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Physio-biochemical and transcriptomic analysis of *Bacillus amyloliquefaciens* PG-4-induced salt stress tolerance in *Macrotyloma uniflorum*

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Introduction: *Macrotyloma uniflorum* is an important legume fodder crop and green fertilizer. Salinity impedes plant growth and productivity of legume crops by disrupting the ionic and osmotic balance and hormonal regulation. Plant growth-promoting rhizobacteria (PGPR) are rhizosphere bacteria that contribute to the improvement of plant growth through diverse physiological mechanisms.

Methods: In this study, the growth promoting characteristics of the isolated strain *Bacillus amyloliquefaciens* PG-4 were analyzed, and to further investigated the possible mechanism of PG-4 in mitigating the damage caused by salt stress in *M. uniflorum* plants through pot experiments.

Results: In presence of different salt levels, PG-4 showed a high potentiality to produce several plant growth promoting metabolites such as NH_3, siderophore, 1-aminocyclopropane-1-carboxylic acid deaminase (ACC-deaminase), and hydrolytic enzymes. Inoculation of the PG-4 significantly enhanced plant tolerance to salt stress, as demonstrated by promotion of plant growth (shoot and root biomass) under salt stress condition. Furthermore, PG-4 improved salt tolerance of *Macrotyloma uniflorum* seedlings by affecting the antioxidant enzymes including peroxidase (POD) and superoxide dismutase (SOD), by increasing the levels of proline, soluble sugars and chlorophyll. Treatment with PG-4 increased the K^+ content while decreased the Na^+ concentration level under salt stress. Transcriptomic analysis revealed 5525 genes were differentially expressed (PG-4-inoculated versus non-inoculated samples) at 0 mM NaCl, of which 3277 were upregulated and 2248 downregulated, while 1298 genes were differentially expressed at 100 mM NaCl, of which 819 were upregulated and 479 were downregulated. GO and KEGG enrichment analyses showed that these DEGs were significantly enriched in several terms and pathways mainly involved in the regulation of the cellular redox state, cell wall modification, metabolic adjustments, hemoglobin, biosynthesis of secondary metabolites and plant hormone signal transduction.

Discussion: These data showed that *Bacillus amyloliquefaciens* PG-4 significantly enhance salt stress tolerance in *Macrotyloma uniflorum* plants during salt stress conditions. Therefore, the results may be useful for explaining the mechanism by which PGPR inoculation regulates the salt tolerance of crops.

KEYWORDS

plant growth-promoting rhizobacteria, *Bacillus amyloliquefaciens*, *Macrotyloma uniflorum*, salt stress tolerance, transcriptome

Introduction

Soil salinity is one of the most serious problems associated with soil and is considered a major abiotic threat to agricultural production worldwide. Currently, on a global scale, 20% of the land used for agriculture is affected, and annually, 2,500–5,000 km^2 of crop production is lost annually due to salinity (Singh et al., 2022). Moreover, it has been estimated that by 2050, nearly 50% of arable land will be affected by salinity (Butcher et al., 2016). Salinity can have various effects on all plants, reducing crop growth and output by altering morphological, biochemical and physiological processes (Ali et al., 2022). An excessive accumulation of sodium (Na^+) and chloride (Cl^-) ions during salt stress causes ionic toxicity and leads to the generation of reactive oxygen species (ROS) in plant cells (Munns and Tester, 2008; Farhangi-Abriz et al., 2020). These ROS include superoxide, hydrogen peroxide, hydroxyl radicals, singlet oxygen, etc., which in excess amounts threaten plant cell integrity by initiating lipid peroxidation, damaging nucleic acids and activating programmed cell death and enzyme inhibition (Farhangi-Abriz et al., 2020). In addition, higher salt concentrations in the soil cause lower water availability, a decrease in plant nutrient uptake, and disturbed soil properties such as porosity, aeration and water conductance (Ibarra-Villarreal et al., 2021). However, in response to salt-stressed environments, plants activate various self-defense mechanisms, including osmotic adjustment, antioxidant metabolism and plant hormones (Liu et al., 2022; Wang Y. et al., 2022; Wang Q. et al., 2022). Specifically, osmotic adjustment occurs through the accumulation of organic solutes and inorganic ions in plants (Wang Y. et al., 2022; Wang Q. et al., 2022; Pistelli et al., 2023). Plant antioxidant metabolism involves nonenzymatic components such as ascorbic acid (AsA), glutathione (GSH), carotenoids and phenolic compounds, as well as enzymes such as dehydroascorbate reductase (DHAR); ascorbate peroxidase (APX); glutathione reductase; catalase (CAT); superoxide dismutase (SOD); glutathione peroxidase (GSH-Px); and glutathione S-transferase (GST), which clear excess ROS (Garcia-Caparros et al., 2021). Furthermore, plant hormones, such as ethylene, abscisic acid (ABA) and 3-indoleacetic acid (IAA), are known to endogenously regulate the homeostasis of plants and lead plants to perceive and respond to salt stress (Singh et al., 2022). However, these mechanisms are insufficient for alleviating salt stress beyond a certain limit.

To boost crop yield in salinized land, a large amount of chemical fertilizer is applied to the soil, but the increase in chemical input is not directly proportional to the increase in crop yield (Wang et al., 2018). This behavior has also led to serious soil ecological and environmental problems, including secondary salinization, soil consolidation, soil acidification and microecological imbalance (Ma et al., 2021). Furthermore, several molecular breeding and genetic engineering methods have been developed for improving crop yield under salt stress based on plant defense mechanisms. Nonetheless, these methods are expensive and time-consuming (Haque et al., 2021; Ashraf and Munns, 2022). To overcome the above issues, a new biocontrol approach has been developed to protect plants from salt stress in soil by utilizing beneficial microorganisms (Etesami and Maheshwari, 2018; Etesami and Glick, 2020). Plant growth-promoting rhizobacteria (PGPR) are an important cluster of beneficial, root-colonizing bacteria thriving in the plant rhizosphere, and positive effects on plant growth, development and metabolism (Li et al., 2020). When combined with roots and other tissues, PGPR improve the nutritional supply of crop plants through several mechanisms. PGPR have direct effects, including nitrogen fixation, phosphorus solubilization, NH_3 production, IAA, and siderophores, and indirect effects, including antioxidant defense, volatile organic compound (VOC) production, exopolysaccharide (EPS) production, and osmotic balance mechanisms for improving plant growth and enhancing tolerance against salt stress (Etesami and Maheshwari, 2018; Mohanty et al., 2021). Therefore, an increasing number of scholars are studying how soil microorganisms play a role in plant growth to achieve eco-friendly and sustainable agriculture.

Diverse members of the *Bacillus* genus, which is one of the most extensively studied genera with beneficial microorganisms in the rhizosphere, have also been shown to be involved in promoting plant growth and tolerance against environmental stresses (Egamberdieva et al., 2019; Ibarra-Villarreal et al., 2021; Patani et al., 2023). Several mechanisms underlying the interactions between plants and *Bacillus* strains under salt stress have been revealed. *B. atrophaeus* WZYH01 protected maize from salt stress by regulating the levels of plant hormones (IAA and ABA) and increasing nutrient acquisition (Hou et al., 2022). Similarly, *B. pumilus* NCT4, *B. firmus* NCT1, *B. licheniformis* LCT4, *B. cereus* LAT3, and *B. safensis* LBM4 enhanced the tolerance of tomato plants to salt stress through increased total soluble sugar, proline and chlorophyll contents (Patani et al., 2023). *B. subtilis* helps mitigate salt-induced oxidative stress in soybean plants by modulating antioxidant defense and glyoxalase systems while maintaining ion homeostasis and osmotic adjustment (Hasanuzzaman et al., 2022). Woo et al. (2020) analyzed the ameliorative effects of the *B. subtilis* strain GOT9 on *Arabidopsis thaliana* and *Brassica campestris* under salt stress. Collectively, these results show that a variety of *Bacillus* strains interact with their specific plant hosts and contribute to enhanced tolerance against salt stress. The acclimation of plants to environmental stress after PGPR induction is a complex and coordinated response involving multiple physiological, biochemical, metabolic, and molecular mechanisms. Hence, a thorough understanding of the mechanisms induced by the strain in plants is imperative for enhancing plant yield and quality, as well as sustainable agricultural development.

Legumes are susceptible to a range of abiotic threats, with salinity being one of the most critical barriers to crop yield (Yilmaz and Kulaz, 2019). Horsegram [*Macrotyloma uniflorum* (Lam.) Verdc] is a dicotyledonous plant that belongs to the Leguminosae family, extensively distributed across South Asia, Australia, West Indies and Africa. This species is cultivated mainly for providing nutritional security in the form of food, livestock supplements and green manure because of its high nitrogen availability, nutritious composition, indomitable pest resistance, and drought tolerance (Bolbhat and Dhumal, 2009), and it has been identified as a potential, future food

source by the U.S. National Academy of Sciences (Kadam and Salunkhe, 1985). Like several other legume crops, *M. uniflorum* is susceptible to salinity stress. Therefore, it is necessary to improve the tolerance of *M. uniflorum* to the deleterious effects of salinity, which could be achieved by using PGPR. However, knowledge of the potential of PGPR isolates and their effects on the growth and physiological characteristics of *M. uniflorum* under salt stress remains quite limited. The present study was carried out to determine the behavior of the selected PGPR strains under salinity stress conditions as well as their role in enhancing *M. uniflorum* growth. Thus, the present study was undertaken. (a) Study of the growth-promoting traits of isolated strains, (b) identification and characterization of the isolated bacteria based on biochemical characteristics and confirmation of the bacterial genera through 16S rRNA sequence analysis, and (c) evaluation of the effects of the bacteria on the growth, physiology, and expression level of stress tolerance genes in *M. uniflorum* seedlings under salt stress. Our results not only provide valuable information for exploring the mechanism by which PGPR alleviate the osmotic and oxidative stress of salt-stressed plants but also provide an eco-friendly method for cultivating high-quality and high-yield *M. uniflorum* in salt environments, and provide theoretical reference for the biological improvement of salt soil.

Materials and methods

Materials

M. uniflorum seeds of the native cultivar Yazhou with a viability >98% were obtained from the Institute of Tropical Crop Genetic Resources, Chinese Academy of Tropical Agricultural Sciences. Healthy seeds were selected and stored in a Kraft paper bag at 4°C until use. The PGPR strain of *B. amyloliquefaciens* PG-4 (16S rRNA GenBank: PP320457) was isolated from *M. uniflorum* rhizosphere soil in Danzhou, Hainan Province, China (19°31'N, 109°34′E). The isolation procedure was the same as that described in Hmaeid et al. (2019) and Patani et al. (2023). The strain was stored in 25% glycerol solution at −80°C until use. The strain was preserved in the China Center for Type Culture Collection, with preservation numbers of CCTCC AB 2024049.

Morphological observation of strains and homology analysis of 16S rDNA sequences

After preparing a 24-h culture, the strain was smeared on slides and subsequently fixed with heat. The samples were treated with crystal violet for 1 min following rinsing with running water. The samples were again flooded with gram iodine solution for 1 min following washing with alcohol. Finally, the samples were flooded with safranin, washed with water, dried, and observed under oil immersion conditions (Masi et al., 2021).

Genomic DNA was extracted using an OMEGA Genomic DNA Extraction Kit. Next, 16S rDNA fragments were PCR-amplified with the universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT). The PCR mixture (30 μL) consisted of 15 μL of 2 × PCR mix, 1 μL of DNA, 1 μL of the upstream primer (10 mM), 1 μL of the downstream primer (10 mM), and 12 μL of ddH_2O. Reaction conditions: 95°C 5 min, 94°C 45 s, 55°C 45 s, 72°C 1 min 15 s, 32 cycles, 72°C 10 min. After the reaction was completed, the size and specificity of the amplified fragments were verified by 1% agarose gel electrophoresis and photographed using a gel imaging system. The PCR amplification products were purified and sequenced by Nanshan Biotech (China) Co., Ltd. The 16S rDNA sequencing results of the obtained strains were analyzed using BLAST, and the related sequences with high homology were selected for relationship analysis. A phylogenetic tree was constructed via the neighbor method in MEGA6.0 software.

