Paraburkholderia sp. GD17 improves rice seedling tolerance to salinity

Ruanming Zhu
Shenyang Normal University
Yuting Cao
Shenyang Normal University
Guangzhe Li
Shenyang Normal University
Ying Guo
Shenyang Normal University
Lianju Ma
Shenyang Normal University
Ning Bu
Shenyang Normal University
Lin Hao (haolinwj2001@163.com)
Shenyang Normal University https://orcid.org/0000-0002-0828-3581

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Ruanming Zhu · Yuting Cao · Guangzhe Li · Ying Guo · Lianju Ma · Ning Bu · Lin Hao*

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College of Life Science, Shenyang Normal University, Shenyang 110034, China

Ruanming Zhu
College of Life Science, Shenyang Normal University, Shenyang 110034, China
303750242@qq.com

Yuting Cao
College of Life Science, Shenyang Normal University, Shenyang 110034, China
1442927461@qq.com

Guangzhe Li
College of Life Science, Shenyang Normal University, Shenyang 110034, China
guangzheli@163.com

Ying Guo
College of Life Science, Shenyang Normal University, Shenyang 110034, China
1287867570@qq.com

Lianju Ma
College of Life Science, Shenyang Normal University, Shenyang 110034, China
malianju@163.com

Ning Bu
College of Life Science, Shenyang Normal University, Shenyang 110034, China
309226126@qq.com

*Lin Hao (Corresponding author)
College of Life Science, Shenyang Normal University
Postal address: No. 253, Huanghe North Street, Huanggu District, Shenyang City, 110034, China
Email: haolinwj2001@163.com Tel: 86-24-86593309

Abstract

Background and aims The plant growth promoting rhizobacteria have been extensively implicated in plant responses to changing environments. However, the action mechanisms still need to be elucidated. This study addressed the effect of *Paraburkholderia* sp. GD17 on rice seedlings in responses to salt stress.

Methods The experiment consisted of GD17-inoculated and non-inoculated plants, with or without NaCl treatment. Physiological and biochemical parameters, and gene expression were analyzed.

Results GD17 efficiently colonized inside roots, and provided a protection against salt stress. Following exposure to 68 mM of NaCl for 48 h, although the accumulation of Na^+ was not affected in GD17-inoculated (+GD17) roots relative to non-inoculated ones, its concentration was substantially reduced in +GD17 shoots. The contents of K and other mineral elements were higher in +GD17 plants. The expression of Na^+ and K^+ transporter-encoding genes generally presented a higher level in +GD17 plants. The antioxidative defense especially related to the removal of H_2O_2 was more strongly activated in +GD17 plants. Correspondingly, salt-induced oxidative damage was significantly ameliorated. A substantial increase in proline content and gene expression was observed in +GD17 plants. Additionally, the cell wall invertase-encoding gene displayed a dramatically higher expression level in +GD17 plants.

Conclusions GD17 efficiently improved rice seedling tolerance to salt stress. The possible mechanisms might be associated with the absorption and redistribution of mineral elements, the vacuolar sequestration of Na^+ as well as exclusion of Na^+, antioxidative defense, the production of proline, and the sucrose catabolism in apoplast.

Keywords Rice · Salt stress · *Paraburkholderia* · Antioxidative defense · Osmoregulation · Invertase

Introduction

Salinity is one of the major abiotic factors limiting plant growth, development, and productivity in agriculture (Pitman and Läuchli 2002). More than 800 million hectares of land (*c.* 6% of the world's total land area) is affected by excess salt concentrations (Munns and Tester 2008), and this problem continues to worsen. Generally, the phytotoxicity of salinity is caused directly by osmotic stress and/or ionic stress, and indirectly by secondary stress (Yang and Guo 2018). For example, excess salts in the soil induce plant osmotic stress by reducing the water potential limiting water uptake (Hasegawa et al. 2000), while the excessive uptake of Na^+ and Cl^- leads to ionic stress by affecting the absorption and distribution of essential elements, therefore interfering with various metabolic processes (Lazof and Bernstein 1999). Either the osmotic or the ionic stress can cause plant secondary stress, typically by producing and accumulating reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$). Excessive ROS can severely damage cellular structures and functions by triggering membrane lipid peroxidation, and destroying biological macromolecules such as enzymes, proteins, and DNA (Mittler 2002). Therefore, the manipulation of increasing plant ROS-scavenging capacity, such as enhancing antioxidative enzyme activities or non-enzymatic antioxidant contents, is always favorable for plant tolerance to salt stress (Abogadallah 2010).

Rice is a major food crop providing the world population > 50% of staple food, especially in Asia (Hussain et al. 2018). However, rice is identified as a salt sensitive glycophytic species, especially during the germination, seedling and reproductive stages (Grattan et al. 2002). For instance, the salinity threshold level is 2 dS m^{-1} for rice, while the corresponding value is 6 and 7.7 for salt-tolerant wheat and cotton, respectively (Joseph and Mohanan 2013). NaCl concentration in growth matrix as low as 50 mM could cause a lethal effect on rice seedlings (Yeo et al. 1990). Due to its glycophytic property, as well as special habitat such as stagnant water, rice production in agriculture often severely affected by salinity stress (for a review see Ganie et al. 2019). Therefore, improving salt tolerance or reducing salinity-induced damage during the whole growth cycle of rice would increase the potential of saline-alkali land and ensure food security (Qin et al. 2020).

In agricultural production, besides genetic breeding of salt-tolerant varieties, improvement of cultivation conditions is an important strategy. For example, optimizing rhizosphere bacterial community has been demonstrated to efficiently enhance plant tolerance to salt stress (Chatterjee et al. 2018a, 2018b). Plant growth-promoting rhizobacteria (PGPR) are

a group of soil microorganisms, either rhizospheric (free-living) and/or endophytic (in symbiotic association with plant root interior). Relative to the former, the latter is considered better in stimulating plant growth because of its sustainability and mutually beneficial impact way on plants. PGPR can improve plant growth, and/or induce plant local and systemic resistance to biotic and abiotic stresses probably by increasing nutrient acquisition especially via nitrogen fixation, inorganic P solubilization and production of siderophores, promoting phytohormone synthesis, limiting pathogens, systemically enhancing the defensive capacity, and so on (Berendsen et al. 2012; Estrada et al. 2013). Increasing evidence has shown that application of PGPR could improve plant (including rice) tolerance to salinity stress (e.g. Khan et al. 2020, and references therein). The involved mechanisms include osmotic balance, ion homeostasis, phytohormone production, improving mineral nutrition, regulation of key genes, and so on (Kushwaha et al. 2020). Therefore, PGPR can be used as a low-cost and eco-friendly technology in agriculture especially in salinity-affected areas to enhance crop productivity (Vaishnav et al. 2019).

The bacteria of genus (*Para-*)*Burkholderia* are a group of important rhizosphere microorganisms with a wide diversity in functions and distributions (Coenye and Vandamme 2003). Some species are human friends, such as helping biodegradation, biological control and plant growth promotion in agriculture, while others are foes, such as causing plant, also animal and human diseases. The effect of (*Para-*)*Burkholderia* species on rice plants in responses to abiotic or biotic stresses has been repeatedly reported. For instance, *Burkholderia* sp. P50 strain can increase rice seedling tolerance to salt stress by improving physiological and biochemical metabolisms, and antioxidative defense probably associating with the reduced ethylene production (Sarkar et al. 2018), while *B.* sp. Y4 enhances rice tolerance to Cd stress by increasing uptake of essential elements and reducing Cd absorption and accumulation in various tissues (Wang et al. 2020). Some species of *Burkholderia*, such as *B. vietnamiensis*, can be used as nitrogen fertilizer with N_2-fixing function in agriculture (Trân Van et al. 2000, and references therein). Others can be used to as chemical alternatives to control, locally or systemically, diseases in plants (for a review see Coenye and Vandamme 2003). However, some members can cause serious disease epidemics in cultivated plants. For example, *B. plantarii* is the causative agent of rice seedling blight, while *B. glumae* causes rot of rice grains and seedlings. In recent years, based on the molecular signatures and phylogenomic analysis, the genus *Burkholderia* is proposed to be divided into the emended genus *Burkholderia* only containing the clinically relevant and phytopathogenic organisms and a new genus *Paraburkholderia* gen. nov. mainly including environmental species (Sawana et al. 2014). Interestingly, the colonization of *Burkholderia vietnamiensis* and *Paraburkholderia kururiensis* in rice roots caused different (or specific) gene expression, even opposite regulatory patterns such as in jasmonic acid-related network (King et al. 2019).

