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Alterations in root proteome of salt-sensitive and tolerant barley lines under salt stress conditions

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ABSTRACT

Salinity is one of the most important abiotic stresses causing a significant reduction of crop plants yield. To gain a better understanding of salinity tolerance mechanisms in barley (*Hordeum vulgare*), we investigated the changes in root proteome of salt-sensitive (DH14) and tolerant (DH187) lines in response to salt-stress. The seeds of both barley lines were germinating in water or in 100 mM NaCl for 6 days. The root proteins were separated by two-dimensional gel electrophoresis. To identify proteins regulated in response to salt stress, MALDI-TOF/TOF mass spectrometry was applied. It was demonstrated that the sensitive and tolerant barley lines respond differently to salt stress. Some of the identified proteins are well-documented as markers of salinity resistance, but several proteins have not been detected in response to salt stress earlier, although they are known to be associated with other abiotic stresses. The most significant differences concerned the proteins that are involved in signal transduction (annexin, translationally-controlled tumor protein homolog, lipoxygenases), detoxification (osmotin, vacuolar ATP-ase), protein folding processes (protein disulfide isomerase) and cell wall metabolism (UDP-glucuronic acid decarboxylase, β-D-glucan exohydrolase, UDP-glucose pyrophosphorylase). The results suggest that the enhanced salinity tolerance of DH187 line results mainly from an increased activity of signal transduction mechanisms eventually leading to the accumulation of stress protective proteins and cell wall structure changes.

Introduction

Crop plants are often exposed to various biotic and abiotic stresses, greatly reducing the productivity of the crop worldwide. Soil salinity is among the main abiotic stresses and affects more than 800 million hectares of land, equivalent to more than 6% of the total global area of the Earth (Munns and Tester, 2008). This is a serious problem, considering the growing global population and, consequently, the increased demand for food. Therefore, improving salt tolerance of crop plants is one of the current issues of global breeding program.

Plants differ considerably in their tolerance to salinity. Cereals exhibit low salinity tolerance, but significant differences in salt sensitivity can be observed within this group of plants. Among the cereals, rice (*Oryza sativa*) is considered the most salinity-sensitive and barley (*Hordeum vulgare*) the most tolerant species (Munns and Tester, 2008).

Plants' response to salt stress occurs in two phases. Initially, the harmful effect of salinity is associated with low water potential of the root medium, resulting in osmotic stress. During this phase abscisic acid (ABA) is accumulated and a range of metabolic processes is inhibited, e.g. cell expansion, cell wall synthesis, protein synthesis, stomatal conductance and photosynthetic activity (Barkla et al., 2013).

In the second phase, salt is transported via the xylem to the shoot. Na^+ and Cl^- ions accumulate in shoot cells to a toxic extent, resulting in ionic stress. Subsequently, ion accumulation enhances the production of reactive oxygen species (ROS). By the additional

Abbreviations: 2DE, two-dimensional electrophoretic protein separation; ABA, abscisic acid; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; DOC, sodium deoxycholate; DTT, dithiothreitol; IEF, isoelectric focusing; HSC 70, heat shock cognate 70 kDa; HSP 90, heat shock protein 90-kDa; MALDI-TOF/TOF, matrix assisted laser desorption ionisation time-of-flight/time-of-flight; MeS, methionine synthase; PFP, pyrophosphate-fructose-6-phosphate-1-phosphotransferase; SAH-hydrolase, S-adenosyl-L-homocysteine hydrolase; SHMT, serine-hydroxymethyltransferase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PDI, protein disulfide isomerase-like protein; PMF, peptide mass fingerprinting; TCA, trichloroacetic acid; TCTP, translationally-controlled tumor protein homolog; UGD, UDP-glucuronate decarboxylase; vATP, vacuolar ATPase.

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production of ROS in cells, the balance between production and removal of ROS is disrupted, which eventually leads to oxidative stress (Fatehi et al., 2012).

Salt tolerance is a multigenic trait demonstrating the complexity at both the genetic and physiological levels. Hence, a profound analysis of the proteome is essential to understand the fundamentals of the physiology of salinity stress. The results may finally contribute to the development of effective plant breeding strategies for salt-affected areas (Shavrukov et al., 2010).

In recent years proteome analyses have gained popularity in plant science and have mostly relied on two-dimensional electrophoretic protein separation (2DE) (Barkla et al., 2013). Significant improvements in 2DE technique and mass spectrometry analysis, including MALDI-TOF/TOF enable deeper and more accurate analysis of the proteome.

Salinity tolerance mechanisms are not yet fully explained, especially for an early stage of barley development as majority of the research describing salinity-induced root proteome changes in barley was conducted on 3-leaf or subsequent stages of barley development (Witzel et al., 2009; Fatehi et al., 2012). Moreover, in the previous proteomic studies, salt stress was implicated at seedling (Witzel et al., 2009, 2014) or 4-leaf stage of development (Fatehi et al., 2012). In the present study, we investigate the root proteome changes of 6-day old barley seedlings. The salt stress is applied directly on barley grains to mimic the germination conditions in saline soil where the plant is exposed to salinity from the very beginning. Salt stress conditions applied at seed stage allows to gain the knowledge about the molecular defense mechanisms activated during the germination under salinity conditions.