Plant growth promotion assays

The plant growth-promoting properties of PG-4, including nitrogen fixation, siderophore production, NH_3 production, ACC deaminase production, cellulase production, pectinase production and amylase production, were evaluated in the presence of 0 mM, 50 mM, 100 mM and 200 mM NaCl. The determination method is described below.

Nitrogen fixation

A nitrogen (N_2) fixation test was performed in malate nitrogen-free mineral media with modifications (Baldani and Döbereiner, 1980). The inoculated media were incubated for 72 h at 30 ± 2°C. A color change from pale green to blue was considered to indicate a positive effect of N_2-fixing activity.

Siderophore production

Siderophore secretion by the isolated bacteria was assessed using blue agar plates containing chrome azurol S (CAS) (Sigma–Aldrich) (Schwyn and Neilands, 1987). A positive reaction was indicated by the formation of an orange zone around the colony, indicating siderophore excretion.

NH_3 production

NH_3 production was checked on peptone water according to Cappuccino and Sherman (1992). Fresh cultures grown in LB broth were inoculated into 10 mL of peptone water and incubated at 30 ± 2°C. After 48 h, 500 μL of Nessler's reagent was added to each tube. Ammonia production is indicated by the development of a color change from brown to yellow.

ACC deaminase production

The 1-aminocyclopropane-1-carboxylic acid deaminase activity of PG-4 was detected by using DF medium and ADF medium (Shanghai Yuanye Bio-Technology Co., Ltd., China). These media were inoculated with 1% (v/v) fresh PG-4 cultures and incubated in a shaker (180 rpm, 28°C) for 5 days. The optical density was measured at 600 nm by a spectrophotometer from three replicates; the optical density result was considered to indicate a positive result for ACC deaminase production for PG-4 that was grown in ADF media and that showed no growth on DF media.

Assay for enzyme production

Cellulase production was tested using carboxymethylcellulose (CMC) agar medium (Gupta et al., 2012). After 24 h of incubation at

30°C, the CMC plates were flooded twice: first, with an aqueous solution of Congo red (1% w/v) for 15 min, followed by 1 M NaCl for 15 min. A clear halo was considered to indicate cellulase production. Pectinase production was tested as described by Plazinski and Rolfe (1985) using ammonium mineral agar medium, with some modifications. After 3 days at 30 ± 2°C, the plates were flooded with 2% hexadecyltrimethylammonium bromide. Pectinase production was detected by clear halos around the colonies. Amylase production was determined by soluble starch agar medium. After 2 days of incubation at 28°C, the ability of PG-4 to hydrolyze amylase was determined by the appearance of a halo zone around the colonies, which was confirmed by immersion in Lugol's iodine solution for 15 min and 70% ethanol (Zhou et al., 2021). The noninoculated plates were used as controls.

Evaluation of the plant growth-promoting ability of *B. amyloliquefaciens* PG-4

The healthy seeds were surface-sterilized by immersion in 70% ethanol for 50 s, followed by rinsing three times with sterile distilled water, submerging in 10% (v/v) sodium hypochlorite for 15 min and subsequent washing 5 times with sterile distilled water. The seed germination experiment was carried out as described by Li et al. (2021) with minor modifications. The sterilized plants were completely soaked in 20 mL of PG-4 suspension ($OD_{600} = 0.6$) for 2 h to allow PG-4 to colonize the *M. uniflorum* seeds, which served as the inoculation treatment. Seeds soaked in LB media or distilled water served as uninoculated controls. Next, seeds subjected to different treatments were air-dried at room temperature and subsequently transferred to Petri dishes (10 × 10 cm) containing 0.7% water agar at 25°C under a 12 h light/12 h dark cycle for germination. Each treatment had five replicates, and each replicate contained 18 seeds. The germinated seeds were counted every day until they reached a constant number (approximately 2 days). The germination percentage, seedling plumule length, radicle length, and fresh weight were measured at 3 days after germination.

Pot experimental design

The experimental treatments included (i) no bacterial inoculum or salt stress (CN), (ii) bacterial inoculum or salt stress (BN), (iii) no bacterial inoculum with salt stress (CS), and (iv) bacterial inoculum and salt stress (BS). Surface-sterilized *M. uniflorum* seeds with normal and healthy appearances were selected. Before sowing, the seeds were inoculated by immersion for 2 h in the respective bacterial inoculum ($OD_{600} = 0.6$) or in sterile distilled water (bacterial inoculum treatment and uninoculated controls). The seeds were subsequently transferred to a separate pot (10 cm × 8 cm × 10 cm) filled with a mixture of autoclaved vermiculite and nutrient soil (1:1, v:v). After 7 and 14 days, the seedlings were thinned to one per pot and then reinoculated with 1.0 mL of the corresponding bacterial inoculum ($OD_{600} = 0.6$) (BN and BS) or sterile distilled water (CN and CS) was applied to the stem base. Salt stress was imposed beginning on day 12 by supplementing the watering solution (water or nutritive solution) with 100 mM NaCl. The plants were grown at 25°C under a 16 h light/8 h dark cycle at Hainan University (Haikou, Hainan, China). Pots were arranged in a randomized block design, and each group consisted of 8 pots. Each treatment was kept in independent trays to avoid contamination, and the trays were rotated weekly to randomize possible position effects. After 14 days of stress, the SPAD values; plant height; plant diameter; and shoot and root fresh weights (FWs) were recorded. Then, all plants were removed from the pots, immediately cleaned with distilled water, and then stored at −80°C to measure physiological characteristics. In the remaining three pots of each treatment, all the plants in the pot were removed from the pot and cleaned immediately with distilled water. Then, the samples were oven dried at 60°C for 72 h to measure their dry weight and Na^+ and K^+ concentrations.

Physiological characterization and determination of ion contents

Prior to harvest, a SPAD LD-YD chlorophyll meter (LANENDE, Shandong, China) was used to directly determine the relative chlorophyll contents of the plant leaves via soil and plant analyzer development (SPAD). The mature leaves of 10 plants were tested 3 times, and the average values were obtained (Li et al., 2020). The contents of proline (Pro), total soluble sugar (TSS) and malondialdehyde (MDA) were measured by a PRO BC0295 assay kit (Beijing Solarbio Science & Technology Co., Ltd), a BC0035 Plant Soluble Sugar Content Assay Kit (Beijing Solarbio Science & Technology Co., Ltd), and a BC0025 MDA assay kit (Beijing Solarbio Science & Technology Co., Ltd.) following the manufacturer's instructions, respectively. The activities of SOD and POD were measured by a BC0175 SOD assay kit (Beijing Solarbio Science & Technology Co., Ltd.) and a POD BC0095 assay kit (Beijing Solarbio Science & Technology Co., Ltd.) following the manufacturer's instructions, respectively. All leaves used in testing were collected from mature plants at a single location.

A fine powder of dry plant leaves was mixed with acetic acid (0.1 N) (w/v) at 90°C for 2 h, and flame emission spectroscopy was used to measure the Na^+ concentrations (Rus et al., 2001), the contents of K^+ were measured by a Potassium Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

RNA sequencing procedures

RNA was extracted from twelve samples (four treatments × three biological replicates) using a TRIzol reagent kit (Invitrogen, Carlsbad, CA, United States). Total RNA was reverse transcribed to cDNA using an NEBNext Ultra RNA Library Prep Kit for Illumina (NEB #7530, New England Biolabs, Ipswich, MA, USA). Library construction, quality detection and Illumina sequencing were carried out by Gene Denovo Biotechnology Co., Guangzhou, China. An Agilent Bioanalyzer 2,100 system was used to evaluate RNA quality, and library sequencing was performed through an Illumina NovaSeq 6,000 platform. After removing adaptors, the unknown nucleotides and those of low quality were filtered from the raw reads to obtain clean reads. All the downstream analyses were based on these clean reads. *De novo* assembly was carried out using the *de novo* assembly software Trinity.[1]

1 http://trinityrnaseq.sourceforge.net/

Differential expression analysis

To compare the read count values of every gene to the original expression of the gene, HTSeq (0.9.1) statistics were used to standardize the expression by using fragments for exon kilobase per million fragments mapped (FPKM) values. Genes with a false discovery rate (FDR) < 0.05 and an absolute fold change ≥2 were defined as differentially expressed genes (DEGs). The software package R language heatmap (1.0.8) was used for bidirectional clustering analysis of all genes for all samples. For various samples, a heatmap was generated using the Euclidean method to measure the distance to and complete relation method to the cluster according to the expression level of the same and different genes in the same sample.

Gene annotation and pathway analysis

For annotations, all unigenes that were proven to be longer than 200 bp were subjected to a BLAST search (E-value $<1e^{-5}$) against the NCBI nonredundant (NR) protein database (Pruitt et al., 2007), manually annotated and reviewed protein sequence database Swiss-Prot (Apweiler et al., 2004), Gene Ontology (GO) (Ashburner et al., 2000), Clusters of Orthologous Groups of proteins (KOG/COG) (Tatusov et al., 2000; Koonin et al., 2004), protein family (Pfam) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Finn et al., 2008; Kanehisa et al., 2008). The Gene Ontology database[2] was used to map all the DEGs; however, Blast2GO software was used to calculate the differentially enriched genes. KEGG analysis of all the DEGs was performed by using KOBAS 2.0 software. For further analysis, significant pathways were selected.

Quantitative real-time PCR

The expression patterns of ten genes were analyzed via qRT-PCR. A pair of primers for each gene was designed using Primer 3.0 and are listed in Supplementary Table S1. qRT-PCR was subsequently performed using a Real-Time PCR System with a total reaction volume of 10 μL, which consisted of 1 μL of cDNA template, 0.5 μL of forward and reverse primers, 5 μL TB Green Premix Ex Taq II (2×) and 3 μL of sterilized ddH_2O. The PCR procedure was as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 59 s, and 60°C for 15 s. The expression levels of ten genes were determined based on the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008), and relative changes in gene expression were calculated from the qRT-PCR data using the TCTP gene as a reference gene.

Statistical analysis

The collected data are presented as the means of the three replicates ± SE (standard error). One-way analysis of variance (ANOVA) was used to determine statistically significant differences between the means of treatments according to Duncan's multiple range test ($p = 0.05$). All statistical analyses of data were performed using IBM SPSS Statistics 26.0. The calculation formula for Z-Score in the heat map is as follows: Z = (FPKM - mean)/standard deviation.

Results

Plant growth-promoting activity of the PG-4 strain

The PG-4 strain showed the potential to secrete several PGP substances in the presence of different salt concentrations (Control, 50, 100 and 200 mM), as reported in Supplementary Table S2. The PG-4 strain has the potential to fix nitrogen and produce siderophores. Spectrophotometric analysis revealed that strain PG-4 grew better on ADF media than on control DF media under different salt stress conditions, suggesting that PG-4 has the ability to produce ACC deaminase. The analysis also showed that the PG-4 strain has a widespread ability to secrete hydrolytic enzymes, including cellulase, pectinase, and amylase, under different salt stress conditions. These results suggest that the PG-4 strain may be an effective agent for supporting plant resistance to salt stress.

Growth-promoting efficacy of the PG-4 strain

The growth-promoting efficacy of PG-4 on *M. uniflorum* seeds was examined, and the results are shown in Table 1. PG-4 had a growth-promoting effect on seed germination and seedling growth, significantly increasing the germination percentage, plumule and radicle length, and fresh weight ($p < 0.05$). The average germination percentage, plumule length, radicle length, and fresh weight were 100 ± 0.00%, 2.35 ± 0.12 cm, 1.99 ± 0.09 cm, and 1.18 ± 0.02 g, respectively, which were equivalent to increases of 7.15, 20.51, 11.17, and 20.4%, respectively, relative to those of the control after PG-4 was inoculated for 3 days. Overall, these results revealed that, compared with that of the noninoculated control seedlings, PG-4 promoted the growth of inoculated *M. uniflorum* seedlings.

