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Hydrolytic and pumping activity of H^+-ATPase from leaves of sugar beet (*Beta vulgaris* L.) as affected by salt stress

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ABSTRACT

Cell wall extensibility plays an important role in plant growth. According to the acid-growth theory, lower apoplastic pH allows extension growth by affecting cell wall extensibility. A lowered apoplastic pH is presumed to activate wall-loosening enzymes that control plant growth. Plasma membrane (PM) H^+-ATPases play a major role in the apoplastic acidification by H^+ transport from cytosol to the apoplast. A salt-induced decrease in H^+-pumping activity of plasma membrane H^+-ATPases in salt-sensitive maize plants has previously been found. This led us to formulate the hypothesis that salt-resistant plant species such as sugar beet (*Beta vulgaris* L.) may have a mechanism to eliminate the effect of higher salt concentrations on plasma membrane H^+-ATPase activity. In the present study, sugar beet plants were grown in 1 mM NaCl (control) or 150 mM NaCl in hydroponics. H^+-ATPase hydrolytic and pumping activities were measured in plasma membrane vesicles isolated from sugar beet shoots. We found that plasma membrane H^+-ATPase hydrolytic and pumping activities were not affected by application of 150 mM NaCl. Moreover, apoplastic pH was also not affected under salt stress. However, a decrease in plant growth was observed. We assume that growth reduction was not due to a decrease in PM-H^+-ATPase activity, but that other factors may be responsible for growth inhibition of sugar beet plants under salt stress.

Introduction

Salinity is a complex abiotic stress that involves osmotic stress 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 for salt stress in plants does not distinguish between the two phases on the basis of time. These two kinds of stress may overlap depending upon the intensity of stress (Sümer et al., 2004). Fortmeier and Schubert (1995) concluded, for maize, that 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 maize genotypes. Earlier, Niu et al. (1993) suggested that control of intracellular ion homeostasis is the key factor for salt resistance. Plants adapted to salinity maintain a lower concentration of Na^+ in the cytosol by active exclusion of Na^+ into the apoplast (Wu and Seliskar, 1998) and inclusion in vacuoles (Blumwald, 2000). Specific Na^+/H^+ antiporters NHX operating in the tonoplast play a major role in Na^+ exclusion from shoots (Neubert et al., 2005; Saqib et al., 2005). Zörb et al. (2005a) found a linear response of ZmNHX transcription to NaCl concentration in the root medium ranging from 1 to 100 mM for the root tissue of salt-resistant maize hybrids. The driving force for this secondary active transport is generated by plasma membrane (PM) H^+-ATPases and tonoplast H^+-ATPases (Palmgren, 1998; Ratajczak, 2000; Sun et al., 2009) and H^+-pyrophosphatses (Rea and Poole, 1993).

PM-localized H^+-ATPases hydrolyze ATP and export H^+ in order to generate an electrochemical gradient across the membrane (Palmgren, 1998). This proton gradient energizes nutrient uptake by channel proteins and carriers (Buch-Pedersen et al., 2009; Duby and Boutry, 2009). Extrusion of H^+ by PM H^+-ATPase into the apoplast is also a major requirement for lower apoplastic pH, which may increase the cell wall extensibility and allows cell growth (Jahn et al., 1996; Peters et al., 1998; Rayle and Cleland, 1992; Stahlberg and Van Volkenburgh, 1999). Findings by Hager (2003) support the idea that lower apoplastic pH stimulates the cell elongation in *Avena coleoptiles* and activation of cell wall-loosening enzymes enhances the plant growth (Cosgrove, 2000). Bogoslavsky and Neumann (1998) also concluded that outward proton pumping by PM H^+-ATPase increased the cell wall extensibility participating in cell expansion, but other researchers have found no correlation between hormone-mediated wall

Abbreviations: AO, acridine orange; ATP, adenosine triphosphate; EGTA, ethylene glycol tetraacetic acid; MES, 2-(*N*-morpholino) ethanesulfonic acid; PM, plasma membrane; SDS, sodium dodecyl sulfate; SE, standard error; ZmNHX, tonoplast Na^+/H^+ antiporter in *Zea mays*

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acidification and organ growth (Kutschera, 1994; Kutschera, 2006).

