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Proteome analysis of sugar beet (*Beta vulgaris* L.) elucidates constitutive adaptation during the first phase of salt stress

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ABSTRACT

Salinity is one of the major stress factors responsible for growth reduction of most of the higher plants. In this study, the effect of salt stress on protein pattern in shoots and roots of sugar beet (*Beta vulgaris* L.) was examined. Sugar beet plants were grown in hydroponics under control and 125 mM salt treatments. A significant growth reduction of shoots and roots was observed. The changes in protein expression, caused by salinity, were monitored using two-dimensional gel-electrophoresis. Most of the detected proteins in sugar beet showed stability under salt stress. The statistical analysis of detected proteins showed that the expression of only six proteins from shoots and three proteins from roots were significantly altered. At this stage, the significantly changed protein expressions we detected could not be attributed to sugar beet adaptation under salt stress. However, unchanged membrane bound proteins under salt stress did reveal the constitutive adaptation of sugar beet to salt stress at the plasma membrane level.

Introduction

Salinity is a complex abiotic stress that involves osmotic and ionic components. Plant growth is impaired by osmotic stress in a first phase, and by specific ion toxicity in a second phase of salt stress (Munns, 1993). The two-phase model of plant growth under salt stress does not distinguish between the two phases on the basis of time period and therefore may overlap depending upon the intensity of stress (Sümer et al., 2004). Ion toxicity in the second phase of salt stress results primarily from Na^+ accumulation in sensitive tissues and may be prevented by Na^+ exclusion in salt-resistant plants (Garthwaite et al., 2005; Fortmeier and Schubert, 1995). Potassium (K^+) homeostasis under salt stress is of the utmost importance, and a high cytosolic K^+/Na^+ ratio seems to be crucial under salt stress (Chen et al., 2007; Shabala and Cuin, 2007).

Most higher plants are salt-sensitive, while certain plants have evolved mechanisms to resist high salt concentrations. Salt-resistant plants such as halophytes and salt-resistant genotypes of glycophytic plant species show a genetic basis of the salt response. Accumulation of compatible solutes in the plant tissue is a strategy to combat salinity in many plants (Zhu, 2002), and their accumulation has a significant role in plant adaptation to stress (Yeo, 1998). Cytoplasmic accumulation of osmolytes plays a crucial role in the salt resistance of plants in order to protect the cytoplasm from detrimental effects of Na^+ on enzymes, as most enzymes of salt-resistant plants such as halophytes are also not adapted to higher concentrations of Na^+ and show the same sensitivity as the enzymes of glycophytes (Glenn et al., 1999).

The complex nature of the response to salinity requires varied approaches to understand it. Proteome analysis is a relatively new approach to study plant responses to biotic and abiotic stresses. Two-dimensional (2D) separation of proteins provides an easy way to study the expression of stress-induced proteins in plants, and may help to indentify the roles of these proteins under a variety of physiological conditions (Komatsu et al., 2003). In the salt-sensitive maize, a proteomic approach did not contribute much to the understanding of resistance mechanisms, due to a large and nonspecific change in the proteins observed (Zörb et al., 2004). Moreover, a dramatic change in protein pattern under low salt concentration does not necessarily affect biomass production and ion concentration. Similar conclusions were drawn by Saqib et al. (2006) for salt-resistant and salt-sensitive genotypes of wheat. On the other hand, only a limited change in the proteomes of *Oryza sativa* and *Nicotiana tobaccum* were found under salt stress (Abbasi and Komatsu, 2004; Razavizadeh et al., 2009). Although changes in the proteome under salt stress are physiologically complex, Wang et al. (2008) indentified 23 variety-specific salt-responsive proteins in a comparative study of salt-resistant and salt-sensitive wheat genotypes. The response to various salinity levels by the proteome of halophytic plants differed from glycophytes due to their ability to survive under saline conditions. Only 3% of proteins were altered at

Abbreviations: ATP, Adenosine triphosphate; MES, 2-(*N*-Morpholino) ethanesulfonic acid; PM, Plasma membrane; SDS, Sodium dodecyl sulfate; Q-TOF, Quadrupole time-of-flight.

