ORIGINAL PAPER

Overexpression of the rice gene *Lsi1* (low silicon gene 1) enhances plant-microbe interactions that result in improved chilling tolerance

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Abstract

Chilling is an environmental phenomenon that hampers the growth of plants. Related studies are primarily based on the aerial plant parts. In contrast, the belowground parts of plant that interact with the rhizosphere microbiome under low temperature stress have not been studied adequately. The overexpression of Low silicon gene 1 (*Lsi1*) in Dular rice (*Oryza sativa* L.) significantly enhances its concentration of proline compared with the wild type Dular. This overexpression maintains the osmotic balance of cells through an influx of Ca^{2+}, K^+, and H^+ and an efflux of Na^+. The deposition of calcium and activity of plasma membrane H^+-ATPase determined in the root tip was consistent with the results of ion flux obtained. In addition, high-throughput sequencing identified significant variation among 84 genera in different rhizocompartments, including the rhizosphere (R), rhizoplane (RS) and endosphere (N). The bacteria identified are associated with photosynthesis, energy metabolism, nitrogen fixation and defense, which increased significantly in the overexpressed plants. In contrast, the number of pathogenic microorganisms associated with the plant were significantly reduced. Our findings suggest that the stability of ion balance in the overexpressed plant roots affects the structure of microbial community in soil. In conclusion, the overexpression of *Lsi1* gene enhanced the cold tolerance of rice, and its underlying mechanisms were comprehensively analyzed, which can provide further evidence for its role in the stress tolerance of rice plants.

Introduction

In China, cold spells in the late spring, particularly in the month of April, causes damage to rice (*Oryza sativa*) seedlings that lead to reductions in yield. (Fang et al. 2017b)

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Novel methods, such as the development of cold tolerant rice varieties, are required to limit these losses. Many studies suggest that elements, such as silicon (Si), play a significant role in strengthening the defense mechanisms of the plant (Mitani et al. 2005, 2009). Silicon absorption in rice is controlled by the Low silicon gene 1 (*Lsi1*) that encodes a Nod26-like major intrinsic protein (NIP) subfamily (Ma et al. 2006). We unexpectedly found a feature of this overexpressed line that occurs when the temperature decrease to below 15°C. Moreover, our early studies confirmed that the overexpression of *Lsi1* gene in Dular rice increased the cold tolerance by mediating both the transcriptional and proteomic regulatory networks Fang et al. 2017a, Azeem et al. 2016). Nevertheless, the study of *Lsi1* gene in rice has focused on the aboveground plant parts, and to our knowledge, no research on the belowground parts has been reported. Therefore, it is crucial to reveal the response of root-associated microbes to cold in the rhizosphere.

Plants that grow in the soil are in contact with diverse microbial communities. There are many microbial communities in the rhizosphere area (Bulgarelli et al. 2013). In addition, the microbial community of a plant determines its health (Berendsen et al. 2012).

These studies unveil a new avenue toward plant microbial interactions, structure and the factors that affect them. However, no studies have been conducted to understand how the microbial community in rice responds to cold. Our early results indicated that the overexpression of *Lsi1* enhances cold tolerance in rice. In addition, previous studies also noted that the root ions in rice correspondingly change under low temperature stress, which mediates an increase in the cold tolerance of rice plants (Ma et al. 2015). Moreover, many studies have confirmed the close relationship between root ion transport and microbial metabolism (Zhang et al. 2017; Shabala et al. 2009).

We studied the connection between ion transport and the associated root-microbes under cold stress. First, we determined the content of proline (Pro) and silicon(Si) in the wild type (WT) and *Lsi1*-overexpression Dular (OS) roots. In addition, to further elucidate the balance of ions required to maintain the cell membrane permeability, we performed Non-invasive Micro-test Technology (NMT) to monitor the influx and efflux of the ions. We primarily focused on Ca^{2+}, K^+, Na^+ and H^+ in the OS roots in comparison with the WT. Moreover, we utilized the deep pyrosequencing of 16 S rDNA microbial genes to detect the operational taxonomic units (OTUs) that were related to three distinct compartments, including the rhizosphere (R), rhizoplane (RS), and endosphere (N). The large datasets from the WT and *Lsi1* rice were used to identify putative microbial interactions involved in the processes to increase cold tolerance. Therefore, we provide insights into the ionic balance and process of the assembly of root microbiome in response to cold.

Materials and methods

Research material

The study was conducted in the experimental field of the Agricultural Ecological Key Laboratory, Fujian, China. Based on the Coding sequence (CDS) of rice *Lsi1*, our research group designed the PCR primers to amplify rice Lsi1 and inserted it into a gene-overexpression vector namely Ubiquitin 1301 to construct the recombinant plasmid (Lsi1-Ubiquitin1301), and then transformed it into Agrobacterium EHA105; Using Agrobacterium-mediated genetic transformation system, we obtained the Dular's mutants with Lsi1 overexpression (Dular-OS).The rice plants were cultivated in pots. The WT (*Oryza sativa* L., accession: Dular) and OS

(cold tolerant) rice lines were used for all the experiments in this study (Azeem et al., 2016; Fang et al. 2017). Plump rice seeds were disinfected with a solution of 25% sodium hypochlorite (NaClO) for 15 min, washed with deionized water, and then soaked for 48 h for incubation at 28 °C in the dark and 85% relative humidity for 24 h. After germination, the seeds were uniformly planted in growing trays (upper surface diameter 13 cm; lower surface diameter 8 cm; height 7.5 cm) containing matrix soil at 85% relative humidity and 28 °C for 14 h with 20,000 lx light intensity, and then 22 °C in dark for 10 h. After the growth of 1.5 leaves, the planting conditions were adjusted to 15 °C for 14 h with 20,000 lx of light intensity, and then 10 °C in the dark for 10 h at 85% relative humidity. Moreover, normal plantings (the control group) were established at regular temperature (28 °C for 14 h with 20,000 lx of light intensity, and then 22 °C in dark for 10 h at 85% relative humidity). After 48 h of treatment, the samples were collected for subsequent analysis.

Determination of proline (pro) content

0.5 g rice roots were taken and placed in glass tubes, and then 5 mL 3% sulfosalicylic acid solution (3% (W/V) sulfosalicylic acid, 100% H_2O) was added to each tube respectively. Afterward, the tube was sealed with the bulb plug before initiation of t extraction in boiling water bath for 10 min. After it was cooled, the filtrate was filtered into clean glass tubes. Two ml extract was taken in another clean glass tube containing 2 ml glacial acetic acid and 2 ml 2.5% acidic ninhydrin reagent (2.5% (W/V) ninhydrin, 60% glacial acetic acid, 40% 6 M phosphoric acid), and then heated it in water bath for 30 min. After it was cooled, it was shaken for 30s after addition of 4mL toluene, and the components in the tube were let to sit for a sometime. The supernatant was shifted to 10mL centrifugal tube, and centrifuged at 3,000 rpm for 5 min. The supernatant was taken in a colorimetric cup. Toluene was used as a blank control, the absorbance value was measured at 520 nm, and the concentration was calculated.