We previously isolated a PGPR from the root interior of wild soybean. It has the properties of N_2 fixation, P solubilizing activity, indole acetic acid (IAA) production, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity (Guo et al. 2018). Furthermore, this strain has a 100% of the 16S rDNA sequence identity to *Burkholderia* sp. KY357336.1. A NCBI search indicates that KY357336.1 has a 99.43% identity to *Paraburkholderia caribensis* strain MNL-133. Therefore, this strain belongs to environmental species, and is named as *Paraburkholderia* sp. GD17. Interestingly, several *Paraburkholderia* isolated from soybean nodules exhibited a salt-tolerant property (Artigas et al. 2019). The present study explored the effect of GD17 symbiosis with rice roots on seedling growth and responses to NaCl stress, with an emphasis on the element uptake and root-to-shoot transportation, antioxidative defense, and osmoregulation by the analyses of physiological and biochemical parameters, and gene expression.

Materials and Methods

Isolation and identification of *Paraburkholderia* sp. GD17

Paraburkholderia sp. GD17 strain was isolated from the root interior of wild soybean (*Glycine soja*) grown near the coastal belt of PuHe river, Shenyang (N41°50′, E123°24′), China (Guo et al. 2018), and deposited in the Microbiology Laboratory of Shenyang Normal University. Briefly, about 5 g fresh roots of wild soybean were collected and surface-sterilized in 5% (*v*/*v*) sodium hypochlorite solution for 10 min and rinsed three times with sterilized water. The

samples were ground using sterilized mortar and pestle, and the powder was placed into a 250 ml conical flask with plug containing 90 ml of sterile distilled water. The flask was shaken at 150 rpm for 30 min at 28 ˚C on a rotary shaker. Subsequently, 1 ml of suspension was continuously diluted in a 10-fold order, obtaining 10^{-4} – 10^{-6} serial dilutions. The dilutions were spread on Ashby agar medium plate consisting of 10 g mannitol, 0.2 g NaCl, 0.2 g $MgSO_4$, 1 g $CaCO_3$, 0.2 g $CaSO_4$, 0.2 g K_2HPO_4 in 1 L volume, pH 7.2. After incubation for 3 d at 28 ˚C, a total of 12 bacterial isolates, each having unique colony morphologies, were selected and further purified three times. The isolates were streaked on Luria-Bertani (LB) plates (Bertani 1951), and stored in a 4 ˚C freezer. These 12 bacterial isolates were screened and identified by 16S rDNA amplification and sequence alignment. Briefly, the full length 16S rDNA gene was amplified using the universal primers 27F: 5′-AGAGTTTGATCCTGGCTCAG-3′, and 1492R: 5′-TACGGTTACCTTGTTACGACTT-3′. The PCR products were sequenced and compared with the available sequences using the basic local alignment search tool (BLAST) at NCBI.

Plant growth, GD17 inoculation and saline treatment

A japonica type rice (*Oryza sativa* L. cv. Meifeng 115) seeds, purchased from Liaoning Dongya Seed Limited Company, Shenyang, China, were used in this study. After surface-sterilized in 5% (*v*/*v*) sodium hypochlorite solution for 10 min and rinsed three times with deionized water, the seeds were germinated in dark at 28 ˚C for 2 day by evenly placing in Petri dish (9 cm) coated with two sheets of Whatman No.1 filter paper moistened with 10 ml of distilled water. The uniformly germinated seeds were selected and cultured in plastic box (12 × 8 × 10 cm, L × W × H) covered with 48 hole PVC plate, with one germinated seed being kept in one hole. The box was filled with 850 ml 1/2 strength of sterilized Hoagland's nutrient solution. One liter of the nutrient solution contains 945 mg $Ca(NO_3)_2{\cdot}4H_2O$, 506 mg KNO_3, 80 mg NH_4NO_3, 136 mg KH_2PO_4, 493 mg $MgSO_4{\cdot}7H_2O$, 13.9 mg $FeSO_4{\cdot}7H_2O$, 18.65 mg EDTA-Na, 2.86 mg H_3BO_3, 1.81 mg $MnCl_2{\cdot}4H_2O$, 0.22 mg $ZnSO_4{\cdot}7H_2O$, 0.051 mg $CuSO_4{\cdot}5H_2O$ and 0.12 mg $Na_2MoO_4{\cdot}2H_2O$. The seedlings were cultured in a chamber under controlled conditions, with 14 h/10 h day/night, an irradiance of approximately 200 μmol quanta m^{-2} s^{-1}, 28 ˚C/22 ˚C (day/night), and 70% relative humidity. For the inoculation, 10 ml of GD17 fermentation liquor (LB medium) at the logarithmic phase (containing about 10^8 CFU ml^{-1}) was added in the nutrient solution (about 1.2 × 10^6 final concentration of bacteria) after 4 days of seed germination. The choice of inoculation time was based on the pre-experiment (supplementary Fig. S1). The blank control group only added LB culture medium. After 4 days of GD17 inoculation, the seedlings were exposed to salt by transferring to 1/2 strength of Hoagland's nutrient solution supplied with NaCl at the final concentrations of 0, 34, 68, and 136 mM, respectively. Therefore, this study was consisted of four groups designed as control, +GD17, +NaCl, and GD17 + NaCl. To assess plant growth, the seedlings were exposed to NaCl for 7 days. Based on the NaCl-induced damage to plants in a dose- and/or time-dependent way, however, the remainder physiological and biochemical parameters, and gene expression were analyzed on the plants exposed to 34 mM NaCl for 48 h. All the experiments were independently replicated 3 times.

Assay of GD17 colonization in root interior

Colonization of strain GD17 in the root interior was assessed using colony forming units (CFU) and the relative expression level of 16S rRNA, respectively. CFU was counted following the description of Yang et al. (2020a) with some modifications. Briefly, root samples were collected after 0, 2, 24, 48 and 96 h of GD17 inoculation, respectively, and surface-sterilized with 5% (*v*/*v*) sodium hypochlorite solution for 10 min and rinsed three times with sterilized water. The surface-disinfected roots were placed on LB plate to elucidate the disinfection effect, and no residual bacteria were found. The size of the endophytic population was measured on 10 plants. Briefly, the surface-disinfected sample was well crushed using sterilized mortar and pestle, and then was mixed with 15 ml of 0.9% NaCl solution. The suspension was collected into a 50 ml conical flask with plug and homogenized on rotary shaker (150 rpm) for 30 min. After settling the tissues debris, serial dilutions were prepared and spread on LB plate. The plates were incubated at 28 ºC for

2 days, and colonies were counted. For the measurement of the accumulation of 16S rRNA, total RNA was extracted from the above-mentioned root tissues, and the levels of 16S rRNA were analyzed using RT-PCR with the primer 5′-AGCGTGCGTAGGTGGTTATT-3′ and 5′- TCCGCTACCCTCTACCACA-3′ (amplified length of 103 bp) as described in following section "Gene expression analysis".

Histochemical detection of H_2O_2, $O_2^{\cdot-}$ and cell death

For the histochemical observation of hydrogen peroxide (H_2O_2), the detached fresh leaves from four tested groups were stained by 3,3′-diaminobenzidine tetrahydrochloride (DAB), respectively, according to the description by Kasten et al. (2016). Briefly, leaves were immersed in 1 mg ml^{-1} DAB solution containing 0.05% Tween 20 (pH 3.8) by vacuum infiltration, and then incubated for 45 min at room temperature under light. For the assessment of superoxide anion ($O_2^{\cdot-}$) accumulation, the detached fresh leaves were vacuum infiltrated with a solution containing NaN_3 (10 mM) and nitroblue tetrazolium (NBT; 0.1% *w*/*v*) by applying three 20-seconds pulses, and then incubated for one hour at room temperature under light. For the detection of cell death, the detached leaves were incubated in 0.25% Evans blue solution for 30 min. All the stained leaves were soaked in 95% ethanol at 80 °C to remove chlorophyll. The each staining experiment consisted of several groups of leaves, and a similar tendency was observed among them. Therefore, a representative group was presented in the text.

