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RL-WG26 mediated salt stress tolerance in rice seedlings: A new insight into molecular mechanisms

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ABSTRACT

Soil salinization has significant deleterious impacts on arable lands and crop productivity worldwide. Plant growth–promoting rhizobacteria (PGPR) have the capability to establish mutualistic associations with plants, resulting in the increase of plant growth under abiotic stress. PGPR strain RL-WG26 was previously isolated from the rhizosphere of rice Sea Rice 86 (SR86). In this study, by genome-based average nucleotide identity (ANI) calculation, RL-WG26 was re-identified as *Pseudomonas promysalinigenes* here instead of *Pseudomonas putida*. SR86 seedlings inoculated with RL-WG26 possessed significant increases in plant biomass (fresh weight and dry weight), root surface area and length, and chlorophyll content under either normal or saline conditions, when compared with those without RL-WG26 inoculation. Ion content analysis demonstrated that seedlings inoculated with RL-WG26 contained lower Na^+ and Cl^- accumulation but higher K^+ and Ca^{2+} uptake under saline condition, which finally alleviated the salt ion toxic in the RL-WG26 inoculated seedlings, when compared with those without RL-WG26 inoculation. In addition, SR86 seedlings inoculated with RL-WG26 also exhibited higher antioxidant activity and higher concentration of osmoregulation factor proline than those without RL-WG26 inoculation. At last, genomic analysis of strain RL-WG26 identified several genes and gene clusters encoding 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase, the tryptophan-dependent indole-3-acetic acid (IAA) synthesis pathway, and the synthesis of betaine. Taken together, PGPR strain RL-WG26 could enhance the growth and salt stress tolerance in SR86 seedlings through various pathways, which provide a foundation for deciphering the molecular mechanisms of strain RL-WG26-mediated plant salt tolerance and promote the application of this strain.

1. Introduction

Soil salinity is one the major abiotic stress that causes reductions of global crop productivity (Kim et al., 2017). Approximately one fifth of global arable land is affected by soil salinization, which has become the major cause of reductions in plant productivity and the degradation of land (Qin et al., 2016; Szymanska et al., 2016). It was estimated that the global salinized regions are increasing by 10% every year because of climate change and inadequate irrigation management (Shrivastava and Kumar, 2015). And, it was claiming that half of the agriculture lands will be affected by salinity by the year 2050 (Jamil et al., 2011). Salinity might affect plant growth via different approaches, such as sodium and chloride toxicity, increases osmotic stress, disrupts nutrient balance, and generates reactive oxygen species (ROS) (Munns and Tester, 2008; Mishra et al., 2013; Farhangi-Abriz et al., 2020). Hence, it is significant to explore the question of how to alleviate the adverse effects of salt stress, enhance plant salt tolerance and eventually increase crop yields in high-salinity soils.

Promoting plant growth under saline conditions is recognized as one of the major routes of saline soil restoration and utilization, especially for the cereal crops (Lian et al., 2020). In the past decades, great efforts have been made to improve the salt tolerance of plants, by breeding salt tolerant plant varieties, through genetic engineering technology, and by application of beneficial microbes (Ke et al., 2021). It is widely

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recognized that plants have adapted naturally to endure various abiotic stresses, a positive attribute that has facilitated the development of cultivars that are more tolerant to salt stress (Ishitani et al., 2004; Breseghello and Coelho, 2013). Nevertheless, the progress for increasing plant salt tolerance by this approach was slow and the potential reasons have been summarized by Qin et al. (2016). As to the application of genetically modified crops, the main issues are the legislative barriers, security evaluation, and the public acceptance (Fedoroff et al., 2010). Thus, the understanding and application of beneficial microbes for increasing plant salt tolerance are becoming more promising. It is worth noting that a growing body of research has demonstrated that plant adaptability is not only related to the plant itself but might also be significantly related to multiple biotic factors of the environment (Numan et al., 2018; van Zelm et al., 2020).

Roots provide three separate rhizo-compartments (endosphere, rhizoplane, and rhizosphere) for the beneficial plant-environment interactions, including nutrient uptake, biotic and abiotic stress defense, etc. (Lundberg et al., 2012; Lareen et al., 2016). For the rhizosphere, plants interact with specific soil microorganisms that inhabit their root vicinity. The rhizosphere is one of the most complex ecosystems inhabited by millions of microbial cells, which might play an important role in the process of plant adaptation to salt stress. Plant growth–promoting rhizobacteria (PGPR) is a group of rhizobacteria that have the capability to establish mutualistic associations with plants resulting in the increase of plant growth as well as enhanced defense against adversity stress. It is also worth mentioning that PGPR has been extensively developed as biofertilizers (Vessey, 2003).

Numerous PGPR have been isolated with the capability of improving plant salt tolerance and scientists have attempted to decipher the mechanisms of PGPR-mediated plant salt tolerance. The mechanisms of microbe-mediated plant salt tolerance have been systematically reviewed by Qin et al. (2016). Briefly, (i) PGPR might participate in the regulation of plant endogenous hormone levels by producing aminocyclopropane-1-carboxylate deaminase (ACC deaminase), indole-3-acetic acid (IAA) (Egamberdieva et al., 2019), abscisic acid (ABA) (Dodd et al., 2010), cytokinins (Cyt) (Qin et al., 2016), and gibberellic acids (GAs) (Kang et al., 2014); (ii) PGPR can induce the expression of reactive oxygen species (ROS) scavenging genes in plants, including superoxide dismutase, catalase, ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase; (iii) PGPR might generate some important organic osmolytes (proline and polyamines) to protect plants from osmotic stress; and (iv) to help the plants maintain ion homeostasis when subjected to saline conditions, PGPR could produce bacterial exopolysaccharides to bind the toxic Na^+ and restrict Na^+ influx into roots. Now, it is well acknowledged that acquiring PGPR capable of enhancing plant salt tolerance and understanding mechanisms underlying PGPR conferred plant salt tolerance will provide novel strategies for improving the salt tolerance of plants.

During our previous study, a PGPR strain RL-WG26, capable of enhancing plant salt tolerance, was isolated from the rhizosphere of Sea Rice 86 (SR86) under saline conditions (Wang et al., 2022). Strain RL-WG26 has been preliminarily identified as *Pseudomonas putida* and exhibited multiple biological activities. Particularly, the survival rate of SR86 was increased to 80.33% with the inoculation of strain RL-WG26 under saline conditions (soil electrical conductivity (EC) = 1800 μS/cm) while the survival rate for the uninoculated treatment was 13.33%. Moreover, our study also indicated that strain RL-WG26 was able to promote the growth of SR86 under normal cultivation conditions. This study will shed more light on the plant growth promoting and plant salt tolerance enhancing capabilities of strain RL-WG26 from the physiological and biochemical aspects.

Hence, the main purposes of this research were: (i) to re-identify strain RL-WG26 with whole genome based method, (ii) to evaluate the influence of strain RL-WG26 on the physiological characteristics of SR86 under normal and saline conditions, such as dry weight, root characteristics, and chlorophyll content, (iii) to investigate the impacts of strain RL-WG26 on the response of SR86 towards salt stress, through variations of malondialdehyde (MDA) content, SOD and catalase activities and so on, (iv) to study the influence of strain RL-WG26 on the ion concentration in different tissues of SR86, and (v) to decipher the related mechanisms in strain RL-WG26 via genome sequencing and analysis.

2. Materials and methods

2.1. Bacterial strain and medium

Strain RL-WG26 was previously isolated from the rhizosphere of Sea Rice 86 (SR86) under saline conditions and has been deposited in Guangdong Microbial Culture Collection Center under accession number of 61,956. The 16S rRNA gene of strain RL-WG26 has been submitted to GenBank with accession of MZ318256. The modified Luria-Bertani (LB) medium (peptone, 10 g/L; yeast extract, 5 g/L; NaCl, 25 g/L; pH 7.0) was used to culture strain RL-WG26. The solid LB medium was prepared by adding agar into the medium with a final concentration of 15 g/L. The liquid and solid LB media were sterilized by autoclaving at 121 °C for 30 min.

