

PROTEIN EXPRESSION AFTER NaCl TREATMENT IN TWO TOMATO CULTIVARS DIFFERING IN SALT TOLERANCE

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Received June 27, 2012; revision accepted September 13, 2012

SDS-PAGE electrophoresis was used to study the effect of NaCl on protein expression in two cultivars of tomato (*Solanum lycopersicum* L.): Edkawi (salt-tolerant) and Castle rock (salt-sensitive). Five-day-old seedlings were grown on MS agar media supplemented with 0, 50, 100, 150, 200 and 300 mM NaCl. Two days after treatment the seedlings were examined to determine the effect of salt on their growth and to relate that to protein banding variations. Gel analysis showed differences in at least 4 protein bands with molecular weights at 20, 25, 45 and 65 kDa. These proteins were induced in the 50 mM NaCl treatment in the salt-sensitive cultivar, then decreasing to undetectability at higher concentrations. In the salt-tolerant cultivar, most of the proteins exhibited a more or less steady expression pattern and maintained expression through the 200 mM NaCl treatment. All proteins gave weak or no expression signals at 300 mM NaCl, the treatment that proved lethal. Differentially expressed bands were identified using MALDI-TOF mass spectrometry. The putative function of each identified protein in relation to salt stress is discussed.

Key words: SDS-PAGE, salinity, 45 S ribosomal protein, Rubisco, MALDI-TOF mass spectrometry, tomato.

INTRODUCTION

Soil and water salinity is considered a major problem worldwide. High salinity reduces the average yields of most major crop plants by more than 50% (Bray et al., 2000). To date, the mechanism of plant sensitivity to salinity remains elusive, mainly because it is controlled by multiple genes that affect different aspects of plant growth and development. In general, plants exhibit a dual response to salt stress, entailing early and late responses. The former is related to osmotic stress resulting from the negative water potential of saline soil. The latter is due to Na⁺ accumulation in leaf tissues (Munns, 1993; Kafkafi et al., 1996). Only plants with modified adaptive mechanisms can avoid the adverse effect of salinity (Blumwald, 2000). It is important to understand how plants develop salt tolerance, so that vast areas of saline soils can be cultivated. One way of studying this is to compare the differential gene expression of salt-tolerant and salt-sensitive plants under saline conditions (Kong-Ngern et al., 2005). Proteins with differential expression under salinity stress can be

used as molecular markers in the work on improving salt tolerance through genetic engineering techniques. Genes involved in salinity adaptation can be divided into two groups: those that directly protect against stress and those that regulate gene expression during stress (Ashraf, 1994; Winicov, 1998; Saki et al., 2003). Extensive research had been done to study gene responses under salinity treatment (Pareek et al., 1997; Ashraf and Harris, 2004) and identify differentially expressed proteins using mass spectrometry (Cho, 2007), but the molecular aspects of how plants can tolerate salinity have not been fully elucidated. Analyzing the mode of expression of salt-inducible genes is one way of revealing the molecular mechanisms of tolerance in higher plants.

In this study, MALDI-TOF mass spectrometry was used to compare the differential protein expression patterns of salt-tolerant and salt-sensitive tomato cultivars treated with different concentrations of NaCl for 48 hours. The aim was to better understand the differences between plants that tolerate salinity and those that are more sensitive.

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MATERIALS AND METHODS

PLANT MATERIAL

Seeds of *Solanum lycopersicum* Mill var. Edkawi (accession no. LA 2711), a salt-tolerant cultivar, and var. Castle rock, a salt-sensitive cultivar, were kindly provided by the Vegetable Crop Department, Agricultural Research Center, Ministry of Agriculture (Giza, Cairo, Egypt). Each experiment used 40 seeds and was done in triplicate. Seeds of uniform appearance were selected manually, washed, and soaked in water for 24 h to induce simultaneous germination. The seeds were surface-sterilized by washing with 10 ml 70% ethyl alcohol, incubated for 15 min in 10 ml 20% solution of Clorox bleach in distilled water, then washed 4 times with sterile distilled water before being plated on MS-agar (Murashige and Skoog, 1962) nutrient medium for plant culture containing 1.5% agar, 0.4% MS and 0.097% MES buffer [2-(N-morpholino) ethanesulfonic acid] and adjusted to pH 5.7 with potassium hydroxide. Cultured plants were kept in the dark for 5 days at room temperature to germinate. Forty simultaneously germinated seedlings were selected and transferred to MS-agar media supplemented with 0, 50, 100, 150, 200 and 300 mM NaCl. Each treatment was represented by 4 plates, ten seedlings per plate. Seedlings were grown on the media for another 2 days in a growth chamber at 25°C under a 12 h photoperiod. All media were autoclaved at 121°C for 30 min, and all steps of the experiment were carried out in sterile conditions under a laminar flow hood.

