyzer 2100 (Agilent, USA). RNA sequencing libraries were constructed using the TruSeq Stranded mRNA Library Prep Kit (Illumina, USA) according to the manufacturer's protocol, and paired-end

sequencing was performed on an Illumina NovaSeq X. Low-quality reads and adapter sequences were removed from the raw sequencing data using Trimmomatic. The cleaned reads were mapped to the *N. benthamiana* reference genome (Nb_KLAB) using HISAT2 (D. Kim et al., 2019). Mapped reads were assembled into transcripts using StringTie, and transcript abundance was obtained as raw read counts (Pertea et al., 2015). Normalization was performed using DESeq2 v1.44.0 in R. For functional annotation of each gene, orthology based functional annotation was performed using eggNOG-mapper (v2.1.12) and eggNOG database (v5.0.2), and GO and KEGG information for each gene was extracted from the annotation results.

Validation of gene expression by qRT-PCR

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). Seven genes with $|\log_2FC| \geq 2$ in PGEL-SA relative to Mock 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: $T_m = 59-61$ °C; GC = 45-55%; amplicon size = 130-200 bp (**Supplementary Table 1**). *F-box* gene was used as the internal reference gene, and relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. Reactions were performed with 3 biological replicates and three technical replicates under the following conditions: Initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 40 s, and extension at 72 °C for 20 s. Melt curve analysis was then performed from 72 °C to 95 °C at a ramp rate of 0.5 °C/s.

Antioxidant enzyme assays

To evaluate changes in plant redox homeostasis following PGEL-SA inoculation, catalase (CAT) and ascorbate peroxidase (APX) were quantified in the samples used for RNA-seq analysis (96 h after treatment). For enzyme extraction, plant leaf samples were snap-frozen in liquid nitrogen and ground with a mortar and pestle. Ground powder (0.5 g) was mixed with buffer (50 mM potassium phosphate buffer, pH 7.0; 1 mM EDTA; 1% (w/v) PVP), transferred to a 2.0 mL tube, and centrifuged at 12,000 rpm for 15 min in a centrifuge cooled at 4 °C. For the APX assay, the

extraction buffer was supplemented with 0.5 mM sodium ascorbate as an electron donor. Only the supernatant was collected and used immediately for the assays, and absorbance in each assay was measured using a UV-VIS spectrophotometer (OPTIZEN Alpha, K Lab Co., Ltd., Korea).

To measure hydrogen peroxide (H₂O₂) decomposition capacity, CAT and APX were assayed. For CAT, 300 μ M H₂O₂ was added to 50 mM potassium phosphate buffer together with 50 μ L of enzyme extract, and the decrease in absorbance at 240 nm was measured at 10 s intervals for 2 min (Lardinois & Rouxhet, 1994). CAT activity was calculated using a molar extinction coefficient for H₂O₂ of 43.6 M⁻¹ cm⁻¹, and one unit (U) was defined as the amount of enzyme decomposing 1 μ mol of H₂O₂ per minute under the assay conditions. For APX, enzyme extract prepared with ascorbate-supplemented reaction buffer was added to 50 mM potassium phosphate buffer containing 3 mM H₂O₂, 0.5 mM sodium ascorbate, and 0.1 mM EDTA, and the decrease in absorbance at 290 nm was measured at 10 s intervals for 2 min. APX activity was calculated using a molar extinction coefficient for ascorbate of 2.8 mM⁻¹ cm⁻¹, and one unit (U) was defined as the amount of enzyme oxidising 1 μ mol of ascorbate per minute under the assay conditions. The activity of each antioxidant enzyme was normalized to the protein content determined with a Bradford protein assay kit (Takara, Japan) and expressed as specific activity (U/mg protein). The experiment was performed with three biological replicates.

Statistical analysis

All data were analyzed using R (v4.6.1). For plant growth promotion by PGEL-SA, the mean of all plants within each plate was first calculated, and the plate means were used as independent observations (n=3 plates per treatment) in Welch's two-sample t-test. Antioxidant enzyme activities were also analyzed with Welch's two-sample t-test. A *p*-value < 0.05 was considered statistically significant. For plant growth data under treatment with the major volatile compounds, the mean of all plants within each plate was calculated for each treatment, and the three plate means were used as independent observations for one-way ANOVA followed by Tukey's HSD post hoc test. Graphs were visualized using ggplot2 (v3.5.1) and pheatmap (v1.0.12).

Results

Complete genome assembly of '*P. ceruminis*' PGEL-SA

Illumina sequencing produced a total of 3,421,858,716 bp of raw data, of which more than 93.5% of bases had quality scores of Q30 or higher. After retaining only reads with Q30 or higher and removing adapters, 2,647,933,638 bp of filtered reads were obtained. PacBio SMRT sequencing produced a total of 719,849,120 bp of reads, with an N50 of 10,033 bp and a mean read length of 8,798 bp (**Table 1**).

Hybrid assembly yielded a single circular contig with a total length of 5,988,567 bp and a G+C content of 61.9% (**Fig. 1, Table 1**). In the BUSCO analysis, all 124 complete and single-copy orthologs were detected, confirming a high-quality closed genome. Gene prediction identified a total of 5,551 genes (5,450 CDS, 78 tRNA, 22 rRNA (5S, 16S, 23S), and 1 tmRNA) (**Table 1**).

Phylogenetic placement and genome-based taxonomic assignment

The 16S rRNA gene sequence showed 100% similarity to '*P. ceruminis*' BML-PP028^T, 99.93% to *P. monteilii* NBRC 103158^T, and 99.93% to *P. plecoglossicida* NBRC 103162^T. In the maximum-likelihood phylogenetic tree, PGEL-SA also branched adjacent to '*P. ceruminis*' BML-PP028^T (**Supplementary Fig. 1**), and additional phylogenetic analyses using the maximum parsimony and neighbour-joining methods showed similar clustering patterns (**Supplementary Fig. 2, 3**). However, multiple close relatives exceeded the interspecies threshold of 98.7% (Yarza et al., 2014), and 16S rRNA marker alone was insufficient for species-level identification.

The ANI value was highest against BML-PP028^T at 99.18%, whereas the values against the remaining close relatives, including *P. monteilii* NBRC 103158^T, *P. plecoglossicida* NBRC 103162^T, *P. defluvii* WCHP16^T, and *P. anuradhapurensis* RD8MR3^T, were all below 90%, far below the species delineation threshold of 95-96%. The dDDH values were likewise below the species delineation threshold of 70% for all strains except '*P. ceruminis*' BML-PP028^T (93.6%). In clustering based on OrthoANI distances, PGEL-SA also formed the same clade as '*P. ceruminis*' BML-PP028^T (**Fig. 2a**). To examine the genomic diversity of PGEL-SA and closely related *Pseudomonas* strains, the distribution of gene clusters was analyzed. In the Roary gene presence/absence matrix, the patterns of PGEL-SA and BML-PP028^T were the most similar, indicating that the two strains share the closest accessory genome composition among the strains compared (**Fig. 2b**). Nevertheless, a considerable number of genes were strain-specific even between these two strains, suggesting that the clinically derived BML-PP028^T and the rhizosphere-derived PGEL-SA are distinguishable at the accessory genome level (**Supplementary Fig. 4**). However, because some of the strains

included in the comparison are draft assemblies, the possibility cannot be excluded that the absence of particular genes reflects assembly gaps or differences in annotation rather than true gene loss.

Plant growth responses under separated and unpartitioned coculture conditions

To evaluate the interaction between PGEL-SA and plants under conditions without physical contact, I-plate coculture was performed. After coculture, a clear difference in plant growth was observed between the PGEL-SA treatment and Mock (**Fig. 3a**). The shoot fresh weight of seedlings exposed to PGEL-SA was 62.91 ± 8.87 mg, compared with 24.05 ± 2.78 mg in Mock, an increase of more than two-fold ($p=0.0112$), and leaf number increased significantly from 5.33 ± 0.12 in Mock to 6.20 ± 0.00 in PGEL-SA ($p=0.0059$). The root system response was examined separately in unpartitioned square-dish coculture (**Fig. 3b**). Under this condition, lateral root number increased significantly from 3.00 ± 0.38 in Mock to 7.56 ± 1.25 in PGEL-SA ($p=0.0173$), whereas primary root length decreased significantly from 47.55 ± 0.76 mm in Mock to 30.69 ± 2.20 mm in PGEL-SA ($p=0.0027$) (**Fig. 3c**). In summary, shoot growth promotion was observed under the partition-separated I-plate condition, and a shift in root system architecture from primary root dominance to lateral root branching dominance was observed under the unpartitioned square-dish. However, because the square dish lacked a physical partition, the distributions of gaseous and diffusible factors to the root responses could not be distinguished.