The measurement of physiological and biochemical parameters

Plant growth was assessed by measuring the dry and fresh weights of the aerial parts and roots, respectively. Plants were dried in an oven at 80 °C for 48 h, and weighed as dry weight. For the determination of element contents, approximately 0.15 g of dried samples of leaves and roots were ground to a fine powder in a mortar and pestle, respectively, and digested with 5 ml of concentrated HNO_3. The total concentrations of Na and eight essential elements (K, P, Mg, Ca, Zn, Fe, Mn, and Cu) in the extracts were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES, iCAP 6000 Series, Thermo Electron Corporation, USA). For the activity detection of superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (PRX; EC 1.11.1.7) and catalase (CAT; EC 1.11.1.6), fresh leaves or roots (0.2 g) were collected and pooled from 20 plants received the same treatment, and homogenized in liquid nitrogen using a mortar and pestle. The powder was suspended in 2 ml of ice-cold extraction solution consisting of 50 mM phosphate-buffered saline (pH 7.8), 0.1 mM EDTA, 1% (v/v) Triton X-100, and 4% (w/v) polyvinylpyrrolidone. Leaving on ice for 10 min, it was centrifuged at 12,000 × *g* and 4 °C for 15 min, and the supernatant obtained was used as crude enzyme extract. SOD was analyzed following the method of Beyer and Fridovich (1987). One unit was defined as the amount of enzyme required to inhibit the photoreduction of nitroblue tetrazolium by 50%. PRX was measured according to the description by Hemeda and Klein (1990). One unit was defined as an increase of 0.01 absorbance at 470 nm per min. CAT was measured by the method of Aebi (1983). One unit was defined as a decrease of 0.01 absorbance at 240 nm per min. Meanwhile, the leaf isozyme activities of SOD, PRX and CAT were analyzed by native polyacrylamide gel electrophoresis (PAGE), respectively, according to the previous description (Hao et al. 2012). The protein concentration in the crude enzyme extract was detected by the method of Bradford (1976). The reduced form of glutathione (GSH) was quantified using the method of Griffith and Meister (1979). The oxidized form of glutathione (GSSG) was calculated from the amount in 1,4-dithiothreitol (DTT)-treated samples minus the amount in non-treated samples. Extraction and quantification of the reduced form (AsA) and oxidized form (DHA) of ascorbate were based on the method described by Wang et al. (1991). This assay is based on the reduction of Fe^{3+} to Fe^{2+} by AsA in acid solution followed by formation of a pink color chelate between Fe^{2+} and bathophenanthroline that absorbs at 525 nm. Content of DHA was calculated from the amount in DTT-treated samples minus the amount in non-treated samples. A standard curve was drawn using 0 – 1 mM of AsA. The H_2O_2 and malondialdehyde (MDA) contents were respectively determined according to the earlier descriptions (Hao et al. 2012, and references therein). Soluble sugars were measured using the anthrone colorimetric method as described by Zhang and Qu (2003). Proline contents were detected by the

method of Bates et al. (1973).

Gene expression analysis

Fresh leaves or roots were harvested from 20 plants of each treatment group, and ground in liquid nitrogen using a mortar and pestle, respectively. Total RNA was isolated from the shoots or roots with Total RNA Isolation System (Promega), and the first-strand cDNA was synthesized using PrimeScript RT Reagen Kit (TaKaRa), both according to the manufacturer's instructions. The gene expression was analyzed using Lightcycler 96 fluorescence real time PCR system (Roche, Basel, Switzerland), and the reaction program setup was referred the previous description of Qu et al. (2018). The relative expression level was calculated according to the $2^{-\Delta\Delta CT}$ method using the Ct geometric average value of three reference genes, *PK* (Os06g0702800), *NABP* (Os06g0215200) and *TCTP* (Os11g0660500) as the internal control. These genes presented a stable expression in *Oryza sativa* using organ, development, biotic and abiotic transcriptome datasets (Narsai et al. 2010). Furthermore, their encoding products belong to different functions as described in Table S1, so their expressions are not likely to be co-regulated. Therefore, it was appropriate that they were used as multiple internal control genes as described by Vandesompele et al. (2002). The short descriptions of the genes analyzed in this study and their primer sequences were presented in Supplementary Table S1 and S2, respectively.

Statistical analysis

All data in the text came from three independent biological experiments, and presented as the mean ± SD. Where applicable, data were subjected to analysis of variance (ANOVA) using SAS software (SAS Institute, Cary, NC, USA), taking $p < 0.05$ as significance according to Duncan's multiple range test.

Results

GD17 colonization and effect on plant growth

To assess the colonization efficiency of GD17 in rice seedlings, the roots were harvested at 0, 2, 24, 48 and 96 h after the inoculation, respectively. The result showed that no GD17 strain was found in root interior before the inoculation, while substantial numbers of GD17 were detected after 2 h of inoculation, and it dramatically increased with the extension of inoculation duration as indicated by colony-forming units (CFU) (Fig. 1), and also by the estimate of 16S rRNA accumulation (Fig. S2). This suggested that strain GD17 efficiently infected and colonized inside rice roots. Therefore, the salt stress was performed at the fourth day after GD17 inoculation. The effect of salt stress on plant growth was elucidated at the seventh day after application of different doses of NaCl, corresponding a 15 d-growth period including sequential 4 d germination, 4 d GD17 infection, and 7 d salt stress. Exposure to NaCl inhibited plant growth (Fig. 2A - E), and also led to leaf chlorosis especially severe at the tips (Fig. 2E) in a dose-dependent way. Inoculation of GD17 efficiently alleviated salt-induced growth inhibition and visible damage. It also improved plant growth under normal growth conditions, such as about 19% and 13% increases in shoot fresh weight (FW) and dry weight (DW), and 23% and 19% increases in root FW and DW, respectively, relative to their respective non-inoculated controls. All reached significant differences at $p < 0.05$ (Fig. 2A - D).

Uptake and root-shoot transportation of Na^+ and nutrient elements, and gene expression of related ion transporters

Exposure to 68 mM NaCl for 48 h increased shoot Na concentrations, to a greater degree in non-inoculated plants than in GD17-inoculated ones (by 1.76 times versus 1.43 times; $p < 0.05$) (Fig. 3A). The root Na concentrations also significantly increased, but there was no statistical difference between GD17-inoculated and non-inoculated plants (by 3.62 times versus 3.95 times; $p > 0.05$) (Fig. 3B). Overall, exposure to salt limited the uptake and root-shoot transportation of macronutrients and micronutrients especially in non-inoculated plants, while +GD17 plants efficiently prevented the negative effect (Fig. 3A and B). For instance, in comparison with the control levels (without both NaCl and GD17), K content decreased by 32.6% and 39.1% in non-inoculated shoots and roots, while by 21% and 31.9% in

+GD17 ones under salt stress, respectively, both of them with a statistical difference ($p < 0.05$) between non-inoculated and GD17-inoculated plants.

To dissect the regulatory mechanisms of GD17 on Na^+ and K^+ uptake and redistribution, gene expression of Na^+ and/or K^+-related transporters was analyzed. The Na^+/H^+ antiporter-encoding gene *NHX* (*Na/H exchanger*) *1* and *-2*, responsible for the coupled exchange of K^+ or Na^+ for H^+ at the tonoplast, presented a NaCl- and/or GD17-induced expression pattern, particularly efficient for *NHX1* under the combined treatment (Fig. 4A - D). The Na^+/H^+ exchanger-encoding gene *SOS* (*salt overly sensitive*) *1* and *2*, mediating Na exclusion from roots and shoots, and Na redistribution between roots and shoots, generally exhibited a NaCl- and/or GD17-induced expression pattern, especially under the combined treatment (Fig. 4E - H). The expression of K^+ transporter-encoding gene *HAK* (*high-affinity K*) *1* and *5* was up-regulated in both shoots and roots by salt stress, especially in combination with GD17 inoculation (Fig. 4I - L).

