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Original Article

Canola Seedling Response to NaCl Stress – a Proteomic Approach

Soheyla MOHAMMADI ALAGOZ, Mahmoud TOORCHI*, Ali BANDEHAGH

*University of Tabriz, Faculty of Agriculture, Department of Plant Breeding and Biotechnology, Imam Khomeini Street, Tabriz, Iran; Soheila.mohammadi1988@gmail.com; mtoorchi@tabrizu.ac.ir (*corresponding author); bandehhagh@yahoo.com*

Abstract

Salinity is a major abiotic stress worldwide, which causes a tremendous loss in the quality and quantity of agricultural products. In order to identify defense mechanisms to salinity-induced stress, a two-dimensional electrophoresis approach was used to investigate proteins achieved from canola cultivar 'Option 500' seedlings, after plants had been treated with NaCl. The 300 mmol NaCl treatment caused a significant decrease in fresh and dry weight as well as plant height and increment of proline content compared with control group. There were no significant changes in the quality of catalase and peroxidase activity. Out of 110 repeatable protein spots, 44 were registering change, according to induction factor index. 7 spots were recognized significant at 1% probability level statistically. These proteins were involved in energy production, ROS scavenging and suppressor, and those involved in photosynthesis. Production of chloroplastic fructose-bisphosphate aldolase enzyme along with carbohydrate metabolisms of Kelvin cycle and glycolysis increases under salinity condition. The amount of Cu/Zn SOD in chloroplast reduces in response to salinity which results from an increase in OH^- radicals. Expression of photosynthesis proteins have been reduced probably as a consequence of the drop in osmotic pressure resulted from water scarcity and stomata closure, which reduces the availability of Co_2.

Keywords: catalase, down-regulation, peroxidase, proline, two-dimensional electrophoresis (2DE), Up-regulation

Introduction

Canola (*Brassica napus* L.) refers to a variety of rapeseed containing low contents of two undesirable constituents' erucic acid and glucosinolate in the seed and meal, respectively (Kandil *et al*., 2012). Canola holds the largest portion of cultivation among oil plants in Iran. Salinity a menace to crop production has afflicted agriculture more than ever. It leaves undesirable effects on the production and quality of the crops, through increasing the osmotic pressure, imbalanced ion exchange, and malnutrition due to impeded uptake of necessary ingredients (Temizel *et al*., 2014; Odabas *et al*., 2014). Plus, it interferes with cellular metabolic pathways and increases the production of reactive oxygen species commonly known as ROS (Zhu, 2002). These are very active molecules and radicals which damage proteins, lipids in the plasma membrane structure and nucleic acids under stress condition which leads to their over production, which eventually kills the cell (Morant-Manceau *et al*., 2004). Plants have developed defense mechanisms to mitigate the harmful effects of stress-induced over-production of ROS, including both enzyme such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX or POD), and Ascorbate peroxidases (APX) and non-enzyme anti-oxidants (such as proline, betaine, ascorbate) (Silva *et al*., 2008). Enzymes scavenge ROS, and organic compounds contribute to balanced osmotic pressure (Rhodes and Hanson, 1993), enzyme activity (Mansour, 2000), ROS detoxification (Ashraf and O'Leary, 1994), and protect plasma integrity (Bohnert and Jensen, 1996).

Salinity stress in early stages can lower attributes related to length and weight of the rootlet and young stem as well as limiting the expansion of leaf in seedling (Jianjie *et al*., 2013; Temizel, 2015). Canola is considered as a moderate tolerant to salinity, next to wheat and barley can keep half of its normal growth and productivity under root-zone condition between 4-8 dSm^{-1} (Houle *et al*., 2001). Canola cultivars show a different percentage of germination and seedling dry weight under salinity stress (Ghasemi and Esmaeilpour, 2008).Canola is more sensitive to salinity at speed germination and seedling growth (Cuartero *et al*., 2006). Also, the variation in response to stress indicates the importance of the genetic role in salt tolerance.

