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Proteomic analysis of salt -responsive proteins in canola leaves

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Abstract

Salinity is one of the major abiotic stresses limit agricultural productivity worldwide. To identify the mechanisms of salt responsiveness in canola, the proteinsexpressed in the leaves of salt-tolerant, Hyola 308 was analyzed. Plants were exposed to 0, 150, and 300mM NaCldruing the vegetative stage. An increase in the Na content and a reduction in K contend of shoot and root was observed. K/Na discriminant ratios were significantly reduced in leaves and roots due to salt stress.Two-dimensional polyacrylamide gel electrophoresis coupled with mass spectrometry analysis could identifyfourteen salt responsive proteins by Coomassie brilliant blue staining. These proteins functionally involved in oxidative stress, photosynthesis, signal transduction, protein kinase, transcription and proteases.These proteins might control the sensitivity of several regulatory genes to short exposure of canola to salt stress.

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Introduction

Soil salinity is a major abiotic stress that severely limits plant productivity worldwide. It is estimated that more than 6% of the world's total land and approximately 20% of irrigated land are affected by salinity (Munns and Tester, 2008). High salt concentrations in the soil or in the irrigation water can have a devastating effect on plant metabolism, disrupting cellular homeostasis and uncoupling major physiological processes. A direct result of salt-induced cellular changes is an enhanced accumulation of reactive oxygen species (ROS), ultimately imposing a secondary oxidative stress in plant cells (Triantaphylides and Havaux, 2009).

Higher plants have developed many strategies to counteract salt stress, including selective ion uptake and exclusion, compartmentation of Na+ in vacuoles, detoxification of reactive oxygen species by the antioxidant system, and accumulation of osmo-protectants in the cytosol (Tuteja, 2007; Shi *et al*, 2000; Apse *et al* 1999). Salt tolerance is a complex phenotype that is controlled by multiple genes. Identifying novel genes/proteins, determining their expression patterns in response to salt stress, and exploring their functions in stress adaptation are the basis for effective engineering strategies to improve salt stress tolerance in plants (Waditee *et al*, 2005).

Brassica oilseed species now hold the third position among the oilseed crops and are an important source of vegetable oil (Ashraf and McNeilly, 2004). Rapeseed is sensitive to salt stress during the early stage of growth (Steppuhn*et al*, 2001), and this explains its classification as sensitive to salinity conditions at the mentioned stage (Francois *et al*, 1999). The response to salinity is a complex trait inherited quantitatively. Large-scale analysis of the transcriptome and proteome can provide global insight into the characteristics of salinity responses in plants, and help to deepen our understanding of the expression patterns and functions of the response-associated genes and proteins (komatsu *et al*, 2009).

To cope with salt stress, plants have evolved complex salt-responsive signaling and metabolic processes at the cellular, organ and whole-plant levels. However, our understanding of these mechanisms is incomplete because of the complexity of salt-induced stress, which has both an ionic component and an osmotic component (Munns and Tester, 2008). Proteomics offers a new platform for studying complex biological functions involving large numbers and networks of proteins and can serve as a key tool for revealing the molecular mechanisms that are involved in interactions between salinity and plant species (Zhang *et al*, 2012).

Variation of the plant proteome under salt stress has already been studied in several plants, among others in soybean (Aghaei *et al*, 2008), rice (Kim *et al*, 2005), wheat (Wang *et al*, 2008) and Arabidopsis (Jiang *et al*, 2007). Bandehagh *et al*. (2011) studied salt responsive proteins in canola leaves using a proteomic technique, in which the differentially expressed proteins were involved in a number of processes including oxidative stress, energy production, electron transport, signal transduction, translation, phosphate metabolic processes, and photosynthesis. As our knowledge, there are a few reports on the effect of salt stress on expression of proteins in canola through proteomics. On the other hand, leaves play major role in transporting essential minerals and water from the roots to aerial parts. Moreover, photosynthesis and cell growth are among the primary processes to be affected by salt stress (Munns and Tester, 2008). Hence, for better understanding of how plants respond and adapt to salt stress, it is important to focus on leaf system.