The re-identification of strain RL-WG26 was accomplished via average nucleotide identity (ANI) calculation while the genomic DNA of strain RL-WG26 was extracted using a MiniBEST Bacterial Genomic DNA Extraction kit (Takara, Japan) following the manufacturer's protocol. The purity, concentration, and integrity of extracted DNA were detected by Nanodrop 2000, Qubit and 0.35% agarose gel electrophoresis. Then, the high-quality genomic DNA was applied to the complete genome sequencing. The genome sequencing was accomplished with a MinION sequencing platform (Oxford Nanopore Technologies, UK) by Biomarker Technologies Corporation (Beijing, China). Based on phylogenetic analysis via 16S rRNA gene, the representative genomes of closest species were withdrawn from NCBI and the ANI value between strain RL-WG26 and selected genomes were calculated with ANI calculator provided by Ezbiocloud (Yoon et al., 2017).

The seeds of strain RL-WG26 for inoculum was prepared by inoculating single colony of strain RL-WG26 into LB liquid medium and incubating under constant shaking (180 rpm, 30 °C) for 24 h. Cells were harvested by centrifugation (6000 × *g*, 3 min) and the cell pellets were washed by PBS buffer (pH 7.8, 100 mM). The centrifugation and washing were repeated for three times, and the cells were resuspended in 1 mL of fresh PBS buffer (pH 7.8, 100 mM). The cell suspension was adjusted to approximately 8×10^7 cell/mL with A_{600} of 0.8. The obtained cell suspension was used as inoculants and the inoculation ratio was maintained at 1 mL per plant unless stated otherwise.

To ensure that strain RL-WG26 colonized in the rhizosphere of SR86, re-isolation was conducted weekly from the rhizosphere soil of SR86 during the incubation period. For the rhizosphere soil sampling, the attached soil was removed by gentle shaking and then the soil attached to the root of SR86 seedlings was collected. The isolation and identification were accomplished as previously described (Wang et al., 2022).

2.2. Plants, soil, and seawater

SR86 is a new rice cultivar domesticated from a wild strain which was first found in 1986 in seawater submerged coastal region of Zhanjiang City, South China (Chen et al., 2017). The seeds of SR86 were kindly provided by Professor Risheng Chen. The seed germination and management were performed as previously described (Wang et al., 2022). Rice seedlings were grown to trifoliate stage while rice seedlings showing similar status were selected for the following assays. To make the experimental conditions close to real situation, diluted seawater was selected as the irrigation water. Seawater was collected from Zhanjiang Bay (Zhanjiang, China) while soil was collected from rice-planting farmland around Guangdong Ocean University (Zhanjiang, China). The detailed information of seawater and soil is presented in Table S1. Seawater was filtered with gauze to remove particles while soil was

sieved with a 4-mmpore size mesh. Prior to pot experiments, soil was mixed and covered with diluted seawater (diluted with double distilled water), and the salinity of soil was adjusted to the target range. The mixture was kept at room temperature overnight, and then, the salinity of soil was recorded. The salinity of soil was maintained at the initial salinity approximately, by adding double distilled water or diluted seawater. Finally, the stable salt stress condition with different salinities was constructed.

2.3. Experimental design

The schematic diagram for the experimental design was shown in Fig. 1. Two treatments were set to verify the plant growth promoting capability of strain RL-WG26, including inoculation (N_{26}) and non-inoculation (N_{ck}) of strain RL-WG26 into the rhizosphere of SR86 under normal conditions. The plant salt tolerance enhancing capability of strain RL-WG26 was analyzed with two salinities (1400 μS/cm and 1600 μS/cm). The pot experiments were conducted in plastic pots (110 cm length by 60 cm width by 50 cm depth) as previously reported (Wang et al., 2022). Briefly, soil with target salinities and seedlings of SR86 were prepared in plastic pots as described above. Twenty seedlings with similar status in trifoliate stage were planted in each pot and three pots for each treatment were prepared. Subsequently, 1 mL of bacterial seeds was inoculated into the rhizosphere of SR86 while the inoculation of same volume of PBS was served as control treatment. Normal seedling management was performed under controlled conditions at Guangdong Ocean University. The status of each plant was recorded daily and the withered seedlings were abandoned. After two weeks of cultivation, the seedlings in all pots were harvested for physiological and biochemical characterization.

2.4. Physiological parameters measurement

After harvesting, the survival rate and plant fresh weight (FW) of each treatment were measured. The total root length, root area, and root volume were scanned with the WinRHIZO software (Regent Instruments, Quebec, Canada) using the Epson Expression V800 scanner (Epson, CA, USA). Subsequently, the dry weight (DW) of plants was determined after incubating each part in an oven at 75 °C for 48 h. To evaluate the impacts of salt stress on the seedlings of SR86, the salt injury index was calculated as previously described (Zhu et al., 2008; A. He et al., 2021). Briefly, the injury degree was categorized into five levels: level 0 (no impairment: leaves healthy), level 1 (slight impairment: withering at leaf tips, leaf margins, or veins or etiolating), level 2 (mediate impairment: half of the leaves and veins withered or etiolated), level 3 (serious impairment: more than 80% of the leaves and veins withered or etiolated), and level 4 (plants dead). Calculation of Salt injury index (Eq. (1)) was shown in supplementary.

2.5. Chlorophyll content

Leaf chlorophyll and carotenoid contents were determined following the method of Wellburn (1994). Briefly, the collected leaves of SR86 seedlings were weighted and ground into fine powder with the help of mortar pestle. Chlorophyll and carotenoid were extracted by 80.0% acetone (*v/v*) for 6 h in the dark. The supernatant was used to determine the content of chlorophyll a (Chla), chlorophyll *b* (Chlb) and total carotenoid (Cart) by recording the absorbance at 663, 647 and 470 nm. The equations used for calculation of Chla (Eq. (2)), Chlb (Eq. (3)) and Cart (Eq. (4)) were shown in supplementary.

2.6. Tissue ion concentrations

The tissue ion concentration of SR86 seedlings in each treatment was determined as previously described (Wang et al., 2022). Prior to ion determination, the leaves and roots were rinse by deionized water for several times. Then, the tissues were dried to constant weight and ground into fine powder. The content of Na^+, K^+ and Ca^{2+} were measured by an inductively coupled plasma mass spectrometry (ICP-MS) (Prodigy XP, Leeman Labs, USA) while the concentration of Cl^- was determined by an ion chromatograph (ICS-4000, Dionex, USA).

2.7. Lipid peroxidation content

MDA content in roots was estimated to evaluate the lipid peroxidation. Fresh roots were fully homogenized with the help of mortar pestle

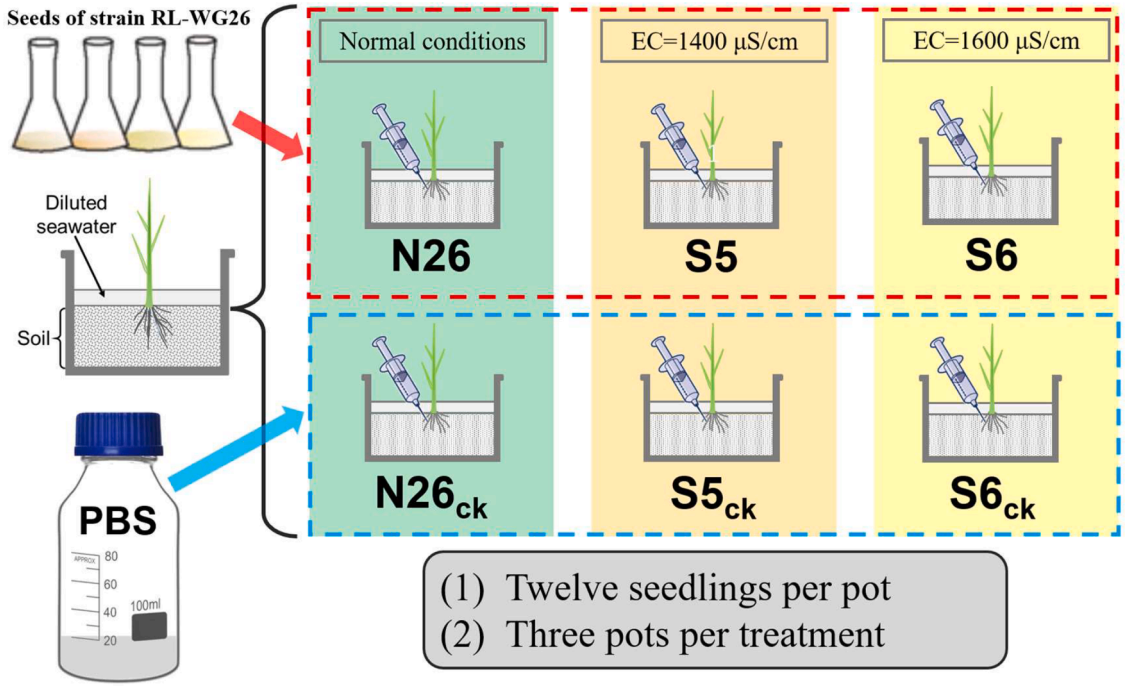

Fig. 1. The schematic diagram for the experimental design.