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150 mM NaCl concentration in *Suaeda aegyptiaca* leaves. However, the proteomic alterations increased with an increase in salinity level, and were 11% of total protein spots detected at 600 mM NaCl. The salt-responsive proteins changed in abundance rather than in presence or absence (Askari et al., 2006).

Sugar beet is a very important crop of halophytic nature and is thus able to survive under high salt concentrations. Analysis of the sugar beet proteome under salt stress may provide useful information regarding more specific adaptive mechanisms to prevent salt stress. The objectives of this study were: (1) to determine the proteomic alterations in sugar beet under salt stress and (2) to identify significantly changed proteins and their possible relevance to salinity adaptation.

Materials and methods

Plant cultivation and harvesting

Sugar beet seeds (*Beta vulgaris* L. cv. Evita) were sown in sand and irrigated with 1 mM $CaSO_4$ solution. After 1 week, seedlings were transferred to 50 L plastic containers (70 plants per container) containing 1/4 full-strength nutrient solution. The following day, the nutrient solution was increased to 1/2 and the plants were transferred to plastic pots having 4.5 L full strength nutrient solution after an additional 2 d. Nutrient solution [N (5.3 mM), K (4.0 mM), P (0.3 mM), Mg (0.5 mM), Ca (2.0 mM), Mn (0.5 μM). Zn (0.1 μM), Cu (0.2 μM), B (10.0 μM), Mo (0.01 μM), and Fe (10.0 μM)] was changed after every 3 d (Wakeel et al., 2009). The NaCl treatment was started 1 week after reaching full-strength nutrient concentration. NaCl was added in 25 mM increments daily until a final concentration of 125 mM NaCl. The experiment was carried out in a climate chamber where light intensity was 200 W m^{-2} for 16 h; the temperature was 22 °C for the light period and 20 °C for the dark period. The relative humidity was 70%. The experiment was replicated three times with 70 plants per treatment. Plants were harvested 7 d after treatment application and, after weighing roots and shoots, half of them were oven-dried at 80 °C and Na and Cl concentrations were measured after dry-ashing (550 °C) using an atomic absorption spectrophotometer (Varian FS 220) and Cl^- concentrations were measured potentiometrically by titrating AgCl (Eppendorf 6610). Half of the roots and shoots were shock-frozen in liquid nitrogen and were then stored at −80 °C for protein analysis.

In a separate experiment, plants were grown in a similar way to isolate the plasma membrane from sugar beet shoots. However, 1 week after reaching full-strength nutrient concentration, NaCl was added in 50 mM (only in salt treatment) increments daily until a final concentration of 150 mM NaCl.

Protein extraction

Proteins were prepared for isoelectric focusing using a DTT–TCA–acetone precipitation method modified by Zörb et al. (2009). Frozen (−80 °C) plant material was ground and homogenized under liquid nitrogen. Afterwards, 1.8 mL lysis buffer (10% TCA in acetone) was added to 200 mg ground plant material mixed thoroughly before sonication for 15 min in ice-cold ultrasonic bath. The samples were incubated overnight at −20 °C and centrifuged at 20,000 × *g* for 15 min at 4 °C. The precipitate was re-suspended in 1 mL 4 °C cold buffer (50 mM DTT; 2 mM EDTA, in acetone) and was incubated for 10 min in an ice-cold ultrasonic bath for another 10 min. This procedure was repeated twice. Pellets were lyophilized under nitrogen. Four replicates were collected and resuspended in 1 mL lysis buffer (8 M urea, 2 M thiourea, 0.5% pharmalyte buffer (v/v, pH 3–10), 4% CHAPS; 30 mM DTT, 20 mM Tris-base, pH 8.8, 5 mM Pefablock). For solubilization of proteins, samples were incubated for 2 h at 33 °C and for 15 min in an ultrasonic ice-cold bath. After vortexing, samples were centrifuged (18,000 × *g*, 30 min) and the supernatant collected and protein concentration was determined in 1:50 dilutions of samples using the 2D QUANT protein determination kit from GE Healthcare.