Measurement of net Ca^{2+}, K^+, Na^+ and H^+ flux with the NMT system

The net fluxes of Ca^{2+}, K^+, Na^+ and H^+ were measured by Non-invasive Micro-test Technology (NMT-YG-100, Younger USA LLC, Amherst, MA 01002, Beijing, China). The influxes were measured with V2.0 (Younger USA LLC, Amherst, MA 01002, USA) Software (Zhao et al. 2015). This technique provides a high level of resolution and non-invasively method to measure different ions and small molecules in situ with working principle detailed in Li et al. (Li et al. 2010). The root of rice plant was calibrated with

measuring solution for 10 min, then transferred to a small petri dish (6 cm diameter) containing 10 ml of measuring solution. While, the roots were immobilized in petri dish containing measuring solution and microelectrode was vibrating between two positions i.e. 30 µm from the root surface and kept perpendicular axis to the root. The background was recorded by vibrating the electrode in measuring solution without containing roots, then the electrode tip was filled with a commercially available ionophore (Ca^{2+}, K^+, Na^+ and H^+). The measuring solution for Ca^{2+}, H^+ and K^+ contained a mixture 0.1mM $CaCl_2$, 0.1mM KCl and 0.3mM 2-(N-Morpholino) ethanesulfonic acid (MES), pH 6.0. The measuring solutions for Na^+ consisted of 0.1mM $CaCl_2$, 0.1mM KCl, 0.1mM NaCl and 0.3mM MES, pH 6.0.

Assay of plasma membrane H^+-ATPase activity

Plasma membrane of rice root was prepared according the method of Xu et al. (Xu et al. 2012) and was stored at -80 °C until analysis. Bradford method was used to determine the protein concentration (Bradford 1976). The H^+-ATPase activity was determined in a reaction volume of 0.5 mL that contained 30 mM BTP/MES, pH 6.5, 5 mM $MgSO_4$, 50 mM KCl and 4 mM Tris-ATP and plasma membrane. The reaction continued for 30 min at 30 °C and was stopped with 1 mL of stopping solution containing 2% (v/v) concentrated H_2SO_4, 5% (w/v) sodium dodecyl sulphate 0.7% (w/v) sodium molybdate, followed by 50 mL of 10% (w/v) ascorbic acid. Absorbance at 700 nm was measured with a spectrophotometer.

Calcium concentration

The selected root tips were washed with ddH_2O before the concentration of calcium was measured as described by Qin (Qin et al. 2017). Briefly, the root tip samples were incubated in a solution that contained 0.2 mM $CaCl_2$, 50 mM sorbitol, 20 µM Fluo-3 AM (Beyotime Biotechnology, Haimen, China), and 1% (v/v) dimethyl sulfoxide. The samples were wrapped in foil and incubated at 4 °C for two hours and washed twice with a solution of 0.2 mM $CaCl_2$ and incubated at room temperature for two hours. The sample slides were then observed under 10 X fluorescence microscopy.

Sampling and collection of the rhizocompartments

The roots were collected from rice plants, and the soil was shaken off the roots to leave 1 mm of soil remaining around the roots. The 1 mm of soil was washed off in phosphate-buffer saline (PBS) and kept as the rhizosphere soil. The clean roots were washed twice to remove remaining soil and placed in clean PBS in a 50 ml falcon tube. The rhizoplane

microbes were extracted by sonicating the roots after the rhizosphere compartment had been removed. The sonicated roots were then placed in a new, clean falcon tube and sonicated twice more. The PBS in tube was discarded and refilled with clean PBS. These roots were then kept as the endospheric sample (Edwards et al. 2015).

DNA extraction, PCR amplification, PCR purification and pyrosequencing

The total DNA of different rhizocompartments, including the rhizosphere, rhizoplane and endosphere soil, was extracted using a Soil Gen DNA Kit (CWBIO, Beijing, China) following the manufacturer's instructions. A Nanodrop 2000 C spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to determine the concentration of DNA. The DNA was then diluted to 1 ng/ul using sterile water. The 515 F (GTGCCAGCMGCCGCGGTAA) and 806 R (GGAC-TACHVGGGTWTCTAAT) primers were used to amplify the bacterial 16 S rDNA V4 fragments (Osborne et al. 2018, Xu et al. 2017). PCR reactions were conducted, and the PCR products were monitored using 2% agarose gel electrophoresis. The samples that contained bands between 400 and 450 bp were selected for further analysis. The sequencing libraries were obtained using a TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. Pyrosequencing was performed on an Illumina HiSeq 2500 platform, and 250 bp paired-end reads were generated.

Operational taxonomic unit (OTU) analysis and statistical analysis

The barcode and primer sequences were removed, and the raw tags of origin tag data were spliced using FLASH (V1.2.7). After the chimeras were removed, the sequences obtained were denoted as effective tags and used to perform OTU cluster and species annotation. Sequences that had ≥97% similarity was assigned to the same OTU using Uparse software v7.0.1001 (Edgar 2013).

The abundance of OTUs in each sample was homogenized, and alpha and beta diversity analyses were performed. The Observed-species and the community richness Chao1, ACE, Shannon, Goods-coverage and PD_whole_tree indices were calculated using QIIME software (Version 1.8). The R software (Version 2.15.3) was used to draw distribution curves, and the differences among groups of alpha diversity indices were analyzed. The function of microorganisms was predicted by the FAPROTAX database and Python 2.7 software. A principal coordinate analysis (PCoA) was conducted using the R language program. A one-way analysis of variance (ANOVA) followed by a Tukey's test (P≤0.05)
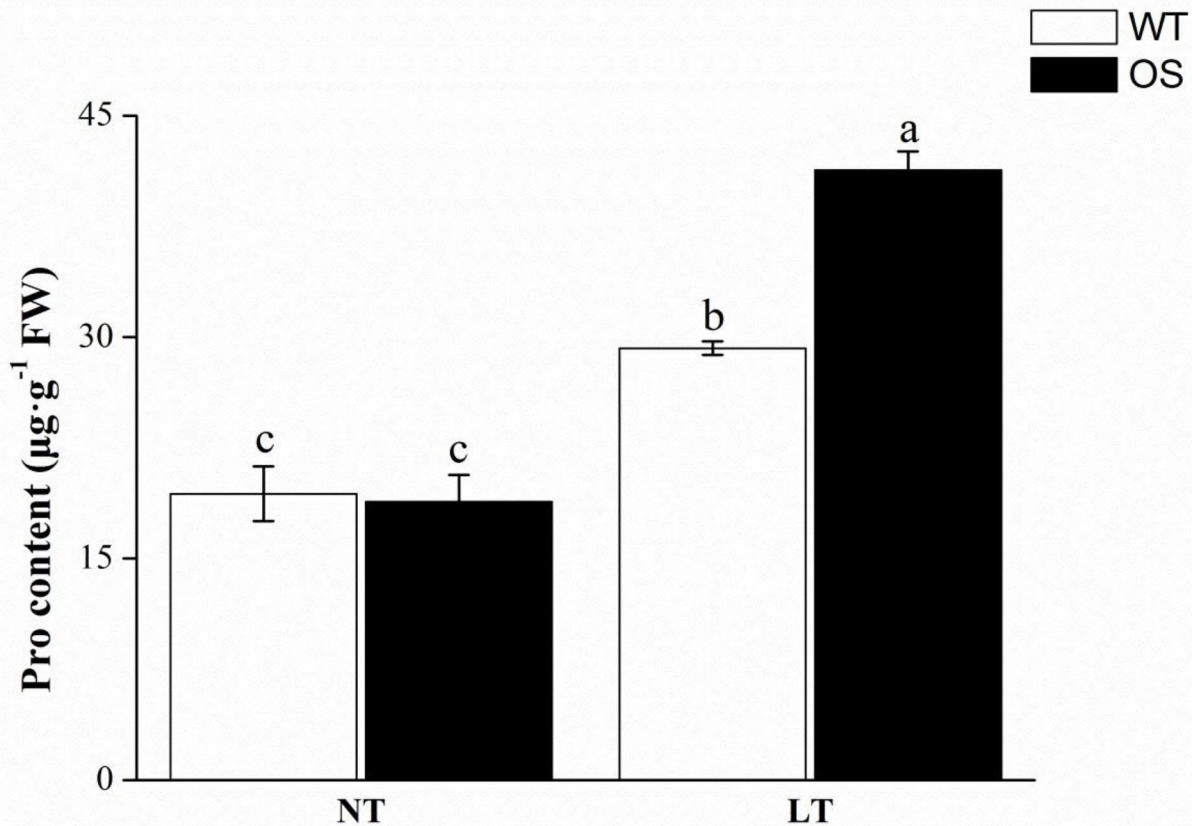
Fig. 1 The proline content in OS and WT roots. WT: Wild type Dular. OS: *Lsi1*-overexpression Dular. NT: Normal temperature. LT: Low Tempera-ture. The small letter indicates significance level were between the 4 treatments (two genotypes and two stress conditions). Columns with different letters are statistically significantly different (LSD test, P < 0.05). Error bars are standard error (±SE) of three biological replications

was performed for multiple comparison using DPS software version 7.51 (Enfield, UK).