ANALYSIS OF ROOT GROWTH

To determine the effect of NaCl treatment on the root elongation rate, five-day-old seedlings were marked from outside the plate at their root tips right after their transfer to media supplemented with 0, 50, 100, 150, 200 and 300 mM NaCl. Changes in root length were also marked after 24 and 48 h. Images for marked plates were captured with a digital camera (8 mpx, Sony). The increase in root lengths (mm) (differences between all marks for each root) was measured using Image Pro Plus (Media Cybernetics, Inc. MD, U.S.A.). Salt tolerance in both cultivars was determined by immersing eight-week-old seedlings in water supplemented with the previously mentioned concentrations for 24 h. The morphological changes of treated plants were recorded and compared. Averages, standard error, and the significance of the data were determined using the tools and the Student t-test in Excel ver. 2007 (Microsoft Corporation, WA, U.S.A.). I took $P = 0.05$ as indicating significance.

PROTEIN EXTRACTION

All steps were carried on ice or at 4°C. Seedlings were collected 48 h after NaCl treatment and prepared for protein extraction on the same day. Soluble protein extraction was done according to the method of Baboul`ene (2003) with some modifications. Seedlings were ground in liquid N₂ and homogenized in lysis buffer [0.05 M Trizma buffer, pH 8, 0.1 mM; protease inhibitor phenylmethyl sulfonylfluoride (PMSF), 100 mM dithiothreitol (DTT) as reducing agent instead of mercaptoethanol]. All reagents were from Sigma-Aldrich, St. Louis, MO, U.S.A. The homogenate was centrifuged at 12,000 g for 30 min. The supernatant was neutralized with potassium hydroxide (5 N KOH), recentrifuged as before, and mixed with activated charcoal to eliminate pigments. The soluble protein concentration was measured spectrometrically using a Pierce Coomassie protein assay kit (Thermo Scientific, U.S.A.) according to the manufacturer's instructions, using bovine serum albumin (BSA) as standard.

SDS-PAGE PROTEIN ELECTROPHORESIS

Proteins were separated on polyacrylamide gel (4% stacking and 15% resolving gels) in the presence of sodium-dodecyl sulphate (SDS-PAGE) following the method of Laemmli (1970). Protein samples were loaded (20 µg/lane) and electrophoresis was performed at 100 V/45 mA with an omniPAGE Mini Wide vertical unit (Cleaver Scientific, UK) following the manufacturer's instructions. The bands were fixed and stained overnight with Coomassie brilliant blue R-250 (CBB) solution, then destained using the same solution without CBB until the gel background became stain-free. The molecular weight of the protein bands was calibrated against a 10-200 kDa PageRuler™ unstained protein ladder (Fermentas, U.S.A.).

SAMPLE PREPARATION FOR MALDI ANALYSIS

Mass spectrometric analysis was conducted in the Protein Sciences Facility, Carver Biotechnology Center, University of Illinois at Urbana, using a Waters Q-ToF API-US mass spectrometer. Prior to LC/MS/MS analysis, the protein band in the gel slice was crushed, destained and dehydrated in 50% acetonitrile containing 25 mM ammonium acetate. The protein was digested using 60 ng/µl proteomics-grade trypsin (G-Biosciences, St. Louis, MO) and a CEM Discover microwave reactor (Matthews, NC) for 15 min at 55AC and 50 watts. Digested peptides were extracted 3 times using 50% acetonitrile containing 5% formic acid, then pooled and dried using a Savant Speedvac concentrator (SDDB1DDA, Thermo Scientific, U.S.A.). The dried peptides were suspended in 13 µl 5%