in thiobarbituric acid (TCA) (0.1%, *w/v*). The extracts were centrifugated for 20 min (6000 × *g*) and an aliquot of 1 mL supernatant was mixed with 2 mL of thiobarbituric acid (TBA) (0.5% and containing 20% of TCA). The mixture was incubated at 95 °C for 30 min, cooled on an ice-water bath, and centrifuged at 8000 × *g* for 10 min. Supernatant absorbance was recorded at 532 nm and 600 nm and the MDA content was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Madhava Rao and Sresty, 2000).

2.8. Proline content

To determine the impacts of strain RL-WG26 on the osmolytes in SR86 seedlings, total free proline content in fresh root was estimated as described by Bates et al. (1973). An aliquot of 0.5 g sample was ground into fine powder in a mortar pestle with liquid nitrogen and extracted by 5 mL of chilled sulphosalicylic acid (3.0%, w/v). Then, 2 mL of the mixture was mixed with an equal volume of ninhydrin reagent and the mixture was incubated at 100 °C for 1 h. The reaction mixture was then cooled in ice-water bath, thoroughly mixed with 4 mL of toluene by vortex, and followed by centrifugation (8000 × *g*, 5 min). The upper phase was applied for the recording of absorbance at 520 nm. Meanwhile, a standard curve of proline with known concentration was established and the content of proline in plant tissue was calculated thereafter.

2.9. Antioxidant enzyme activities

The antioxidant enzyme (CAT, POD and SOD) activities in fresh roots were determined to evaluate the influences of strain RL-WG26 on the antioxidant systems in SR86. Approximately 0.1 g of fresh roots were ground into fine powder in a mortar pestle with the help of liquid nitrogen, and corresponding extraction solution (1 mL) was added into the mortar. The mixture was centrifuged at 10,000 × *g* for 10 min at 4 °C and the supernatants were applied for determination of antioxidant activities. CAT, POD, and SOD activity were determined using the method described by He et al. (2018), Chance and Maehly (1995), and Giannopolitis and Ries (1977), respectively. Enzyme activity unit (U) is defined as the reaction of one nmol of substrate per minute. CAT activity was estimated by the decrease in H_2O_2 according to the absorbance at 240 nm using the spectrophotometer. POD activity was determined based on the guaiacol oxidation using H_2O_2 at 470 nm. SOD activity was measured by recording the absorbance at 560 nm according to the method of nitro blue tetrazolium chloride. The activities of CAT, POD and SOD were expressed as Unit per mg of fresh weight (U/mg FW).

2.10. Insights into the plant salt tolerance promoting mechanisms

To have an insight into the molecular mechanisms of plant growth promoting and plant salt tolerance enhancing in strain RL-WG26, the complete genome sequencing and analysis were conducted. Strain RL-WG26 was inoculated into fresh LB liquid medium and incubated under constant shaking (180 rpm, 30 °C) for 24 h. An aliquot of 2 mL culture was applied for centrifugation (10,000 × *g*, 8 min) and the cell pellets were washed by PBS buffer (pH 7.8, 100 mM). The washing and centrifugation were repeated for three times and the obtained cell pellets were applied to genome extraction with a MiniBEST Bacterial Genomic DNA Extraction kit (Takara, Japan) following the manufacturer's protocol. DNA integrity, quality and concentration were determined by agarose gel electrophoresis, a Qubit fluorometer (Thermo, USA) and a NanoDrop 1000 spectrophotometer (Thermo, USA), respectively. The purity, concentration, and integrity of extracted DNA were detected by Nanodrop 2000, Qubit and 0.35% agarose gel electrophoresis. Then, the high-quality genomic DNA was applied to the complete genome sequencing. The genome sequencing was accomplished with a MinION sequencing platform (Oxford Nanopore Technologies, UK) by Biomarker Technologies Corporation (Beijing, China). The obtained genome sequence was submitted to GenBank database of NCBI and the prediction of coding sequences (CDSs) was conducted with Prodigal v2.6.3. The general annotation of predicted CDSs was accomplished using Gene Ontology database (GO), Kyoto Encyclopedia of Genes and Genomes database (KEGG), eggCOG database, Pfam database, and SwissProt database. Further, the genome was submitted to Prokaryotic Genome Annotation Pipeline (PGAP, https://www.ncbi.nlm.nih.gov/genome/annotation_prok/) and Rapid Annotation using Subsystem Technology server (RAST, https://rast.nmpdr.org/) for in-depth annotation. Genes and gene clusters potentially involved in the plant growth promoting and plant salt tolerance enhancing progresses were manually checked.

2.11. Statistical analysis

All assays in study were performed in three replicates and the statistics analysis was performed with SPSS (version 19.0, SPSS Inc., Chicago, USA). All data was subjected to one-way analysis of variance (ANOVA) followed by Tukey's test. All values are expressed as the mean ±SE. '*' denotes $P < 0.05$; '**' denotes $P < 0.01$ and '***' denotes $P < 0.001$. Box and bar charts were drawn via origin software.

2.12. Data availability

Strain RL-WG26 has been deposited in Guangdong Microbial Culture Collection Center under accession number of 61,956 and the 16S rRNA gene of strain RL-WG26 has been submitted to GenBank with accession of MZ318256. The complete genome sequence of strain RL-WG26 is available in GenBank under accession number CP104557 (BioProject: PRJNA880516; BioSample: SAMN30845004).

3. Results and discussion

3.1. Re-identification of strain RL-WG26

According to the phylogenetic analysis of 16S rRNA gene (Fig. S1), strain RL-WG26 was identified as *Pseudomonas* sp. and clustered with *Pseudomonas juntendi, Pseudomonas monteilii, Pseudomonas putida, Pseudomonas promysalinigenes*, and *Pseudomonas plecoglossicida*. Thus, the representative genomes of these species were withdrawn from NCBI. The detailed information of these genomes was shown in Table S2. The ANI values between the selected genomes were shown in Fig. 2. Strain RL-WG26 was identified as *Pseudomonas promysalinigenes* since they share the highest ANI value of 98.58% and the closet GC content.

DNA-DNA hybridization tests have been applied to determine the relatedness between bacteria since 1960s and are still one of the most valuable criterions in the delineation of relationships between taxa (Oren and Garrity, 2014). Although the advantages and applications of this technique have been widely reported, there are still several important drawbacks (Klappenbach et al., 2007). Thus, taxonomists are actively searching for alternative methods to advance the species definition for prokaryotes. The availability of complete sequences of a number of prokaryotic genomes has made it possible for us to perform DDH analyses in silico. As a representative genome-based taxonomy, average nucleotide identity (ANI) assay is a robust means to compare genetic relatedness between strains and it has been proved that the traditional cut-off value of 70% DDH for species definition corresponded to 95% of ANI values (70% DDH ≈ 95% ANI) (Klappenbach et al., 2007; Kim et al., 2014). Hence, ANI analysis was adopted for phylogenetic analysis of strain RL-WG26 at the genomic level. The type strain of *Pseudomonas promysalinigenes* is RW10S1^T (LMG 32028^T = CFBP 8844^T) which was isolated from the exorhizosphere of rice at Kurunegala, Sri Lanka in 1990 (Vlassak et al., 1992; Girard et al., 2021; Oren and Garrity, 2022). Strain RW10S1^T was found to be able to produce promysalin to promote its own swarming, biofilm formation and surface colonization (Li et al., 2011). These indicated that *Pseudomonas*