Volatile compound profile of the culture supernatant

To search for candidate mediators of the growth responses observed under the separated condition, SPME-GC-MS analysis was performed on culture supernatant grown in TSB medium for 18 h. Using uninoculated TSB medium as the control, medium-derived and culture-derived peaks were compared, and multiple volatile compound peaks distinguishable from the control were detected in the PGEL-SA culture supernatant (**Fig. 4**). Qualitative analysis using the NIST11 mass spectral library tentatively identified 3-methyl-1-butanol, DMDS, and MeSA as major volatile compound candidates. The relative peak area was highest for DMDS at 13.19%, followed by MeSA at 6.68% and 3-methyl-1-butanol at 3.74% (**Table 2**). However, because this analysis was performed under TSB liquid culture conditions and is based on relative quantification, it cannot be assumed that the composition and abundance of the detected compounds are identical to those under the TSA solid medium condition of the I-plate. These compounds are therefore presented as candidate mediators of the growth responses observed under the separated condition.

Effects of individual volatile compounds on plant growth

To examine the individual effects of DMDS, MeSA, and 3-methyl-1-butanol, the compounds with high relative abundance among those detected, each compound was applied at different concentrations and the growth responses of *N. benthamiana* were examined. In the live PGEL-SA treatment, fresh weight and leaf number increased significantly relative to the controls, whereas the DMDS and MeSA treatments showed no significant differences from the solvent control (EtOH) or Mock at any of the concentrations tested (**Supplementary Fig. 5**). In contrast, 3-methyl-1-butanol alone showed significant growth promotion relative to EtOH and Mock, but the magnitude of the effect did not reach the level observed in coculture with live PGEL-SA (**Supplementary Fig. 5**). This suggests that 3-methyl-1-butanol may contribute partially to the observed growth promotion but does not account for the full phenotype on its own, and that other volatile factors, their combinations, or complex factors such as CO₂ derived from bacterial respiration may be involved.

Genome-encoded plant growth-promoting traits

To assess the plant growth-promoting potential of PGEL-SA at the genome level, the predicted proteome was annotated with PGPT-Pred (**Supplementary Table 2**). A total of 6,369 PGPT annotations were identified; because individual proteins could receive multiple ontology assignments, the number of annotations exceeded the number of predicted CDSs. Indirect effects accounted for the largest share (4,209 annotations), followed by the direct effects (2,145) and a small number of putative PGPTs (15). Among the direct effects, biofertilization was the most represented (875 annotations), dominated by phosphate solubilization, potassium solubilization, iron acquisition, and nitrogen acquisition. Phytohormone/plant-signal production (638) and bioremediation (633) were also identified. Among the indirect effects, plant colonization was the largest category (1,521 annotations), including genes related to the use of plant-derived substrates, motility/chemotaxis, surface attachment, and root colonization. Competitive exclusion (1,305) comprised genes related to bacterial fitness, quorum sensing/biofilm formation, and exopolysaccharide production, and stress control/biocontrol (1,294) was dominated by genes for neutralizing abiotic and biotic stresses. A small number of genes were assigned to plant immune response stimulation (89, including ISR induction).

Among volatile compound metabolism categories, the largest group comprised genes assigned to sulfur-containing volatile metabolism, including the cysteine/sulfate assimilation module (*cysA-cysZ*), *aprA/aprB*, and *metB*, which

were mapped to plant-signalling and salinity-stress volatile branches. *mdeA*, involved in DMDS biosynthesis, was also observed. In addition, genes involved in the biosynthesis of acetoin/2,3-butanediol volatiles, which were not detected by SPME-GC-MS, were identified, suggesting that the plant growth-promotion response may have been driven by multiple compounds beyond the principal factors detected.

Transcriptome responses to rhizosphere inoculation

To explore molecular-level responses to bacterial inoculation, transcriptome analysis was performed under direct rhizosphere inoculation with PGEL-SA. Sequencing yielded an average of approximately 6.76 Gb of reads per sample. The GC content of the samples ranged from 43.2% to 45.4%, and the proportion of bases with Q30 or higher was 97%. Alignment of the trimmed reads to the reference genome gave mapping rates of 97.11-99.26% (mean approximately 98.6%), confirming sufficient data quality (**Supplementary Table 3**).

Analysis of DEGs between Mock and PGEL-SA at 18 h and 96 h after treatment showed that at 96 h the transcriptome profiles were clearly separated in principal component analysis (**Supplementary Fig. 6a**). Across all time points and comparisons, a total of 1,097 DEGs with $|\log_2FC| > 2$ and adjusted p -value < 0.05 were identified, and 34 DEGs were unique to the PGEL-SA 96 h versus Mock 96 h comparison (**Supplementary Fig. 6b**).

GO analysis of the 96 h DEGs showed that upregulated genes were significantly enriched in chaperone/HSP-related terms, including protein folding, ATP-dependent protein folding chaperone, unfolded protein binding, and protein refolding, as well as translation- and ribosome biogenesis-related terms, including small/large ribosomal subunit, ribosome biogenesis, rRNA binding, and ribonucleoprotein complex (**Fig. 5a**). KEGG pathway enrichment analysis also showed significant enrichment in pathways closely related to protein biosynthesis, including biosynthesis of amino acids, ribosome biogenesis in eukaryotes, aminoacyl-tRNA biosynthesis, and nucleocytoplasmic transport, and carbon fixation by the Calvin cycle was also identified as a top pathway (**Fig. 5b**).

In contrast, the downregulated gene set was significantly enriched in sulfate transport, sulfate transmembrane transporter activity, and secondary active sulfate transmembrane transporter activity (**Fig. 5c**). Individual examination of sulfur metabolism-related genes showed that expression of all sulfate transport-related genes decreased, whereas expression of genes related to glutathione biosynthesis, methionine biosynthesis, and stress responses increased (**Fig. 5d**). Thus, under the rhizosphere inoculation condition, genes related to the uptake and transport of inorganic sulfur

and genes related to the downstream utilization of reduced sulfur were regulated in opposite directions.

In addition, changes in the expression of defense-related functional categories, specifically antioxidant response genes, were analyzed. Examination of DEGs belonging to antioxidant-related GO terms, including oxidoreductase activity (GO:0016491), superoxide dismutase activity (GO:0004784), peroxidase activity (GO:0004601), and superoxide metabolic process (GO:0006801), identified a total of 41 antioxidant-related DEGs, of which 37 were upregulated and 4 were downregulated in PGEL-SA treatment (**Supplementary Fig. 7**). Among the upregulated genes, oxidoreductase activity-related genes were the most numerous at 23. Meanwhile, genes belonging to superoxide metabolic process (g44273, g44274, g44275; *csdI*) and a peroxidase (g26278) were downregulated. In the antioxidant enzyme activity assays, CAT activity was slightly higher in the PGEL-SA treatment but not significantly ($p = 0.3305$), whereas APX activity decreased significantly ($p = 0.04741$) (**Supplementary Fig. 8**). Antioxidant-related gene expression and enzyme activities did not change uniformly, and the results differed among enzymes.

To verify the reliability of the RNA-seq results, qRT-PCR was performed on major DEGs. The direction of expression changes measured by qRT-PCR for the validated genes was consistent with the RNA-seq data, with $R^2 = 0.8252$ (**Supplementary Fig. 9**).

Discussion

In this study, we sequenced the complete genome of PGEL-SA, isolated from the *N. benthamiana* rhizosphere, and confirmed through genome-based metrics that this strain belongs to the lineage proposed as '*P. ceruminis*' by Tohya et al. (2023) within the *Pseudomonas putida* group. The 16S rRNA gene alone could not distinguish it from close relatives such as *P. monteilii* and *P. plecoglossicida*, but the ANI (99.18%) and dDDH (93.6%) values supported the conclusion that it belongs to '*P. ceruminis*'. This reconfirms the limitations of single markers and the necessity of genome-based metrics for the identification of closely related species within the genus *Pseudomonas* (Goris et al., 2007; Yarza et al., 2014). Indeed, among *Pseudomonas* strains isolated from Korean agricultural soils, a case has been reported in which the 16S rRNA sequence matched '*P. ceruminis*' and its close relatives equally well and the isolate was identified only at the genus level (Sim et al., 2025). This suggests that 16S sequences similar to this lineage may also exist in non-clinical environments, while showing that genome-based metrics are required for species-level

confirmation. Genome-based metrics are therefore also required for the identification of lineages derived from plant-associated environments. This study identifies a plant rhizosphere-derived '*P. ceruminis*' lineage by complete-genome metrics and presents its plant-interaction characteristics.