Statistical analysis

All the data presented in the paper are the means of three biological replicates. The differences among the treatments were calculated and statistically analyzed using the variance and the least-significant-difference multiple-range test (LSD, P < 0.05). The Statistical package for OriginPro 8.0 and the Data Processing System (DPS) version 7.05 were used for the statistical analysis.

Results

The OS and WT under chilling stress

To gain insight into the ability of rice plants to eliminate reactive oxygen species (ROS), we measured the contents of Pro and Si in both the WT and OS plants at low and normal temperatures. The content of Si in OS was significantly increase compare to that in WT both in low and normal temperature (Figure S1). There was no significant difference in the content of Pro between the OS (18.85 µg·g^{-1}·FW) and WT (19.39 µg·g^{-1}·FW) at normal temperatures (Fig. 1). The contents of Pro in the OS and WT significantly increased at low temperature compared with normal temperature, and the content of Pro in the OS (41.32 µg·g^{-1}·FW) was significantly higher than that of the WT (29.25 µg·g^{-1}·FW), which increased by 41.26% at low temperature. In addition, the content of Pro and Si increased in the OS plants under low temperature stress, which protected the cell membrane system from damage and enhanced the cold resistance of the plants.

The fluxes of Ca^{2+}, K$^+$, Na$^+$ and H$^+$ in the WT and OS

To estimate the flow of some important ions involved in the response to cold, maintenance of the osmotic balance
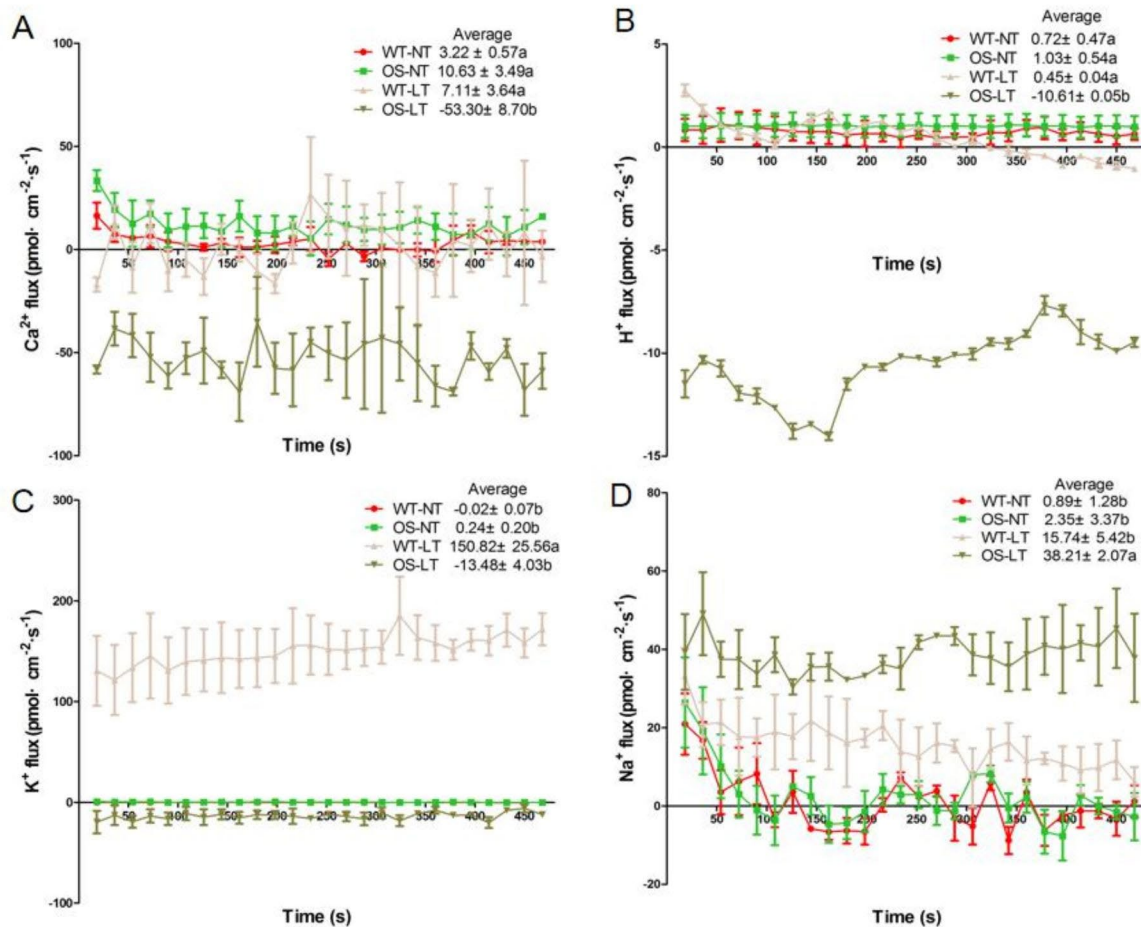
Fig. 2 The influx and efflux of Ca^{2+} (A), H^+ (B), K^+ (C) and Na^+ (D) in WT and OS. NT: Normal temperature. LT: Low Temperature. Error bars are standard error ($\pm$ SE) of three biological replications

and defense signaling in roots, we used NMT technology. There was no significant difference in the concentrations of Ca^{2+}, K^+, Na^+ and H^+ between the WT and OS plants at normal temperature, and a small amount was excreted. There were significant differences observed in the fluxes of ions between the WT and OS (Figs. 2 and 3). The flow rate of Ca^{2+} revealed that the roots of the OS plants assimilated higher amounts of Ca^{2+} compared with the WT. In addition, the roots of WT plants occasionally absorbed and released Ca^{2+}. Furthermore, the OS plants absorbed Ca^{2+} as a whole, and the rate reached 54.31 pmol·cm^{-2}·s^{-1}. However, the WT plants showed an efflux of Ca^{2+} as a whole, and the rate reached 0.92 pmol·cm^{-2}·s^{-1}. In addition, the OS roots significantly absorbed K^+, and the rate reached 12.78 pmol·cm^{-2}·s^{-1}. However, the roots of the WT plants emitted a larger quantity of K^+, and the rate reached 157.02 pmol·cm^{-2}·s^{-1}. The flow rate of Na^+ showed that the roots of the OS plants emitted more Na^+, and the rate reached 43.02 pmol·cm^{-2}·s^{-1}, which was significantly different

from the efflux in WT, which was 14.04 pmol·cm^{-2}·s^{-1}. Similarly, there was a distinct difference in the efflux of H^+ ions between the OS (10.21 pmol·cm^{-2}·s^{-1}) and the WT (0.09 pmol·cm^{-2}·s^{-1}). These results indicate that the OS plants absorbed Ca^{2+}, K^+ and H^+ under low temperature stress, while the efflux of Na^+ was observed, which maintains the stability of cell membrane function, and enhances the cold resistance of the plants.