Oxidative stress and antioxidative defense

Exposure to NaCl caused oxidative stress to a greater degree in non-inoculated plants than in GD17-inoculated ones, as indicated by the production of H_2O_2 and MDA (Fig. 5A - D). For example, increases of 62% and 55% in the content of H_2O_2 were detected in non-inoculated shoots and roots, while only 13% and 5% increases in +GD17 partners, respectively, both of them with a significant difference ($p < 0.05$) between the inoculated and non-inoculated plants. Similarly, a significant difference occurred in MDA contents which increased by 61% and 53% in non-inoculated shoots and roots, while only by 17% and 27% in +GD17 ones, respectively. The GD17-conferred ameliorative effect on salt-induced oxidative stress was also exhibited by *in situ* staining reactions with DAB and NBT shown the accumulation and distribution of H_2O_2 and $O_2^{\cdot-}$, respectively, and with Evans blue shown dead cells (Fig. S3).

Relative to the control levels (without both NaCl and GD17), the activity of SOD increased by 11% ($p > 0.05$) and 21% ($p < 0.05$) in non-inoculated shoots and roots following salt stress, respectively. However, it was obviously down-regulated in +GD17 plants, such as decreases of 23% and 26% in shoots and roots under normal conditions (both at $p < 0.05$), and 49% and 23% under salt stress (both at $p < 0.05$), respectively (Fig. 6A and B). In comparison with the control levels, the activity of PRX was elevated by 24% and 60% in shoots and roots under salt stress, by 50% and 44% in +GD17 partners, and by 82% and 70% in combined treatment, respectively, all of them with a significant difference at $p < 0.05$ (Fig. 6C and D). A significant increase was also detected for CAT activity in shoots and roots, such as by 36% and 36% following salt stress, 31% and 53% upon GD17 inoculation, and 58% and 87% under combined treatment, respectively (Fig. 6E and F). The change patterns of these enzymatic activities in leaves were also exhibited by their respective isozyme expression profiles (Fig. S4).

The expressions of Cu-Zn SOD-encoding gene *CSD1* and *CSD2* were generally up-regulated in non-inoculated shoots and roots following salt stress, while they presented dramatic down-regulations in +GD17 shoots and roots regardless of salt stress (Fig. 6G – J). Two PRX-encoding gene *PRX41* and *PRX27* generally exhibited a salt- and/or GD17-induced expression pattern (Fig. 6K – N). Similarly, two CAT-encoding gene *CATA* and *CATC* displayed a salt- and/or GD17-induced expression (Fig. 6O – R).

As two of the most important antioxidants, glutathione and ascorbic acid including their contents and redox status (GSH/GSSG and AsA/DHA) presented a similar change pattern under salt stress or in GD17-inoculated plants. Generally, higher levels of them were detected in +GD17 shoots and roots than in non-inoculated ones under either normal conditions or salt stress, even though most of them were prone to decrease following salt stress (Fig. 7A – H). The change tendency of these parameters was also reflected on the transcriptional levels of their reductase-encoding genes, *GR1* and *-2* (*glutathione reductase 1* and *2*), and *MDAR1* and *-2* (*monodehydroascorbate reductase 1* and *2*), respectively (Fig. 7I - P).

Osmoregulation substance and related gene expression

In comparison with the control level (without both NaCl and GD17), proline contents were enhanced in shoots and roots by 42% and 52% under salt stress, by 40% and 69% upon GD17 inoculation, and by 90% and 130% under combined treatment, all of them with a statistically significant difference at $p < 0.05$ (Fig. 8A and B). The expression of *P5CS*, encoding delta-l-pyrroline-5-carboxylate synthetase, a key enzyme in the synthesis of proline, was analyzed. Though GD17 inoculation did not affect the expression of *P5CS1* under normal conditions (without NaCl), it substantially promoted the expression level in both shoots and roots under salt stress (Fig. 8C and D). The expression of *P5CS2* was up-regulated in shoots and roots following NaCl stress or +GD17, especially with the combined treatment (Fig. 8E and F).

Soluble sugar increased by 24% and 19% (both at $p < 0.05$) in non-inoculated shoots and roots following salt stress, respectively, but it seemed unaffected in +GD17 plants (Fig. 9A and B). The expression of several invertase-related genes was analyzed. The vacuolar invertase-encoding *VIN2* displayed a mild up-regulation in shoots under salt stress and/or +GD17 (Fig. 9C), but down-regulated in roots (Fig. 9D). The cell wall invertase-encoding gene *CIN5* was expressed at a dramatically higher level in shoots under salt stress and/or +GD17 compared with control (without both salt and GD17) (Fig. 9E), and also at a statistically high level in roots ($p < 0.05$) (Fig. 9F). However, the expression of neutral/alkaline invertase-encoding gene *NIN1* was down-regulated in +GD17 shoots and roots especially under salt stress, relative to the non-inoculated parallels (Fig. 9G and H).

Discussion

Plant photosynthetic tissues are the main target for Na-induced phytotoxicity. Plants evolve various mechanisms to alleviate Na^+ toxicity in leaves, including limiting Na^+ uptake from the soil, reducing the root-shoot transportation, sequestrating Na^+ into vacuoles, cycling Na^+ from shoots to roots, and so on (Zhang et al. 2018). In this study, although GD17 inoculation did not obviously affect the root uptake of Na^+ from culture solution (Fig. 3B), it substantially reduced Na^+ transportation from roots to shoots (Fig. 3A). Additionally, because one of the negative impacts of excess rhizosphere Na^+ is to limit plant uptake of nutrient elements especially K^+ due to Na^+ competition at K^+ binding sites (Maathuis and Amtmann 1999), the maintenance of a high cytosolic K^+/Na^+ ratio is one of the pivotal determinants in plant tolerance to salt stress (Kader et al. 2006). In this study, +GD17 indeed ameliorated Na^+-induced limitation of K^+ uptake (Fig. 3B) and transportation from roots to shoots (Fig. 3A). This led to higher K^+/Na^+ ratio in +GD17 leaves (10 : 1) and roots (2.3 : 1) compared with non-inoculated leaves (7.5 : 1) and roots (1.9 : 1) under salt stress. These data suggested that GD17-conferred plant tolerance to salt stress might be correlated with the regulation of the uptake and root-shoot transportation of Na^+ and K^+. Similarly, the GD17-mediated element uptake and redistribution patterns were also observed for P, Mg, Mn, Ca, Zn, and Fe but not Cu (Fig. 3A and B). The regulatory effect on the balance of Na^+ and K^+, and the absorption and redistribution of mineral elements has been implicated in rice plant adaptability to salt stress (e.g. Zhang et al. 2018). Furthermore, *Burkholderia*-mediated rice plant tolerance to abiotic stress such as heavy metal Cd was also correlated with essential nutrient uptake (Wang et al. 2020). *B. phytofirmans* PsJN-stimulated growth and yield of quinoa under salinity stress was accompanied by decreased leaf Na^+ and increased leaf K^+ (Yang et al. 2020a).

Many cytosolic enzymes are activated by K^+ and inhibited by Na^+ (Flowers et al. 1977). Therefore, Na^+ isolation in vacuoles is an another adaptive mechanism in plant responses to salt stress. Na^+/H^+ antiporters (NHXs) extensively exist in organisms from prokaryotes to higher eukaryotes, responsible for the coupled exchange of K^+ or Na^+ for H^+ at the tonoplast, therefore maintaining K homeostasis in the cytoplasm and Na compartmentation in vacuoles (for a review see Bassil et al. 2012). For instance, AtNHX1 contributes Arabidopsis plant tolerance to salt stress by mediating K^+ and Na^+ sequestration in vacuoles, also regulates plant growth and development under normal growth conditions by controlling the turgor generation, cell expansion, stomata movement, and vesicular trafficking (Apse et al. 2003; Barragan et al. 2012). The overexpression of *AtNHX1* in tomato plants improved salt tolerance by partitioning more K^+ in vacuoles, therefore activating K uptake by roots, and increasing K^+ contents in plant tissues (Leidi et al. 2010). In rice,

four NHXs (OsNHX1 - 4) have been reported, and they have some similarities in construction or functions. Like AtNHK1 in Arabidopsis, OsNHX1 is the most abundant vacuolar K^+/H^+ and Na^+/H^+ antiporter in rice. Its expression in rice shoots and roots was induced to a peak at 2 - 4 h after NaCl stress (Almeida et al. 2017). In this study, the expression of *OsNHX1* was markedly up-regulated in +GD17 roots particularly under NaCl stress, and was also statistically significantly induced in shoots (Fig. 4A and B), suggesting that the GD17-conferred rice seedling tolerance to NaCl stress might be associated with the NHX-mediated K^+ and Na^+ vacuolar transportation. This was also suggested by the expression pattern of *OsNHX2* (Fig. 4C and D). In Arabidopsis, AtNHX2 shares the greatest similarity with AtNHX1 by 87% identical residues. Furthermore, *AtNHX2* is also one of the most abundantly transcribed genes in various tissues of *A. thaliana*, and its expression is regulated by salt stress driving Na^+ or K^+ into the vacuole (Aharon et al. 2003). The well-known PGPR *B. phytofirmans* PsJN-conferred Arabidopsis plant tolerance to NaCl was correlated with increased expression levels of *AtNHX2* in roots and leaves (Pinedo et al. 2015).