In this study, rapeseed protein profiles from NaCl-treated plants were monitored by a proteomics approach in order to elucidate the mechanisms by which plants respond to salt stress. So, proteins were separated by two-dimensional polyacrylamide gel electrophoresis and the responsive proteins were detected by mass spectrometry.

Materials and methods

Plant materials and growth conditions

The experiment was conducted in hydroponic culture system under greenhouse condition. Salt-tolerant *Brassica* genotype (Hyola 308) was subjected to 0, 150, and 300mM NaCl concentrations with three replicates. NaCl treatment was imposed gradually to 7-day-old seedlings. Three weeks after starting salt stress, plants were harvested from three independent biological replicates for physiological and proteomic analysis.Potassium and sodium content of leaves and roots were determined with a flame photometer.

Protein extraction

A portion (400 mg) of canola leaves was homogenized with 4- fold of phosphate buffer (pH 7.6) containing 65 mM K2HPO4, 2.6 mM KH2PO4, 400 mMNaCl and 3 mM NaN3 using glass mortar and pestle on ice. The homogenate was centrifuged 2 times at15,000g for 10 min at 4 8C. The supernatant after second centrifugation was incubated on ice by adding trichloroacetic acid to a final concentration of 10% to precipitate the proteins. After 30 min the solution was centrifuged at 15,000g for 10 min. The resultant precipitate was washed twice with pre-chilled ethanol and was resuspended in lysis buffer containing 8 M urea, 2% Nonidet P-40, 0.8% ampholine (pH 3.5–10.0) (GE Healthcare, Piscataway, NJ, USA), 5% 2-mercaptoethanol and 5% polyvinylpyrrolidone-40.

Two-dimensional polyacrylamide gel electrophoresis

The crude protein (500 mg, 100 mL) was separated by 2-DE [O'Farrell, 1975] in the first dimension by isoelectric focusing (IEF) tube gels and in the second dimension by SDS-PAGE. A prepared IEF tube gel of 11 cm length and 3 mm diameter consisted of 8 M urea, 3.5% acrylamide, 2% NP-40, 2% ampholytes (pH 3.5–10.0 and 5.0–8.0), ammonium persulfate, and N,N,N0 ,N0-tetramethylethylenediamine.

Electrophoresis was carried out at 200 V for 30 min, followed by 400 V for 16 h, and 600 V for 1 h. After IEF, SDS-PAGE was performed in the second dimension using 15% polyacryleamide gels with 5% stacking gels, followed by Coomassie brilliant blue (CBB) staining before drying of the gels. The isoelectric point (pI) andMr of each protein were determined using 2-DE Markers (Bio- Rad, Hercules, CA, USA).

Image analysis and data analysis

The analytical gels were scanned using GS-800 calibrated densitometer (BioRad) at 600 dpi resolution and analyzed using Melanie 4 software (GeneBio, Geneva, Switzerland). Quantitative comparison of protein spots was based on their percent volumes. One 2-DE gel per sample was run and percent volume of each spot was analyzed. The one-way ANOVA and comparison of treatment means were carried out by statistical analysis system (SAS) programs (version 9.1, SAS/STAT Software for PC. SAS Institute, Cary, NC, USA).

Protein identification

Protein spots were excised from CBB-stained preparative polyacrylamide gels. Proteins were identified using MALDI TOF/TOF MS (Applied Biosystems 4700, San Francisco, CA, USA) as previously described [Torabi*et al*,]. Combined MS-MS/MS searches were conducted with the selection of following criteria: NCBInr database (Release 28.10.2005; 2 928 294 sequences; 1 009 792 487 residues), all entries, parent ion mass tolerance at 50 ppm, MS/MS mass tolerance of 0.2 Da, carbamidomethylation of cysteine (fixed modification), and methionine oxidation (variable modification). The probability score (95% confidence level) calculatedby the software was used as criteria for correct identification.

Results

Effect of salinity on ionic relations

ANOVA showed a significant effect of salinity levels on the Na and K contents of leaves and roots, except k content of leaves (Table 1). Leaves and root K/Na ratios were significantly reduced due to salt stress Table 1). In order to investigate the effects of NaCl on the leaf of 300 mMNaCl-treatment was used for proteome analysis.