Measurement of calcium deposition and H^+-ATPase activity

We identified that many Ca^{2+} ions flowed in both genotypes under low and normal temperature. To further confirm this finding, we measured the deposition of Ca^{2+} in the root tips of WT and OS. In addition, we found that more Ca^{2+} was deposited in the OS compared with the WT under chilling stress, thus, indicating the involvement of Ca^{2+} in the cold response, while it is no difference in normal temperature
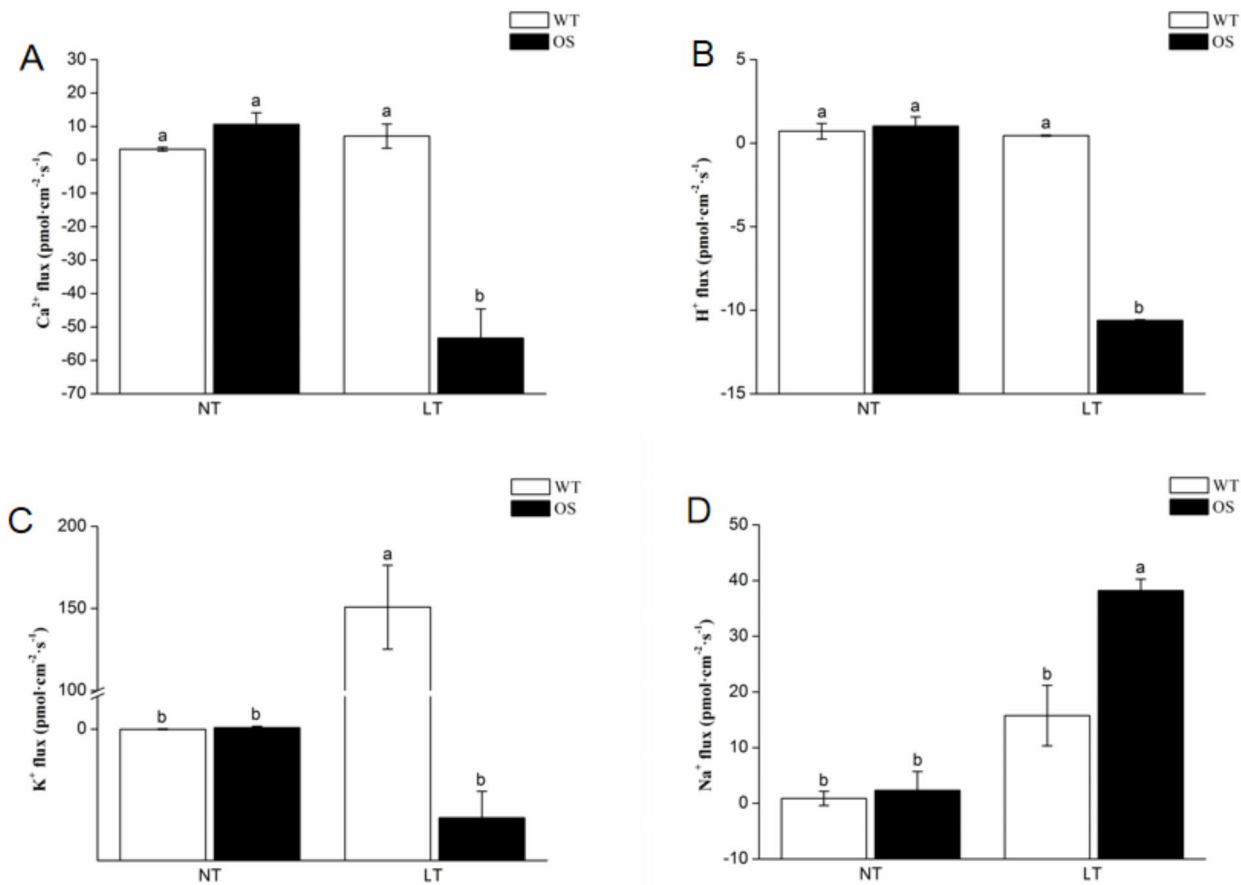
Fig. 3 The average influx and efflux rates of Ca^{2+} (A), H^+ (B), K^+ (C)and Na^+ (D)in WT and OS. NT: Normal temperature. LT: Low Temperature. Columns with different letters are statistically significantly different (LSD test, $P < 0.05$). Error bars are standard error ($\pm$ SE) of three biological replications

(Fig. 4 A). The results of ion absorption in this study also indicated that OS plants could absorb large amounts of Ca^{2+} under low temperature stress. The root calcium deposition experiment confirmed that the calcium content of OS roots was significantly higher than that of the WT under low temperature. Moreover, the H^+-ATPase activity was also measured in the roots of both genotypes. The OS exhibited higher H^+-ATPase activity than the WT under low temperature, which increases the energy level for the influx and efflux of ions in the OS (Fig. 4B).

OTU cluster and species annotation

The soil bacterial community related to the defense of the WT and OS against cold was identified by utilizing deep 16s rDNA pyrosequencing. Across all the soil samples, 1,457,835 effective tags that contained species annotation were identified, with an average of 76,606 effective tags per sample. Among the total effective tags, 40,400 singletons that accounted for 2.9% of total tags were removed before

further analysis (Figure S2). Rarefaction curves demonstrated the sequencing depth, and the number of observed species numbers is shown in Figure S3. At a 97% sequence similarity cut-off, we obtained a sum of 54,655 OTUs across the 18 samples. The OTUs numbers in the WT R, WT RS, WT N, OS R, OS RS and OS N plots were 3,132, 3,023, 2,905, 3,535, 3,411 and 2,212, respectively. On average, we were able to classify approximately 99.4%, 97.9%, 94.3%, 85.3%, 62.8% and 23.2% of effective sequences at the phylum, class, order, family, and genus and species level, respectively.

Alpha diversity indices

The alpha diversity was determined to further investigate the diversity of species within a sample. The Chao 1 and abundance-based coverage estimator ACE indices were calculated to estimate the abundance of each sample. Both the Shannon and Simpson indices were determined to estimate the diversity within each individual sample. In addition, the
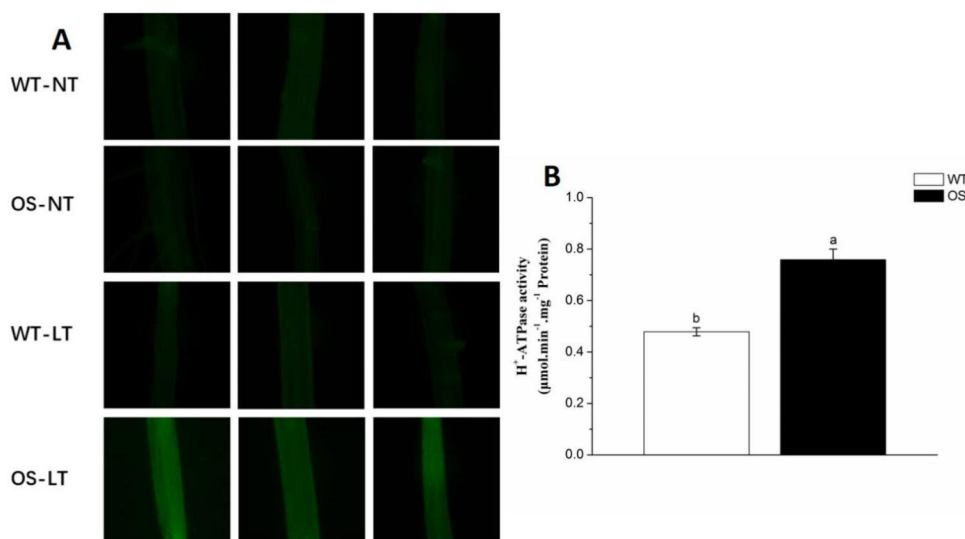
Fig. 4 The Calcium deposits (A) and H⁺-ATPase (B) activity in the root at low temperature. Columns with different letters are statistically significantly different (LSD test, P < 0.05). Error bars are standard error (± SE) of three biological replications