Plasma membrane isolation

Plasma membranes of sugar beet shoots were isolated with two-phase partitioning according to Zörb et al. (2005) and the microsomal membranes were fractionated by partitioning in aqueous dextran T-500 (Sigma) and polyethylene glycol 3350 (Sigma) according to the method of Larsson (1985). Stock solutions of polymers were prepared with concentrations of 20% and 40% (w/w) for dextran and polyethylene glycol, respectively. The concentration of the dextran stock solution was determined by optical rotation. The phase stock was weighed and diluted to 6.1% (w/w, each polymer) with phase buffer to a final weight of 32 g. Polymers in "start tubes" were diluted to 26 g. Six grams of microsomal re-suspension (in phase buffer) were added to the upper phase of each start tube. The tubes were sealed with Parafilm and mixed by inversion (30 times).

Phase separation was achieved at 4 °C by centrifugation at 720 × *g* (Sorvall AH-629 rotor, 36 mL) for 23, 15 and 10 min, respectively. The upper phases obtained after two separations were diluted with phase buffer and centrifuged at 4 °C and 151,000 × *g* for 40 min. The pellets were resuspended and diluted with resuspension buffer (250 mM sucrose, 3 mM KCl, 5 mM BTP/MES, pH 7.8) and centrifuged again at 4 °C and 151,000 × *g* for 40 min. The pellets were resuspended in resuspension buffer, divided into aliquots and immediately stored in liquid nitrogen. To determine the purity of the plasma membrane (PM) fraction isolated from sugar beet shoots, the ATPase hydrolytic activity was analyzed in the presence of nitrate, azide, molybdate or vanadate, which are inhibitors of tonoplast H^+-ATPase, mitochondrial H^+-ATPase, unspecific phosphatases and PM-H^+-ATPase, respectively (Wakeel et al., 2010a). The vanadate-inhibited H^+-ATPase activity was 92.5%, which represents a high purity of plasma membrane vesicles.

For 2-DE, plasma membrane vesicles were resuspended in 1 mL lysis buffer, and for solubilization of proteins, samples were incubated for 2 h at 33 °C and for 15 min in an ultrasonic ice-cold bath. The samples were centrifuged (18,000 × *g*, 30 min) and the supernatant was collected. Protein concentrations were determined in 1:50 dilutions of samples using the 2D QUANT protein determination kit from GE Healthcare.

Two-dimensional gel electrophoresis (2-DE)

2-DE was performed following the modified protocol of Zörb et al. (2009). For each sample, one IPG strip (11 cm, pH 3–10, GE Healthcare) was placed into a strip holder, 125 μg protein was added and the strip holder was covered with paraffin oil. IEF was carried out in an IPGphore chamber (GE Healthcare) under the following conditions: 10 h rehydration; 100 V, 2 h; 500 V, 1 h; 100 V, 2 h; 8000 V, 2 h, approx. 34,000 V h per run. The temperature was 20 °C and the current was 45 μA per strip. After running the first dimension, strips were placed in equilibration buffer (50 mM Tris–HCl, pH 8.8; 6 mM urea; 30% glycerol; 2% (w/v) SDS; bromophenol blue, 0.001%(w/v) containing 1% DTT (w/v)) and carefully shaken for 10 min. Thereafter, the strips were incubated for an additional 10 min in equilibration buffer with 4% (w/v) iodoacetamide without DTT under slow agitation. Strips were stored at −20 °C. After thawing, the strips were rinsed several times with SDS-PAGE running buffer (25 mM Tris-base; 192 mM glycine; 0.1% (w/v) SDS).

The second dimension SDS gels contained 12.5% (v/v) acrylamide. Molecular weight standards in a range from 10 to 220 kDa