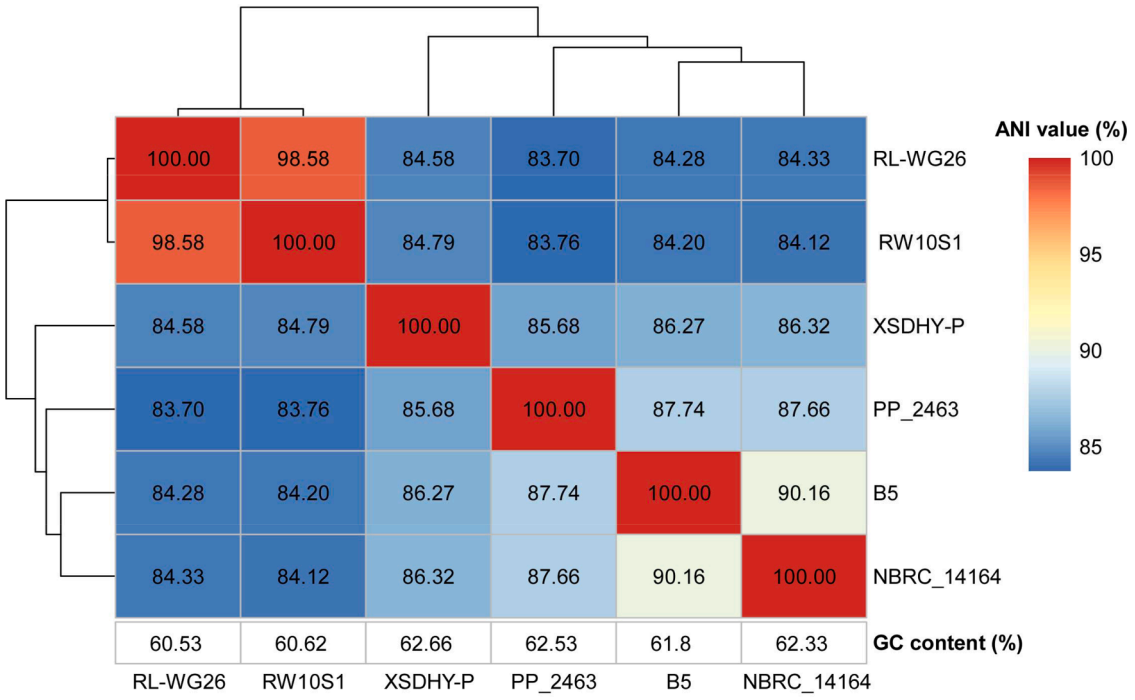

Fig. 2. The ANI values between the selected genomes. (PP_2463: *Pseudomonas juntendi* PP_2463; B5: *Pseudomonas monteilii* B5; NBRC_14,164: *Pseudomonas putida* NBRC 14,164; RW10S1: *Pseudomonas promysalinigenes* RW10S1; *Pseudomonas plecoglossicida* XSDHY-P).

promysalinigenes might have close relationship with rice and rhizosphere colonization.

3.2. Effects of RL-WG26 on root morphology and plant growth of SR86 seedlings under normal and salt stress conditions

After 15 days cultivation, the survival rates of SR86 seedling plants were detected as 100.00% (N26), 100.00% ($N26_{ck}$), 91.67% (S5), 86.11% ($S5_{ck}$), 80.56% (S6), and 50.00% ($S6_{ck}$), which indicated that the inoculation of strain RL-WG26 could significantly improve the survival rate of SR86 under salt stress. Strain RL-WG26 promoted root development of SR86 under normal and saline conditions (Fig. 3A and 3B). Briefly, the inoculation of strain RL-WG26 can promote the development of roots and increase the number of lateral roots under both normal and salt stress conditions. The salt injury indexes of all treatments were calculated and presented in Fig. 3C. Under salt stress condition, the salt injury indexes were decreased from 64.58% ($S5_{CK}$) to 36.11% (S5) and 80.56% ($S6_{CK}$) to 51.39% (S6). Further, the detailed parameters of roots also demonstrated that strain RL-WG26 should be recognized as a plant growth promoting rhizobacteria as well as plant salt tolerance enhancing rhizobacteria. The average total root area (Fig. 3D) of the treatments inoculated with strain RL-WG26 (N26 = 55.08 cm^2, S5 = 27.79 cm^2, S6 = 27.65 cm^2) is significantly higher than the treatments without inoculation ($N26_{ck}$ = 32.50 cm^2, $S5_{ck}$ = 21.45 cm^2, $S6_{ck}$ = 16.11 cm^2). Similarly, the inoculation of strain RL-WG26 could significantly promote the development of root length compared to the control treatment (Fig. 3E) with an average total root length increased from 471.32 cm ($N26_{ck}$), 181.46 cm ($S5_{ck}$) and 135.68 cm ($S6_{ck}$) to 662.43 cm (N26), 273.47 cm (S5) and 202.43 cm (S6), respectively. The measurements of plant FW and DW also demonstrated that the inoculation of strain RL-WG26 could increase the biomass of SR86 seedling plants under both normal and salt stress conditions (Fig. 3F and 3G).

Our results demonstrated that the inoculation of strain RL-WG26 under normal conditions could significantly promote the growth of SR86 seedlings. Sensitivity index has been extensively adopted to describe the relative sensitivity of plants to specific adversity stress (A. He et al., 2021). Meanwhile, sensitivity index was also widely used to evaluate the plant growth promoting effects of PGPR during the alleviation plant sensitivity to abiotic aggressions (Masmoudi et al., 2021). In the present study, salt injury index was used to evaluate the promotion of plant salt tolerance by strain RL-WG26 and the results indicated that the inoculation of strain RL-WG26 could decrease the salt injury index of rice seedlings under stress compared with treatment without inoculation and therefore alleviate plant salt stress. Results also suggested that high salinity could inhibit the growth, architecture traits and biomass of rice seedlings, which is in consist with known reports by Kausar et al. (2013) in wheat, Abdel Latef and Chaoxing He (2011) in tomato, Abdel Latef and Chaoxing He (2014) in pepper, Mostofa et al. (2015) in rice, Ahmad et al. (2016) in chickpea and Egamberdieva et al. (2017) in soybean. Thus, the inoculation of strain RL-WG26 could mitigate the inhibition intensity of salt on the growth of SR86 seedlings by promoting the root development and increasing the biomass of plants.

3.3. Effects of RL-WG26 on the content of photosynthetic pigments of SR86 seedlings under normal and salt stress conditions

As expected, salt stress reduced the content of selected photosynthetic pigments in SR86 seedlings. Without inoculation of strain RL-WG26, the average content of chlorophyl a was about 1.86 mg/g under normal conditions while reduced to 1.22 mg/g and 1.19 mg/g for EC = 1400 μS/cm and EC = 1600 μS/cm, respectively (Fig. 4A). Similarly, the average contents of chlorophyl b and total carotenoid were decreased by 36.0 - 36.2% and 37.7–39.1% for EC = 1400 μS/cm and EC = 1600 μS/cm, respectively (Fig. 4B and 4C). However, RL-WG26 treated plants exhibited effective restoration of chlorophyll and significantly enhanced for all treatments by: (i) 22.0% (normal conditions), 34.1% (EC = 1400 μS/cm), and 33.9% (EC = 1600 μS/cm) for chlorophyl a; (ii) 27.8% (normal conditions), 35.7% (EC = 1400 μS/cm), and 31.3% (EC = 1600 μS/cm) for chlorophyl b; and (iii) 26.9% (normal conditions), 31.7% (EC = 1400 μS/cm), and 30.6% (EC = 1600 μS/cm) for total carotenoid. It is noteworthy that the inoculation of strain RL-WG26 almost restored the photosynthetic pigments of plants under salt stress to the level of plants under normal conditions (the treatment