One way to identifying genetically potentially tolerant cultivars is proteomics. Its use has been promising in tracking down genes and proteins involving in salt tolerance, by

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differentiating the proteins through two dimensional electrophoresis (2DE) (Chen and Harmon, 2006). It also makes the investigation of dynamic expression of proteins possible (Guo *et al*., 2012). Bandehagh *et al*. (2011), studied salt tolerance in canola by extracting proteins from seedling′s leaves. The results indicated that Na accumulation in sensitive cultivars is higher than tolerant cultivars. From 900 detected protein spots, 44 and 31 reproducible spots were found in tolerant and sensitive cultivars, respectively.

Considering the increasing demand for organic oil, there is an urgent need for study of canola response to salinity stress with an aim to narrow the gap currently existing between the oil production and consumption.

Materials and Methods

Plant materials and traits measurement

Plant materials used in this experiment included canola cultivar, 'Option 500'. Plants were grown in hydroponic culture system as explained by Bandehagh *et al*. (2008). 34 day-old seedlings were treated with 300 mM of NaCl. After 10 days imposing NaCl stress treatment, collected leaves immersed in liquid nitrogen and stored at -80 °C until protein extraction. Fresh weight (PFW) and plant height (PH) were measured besides sampling. Samples were dried by the oven at 70 °C for 48h then dry weight measured.

Measurement of free proline

Proline content was estimated by using ninhydrin reaction method (Bates *et al*., 1973). 0.2 g of stored leaves was homogenized with 5 mL, 3% sulphosalicylic acid and centrifuged at 25 °C for 7 minutes. Then, 1 mL of the filtered extraction was taken for the analysis to which 1 mL of ninhydrin reagent and 1 mL glacial acetic acid were added. The mixture was incubated in 100 °C water bath. The reaction mixture was extracted with 2 mL toluene, and absorption of chromophore was measured at 250 nm against toluene as blank using spectrophotometer (Beckman, Fullerton, CA, USA). Proline content was calculated from an L-proline standard curve.

Enzyme extraction

0.5 g of the fresh leaf was ground in liquid nitrogen to a fine powder, to which, then, 1.5 ml buffer phosphate 50 mol (pH=7.5) containing PVP 1% and 2 mmol EDTA was added, and ground some more. The obtained extract was spun a centrifuge set at 20000 g at 40 °C for 20 minutes. The clear, floating supernatant was used assay catalase (CAT) and peroxidase (POX).

Peroxidase assay

POX's activity was evaluated according to (MacAdam *et al*., 1992). 0.5 g of the root specimen was homogenized in cold potassium phosphate buffer 0.1 mol (PH=7.5) containing 0.5 m mol EDTA. Homogenized samples were centrifuged at 15000 g for 15 min. 20 µl of the homogenized supernatant was added to 0.81 ml potassium phosphate buffer 0.1 mol (pH=6.6). 90 µl of 1% guaiacol was added to the result. The solution was poured in a cuvette. And, 90 µl of 3% H_2O_2 was added before measuring the reaction speed as electron receptor. Absorbance was measured at 470 nm at 25 °C for 60 seconds in a spectrophotometer. Enzymatic changes recorded as the change in absorption in minute mg^{-1} protein.

CAT assay

For CAT, the assessment carried out as described by (Pereira *et al*., 2002). First, 3.5 ml of peroxide hydrogen 3% was taken and mixed with 50 ml double distilled water. Then From the solution, 17.5 µl was taken and mixed with 707.5 µl potassium phosphate buffer free of PVP and EDTA. Afterwards, 20 µl of the sample was added. Finally, absorption was recorded at 240 nm at 25°C for 60 seconds in the spectrophotometer.

Leaf proteins extraction and quantitation

Total protein were extracted from approximately 0.5 g of frozen leaf per each biological replicate and ground to a fine powder in cold acetone containing 10% TCA and 0.07% 2-Mercaptoetanol. The resultant powder dissolved in Lysis buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 60 mM DDT and 1% ampholyte (pH: 3-10). Protein concentration was determined by Bradford assay (Bradford, 1976).

