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A proteomic analysis of salt stress response in seedlings of two African rice cultivars

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ABSTRACT

Salt stress is one of the key abiotic stresses threatening future agricultural production and natural ecosystems. This study investigates the salt stress response of two rice seedlings, which were screened from 28 Kenya rice cultivars. A proteomic analysis was carried out and Mapman bin codes employed in protein function categorization. Proteins in the redox, stress, and signaling categories were identified, and whose expression differed between the salt tolerant and the salt sensitive samples employed in the present study. 104 and 102 root proteins were observed as significantly altered during salt stress in the tolerant and sensitive samples, respectively and 13 proteins were commonly expressed. Among the 13 proteins, ketol-acid reductoisomerase protein was upregulated in both 1 and 3 days of salt treatment in the tolerant sample, while it was down-regulated in both 1 and 3 days of salt treatment in the sensitive sample. Actin-7, tubulin alpha, V-type proton ATPase, SOD (Cu-Zn), SOD (Mn), and pyruvate decarboxylase were among the observed salt-induced proteins. In general, this study improves our understanding about salt stress response mechanisms in rice.

1. Introduction

Environmental stresses such as high salt levels, drought, and low temperature greatly affect plant productivity [1–5]. Despite these challenges, demand for food by a growing population is increasing tremendously. A large increase in the global population is anticipated by the year 2050 [6,7]. Land clearing and/or irrigation with brackish water contribute to increases in secondary soil salinity [5,8]. Soil is described as saline when its electrical conductance is greater than four dS m^{-1} [8], with high soluble salt concentrations. Consequently, to guarantee sustainable food production, agricultural land crops ought to exhibit greater tolerance to salt stress [8]. Plants are classified as sensitive or tolerant depending on the rate at which salt in the leaf tissues reach toxic levels [5].

Plants employ numerous physiological and biochemical strategies and responses at both molecular and cellular levels to survive harsh environments [1]. There is considerable variation in salt stress tolerance levels among rice genotypes involving intricate physiological mechanisms [9] and regulated by numerous quantitative trait loci (QTLs) [10–12]. Additionally, rice could be sensitive to salt stress in both the young seedling stages and the reproductive stages [13].

A large number of genes functioning in ion transport, salt signaling, transcript regulation, and the biosynthesis of specific metabolites involved in plant responses to salt stress have been isolated at the transcriptomic level [14–17]. A study on gene expression profiles during the initial phases of salt stress in rice revealed approximately 10% significant upregulation or downregulation of transcripts in salt tolerant rice [18]. However, salt stress studies at the gene level do not provide clear insights on the quantity and quality of gene products (proteins). The protein amount does not always correlate with mRNA, particularly in low abundance proteins. Post-translational modifications undergone by proteins, including phosphorylation and glycosylation, removal of signal peptides, crucial processes for protein activities and subcellular localization could be the reason for these notable differences. An approach at the protein level, therefore, is necessary; to evaluate protein dynamics in the course of salt stress [18–20].

Numerous constitutive and stress-induced variations in root proteins have been revealed through proteomic comparisons between salt stress tolerant and sensitive genotypes [21]. Induction of nine salt-responsive proteins in rice roots exposed to salt stress has been reported, which included a 14.5 kDa SALT protein [22]. Additionally, six novel salt-responsive proteins were identified in a study by Yan et al. [20], including UDP-glucose pyrophosphorylase, cytochrome *c* oxidase subunit 6b-1, glutamine synthetase root isozyme, a putative nascent polypeptide associated complex alpha chain, and a putative splicing factor-like

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protein and putative actin-binding protein. The proteins above were associated with the regulation of carbohydrate, nitrogen, and energy metabolism, reactive oxygen species scavenging, mRNA and protein processing, and cytoskeleton stability [23]. Photosystem II (PSII) oxygen-evolving complex protein, fructose bisphosphate aldollases, and superoxide dismutase are among the proteins that demonstrated a significant change in abundance in response to salt stress in rice leaf sheaths [24]. In vitro salt stress studies of rice cells cultured in media containing a high concentration of NaCl further demonstrated an accumulation of 26 and 27 kDa proteins [25].

Cultivation of rice dates back more than 3,000 years, and it has been shown that African and Asian rice differs in their origin [26]. To the best of our knowledge, classification of the varieties and the developed lines of rice grown in Kenya into either salt sensitive or salt tolerant have not been carried out. In this study, 28 rice samples from Kenya were classified into either salt tolerant, intermediate or salt sensitive. A proteomic approach was employed in investigating the protein dynamics during salt stress in seedlings of different genotypes. This study could offer greater insights on how varying protein expression patterns influence salt sensitivity or salt tolerance.

2. Materials and methods

2.1. Salt stress screening

A total of 48 rice samples obtained from different parts of Kenya were collected from Jomo Kenyatta University of Agriculture and Technology (a mixture of traditional cultivars, landraces, and developed varieties) and screened for salt resistance. Five selected rice samples from Kenya (Pumba ya muwa, Moshi, Cushe, Kibawa, Basmati 217) whose salt sensitivity were not originally known were subjected to various salt concentrations to determine the appropriate concentration for subsequent analysis. The samples were germinated in sterile sand and grown for ten days. Ten-day-old rice seedlings were subjected to 25, 50, 100, 200, 300, and 500 mM NaCl and morphological changes monitored and recorded for 15 days. The experiment scheme is illustrated in Supplementary Fig. 1.