In the SPME-GC-MS analysis of the culture supernatant, DMDS, MeSA, and 3-methyl-1-butanol were tentatively identified as major volatile compound candidates. All of these are compounds with reported biological activity in interactions with plants. DMDS is produced through methionine catabolism and is known to promote growth, including leaf area, chlorophyll, and lateral root number, by supplying reduced sulfur to plants under sulfur-deficient conditions (Meldau et al., 2013). In addition, Kai et al. (2009) reported that *P. fluorescens* B-4117 produces VOCs including DMDS with antimicrobial activity, and Huang et al. (2012) reported that DMDS produced by *Bacillus cereus* C1L induces ISR against *Botrytis cinerea* in *N. benthamiana*. MeSA production has been reported in several *Pseudomonas* species (Mhlango et al., 2022), and after uptake by plants it is converted to salicylic acid by MeSA esterase (SABP2), inducing defense gene expression (Park et al., 2007). 3-methyl-1-butanol is known to potentially contribute to lateral root formation through jasmonic acid signalling (Gamboa-Becerra et al., 2021). However, the detected compounds were based on qualitative identification using the NIST11 mass spectral library; confirmation using authentic standards and retention indices was not performed, and only relative peak areas without absolute quantification using internal standards were presented. Moreover, the analysis remained at the qualitative level and did not include statistical comparison of abundance across replicate samples. The composition and abundance of the detected compounds should therefore be interpreted tentatively.

In the partition-separated I-plate coculture, *N. benthamiana* showed a more than two-fold increase in shoot fresh weight and an increase in leaf number, and in the unpartitioned square-dish coculture, increased lateral root number and inhibited primary root elongation were observed. The restructuring of root system architecture from primary root dominance to lateral root branching dominance is interpreted as a response that increases root surface area per unit volume and optimizes water and nutrient uptake, and is a feature repeatedly reported in interactions with various rhizosphere bacteria. In our experiment, the two compartments were separated by the medium, so that physical contact between bacteria and plants was blocked and only the headspace was shared; the observed responses are therefore judged to result from factors transmitted through the gas phase. However, DMDS and MeSA did not reproduce the phenotype observed in coculture with live bacteria, whereas 3-methyl-1-butanol showed a partial growth-promoting

effect but did not account for the full phenotype. Because the gas phase contains not only bacterially produced volatile organic compounds, but also various gases including CO₂ derived from respiration (Kai & Piechulla, 2009), verification using a CO₂-only control is needed to confirm the contribution of VOCs to the observed promotion effect.

In the DEG analysis performed under direct rhizosphere inoculation, ribosome biogenesis, enhanced folding capacity, and carbon fixation-related pathways were upregulated. The upregulated genes represent metabolic processes essential for active plant growth, suggesting an overall enhancement of protein synthesis capacity and an increased supply of energy required for biosynthesis. Although obtained under a different interaction condition, these transcriptional changes are consistent with enhanced biosynthesis activity. Sulfate transport-related genes were consistently downregulated, whereas methionine and glutathione biosynthesis-related genes were upregulated. This directionality is consistent with reports of decreased expression of sulfate transporter (*SULTR*) and sulfur assimilation-related genes in DMDS-treated *N. attenuata* (Meldau et al., 2013). These expression patterns raise the possibility that bacterially derived reduced-sulfur compounds contributed to sulfur-metabolic readjustment; however, neither sulfur uptake nor reduced sulfur transfer was directly measured. Among antioxidant-related genes, many were upregulated, but at the enzyme activity level, CAT activity did not change significantly, whereas APX activity decreased significantly. This discrepancy may have arisen from post-transcriptional regulation, enzyme-specific substrate specificity, or differences in sampling time points. However, elucidating causal relationships at the level of individual enzymes will additionally require time-course analyses and protein quantification.

The genome-level PGPT-Pred annotation showed that PGEL-SA possessed a broad repertoire of plant growth-promoting genes across the direct and indirect categories. In particular, genes involved in sulfur-containing volatile metabolism and DMDS biosynthesis, including *mdeA*, were identified, consistent with DMDS detected in the culture supernatant and the readjustment of sulfur metabolism observed under the inoculation condition. In addition, genes of other volatile pathways not detected by SPME-GC-MS, such as acetoin/2,3-butanediol, were also present, supporting the possibility that factors other than the detected compounds contributed to the observed growth promotion. However, because gene prediction indicates functional potential without guaranteeing actual expression and activity, genetic verification of the causal relationships between the production of individual compounds and plant responses is required as follow-up work. The growth-promoting effects of the major volatile compounds, which were not fully elucidated in this study, are likewise subject to this follow-up verification.

Conclusion

In this study, we sequenced the complete genome of PGEL-SA, isolated from *N. benthamiana* rhizosphere, and confirmed through genome-based metrics that this strain belongs to the lineage proposed as '*P. ceruminis*' within the *P. putida* group. This study identifies a strain of this lineage isolated from the plant rhizosphere at the complete-genome level and shows that it can interact with plants. Under the I-plate coculture condition in which physical contact was blocked, seedlings exposed to PGEL-SA showed increased shoot fresh weight and leaf number, and under the unpartitioned condition, increased lateral root number and inhibited primary root elongation were observed together. Among DMDS, MeSA, and 3-methyl-1-butanol, tentatively identified as major volatile compound candidates in the culture supernatants, DMDS and MeSA did not reproduce the above phenotype when applied individually and 3-methyl-1-butanol showed only a partial effect. In order to confirm the contribution of bacterially derived VOCs, verification including a CO₂-only control and genetic verification of individual compound-plant response relationships are required as follow-up work. Transcriptome analysis under the rhizosphere inoculation condition showed activation of protein synthesis-related pathways together with downregulation of genes associated with inorganic sulfate transport and increased expression of genes related to the utilization of reduced sulfur, suggesting that readjustment of sulfur metabolism may be a factor involved in this interaction. Taken together, this study expands the complete genome resources of '*P. ceruminis*' and, by presenting its occurrence and functional characteristics in the rhizosphere environment, provides a foundation for follow-up studies aimed at identifying the mediating factors and elucidating their mechanisms of action.

Statements and Declarations

Author contributions

Seung-A Kim conceived and designed the study, performed the experiments, analyzed and interpreted the data, and drafted the manuscript. Kwantae Park contributed to performing the experiments. Soohyun Oh assisted with experiments and data acquisition. Beom Seok Kim and Seungill Kim provided resources and contributed to technical support and acquired funding. Hualin Nie contributed to study design and supervised parts of the work. Sun-Hyung Kim supervised the study and critically revised the manuscript. All authors reviewed and approved the final manuscript. Sun-Hyung Kim is the corresponding author.

Funding

This work was supported by the 2025 sabbatical year research grant of the University of Seoul to Seungill Kim and by the Rural Development Administration of the Republic of Korea (Project No. RS-2021-RD009837).

Data availability

The whole-genome sequence of '*Pseudomonas ceruminis*' PGEL-SA has been deposited in DDBJ/ENA/GenBank under the accession no. JBXVOE000000000. This project has been assigned BioProject no. PRJNA1456818 and BioSample no. SAMN57425505. The RNA-seq data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) and are accessible through the BioProject accession number PRJNA1510831.

Tables

Table. 1 Statistics of genome sequencing, assembly and annotation of '*Pseudomonas ceruminis*' PGEL-SA. As the assembly is a single circular contig, the contig N50 equals the genome size.