SOS1 is critical for Na^+ exclusion from plant tissues and/or controlling long-distance Na^+ transport from roots to shoots, therefore effectively improving plant tolerance to salt stress (Shi et al. 2002). In this respect, the molecular decipherment of SOS1/SOS2/SOS3 complex provides invaluable information (Zhu 2000). In Arabidopsis, the action model of SOS-complex has been well established, namely that the activity of SOS1 is regulated through the protein phosphorylation by SOS2 (kinase), and the latter is activated by SOS3 (a calcineurin B-like Ca^{2+}-binding protein) (Qiu et al. 2002). In rice, the homologs of AtSOS1, -2 and -3 have been identified, such as OsSOS1, OsCIPK24, and OsCBL4, respectively. Like its Arabidopsis partner, OsSOS1 is activated by SOS2-SOS3 protein kinase complex leading to plant tolerance to salt stress (Martínez-Atienza et al. 2007). OsSOS1 plays important roles to enhance salt tolerance in rice by mediating Na^+ exclusion and controlling Na^+ transport from roots to shoots (Mahi et al. 2019). In this study, the expression patterns of *OsSOS1* and *-2* (Fig. 4E - H), together with the reduced Na^+ accumulation in shoots (Fig. 3A) implied that the SOS pathway might be involved in GD17-conferred rice seedling tolerance to salt stress.

Besides controlling Na^+ absorption and redistribution as described above, increasing K^+ acquisition is an important mechanism to maintain a high ratio of cytosolic K^+/Na^+ leading to plant tolerance to salt stress. In plants, there are many transporters for K acquisition and distribution (Wang and Wu 2013). Among them, *KT/KUP/HAK* comprises a large gene family in plant genome, such as 27 in rice (Yang et al. 2009). Most members of this family function in high-affinity K absorption and translocation under low K concentration ranges and/or salt stress transport (Yang et al. 2014). In this study, the expression patterns of *HAK1* and *HAK5* (Fig, 4I - L) both belonging to the same phylogenetic group, as well as the K^+ accumulation patterns in shoots and roots (Fig. 3A and B), suggested that GD17-conferred rice seedling tolerance to salt stress was correlated with K^+ absorption and root-shoot transport. The OsHAK1- and OsHAK5-mediated K acquisitions, thus increasing K^+/Na^+ ratio and salt tolerance, have been reported (Horie et al. 2011; Yang et al. 2014; Chen et al. 2015).

The production and accumulation of ROS, such as $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$, are a common consequence in plants subjected to high-intensity stresses including salinity. The excess ROS could disrupt cell structures and functions, such as causing membrane lipid peroxidation, biomacromolecule (e.g. protein, DNA) degradation (Mittler 2002). In this study, the protective effect of +GD17 on rice seedlings against salt stress was associated with a mitigated oxidative damage as indicated by the formations of $O_2^{\cdot-}$, H_2O_2, MDA and dead cells (Fig. 5 and S3). To avoid the ROS-induced destructive oxidative damage, plants activate antioxidant defense systems including antioxidases such as SOD, PRX, CAT, glutathione reductase (GR) and dehydroascorbic acid reductase (DHAR), and non-enzymatic antioxidants such as glutathione and ascorbic acid (Ahanger et al. 2017). In this study, SOD activity presented a up-regulated tendency in non-inoculated shoots and roots following salt stress, while it was down-regulated in +GD17 plants (Fig. 6A and B). The change pattern of SOD activity was also reflected by the isozyme expression profiles (Fig. S4). This might be due to a reduced accumulation of $O_2^{\cdot-}$ in +GD17 plants particularly under salt stress as indicated by NBT staining (Fig. S3), suggesting an amelioration role of GD17 in rice plants in responses to salt stress. A previous study also found that

Burkholderia sp.-conferred rice plant tolerance to salt stress was accompanied by a reduced SOD activity (Sarkar et al. 2018). However, the activities of PRX and CAT, both functioning in H_2O_2 scavenging, were up-regulated in +GD17 plants especially under salt stress as measured by spectrophotometry (Fig. 6C – F) and isozyme expression (Fig. S4). Although only a few of the genes encoding SOD, PRX and CAT were analyzed at the transcriptional level in this study, such as *CSD1* and *CSD2* (Fig. 6G – J), *PRX27* and *PRX41* (Fig. 6K – N), *CATA* and *CATC* (Fig. 6O – R), their expression patterns essentially reflected the change tendency of their enzyme activities. This suggested that the involvement of GD17 in plant antioxidative defense against salt stress was correlated with the transcriptional regulation of antioxidases. The similar finding was also reported in *Burkholderia*-mediated plant responses to heavy metal Cd (Khanna et al. 2019).

The ascorbate (AsA)-glutathione (GSH) cycle plays a crucial role in overcoming the environmental stress-induced oxidative damage to plants by removing excess ROS (especially H_2O_2). This depends on either the cellular concentrations of AsA and GSH, or their redox status (AsA/DHA and GSH/GSSG). In this cycle, glutathione reductase (GR) and monodehydroascorbate reductase (MDAR) are involved in the regeneration of GSH and AsA from their oxidized forms, respectively (Khan et al. 2020). In this study, the change tendency of GSH and AsA contents and their redox status, as well as the expression patterns of GR- and MDAR-encoding genes suggested that GD17-conferred rice plant tolerance to salt stress was associated with H_2O_2 removal. It was supported by the substantial reduction of H_2O_2 levels in +GD17 plants under salt stress as shown by both spectrophotometry (Fig. 5A and B) and DAB staining (Fig. S3).

Salt stress generally leads to cellular hyperosmotic situation, which triggers the production and accumulation of compatible osmolytes such as proline and sugars (Ahanger et al. 2017). These substances play multiple physiological functions in plant responses to stress conditions, such as improving plant adaptation to salt-induced osmotic stress, maintaining cell homeostasis, stabilizing the structures and functions of proteins, regulating cytosolic acidity, and balancing cell redox status (Verbruggen and Hermans 2008; Ahanger et al. 2017). In this study, proline was substantially elevated in both shoots and roots by salt stress and GD17 inoculation, alone and especially in combination (Fig. 8A and B). This suggested that GD17-conferred rice plant tolerance to salt stress was linked to increased proline levels. Several lines of evidence have shown that (*Para*)*Burkholderia*-mediated plant tolerance to salt was coupled with enhanced proline accumulation (e.g. Pinedo et al. 2015; Sarkar et al. 2018).

Plant synthesis of proline is mainly by the glutamate pathway, in which glutamate is converted to proline by two successive reactions catalyzed by pyrroline-5-carboxylate synthase (P5CS) and pyrroline-5-carboxylate reductase (P5CR), respectively. As a rate-limiting enzyme in proline synthesis, P5CS activity is controlled by feed-back inhibition and transcriptional regulation (Verbruggen and Hermans 2008). In this study, both *P5CS1* and *P5CS2* presented a higher expression level in +GD17 shoots and roots than in non-inoculated ones following salt stress (Fig. 8C - F). This was consistent with the change tendency of proline contents (Fig. 8A and B), suggesting that the GD17-mediated proline production and accumulation was at least in part associated with the transcriptional regulation of the biosynthesis-related genes. The tight correlation between *P5CS* expression and proline production has been repeatedly observed in rice plants especially under stress conditions. For instance, the expression level of *OsP5CS* increased in rice plants upon NaCl stress, wherein a salt-tolerant cultivar presented higher *P5CS* transcripts and proline contents than did a sensitive one under salt stress (Igarashi et al. 1997). The increased expression levels of *OsP5CS1* and *OsP5CS2* coupled with enhanced proline accumulation, as well as tolerance to drought and osmotic stress was found in transgenic rice over-expressing ethylene response factor-encoding gene *JERF3* (Zhang et al. 2010). Additionally, heterologous overexpression of cDNA clone encoding VaP5CS (*Vigna aconitifolia*) or PvP5CS (*Phaseolus vulgaris*) efficiently improved the proline contents and tolerance to salt stress in transgenic rice plants (Zhu et al. 1998; Chen et al. 2009).