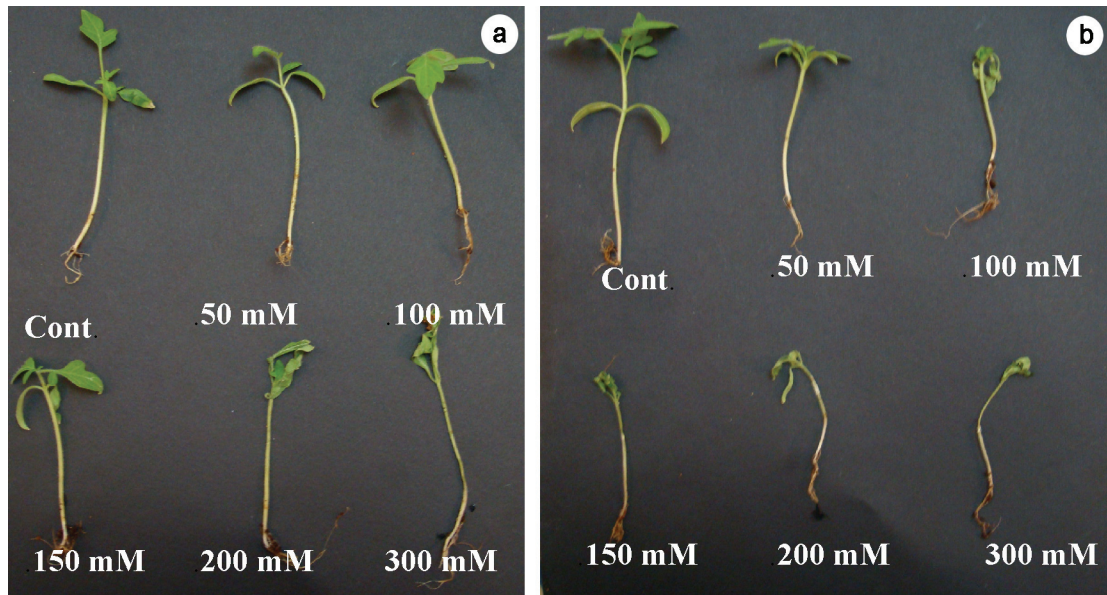


Fig. 1. Effect of different NaCl concentrations on 8-week-old tomato plant seedlings immersed in NaCl for 24 h. (a) Edkawi, (b) Castle rock.

acetonitrile containing 0.1% formic acid, and 10 μ l was injected for LC/MS/MS.

HPLC for the trypsin-digested peptides was performed with a Waters nanoACQUITY Ultra Performance UPLC™ System using a Waters Atlantis dC18 nanoACQUITY column (3 μ beads, 75 μ inner diameter, 150 mm length). The solvents were water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The flow rate was 250 nl/min, and the gradient was from 100% A to 60% B in 60 min. The effluent from UPLC was infused directly into a Waters Q-ToF using a Waters nano-ESI ion source.

Mass spectrometer control and data acquisition were done using a Waters Mass Lynx 4.1 in data-dependent acquisition mode. After an initial full scan, the four most intense ions were subjected to MS/MS fragmentation by collision-induced dissociation.

Samples were analyzed using a TOF-TOF 4800 Maldi Analyzer (ABSciex, MA, U.S.A.) in reflector-positive mode. A six-peptide mixture from ABSciex was used for calibration of MS and MSMS mode. Automated acquisition of MS data followed by MS/MS data was controlled with 4000 Explorer software (Applied Biosystems, CA, U.S.A.). Acceleration voltage of 20 kV was used in MS reflector mode, and 8 kV in MSMS mode.

DATABASE SEARCH FOR PROTEIN IDENTIFICATION

The protein search was performed using Protein Pilot 4.0 (ABSciex, MA, U.S.A.). This software incor-

porates the Paragon™ database search engine and uses the ProGroup™ algorithm for results compilation. The minimum acceptance criterion was 99% confidence level. Proteins were identified based on the presence of at least four peptides from a protein identified by the ProGroup algorithm. Proteins were identified based on searches against the ITAG2.3 database available at http://solgenomics.net/organism/solanum_lycopersicum/genome. The result was searched against the NCBI NR Protein database. The search was specific to green plants. The raw data were processed with Waters Protein Lynx Global Server ver. 2.2.5 for noise filtering and deisotoping, and then with Mascot ver. 2.3 (Matrix Sciences, London, UK).