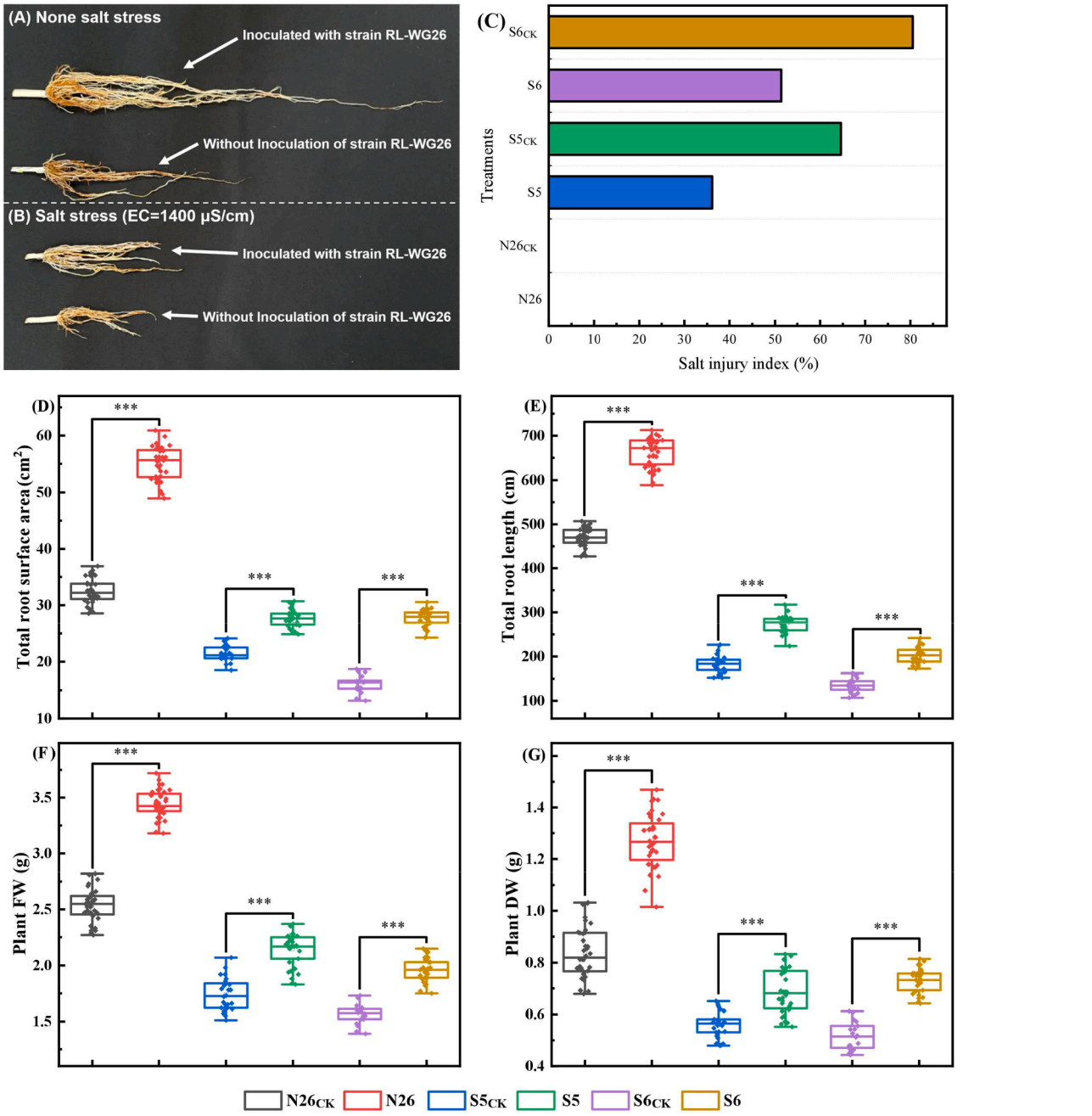

Fig. 3. Effects of strain RL-WG26 on the root development of SR86 (A: effects of strain RL-WG26 on the root morphology of SR86 under normal conditions; B: effects of strain RL-WG26 on the root morphology of SR86 under salt stress (EC=1400 μS/cm); C: Salt injury index (%) of SR86 under salt stress. D: effects of strain RL-WG26 on the total root surface area of SR86 under salt stress; E: effects of strain RL-WG26 on the total root length of SR86 under salt stress; F: effects of strain RL-WG26 on the fresh weight (FW) of SR86 under salt stress); G: effects of strain RL-WG26 on the dry weight (DW) of SR86 under salt stress).

without inoculation).

Photosynthetic pigments play a pivotal role in photosynthesis process which could significantly affect plant growth and biomass accumulation. Therefore, the content of photosynthetic pigments has been extensively employed as a relevant parameter to evaluate the photosynthetic efficiency and to determine the response of plants to environmental stress (Kalaji et al., 2016). In the present study, the content of photosynthetic pigments of SR86 seedlings was significantly declined when exposed to salt stress conditions (Fig. 4). Similar results were observed in previous investigations in which the content of photosynthetic pigments was significantly decreased under salt stress conditions (Kumar et al., 2017; Kumar and Verma, 2018; Wang et al., 2018). The decrease of photosynthetic pigments could cause the damage of photosynthesis apparatus and therefore lead to photosynthesis inhibition (Abeer et al., 2015). Known reports have demonstrated that the decrease of photosynthetic pigments might be due to a series of molecular and physiological changes. As reported by Zörb et al. (2009), the reduction of chlorophyll contents is attributed to the destructive effect of salt stress on chloroplasts. In addition, the modification of pigment-protein complex stability under salt stress could result a decreasing of chlorophyll concentration (Abogadallah, 2010). However, higher content of photosynthetic pigments in strain RL-WG26 treated SR86 seedlings was observed under both normal conditions and salt stress when compared to those of control treatments. Many works have illustrated that PGPR could enhance the uptake of water and nutrients by plants, might produce higher concentration of antioxidative

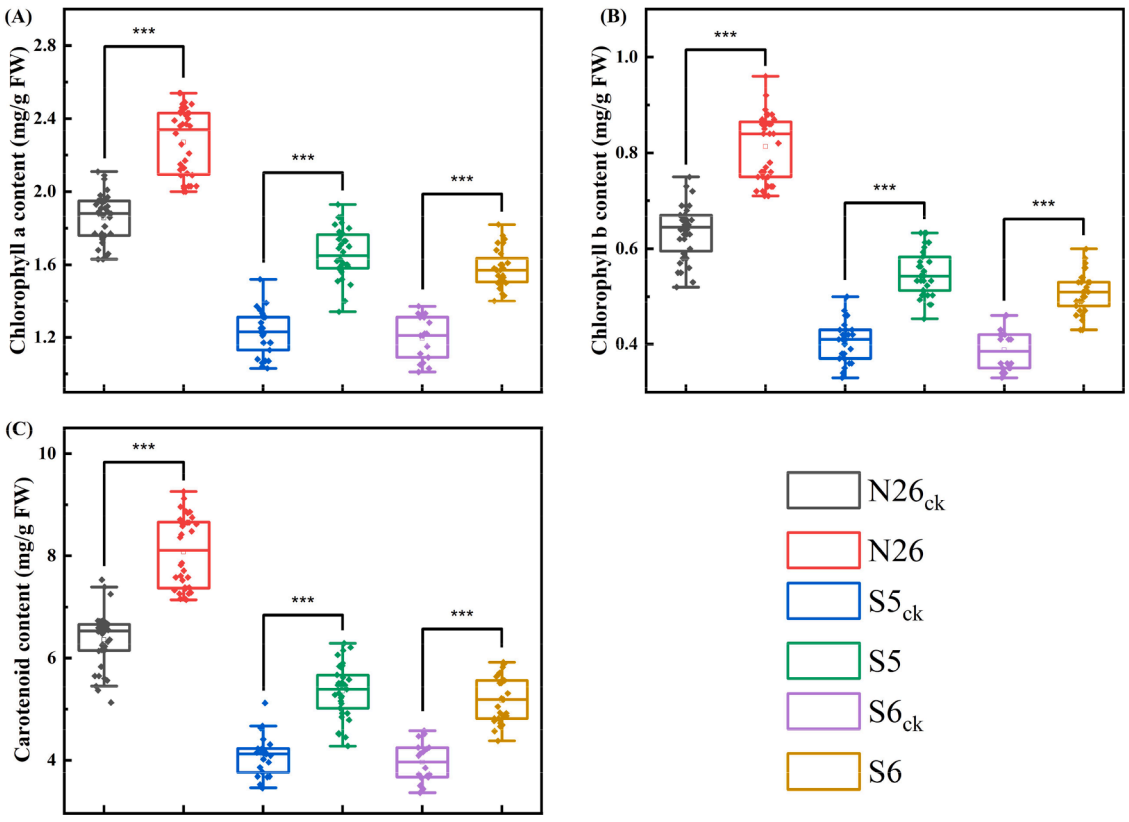

Fig. 4. Effects of strain RL-WG26 on the content of photosynthetic pigments in SR86 seedlings (A: effects of strain RL-WG26 on chlorophyll a content of SR86 seedlings under salt stress; B: effects of strain RL-WG26 on chlorophyll *b* content of SR86 seedlings under salt stress; C: effects of strain RL-WG26 on carotenoid content of SR86 seedlings under salt stress).

compounds, and therefore might protect the photosynthetic apparatus (Evelin et al., 2009; Cappellari and Banchio, 2020). Consequently, the variation tendency of the content of photosynthetic pigments is in consistent with the tendency of plant physiological index (e.g., root length and biomass) in this study.

