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Proteomics reveals the effects of gibberellic acid (GA_3) on salt-stressed rice (*Oryza sativa* L.) shoots

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ABSTRACT

Salt decreases gibberellic acid (GA_3) content in rice. Nevertheless, GA_3 may exert a beneficial effect on salt-stressed rice. In our analysis, GA_3 reduced NaCl-induced growth inhibition in rice (*Oryza sativa* L. cv. Nipponbare) in a concentration-dependent manner, including the length of root tissue. However, remains unclear. We employed a comparative proteomic analysis to elucidate the mechanism of GA_3 activity in this phenomenon. 5-Day-old seedlings treated with salt and gibberellic acid (H_2O, 0.5% NaCl, 0.5% NaCl + 100 μM GA_3, and 100 μM GA_3) for 48 h were used for the proteomics analysis. Eleven proteins differently regulated by salt and GA_3 were revealed by 2D PAGE and were identified by MALDI-TOF MS. These proteins were identified as glutamyl-tRNA reductase, enolase, salt stress-induced protein (SALT protein), Os09g0249700, hypothetical protein OsJ_014066, putative chaperonin 21 precursor, Os04g0659300, hypothetical protein OsJ_025258, ribulose bisphosphate carboxylase, isoflavone reductase-like protein and phosphoglucomutase. Some of these proteins are involved in biochemical pathways such as photosynthesis and glycolysis, but others were found to be novel proteins involved specifically in the response to salt in rice. GA_3 had a significant influence on the abundance of some salt-regulated proteins. Our studies have provided new insight to reveal the modulating effect of GA_3 on salt stress in rice.

1. Introduction

Salt stress is one of the most serious restrictions on grain production in many countries. It causes water deficit, ion toxicity, and nutrient deficiency, leading to growth and yield reduction, and even to plant death. Recently, it has been reported that many proteins are regulated in root [1], panicles [2], leaf lamina [3,4] and plasma membrane [5] of rice subject to salt stress. Protein phosphorylation also occurs during salinity stress in rice [1] and hundreds of genes exhibit altered expression during salt stress [6,7]. Sahi et al. [8] noted that transcriptional and translational machinery are important determinants in controlling salt stress response. Hormone-related signal transduction plays an important role in plant response to adversities. As a group of essential endogenous regulators of plant growth, active gibberellins (GAs) influence seed germination, stem elongation, leaf expansion and reproductive development [9,10]. Proteomics studies show that the expression levels of some proteins in rice root [11,12], leaf sheath [13] and vacuolar membrane [14] are changed when treated with GA_3. In salt-stressed rice, exogenous GA_3 counteracts the effect of salt on the length and dry weight of the shoot, and on carotenoid and chlorophyll B contents [15]. Moreover, reduced GA accumulation causes increased accumulation of DELLAs, a family of nuclear growth-restraining proteins which function as negative regulators of GA signaling, and consequently causes growth inhibition [16,17]. Recent work has demonstrated that rice gibberellin insensitive dwarf1 (GID1) and three *Arabidopsis* GID1 homologs, AtGID1a, b, and c [18,19], can function as soluble GA receptors. Of particular interest is that salt stress was found to induce DELLA protein stabilization [20] and decrease GA_3 content in rice [21]. These results suggest that by a mechanism dependent on DELLA and/or other proteins, salt can decrease GA content in rice and GAs could exert a natural beneficial effect on salt-stressed rice. However, the mechanism through which GA_3 has an attenuating effect on salt stress of rice remains unclear.

Abbreviations: IEF, isoelectric focusing; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; GA_3, gibberellic acid; 2D PAGE, two-dimensional poly-acrylamide gel electrophoresis.

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Advances in proteomics and genomics technologies have significantly facilitated studies of plant response to salinity stress. In previous reports, studies concerning the effects of GA_3 on salt stress in rice were performed mainly at the level of differences in gene expression. As proteins play important roles in the response to phytohormones and stresses in plants, the study of proteins altered by salt and GA_3 is necessary to understand the role of GA_3 in NaCl-induced growth inhibition in rice. [In this report, we investigated the morphological changes of salt-stressed rice caused by GA_3, and traced the change of proteins among the four treatments.]

2. Materials and methods

2.1. Rice culture and treatments

Rice (*Oryza sativa* L. cv. Nipponbare) seeds were kindly provided by Prof. Shen Shihua (Institute of Botany, Chinese Academy of Sciences). Seeds were soaked in deionized water for 36 h and surface sterilized with 10% sodium hypochlorite for 10 min, then sowed in Petri dishes with filter papers saturated in treatment solutions (30 seeds in each dish). To determine the effects of GA_3 on salinity tolerance in rice, seeds were cultured in treatment solutions with different concentrations of GA_3 (0, 5, 25, and 100 μM) with 0.5% NaCl, and in deionized water only as a control. Petri dishes were placed in an incubator without light at 25 °C and the treatment solutions were refreshed daily. The germination energy was determined with 3-day-old seedlings, and the length of rice shoot and root were evaluated with 7-day-old seedlings.