Feature	Illumina	PacBio
Total reads	17,539,712	81,815
*Mapped reads	17,534,273 (99.97%)	81,815 (100%)
*Coverage	100%	100%
Depth	423.64	120.13
Insert size (Std.)	457.48 (101.64)	-
Assembly		
Contigs	1 (circular)	
Genome size (bp)	5,988,567	
G + C content (%)	61.9	
*BUSCO completeness	124 (100%)	
Total genes	5,551	
CDS	5,450	
tRNA	78	
rRNA	22	
tmRNA	1	

*Mapped reads and coverage were obtained by remapping raw reads to the completed assembly; completeness was assessed independently with BUSCO

462 **Table. 2** Major volatile compound candidates tentatively identified in the culture supernatant of '*Pseudomonas*
463 *ceruminis*' PGEL-SA by SPME-GC-MS

No.	Retention		CAS No.	Quality	Area (%)
	Time (min)	Compound Name			
1	3.768	3-Methyl-1-Butanol	000123-51-3	91	3.74
2	3.952	Dimethyl disulfide	000624-92-0	98	13.19
3	12.991	Methyl salicylate	000119-36-8	97	6.68

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Figure legends

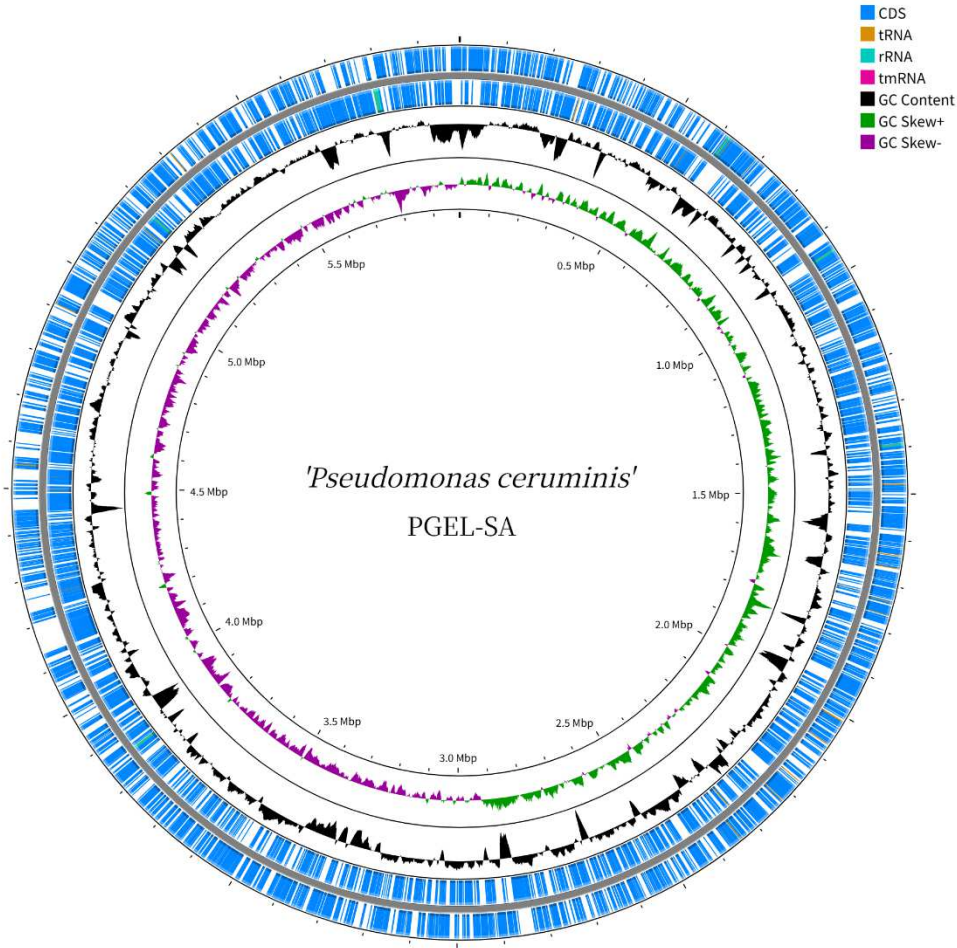


Fig. 1 Circos plot of the completed genome assembly and annotation, generated using Proksee. From the outermost to the innermost ring: coding sequences (CDS), tRNA genes, rRNA genes, tmRNA genes, GC content, GC skew, and genome coordinates in base pairs (bp)

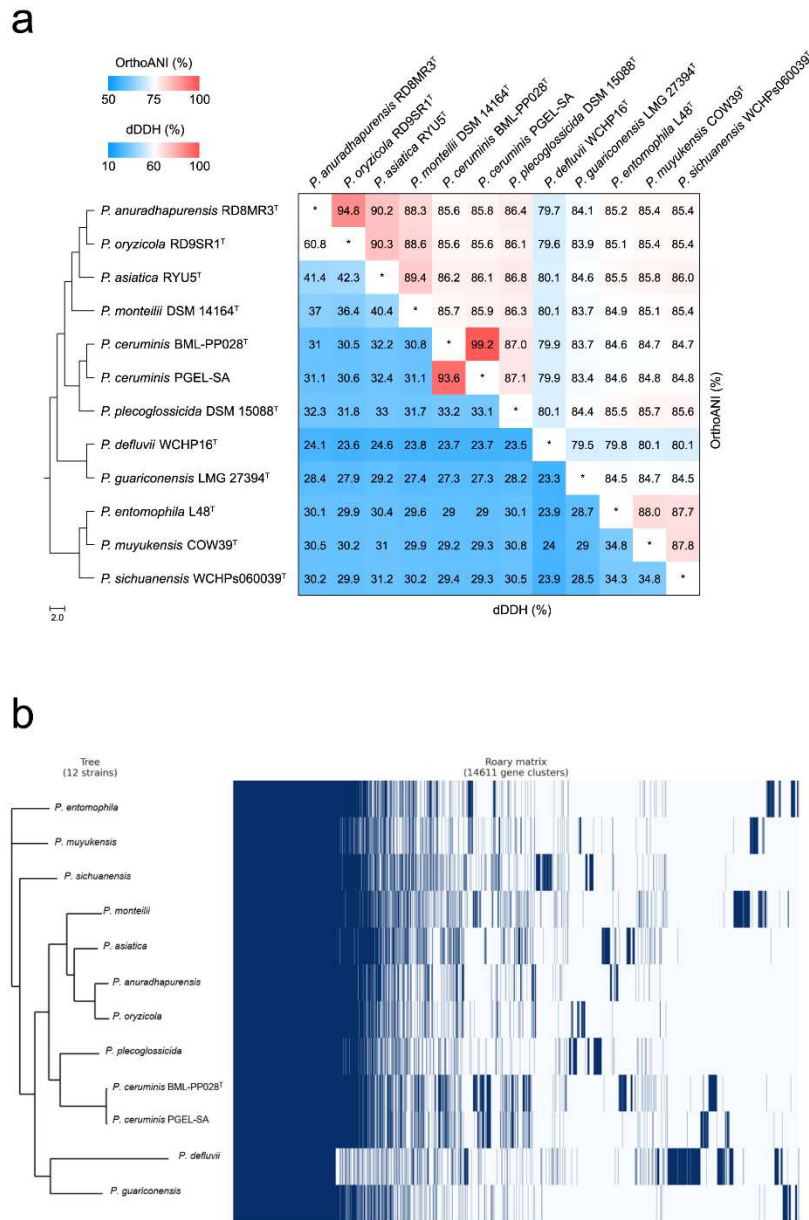


Fig. 2 Genome-based relatedness of PGEL-SA and closely related *Pseudomonas* strains. **(A)** Heatmap shows OrthoANI values created using OAT software and the formula 2 equation for dDDH created in GGDC. The dendrogram on the left was generated by hierarchical clustering of the OrthoANI distance matrix. **(B)** Clustered gene presence/absence and accessory genome distribution based on pan-genome analysis using Roary