To date, there is lack of studies describing the proteome changes under seeds-applied salt stress in the roots of two barley lines contrasting in salinity tolerance.

Materials and methods

Germinating conditions

Seeds of salt-tolerant (DH187) and salt-sensitive genotype (DH14) were obtained from Leibniz Institute of Plant Genetics and Crop Plant Research (Gatersleben, Germany). Salinity sensitive and tolerant lines of barley belong to the Steptoe/Morex mapping population, created in terms of salinity tolerance (Sadeghil et al., 2013).

Seeds germination was conducted in a growth chamber at the constant temperature of 20 °C for 6 days on the tissue-paper placed in glass cylinders. The seeds that constituted the control samples of sensitive (Cs) and tolerant (Ct) barley lines were germinated in Mili-Q water. Salt stressed samples of sensitive (Ss) and tolerant (St) barley lines were germinated in 100 mM NaCl solution. One hundred seeds were sown per every biological replication. After the appointed time the roots were cut and immediately frozen in liquid nitrogen. All experiments were performed in three biological replicates ($n = 3$).

Fresh and dry weight content measurement

For the measurement of the fresh and dry weight content, approximately 100 roots from at least three independent biological replicates of each sample were examined. For the fresh weight content measurement, roots were weighed immediately after cutting. For the dry weight content measurement, roots were weighted, dried at 105 °C for 24 h and weighted again. The obtained data were analyzed with Statistica 10.0 (StatSoft, Poland), using Student's *t*-test and reported as means ± SD. Differences between the control samples and stress samples were considered statistically significant at a significance level $p \leq 0.05$.

Protein extraction

Harvested roots were grounded in liquid nitrogen using mortar and pestle. Proteins were extracted with a buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG buffer pH 3–11 NL (GE Healthcare), 120 mM dithiothreitol, protease inhibitors cocktail (Sigma). The protein extraction was carried out for 1 h on the laboratory shaker at 4 °C. After the incubation time samples were centrifuged (14,000 × *g*, 10 min, 4 °C) and protein extracts were purified using DOC/TCA precipitation (Gómez-Vidal et al., 2008) with some modifications described below. One ml of the protein extract was mixed with 100 μL of 0.2% sodium deoxycholate (DOC). The mixture was incubated on ice for 15 min. Then 340 μL of 24% trichloroacetic acid (TCA) was added and the entire mixture was incubated on ice for 1 h. After the appointed time the samples were centrifuged (14,000 × *g*, 10 min, 4 °C). The precipitates were subsequently washed twice with ice-cold acetone supplemented with 0.07% [w/v] DTT. Air-dried pellets were dissolved in rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer pH 3–11 NL (GE Healthcare), 80 mM DTT and 0.002% bromophenol blue. The protein concentration was quantified using 2D-PAGE adapted Bradford assay (Ramagli and Rodriguez, 1985) with BSA dissolved in rehydration buffer as a protein standard. The BSA standards were prepared in the volume of 50 μL to cover the range of 0–12 μg ml^{-1}. BSA dilutions and the examined samples were acidified with 10 μL of 0.1 M HCl. Afterwards, 940 μL of Bradford reagent was added. The measurements were carried out at a wavelength of 595 nm using Infinite M200 PRO multimode microplate reader (Tecan).

Two-dimensional electrophoresis

Extracted proteins were separated using IEF/SDS-PAGE (Görg et al., 2004). Each sample, containing 400 μg of protein in rehydration buffer, was loaded onto 24 cm Immobiline DryStrip Gels (GE Healthcare) with the non-linear pH gradient 3–11. Isoelectric focusing was performed in the Ettan IPGphor 3 system (GE Healthcare). The running conditions were as follows: 30 V/10 h (active rehydration at 20 °C), 500 V/1 h (step and hold), 1000 V/1 h (gradient), 8000 V/3 h (gradient), 8000 V/4:30 h (step and hold).

After the isoelectric focusing, the IPG strips were equilibrated twice for 15 min in 15 ml of equilibration solution. The first equilibration solution contained 75 mM Tris–HCl buffer (pH 8.8), 6 M urea, 29.3% (v/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol blue and 65 mM DDT. The second equilibration solution was modified by replacing DDT with 135 mM iodoacetamide.

The second dimension (SDS-PAGE) was performed using 12.5% SDS polyacrylamide gels and was carried out in the Ettan DALTsix electrophoretic unit (GE Healthcare). Electrophoresis was performed at 30 °C (2.5 W/gel for 30 min, 17 W/gel for 4:30 h). The protein spots were stained with colloidal CBB G-250. Each treatment was replicated thrice.

Gels were scanned at 300 dpi using Image-Scanner III (GE Healthcare). Gel analysis was performed with Image Master 2D Platinum 7.0 software (GE Healthcare). The volume of each spot from each replicate was normalized against total spot volume and quantified. In order to investigate the salt-responsive proteins of both lines, the control samples and salt-stressed samples of both lines were matched. Proteins exhibiting at least 1.75-fold reproducible abundance changes between compared samples were subjected to statistical analysis using the *t* test ($p \leq 0.05$). Only the spots that were present on all three replicate gels were quantified. The proteins which abundance changed significantly were designated to mass spectrometry for identification.