Two-dimensional electrophoresis and image analysis

The crude protein (400mg, 100 µL) was separated by 2-DE (O'Farrell, 1975). First dimensional separation was by isoelectric focusing (IEF) in tube gels and the second stage, by SDS-PAGE. An IEF tube gel of 11 cm length and 3 mm diameter consisted of 8 M urea, 3.5% polyacrylamide, 2% NP-40, 2% ampholyte (pH: 3-10 and 5-8), ammonium persulfate and TEMED was prepared. The voltage set for the IEF were 200 V for 30 min, 400 V for 16 h and 600 V for 1 h. Tube gels were, then, retrieved from the glass tubes and subjected to the second dimensional (SDS-PAGE) by transferring on to a 15% acrylamide separating gel and 5% acrylamide stacking gel. The gels were stained with coomassie brilliant blue (G-250).

The analytical gels were scanned using GS-800 calibrated densitometer (Bio-Rad) at 600 dpi resolution. The scanned gels were saved as TIFF images for subsequent analysis using PDQuest software (version 8.0, Bio-Rad). Spot detection, spot measurement, background subtraction and spot matching were performed. Following automated spot detection, gel images were carefully edited. Three well-separated gels of each treatment were used to create replicate groups. Statistic, quantitative and qualitative "analysis sets" were created between each control group and corresponding treated group. The quantity of the protein spot was expressed as the intensities of all the proteins on gels determined by the migration of protein spots along the 11 cm IEF (5-8 linear).Protein identification was performed using searching program against the NCBI canola protein database, matching the pI and MW values of the changed spots with those from the protein databank.

Statistical analysis

Statistical analysis was performed based on t-test for all the traits studied using IBM.SPSS Statistics v21 computer software package. Significant differences among means were shown with different letters at the 1% probability level. Three repeats were applied to measure proline characteristics and enzymes whereas seven repeats were used to measure the other characteristics.

Results and Discussion

Comparison of the means revealed that shoot fresh weight (g), dry weight (g), plant height (cm) and proline content (mg g^{-1}FW) were significantly changed at 1% (Table 1). Also, CAT and POX did not show significant changes.

Protein identification

The results indicated that out of 110 repeatable spots in cultivar 'Option 500', 44 spots showed significant changes in expression, according to IF index. After performing T-test, 7 spots proved significant. Spot A was up-regulated, while B, C, D, E, F, and G were down-regulated. The results for Two-dimensional Electrophoresis are illustrated in Fig. 1. Information about the protein spots is provided in Table 2. Fig. 2 provides information on changes in expressions of the proteins in the treatments and control.

Salt stress causes a decline in crop growth in numerous ways such as; oxidative stress, particular ion toxicity, nutritional disorders and osmotic stress (Ashraf, 2009). Salt stress in seedling stages results decrease in growth related traits like rootlet length, plumule length, the fresh and dry weight of rootlet, plumule and leaf weight and area (Jianjie *et al*., 2013). It's believed that measure of variation in dry weight in stress condition to dry weight in normal condition (no stress) or relative root/shoot dry weight can be used as most important measurement of selection and classification of tolerant and sensitive genotypes (Kandil *et al*., 2012). In early stages of salinity, plant experiences water stress that reduces leaf expansion. Osmotic effects of salt stress frequently seen after use of salt, by the resumption of stress it affects cell growth and development and causes stomata cells closure (Flowers, 2004). In long -range exposure to salinity plant faces ion stress that can cause early aging in elder leaves with a decline in photosynthesis of growth supporting available area. Early aging by ion stress in elder leaves and toxicity signs in mature leaves because of high sodium concentration results disorders in protein producing and enzymes activity (Munns, 2002), thus by the negative effect on photosynthesis and CO_2 fixation reduces nutrients absorption and finally plant growth (Cha-um and Kirdmanee, 2009). Salinity negative effect through osmotic potential is stimulated by salt stress in the plant because of root cells disability in water absorbance (Mer *et al*., 2000).