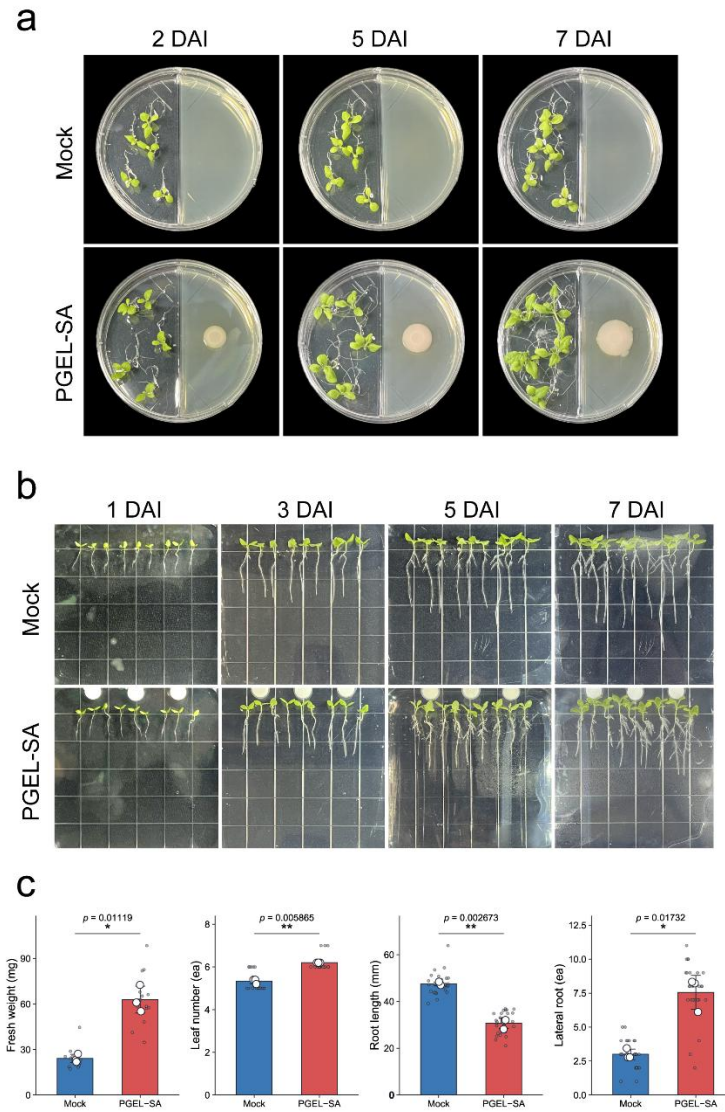


Fig. 3 Growth responses of *Nicotiana benthamiana* exposed to PGEL-SA. **(A)** Shoot growth in I-plate coculture in which the compartments were separated by a central partition. **(B)** Root development under the square-dish condition. **(C)** Shoot fresh weight and leaf number (n = 3 plates), primary root length and lateral root number (n = 3 plates). Small grey points represent individual plants, whereas large open circles represent the plate means used for statistical analysis. Bars indicate the mean $\pm$ SD of three plate means. Statistical significance was assessed using Welch's two-sample t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n=3 independent plates per treatment; each value is the mean of 5 (shoot) or 9 (root) plants within a plate

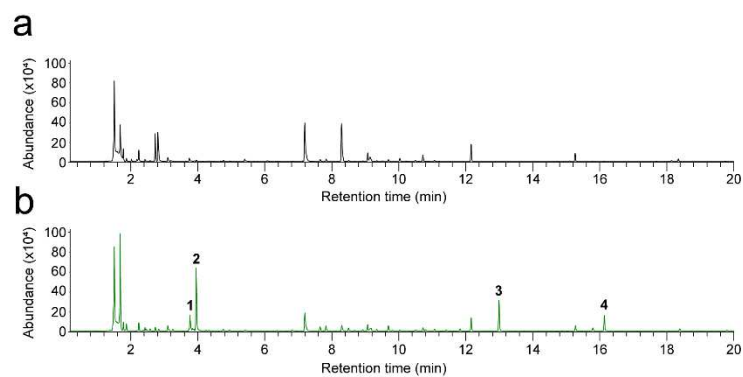


Fig. 4 Headspace VOC profile of the cell-free culture supernatant of '*Pseudomonas ceruminis*' PGEL-SA grown in tryptic soy broth (TSB) for 18 h. Peaks 1-3 correspond to 3-methyl-1-butanol, dimethyl disulfide, and methyl salicylate, respectively. Upper, TSB medium control; lower, '*P. ceruminis*' PGEL-SA