Table 1 Calculations of observed species, richness and diversity in different soil samples

Treatments	Observed species	*Shannon*	*Simpson*	*Chao 1*	*ACE*
WT R	2686.33b	8.46b	0.992a	3058.32b	3126.70bc
WT RS	2604.67b	8.45b	0.991a	2966.30b	3038.65b
WT N	2483.00b	7.85c	0.982b	2827.76b	2900.83b
OS R	3116.33a	9.01a	0.994a	3485.23a	3536.36a
OS RS	2976.33a	8.67b	0.992a	3329.85a	3400.02ab
OS N	1873.67c	6.33d	0.934c	2222.85c	2290.70c

Note: WT and OS represent the wild type and *Lsi1*-Overexpression Dular. R、RS and N represent rhizosphere, rhizoplane and endosphere, respectively. Whereas, "a", "b", "c" and "d" shows significance level at P ≤ 0.05. Different letters in columns show significant differences determined by Tukey's test (P ≤ 0.05, n = 3)

bacterial community analysis showed that a significantly higher bacterial diversity was observed in OS R and OS RS than WT R and WT RS. In contrast, less bacterial diversity was observed in the OS N than WT N (P ≤ 0.05). In addition, there were no significant differences in the bacterial species identified and the Shannon, Simpson, Chao 1 and ACE diversity indices between WT R and WT RS (P > 0.05). The richness indices, including the Shannon, Simpson, Chao 1 and ACE indices, in OS N were lower than those of the other treatments. Both the species observed and the Shannon, Simpson, Chao 1 and ACE diversity indices were significantly reduced in the change from OS R to OS N (P ≤ 0.05). However, only the Shannon and Simpson diversity indices were significantly reduced from WT R to WT N (Table 1).

Beta diversity indices

The beta diversity indices were determined by the differences in bacterial community composition and structure between the samples. In this study, the weighted UniFrac distance between the WT R and WT RS was a minimum of 0.074, but it was 0.151 in the OS R and OS RS. This indicated that the soil bacterial community between WT R and WT RS was more similar than that of OS R and OS RS. The weighted UniFrac distances between OS N vs. OS R (0.322) and OS N vs. OS RS (0.290) were greater than those of the WT N vs. WT R (0.268) and WT N vs. WT RS (0.239). This indicated that the soil bacterial community from the R to N in OS than WT had a greater difference. The weighted UniFrac distances between OS R vs. WT R, OS RS vs WT RS and OS N vs. WT N were 0.093, 0.108 and 0.231, respectively. This could explain why the highest difference in the soil bacterial community was observed in N compared with the other treatments (R and RS) as shown in Figure S4.

PCoA analysis

A PCoA was performed based on the weighted UniFrac distance. In addition, the PCoA analysis identified distinct differences in the soil bacterial community structure between different treatments. Nevertheless, the first two components (PC1 and PC2) of PCoA explained 60.17% and 18.34% of the total bacterial community variation, respectively, as shown in Fig. 5. Furthermore, the PCoA analysis showed that the bacterial communities of WT N and OS N were far from the other groups, and OS N was the most diverse among all the groups.
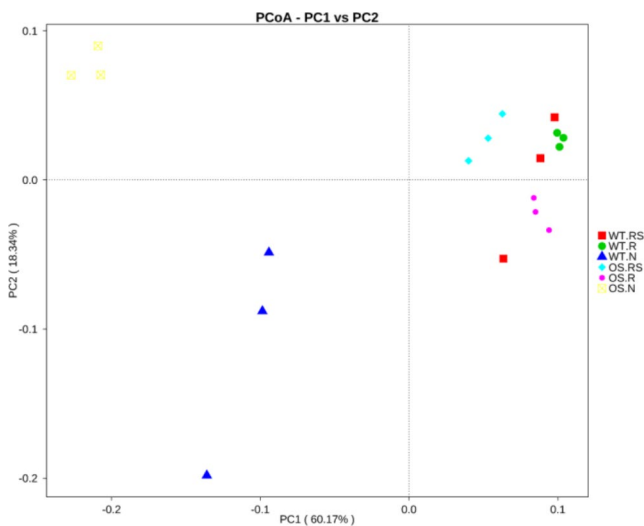
Fig. 5 Principal coordinate analysis (PCoA) of bacterial communities based on weighted Unifrac algorithm for different soil samples. R: Rhizosphere. RS: Rhizoplane. N:Endosphere

Analyses of bacterial communities

The cold significantly changed the bacterial communities in the rhizosphere compartments of OS and WT. We identified 52 phyla. However, more than 95% of the OTUs were members of 10 phyla, including *Proteobacteria, Firmicutes, Bacteroidetes, Cyanobacteria, Acidobacteria, Chloroflexi, Verrucomicrobia, Actinobacteria, Planctomycetes* and *Gemmatimonadetes*. The relative abundance of these phyla is shown in Figure S5. There were significantly fewer members of *Proteobacteria* in the WT N than in R and RS, but there were no significant differences identified between R and RS. In contrast, the number of *Proteobacteria* in the OS was higher in N and decreased significantly from RS to R. The numbers of *Firmicutes* and *Cyanobacteria* in the WT increased significantly from R to N. Nevertheless, the *Firmicutes* in the OS decreased significantly from R to N, while the *Cyanobacteria* increased from R to N as shown in Table S1.

Venn diagram of the genera identified in the OS compared with the WT

We not only identified 84 bacterial genera from R, RS and N but also used a Venn diagram to detect the exclusive and shared genera between six different soil samples (Fig. 6). The exclusive genera were 20, 18 and 30 in R, RS and N respectively. There were eight, one and four genera in common in R and RS, RS and N, R and N respectively, and three genera in common across R, RS and N.

Analysis of the dominant genera

In this study, 84 bacterial genera that were identified in six different treatments are shown in the heatmap in Fig. 7. We classified the 84 genera identified into 10 functional categories, including denitrification (7.14%), defense (5.95%), azotification (8.33%), photosynthesis (4.76%), chemotrophy (11.9), energy (1.19%), biodegradation (9.52%), carbon metabolism (4.76%), pathogenicity (26.19%) and unknown (20.24%). The distribution of microbial communities in R, RS and N differed significantly (Fig. 8 and Table S2).

The microorganisms for denitrification and biodegradation accumulated in WT N. In contrast, the microorganisms related to azotification and energy accumulated in OS N, while the microorganisms for defense and photosynthesis accumulated in OS R, RS and N. Moreover, the pathogenic microorganisms accumulated in WT RS and N. These results indicate that rice plants have a significant effect on shaping the composition and structure of microbial communities in the root environment. In contrast, the results of microbial community in the root environment also affect the growth and development of plants. The OS plants can select many beneficial microorganisms, such as those involved in defense, azotification, photosynthesis and energy, and block the invasion of harmful microorganisms under low temperature stress. These results indicate that rice plants have a significant effect on shaping the composition and structure of microbial communities in the root environment. Moreover, the results of microbial community in the root environment also affect the growth and development of plants.

