

The Proteome Response of Salt-Sensitive Rapeseed (*Brassica napus* L.) Genotype to Salt Stress

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Abstract

Productivity of rapeseed (*Brassica napus* L.), the third most important oilseed crop, was reduced more than other crops under the salt stress higher than the threshold. Thus, breeding, especially at seedling stage, seems necessary. Plants under salt stress, by synthesis of essential metabolites, specific structural proteins or enzymes of metabolic pathways deal with the stress. To identify the molecular mechanisms of salt responsiveness in rapeseed, 'Option500' a salt-sensitive genotype was exposed to 0, 150, and 300mM NaCl during the seedling stage. An increase in proline and the Na⁺ content of leaf and a reduction in shoot dry weight, plant height, K⁺ content and K⁺/Na⁺ ratio were observed. Protein expression changes were examined by two-dimensional electrophoresis (2-DE). Out of 110 protein spots identified by 2-DE gels, 37 spots showed significant abundant changes based on induction factor (IF), and 7 spots were recognized significantly at 5% probability level, which 1 and 6 spots were up and down-regulated, respectively. By using LC-MS/MS mass spectrometry analysis, proteins were identified which are involved in energy production and photosynthesis. Activity of enzymes involved in energy production decreased under stress, while the abundance of Phosphoribulokinase (PRK) -an important enzyme in the pentose phosphate pathway- increased.

Keywords: abiotic stress; proteomics; rapeseed; salinity; two-dimensional electrophoresis

Introduction

Soil salinity is a global problem that through the effect on plant growth and limiting exploitation of agricultural lands has restricted the productions of agricultural crops (Joseph *et al.*, 2010). Salinity as one of the most important abiotic stresses in arid and semi-arid areas reduces the average of crop yield about 50% (Kandil *et al.*, 2012). Oil seeds have the third place in the human food needs after cereals and beans. And in the meantime, oily species of *Brassica* are allocated the third place among oil seeds (Shirazi *et al.*, 2011). Rapeseeds with high proteins and lipids and due to the low amount of saturated fatty acids (less than 4% palmitic acid) and a relatively large amount of oleic acid (60%) and α -Linolenic acid (9%), in comparison to sunflower, maize and soybean oils have high nutritional quality (Gunstone, 2004). For optimal use of the saline soils, salinity control through planting tolerant genotypes is one of the basic strategies, because soil remediation needs more time and it is not economical (Purty *et al.*, 2008). Since soil and water salinity are the most important limiting factors of Rapeseed production, breeding and using the tolerant genotypes to salt stress can be a good strategy to preserve the

production levels in the face of salinity stress (Ashraf and Akram, 2009).

Salinity reduces or delays the germination of most crops (Farhoudi and Sharifzadeh, 2006; Benincasa *et al.*, 2013). It also reduces some traits such as root length, shoot height, root and shoot dry weight and leaf area index at seedling stage (Miyamoto *et al.*, 2012; Haq *et al.*, 2014). Although, salinity affects all stages of plant growth, but germination and seedling stages are known as the most critical stages in most crop species (Munns, 2002).

The rate of photosynthesis and transpiration, stomatal conductance, chlorophyll content and leaf area decreased while sodium content of soil increasing. Reducing the rate of photosynthesis under salt stress can be happen due to a reduction in stomatal conductance, impaired metabolic processes, especially in carbon absorption, inhibition of photochemical capacities or a combination of all items (Ashraf and McNeilly, 2004). Losses of cell turgor, as the result of decreasing soil water potential, cause stomata closure and therefore reduce the rate of photosynthesis (Gul *et al.*, 2014). The synthesis of chlorophyll is affected by high salt concentration and the salt effects on the synthesis of chlorophyll (*a*) are much greater (Jamil *et al.*, 2014).