Identification of the PG-4 strain

The colony morphology of the PG-4 strain is shown in Figures 1A,B, and the 16S rDNA gene sequences were obtained and compared with GenBank sequences. The strain was identified as gram-positive and facultative anaerobic following the protocol described in the "Common Bacterial System Identification Manual" and "Bergey's Manual of Determinative Bacteriology" with slight modifications (Dong and Cai, 1999). The 16S rRNA amplicon sequencing results revealed a total of 1,404 bases with homology between PG-4 and *Bacillus amyloliquefaciens* NBRC 15535 (NCBI accession number: NR041455.1) of 99.3%. Their close relationship was further supported by a phylogenetic tree (Figure 1C) (Nautiyal et al., 2013; Kim et al., 2017; Liu et al., 2017; Tanaka et al., 2017; Kazerooni et al., 2021). Accordingly, the PG-4 strain was identified as *B. amyloliquefaciens*.

2 http://bioinfo.cau.edu.cn/agriGO/

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TABLE 1 *Macrotyloma uniflorum* seed growth-promoting effects of *Bacillus amyloliquefaciens* PG-4.

Treatment	Germination (%)	Plumule length (cm)	Radicle length (cm)	Fresh weight (g)
Control	93.33 ± 2.72b	1.95 ± 0.10b	1.79 ± 0.07b	0.98 ± 0.01c
LB	96.58 ± 1.40ab	1.98 ± 0.09b	1.85 ± 0.08ab	1.12 ± 0.04b
PG-4	100 ± 0.00a	2.35 ± 0.12a	1.99 ± 0.09a	1.18 ± 0.02a

Different letters indicate significant differences at the level $p < 0.05$.

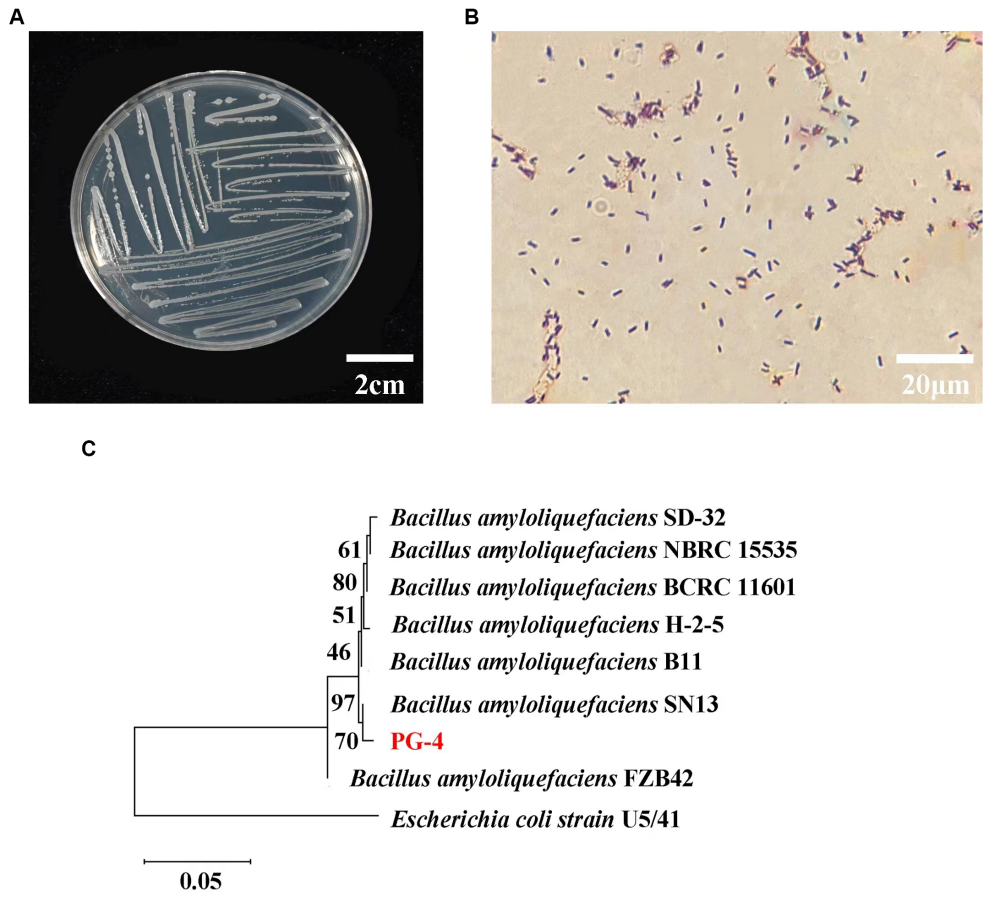

FIGURE 1
Molecular identification and biological characteristics analysis of strain PG-4. **(A)** Morphological characteristics of colony of strain PG-4. **(B)** Gram staining of strain PG-4. **(C)** A phylogenetic tree among eight *Bacillus* strains was derived from alignment of the 16S rRNA gene sequence. Scale bar, 0.05 substitutions per nucleotide position. Information on their isolation sources was obtained from the references and NCBI (BioSample).

Effects of *B. amyloliquefaciens* PG-4 on *M. uniflorum* seedlings under salt stress

After the interaction of *B. amyloliquefaciens* PG-4 with *M. uniflorum* under control conditions (without salt stress) and stress conditions (100 mM NaCl), the growth patterns of the plants were evaluated for 14 days. The growth of the plant seedlings under all treatments (CN, BN, CS, and BS) is shown in Figure 2. Greater plant growth was observed in the plants treated with PG-4 under salt stress (Figure 2A). The shoot length of the treated plants (BS) under salt stress was significantly ($p < 0.05$) different from that of the untreated control (CS) plants. The shoot length of the plants treated with CS was approximately 9.65 cm, whereas the shoot length of the BS-treated plants was approximately 10.05 cm (Figure 2B). Moreover, there was no significant ($p > 0.05$) difference in the roots of the untreated and treated plants under salt stress conditions (Figure 2C). In addition, under salt stress, compared with those in the control group without inoculation (CS), the shoot and root fresh weights of the plants treated with BS increased by 2.7 and 1.8 times, respectively (Figures 2D,E), and the shoot and root dry weights increased by 2.9 and 1.5 times, respectively (Figures 2F,G).

The chlorophyll content is an important index for predicting the health and photosynthetic capacity of plants under salt stress. Therefore, we measured the leaf chlorophyll content to examine the impact of PG-4 on the photosynthetic capacity of plants under salt stress. The results showed that salinity significantly ($p < 0.05$) reduced the chlorophyll content in the leaves of *M. uniflorum*. However, our findings also showed that, compared with that of the uninoculated control plant, PG-4 inoculation significantly increased the chlorophyll content in the plants under salt stress or those under no-salt conditions (Figure 3A).

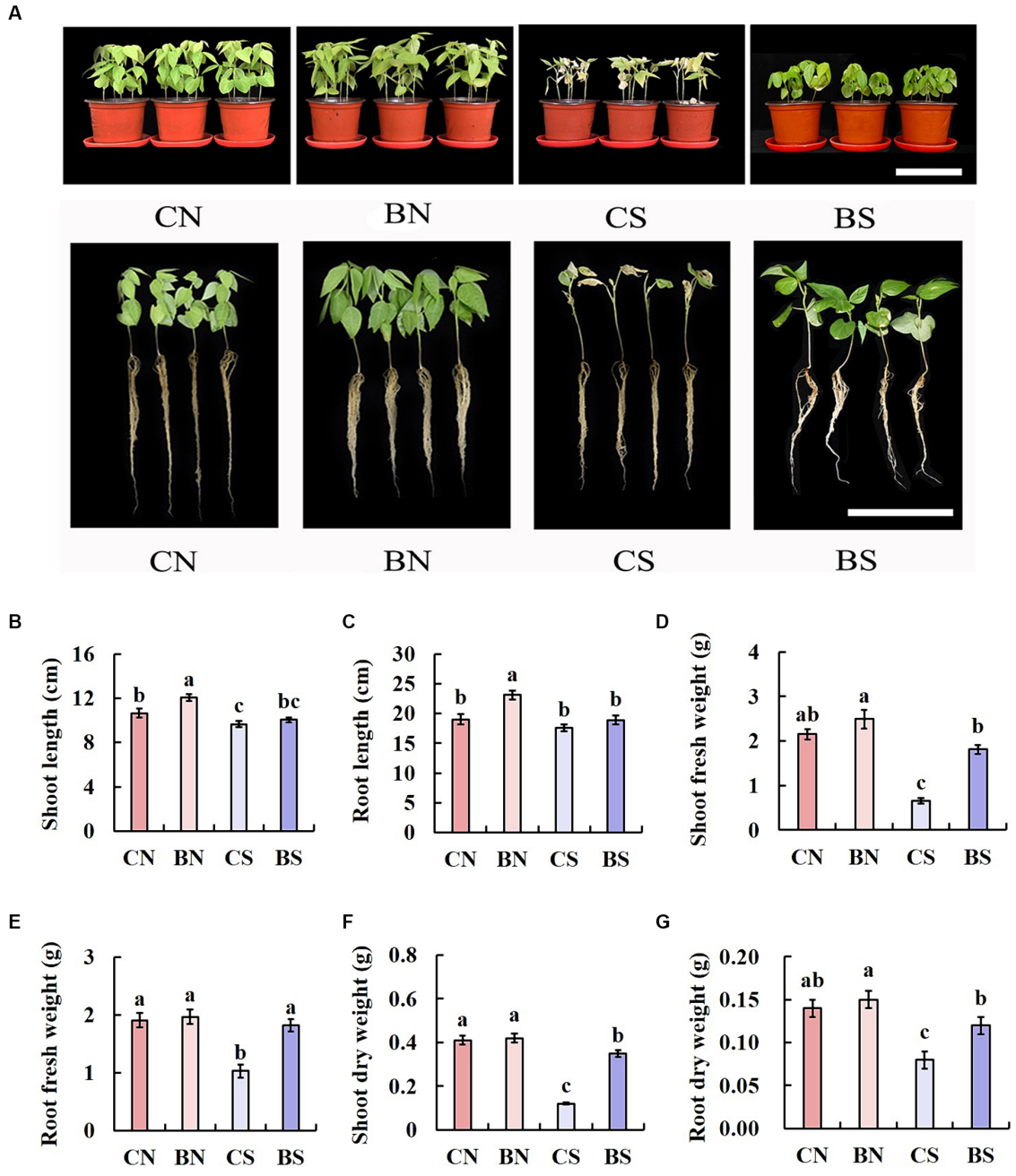

FIGURE 2
Effect of PG-4 inoculation on the growth of *Macrotyloma uniflorum* seedlings under salt stress in a pot experiment. **(A)** Growth status of *M. uniflorum* seedlings under the four treatments. Scale bar, 10 cm. **(B)** Shoot length. **(C)** Root length. **(D)** Shoot fresh weight. **(E)** Root fresh weights. **(F)** Shoot dry weight. **(G)** Root dry weight. CN, CS, BN, and BS represented the treatments of control and non-salt, control and salt, PG-4 inoculated and non-salt, and PG-4 inoculated and salt, respectively.