For biosynthesis of particular physiological proteins, plant increases proline amount. Effects of salinity on increasing praline concentration has been reported in rice, wheat and canola (Farshadfar and Sutka, 2002). Proline is an amino acid as a radical absorbent osmolyte, electron source for macro-molecules fixation and member of the cell wall (Matysik *et al*., 2002). Compatible solutes as osmotic protections provide: 1) positive effects on cellular units 2) the conversation of subcellular construction 3) increasing cell osmotic potential in the plant under stress condition (Ashraf and Foolad, 2007). These protective osmotic compounds include proline, betaine glycine compounds (Belkheiri and Mulas, 2011). It's believed that proline has compatibility role in plant tolerance to stress. Proline is known as; 1) compatible osmolyte 2) molecular chaperons stabilizer in protein structure 3) carbon and nitrogen storage mechanism 4) cellular reduction condition equilibrium 5) part of stress signals effective on compatibility responses (Verbruggen and Hermans, 2008). Accumulation of proline might cause proteolysis increasing or protein synthesis reducing. For plant high concentration of proline in salt stress condition is proper as interferences with osmotic potential of leaf and osmotic adjustment. In addition to having a role as an osmolyte, proline is able to increase stability and solidity of the membrane in different situations as an enzymatic protector. Also, accumulation of proline has a non-enzymatic role in detoxification of free radicals (Khan *et al*., 2002). It seems increasing in proline concentration is not the reason for salt

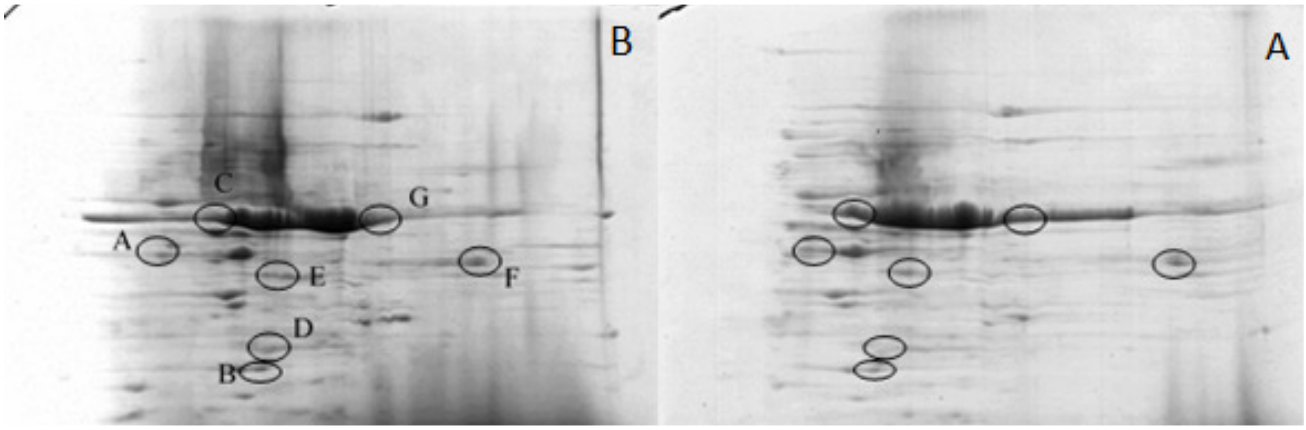

Fig. 1. Protein spots detected using 2DE performed on proteins extracted from canola leaves under (A) control, (B) treatment (300 mmol NaCl) conditions

Table 1. Comparison of the means of NaCl stresses on studied attributes in canola

Treatment	Fresh weight (g)	Dry weight (g)	Plant height (cm)	Proline (mg g^{-1}FW)	CAT (M min^{-1})	POX (µmol min^{-1})
Normal	8.3642^{a}	0.7121^{a}	24.8571^{a}	16.5577^{b}	2.7467	0.1633
Stress	4.2714^{b}	0.3850^{b}	16.3928^{b}	561.7558^{a}	0.9100	0.6367
T	3.171^{**}	3.174^{**}	4.531^{**}	-5.853^{**}	1.195^{ns}	-1.510^{ns}

** And ns represent significant at 1% and non-significant, respectively

Table 2. Detailed information about proteins undergone changes under 300 mmol NaCl influence