Protein identification

The MS analysis of expressed proteins resulted in the identification of 14proteins (Table 2). The identified proteins were involved in a number of processes including oxidative stress, photosynthesis, signal transduction, protein kinase, transcription and proteases.

Proteins involved in the response to oxidative stress included the copper/zinc SOD (spot 3301), Thioredoxin superfamily protein (spot 5302) and Peroxiredoxin Antioxidant (7401). Proteins involved in the photosynthesis included OEE2 (spot 6301), Ribulosebisphosphate carboxylase/oxygenase small subuni (7301) and Fructose-1,6-Bisphosphate Aldolase (spot 4501). Proteins involved in Signal transducthion included ABI2 (spot 3503), Response regulator 10 (spot 3602)and phospholipase d alpha 1(spot 1802). Proteins involved in protein kinase included CDPK-related kinase 3 (spot 3505) and AGC kinase family (spot 8703). RING/U-box protein (spot 2501) was identified as transcription factor. We also identified two proteins involved in ProteaseCLPP2 (caseinolyticprotease)(spot 5203) and RING-finger type ubiquitin ligases (spot 4702).

Table 1. Analysis of variance of Na content,K content and K/Na ratio of leaves and roots Hyola 308 under salinity stress.

		Mean square					
Source of variation	Degree of freedom	shoot Na	Shoot K	Shoot K/Na	Root Na	Root K	Shoot K/Na
Salinity	2	7887.079	785.931	60.567	923.4	36.425	1.116
Error	6	285.057	3.476	0.873	20.846	7.351	0.042
CV		27.12	6.54	32.92	13.07	13.7	26.88

**p<0.01, ***p<0.001, ns=non-significant

Discussion

In this study, Na content was increased under salt stress, but K content decreased. This result suggests that Na toxicity leads to damaging effects of NaCl in canola. Under salt conditions, Na+ enters roots passively through nonselective cation channels (Munns and Tester, 2008; Tester and Davenport,2003). Most of the Na+ can be pumped out of the root cells via root plasma membrane (PM) Na+/H+ antiporters (NHXs)(Tester and Davenport,2003). The remaining Na+ may be sequestered into vacuoles via tonoplast NHXs or transportedto the shoots through xylem (Munns and Tester,2008).

The K/Na ratio in the leaves and roots of control plants were higher than in salt-stressed plants .Therefore, the K/Na ratio decreased in the leaves and shoots in relation to salinity. The increase of Na+, Na+/K+ ratio, have been found in leaves and roots of canola under salinity (Bandehagh,2011;Ashraf, 2008-Na).

A high K+/Na+ ratio in the cytosol is essential for the normal cellular functions of the plants. Na+ competes with the K+ uptake through Na+–K+ cotransportersand may also block the K+ specific transporters of root cells under saline conditions (Zhu, 2003). In view of some reports, high K+/Na+ ratios and K+ vs. Na+ selectivityin plants under saline conditions have been suggested as one of the important selection criteria for salt tolerance (Wenxue*et al*, 2003).

The number of identified proteins was involved in ROS detoxification. These include chloroplastic copper/zinc SOD (spot 3301), Thioredoxinsuperfamily protein (spot 5302) and PeroxiredoxinAntioxidant (7401). Besides the primary ionic and osmotic stresses, salt inducesseveral secondary stresses including an oxidative stress through the accumulation of reactive oxygen species (Munns and Tester, 2008). This stress can cause oxidative damage to membrane lipids, proteins, and nucleic acids (Pang and Wang, 2008).