RESULTS

EDKAWI AND CASTLE ROCK GROWTH RATES AT DIFFERENT NaCl CONCENTRATIONS

Eight-week-old seedlings having their roots immersed for 24 h in solutions containing 50, 100, 150, 200, 300 mM NaCl or water as control exhibited different responses. The first signs of wilting appeared at 100 mM NaCl in Castle rock and 200 mM NaCl in Edkawi (Fig. 1). The trend was similar for plants growing on MS-agar media at the same concentrations (Fig. 2). Generally the seedling growth rate was faster for Edkawi than for Castle rock under the same growth conditions (Tab. 1, Fig. 2). The root elongation rates of MS-agar media

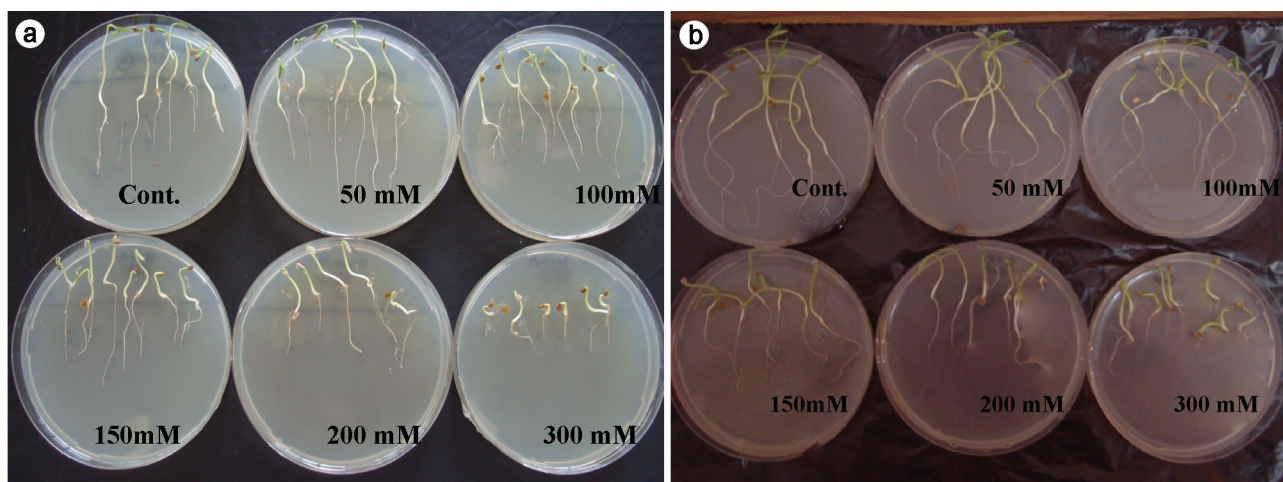


Fig. 2. Seedling germination on media supplemented with different concentrations of NaCl. (a) Castle rock, more sensitive to salt treatment and growing at a much slower rate on salt-free medium, (b) salt-tolerant Edkawi.

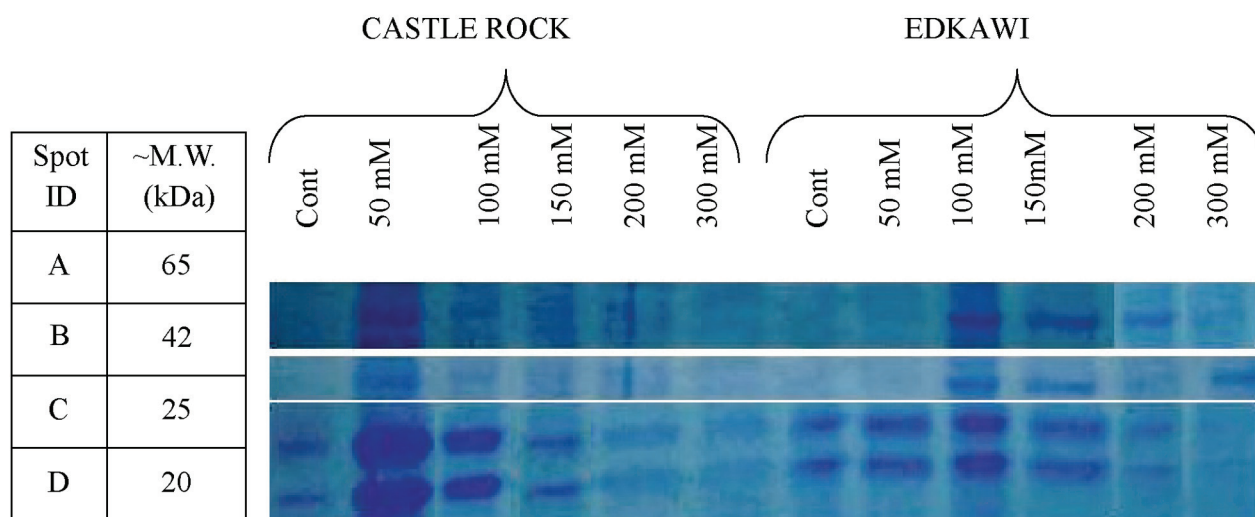


Fig. 3. Differentially expressed protein banding patterns on 1D SDS-PAGE gel for castle rock and Edkawi plants treated with 0, 50, 100, 150, 200 and 300 mM NaCl. At left, molecular weights of each band (kDa).

grown plants were comparable until 100 mM NaCl in both cultivars. The root elongation rate of Castle rock dropped markedly at 150 and 200 mM NaCl. NaCl at 300 mM inhibited the growth of both varieties (Tab. 2).