In this study, although soluble sugar was enhanced in both shoots and roots following salt stress, it was not affected by GD17 inoculation (Fig. 9A and B). However, invertase-related genes presented divergent expression patterns (Fig. 9C - H), suggesting that sucrose hydrolysis in different cellular compartments might exert specific biological roles in

GD17-mediated rice seedling adaptation to salt stress. The invertase-mediated sucrose irreversible hydrolysis into glucose and fructose not only provides carbon sources, energy, and osmoregulation substances, but also plays signaling especially in sink tissues involving in plant growth, development, and adaptation to changing environments (Ruan et al. 2010). Based on their pH optimum and sub-cellular localization, invertases are classified as acidic vacuolar and cell wall isoforms, and alkaline/neutral cytoplasmic isoforms. In rice genome, at least 19 invertase-encoding genes have been identified including 9 cell-wall (*CIN1 - 9*), 2 vacuolar (*VIN1 - 2*), and 8 neutral/alkaline (*NIN1 - 8*) (Ji et al. 2005). In this study, one representative of each subclass, namely *CIN5*, *VIN2* and *NIN1*, was respectively analyzed at the transcriptional level. The transcripts of these three genes can be detected in both shoots and roots of rice plants (Ji et al. 2005). Previous study showed that ectopic overexpression of the cell wall invertase gene *CIN1* enhanced drought tolerance in tomato plants, which was associated with the integration of metabolic, hormonal, and stress signals (Albacete et al. 2015). Furthermore, the overexpression of the genes encoding proteinaceous inhibitors of the cell wall/vacuolar acid invertases reduced plant tolerance to salt stress, while the loss-of-function mutation increased salt tolerance, which was correlated with the ABA response (Yang et al. 2020b). In this study, based on the expression patterns of invertase-encoding genes (Fig. 9C - H), it was proposed that the acidic invertase (especially the cell wall isoform)-mediated sucrose catabolism might be involved in GD17-conferred salt stress. However, the involved mechanism is yet to be deciphered.

Conclusion

The current study provided evidence that the root inoculation of *Paraburkholderia* sp. GD17 strain efficiently improved rice seedling growth and tolerance to salt stress. The involved mechanisms might be associated with ameliorating Na^+-induced limitation in the uptake and root-shoot transportation of K^+ and other essential nutrient elements, facilitating Na^+ and/or K^+ compartmentation in the vacuole, increasing antioxidative defense and decreasing oxidative damage, enhancing the production and accumulation of proline, and promoting sucrose catabolism in apoplast. These findings would be beneficial to understand the involvement of PGPR in improving plant growth and systemic adaptation to changing environments. Furthermore, because GD17 strain can form symbionts with rice plant roots, it might be utilized in salt-affected agricultural fields to improve rice growth and yield in a sustainable way.

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Author contributions

HL and LGZ designed and carried out the research. ZYM, CYT, GY, MLJ and BN contributed to carry out the physiological, biochemical and the qRT-PCR analyses. HL and LGZ analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

Author statement

The authors declare that they have no conflict of interest.

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Figures

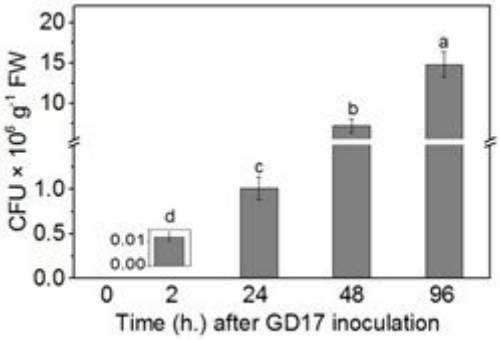

Figure 1

Colonization efficiency of GD17 inside roots as indicated by colony-forming units (CFU). GD17 inoculation was performed at 4 d after seed germination. The data were collected from three replicated experiments (n = 3) with 10 plants used in each batch of experiment, and represented as means ± SD. Bars with different lower-case letters indicate significant differences at $p < 0.05$

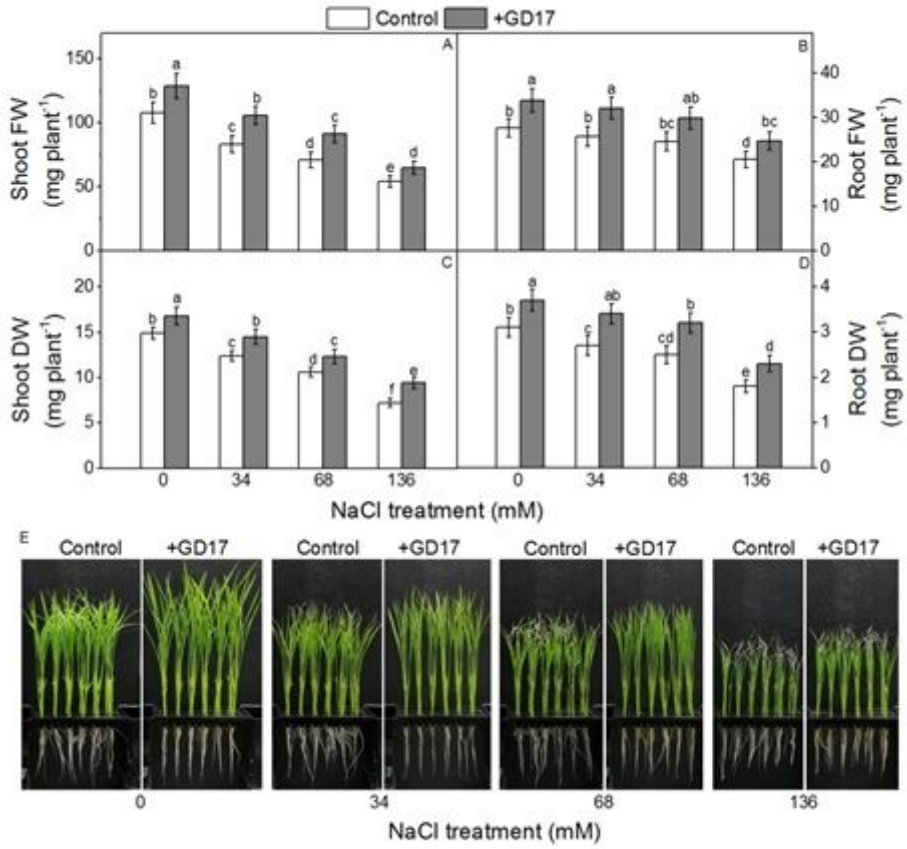

Figure 2

Effect of salt stress and/or GD17 inoculation on plant growth. Fresh weight of shoots (A) and roots (B), dry weight of shoots (C) and roots (D), and representative pictures (E). Fifteen-day-old plants (sequential 4 d germination, 4 d GD17 infection, and 7 d salt stress) were evaluated. The data were collected from three replicated experiments (n = 3) with 30 plants used in each batch of experiment, and represented as means ± SD. Bars with different lower-case letters indicate significant differences at $p < 0.05$