The OS plants absorb Ca^{2+}, K^+, H^+ and expel Na^+, thus, better equipping the OS to maintain the stability of membrane function. Ion transport in the root system of rice is not only an important way to regulate the plant response to external low temperature stress but also an essential carrier material to mediate material exchange and energy flow between the root system and soil (Fig. 9). In brief, it can be considered as one of key factors that contributes to the microecological characteristics in different root environments of plants, and it could also be the basis for the establishment of plant-microorganism interactions.

Discussion

The increase in proline content enhanced the cold resistance of rice

Cold adversely effects the growth of plants and causes physiological dysfunction (Lukatkin et al. 2012; Kroemer et al. 2004). Plants may develop cold tolerance to some extent during the initial responses to cold (Fowler and Thomashow
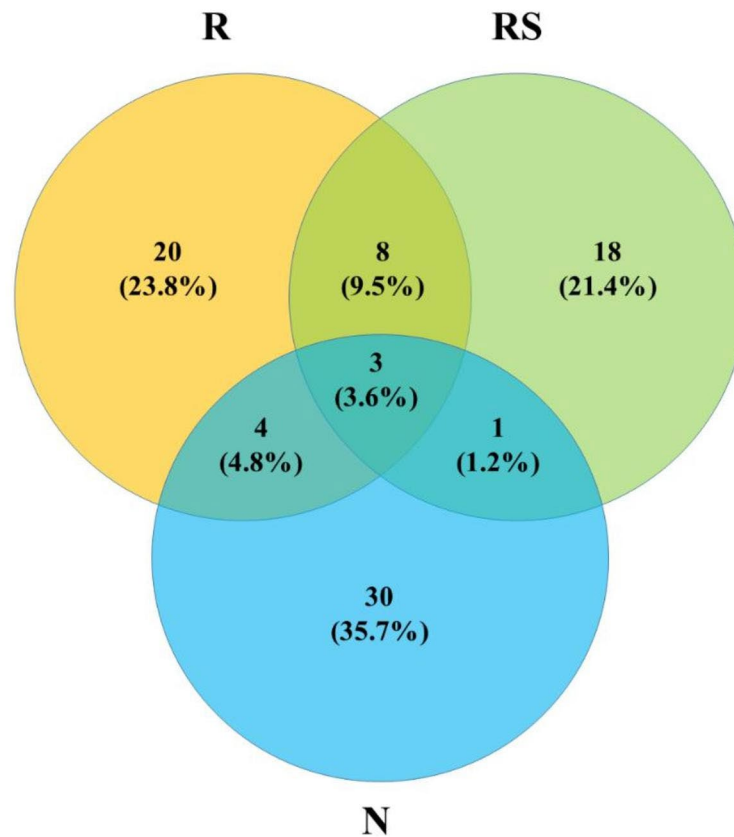
Fig. 6 Venn diagram analysis of identified exclusive and shared genera between the R, RS and N of OS compared with WT. R: Rhizosphere. RS: Rhizoplane. N: Endosphere

2002). However, continued exposure to cold stress is disastrous for plant life. Silicon has been reported to help plants resist abiotic stresses, such as cold, drought and metal toxicities (Muneer et al. 2017). Previous studies have found that the overexpression of *Lsi1* in rice resulted in significant resistance against cold Fang et al. 2017a, Azeem et al., 2016). Under stress, a large amount of ROS are produced in plants. ROS serve as important signaling molecules in plant growth and development and the responses to various stresses (Apel and Hirt 2004). When low temperature stress occurs, the ROS in rice starts to accumulate in large amounts, and the rice antioxidant system begins to function. Our previous study showed that the OS plants produce large amounts of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) (Azeem et al. 2016). It has been reported that Pro plays roles in protecting enzymes from denaturation and helps to stabilize the machinery of protein synthesis, regulate cytosolic acidity, enhance the capacity to bind water, and act as a reservoir of carbon and nitrogen (Liu et al. 2007). In this study, the Pro content of the OS plants was significantly higher than that of the WT plants, which protected the roots of rice system. In addition, it enhanced the activity of rice root protective enzymes, stabilized the

function of the cell membrane, improved the storage of nutrients in the root system, effectively eliminated the damage of ROS to the plant, and improved the cold resistance of the plants.

The OS root ion dynamics and maintenance of the ion balance in response to cold

The cold severely damages the plant cell membrane, and as a result, the lipid membrane permeability increases, and solutes leak out of the cells, which causes water loss. This deleteriously affects the balance of exchange of plant cell material with the surrounding environment and thus, destroys it (Lyons and Raison 1970). Therefore, it is important to know how OS maintains the activity of plasma membrane proton pump and regulates the intracellular influx and efflux of important cold responsive ions, such as Na^+, K^+, Ca^{2+}, and H^+. Additionally, the balance of intracellular potassium and sodium depends on the "sodium potassium pump" on the cell membrane, namely the Na^+-K^+ATP enzyme. Moreover, an increase in the concentration of extracellular Ca^{2+} can inhibit the efflux of K^+, reduce the intracellular Na^+ concentration, and improve the activity
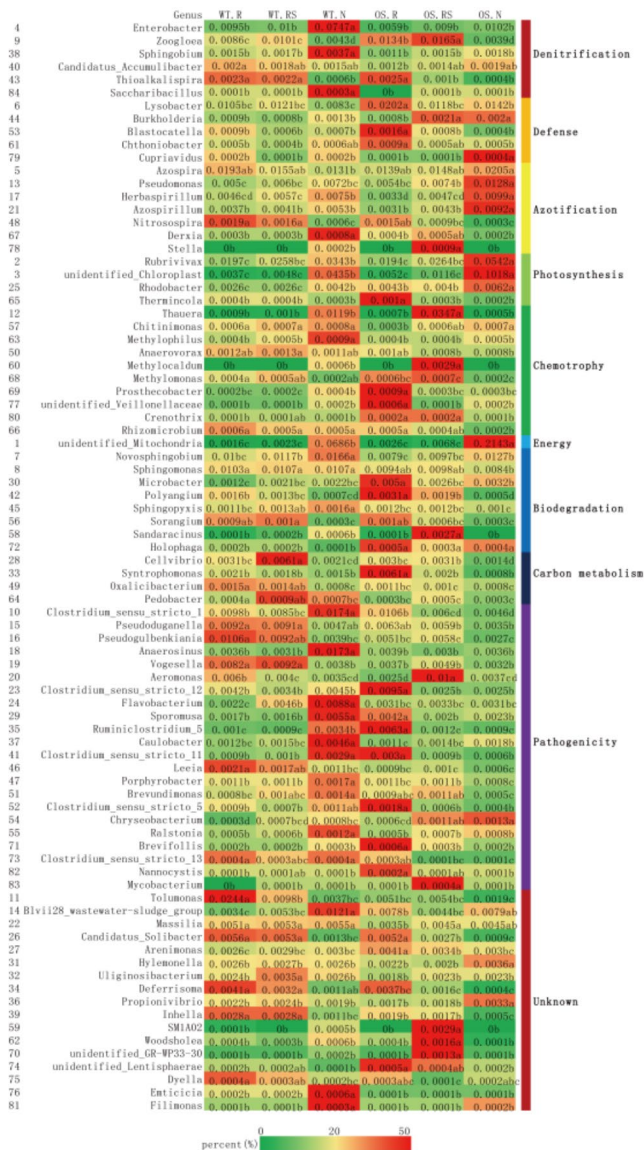
Fig. 7 Heat map analysis of the 84 genera in six different treatments. Columns with different letters are statistically significantly different (LSD test, P < 0.05). Error bars are standard error (±SE) of three biological replications. R: Rhizosphere. RS: Rhizoplane. N: Endosphere