TABLE 1. Growth rate of Edkawi and Castle rock seedlings growing on soil without any treatments. Measurements were taken for 8-weeks-old seedlings; n= 60

8-week-old plants in soil before treatments	Root length (cm)	Shoot length (cm)
Edkawi	3.4 ± 0.8	5.02 ± 0.92
Castle rock	3.7 ± 1.2	4.2 ± 1.1

PROTEIN PROFILES OF EDKAWI AND CASTLE ROCK

Gel analysis showed at least four bands differing in expression level between the two tomato cultivars at the same salt concentration. The expression level of these proteins generally increased in Castle rock, and was steady or decreased in the salt-tolerant Edkawi plants (Fig. 3). These bands, labeled A, B, C and D here, separated at 65, 43, 25 and 20 kDa respectively. The proteins in these four bands were identified by MALDI-TOF mass spectrometry.

IDENTIFICATION OF PROTEIN BAND CONSTITUENTS

Each differentially expressed band gave 2–3 hits when analyzed using MALDI TOF LC/MS/MS spec-

TABLE 2. Root growth rate for plants grown on media supplemented with different NaCl concentrations. Root length is measured in cm and the average of $n = \sim 20$ is shown. (*) indicates the degree of significance in respect of control according to the Student t-test

Plants on culture media supplemented with	No NaCl	50 mM	100 mM	150 mM	200 mM	300 mM
Edkawi						
Day 1	1.03± 0.3	1.2 ±0.14*	0.83±0.15	0.63±0.12*	0.19 ± 0.06*	0.12 ± 0.06*
Day 2	1.6 ± 0.3	1.3 ± 0.48	1.04 ± 0.21	1.22 ±0.25**	0.3 ± 0.02***	0.23 ± 0.03***
Castle rock						
Day 1	0.94 ± 0.31	1.49 ± 0.45	1.1 ± 0.28	1.01 ± 0.41	0.99 ± 0.3*	0.24 ± 0.07*
Day 2	1.42 ± 0.44	1.09 ± 0.43	0.82 ± 0.27	0.6 ± 0.29*	0.57 ± 0.22*	0.35 ± 0.19*

trometry (Tab. 3). Band A contained a large ribulose-1,5-bisphosphate carboxylase/oxygenase subunit, a structural maintenance of chromosomes (SMC) protein, and a hypothetical protein. Band B contained a hypothetical protein, a basic helix-loop-helix (bHLH) domain-containing protein, and a myosin heavy chain-like protein. Band C included uncharacterized proteins but one of them localizes to the plasma membrane. Band D contained 40S ribosomal protein S28-1 and a pentatricopeptide repeat-containing protein in addition to a hypothetical protein.

DISCUSSION

Salt stress is a pervasive and economically damaging environmental factor that prevents crop plants from realizing their full genetic potential in early germination stages (Sairam and Tyagi, 2004; Rani, 2011). Under saline stress the response of plants to protein accumulation depends on the plant species and cultivar. In barley, accumulation of soluble proteins was higher in a salt-tolerant than in a salt-sensitive cultivar (Hurkman et al., 1989) while the reverse was found in wheat plants (Ashraf and Oleary, 1999). Findings differ on the response of tomato cultivars in accumulation of soluble proteins under salinity stress (Amini and Ehsanpour, 2005; Khosravinegad et al., 2009). My work was done mainly to compare the performance and soluble protein accumulation patterns of two tomato cultivars differing in their salt stress tolerance.