High concentrations of cations such as Na⁺ which is the most common element of salinity can disrupt the biochemical processes in plant tissues, or they can prevent their works and change the synthesis of required proteins quantitatively and qualitatively, which in turn disrupt growth process (Ashraf and McNeilly, 2004). On the other hand, a high concentration of sodium disturbs the nutritional balance and also causes ionic toxic and osmotic regulation disorder. In response to salt stress, osmotic adjustment or the accumulation of organic compounds (e.g., proline) and minerals (such as Na⁺ and K⁺) occurs in cells that causes absorption of water into the cells and saves cells turgor (Nayyar, 2003; Shirazi *et al.*, 2011). Soluble organic compounds such as sugars, organic acids, polyols and many compounds containing nitrogen such as amino acids, amides, proteins and ammonium compounds are involved in osmotic adjustment and enzymatic activity of plant species. Therefore, such organic osmotic compounds can play a key role in the tolerance to salt stress (Ashraf and McNeilly, 2004). By studying the effect of salinity on plants and plant's response to stress, it can be assumed that the free amino acids have more critical role than other organic soluble osmotic compounds (Giannakoula and Ilias, 2013). Also proline has found more than other amino acids in plants under stress and this amino acid plays an important role in adjusting the accumulation of usable nitrogen, osmotic adjustment and membrane stability (Parida and Das, 2005).

Environmental stresses usually cause protein disorders (Joseph and Jini, 2010). Plants that face stress conditions, synthesize some essential metabolites to overcome the situation (Turan *et al.*, 2012). This action is performed by changing the expression of genes in order to reduce or increase the amount of specific structural proteins or enzymes of metabolic pathways (Joseph *et al.*, 2010). Proteomics approach is a powerful and suitable tool to examine the expression changes and to identify the proteins that respond to abiotic stresses (Sobhanian *et al.*, 2011). Proteomics analysis has been reported as one of the best strategies to study the dynamic expression of proteins under salt stress (Guo *et al.*, 2012). Comparative proteomics of plants before and after stress have given a lot of information about the tolerant mechanisms of plants against environmental stresses (Kamal *et al.*, 2010).

This study was designed and performed to improve our understanding of physiological mechanisms of salt tolerance in rapeseed at seedling stage by exploring and identifying changes that occur in the structural proteins or enzymes in metabolic pathways under salinity by use of proteomic approaches. For identification of proteins and molecular pathways involved in the development of tolerance to salinity and determining candidate genes, the LC-MS/MS mass spectrometry technique was used.

Materials and Methods

Plant material and growth conditions

In this study, susceptible spring rapeseed 'Option500' (Dolatabadi *et al.*, 2016) which obtained from Seed and Plant Improvement Institute (SPII) Karaj-Iran, was evaluated under salt stress at seedling stage by using hydroponic culture which they were irrigated four times daily with a modified Hogland nutrient solution. Three

levels of NaCl salinity (zero (control), 150 and 300 mM) was applied gradually (50mM per day) one week after planting the plantlets.

Physiological traits analysis

Twenty-eight days after exposure to salt stress, and at the end of the seedling stage, shoot dry weight, plant height, content of leaf proline, sodium and potassium were measured. To measure proline, samples of leaf number three were frozen by liquid nitrogen and were transferred into the -80 °C freezer. Proline content of leaf was measured by ninhydrin method (Bates, 1973). Dry weight of samples was determined after drying at 75 °C for 48 hours. The dried samples were used to measure Na⁺ and K⁺ content. The samples were solved in nitric acid (7.2N) and heated on hot plate. Sodium and potassium ions were measured by flame photometer.

Statistical analysis

Analysis of variance was performed in randomized complete block design with three replications and Duncan's multiple range tests were used for the means comparison. Statistical analyses were performed by MSTATC, IBM SPSS Statistics v21 and Excel computer programs.