3.4. Effects of RL-WG26 on ion uptake of SR86 seedlings under normal and salt stress conditions

The detected ion concentrations in the roots and leaves of SR86 seedlings are shown in Table 1. The concentration of Na^+ in the roots and leaves significantly increased when the seedlings of SR86 were subjected to saline conditions, even in the treatments inoculated with strain RL-WG26. The content of K^+ in the roots of SR86 seedlings was significantly decreased when treated with osmotic stress while there is no significant difference between inoculated and un-inoculated groups. Although the content of K^+ in the leaves of SR86 seedlings was also decreased under saline conditions, the results suggested that the inoculation of strain RL-WG26 could mitigate the decrease of K^+ in leaves under saline conditions in comparison with the un-inoculated treatments. Furthermore, the Na^+/K^+ molar ration in each treatment was calculated and the results demonstrated that the inoculation of strain RL-WG26 could decrease the Na^+/K^+ molar ratio in both roots and leaves, when compared with un-inoculated groups under salt stress. Although the Ca^{2+} content in leaves significantly increased under salt stress, no significant difference between inoculated and un-inoculated treatments was observed. Thus, the inoculation of strain RL-WG26 could increase the content of Ca^{2+} in roots under saline conditions when compared with the un-inoculated treatments. In addition, the results indicated that strain RL-WG26 is capable of reducing the

Table 1
Effects of salt stress and inoculation of RL-WG26 on ion concentration in SR86 seedlings.

Treatments	Roots					Leaves				
	Na^+	K^+	Na^+/K^+ molar ration	Ca^{2+}	Cl^-	Na^+	K^+	Na^+/K^+ molar ration	Ca^{2+}	Cl^-
$N26_{CK}$	2.02±0.29a	13.184 ±0.83a	0.26±0.04a	3.98±0.12a	1.04 ±0.11a	3.36±0.35a	25.46 ±0.63a	0.23±0.03a	3.03 ±0.20a	0.49 ±0.07a
N26	1.93±0.27a	13.52 ±0.58a	0.24±0.04a	4.01 ±0.15ab	1.05 ±0.12a	3.05±0.28a	26.25 ±0.73b	0.20±0.02a	3.11 ±0.19a	0.51 ±0.07a
$S5_{CK}$	16.91 ±1.33b	7.05±1.07b	4.17±0.67b	4.13±0.20b	3.52 ±0.10b	10.61 ±0.99b	10.88 ±1.56c	1.70±0.32b	4.07 ±0.15b	1.52 ±0.21b
S5	10.13 ±0.92c	7.46±0.60b	2.32±0.27c	4.86±0.28c	1.96 ±0.14c	6.81±0.48c	15.39 ±0.89d	0.76±0.08c	4.06 ±0.16b	0.96 ±0.11c
$S6_{CK}$	19.04 ±0.9d	6.96±0.92b	4.72±0.57b	4.15±0.26b	4.01 ±0.16d	15.04 ±0.79d	10.33 ±1.49c	2.53±0.40d	4.04 ±0.14b	1.68 ±0.12d
S6	10.45 ±0.68c	7.12±0.63b	2.52±0.32c	5.24±0.24d	2.35 ±0.20e	7.05±0.55c	14.42 ±0.80e	0.83±0.08c	4.05 ±0.14b	1.02 ±0.10c

concentration of Cl^- in the roots and leaves of SR86 seedlings under salt stress. Lower Na^+/K^+ratio and Cl^-accumulation in SR86 seedlings inoculated with strain RL-WG26 contributed less salt stress injury of the seedlings.

Generally, salt stress has deleterious effects on the plant growth and development, which could alter water relations of plants' tissues, inhibit enzyme activity, cause nutrient imbalance, etc. (Lovelli et al., 2012; Mostofa et al., 2015). Excess concentration of Na^+ and Cl^- is always considered as the major reason causing salt toxicity to plants. Therefore, the typical approaches to mitigate salt stress include (i) decreasing the concentration of Na^+ and Cl^- in plants by restricting the influx of them or accelerating the efflux of them and (ii) decreasing the Na^+/K^+ or Na^+/Ca^{2+} ratio in plant cells. To maintain ion homeostasis in plants when subjected to saline conditions, several studies reported that PGPR could generate bacterial exopolysaccharides and lipopolysaccharides to bind the toxic Na^+ and restrict Na^+ influx into roots (Natera et al., 2016; Costa-Gutierrez et al., 2020). Some PGPR can decrease the Na^+/K^+ and Na^+/Ca^{2+} ratio by selectively enhancing K^+ and Ca^{2+} uptake and avoiding translocation of toxic Na^+ under salt stress (Qin et al., 2016; Masmoudi et al., 2021). In addition, some PGPR can modulate the expression of genes encoding membrane porin, such as ion channels and aquaporin, and eventually regulate the ion homeostasis and water relation in plant cells (Marulanda et al., 2010). In consist with known repots, although the inoculation of strain RL-WG26 could significantly reduce the contents of Na^+ and Cl^- under saline conditions, the contents of Na^+ and Cl^- are still higher than those of normal conditions. These results suggested that most of PGPR could only mitigate, but not totally eliminate the salt stress. Similar with strain RL-WG26, Masmoudi et al. (2021) reported that FMH2-treatment mitigated salt stress by decreasing Na^+/K^+ and Na^+/Ca^{2+} ratios in the salinized tomato plants. Interestingly, although the inoculation of strain RL-WG26 could promote the accumulation of Ca^{2+} in roots, no significant enhancement of Ca^{2+} content was observed in leaves under salt stress, compared with the un-inoculated treatments.

3.5. *Effects of RL-WG26 on salt induced MDA and proline contents in SR86 seedlings*

The content of MDA in the roots of SR86 seedlings was determined and the results were presented in Fig. 5A. When plants were exposed to abiotic stresses, excessive ROS was generated, such as superoxide anion radicals (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH),