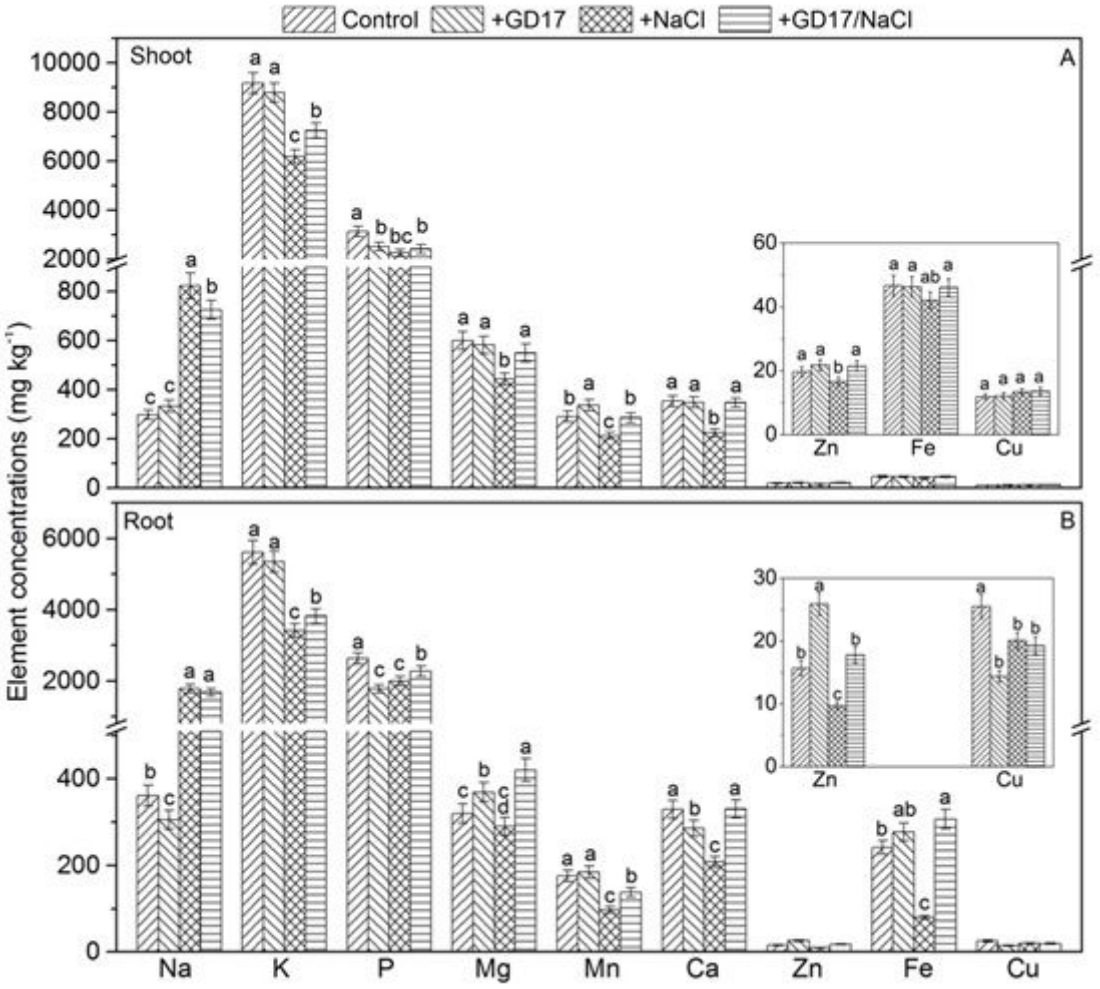

Figure 3

Effect of salt stress and/or GD17 inoculation on the accumulation of Na and nutrient elements in rice shoots (A) and roots (B). Eight-day-old plants (sequential 4 d germination, 4 d GD17 infection) were exposed to 68 mM NaCl for 48 h. The data were collected from three replicated experiments (n = 3), and represented as means ± SD. Bars with different lower-case letters indicate significant differences at $p < 0.05$

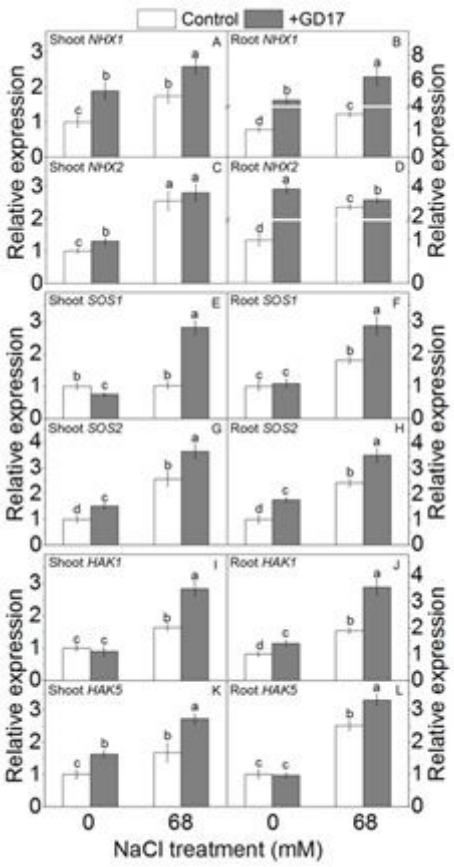

Figure 4

Effect of salt stress and/or GD17 inoculation on the expression of Na+/H+ antiporter-encoding gene NHX1 and NHX2 (A - D), Na+/H+ exchanger-encoding gene SOS1 and SOS2 (E - H), and K+ transporter-encoding gene HAK1 and HAK5 (I - L). Eight-day-old plants (sequential 4 d germination, 4 d GD17 infection) were exposed to 68 mM NaCl for 48 h. The data were collected from three replicated experiments (n = 3) with 20 plants used in each batch of experiment, and represented as means ± SD. Bars with different lower-case letters indicate significant differences at $p < 0.05$

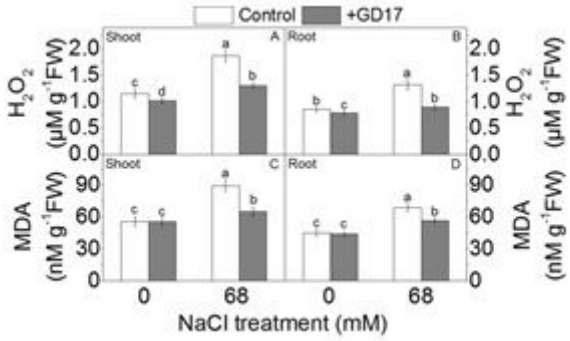

Figure 5

Effect of salt stress and/or GD17 inoculation on the contents of H2O2 (A, B) and malondialdehyde (MDA) (C, D). Eight-day-old plants (sequential 4 d germination, 4 d GD17 infection) were exposed to 4 mM NaCl for 48 h. The data were collected from three replicated experiments (n = 3) with 20 plants used in each batch of experiment, and represented as means ± SD. Bars with different lower-case letters indicate significant differences at $p < 0.05$