Protein extraction and two-dimensional electrophoresis

Samples of leaf number three under the zero and 300 mM treatment of sodium chloride, in three replications were packed in aluminum foil separately and frozen in liquid nitrogen and kept in a -80 °C freezer until the protein extraction. Leaves total protein extraction were done by TCA/acetone method (Salekdeh *et al.*, 2002). In this method, 0.5 g of frozen leaf samples were powdered and dissolved in lysis buffer (7M urea, 2M thiourea, 2% CHAPS, 1% ampholyte (pH: 3-10) and 60 mM DDT) by centrifuging at 20000g for 20 min at 25°C. To determine protein concentrations Bradford method (Bradford, 1976) was used. The protein extract (400 µg, 100 µL) was separated by 2-DE. The first dimension electrophoresis was performed by 11 cm length and 3 mm diameter with 8 M urea, 3.5% polyacrylamide, 2% NP-40, 2% ampholyte (pH: 3-10 and 5-8), ammonium persulfate and tetramethylethylenediamine (TEMED) IEF tube gels. The first dimension electrophoresis was done in three-phase (200 V for 30 minutes, 400 V for 16 hours and 600 volts for 1 hour). The second dimension was done by SDS-PAGE method using 15% polyacrylamide separating gel and 5% acrylamide stacking gel for about 3 hours at 35 mA. The gels were stained with Coomassie brilliant blue (G-250).

Image analysis and protein identification

The 2-DE gels were scanned with GS-800 (Bio-Rad) and analyzed with PDQuest ver. 8.0.1 (Bio-Rad). Spots with significant expression changes were identified by Student's *t*-test. After identifying protein spots with significant differential abundant, the spots were sent to Alberta Proteomics and Mass Spectrometry Facility for mass spectrometry analyzing. Protein spots were identified by LC-MS/MS mass spectrometry method and Sequest software. Combined LC-MS/MS selections with the searches of UniprotKB database were used to the final identification of the proteins.

Results

Between salinity levels, in all traits significant differences were observed (Table 1). Variance analysis showed that the differences between salinity levels in all traits were significant at 5% probability. Changes in the traits under salinity are shown in Fig. 1. In 300 mM, highest content of proline and, in control condition, lowest content have been observed (Fig. 1). Two-dimensional electrophoresis of genotypes and protein spots analysis by using PDQuest software led to the identification of 110 repeatable protein spots in gels (Fig. 2). 44 spots among identified 110 spots, based on IF index, showed significant abundant changes, and after Student's *t*-test, seven protein spots were significantly changed at 5% probability level, which spot 2405 was up-regulated and spots 3102, 3502, 4104, 4303, 8404 and 8501 were down-regulated (Fig. 3). The detected proteins which were identified by LC-MS/MS mass spectrometry were involved in energy production and photosynthesis (Table 2). Spot 2405 which had increased

the abundant under salt stress was probably Phosphoribulokinase (PRK) that was involved in the production cycle of carbohydrates (Caruso *et al.*, 2008). Spots 3502 and 4303, respectively, were related to beta and alpha subunits of chloroplast ATP synthase which involve in the production of ATP (Kang *et al.*, 2012). Spot 4104 was related to Triosephosphate isomerase (TPI) which plays a key role in the energy production cycle (Sharma *et al.*, 2012). Spot 8404 which related to Glyceraldehyde-3-phosphate dehydrogenase is involved in carbon dioxide pathway (Tanou *et al.*, 2009). Spot 8501 which was identified as ribulose -1,5 - biphosphate carboxylase /oxygenase (RuBisCO), has a key role in the stabilization cycle of carbon dioxide (Guo *et al.*, 2012). Spot 3102 which showed an expression reduction under salt stress, although have been reported on rapeseed previously, and it has an access code in UniprotKb database, but still not identified very well (Table 2). Identifying its structure and function precisely, certainly will help to better understand the rapeseed's tolerance mechanism to salt stress

Table 1. Analysis of variance for studied traits in 'Option500' under salinity treatments

Source of Variance	Degrees of Freedom	Mean Square					
		Shoot Dry Weight	Shoot Height	Leaf Na ⁺	Leaf K ⁺	Leaf K ⁺ /Na ⁺	Leaf Proline
Replication	2	0.008 ^{ns}	5.7 ^{ns}	271.3 ^{ns}	217.9 ^{ns}	0.488 ^{ns}	468.7 ^{ns}
Salinity (S)	2	0.264 [*]	136.7 [*]	16856.8 ^{**}	805.6 [*]	22.2 ^{**}	2056.6 ^{**}
Error	4	0.022	12.9	323.712	67.040	0.431	105.312
CV%		38.78	19.27	21.02	28.95	37.92	26.96

* Significant at the 5% probability level; ** significant at the 1% probability level; ns: Not significant.