Salinity significantly ($p < 0.05$) increased the TSS content in the leaves of *M. uniflorum*. However, whether the plants were under salt stress or control conditions, the TSS and Pro content in the leaves of *M. uniflorum* inoculated with PG-4 was significantly higher than that in the leaves of the uninoculated control plants (Figures 3B,C). Salt stress significantly ($p < 0.05$) increased the MDA content. However, under salt stress, inoculation with PG-4 significantly reduced the MDA content in *M. uniflorum* leaves compared with that in uninoculated control plants (Figure 3D). Overall, these results indicated that inoculation with PG-4 can significantly ($p < 0.05$) reduce oxidative stress-related MDA levels and improve the salt tolerance more effectively in plants under salt stress. Furthermore, the data analysis showed that SOD and POD activity was increased by PG-4 inoculation and salinity stress, with the highest activity in BS-treated *M. uniflorum* leaves (Figures 3E,F). The analysis of Na^+ and K^+ content showed that salt stress strongly increased the absorption of Na^+ and decreased K^+ uptake. However, under salt stress, inoculation with PG-4 significantly ($p < 0.05$) increased the accumulation of potassium in plant leaves, while reduced the accumulation of sodium (Figures 3G,H). These data suggest the positive effect of *B. amyloliquefaciens* PG-4 on K^+ absorption under salt conditions.

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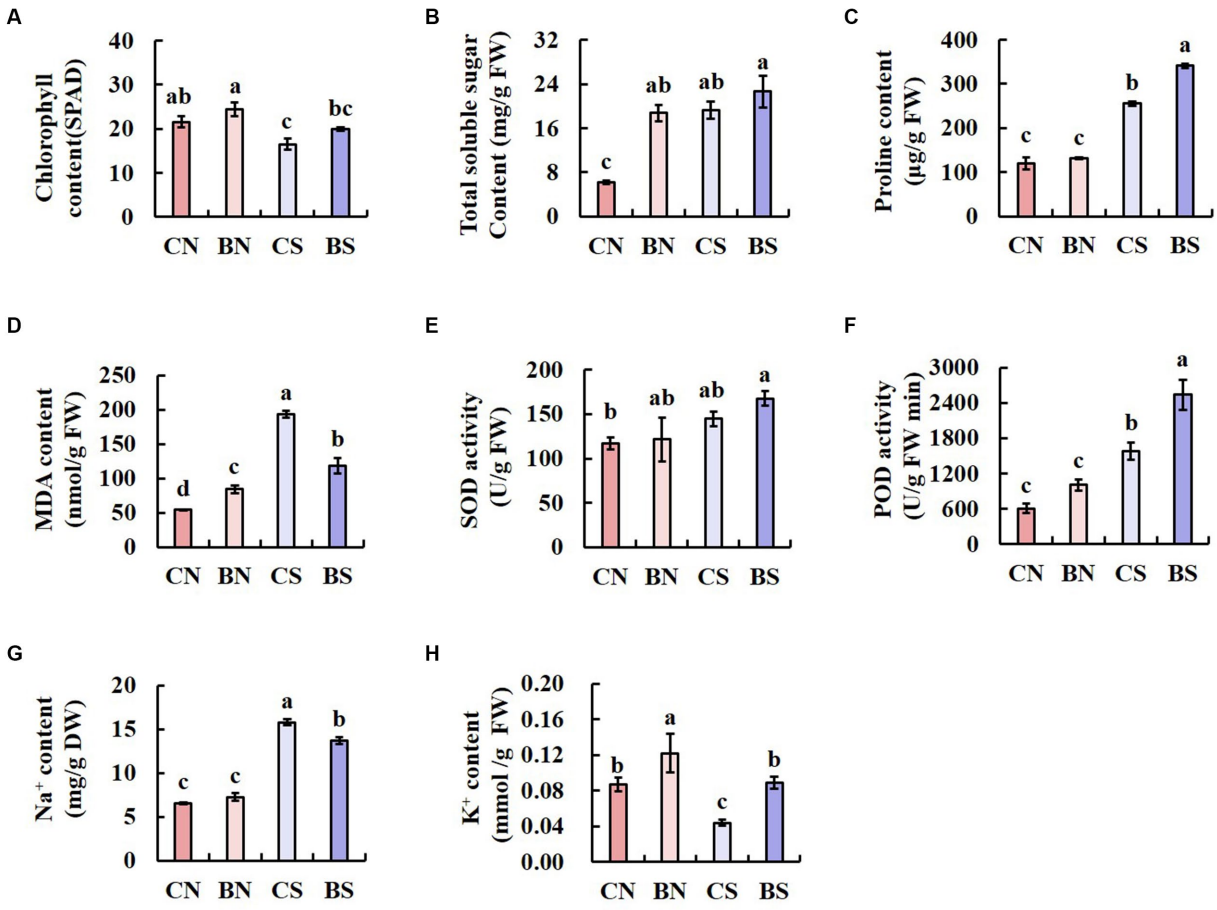

FIGURE 3
The physiological parameters of *M. uniflorum* seedlings under different treatments. Bars represent means ± SE. Different letters indicate significant differences at the level $p < 0.05$ according to Duncan's multiple range test test (n = 3 independent replicates). **(A)** Chlorophyll content. **(B)** Total soluble sugar content. **(C)** Proline content. **(D)** MDA content. **(E)** SOD activity. **(F)** POD activity. **(G)** Na^+ content. **(H)** k^+ content.

Overview of the transcriptome analysis and read assembly

To unravel the mechanism of *B. amyloliquefaciens* PG-4-mediated salt tolerance in *M. uniflorum* plants, transcriptome profiling was carried out using an RNA-Seq approach. In this study, RNA libraries were constructed and sequenced for a total of 12 samples from four groups of *M. uniflorum* samples, namely, CN, BN, CS, and BS. Among them, there were 547,313,032 original fragments. Adapter sequences and low-quality reads, at least 544,006,108 clean reads remained. Q30 (sequences with a sequencing error rate of less than 0.1%) was ≥94%, and the average GC content was 43.95% (Table 2). Trinity software was used to generate 42,179 unigenes via *de novo* transcriptome assembly. The average length of unigene is 1,314 bp (Supplementary Table S3; Figure 4A).

Functional annotation

To analyze the comprehensive functional annotation of the 42,179 unigenes, we used BLAST against four databases, namely, the NR, KEGG, KOG, and SwissProt databases. A total of 28,231 unigenes were annotated in at least one database (Figure 4B). Among them, 16,796 unigenes were assigned to 25 functional clusters according to the KOG database. "General function prediction only" (17.4%, 3,611) was the largest category (Figure 4B; Supplementary Figure S1A). In addition, we obtained functional information on the similarity between the sequences of *M. uniflorum* and related species by blasting against other plant species via the NR database. The *M. uniflorum* isoforms had the highest number of hits to *Phaseolus vulgaris* (5,810 hits), followed by *Vigna unguiculata* (4,292 hits) and *Vigna angularis* (3,364 hits) (Figure 4C).

To study the function of these genes in biological pathways, the unigenes were identified via the KEGG pathway database. A total of 6,938 unigenes were grouped into five main KEGG functional categories and 138 KEGG pathways (Figure 4D; Supplementary Table S4). A high proportion of unigenes were distributed in "Metabolism" (7,730) (Figure 4D), such as "Global and overview maps" (3,259), "Carbohydrate metabolism" (1,133), and "Amino acid metabolism" (627). To further classify the *M. uniflorum* unigenes, GO annotation was performed based on three major categories, namely, biological process, cellular component, and molecular function (Supplementary Figure S1B; Supplementary Table S5). For biological process classification, "cellular process" (14,775), "metabolic process" (12,742), and "biological

TABLE 2 Illumina-seq output statistics of 12 samples.

Sample	Raw reads	Clean reads	Clean bases/bp	Q20/%	Q30/%	GC content/%
CN 1	39,696,570	39,438,428	5,883,398,498	98.19	94.50	43.91
CN 2	45,117,204	44,824,702	6,673,992,341	98.46	95.17	44.09
CN 3	44,289,272	44,021,598	6,554,995,430	98.25	94.70	44.20
BN 1	44,653,594	44,370,548	6,596,504,239	98.04	94.18	44.11
BN 2	42,546,186	42,304,678	6,309,086,300	98.05	94.17	44.22
BN 3	50,120,930	49,829,064	7,426,054,656	98.30	94.84	44.06
CS 1	54,736,570	54,386,662	8,109,747,709	98.22	94.60	43.83
CS 2	39,172,702	38,944,782	5,803,659,457	98.12	94.34	43.37
CS 3	46,122,110	45,837,128	6,834,092,401	98.06	94.21	43.90
BS 1	39,288,066	39,072,358	5,823,173,139	98.44	95.19	44.01
BS 2	53,720,896	53,414,096	7,947,344,030	98.57	95.58	43.93
BS 3	47,848,932	47,562,064	7,094,648,863	98.31	94.84	43.82
Total	547,313,032	544,006,108	81,056,697,063	–	–	–

regulation" (4,309) were the three major categories. Unigenes involved in "binding" (12,266), "catalytic activity" (11,136) and "transporter activity" (1,807) were highly represented in the molecular function subgroups. The major categories of cellular component were "cellular anatomical entity (10,809)," "protein-containing complex (3,876)" and "virion component (101)."

Differential expression gene (DEG) and functional enrichment analysis among different treatments

The expression of these unigenes was subjected to principal component analysis (PCA) (Supplementary Figure S2A). The first two components, which explained 49.6 and 22.8% of the variation, respectively, were able to distinguish the four different treatment groups, with three biological replicates clustering together, and each group had certain differences in their gene expression profiles. A heatmap of Pearson's correlation coefficients between all sample pairs also showed that each sample was reliable with good reproducibility (Supplementary Figure S2B). Based on the read counts, DEGs were assessed with DESeq2 software. In this study, DEGs between treatments were defined on the basis of an absolute fold change ≥ 2 and a false discovery rate (FDR) < 0.05. Hierarchical clustering analysis was used to determine the expression patterns of DEGs under different experimental conditions (Figure 5A). Four different comparison groups were obtained by comparing the DEGs between different treatments: BN vs. CN, BS vs. BN, CS vs. CN, and BS vs. CS. The expression trends and numbers of DEGs identified in the different comparisons are shown in Figure 5B. There were 5,525 DEGs between the BN vs. CN groups, 3,277 upregulated genes and 2,248 downregulated genes. A total of 1,473 DEGs were found between BS vs. BN, which included 859 upregulated genes and 614 downregulated genes (Figure 5C). A total of 3,783 DEGs were found between CS vs. CN; these included 2,280 upregulated genes and 1,503 downregulated genes (Figure 5C). A total of 1,298 DEGs were found between BS vs. CS groups, which included 819 upregulated genes and 479 downregulated genes (Figure 5C). The total number of unique downregulated DEGs in the BS vs. BN comparison was 443, with 1,332 in the CS vs. CN comparison, while 171 DEGs were common between the treatments; 819 unique upregulated DEGs were found in the BS vs. BN comparison; and 1,827 were found in the CS vs. CN comparison, while 40 DEGs were common between the two treatments (Figure 5D).

Functional annotation and classification of differentially expressed genes

To further predict the DEGs induced by PG-4 inoculation and salt stress, functional annotation of the DEGs in the four groups was performed based on GO and KEGG analyses. Among the four comparative groups, BN vs. CN, BS vs. BN, CS vs. CN, and BS vs. CS, 3,904, 1,038, 2,763 and 905 DEGs, respectively, were subdivided into at least one GO term to describe their biological processes, molecular functions, and cellular components (Supplementary Figure S3). Under no-salt stress, a higher percentage of the PG-4-induced DEGs involved in plant growth regulation were found; these DEGs were associated mainly with "Cellular process," "metabolic process" and "response to stimulus" in the biological process category (Supplementary Figure S3A). According to the "response to stimulus" classification, 832 genes were changed by PG-4 inoculation under no-salt stress, whereas only 203 DEGs were regulated by salt stress in PG-4-inoculated seedlings (Supplementary Figures S3A,B). In addition, all DEGs induced by salt stress and PG-4 inoculation were mapped via KEGG to reference canonical pathways to identify the associated biological pathways (Supplementary Figure S4). A total of 347 salt stress-related DEGs in PG-4-inoculated plants were assigned to 109 pathways, which were classified into 19 KEGG pathway subcategories, of which the most common DEGs were "global and overview maps" followed by "carbohydrate metabolism," "biosynthesis of other secondary metabolites," and "translation" (Supplementary Figure S4A; Supplementary Table S6). The DEGs induced by PG-4 in salt-treated plants were assigned to 104 pathways, mainly "global and overview maps," "carbohydrate metabolism," "biosynthesis of other secondary metabolites" and "amino acid metabolism" (Supplementary Figure S4B; Supplementary Table S7).