Antioxidant enzymes are the most important components in the ROS scavenging system [Meloni]. SOD is a major scavenger of O_2 , and its enzymatic action results in the formation of H2O2. In our experiment the abundance of copper/zinc SOD (spot 3301) and Thioredoxin superfamily protein (spot 5302) were increased. Therefore, these enzymatic systems mitigate the damaging effects of ROS. Accumulation of SOD in response to salt stress plays a protective role, and has been reported to occur in rice [Komatsu, 2004] and sugar beets [Hajheidari, 2005] in response to abiotic stresses. The abundance of another protein spot was identified as of Peroxiredoxin Antioxidant (spot 7401) decrease in response to salinity.This reduction might be due to production of high levels of hydroxyl radicals in this genotype. When plants are subjected to environmental stress conditions, such as high salinity, the balance between the production of ROS and the quenching activity of antioxidant enzymes is upset, and the degree of this imbalance shows the degree of sensitivity to stress [Sun,2006]. Polyphenol oxidase (spot 2602) can accelerate production of ROS in plant cells. Plant may use ROS as signaling molecules for increasing the production of oxidative stress tolerance enzymes during acclimation to high salt levels. As salt levels increase further, the production of detoxification oxidative tolerance enzymes may dominate ROS signaling effects [Mittler, 2004].

Table 2. Identification of salt stress-responsive proteins in leaves of canola.

ID on gel	Homologous protein	Exp.pI/MW (kDa)	Theo.pI/MW (kDa)	change	Accession no.[a])
3503	ABI2/ ABA INSENSITIVE 2	6.24/46.61	6.25/46.30	Increas	1009134375
3301	copper zinc superoxide dismutase	6.36/21.36	6.13/20	Increas	5689611
5302	Thioredoxin superfamily protein	7.35/26.23	7.14/26.05	Increas	1009110035
2501	RING/U-box protein	5.91/44.63	5.78/44.55	Increas	5019480528
3505	CDPK-related kinase 3	6.79/41.22	6.74/42.12	Increas	5019480528
4501	Fructose-1,6-Bisphosphate Aldolase	7.01/41.91	9.01/42.0	Increas	Q9LLD
3602	response regulator 10	6.36/61.77	6.23/61.65	Increas	1009125747
1802	phospholipase d alpha 1	5.85/91.4	5.70/91.84	Increas	1009123084
5203	CLPP2 (caseinolytic protease(	7.42/16.10	7.27/26.28	Decrease	1009128668
7401	Peroxiredoxin Antioxidant	7.88/35.37	8.20/32.22	Decrease	TC563
4702	RING-finger type ubiquitin ligases	6.93/64.62	6.97/64.51	Decrease	1009133717
7301	Ribulosebisphosphate carboxylase/oxygenase	7.80/23.95	8.8/20	Decrease	11990897
	small subuni				
6301	Oxygen-evolving enhancer protein 2(OEE2)	7.49/21.28	6.88/28	Decrease	131391
8703	AGC kinase family	8.59/63.32	8.54/63.17	Increas	1009127582

We observed downregulation of OEE2 (spot 6301) and Ribulosebisphosphate carboxylase/oxygenase small subuni in respons to salt stress.These proteins play important roles in photosynthesis, and their downregulationrevealed that photosynthesis is vulnerable to salt stress in canola.Downregulation of OEE2 in canola under salt stress has also been reported by Bandehagh*et al* (2011).

Salt stress causes reduced stomatalconductancethat leads to an anaerobic condition. Fructose-1,6-bisphosphatealdolase is upregulated, suggesting that this protein plays a role in acclimation to anaerobic conditions created by salt stress. Acclimated seedlings maintain a higher energy status during anoxia, and this is associated with a greater ability to synthesize ATP through glycolysis andethanolic fermentation [Abbasi, 2004], thereby increasing intracellular ATP formation.