Table 2. Identification of differentially abundant proteins of 'Option500' after salt stress

Spot ID	Protein name	Accession number ^a	Reference species	Cov. (%) ^b	Score ^c	pI/MW		IF ^d	UPs ^e / P _s ^f	PSMs ^g / AA ^h	Function
						Theo.	Exp.				
2405	Phosphoribulokinase	A0A078GC16	<i>Brassica napus</i>	72.29	762.17	5.77/44.4	5.5/41.30	14.46	3/21	301/397	Carbohydrate metabolic
3102	BnaA09g07850D protein	A0A078FW31	<i>Brassica napus</i>	40.93	21.27	5.72/23.5	5.92/22.05	0.48	6/6	7/215	Unknown
3502	ATP synthase subunit beta, chloroplastic	D1L8P5	<i>Brassica napus</i>	36.75	42.02	5.26/53.7	5.82/50.68	0.19	12/12	14/498	ATP synthesis
4104	Triosephosphate isomerase	A0A078CJ83	<i>Brassica napus</i>	65.35	94.13	5.73/27.2	6.03/24.96	0.39	4/13	37/254	Glycolytic
4303	ATP synthase subunit alpha, chloroplastic	D1L8M3	<i>Brassica napus</i>	13.41	11.81	5.20/55.3	6.08/38.02	0.32	5/5	5/507	ATP synthesis
8404	Glyceraldehyde-3-phosphate dehydrogenase	A0A078EFC4	<i>Brassica napus</i>	40.85	591.80	7.75/42.7	7.31/43.20	0.20	5/15	215/399	Glucose metabolic
8501	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Q71SX0	<i>Brassica napus</i>	56.58	637.91	6.29/52.9	6.62/52.76	0.22	27/27	236/479	Carbon fixation

a Accession number according to UniprotKb data base (www.uniprot.org).

b Percentage of the protein sequence covered by the matching peptides.

c Sequest Score

d Induction Factor (%volume of protein in stress condition / % volume of protein in control condition).

e Unique peptides identified which only occur in the protein identified.

f All unique peptide plus peptides that may be common between two or more proteins.

g Peptide Spectral Matches. PSMs are roughly proportional to protein abundance.

h Number of amino acids in the intact protein (theoretical).