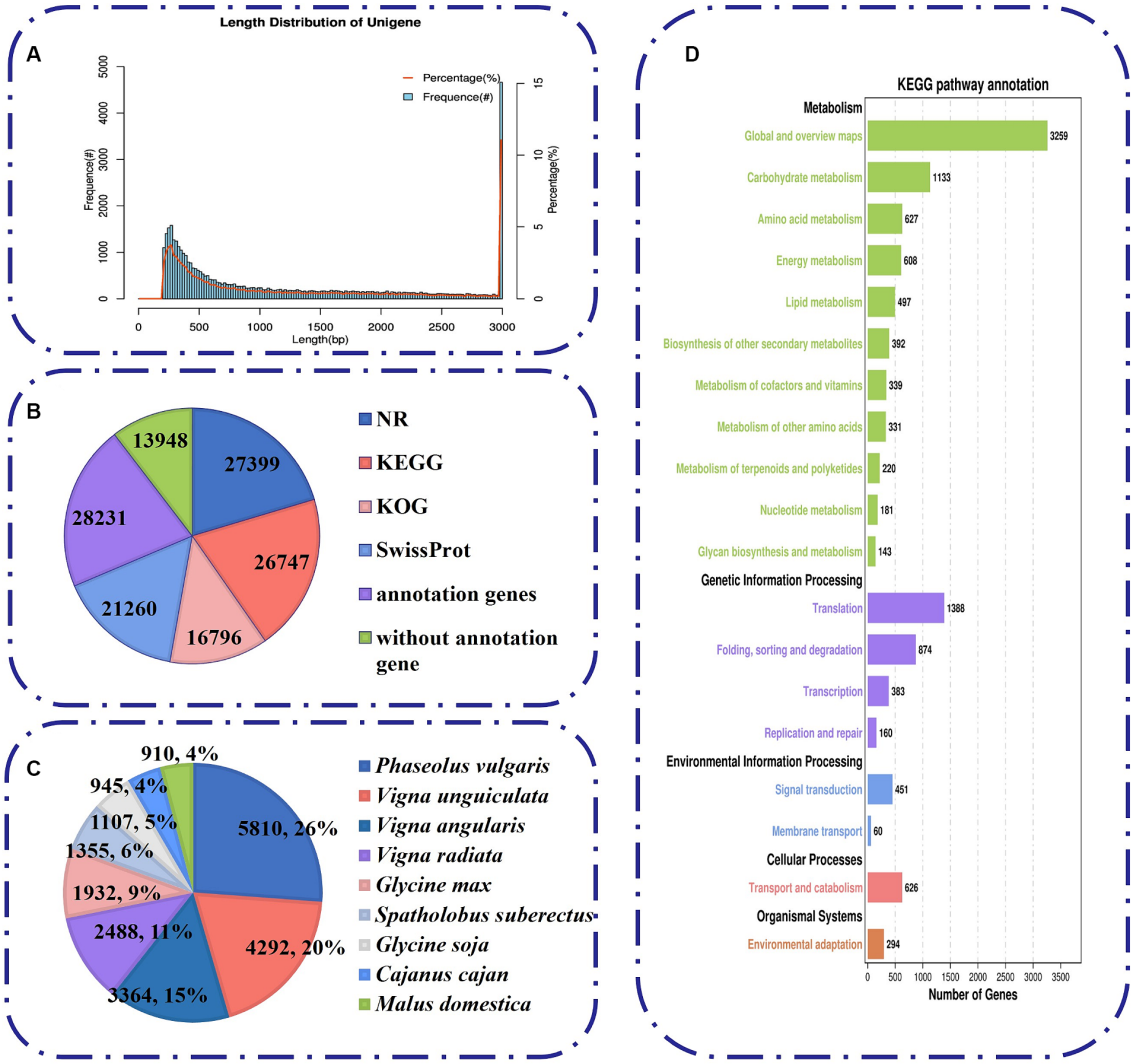

FIGURE 4
Characteristics of unigenes generated by Illumina sequencing. **(A)** The length distribution of all assembled unigenes. **(B)** The number of unigenes annotated by different databases, including NR, KEGG, KOG and Swiss-Prot. **(C)** Species distribution of the top BLAST hits for all homologous sequences. **(D)** A total of 6,938 unigenes were assigned to different KEGG terms.

Go and KEGG pathway enrichment analysis of differentially expressed genes

To elucidate the biological functions of these DEGs, the enriched GO terms were analyzed. The top 20 enriched GO terms are shown in Supplementary Figure S5. The DEGs of the different comparison groups were investigated through GO classification and KEGG enrichment. According to the GO enrichment analysis, 2,763 DEGs in the CS treatment group compared with those in the CN treatment group were significantly enriched (q-value <0.05) in 246 terms, and the most enriched GO terms that involved DEGs between CS vs. CN were related to "catalytic activity," "oxidoreductase activity," and "Small molecule metabolic process" (Supplementary Figure S5A). Moreover, 1,038 DEGs in the BS group compared with those in the BN group were significantly (q value <0.05) enriched in 39 terms, and the DEGs between the BS vs. BN groups were associated mainly with "heme binding," "calcium ion binding," "carbohydrate metabolic process" and the activity of various enzymes, such as oxidoreductase, peroxidase and glycosyltransferase (Supplementary Figure S5B). The lists of all identified significantly (q-value <0.05) enriched GO pathways are shown in Supplementary Tables S8, S9. KEGG pathway analysis was performed to explore the biological pathways represented by DEGs in the four comparison treatments. Several significant pathways were identified in the different comparisons (Figure 6). KEGG pathway enrichment revealed that the DEGs between CS vs. CN were involved primarily in "metabolic pathways," "biosynthesis of secondary metabolites," "phenylpropanoid biosynthesis," "glycosylphosphatidylinositol (GPI)-anchor biosynthesis," "circadian rhythm-plant," "isoflavonoid biosynthesis," "cyanoamino acid metabolism," "alpha-linolenic acid metabolism" and "glycerolipid metabolism" (Figure 6A). The DEGs in the BS vs. BN comparison were assigned to "phenylpropanoid biosynthesis," "plant-pathogen interaction," "metabolic pathways," "biosynthesis of secondary metabolites," "pentose and glucuronate interconversions," "starch and sucrose metabolism" and "plant hormone signal transduction" (Figure 6B). The lists of all identified KEGG pathways are shown in Supplementary Tables S10, S11.

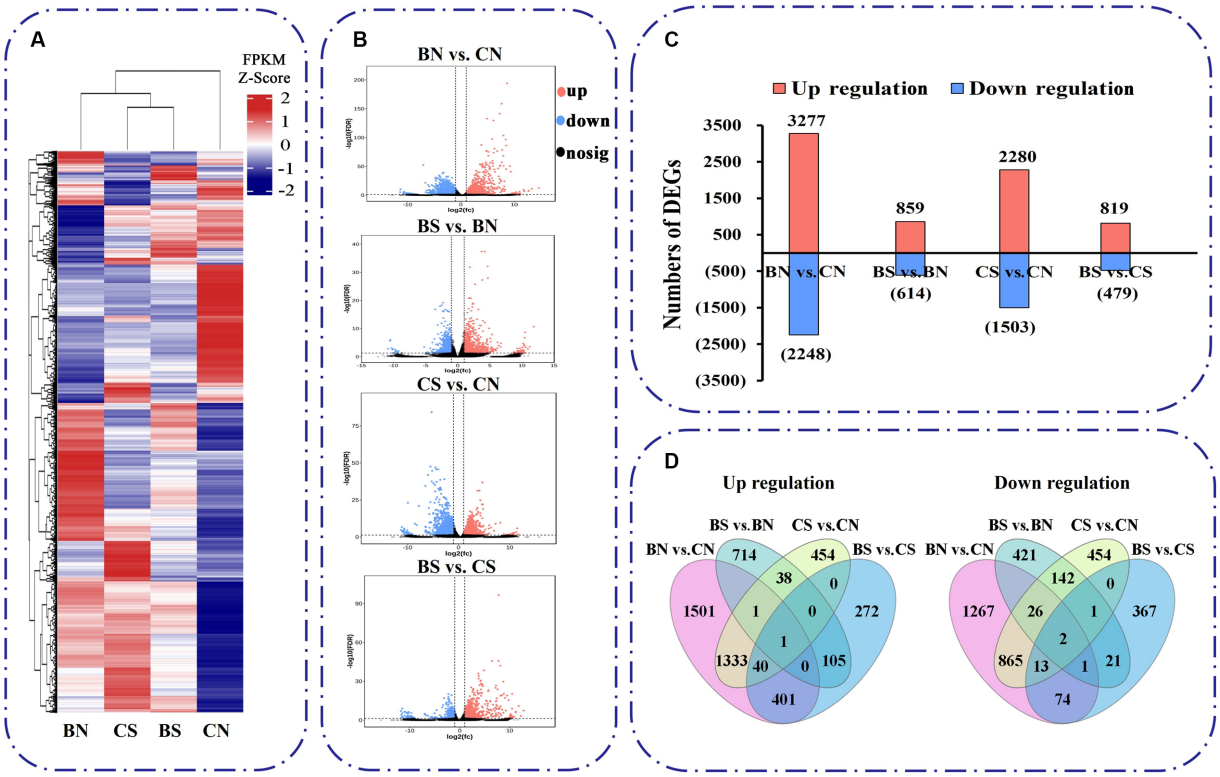

FIGURE 5
Transcriptional variations in *M. uniflorum* roots under different treatments. **(A)** Hierarchical clustrering analysis of the DEGs under different treatments. The color scale represents FPKM values in each treatment, normalized using z-score. **(B)** Significance analysis of the DEGs in different treatments by Volcano plots. **(C)** The number of up- and down-regulated genes in different treatments. **(D)** Venn diagrams showed the proportions of the up-regulated and down-regulated genes in four treatments.

Response of key unigenes to hormone signal transduction

Phytohormones, such as ethylene, IAA, cytokinin and ABA, play critical roles in eliciting a salinity stress adaptation response in plants (Singh et al., 2022). The three protein components related to auxin signal transduction are the auxin receptor-associated SCF complex (SKP1, Cullin and F-box complex), the auxin protein (Aux/IAA), which has regulatory functions, and the auxin response factor (ARF). Early auxin response genes can be divided into three categories: Aux/IAAs, GH3s, and small auxin-up RNAs (SAURs) (Hagen, 2015). The AUX/IAA, ARF, SAUR, GH3, and SAUR genes in *M. uniflorum* under salt stress were significantly downregulated to varying degrees, while these genes were upregulated by PG-4 in *M. uniflorum* under salt stress (Figure 7A; Supplementary Table S12). Three AUX/IAA genes (Unigene0023452, Unigene0023453 and Unigene0035539), one ARF gene (Unigene0030592), one SAUR genes (Unigene0028233) and one GH3 genes (Unigene0015319) were downregulated under salt stress, while they were significantly upregulated by PG-4 under salt stress. The genes Unigene0023453, Unigene0023452, and Unigene0015319 were down regulated by 2.9-, 2.5- and 2.8-fold, respectively, by salt stress, while they were significantly downregulated 2.4-, 2.4- and 1.8-fold, respectively, by PG-4 under salt stress conditions. In the ABA signaling pathway, only one PYL gene (Unigene0002166), three protein phosphatase 2C (PP2C) genes (Unigene0030091, Unigene0031036 and Unigene0031406) and one ABF gene (Unigene0003719) were differentially expressed in the BS vs. CS comparison (Figure 7B; Supplementary Table S12). The protein phosphatase 2C 51 isoform X2 (Unigene0031406) were significantly downregulated by salt stress but upregulated by PG-4 inoculation. In the BR signaling pathway, three TCH4 genes (Unigene0036355, Unigene0022458, and Unigene0027059) were significantly downregulated by 1.3-, 1.2- and 1.2-fold under salt stress, respectively, while they were significantly upregulated by 1.6-, 2.6- and 2.3-fold after PG-4 inoculation under salt stress (Figure 7C; Supplementary Table S12). A differentially expressed JAZ genes (in JA signaling pathway) were present in BS vs. CS (Figure 7D; Supplementary Table S12), and two differentially expressed TGA genes (in SA signaling pathway) were present in BN vs. CN (Figure 7E; Supplementary Table S12). Overall, these results indicated that PG-4 can affect the signal transduction process of plants in response to salt stress, which can help plants prepare for future salt stress.