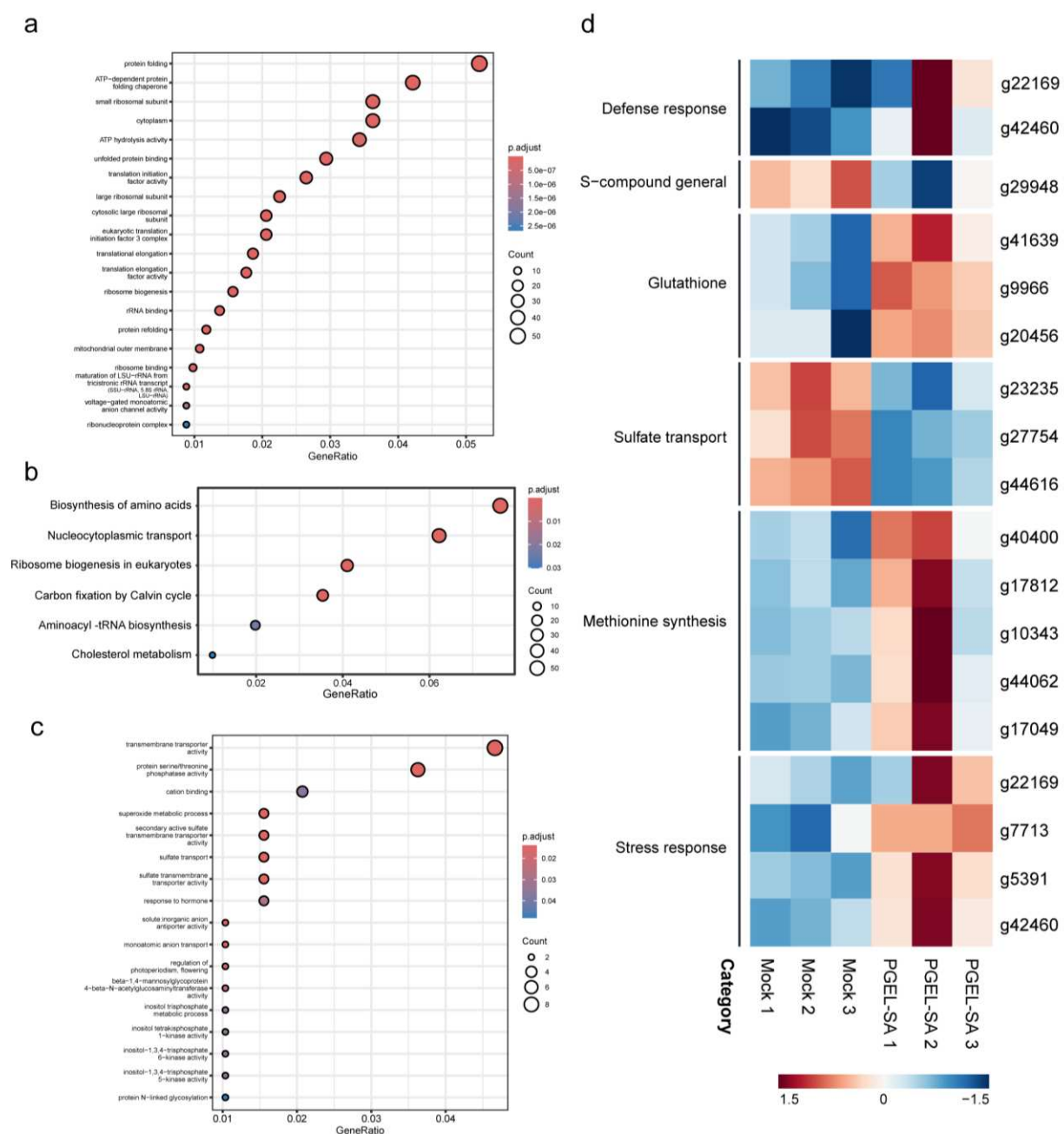


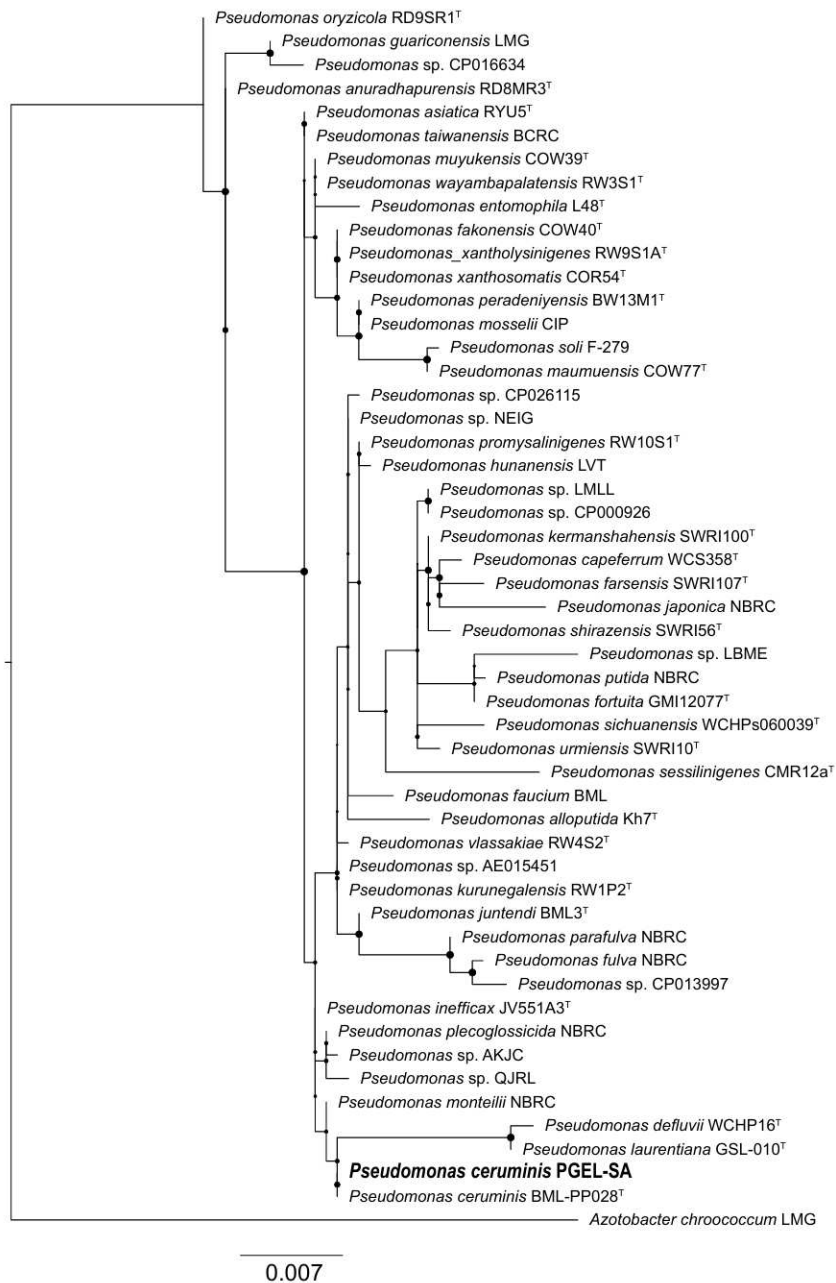
Fig. 5 Transcriptome analysis of *Nicotiana benthamiana* under direct rhizosphere PGEL-SA inoculation. (A) GO enrichment of upregulated DEGs. (B) KEGG pathway enrichment. (C) GO enrichment of downregulated DEGs. (D) Expression heatmap of sulfur metabolism-related genes

495 **Supplementary Information**

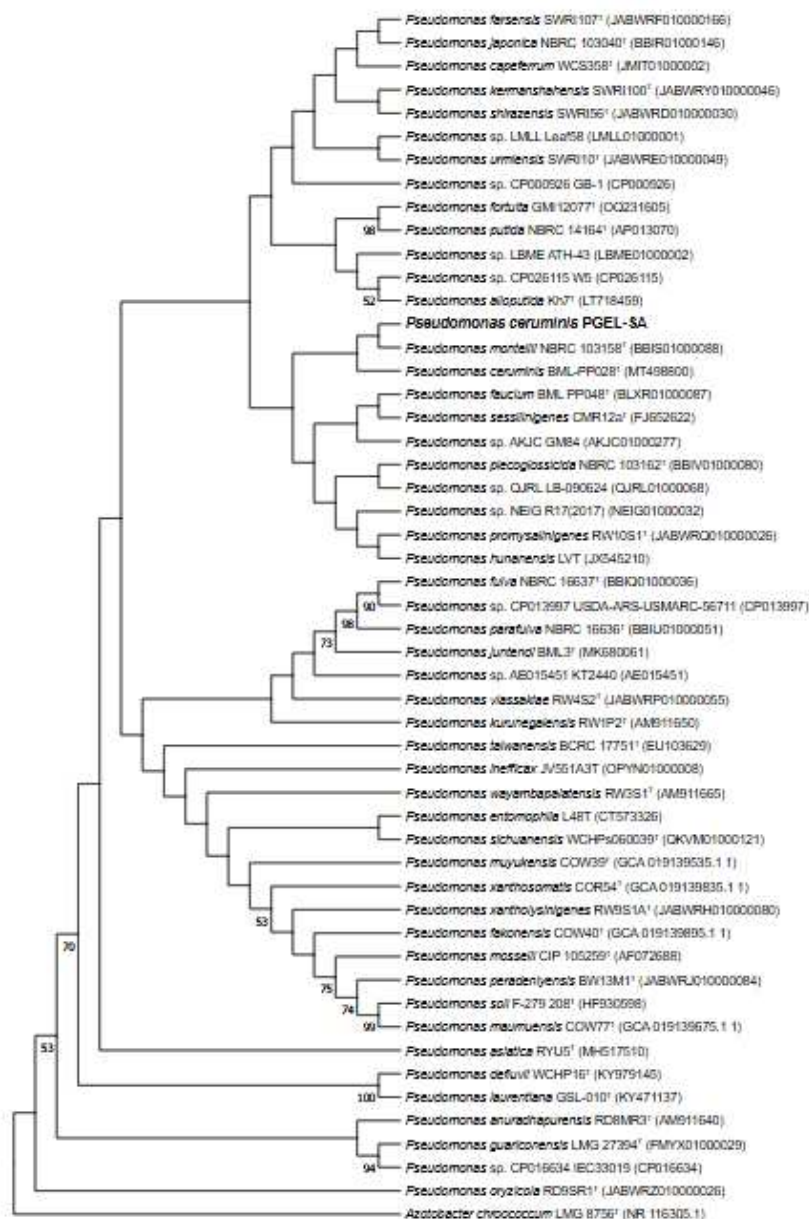
496 **Supplementary Table 1** qRT-PCR primer list

497 **Supplementary Table 2** PGPT-Pred annotation summary

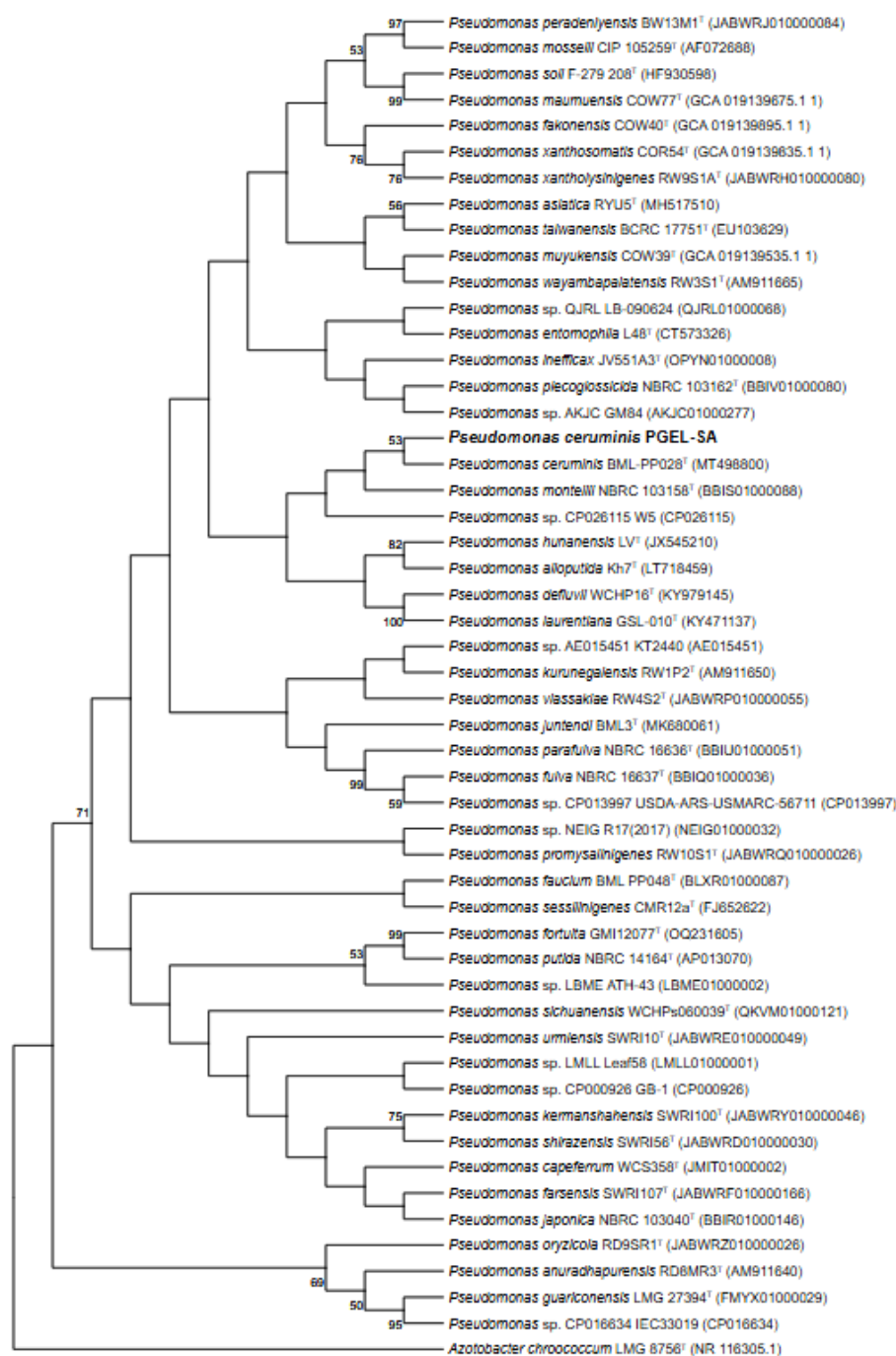
498 **Supplementary Table 3** RNA-seq read mapping statistics