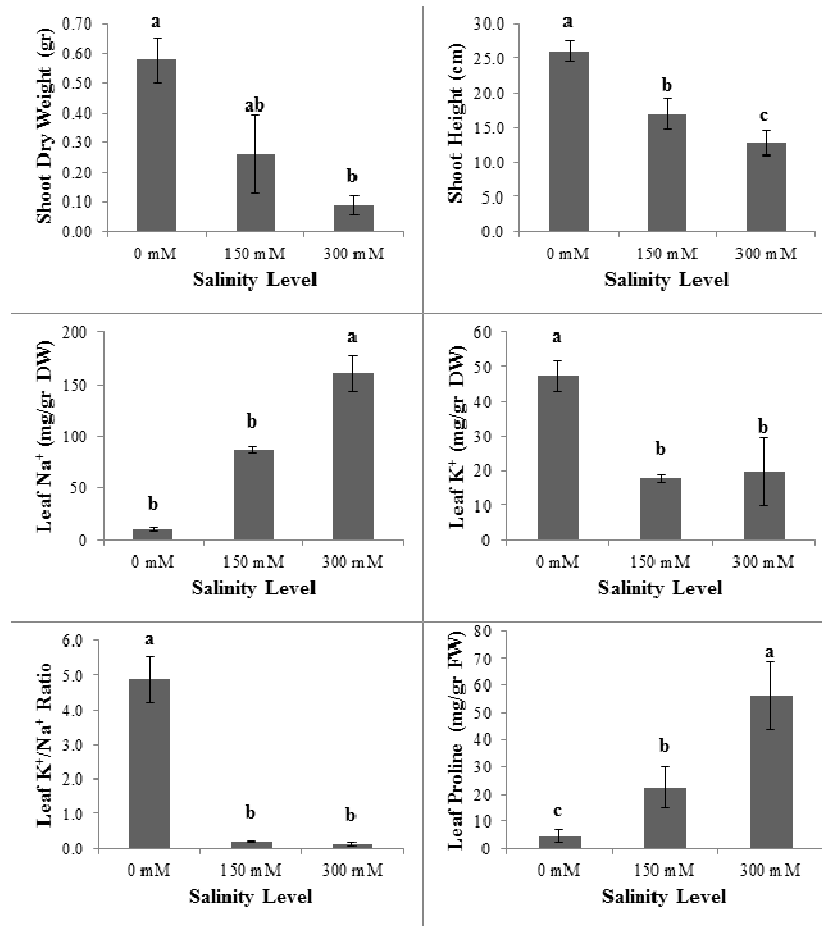


Fig. 1. Effects of salt stress (0, 150 and 300 mM NaCl) on shoot dry weight, shoot height, Leaf Na⁺, Leaf K⁺, Leaf K⁺/Na⁺ ratio and Leaf Proline of option500. Columns with the same letters are not significantly different at (P>0.05) levels by Duncan's multiple range test

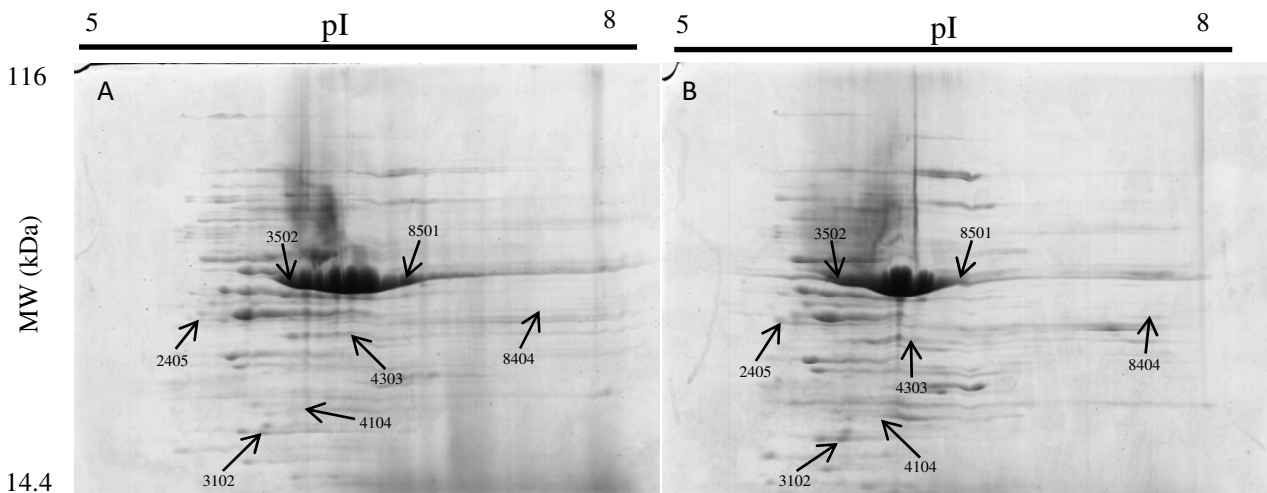


Fig. 2. Representative 2-DE gels of 'Option500' leaves under 0 (A) and 300 mM (B) NaCl treatment. Numbered spots correspond to salt-responsive proteins, which analyzed by LC-MS/MS