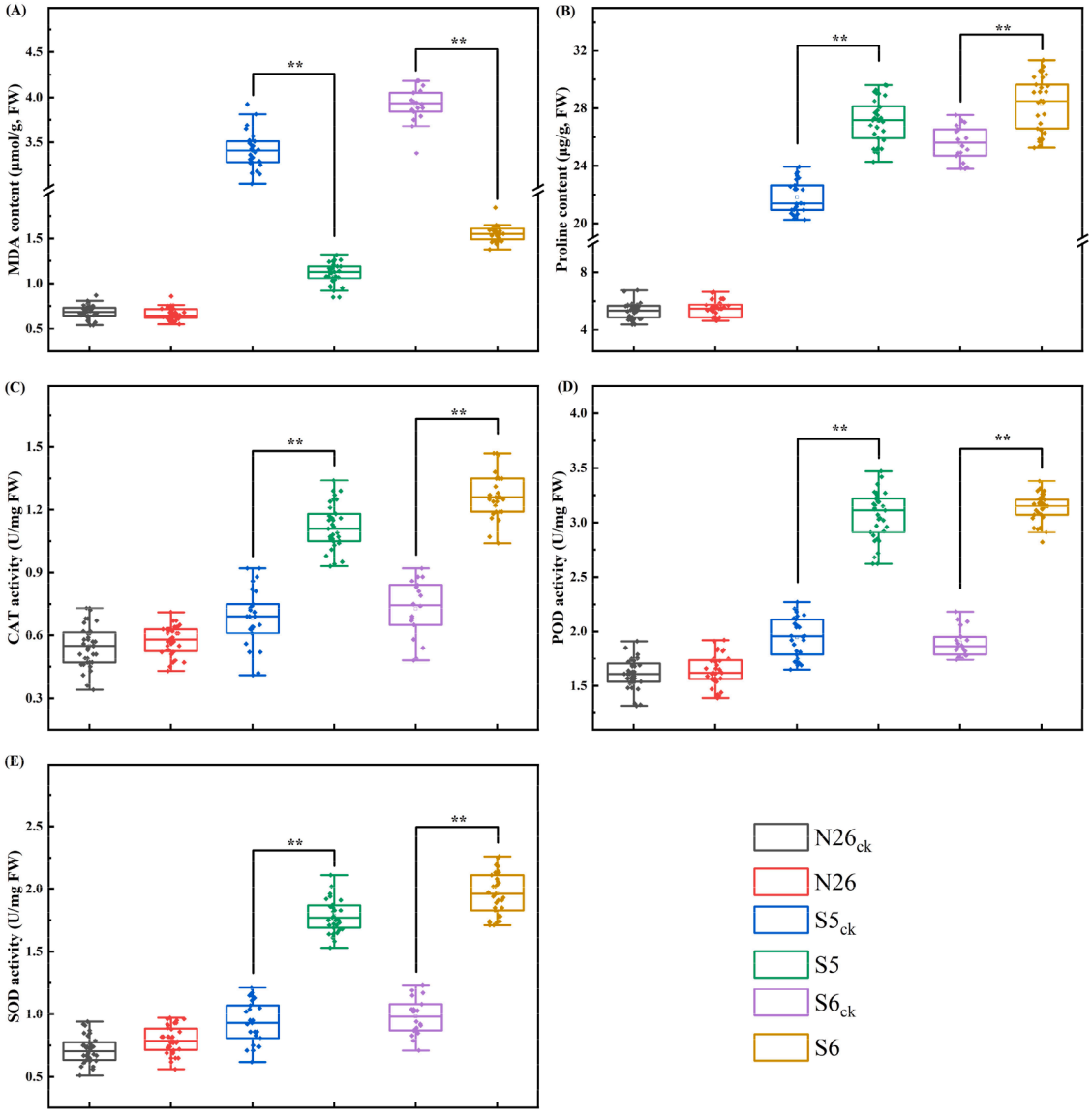

Fig. 5. Effects of the inoculation of strain RL-WG26 under salt stress on MDA content (A), proline content (B), and the activities of CAT (C), POD (D) and SOD (E) in SR86 seedlings.

which could damage the integrity of membranes, then increase the content of MDA, and eventually lead to various physiological and biological changes in plants. MDA could indicate the damage effect of salinity, and it also found that the MDA content in SR86 seedlings without RL-WG26 inoculation increased in parallel with increasing of salt concentration. Interestingly, the average MDA concentration in strain RL-WG26 treated groups decreased by 67.4% (S5) and 60.4% (S6) as compared with their control groups ($S5_{CK}$ and $S6_{CK}$, respectively). These results proved the inoculation of strain RL-WG26 could significantly alleviate the peroxidation of membrane lipid and therefore maintain the stability and integrality of cell membranes. Our results are in accord with previous studies, where PGPR could mitigate the osmotic stress together with the MDA content in plants (A. He et al., 2021). It is well known that excessive ROS (e.g., superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals) generated when plants were exposed to abiotic stresses. These ROS could damage the membrane integrity, increase the content of MDA, and eventually lead to various physiological and biological changes in plants (Hinojosa et al., 2019; Xie et al., 2019). Therefore, the activities of several antioxidant enzymes were determined in the following assays.

Additionally, plants could generate important osmolytes (e.g., proline and polyamines) to counteract the effects of salt stress (Zhu et al., 2008; Qin et al., 2016). Changes of proline contents in seedlings treated by salt and inoculation of strain RL-WG26 were shown in Fig. 5B. Plants could accumulate proline under saline conditions to counteract osmotic stress, in which salt stress significantly boosted proline content by 4.1-fold and 4.82-fold at $S5_{CK}$ and $S6_{CK}$ treatments, respectively, when compared to the control treatment of $N26_{CK}$. Meanwhile, plants inoculated with strain RL-WG26 had significantly higher proline content than the un-inoculated treatments. Specifically, the average contents of proline in the roots of SR86 seedlings were increased from 21.82 μg/g ($S5_{CK}$) and 25.57 μg/g ($S6_{CK}$) to 27.12 μg/g (S5) and 28.34 μg/g (S6), which indicated that strain RL-WG26 might promote the salt tolerance of SR86 seedlings by inducing the generation of proline in plants. Similar with strain RL-WG26, *Bacillus licheniformis* AP6 and *Pseudomonas plecoglossicida* PB5 could enhance growth and salt tolerance in sunflower plants by stimulating the generation of proline (Yasmeen et al., 2020). Kumar et al. (2021) also reported that *Bacillus pumilus* strain JPVS11 could improve the content of proline in rice and eventually enhance plant growth attributes of rice under salinity stress.

3.6. Effects of RL-WG26 on antioxidant enzyme activity in SR86 seedlings under normal and salt stress conditions

Generally, abiotic stress triggers the generation of ROS in plants, which could modulate plant growth and physiology, while plants always enhance the activity of antioxidant enzymes to eliminate the excessive ROS and therefore protect plants from oxidative damage. Meanwhile, almost all known PGPRs are capable of enhancing the antioxidative systems in plants for ROS scavenging. As shown in Fig. 5, strain RL-WG26 significantly stimulated the CAT, POD and SOD activity in SR86 seedlings under salt stress. SOD is known to be able to transform O_2^- into H_2O_2 while H_2O_2 could be converted into water and oxygen by CAT and POD. In the present study, inoculation of strain RL-WG26 enhanced the average activities of SOD in the roots of SR86 seedlings from 0.93 U/mg ($S5_{CK}$) and 0.98 U/mg ($S6_{CK}$) to 1.79 U/mg (S5) and 1.96 U/mg (S6), respectively. Accordingly, CAT and POD activities followed similar trends with SOD. Specifically, (i) CAT activity in inoculated plants increased significantly by 67.7% and 74.1% for 1400 μS/cm and 1600 μS/cm, respectively, as compared with non-inoculated plants, and (ii) the average activities of POD in the roots of SR86 seedlings inoculated with RL-WG26 were enhanced from 1.95 U/mg ($S5_{CK}$) and 1.9 U/mg ($S6_{CK}$) to 3.06 U/mg (S5) and 3.14 U/mg (S6), respectively, as compared to the treatments without inoculation. Similarly, Yasmeen et al. (2020) reported that biofilm forming rhizobacterial strain *Bacillus licheniformis* AP6 and *Pseudomonas plecoglossicida* PB5 could mitigate osmotic stress in sunflower plants by stimulating antioxidant enzymes activity, including CAT, SOD and guaiacol peroxidase. Overall, PGPR could promote growth and stress tolerance of SR86 seedlings by triggering these beneficial plant defense-related enzymes and therefore eliminating the excessive ROS.

