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Research article

Metabolite profiling and gene expression of Na/K transporter analyses reveal mechanisms of the difference in salt tolerance between barley and rice

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ABSTRACT

Barley (*Hordeum vulgare*) and rice (*Oryza sativa*) differ greatly in their salt tolerance, although both species belong to the Poaceae family. To understand the mechanisms in the difference of salt tolerance between the two species, the responses of ionome, metabolome and gene expression of Na and K transporters to the different salt treatments were analyzed using 4 barley and 4 rice genotypes differing in salt tolerance. In comparison with 4 rice genotypes, four barley genotypes showed better plant growth, lower shoot Na concentration and higher K concentration at the 9 day after salt treatments. There was a dramatic difference in absolute expression levels of *SOS*, *HKT* and *NHX* family genes between barley and rice, which might account for their difference in Na/K homeostasis and salt tolerance. Moreover, rice leaves accumulated excess Na under salt treatments, which caused serious damages to physiological metabolisms based on metabolomic analysis, but barley leaves had lower Na concentration and small changes in the most metabolites. These results provide useful insights into the molecular mechanism in the difference of salt tolerance between rice and barley.

1. Introduction

Soil salinity is one of major abiotic stresses, leading to a remarkable decrease of crop production worldwide (Munns and Tester, 2008). Currently, the total area of salt affected soils is up to 9.0×10^8 hm^2, including 20% of the arable lands and nearly one-half of the irrigated lands (FAO, 2015; Rengasamy, 2010). China has more than 1.0×10^8 hm^2 saline soils, much larger than other countries and regions (Zhang et al., 2010). Unfortunately, soil salinization is still expanding due to excessive emission of industrial pollutants and irrational farming activities (Zhu, 2001), posing a serious threat to agricultural production and food security. Therefore, it is essential to reveal molecular mechanisms of crop salt tolerance so as to develop new crop cultivars with high salt tolerance.

There is a large difference in salt tolerance among crop species. Rice is one of the most important food crops in the world, but its poor salt tolerance limits its planting in the saline soil (Ligaba and Katsuhara, 2010). On the other hand, barley ranks the fourth in terms of planting area in the world and has higher tolerance to multiple abiotic stresses, in particular salinity, thus commonly used as a model crop in abiotic tolerance studies (Nevo and Chen, 2010; Hamam et al., 2016). Both barley and rice belong to the Poaceae family, and are diploid in genomic composition. Obviously the excellent salt tolerance in barley could provide valuable references for salt tolerance improvement in rice. Therefore, it is imperative to reveal physiological and molecular mechanisms underlying the differences in salt tolerance between barley and rice.

Salt tolerance is a complex quantitative trait in plants (Urano et al., 2010). During long-term adaptation to various abiotic stresses, plants have evolved adaptive mechanisms for fighting against the stresses (Adem et al., 2014; Chen et al., 2007; Deinlein et al., 2014; Nevo, 1997). Up to date, osmotic adjustment (such as compatible solute accumulation), ionic balance (especially for K^+/Na^+ homeostasis) and anti-oxidation are considered as the main mechanisms of salt tolerance in plants. Especially for ionic balance, it was considered as an important mechanism of plant salt tolerance and was associated with the roles of Na/K transporters under salt stress conditions, including SOS, HKT and NHX transporter families (Flowers, 2004; Munns, 2005; Munns and Tester, 2008). For instance, three genes (*SOS1*, *SOS2* and *SOS3*) form the SOS pathway for root Na^+ excretion (Shi et al., 2000; Munns and

Abbreviations: HKT, High affinity potassium transporter; NHX, Na^+/H^+ antiporter; PCA, Principal component analysis; PC1, The first principal component; PC2, The second principal component; SOS, Salt overly sensitive; TCA, Tricarboxylic acid cycle

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Tester, 2008; Zhu, 2003). HKT transporters are categorized into K^+/Na^+ uniporters or K^+-Na^+ symporters (Himabindu et al., 2016). Besides, NHX transporters are involved in the exchange of cations across tonoplast membranes through vacuolar H^+-ATPase and H^+-PPase (Blumwald et al., 2000; Himabindu et al., 2016). Many studies have been done to compare the difference in salt stress responses among genotypes within a certain crop species (Tavakkoli et al., 2011), but few studies were involved in the comparison of different crops. Nakamura et al. (1996) compared relative dry weight, tissue Na content and ATPase activity between barley and rice in responses to different salt treatments, and found that the higher salt tolerance of barley was attributed to its less shoot Na accumulation. It may be hypothesized that lower expression of the genes related to Na^+ transporters from roots to shoots causes less Na accumulation in shoots, leading to higher salt tolerance. However, all previous studies cannot provide the detail molecular evidences for us to understand the difference of salt tolerance between barley and rice, because of uncompleted release of barley genomic sequences. With the completion of barley genome sequences, it is time for us to resolve their molecular differences in salt tolerance.

In this study, we analyzed physiological and molecular responses of 4 barley genotypes (including wild and cultivated barleys) and 4 rice genotypes (including *Indica* and *Japonica*) to salt stress, in order to reveal the mechanisms of the difference in salt tolerance at ion, metabolite, gene expression levels, respectively. The objective of the present study is to reveal the mechanisms of salt tolerant difference between rice and barley.

2. Materials and methods

2.1. Plant materials and growth conditions

Four barley genotypes, including two cultivars (Hua 30 and ZD 9) and two Tibetan wild accessions (XZ26 and XZ169) and four rice genotypes, including two *Japonica* rice (Nipponbare and 02428) and two *Indica* rice (9311 and JHSM), were used in the current study.