of H$^+$-ATPase on vacuolar membranes. In addition, studies have confirmed that the proton pump plays an important role in maintaining the K$^+$/Na$^+$ balance in plants (Niu et al. 1995). Higher proton pump activity can provide an H$^+$ electrochemical potential gradient for the reversed transport of Na$^+$/H$^+$, reduce the depolarization of plasma membrane, and limit the efflux of K$^+$ (Chen et al. 2010; Maathuis 2006). The results obtained during this study led us to hypothesize that under cold stress, OS maintains the mechanism of balancing the intracellular K$^+$/Na$^+$ by Na$^+$ efflux, which helps to avoid the excessive accumulation of Na$^+$ in the cytoplasm. Another way could be owing to the loss of inhibition of K$^+$. Ca^{2+} plays an important role in the process of plant

resistance, such as stress signal transduction, metabolic control and the protection of film stability (Polisensky and Braam 1996, Knight et al., 1997). The results of this study show that under cold stress, the Ca^{2+} in the OS rice roots tended to flow internally, which resulted in an increase in free Ca^{2+} in the roots. Therefore, it prevents the depolarization of plasma membrane H$^+$-ATPase and limits the K$^+$ efflux to maintain the balance of the intracellular Na$^+$/K$^+$. Nevertheless, the calcium deposits and H$^+$-ATPase activity in the OS root were significantly higher than those in the WT, which confirms the ion dynamics data. Moreover, we found that the plasma membrane H$^+$-ATPase activity was significantly higher than that of the WT, which indicates that the plasma membrane H + pump activity of the OS plants was significantly higher than that of the WT plants under low temperature stress. However, under chilling stress, the OS plants promote Na$^+$ efflux and increase a small amount of K$^+$ by increasing the activity of plasma membrane H$^+$ pump, which negates the loss of excessive K$^+$. This shows that the proton concentration gradient involves Na$^+$ after improving the activity of plasma membrane H$^+$ pump. Furthermore, the H$^+$ transmembrane reverses transport or can also reduce the depolarization of plasma membrane and thus, limit K$^+$ outflow. Numerous studies have indicated that exogenous Ca^{2+} can regulate the Na$^+$/H$^+$ reverse transport system of plant cells to maintain the intracellular K$^+$/Na$^+$ balance (Sun et al. 2010). The results suggest that that absorption of more Na + inhibited Ca^{2+} in the WT. Membranes with an increasing ratio of Na$^+$/Ca^{2+} ions lead to the damage of membrane integrity and functions, ultimately increasing the cell membrane permeability.

In brief, these results further confirm that *Lsi1* mediates ion transport and enhances the chilling tolerance of plants.

The response of rice microbial communities to cold

The formation of cold resistance in plants is a complex biological process. Multiple factors interact and mutually influence the plant and its underground soil microbiome. Exploring the process of plant-microbe interactions and its regulatory mechanisms play an important role in studying the interrelationship between soil microbes and plant growth and development (Bais et al. 2004). In addition, studies have shown that different plants exhibit unique and representative rhizosphere microbial communities (Berendsen et al. 2012). The difference in the ion absorption of root leads to differences in plant growth and development, which, in turn, affect the composition of microbial community.

In Arabidopsis, characterization of the diversity of core root microbiome confirmed that the microbial population in the endosphere is less than those in the rhizosphere and rhizoplane microbiomes (Haas et al. 2011). The OS has a
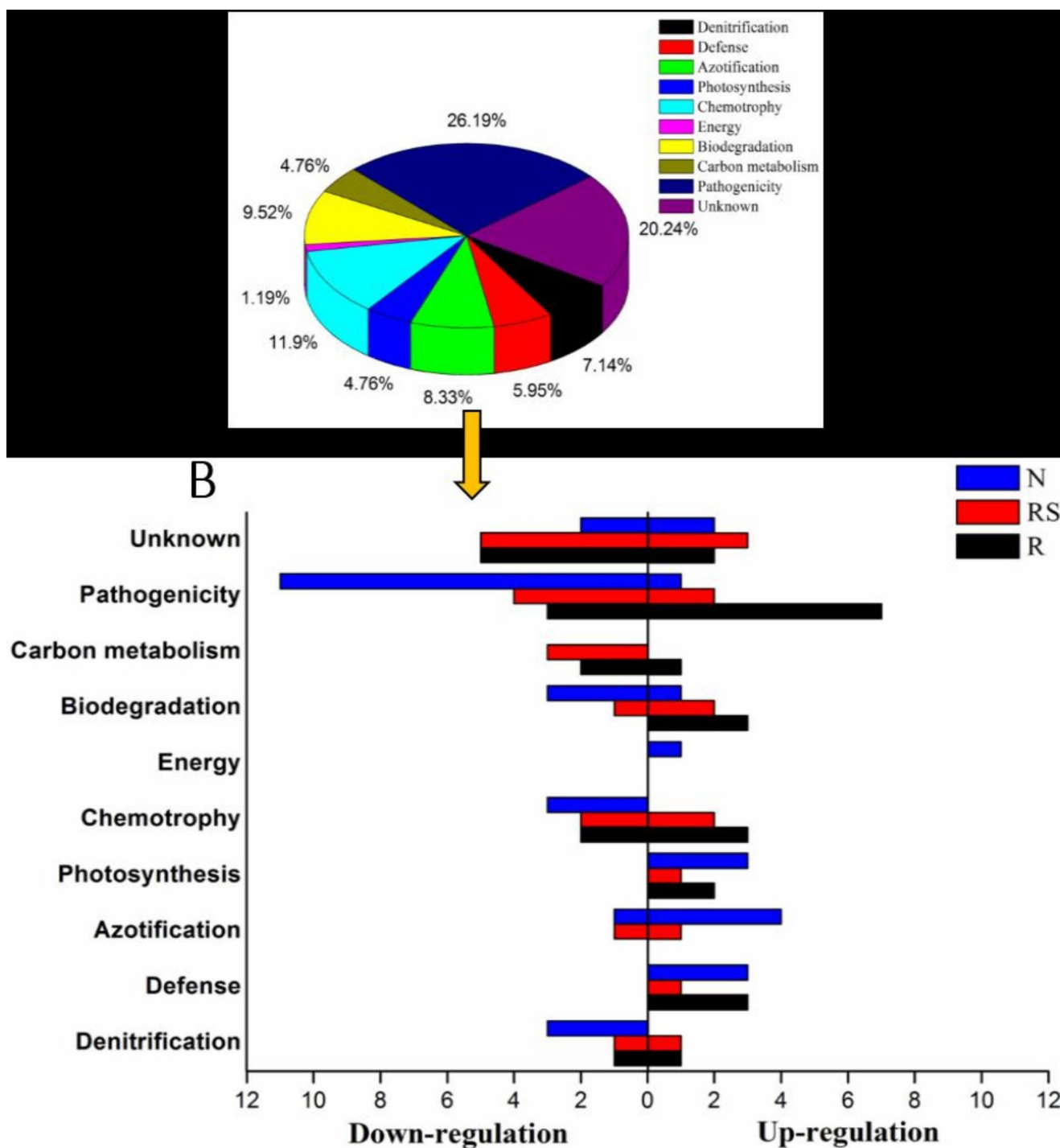
Fig. 8 Functional classification of identified 84 bacterial genera (A). The expression of identified 84 bacterial genera in six different soil samples (B)

higher abundance of *Proteobacteria* in N than in R and RS, while its abundance in the WT was higher in R and RS and less in N. This could be one of the reasons that helped the OS to develop resistance against the cold. Furthermore, a PCoA analysis showed a distinct separation among all the soil samples and particularly those between the WT N and