Under saline and drought conditions, leaf elongation is a very sensitive stress parameter (Neumann, 1993; Schmidhalter et al., 1998). Leaf elongation or cell extension is not limited by turgor, but by a reduction in cell wall extensibility (Van Volkenburgh and Boyer, 1985). Salt-sensitive plants such as maize (*Zea mays* L.) respond with rapid shoot growth reduction after a few days under moderate salt treatment (Fortmeier and Schubert, 1995; Maas et al., 1983; Zörb et al., 2005a). Extrusion of H^+ from the cytosol across the PM is responsible for cell extension via cell wall acidification. Therefore, PM H^+-ATPase activity is expected to play an important role in the salt-stress response (Janicka-Russak and Klobus, 2007). Chelysheva et al. (2001) found that osmotic stress changed neither the amount of H^+-ATPase molecules in the plasma membrane nor hydrolytic activity, but it decreased the H^+-ATPase pumping activity through its influence on the coupling between H^+-transport and ATP hydrolysis. The reduced H^+ pumping by PM H^+-ATPase may lead to an increase of apoplastic pH. Felle et al. (2005) also detected an increase in apoplastic pH of barley leaves under short-term salinity, which suggested that the pH gradient across the PM was reduced. Recently, similar results in long-term experiments have been found in a salt-sensitive maize cultivar (Pitann et al., 2009a). Salt stress-triggered reduction of ATPase H^+-pumping efficiency in the PM of leaf cells during the first phase of salt stress would thus limit leaf elongation. PM-H^+-ATPase-mediated ATP hydrolysis was not affected by NaCl treatment for maize plants. However, H^+-pumping activity of PM H^+-ATPase was strongly decreased (Pitann et al., 2009b; Zörb et al., 2005b). The decrease in H^+-pumping efficiency may be responsible for increased apoplastic pH and growth reduction due to limited cell wall extension. Contrary to maize, sugar beet has a better ability to exclude Na^+ from the cell cytoplasm and is more resistant to salinity compared to glycophytes such as maize (Blumwald and Poole, 1987). Moreover, higher concentrations of Na^+ also stimulate the growth of this plant species (Wakeel et al., 2009a). Therefore, it is assumed that a major mechanism, which contributes to salt resistance, is located at the PM. The present study was designed to investigate the H^+ transport by PM H^+-ATPase in sugar beet shoots grown under salt stress. We hypothesized that PM H^+-ATPase shows an adaptive response to salt stress and the hydrolytic and H^+-pumping activities of PM H^+-ATPase from sugar beet leaves are not affected, leaving the apoplastic pH undisturbed.

Materials and methods

Plant cultivation

Sugar beet seeds (*Beta vulgaris* L. cv. Felicita) 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 $\frac{1}{4}$ full-strength nutrient solution. The following day, the nutrient solution was increased to $\frac{1}{2}$ and to full concentration 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., 2009b). The NaCl treatment was started 1 week after reaching full-strength nutrient concentration. NaCl was added in 50 mM increments daily until a final concentration of 150 mM NaCl was achieved. The experiment was carried out in a climate chamber where light intensity was 200 Wm^{-2} for 16 h; 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.

Plant harvest

Plants were harvested 7 d after application of 150 mM NaCl and separated into roots and shoots. Harvested root material was washed with 1 mM $CaSO_4$. Fresh weights of roots and shoots were determined. Plant material (growing leaf blades) for membrane isolation was cooled to 4 °C and processed immediately.

Plasma membrane isolation

Microsomal cell membranes were isolated according to Yan et al. (2002) and microsomal plasma membrane was fractionated using two-phase partitioning in aqueous dextran T-500 and polyethylene glycol according to 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 (Larsson, 1985). 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 with centrifugation at 4 °C and 720*g* (Sorvall AH-629 rotor, 36 mL) for 23 min followed by two washing steps in identical phases. Centrifugation time for the second separation was 15 min. The upper phases obtained after two separations were diluted with phase buffer (see above) and centrifuged at 151,200*g* for 40 min. The pellets were washed with re-suspension buffer (250 mM sucrose, 3 mM KCl, 5 mM BTP/MES, and pH 7.8) and pelleted again. The pellets were re-suspended in a modified re-suspension buffer (250 mM sucrose, 3 mM KCl, 5 mM BTP/MES, pH 7.8, and 1 mM dithiothreitol), divided into aliquots, and immediately stored in liquid nitrogen. Protein was quantified according to the method of Bradford (1976) using bovine serum albumin (Sigma) as a standard.

Enzyme assay

Plasma membrane ATPase hydrolytic activity was determined in 0.5 mL of 30 mM BTP/MES buffer containing 5 mM $MgSO_4$, 50 mM KCl, 0.02% (w/v) Brij 58 (Sigma), 5 mM Bis-Tris-ATP in the presence and absence of inhibitors: 50 mM KNO_3 (for tonoplast ATPases), 1 mM Na_2MoO_4 (for nonspecific phophatases), 1 mM NaN_3 (for mitochondrial membrane ATPases), or 0.3 mM Na_3VO_4 (for plasma membrane ATPases). The reaction was initiated by the addition of 2.5 μg of membrane protein at 30 °C and stopped after 30 min with 1 mL of stopping reagent [2% (v/v) concentrated H_2SO_4, 5% (w/v) SDS, and 0.7% (w/v) $(NH_4)_2MoO_4$)] followed immediately by 100 μL of 10% (w/v) ascorbic acid. After 10 min, 1.45 mL of arsenite–citrate reagent (2% [w/v] sodium citrate, 2% [w/v] sodium *m*-arsenite and 2% [w/v] glacial acetic acid] were added to prevent the measurement of phosphate liberated by ATP hydrolysis under acidic conditions. Color development was completed after 30 min and A_{820} was measured spectrophotometrically. ATPase activity was calculated as phosphate liberated in excess of a boiled-membrane control. The vanadate-inhibited hydrolytic activity served as a measure of plasma membrane ATPase activity. Hydrolytic activity was measured at 20 and 30 °C and 100 mM K^+ or Na^+ concentrations in the assay were provided as KNO_3, KCl, $NaNO_3$, and NaCl.