2.2. Sample extraction

For proteomic analysis, 5-day-old seedlings were treated either with H_2O, 0.5% NaCl, 0.5% NaCl + 100 μM GA_3, or 100 μM GA_3 for 48 h, respectively. Shoots of seedlings were harvested for protein extraction after treatment. Proteins were extracted using a two-step trichloroacetic acid/acetone protein extraction protocol. Briefly, shoots were ground in liquid nitrogen with mortar and pestle (pre-cooled) into a fine powder. The powder was precipitated with ice-cold acetone containing 10% (w/v) trichloroacetic acid and 0.07% (v/v) 2-mercaptoethanol (acetone–TCA–2-ME) for 2 h at −20 °C, and then centrifuged at 40,000 × *g* for 25 min at 4 °C. The pellets were washed with ice-cold acetone containing 0.07% (v/v) 2-ME, for 6 h at −20 °C and centrifuged again at 4 °C. Then pellets were lyophilized to powder and were stored at −80 °C until further use. Three biological replicates were extracted independently for each treatment.

2.3. 2D PAGE separation

Samples were dissolved in rehydration buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (v/v) IPG buffer, 1% (w/v) DTT], and the protein concentration was determined by 2D Quant kit (GE Healthcare) according to the manufacturer's instructions. Quantified proteins were then used for 2D PAGE. Isoelectric focusing (IEF) was carried out using an Ettan IPGphor III (GE Healthcare) on 24 cm IPG strips (pH 4–7, GE Healthcare). 1.4 mg of protein in 450 μl of rehydration buffer was loaded into the IEF tray and active rehydration was carried out at 20 °C for 12 h (30 V for 8 h and 50 V for 4 h), followed by 300 V for 1 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 12 h.

For separation in the second dimension, the focused strips were equilibrated twice for 15 min in 10 ml of equilibration buffer. The first equilibration was performed in equilibration buffer containing 6 M urea, 2% (w/v) SDS, 30% (w/v) glycerol, 1% (w/v) DTT, and 50 mM Tris–HCl (pH 8.8). During the second equilibration, DTT was replaced by 2.5% (w/v) iodoacetamide in equilibration buffer. Equilibrated IPG gel strips were then loaded on top of vertical SDS poly-acrylamide gels (12.5% total monomer, with 2.6% crosslinker) using Ettan DALT six (GE Healthcare). After SDS-PAGE separation, the gels were stained with colloidal CBB R-350 (GE Healthcare) and scanned with an Image Scanner (GE Healthcare). Data analysis of the scanned gels was performed using ImageMaster 2D Platinum Elite software ver. 6.0 (Amersham Pharmacia Biotech, Uppsala, Sweden). Differentially expressed protein spots (more than 1.5-fold change in intensity in three biological replicate gels) were excised for trypsin digestion and mass spectrometry analysis. The effect of treatments was analyzed by the two-tailed paired *t*-test.

2.4. In-gel digestion and MALDI-TOF MS analysis

The excised protein spots were washed with ultrapure water twice at room temperature, and destained with 100 μl of 100 mM NH_4HCO_3/acetonitrile (50:50, v/v) for 1 h. Gel fragments were dehydrated with 50 μl of acetonitrile for 10 min and dried at room temperature. 10 μl of 20 ng/μl trypsin (Promega) was added to each dried gel fragment and incubated for 45 min at 4 °C. 10 μl NH_4HCO_3 (50 mM) was added and fragments were incubated at 37 °C overnight (about 16 h). After digestion of proteins, peptides were desalted with C18 ZipTips (Millipore Corp., Bedford, MA, USA), then spotted on MALDI plates (Bruker Daltonics, Germany) in 50% acetonitrile and 0.1% trifluoroacetic acid (TFA). Finally, peptides were co-crystallized with saturated α-cyano-4-hydroxycinnamic acid (CHCA) prepared in 50% (v/v) acetonitrile containing 1% TFA.

To obtain peptide mass fingerprint (PMF) of protein spots, peptide masses were measured using autoflex MALDI-TOF MS (Bruker Daltonics, Billerica, MA, USA). External calibration was performed with a peptide calibration standard (Bruker Daltonics, Part No.: 206 195). All obtained PMFs were analyzed with the protein search engine MASCOT (Matrix Science, U.K.) against NCBI's database. Search parameters were set as follows: peptide tolerance (0.2 Da), NCBInr database, *O. sativa* (taxonomy), carbamidomethylation of cysteine (fixed modification), and methionine oxidation (variable modification). Molecular function of proteins was annotated using the database at http://www.uniprot.org/uniprot.

3. Results

3.1. Salt-stressed rice growth response to GA_3

Rice seeds were germinated in different concentrations (0, 5, 25, and 100 μM) of GA_3 combined with 0.5% NaCl, and in water only as control. 0.5% NaCl treatment alone significantly reduced the germination energy, root length and shoot length of rice, but the reductions could be partially rescued by adding GA_3 (Fig. 1). Our results showed that among the treatments of adding GA_3 in concentrations of 5, 25, and 100 μM, there were no significant differences of the reduce-rescuing effects on germination energy and root length (Fig. 1A and B), whereas on shoot length, there were significant difference between 25 μM GA_3 and 100 μM GA_3 (Fig. 1C).

3.2. Proteins regulated by different treatments

5-Day-old seedlings were treated either with H_2O, 0.5% NaCl, 0.5% NaCl + 100 μM GA_3 or 100 μM GA_3 for 48 h, respectively. Shoot proteins of three biological replicates were extracted and separated independently by 2D PAGE (shown in Fig. 2). Eleven differentially expressed proteins were revealed by 2D PAGE (shown in Figs. 2 and 3), and were identified with high confidence