Salt treatments were carried out following a modified procedure as describe by Glenn et al. [27] by obtaining about 40 seeds per sample for the salt sensitivity assay among the 30 rice samples (including 9311 [28] and Nipponbare [29]). They were surface sterilized by soaking in 10% hydrogen peroxide for 20 min, followed by washing three times with distilled water and two times with deionized water. The sterilized seeds were then germinated in sterile sand in a growth chamber maintained at 27 °C and 22 °C day and night temperatures, respectively. The seedlings were allowed to grow for ten days. Uniform seeds were selected for salt treatment. Ten-day-old seedlings were uprooted from the sand and their roots rinsed with distilled water. They were transplanted in 1/2-strength Hoagland nutrient solution (Macronutrients, KNO_3, $Ca(NO_3)_2$ $4H_2O$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, Micronutrients, H_3BO_3, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, MoO_3, Fe-EDTA) [30] for the control and for the treatment, the Hoagland nutrient solution was supplemented with NaCl to a concentration of 500 mM. The 1/2-strength Hoagland solution supplemented with NaCl and the control were changed every three days. There were three replicates with ten seedlings per replicate, including for the controls. We recorded the number of days it took for each sample to die. Seedlings exhibiting dried yellowed leaves and stems were deemed dead. They were not re-cultured in 1/2 Hoagland nutrient solutions to check if they could revive since the aim of this experiment was to select the sensitive and tolerant genotypes from the total sample batch. When more than four seedlings in at least two replicates died, the number of days was recorded as the death time for that particular sample. From this experiment, two rice samples, from the most tolerant group, and the sensitive group were selected for further analysis. Subsequent experiments were based on the two selected samples.

2.2. Protein extraction and quantification

Sensitive and tolerant seeds were surface sterilized and grown in sterile sand for ten days. Ten-day-old seedlings were subjected to 300 mM salt concentrations for 0, 1, and 3 days. The roots were harvested, rinsed in distilled water, and snap frozen in liquid nitrogen. The samples were stored in −80 °C for subsequent experiments. Protein from rice roots was extracted according to the method described by Li et al. [31]. Briefly, the frozen roots (1 g) were ground into powder in liquid nitrogen using a mortar and pestle. They were then dissolved in a homogenization buffer (20 mM Tris-HCl (pH 7.5), 250 mM sucrose, 10 mM ethylene glycol bis (2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1% Trion −100, 1 mM phenylmethane sulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DDT)), followed by centrifugation at 12,000 *g* for 20 min at 4 °C. Afterwards, an equal volume of tris-phenol (pH ≥ 8.0) was added to the supernatants, vortexed, and centrifuged at 12,000 *g* for 20 min at 4 °C. The obtained top phenol phase was carefully transferred into another tube. A small amount of homogenization buffer was then added, mixed thoroughly, and centrifuged at 12,000 *g* for 20 min at 4 °C. Subsequently, five equal volumes of 0.1 M methanolic ammonium acetate in 100% methanol were mixed with the phenol phase and incubated overnight at −20 °C. The precipitated proteins were washed once with 0.1 M methanolic ammonium acetate and twice with cold acetone. The protein pellets were vacuum-dried and then stored at −80 °C. The Bradford method [32] was used to quantify proteins in a spectrophotometer (DU®640, BECKMAN, USA).

2.3. Protein digestion

The protein samples were resolved in sample buffer (40 mM Tris–HCl, pH 6.8, 2 mM DTT, 4% glycerol, 2% SDS, 0.1% Bromophenol blue) for electrophoresis. 100 μg proteins were loaded into a single well of the vertical gel for each run. The gel was stained with Coomassie Brilliant Blue (CBB) R-250 after the full-length run to visualize the proteins. The gel was then cut into five pieces and proteins in each piece were digested as follows: each small gel strip was distained with the distaining solution (50 mM NH_4HCO_3 in 50% v/v ACN) for 1 h at 37 °C. The distaining step was repeated until the gel was colorless. The gel pieces were washed with HPLC grade water and lyophilized. Before trypsin digestion, the proteins were reduced by DTT, which had a final concentration of 5 mM and incubated at 56 °C for 30 min to reduce the disulfide bonds. The protein mixture was allowed to cool to room temperature and briefly spun to collect condensation. Iodoacetamide was added to a final concentration of 14 mM and incubated in the dark for 30 min at room temperature to alkylate cysteins. The unreacted iodoacetamine were quenched by 0.5 M DTT to additional 5 mM and incubated in the dark for 15 min at room temperature. This protein mixture was then digested with grade modified trypsin (Promega, Madison, WI, USA) at a 1:100 enzyme/protein concentration at 37 °C overnight [33]. After digestion, the protein peptides were collected, and the gels washed with 0.1% TFA in 50% acetonitrile (ACN) three times to collect the remaining peptides. Peptides were desalted using ZipTip C18™ (Millipore Sigma, Darmstadt, Germany). Isotope dimethyl labeling on the trypsin-digested peptides was performed where 0 day was labeled with (D_2CO, $NaBH_3CN$), 1 day ($D_2^{13}CO$, $NaBD_3CN$) and 3 days with (H_2CO, $NaBH_3CN$) as described by Yue et al. [34] and mixed. Three biological replicates were used for the LC-MS/MS (Supplementary Fig. 1).

2.4. Mass spectrometry analysis

RPLC-ESI-MS/MS was used to analyze the samples. LC-MS/MS analyses were carried out on a hybrid quadrupole-TOF LC-MS/MS mass spectrometer (TripleTOF 5600+, AB Sciex) equipped with a nanospray source. Peptides were first loaded onto a C18 trap column