Edkawi is more salt-tolerant than Castle rock, and this was further confirmed by my results for the root elongation rates of 5-day-old seedlings grown on media supplemented with different salt concentrations and 8-week-old plants immersed by their roots for 24 hours along the same concentration gradient. The stimulatory effect of NaCl at low concentrations has been noted in other work (Ziemienowicz et al., 2011). The expression of salt-induced

TABLE 3. Band analysis of differentially expressed proteins using MALDI-TOF/MS

Band ID	Protein ID	Accession #
A	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	gi 7525041
	structural maintenance of chromosomes protein 4	gi 15239023
	hypothetical protein	gi 110737807
B	hypothetical protein	gi 4467113
	basic helix-loop-helix domain-containing protein	gi 42567496
C	myosin heavy chain-like protein	gi 15238179
	uncharacterized protein localized into the plasma membrane	gi 15230373
	F15O4.23	gi 8778370
D	uncharacterized protein	gi 15219662
	40S ribosomal protein S28-1	gi 15232831
	At5g03850	gi 166007601
	pentatricopeptide repeat-containing protein	gi 15226879

genes is more pronounced in salt-sensitive cultivars (Sarhan and Perras, 1987; de Lorenzo et al., 2007). I found no difference between the protein banding patterns of the two cultivars in the absence of NaCl. Only the bands with differential expression in the two cultivars after salt treatment were subjected to further analysis by MALDI-TOF mass spectrometry. Four bands separated in 1D SDS-PAGE at 65, 43, 25 and 20 kDa, labeled A, B, C and D, respectively.

The components of bands A and B exhibited low expression in the control treatment of both cultivars, accumulated at 50 mM NaCl, then declined at higher salt concentration in Castle rock, while remaining more or less steady in Edkawi at 100–200 mM NaCl

(Fig. 3). Rubisco is an enzyme composed of two subunits: the small nuclear subunit (RbcS) and the large chloroplast subunit (RbcL). RbcL identified in band A is the catalytic domain which participates in CO₂ assimilation and mobilization in plants. Rubisco is degraded under stress (Thoelen et al., 2007) via ATP-dependant ubiquitination (Shanklin et al., 1995; Vierstra, 1996) and usually is present as fragments rather than intact protein. The pronounced accumulation of Rubisco as the RbcL fragment in Castle rock plants at 50 mM NaCl may indicate increased degradation of it under salt stress (Chattoopadhyay et al., 2011). Because Rubisco represents 50% soluble protein and 30% total N₂, its degradation can be used as an indicator of a plant's tolerance threshold after which the plant needs to provide essential amino acids to maintain protein synthesis at stressed sites (Feller et al., 2008). The second protein in band A was identified as a structural maintenance of chromosomes (SMC) protein which is essential for chromosome replication and segregation in all organisms (Harvey et al., 2003). The third protein identified in band A was a hypothetical protein that localizes to the plasma membrane, which might indicate its involvement in regulation of NaCl uptake and management within the cell. The expression patterns of these proteins may indicate the ability of Edkawi to keep Rubisco content in balance, maintain normal chromosome segregation, and up-regulate ion pump proteins under salt stress.

In band B, the basic helix-loop-helix (bHLH) domain-containing proteins are transcription factors (Massari and Murre, 2000). They are involved in vesicle trafficking (Yokota et al., 1995) and transcription of ribosomal proteins (Philimonenko et al., 2010). The myosin heavy-chain protein subunit is salt-responsive and is directly influenced by the vacuolar Na⁺/H⁺ antiporter that mediates transport of Na⁺ and K⁺ into the vacuole (Sottosanto et al., 2007). Myosin mutants are defective in both organelle movement and polar auxin transport through the action on several vesicle-mediated processes Holweg and Nick (2004). The expression patterns of the protein components of band B in both cultivars suggest that vesicle trafficking is more active in Edkawi than in Castle rock at high salt concentration.

In band C, three proteins with no clear molecular function were detected. However, the localization of AT3G19370 protein within the plasma membrane may indicate its involvement in ion influx/efflux processes. This possibility needs further investigation.

Ribosomal proteins are overexpressed in stress-tolerant plants under stress (Kerri et al., 2006; Raquel et al., 2007). This overexpression probably maintains translational processes and proper ribosome assembly. S28-1 and pentatricopeptide

repeats (PPR) were identified in band D. 40S ribosomal proteins are involved in binding the small ribosomal subunit with RNA transcripts in the cytoplasm (Bernstein et al., 2004), while PPR is responsible for posttranslational processes in mitochondria and chloroplasts (Meierhoff et al., 2003; Milli and Roma, 2003; Nakamura et al., 2004; Delannoy et al., 2007), RNA stabilization and processing (Barkan et al., 1994; Manthey and McEwen, 1995). The steady expression of these proteins in Edkawi may indicate the ability of this cultivar to maintain normal transcription and translation, with a potential for posttranslational modifications for adaptation to salinity. Most of these suggestions need to be verified, and are offered here as preliminary inferences about some mechanisms of salinity adaptation in salt-tolerant plants.

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