OS N samples. This suggests that there is a strong relationship between plant performance and the soil microbial community. In addition, the OS N had significantly higher microbial diversity than the WT N. A higher microbial diversity is thought to be responsible for the development and maintenance of disease suppressive soils (Qiu et al.
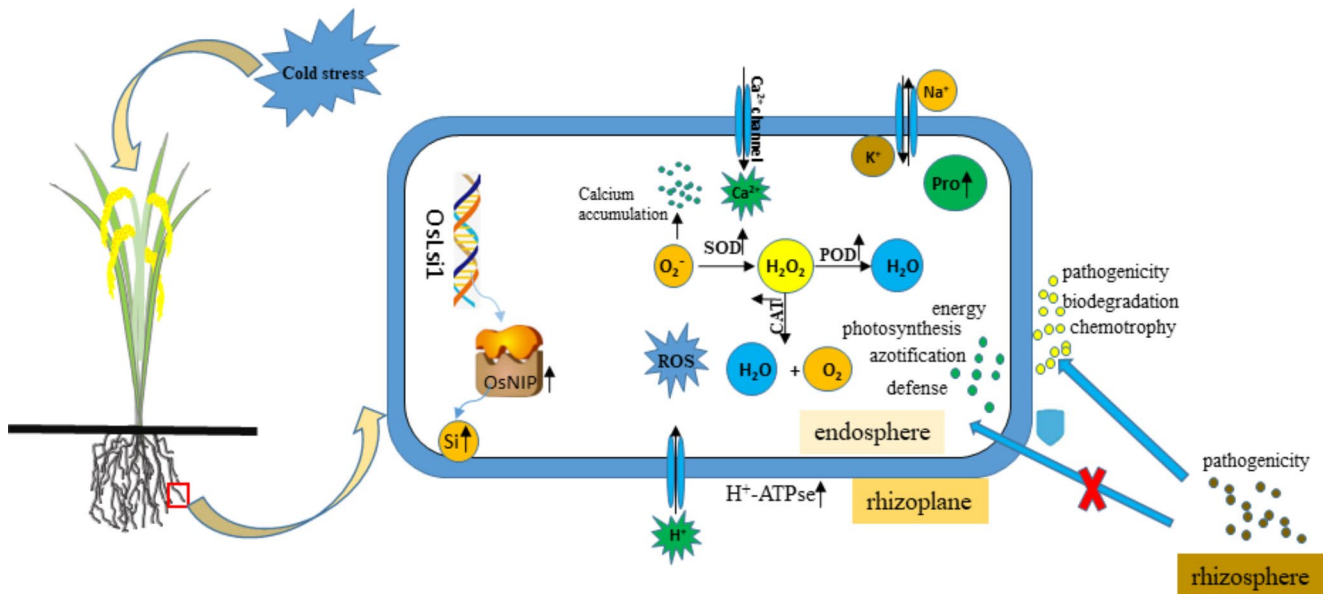
Plant-Microbe interaction

Fig. 9 Schematic pathway is depicting the overexpression of rice gene *Lsi1* mediate the plant- microbe interaction resisting to chilling

2012; Yuan et al. 2014, Bruggen and Semenov, 2000). The OS rice plants had significant effects on the microbial community composition and structure in different soil layers under low temperatures.

Moreover, we found significant variability in the patterns of relative abundance of genera between the soil compartments of WT and OS. The 84 bacterial genera identified have been classified in 10 functional classes. Among these soilborne microorganisms, some groups of microorganisms are known to benefit the plants. Examples include supplying key plant nutrients, such as nitrogen and phosphorus, protecting against soilborne diseases, and promoting resistance to abiotic stress (Berendsen et al. 2012). These beneficial microorganisms are collectively referred to as plant growth promoting rhizobacteria (PGPR). *Clostridium* is a pathogenic bacterium that produces exotoxins and invasive enzymes (Kasai et al. 2005). *Flavobacterium* is a genus of Gram-negative, non-motile and motile, rod-shaped bacteria that consists of 130 species that have been recognized as causing disease (Bernardet et al. 1996). The OS N accommodated a lower abundance of pathogenic bacteria, and most of them were found in the OS R compared to the WT. As a result of that, the OS plants effectively avoided the invasion of pathogenic bacteria and ensured the stability of organism functions. *Lysobacter* can not only colonize the rhizosphere of many plants but also secrete a variety of antibiotics (Md Tofazzal et al., 2005) and bioactive substances (Folman et al. 2003) that inhibit the growth of bacteria and control plant diseases (Jochum et al. 2006). It led to the large presence of pathogenic bacteria in OS R and RS. The bacteria in OS N

that are associated with energy and azotification increase the nutrients and enhance the circulation of energy in rice roots.

In brief, OS N accommodates a lower abundance of pathogenic bacteria, and most were found in OS R compared with the WT. In contrast, OS N has higher proportions of microorganisms involved in photosynthesis, energy, azotification and defense than RS and R, which ultimately enhances its ability to tolerate cold. These results indicate that rice plants have a significant ability to shape the composition and structure of microbial communities in the root environment. In contrast, the results of microbial community in the root environment also affect the growth and development of plants. Based on microbial studies of R and N, it has been proposed that the plants might accommodate the microbes in two steps. The first is a general recruitment around the root, and the second step is on the genetic basis (species specific) entry of microbes inside the root. We suggest that the rhizoplane plays a critical role in regulating the transport of microbes that have adhered to the rhizosphere. Few microbes can bind to the rhizoplane, and only a fraction of them can enter and proliferate in the endosphere (Caporaso et al. 2010). Each of these events involves complex molecular signals from plant, possibly the root exudates, cell membrane proteins, plant metabolites or cell wall components (Lundberg et al. 2012).

Our study indicates that changes in the microbial community structure on the roots also have important effects on plant root absorption, energy flow, and information transfer, which, in turn, affect plant growth and development. However, the rhizocompartments showed the largest

variation during the comparison of OS and WT, suggesting the involvement of genotypic factors for the recruitment of microbes. In summary, the shift in microbial diversity among each compartment indicates the two to three selective steps from the root to interior and response to multiple signals from the plant, which coordinate the assembly of the root microbiome.

Conclusion

Our results suggest that the *Lsi1* gene plays a pivotal role in the response to chilling tolerance. The NMT analysis showed that the OS absorbed a significant amount of Ca^{2+} and H^+ and a minute amount of K^+. However, there was a large efflux of Na^+ to maintain the balance of cell osmotic pressure. The calcium deposition method was used to locate the calcium content in the root of OS, and it was significantly higher than that of the WT, which confirmed our root ion absorption results of Ca^{2+}. Additionally, the H^+-ATPase activity on the root membrane in the OS was significantly higher than that of the WT, which indicated that the plant had stronger energy metabolism and helped it to resist the low temperatures of external environment. Further, the results of high-throughput sequencing showed that the number of microorganisms associated with photosynthesis, energy metabolism, nitrogen fixation and defense was significantly up-regulated at low temperature, while the number of microorganisms associated with pathogenicity was significantly down-regulated. Moreover, the overexpressed plants can respond to stress in a timely manner under low temperature, and the plant itself can tolerate adverse conditions through a series of reactions. In conclusion, the overexpression of the *Lsi1* gene enhanced the cold tolerance of rice, and its mechanism was comprehensively analyzed, providing a theoretical basis for the production of rice under events of sudden temperature decrease.

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