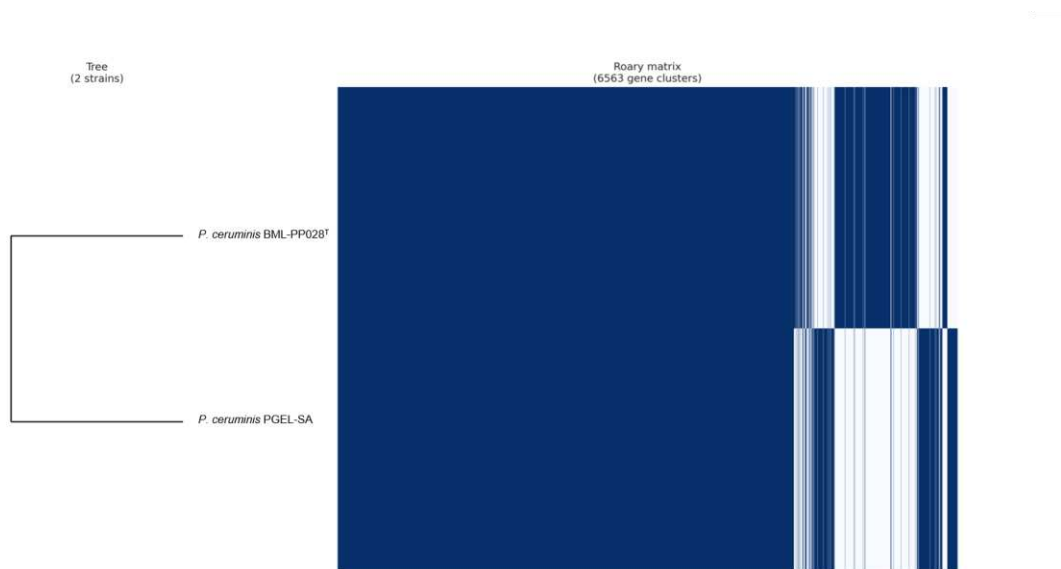
Supplementary Fig. 1 Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences



Supplementary Fig. 2 Maximum parsimony phylogenetic tree based on 16S rRNA gene sequences



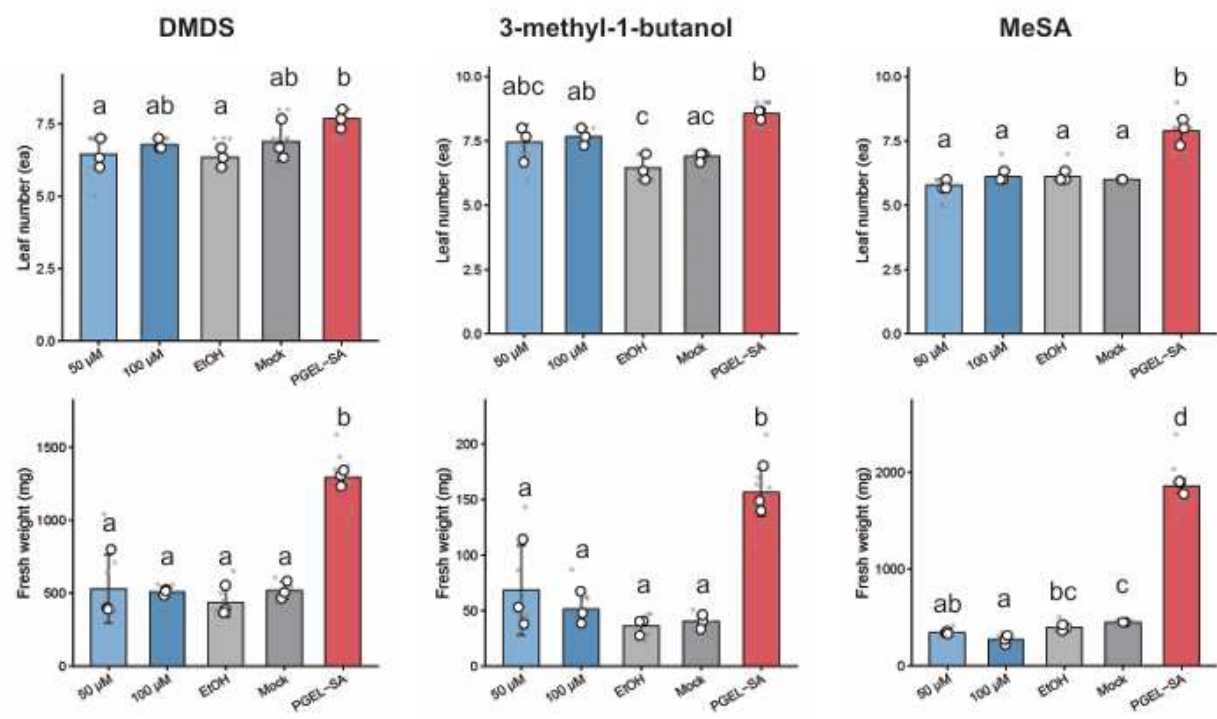
Supplementary Fig. 3 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences



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506 **Supplementary Fig. 4** Clustered gene presence/absence and accessory genome distribution

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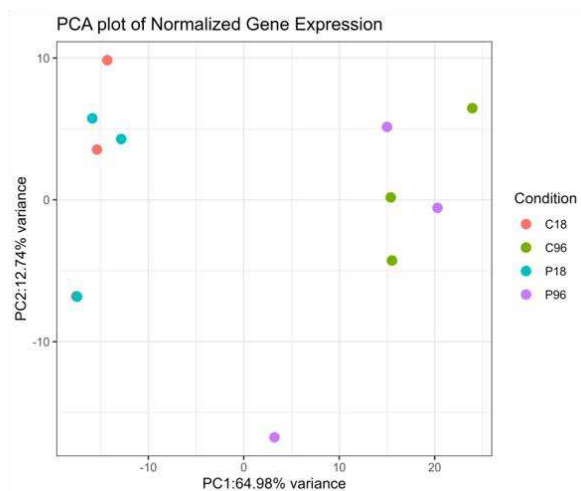
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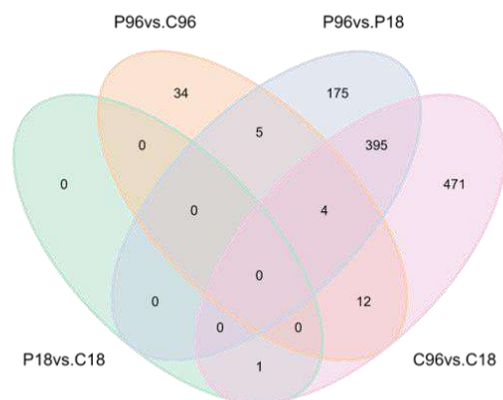
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Supplementary Fig. 5 Effects of individual treatments with dimethyl disulfide (DMDS), 3-methyl-1-butanol and methyl salicylate (MeSA) on plant growth