3.7. Insights into the plant salt tolerance promoting mechanisms of strain RL-WG26

The complete genome of strain RL-WG26 is consisted of one circular genome with a length of 5203,533 bp and G + C content of 60.53 mol%. The circular representation of the genome is shown in Fig. 6A and the genomic features of strain RL-WG26 is presented in Table S3. In total, 4726 genes are predicted in the genome of strain RL-WG26, including 163 RNA genes, 22 ribosomal RNA genes, 75 transfer RNA genes, and 66 noncoding RNA genes. To decipher the plant salt tolerance promoting mechanism, the genome of strain RL-WG26 was submitted to Prokaryotic Genome Annotation Pipeline (https://www.ncbi.nih.gov/genome/annotation_prok/) and Rapid Annotation using Subsystem Technology server (https://rast.nmpdr.org/) for automatic gene prediction and in-depth annotation. Several genes and gene clusters of strain RL-WG26 contributed to growth promotion and enhancing salt tolerance of plants were shown in Table 2 and the potential strategies of these processes were summarized in Fig. 6B. A tryptophan-dependent IAA synthesis pathway was identified in the genome of strain RL-WG26, encoded by gene cluster *trpABCDE* and *iaaHM,* which are involved in the biosynthesis of tryptophan and IAA, respectively. IAA is known as an important phytohormone for plant growth and development, and could affect many vital physiological processes in plants, such as cell elongation and division, which has been proved to be able to improve salt stress tolerance in kinds of crops (Singh et al., 2015; Orhan, 2016; Aslam and Ali, 2018). The coding sequence of an ACC deaminase was identified in the genome of strain RL-WG26 with a length of 891 bp. ACC is known as the precursor for the biosynthesis of ethylene while the ACC deaminase producing microbes are capable of transforming ACC into ammonia and α-ketobutyrate, which are further utilized as carbon and nitrogen sources for growth (Orozco-Mosqueda et al., 2020). Consequently, the inoculation of PGPR capable of producing ACC deaminase enzyme can help to inhibit the synthesis of excessive ethylene and therefore promote plant growth under salt stress (Singh et al., 2022). In addition, a gene cluster (*betABCTI*) encoded the pathway of betaine biosynthesis was identified in the genome of strain RL-WG26. Betaine is one of the major organic osmolytes that accumulate in a variety of plant species in response to environmental stresses (Zhu et al., 2022). It has been reported that the up-regulation of glycine betaine synthesis is associated with choline-induced salt tolerance in halophytic seashore paspalum (Gao et al., 2020). Overall, the genome sequencing and analyzing preliminarily elucidated the molecular mechanisms of strain RL-WG26 contributed enhancement of salt tolerance in SR86 seedlings.

4. Conclusion

The presented study highlights the beneficial plant-microbe interactions between a PGPR strain *Pseudomonas promysalinigenes* RL-WG26 and SR86 seedlings under salt stress. Strain RL-WG26 was identified as a PGPR strain since it could improve the physiological characteristics of SR86 seedlings under normal conditions, including the biomass of plant, root surface area and length, and chlorophyll contents. Further, the capabilities of strain RL-WG26 for enhancing the salt tolerance of SR86 seedlings were demonstrated by improving the physiological characteristics of SR86, decreasing the content of MDA, improving the content of proline, regulating the ion concentration in different tissues of SR86 seedlings, and enhancing the activities of CAT, POD and SOD. Finally, the molecular mechanisms for enhancing plant growth and salt tolerance in strain RL-WG26 were deciphered by genome sequencing and analyzing. These results suggested that strain

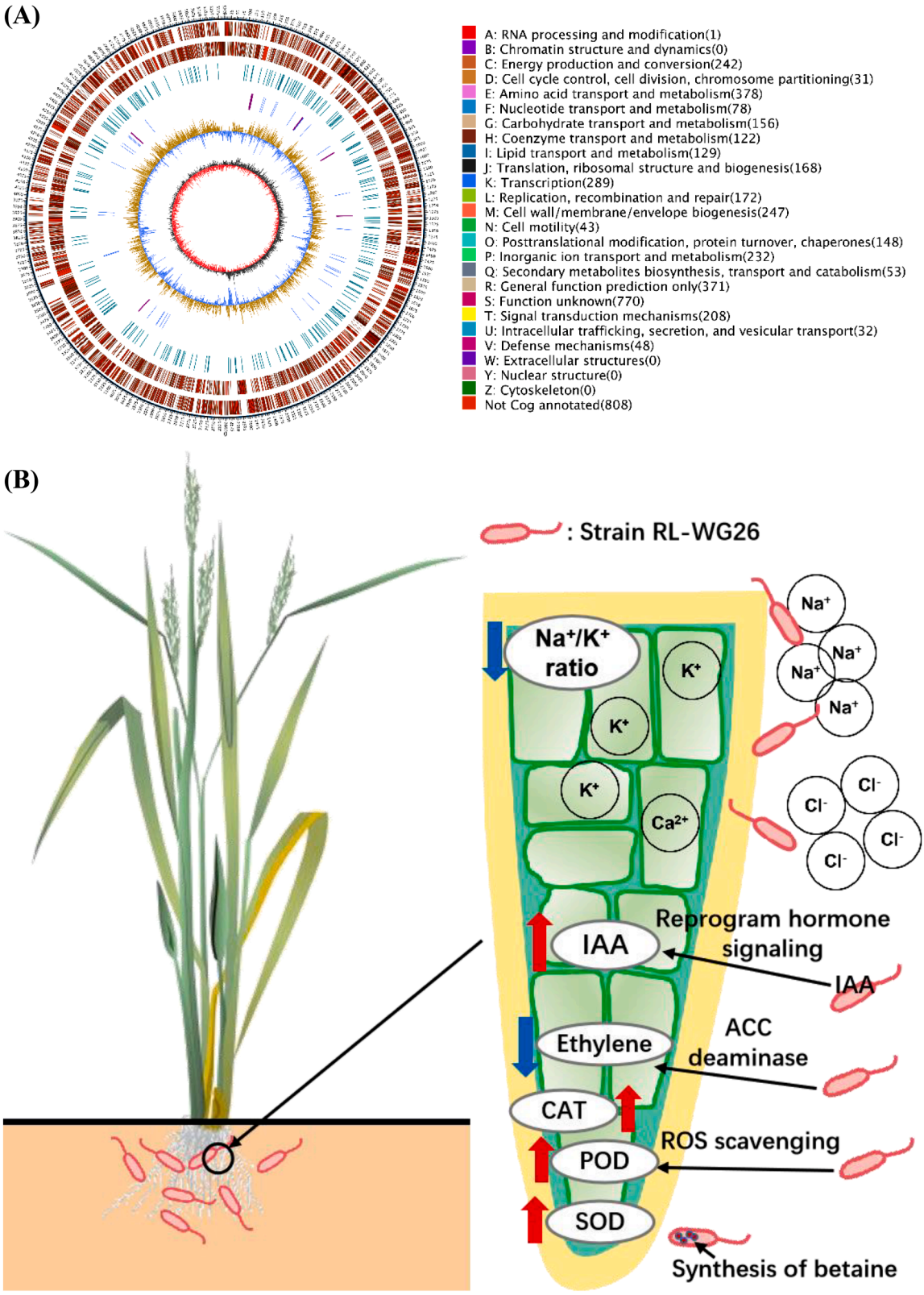

Fig. 6. The circular representation of genome of strain RL-WG26 (A) and the summary of the potential strategies for strain RL-WG26 contributed enhancement of plant growth and salt tolerance (B).

Table 2
Genes contributed to the enhancing plant growth and salt tolerance in strain RL-WG26.

Function	Genes	Enzymes	Locus_tag*
Synthesis of tryptophan	*trpAa*	Anthranilate synthase, aminase component	N5C08_02370
	trpAb	Anthranilate synthase, amidotransferase component	N5C08_02380
	trpB	Anthranilate phosphoribosyl transferase	N5C08_02385
	trpC	Phosphoribosyl anthranilate isomerase	N5C08_07950
	trpD	Indole-3-glycerol phosphate synthase	N5C08_02390
	trpEa	Tryptophan synthase alpha chain	N5C08_00445
	trpEb	Tryptophan synthase beta chain	N5C08_00450
Synthesis of IAA	*iaaM*	Tryptophan 2-monooxygenase	N5C08_02195
	iaaH	Indole-3-acetamide hydrolase	N5C08_02190
Synthesis of betaine	*betA*	Choline dehydrogenase	N5C08_22,585
	betB	Betaine aldehyde dehydrogenase	N5C08_22,580
	betC	Choline-sulfatase	N5C08_00430
	betT	High-affinity choline uptake protein	N5C08_22,570
	betI	Transcriptional regulator	N5C08_22,575
Inhibition of ethylene biosynthesis	*acdS*	1-aminocyclopropane-1-carboxylate deaminase	N5C08_08030

RL-WG26 is an attractive candidate for the restoration and utilization of salinized land and might provide important clues to decipher the plant-microbe interactions mediated improvement of plant salt tolerance.

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Author contributions

Lei Ren and Yanyan Wang conceived and designed the project; Yi Zhang, Guan Wang and Yu Ling prepared the plant samples and conducted physiological experiments; Lei Ren, Yanyan Wang, Yi Zhang and Yongxiang Huang analyzed the data; Lei Ren and Yi Zhang wrote the manuscript; Hanqiao Hu, Yujian Mo and John Zhou revised the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2023.100306.

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