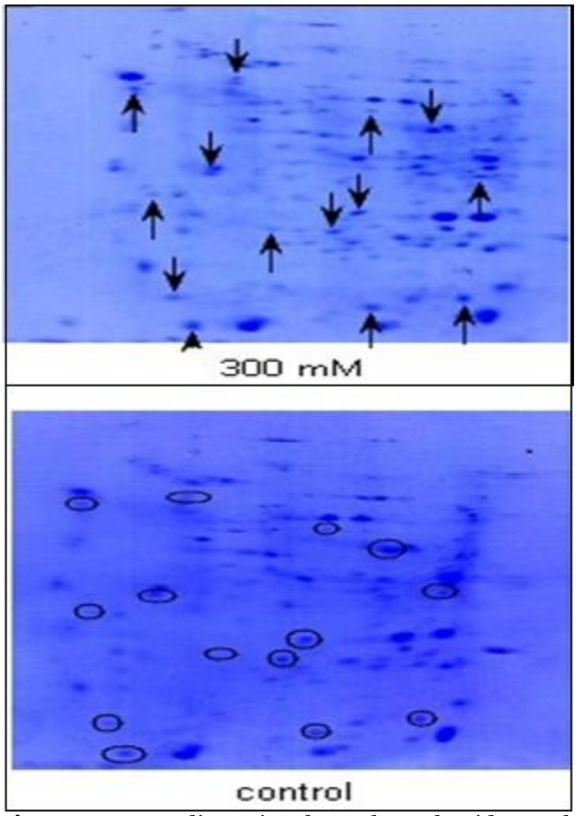

Fig. 1. Two-dimentional polyacrylamide gel electrophoresis of proteins extracted from leaves of Hyola 308 at 0 and 300mM NaCl and stained with Coomassie brilliant blue.

In our experiment, salinity stress resulted in anincreas in the abundance ofProteins involved in Signal transducthion included ABI2 (spot 3503), Response regulator 10 (spot 3602)and phospholipase d alpha-1(spot 1802).

Protein Abscisic acid-insensitive 2 (ABI2) (spot 3503) is a represor of the ABA signaling pathway, regulating various ABA responses like: stomatal closure, osmotic waterpermability of the plasma membrane, high light stress, response to glucose, seed germination and inhibition of vegetative growth. Involved in acquired thermotolerance (Dong *et al*, 2005).

Response regulator 10 (spot 3602) is a component of ARR proteins and it is transcriptional activator that binds specifically to the DNA sequence 5'-[AG]GATT-3'. Functions as a response regulator involved in His-to-Asp phosphorelay signal transduction system. Phosphorylation of the Asp residue in the receiver domain activates the ability of the protein to promote the transcription of target genes. Could directly activate some type-A response regulators in response to cytokinins (Hwang I and Sheen J, 2001).

Phospholipase D alpha-1 (spot 1802)plays an important role in various cellular processes, including phytohormone action and response to stress, characterized by acidification of the cell. Phospholipase D alpha 1- regulates abscisic acid signaling (Zhang *et al*,2004).

CDPK-related kinase 3 (spot 3505)and AGC group (spot 8703)upregulated in salt stress.CDPK-related kinase 3 (spot 3505) may play a role in signal transduction pathways that involve calcium as a second messenger By similarity. Serine/threonine kinase that phosphorylates histone H3. Confers thermotolerance; involved in the heat-shock-mediated calmodulin-dependent signal transduction leading to the activation of heat-shock transcription factors (HSFs); phosphorylates HSFA1A (Wang *et al*, 2004).

The AGC group (spot 8703) is named after the protein kinase A, G, and C families (PKA, PKC, PKG) which have a long history as cytoplasmic serine/threonine kinases that are regulated by secondary messengers such as cyclic AMP (PKA) or lipids (PKC) (Pearce *et al*, 2010).

RING/U-box superfamily protein(spot 2501) which acts as zinc ion bindingis expressed in 22 plant structures and during 14 growth stageswas identified as transcription factor. These proteins upregulated in response to salt stress.Hwang *et al* (2009), showed that RING/U-box superfamily protein, is involved in salt and drought stress tolerance of rice.

Two proteins involved in ProteaseCLPP2 (caseinolyticprotease)(spot 5203) and RING-finger type ubiquitin ligases (spot 4702)downregulated in response to salt stress thatmay be appropriate to maintain the structure of proteins under stress.

Caseinolytic proteases (ClpPs) are barrel-shaped self-compartmentalized peptidases involved in eliminating damaged or short-lived regulatory proteins (Benaroudj *et al,* 2011).

In this study a significant fraction of the proteins we detected are involved in photosynthesis, signal transduction and oxidative stress responses. These results suggest that photosynthesis-related proteins play an important biochemical role in the adaptation of canola leaves to high salinity conditions. Our results also suggest that these proteins are responsible for dealing with salt-inducedoxidative stress in the leaves of canola exposed to high salinity conditions.

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