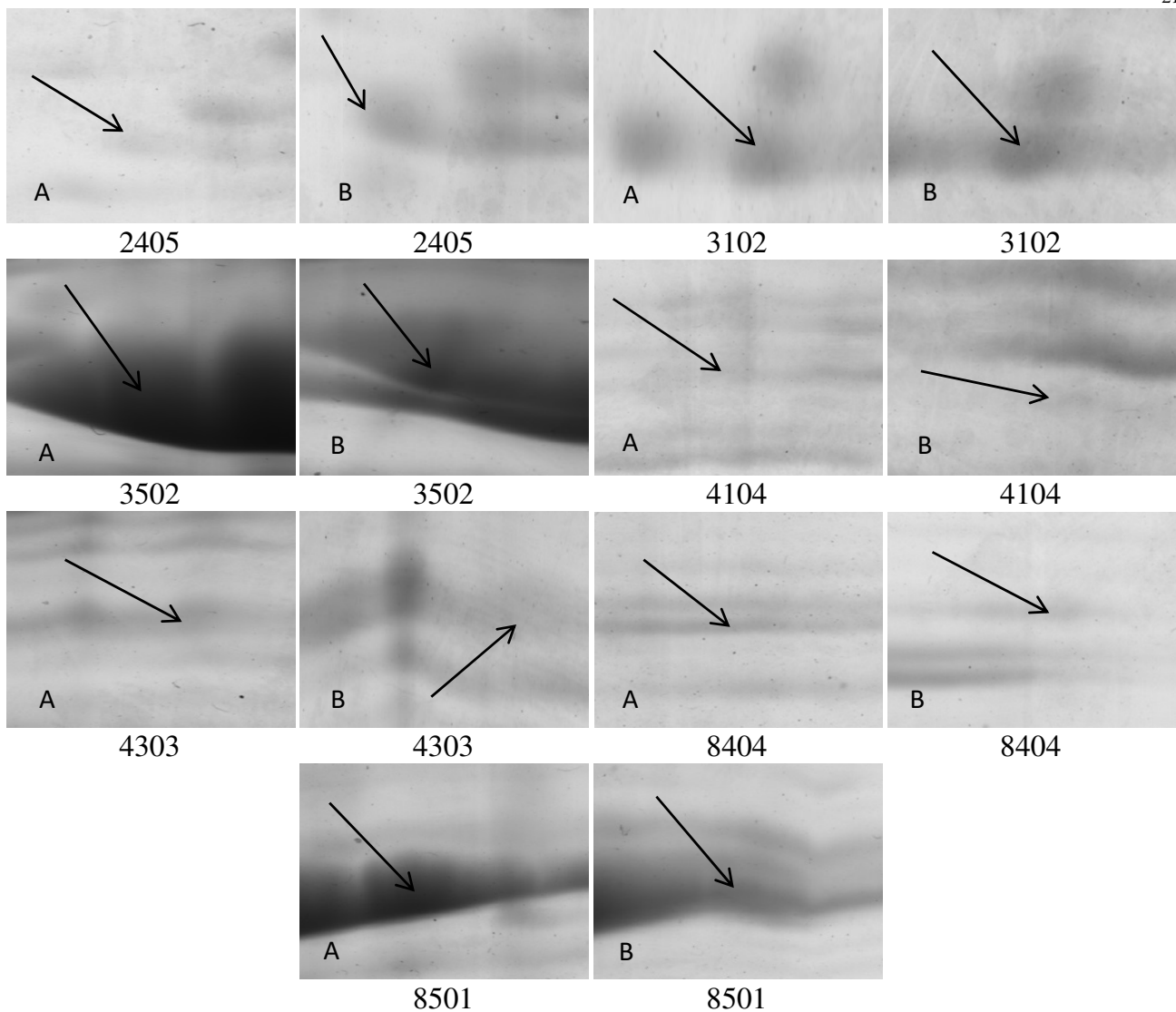


Fig. 3. Representative zoomed-in-gel sections, showing changes in protein abundance to 0 (A) and 300 mM (B) NaCl treatment

Discussion

Reduction in shoot dry weight and plant height by the effects of salinity has also been reported in previous studies (Mer *et al.*, 2000; Bandeh-hagh *et al.*, 2008). Under salt stress, leaf area expansion and plant height reduced much faster than other morphological traits (Hajiaghahi Kamrani *et al.*, 2013). Proline is one of the most important Osmolytes that synthesis in plants exposed to salt stresses and acts as an osmoprotectant. Proline concentration in plants under salt stress happens due to low activity of oxidative enzymes (Parihar *et al.*, 2014). The increments in amount of sodium in shoot and root, as well as sodium to potassium ratio in shoot and root under salt stress have been reported previously (Dolatabadi *et al.*, 2012). The same reports have shown a reduction in potassium level of shoot and root in salinity which is happened due to an antagonistic relationship between Na^+ and K^+ (Parida and Das, 2005). So that Na^+ ion abundance in the environment prevents K^+ ion's absorption (Bahrani, 2013).