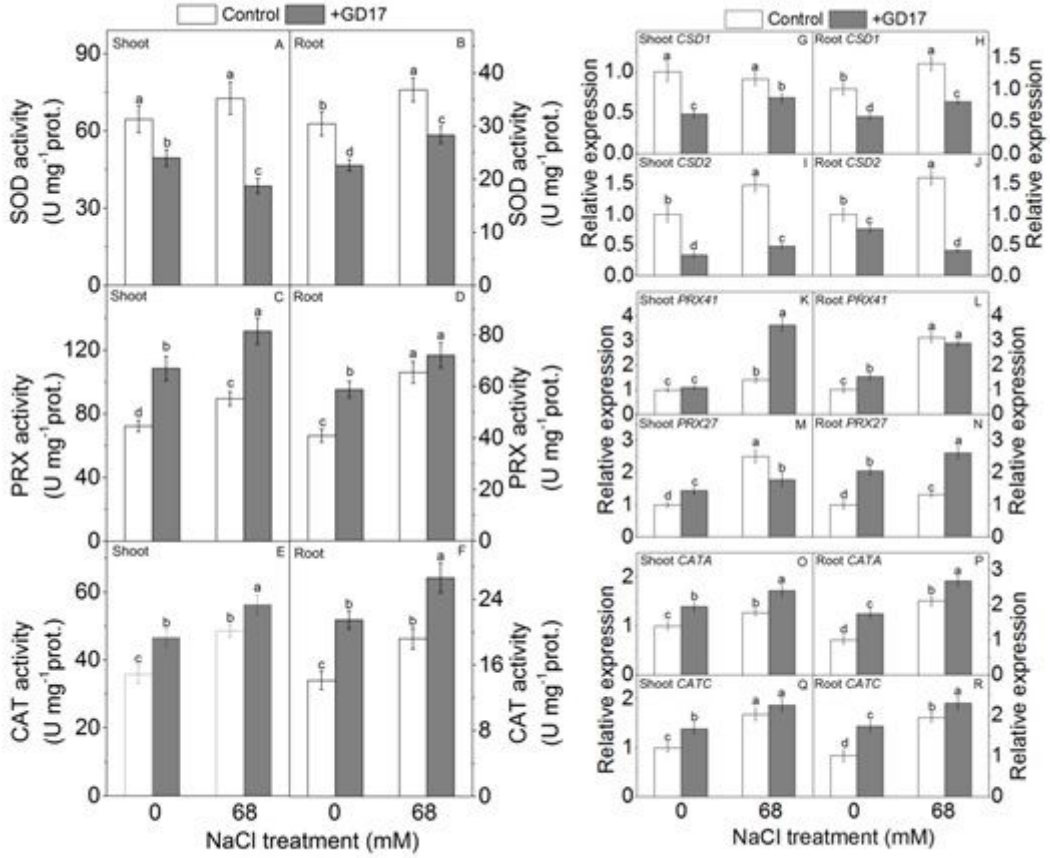

Figure 6

Effect of salt stress and/or GD17 inoculation on the activities of superoxide dismutase (SOD) (A, B), peroxidase (PRX) (C, D) catalase (CAT) (E, F), and the relative expression of SOD-encoding gene CSD1 (G, H) and CSD2 (I - J), PRX-encoding gene PRX41 (K, L) and PRX27 (M, N), and CAT-encoding gene CATA (O, P) and CATC (Q, R). Eight-day-old plants (sequential 4 d germination, 4 d GD17 infection) were exposed to 68 mM NaCl for 48 h. The data were collected from three replicated experiments (n = 3) with 20 plants used in each batch of experiment, and represented as means ± SD. Bars with different lower-case letters indicate significant differences at $p < 0.05$

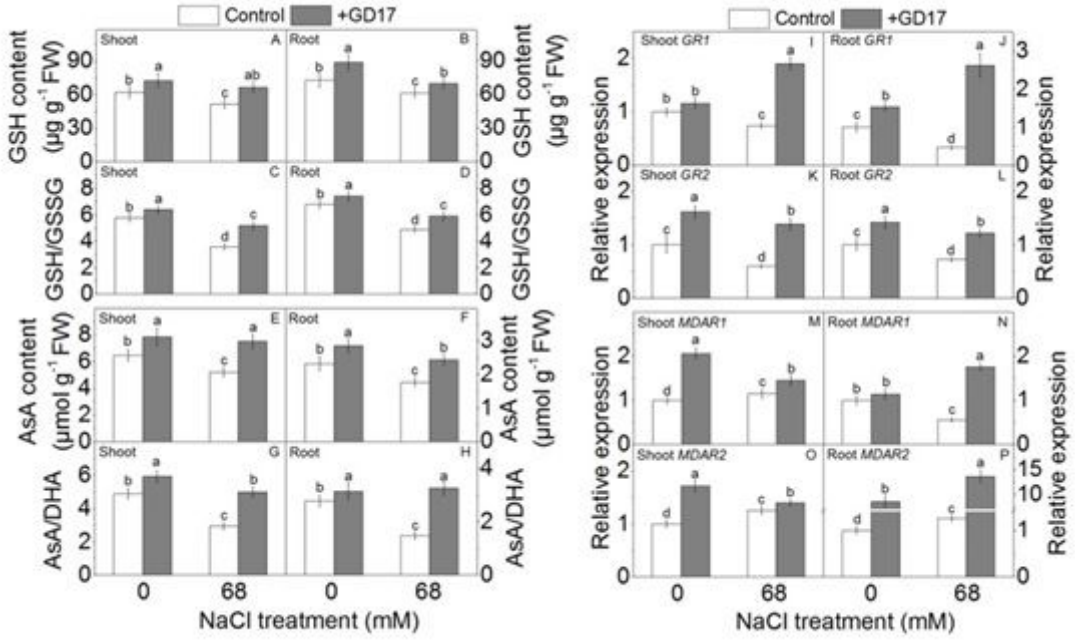

Figure 7

Effect of salt stress and/or GD17 inoculation on contents of reduced glutathione (GSH) (A, B), the ration of GSH/GSSG (oxidized form of glutathione) (C, D), contents of reduced ascorbic acid (AsA) (E, F), the ration of AsA/DHA (oxidized form of ascorbic acid) (G, H), and the relative expression of glutathione reductase-encoding gene GR1 (I, J) and GR2 (K, L), and monodehydroascorbate reductase-encoding gene MDAR1 (M, N) and MDAR2 (O, P). Eight-day-old plants (sequential 4 d germination, 4 d GD17 infection) were exposed to 68 mM NaCl for 48 h. The data were collected from three replicated experiments (n = 3) with 20 plants used in each batch of experiment, and represented as means ± SD. Bars with different lower-case letters indicate significant differences at $p < 0.05$

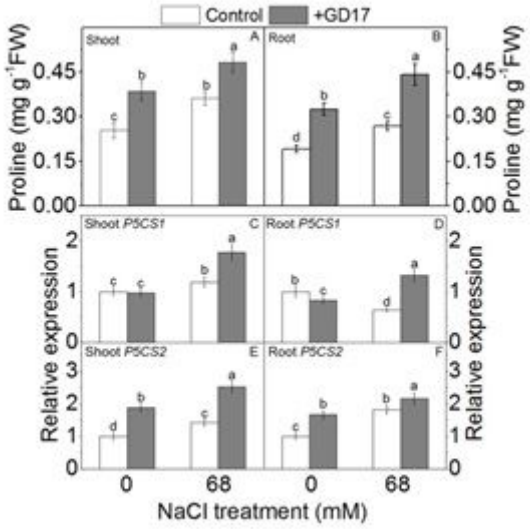

Figure 8

Effect of salt stress and/or GD17 inoculation on contents of prolne (A, B), and the relative expression of delta-l-pyrroline-5-carboxylate synthetase-encoding gene P5CS1 (C, D) and P5CS (E, F). Eight-day-old plants (sequential 4 d germination, 4 d GD17 infection) were exposed to 68 mM NaCl for 48 h. The data were collected from three replicated experiments (n = 3) with 20 plants used in each batch of experiment, and represented as means ± SD. Bars with different lower-case letters indicate significant differences at $p < 0.05$

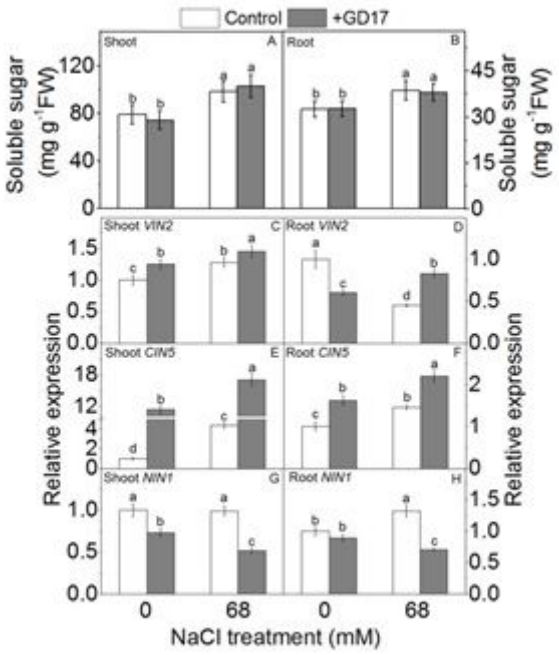

Figure 9

Effect of salt stress and/or GD17 inoculation on contents of soluble sugar (A, B), and the relative expression of vacuolar invertase-encoding VIN2 (C, D), cell wall invertase gene CIN5 (E, F) and neutral/alkaline invertase gene NIN1 (G, H). Eight-day-old plants (sequential 4 d germination, 4 d GD17 infection) were exposed to 68 mM NaCl for 48 h. The data were collected from three replicated experiments (n = 3) with 20 plants used in each batch of experiment, and represented as means ± SD. Bars with different lower-case letters indicate significant differences at $p < 0.05$

Supplementary Files

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