a

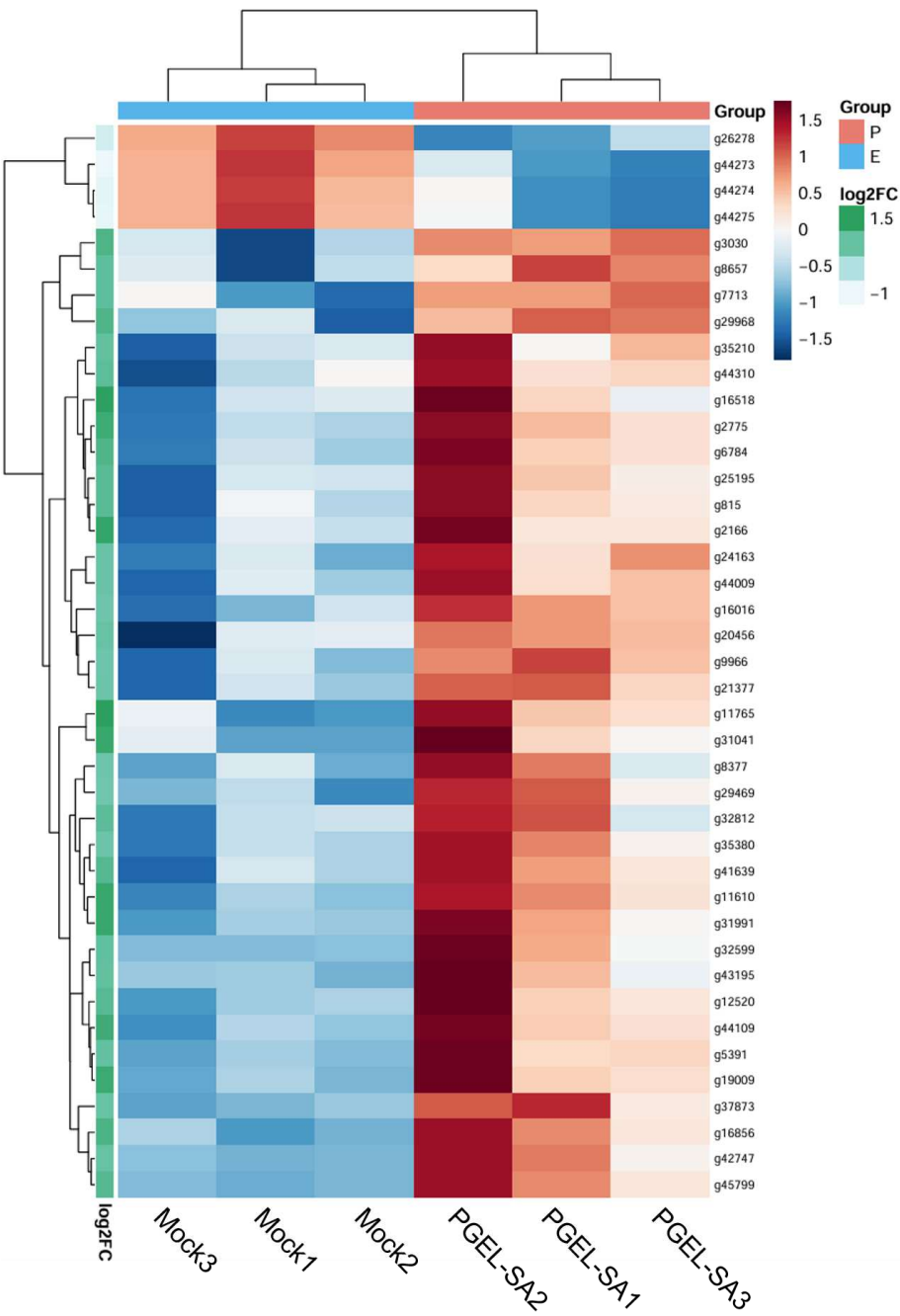


b



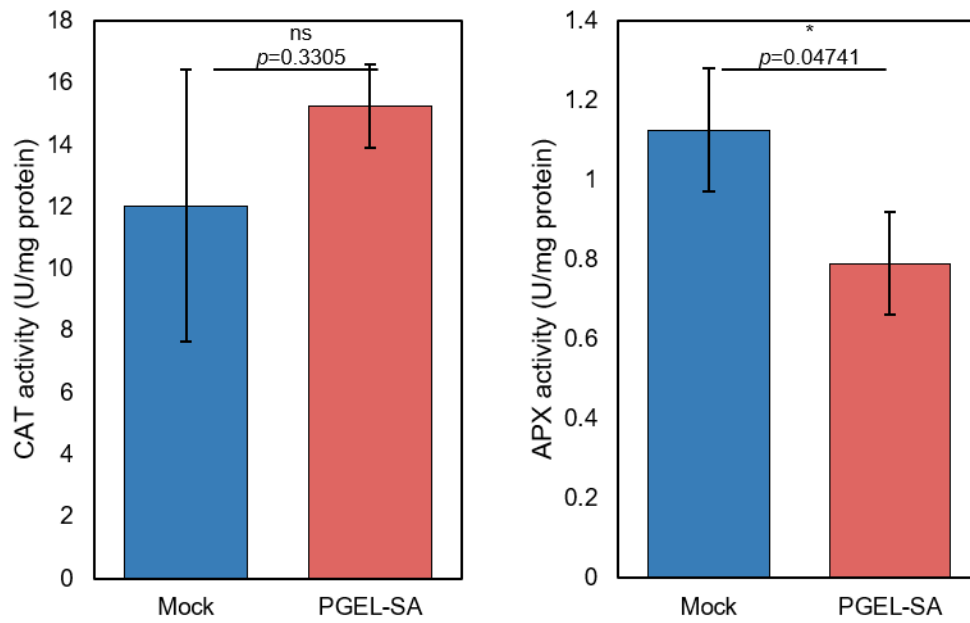
Supplementary Fig. 6 Principal component analysis and differential expression overview of the transcriptome profiles

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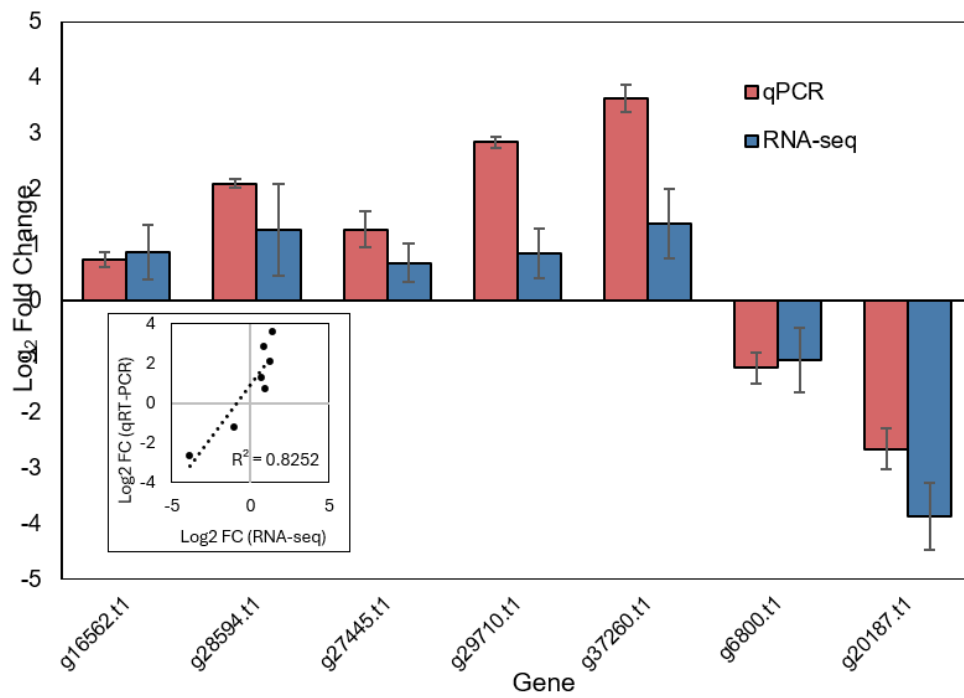
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516 **Supplementary Fig. 7** Antioxidant-related DEGs



517

518 **Supplementary Fig. 8** Antioxidant enzyme activity measurements



Supplementary Fig. 9 Comparison of qRT-PCR validation results with RNA-seq log₂FC values

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