Expression change	Protein	pI/MW Theo.	pI/MW Exp.	Accession number	Spot
Increase	Chloroplastic fructose-bisphosphate aldolase	5.9/42.21	5.5/41.3	ACM78035	(A)
Decrease	Copper/Zinc SOD	6.74/22	5.92/22.05	5689611	(B)
Decrease	subunit Ribulose 1,5-bisphosphate carboxylase, larg	6.09/52	5.28/50.68	52001641	(C)
Decrease	Copper/Zinc SOD	6.28/22	6.03/24.96	3273753	(D)
Decrease	PSBO-2/PSBO$_2$(PhotosystemII subunit O-2)oxygen evolving	5.92/35	6.08/38.02	15230324	(E)
Decrease	PRK(Phosphoribulokinase); ATP binding	5.71/44	7.31/43.2	15222551	(F)
Decrease	subunit Ribulose 1,5-bisphosphate carboxylase, larg	6.09/52	6.62/52	52001641	(G)

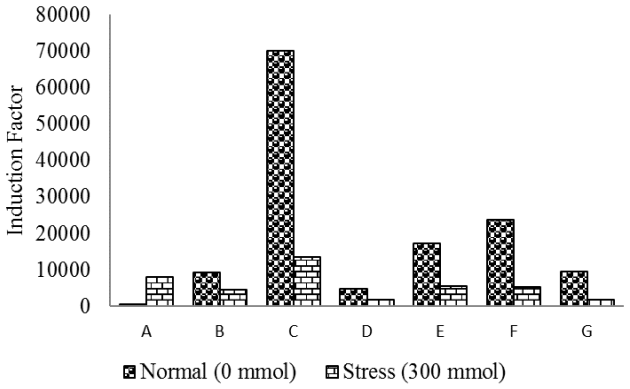

Fig. 2. Changes in expression drown from protein spots detected in canola in control and treatment (300 mmol NaCl)

stress tolerance but consequences of salinity (Lacerda *et al*., 2003). Likewise, proline content has significantly increased in our study. Research has shown that proline is involved in setting osmotic pressure intra-cellular, intercellular as well as in vacuoles and it is suggested that proline can help in stabilizing cell structure, or even scavenge ROS (Ashraf and Foolad, 2007) such as H_2O_2, OH^- and O^-_2.

ROS are by-products of vital metabolism pathways, and play important roles in signaling and transfer (Foyer and Noctor, 2000). However, when the plant is under stress, their production surpasses their normal scavenging. It is called ROS stress.

Besides proline, many other mechanisms are used by plants to stop ROS-stress from escalation including enzymes such as SOD, POX and CAT (Apel and Hirt, 2004). SOD is the first anti-oxidant defense factor called upon by plant. It changes superoxide to H_2O_2 and oxygen with less negative effects (Sigaud-Kutner *et al*., 2002). CAT and POX constitute a more efficient system to scavenge H_2O_2, which may contribute to salt tolerance (Lin and Kao, 2000). Increased activity of antioxidant enzymes upon salt stress, their involvement in salt tolerance in plants (Gao *et al*., 2008). However, influenced by some mechanisms, unknown to us, amount and activity of these enzymes remain unchanged. In experiments on potatoes (Benavdes, 2000), and rice (Lin and Kao, 2000), for example, researchers found no change in CAT activity, or in cultivars Slm and Opera of canola, there was no change in the activity of POX under salinity stress (Farhoudi *et al*., 2015).

In this experiment, some proteins spotted in more than one stain; among which are Cu/Zn SOD (spots B and D) and large subunit of Ribulose 1,5-bisphosphate carboxylase, (C and G). Proteins with more than one occurrence have previously been detected by other researchers (Caruso *et al*., 2008; Wang et al. 2008; Gao *et al*., 2011 and Bandehagh *et al*., 2011) the reason for which has been explained as existing protein isoforms, post-translation modifications, alternative splicing of mRNA and protein breakage. Post-translation modification such as glycosylation and phosphorylation can change molecular weight and isoelectric focusing of the proteins (Caruso *et al*., 2008).