Response of key unigenes to transporter proteins and key cellular metabolic processes

Among the thirteen DEGs related to ABC transporters expressed in seedlings, seven ABC transporter DEGs (Unigene0003435,

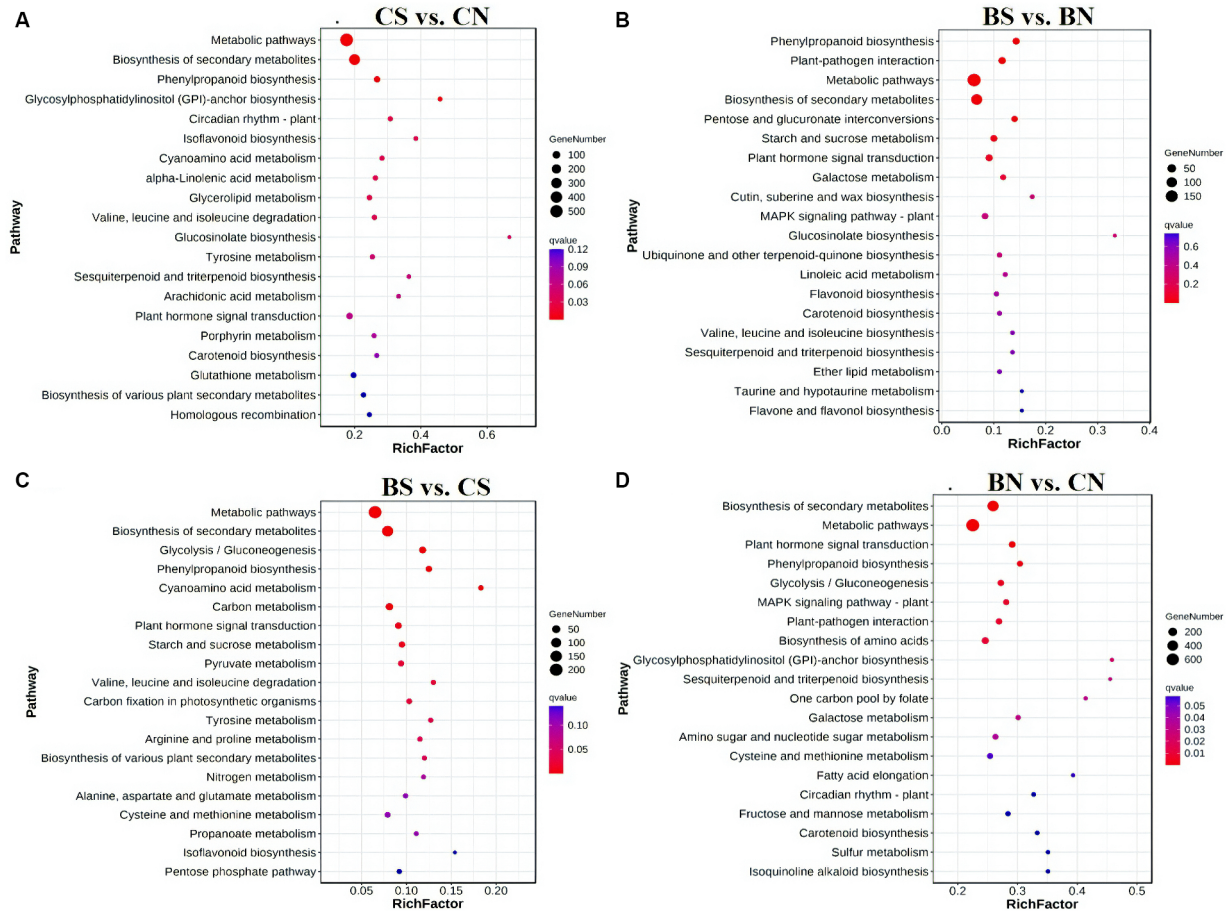

FIGURE 6
Enriched KEGG pathways analysis on of genes with significant differences between groups. **(A)** Enriched KEGG pathways of differential genes between CS and CN. **(B)** Enriched KEGG pathways of differential genes between BS and BN. **(C)** Enriched KEGG pathways of differential genes between BS and CS. **(D)** Enriched KEGG pathways of differential genes between BN and CN. False discovery rate (FDR) is the corrected *p*-value.

Unigene0006341, Unigene0019507, Unigene0022111, Unigene0022624, Unigene0027432, and Unigene0029184) were significantly downregulated, and three (Unigene0000140, Unigene0033713, and Unigene0037140) were significantly upregulated in the CS vs. CN comparison (Supplementary Figure S6A; Supplementary Table S13). In the BS vs. CS comparison, three ABC transporter DEGs (Unigene0003435, Unigene0006339, and Unigene0022513) were significantly upregulated, while only one (Unigene0020052) was significantly downregulated. In addition, the expression of the ABC transporter B family member 11 (Unigene0003435) was significantly downregulated by 1.94-fold in response to salt but significantly upregulated by 6.50-fold in response to PG-4 inoculation under salt stress (Supplementary Figure S6A; Supplementary Table S13). In soluble sugar metabolism, Unigene0038428 and Unigene0038427, which encode invertase, were significantly downregulated, and Unigene0014202, Unigene0010635, and Unigene0019276, which encode sucrose synthase, were significantly upregulated in the CS vs. CN comparison. In the BS vs. CS comparison, the granule-Unigene0003715, glucan endo-1,3-beta-glucosidase 1 (Unigene0042124) and 12 (Unigene0016553) genes were significantly upregulated (Supplementary Figure S6B; Supplementary Table S14).

Pectinesterase (EC 3.1.1.11) is an enzyme that catalyzes the hydrolysis of pectin to produce pectinic acid and methanol and can regulate cell wall strength, cell growth and stress resistance. The expression of DEGs related to pectin metabolism was significantly upregulated by PG-4 inoculation under salt conditions. The pectinesterase/pectinesterase inhibitor 47 (Unigene0031614) and pectinesterase (Unigene0035002) were significantly downregulated (1.50-fold and 1.48-fold, respectively) by salt stress, while they were significantly upregulated (1.79-fold and 1.74-fold, respectively) by PG-4 under salt stress (Supplementary Figure S6C; Supplementary Table S15). In addition to the above transcripts encoding carbohydrate-active enzymes, the xyloglucan endotransglucosylase/hydrolase protein (XTH) transcript required for cell wall synthesis and modification was also shown to be induced by PG-4 inoculation under salinity stress (Supplementary Figure S6D; Supplementary Table S16). In the BS vs. CS comparison, seven XTH DEGs (Unigene0007670, Unigene0036223, Unigene0022458, Unigene00036355, Unigene0022457, Unigene0022459 and Unigene0027059) were significantly upregulated, and only two XTH DEGs (Unigene0033881 and Unigene44199) were significantly downregulated. Overall, these results suggested that the changes in the cell wall structure of *M. uniflorum* in response to salt stress could be adjusted by the inoculation of PG-4 (Supplementary Figure S6D; Supplementary Table S16).

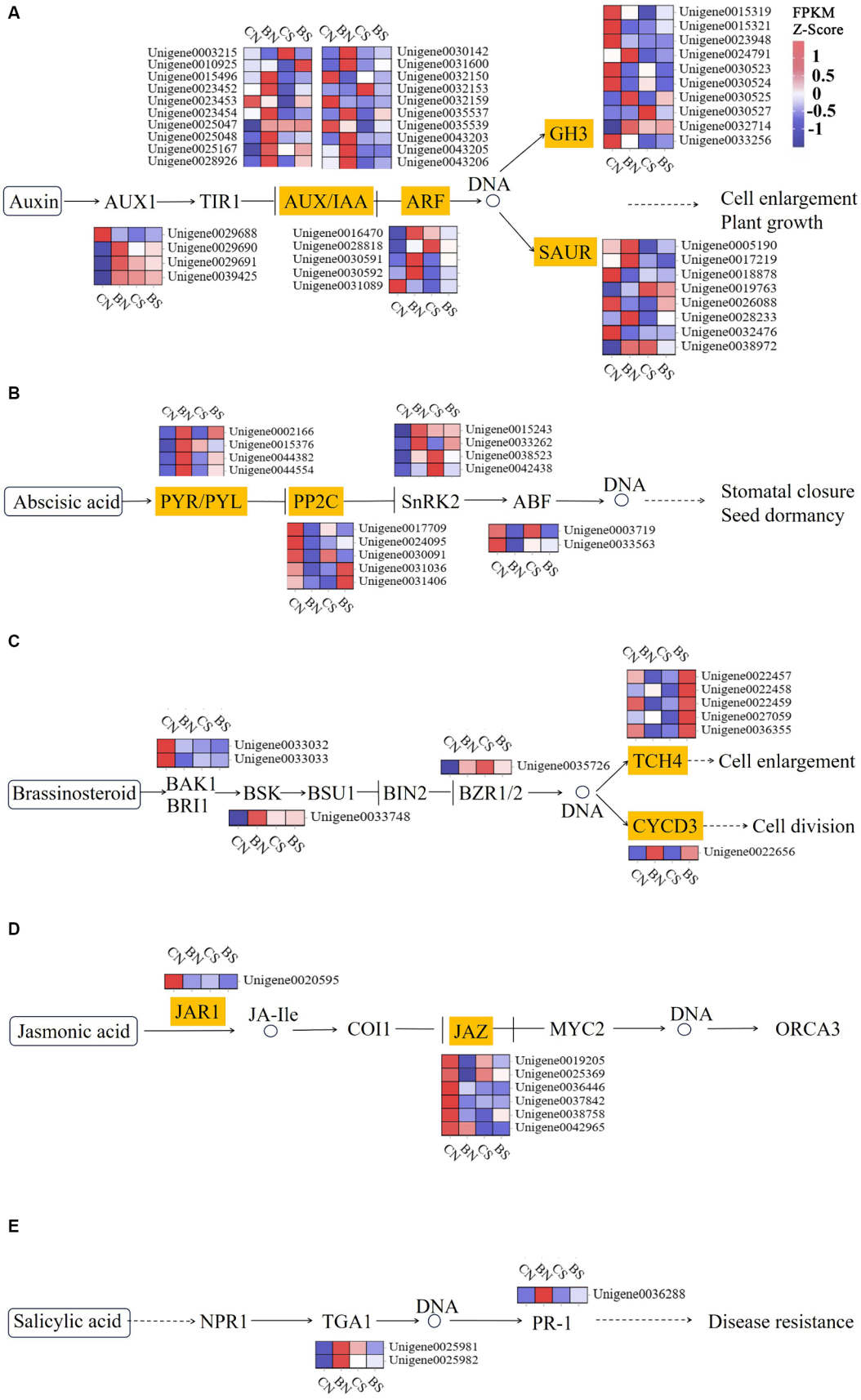

FIGURE 7
PG-4 activated plant hormone signal transduction pathway under salt stress. **(A)** Auxin signaling pathway. **(B)** ABA signaling pathway. **(C)** BR signaling pathway. **(D)** JA signaling pathway. **(E)** SA signaling pathway. The color scale represents FPKM values in each treatment, normalized using z-score.