Decreased photosynthetic activity of enzymes involved in Calvin cycle like PRK (Tanou *et al.*, 2009), has been

reported as an effective strategy to deal with salinity in various studies (Bandehagh *et al.*, 2011; Podda *et al.*, 2013). It seems that, the increment in the activity of PRK enzyme on assimilation pathway of carbon dioxide under salt stress is one of the main reasons for the sensitivity of this genotype to salinity.

Alpha and beta subunits of chloroplast ATP synthase are involved in the production of ATP from ADP in the presence of protons (H^+) across the membranes of chloroplast. The decrement in the activity of this enzyme under salt stress has been reported in previous experiments (Kang *et al.*, 2012; Banaei-Asl *et al.*, 2015). Increase the activity of this enzyme under salt stress is known as a coping strategy to salt stress tolerance (Guo *et al.*, 2012) and sensitive genotypes showed a further reduction of activity under stress (Huseynova *et al.*, 2007).

TPI plays a key role in other metabolic pathways, such as gluconeogenesis, fatty acid biosynthesis, pentosephosphate pathway, and photosynthetic carbon dioxide fixation. Reducing the abundant of this protein has been reported in the stresses that lead to the production of oxygen free radicals (Sharma *et al.*, 2012).

Glyceraldehyde-3-phosphate dehydrogenase was one of the most important enzymes of the assimilation of carbon dioxide pathway whose activity increment as an effective strategy to tolerate the stress condition (Tanou *et al.*, 2009) has also been reported in wheat (Kang *et al.*, 2012), soybean (Sobhanian *et al.*, 2010) and rapeseed root (Banaei-Asl *et al.*, 2015).

RuBisCO played a key role in the stabilization cycle of carbon dioxide. Decrement of this enzyme's activity under salt stress in wheat (Gao *et al.*, 2011) and rapeseed (Toorchi *et al.*, 2014) has been reported. Salinity has a major effect on plants energy metabolism. Osmotic stress causes stomatal closure and reduces the access to carbon dioxide. Reduction of assimilation level under salt stress in glycophyte plants causes the decrement in the abundance of large/small subunit of RuBisCO, RuBisCO activity and an increase in RuBisCO subunits destruction (Bandehagh *et al.*, 2011; Sobhanian *et al.*, 2011).

Plants facing salt stress decrease the rate of ATP production in order to reduce reactive oxygen species (ROS) productions which are regarded as the main source of damage to cells under biotic and abiotic stresses (Jiang *et al.*, 2007). However, high energy production in the early stages helps plants to grow rapidly (Murad *et al.*, 2014). On the other hand, activation of energy production pathways in order to supply the energy that is needed for biosynthesis of responded proteins, osmolytes and active salt ions transportation in the same way, seems necessary (Banaei-Asl *et al.*, 2015). While the genotype 'Option500' by reducing the production of these enzymes tried to reduce energy production and, consequently, ROS productions to keep itself away from destructive effects of salt stress. This can be the reason of 'Option500' sensitivity to salt stress.

Conclusions

'Option500' genotype by decreasing the abundance of enzymes involved in the production of energy tried to deal with the damaging effects of ROS as byproducts of energy production process. And by increasing the abundance of PRK which is an important enzyme in the pentose phosphate pathway, the production of NADPH has been increased as a reducing agent. Since this pathway is dependent on glucose 6-phosphate, and by the fact that by closure of stomata in response to osmotic stress and reduction of available carbon dioxide and decreased in the abundant of RuBisCO enzyme, glucose production was limited. Thus, this approach failed to deal with stress and it faced an initial decline in performance.

Acknowledgements

The authors like to show their gratitude to the members of Alberta Proteomics and Mass Spectrometry Facility of University of Alberta, Canada.

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