Protein A probably represents Chloroplastic fructose-bisphosphate aldolase with up-regulation (Fig. 1). There are two known forms of this enzyme namely chloroplastic and cytosolic. The former is involved in Kelvin cycle by transmuting D-glyceraldehyde 3-phosphate and dihydroxyacetone into Fructose 1, 6-bisphosphate. Also, Fructose bis phosphatase hydrolyses Fructose 1, 6-bisphosphate into fructose 6-phosphate. Fructose bisphosphate aldolase transmutes Fructose 1, 6-bisphosphate into D-glyceraldehyde 3-phosphate and dihydroxyacetone. Production of this enzyme along with carbohydrate metabolisms of Kelvin cycle and glycolysis increases under salinity condition. As has been reported by researches in rice where Fructose-bisphosphate aldolase peaked with increase in glycolytic enzymes and enzymes involved in anaerobic glycolytic and glycolate metabolism (Abbasi and Komatsu, 2004), as well as a peak in Alcohol dehydrogenases in canola (Sobhanian *et al*., 2010) under salinity influence. It has also been hypothesized that Fructose-bisphosphate aldolase can contribute to ion division in vacuoles, since it is directly interact with H^+-ATP and active transportation (Barkla *et al*., 2009).

Spots B and D are probably Cu/Zn SOD. They experienced down-regulation (Fig. 1). There are three distinct groups of SODs. One of them is associated with Zn and Cu for its activity, mostly it depends on Zn; scarcity of Zn for any reason reduces the enzyme's activity (Sun *et al*., 2006). It has been reported that the amount of Cu/Zn SOD in chloroplast reduces in response to salinity (Komatsu and Tanaka, 2004), which results from an increase in OH^- radicals (Sun *et al*., 2006).

Protein spots C, E, F and G presumably involved in photosynthesis, showed down-regulation (Fig. 1). C and G are likely to be related to large/small subunits of RubisCo; their expression have been reduced probably as a consequence of the drop in osmotic pressure resulted from water scarcity and stomata closure, which reduces the availability of Co2. There, reduction in abundance of RubisCo large/small subunit, chloroplast precursor (OEE), Oxygen-evolving enhancer protein 2, carbonic anhydrases as well as Rubisco's activity leads to short assimilation. Fall in assimilation has been reported for many glycophyte plants subjected to salinity stress (Aghaei,

2008; Pang *et al*., 2010; Sobhanian *et al*., 2010; Chatlopadhyay 2011 and Bandehagh *et al*., 2011). However, in some sensitive plants, including durum wheat, an increase in activity of RubisCo is thought to be a response rendered to support the carboxylase activities of RubisCo (Caruso *et al*., 2008).

The F spot is probably related to Phosphoribulokinase (PRK), which has been down-regulated under salinity influence (Fig. 1). The same results have been reported for durum wheat (Caruso *et al*., 2008) and Canola (Bandehagh *et al*., 2011). The CO2 assimilation is said to be hampered in response to the reduction in the amount of enzymes related to thereof; PRK accelerates the ATP phosphorylation dependent on Ribulose bis-phosphate into Ribulose 1,5-bisphosphate, which is considered an important point in pentose phosphate pathway towards CO_2 assimilation. The fall in the amount of RubisCo under salinity could be also due to salinity-induced ROS stress which culminates in protein damage (Kingston-Smith and Foyer 2000 and Feller *et al*., 2008). Plus, a peak in the amount of OH^- leads to breaking down of large subunits the same can happen during protein extraction process (Salekdeh *et al*., 2002).

The E spot in cultivar 'Option500' is Photosystem II subunit O-2 oxygen evolving (PSBO-2/PSBO2), which showed down-regulation (Fig. 1). This protein belongs to the Oxygen evolving family and works to stabilize Mg group as the principal location for breaking down water molecules (Kumar *et al*., 2003).Any reduction in activity of this enzyme may indicate the serious influence of salinity a basic component of photosynthesis namely oxygen release and PSII complex, which eventually leads to leaf senescence and death (Komatsu and Tanaka, 2004).

Conclusions

In this research, application of salt stress on a Canola cultivar created significant changes in dry and fresh weight, plant height as well as proline content. No significant changes were found in CAT and POX. It seems that protein expression increase of Chloroplastic fructose-bisphosphate aldolase to be due to increased carbohydrates metabolism in the Calvin cycle and glycolysis, the frequency of SOD has dropped due to higher production of ROS. Osmotic stress of salinity causes stomatal closure, reduction of access to CO_2 and finally led to reduction of photosynthetic proteins PRK, PSBO-2 / PSBO2 and RubisCo large subunit.

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