Classification of transcription factors (TFs) under PG-4 inoculation and salt stress

In this study, 74 and 166 differentially expressed TFs were identified in the CS vs. BS comparison and the CN vs. CS comparison, respectively, and mainly belonged to the TF families APETALA2/ethylene responsive factor (AP2/ERF), WRKY, myeloblastosis protein (MYB) superfamily, NAC domain protein (NAC), basic helix–loop–helix (bHLH), and basic leucine zipper (bZIP), which are known to be associated with plant abiotic and biotic stress response and regulation (Figure 8; Supplementary Table S17). As shown in Figure 8A, these TFs were extensively upregulated by PG-4 under salinity stress, suggesting the special role of PG-4 in the transcriptional regulation of stress responses. In contrast, 60.64% of the TFs were downregulated by salinity stress, which may reflect the compromised defense response in *M. uniflorum* plants. AP2/ERF is one of the largest TF families involved in the response to salt stress (Xie et al., 2019). Among these TFs, ERF106 (Unigene0018746), PTI5 (Unigene0023663) and ERF095 (Unigene0025360) were significantly downregulated by salt treatment but significantly upregulated upon PG-4 inoculation (Figure 8B; Supplementary Table S17).

RNA-seq results validated by qRT–PCR

To verify the reliability of the RNA-seq data, we randomly selected 10 unigenes (Unigene0023113, Unigene0029497, Unigene0027804, Unigene0022458, Unigene0022457, Unigene0036457, Unigene0004787, Unigene0030152, Unigene0037512, and Unigene0027059) for further investigation via qRT-PCR (Supplementary Table S1; Supplementary Figure S7A). The results showed that the expression patterns determined via qRT-PCR were highly consistent with the RNA–seq data, with a relative R2 of 0.94 (Supplementary Figure S7B).

Discussion

To address the increasing the demand for food due to the growing world population under various abiotic stresses, a more sustainable and environmentally friendly approach is required. The use of PGPR has recently become a new option for improving salt stress tolerance in plants due to its beneficial interactions with plants (Bhat et al., 2020; Chen et al., 2021). *Bacillus* is the most common genus found in PGPR and represent the most widely used and well-studied bacteria in agricultural production (Shultana et al., 2022; Singh et al., 2022; Ditta and Ullah, 2023; Pistelli et al., 2023). Many *Bacillus* spp. have been reported to enhance plant tolerance to salinity and mitigate the salt stress-induced inhibition of plant growth. For example, Akbar et al. (2022) reported that *B. subtilis* and *B. pumilus* significantly enhanced salt stress tolerance in cotton plants under salt stress conditions. Ansari et al. (2019) suggested that the *B. pumilus* strain FAB10 contributes to salt stress alleviation in wheat plants through multiple modes of action. Similarly, *B. amyloliquefaciens* has been demonstrated to increase salt tolerance in a variety of host plants, such as *Arabidopsis* (Liu et al., 2020), maize (Chen et al., 2016), rice (Nautiyal et al., 2013) and *Codonopsis pilosula* (Han et al., 2017). In the present study, the PGP activity of *B. amyloliquefaciens* PG-4 was evaluated under detached and greenhouse conditions (Supplementary Table S2). The results indicated that the plant-beneficial effects were supported by the successful colonization of *M. uniflorum* by PG-4 under both mock and salt conditions. The ability of PGPR to alleviate salt stress damage and improve plant growth could result from the production of bioactive secondary metabolites or compounds. PG-4 produced ACC deaminase, siderophores, nitrogen-fixing enzymes and major enzymes, such as protease, amylase, and cellulase. ACC is a precursor for ethylene, one of the most important regulatory hormones for plant growth (Glick, 2014). The production of siderophores is one of the most vital mechanisms for preventing plant pathogens (Etesami and Maheshwari, 2018), suggesting the potential biocontrol function of PG-4.

Salt stress usually triggers ROS accumulation by reducing CO_2 assimilation, interfering with plant metabolism and causing ion toxicity (Chen et al., 2022; Wang Y. et al., 2022; Wang Q. et al., 2022). The rapid and excessive accumulation of ROS causes lipid peroxidation, protein denaturation, membrane damage and nucleic acid dysfunction in plants (Gupta and Pandey, 2020; Wang Y. et al., 2022; Wang Q. et al., 2022). The level of malondialdehyde (MDA), produced during oxidative damage caused by lipids (lipid peroxidation), is regarded as the main indicator of salinity-induced oxidative stress injury in plant tissues (Gupta and Pandey, 2020; Wang et al., 2021). Compared with that of control plants, the PG-4-inoculated plants presented a lower content of MDA under salt stress (Figure 3D), which agrees with the findings of previous studies (Hmaeid et al., 2019; Gupta and Pandey, 2020; Masmoudi et al., 2021). Wang Y. et al. (2022) and Wang Q. et al. (2022) also showed that *B. cereus* G2 alleviated the toxic effect of oxidative bursts in *Glycyrrhiza uralensis* Fisch. resulting from salt stress by inhibiting the production rate of $O2^-$ and correspondingly decreasing the concentration of MDA. Therefore, we speculate that the inoculation of plants with PG-4 reduces the MDA content and reduces ROS production and accumulation at the initial stage of salt stress, which might be an important mechanism for enhancing the salt tolerance of plants. Antioxidant enzymes play an important role in maintaining the normal functioning of plants by repairing and detoxifying the oxidative damage caused by stress-generated ROS (Akhter et al., 2022; Malea et al., 2022). In the antioxidant enzyme system, the conversion of superoxide anions ($O2^-$) into H_2O and H_2O_2 by SOD is the "first line of defense" for plants to cope with oxidative stress. Peroxidase (POD) is an H_2O_2 scavenging enzyme (Akhter et al., 2022; Wang Y. et al., 2022; Wang Q. et al., 2022). In the present study, compared to that in non-salt -stressed plants, the POD activity in plant leaves significantly increased after 2 weeks of salt stress (Figure 3F), indicating that salinity increases the activities of antioxidants involved in scavenging ROS (Abd_Allah et al., 2018; Wang et al., 2020). Most notably, inoculation with PG-4 significantly increased SOD and POD activity in salt-stressed *M. uniflorum* plants, enabling them to clear excess ROS and alleviate oxidative stress in plants. Similar studies by Hegazi et al. (2017) and Masmoudi et al. (2021) have shown that inoculation with beneficial microorganisms can trigger antioxidant defense mechanisms in plants to induce the synthesis of antioxidant enzymes such as SOD, POD, and CAT. Moreover, osmotic adjustment occurs by reducing osmotic potential to maintain cell volume and turgor, thereby reducing the adverse effects of salt stress by balancing

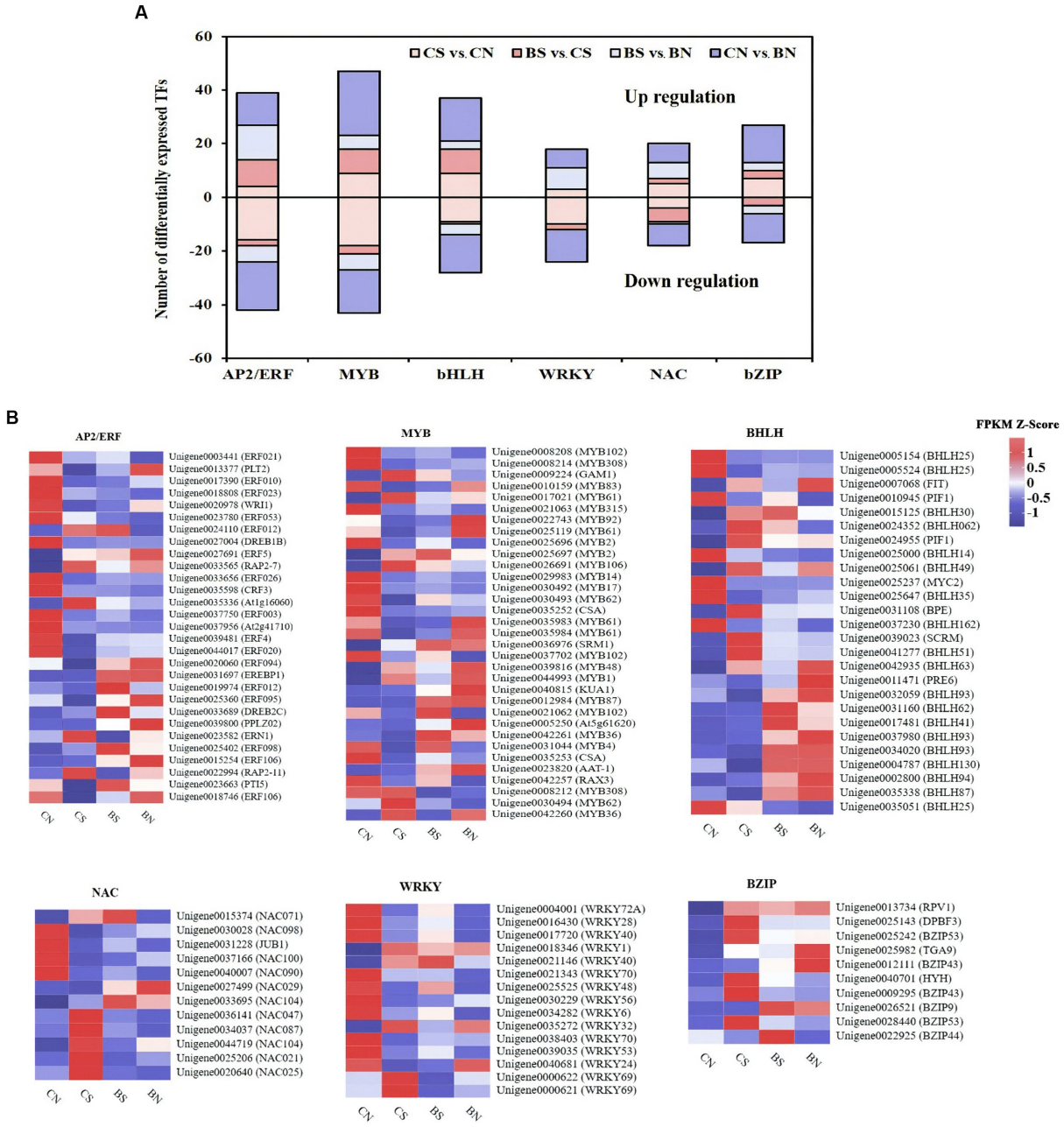

FIGURE 8
The differential expression pattern of the six TF families. **(A)** The number and expression pattern of differentially expressed TFs. The positive numbers indicate the up-regulated TFs and the negative numbers indicate the down-regulated TFs. **(B)** The differential expression pattern of the six TF families were depicted by a heatmap. The color scale represents FPKM values in each treatment, normalized using z-score.

solute potential, which subsequently promotes cell growth (Zhang et al., 2019). In the present study, the soluble sugar and proline contents in *M. uniflorum* leaves also significantly increased under salt stress (Figures 3B,C), which was consistent with previous findings in soybean (Soliman et al., 2020), maize (Wang Y. et al., 2022; Wang Q. et al., 2022) and *Pisum sativum* (Gupta et al., 2022). In particular, PG-4 further increased the proline and soluble sugar contents in salt-stressed *M. uniflorum* plants, suggesting that PG-4 could lower the osmotic potential and maintain cell turgor by promoting proline and soluble sugar accumulation, eventually helping *M. uniflorum* respond to the osmotic stress caused by salt stress. Soluble sugars and proline are involved not only in osmotic adjustment but also in scavenging ROS and protecting cells from oxidative damage (Zouari et al., 2019).