After germination, the seedlings of barley and rice were transplanted into 15 L black plastic containers filled with hydroponic solution as described by Wu et al. (2016). During pre-culture stage, barley seedlings were cultured in aerated one-fifth Hoagland solution (pH 6.0) and rice seedlings were cultured in one-half-strength Kimura B solution (pH 5.6), respectively. All plants were grown in a controlled growth room at 26 °C of 14 h day/20 °C of 10 h night, supplying lights with fluorescent lamps at 250 μmol m^{-2} s^{-1}. The solution was renewed every 3 d. To obtain the same stage plants according to our preparation experiments, four rice genotypes were germinated 10 d earlier than barley. At the same day, salt treatment was initiated to plants of barley (20 d old) and rice (30 d old) by adding NaCl in the solution at a rate of 50 mM per day, to reach final concentrations, i.e. 100 mM and 150 mM, respectively. During salt treatment, all plants were cultured by one-fifth Hoagland solution. The solution without NaCl addition was used as control.

2.2. Determination of transpiration rate

At 24 h of salt treatment, the middle parts of the latest completely expanded leaves in both salt-treated and control plants were used to measure the transpiration rate (TR) and photosynthetic rate (PR) by a portable photosynthesis system (Li-6400XT), Six biological replicates were conducted for each treatment and control. TR was calculated by the following formula: TR = net loss of H_2O (mmol)/[measured leaf area (m^2) × measured time (s)]. PR was calculated using the following formula: PR = net loss of CO_2 (mmol)/[measured leaf area (m2) × measured time (s)].

2.3. Determination of fresh weight and element concentration

At 9 d of salt treatment, plants of each treatment and control were sampled and separated into shoots and roots. Roots were rinsed with deionized water for several times and dried softly by absorbent papers. Fresh weight of shoots and roots was weighed immediately after sampling. There were three biological replicates for each measurement. Then all samples were dried at 80 °C for 3 days, and weighted. Dried samples were digested in boiling tubes containing 6 ml HNO_3 and 200 μl 30% H_2O_2, by a microwave (Multiwave 3000, Anton Paar GmbH, Australia) according to Shen et al. (2016). After that, the concentrations of Na, K, Ca and Mg in the digested solution were determined by an ICP-OES spectrometer (Optima 6000 series, PerkinElmer Inc, USA).

2.4. Metabolite extraction and metabolite profiling analysis

At 9 d of salt treatment, roots and the latest completely expanded leaves of each treatment and control were sampled according to Kim and Verpoorte (2010), and then frozen immediately in liquid nitrogen. For metabolite extraction, sampled tissues (100 mg) with 4 replicates were put into 2 ml EP tubes, adding 0.5 ml extraction solution ($V_{methanol}/V_{chloroform}$ = 3:1) and 25 μl (0.2 mg ml^{-1} stock in dH_2O) ribitol as an internal standard. After vortex for 10 s, mixtures were homogenized by a ball mill for 5 min at 55 Hz, and then centrifuged for 15 min at 12,000 rpm at 4 °C. The supernatant (0.4 ml) was transferred into a new 2 ml glass vial and dried by a vacuum concentrator. After that, 80 μl of methoxyamine hydrochloride reagent (20 mg ml^{-1} stock in pyridine) was added and the glass vials were shaked for 2 h at 37 °C. Then, 0.1 ml Bis(trimethylsilyl)trifluoroacetamide regent (containing 1% TMCS, v/v) was added, and the mixture was shaked for 1 h at 70 °C. After cooling to a room temperature, 10 μl standard mixture of fatty acid methyl ester was added. Then GC-TOF/MS analysis was performed using an Agilent 7890 gas chromatograph system coupled with a Pegasus HT time-of-flight mass spectrometer (Agilent, USA) as described by Wu et al. (2013b).

The processes of raw peaks exacting, data baselines filtering, peak alignment, deconvolution analysis, peak identification and integration of the peak area were performed using Chroma TOF 4.3X software and LECO-Fiehn Rtx5 database (LECO, USA) according to the operation manual. Metabolites were searched from commercial databases such as NIST (http://www.nist.gov/index.html) and KEGG (http://www.genome.jp/kegg). After that, all data was analyzed using Metaboanalyst version 3.0 (http://www.metaboanalyst.ca/), combining with Student's *t*-test ($P < 0.05$) to find significantly changed metabolites. The changes of metabolites were mapped to metabolic pathways according to MapMan software version 3.5.1 (http://mapman.gabipd.org/).

2.5. RNA extraction and qRT-PCR analysis

Fresh tissues of the roots and shoots from barley genotype XZ26 and rice genotype Nipponbare were sampled for RNA extraction after 0, 1, 2, 4 and 7 d of 100 mM salt treatment. Total RNA was extracted from approximately 0.1 g fresh tissues using TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa). The total RNA was then converted to cDNA using PrimeScript™ RT reagent Kit (TaKaRa). Na/K transporter genes were selected including *SOS1* (MLOC_51932 for barley; AY785147 for rice), *SOS2* (MLOC_4961; EU703803), *SOS3* (MLOC_15826; LOC_Os05g45810), *HKT1;1* (MLOC_55066; AJ491816), *HKT1;3* (MLOC_68594; AJ491818), *HKT1;4* (MLOC_58742; AK109852), *HKT1;5* (DQ912169; DQ148410), *HKT2;1* (MLOC_13204; AB061311), *HKT2;3* (MLOC_7152; AJ491820), *NHX1* (MLOC_4602; AB021878), *NHX2* (AK359097; LOC_Os11g42790), *NHX3* (MLOC_10701; AY360145), *NHX4* (AK376115; LOC_Os06g21360), *NHX5* (MLOC_4504; LOC_Os09g11450) and *NHX6* (AK374624;