Salt stress results in the accumulation of Na^+ ions within plants (Joshi et al., 2022). Excessive Na^+ in plants competes with other ions to bind proteins, which leads to enzyme inactivation and affects the uptake of K^+ and other ions through the impact of ion balance between the two sides of the cytoplasmic membrane, causing severe ion toxicity (Zhu, 2016; Liu et al., 2022). Therefore, reducing the accumulation of Na^+ in the cytoplasm to maintain ion balance in

plants is necessary for plants to reduce ion toxicity and enhance salt tolerance. We observed that salt stress led to a decrease in the K^+ concentration in the *M. uniflorum* seedlings, while inoculation with PG-4 significantly increased the K^+ concentration in the seedlings (Figures 3G,H). Our findings indicated that PG-4 reconstructs the ion balance of host plants by increasing K^+ and/or reducing Na^+ in the cycloplasm, thereby reducing the harmful effects of Na^+ on plants (Chen et al., 2016, 2017; Yilmaz et al., 2020; Shultana et al., 2022). Tank and Saraf (2010) suggested that PGPR inoculation increases the production of exopolysaccharides, which retain Na^+ in salt soil and prevent absorption by plant roots. Moreover, after inoculation with PG-4, the chlorophyll content of the *M. uniflorum* plants was significantly higher than control plants under salt stress (Figure 3A). These results may be due to the increased absorption and concentration of K^+ and reduced absorption of Na^+ by inoculation of *B. amyloliquefaciens* under salt stress, which increase the content of antioxidant compounds and improve photosynthesis (Ruíz-Sánchez et al., 2011; ALKahtani et al., 2021). PGPR can generate exopolysaccharides, which enhance soil structure, increase the availability of soil water, improving plant physiological characteristics, such as chlorophyll content, particularly under stress conditions (Tank and Saraf, 2010; ALKahtani et al., 2020).

In recent years, researchers have gradually begun to study the effect of PGPR on plant molecular responses through the use of the transcriptome method, with the final objective of improving plant salt tolerance (Wang Y. et al., 2022; Wang Q. et al., 2022; Xiao et al., 2022). The investigation of the transcriptome-wide response of *M. uniflorum* leaves revealed that PG-4-initiated salt tolerance is likely attributed to the regulation of several key biological processes, such as stress signaling components and regulators, plant hormone signal transduction and putative regulators of cell wall organization. Previous studies have shown that PGPR can enhance antioxidant enzyme activity and enhance host plant antioxidant capacity by inducing the upregulation of antioxidant enzyme-encoding genes (Khanna et al., 2021; Nivetha et al., 2021; Maleki et al., 2023). In the present study, GO terms related to the cellular redox state, such as "oxidoreductase activity," "response to oxidative stress," "antioxidant activity" and "catalytic activity," were significantly enriched with DEGs in the BS vs. CS comparison, which was in line with the effects of PG-4 on the enhancement of antioxidant enzyme activity in *Macrotyloma uniflorum* under salt stress. Moreover, GO terms related to hemoglobin, such as "iron ion binding," "heme binding" and "tetrapyrrole binding," were significantly enriched (Supplementary Figure S5). Hemoglobin and its catalyzed products can act as strong antioxidant enzymes against various abiotic stresses, as well as inducers that function in different developmental processes, including adventitious and lateral root development (Zhao et al., 2008; Xu et al., 2011). These results suggested that PG-4 may ameliorate the negative effects of salinity stress on *Macrotyloma uniflorum* by enhancing its ROS scavenging capacity.

The plant cell wall is the first site at which plants perceive and respond to salt stress (Kaashyap et al., 2018). The stress signal triggers cell wall remodeling to maintain flexibility and protect the cell against ionic imbalance (Kaashyap et al., 2018; Colin et al., 2023). Cell wall modification under abiotic stress conditions is accomplished by modulating the cross-linking of cellulose, pectins and lignin. Xyloglucans are involved in modulating these cross-links and regulating cell wall metabolism processes (Kaashyap et al., 2018; Ganie and Ahammed, 2021). Xyloglucans are linked to at least two cellulose molecules and are reported to remodel stomatal cell walls to prevent excess water loss during osmotic stress (Gall et al., 2015; Byrt et al., 2018). Recently, studies have indicated that XTH participates in plant cell wall construction and modification and that the overexpression of XTH confers increased plant tolerance under high salinity conditions (Zhang et al., 2021). Xyloglucan-modifying enzymes reportedly impart salt and drought tolerance when they are overexpressed in pepper and wheat plants in response to osmotic stress and salt stress (Abuqamar et al., 2013). Kaashyap et al. (2018) reported that the upregulation of xyloglucan genes in the tolerant genotype clearly suggested that their activity in 'cell wall loosening' may impart salt stress tolerance to chickpea plants. In the present study, GO terms related to the cell wall, such as "pectinesterase activity," "cell wall organization," "cell wall modification" and "cell wall organization or biogenesis," were also significantly enriched with DEGs in the CS vs. BS comparison, "xyloglucan: xyloglucosyl transferase activity" and "xyloglucan metabolic process" were significantly enriched with DEGs in the CS vs. BS comparison (Supplementary Figure S5). Under salinity stress, the expression levels of DEGs encoding pectinesterase and xyloglucan endotransglucosylase/hydrolase proteins were mainly downregulated. However, the addition of PG-4 facilitated the upregulation of these transcripts under salinity stress (Supplementary Figures S6C,D). This result indicated that there was a connection between the cell wall synthesis and modification induced by PG-4 inoculation and plant tolerance of salt stress.

PGPR play a key role in regulating plant signal transduction under salt stress. Liu et al. (2017) reported that FZB42 might use the ethylene and jasmonic acid transduction pathways to induce systemic salt tolerance in *Arabidopsis* plants. Lastochkina et al. (2017) reported that *B. subtilis* enhances salt stress tolerance in wheat by enhancing JA signaling. *B. pumilus* enhances the salt tolerance of cotton plants and is related to SA signaling (Akbar et al., 2022). In this study, the auxin, cytokinin, gibberellin, abscisic acid, ethylene, brassinosteroid, JA and salicylic acid signaling pathways were triggered, as reflected in the DEGs (Figure 7). These genes might be involved in the plant mechanism underlying the salt stress adaptation conferred by PG-4.

TFs play important roles in regulating gene expression, biotic and abiotic stress responses, and signal transduction by acting as gene activators, repressors, or both (Zhang et al., 2021; Wang Y. et al., 2022; Wang Q. et al., 2022). The roles of TFs in salt stress tolerance have been clearly demonstrated in important plants, such as pepper (*Capsicum frutescens* L.) (Wang Y. et al., 2022; Wang Q. et al., 2022), asparagus (*Asparagus officinalis*) (Zhang et al., 2021), rice (Tang et al., 2019), and tomato (*Solanum lycopersicum*) (Devkar et al., 2020). For example, Gruber et al. (2009) identified 46 TFs as salt-inducible genes in *Medicago truncatula* roots, including members of the AP2/ERF, WRKY, zinc-finger bZIP, zinc finger, GRAS, ZIM, Bhlh, MYB and bZIP families. In this study, several of the TFs belonging to the AP2/ERF, MYB, bHLH, WRKY, NAC, and bZIP families were downregulated by salt stress and upregulated by PG-4 under salt stress (Figure 8; Supplementary Figure S17), indicating the critical involvement of

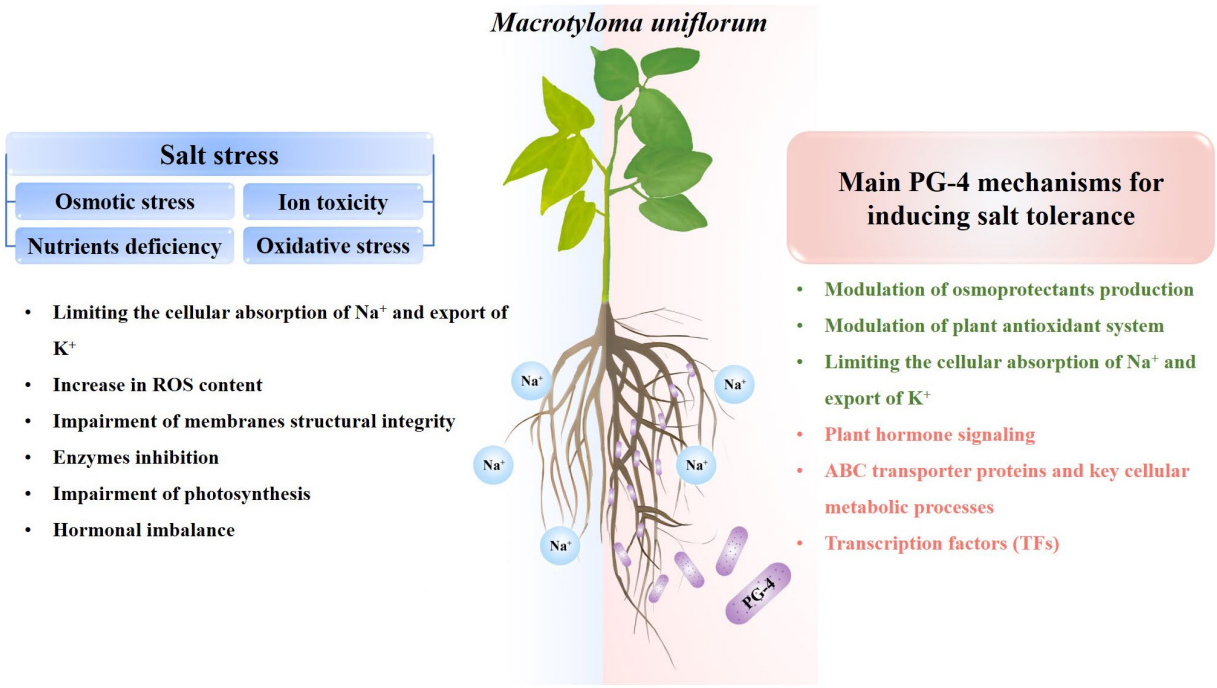

FIGURE 9
General overview of salt stress effects on plants (left) and PG-4 mechanisms involved in salt-stress tolerance (right) in *Macrotyloma uniflorum* seedlings. Green texts represent the physiological mechanism, while red texts represent the molecular mechanism by which PG-4 enhances the salt tolerance of *M. uniflorum* seedlings.

these TFs in the salt stress response and the important roles of PG-4 in the development of salt tolerance in plants.

Conclusion

The application of PGPR plays a crucial role in advancing agricultural resilience and productivity. PGPRs markedly enhance soil health, nutrient assimilation, and plant development, culminating in increased crop yields and a diminished dependency on chemical fertilizers, and its application is of great significance for sustainable agricultural. Furthermore, PGPRs are promising tools for increasing crop salt stress resistance. In the present study, the beneficial effects of *B. amyloliquefaciens* PG-4 on *M. uniflorum* plants under salt stress were evaluated. As shown in Figure 9, PG-4 significantly mitigated the deleterious effects of salt stress on *M. uniflorum* plants by improving biomass growth, increasing chlorophyll content, limiting the cellular absorption of Na^+ and export of K^+," and maintaining membrane integrity by reducing lipid peroxidation and increasing antioxidant production. Additionally, our transcriptome analysis revealed several key mediators of PG-4-induced transcriptional regulation in *M. uniflorum*, including plant hormone signal transduction, cell wall synthesis and modification and transporters. Moreover, transcription factors may also play important roles in mitigating the detrimental effects of salt stress on *M. uniflorum*, such as MYB, AP2/ERF, and bHLH. Our findings are helpful for providing important insights into the salt tolerance mechanism induced by PGPR and for providing a solid basis for future studies on the enhancement of legume forage tolerance to salinity stress, and providing theoretical references for bio-mediated soil improvement.

Data availability statement

The data presented in the study are deposited in the SRA: https://www.ncbi.nlm.nih.gov/sra/PRJNA1075426, accession number PRJNA1075426.

Author contributions

YW: Writing – original draft, Writing – review & editing. CG: Writing – review & editing. YX: Writing – review & editing. XL: Resources, Supervision, Writing – review & editing. DY: Resources, Supervision, Writing – review & editing. QW: Writing – review & editing. HL: Writing – review & editing. YZ: Conceptualization, Supervision, Writing – review & editing. RZ: Conceptualization, Supervision, Writing – review & editing. KL: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2024.1386079/full#supplementary-material

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