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Expression pattern of two sugar transporter genes (*SuT4* and *SuT5*) under salt stress in wheat

Fahimeh Charkazi, S. Sanaz Ramezanpour*, Hassan Soltanloo

Department of Plant Breeding and Biotechnology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran

***Corresponding author: ramezanpours@gau.ac.ir**

Abstract

Salinity stress is important abiotic factor, limiting wheat yield around the world and it is known to induce the accumulation of water soluble carbohydrates. These changes were accompanied by alteration in expression levels of a large number of carbohydrate metabolic genes. In present study, regulation of two wheat carbohydrate transporter genes, namely *SuT4* and *SuT5* during salinity stress at seedling stage were elucidates using quantitative RT-PCR. Salinity stress was induced using NaCl. Measurement on germination and seedling growth revealed Kavir as salt tolerant and Falat as salt susceptible cultivar. Expressions of two sugar transporter genes were differentially regulated during salt stress. Transcripts levels of both genes were almost higher in tolerant cultivar. Highest level of *SuT4* and *SuT5* transcripts was observed in -0.75 MPa of NaCl in salt tolerant cultivar. Transcript level of both genes down regulated in salt susceptible cultivar by increasing NaCl osmotic potential up to -1 MPa. Therefore, it seems that accumulation of sugars was necessarily correlated with accumulation of sugar transporter genes transcripts and salinity tolerance.

Keywords: Salinity, wheat, sugar transporter, expression, carbohydrate
Abbreviations: SuT: Sugar Transporter, MPa : Mega Pascal, PCR: Polymerase Chain Reaction, QRT-PCR: Quantitative Real-Time PCR, *GAPDH:* Glyceraldehyde-3-phosphate dehydrogenase, REST: Relative Expression Software Tools

Introduction

Wheat (*Triticum aestivum* L.) is a major crop for more than one third of the world population. It is originated in south western Asia and has been a major agricultural commodity since pre historic times. Wheat productivity depends upon the production, translocation, storage and utilization of carbohydrates. Carbohydrates serve as a source of energy and also act as signaling molecule in regulation of metabolic pathways under normal and stressed conditions (Gupta and Kaur, 2005). During germination, starch present in the cereal endosperm is hydrolyzed to glucose by amylases and is then converted to sucrose by the sucrose phosphate synthase. Sucrose, thus formed is then transported to the growing embryonic axis, where it is hydrolyzed and the products so formed are used as energy source for the growth of seedlings (Smith et al., 2005). Sugars interact with the sensor proteins and initiate a signal transduction cascade that results in cellular responses such as altered gene expression and enzymatic activity (Gupta and Kaur, 2005). Therefore the sugar transporter proteins play an important role in abiotic stress because they transport carbohydrate within the cells. The tolerance to salt stress is a complex process that involves morphological physiological and biochemical modifications. Survival and growth under saline environments are the result of adaptive processes such as ion transport and compartmentalisation, synthesis and accumulation of organic solutes, bearing to an osmotic adjustment (Fougere et al., 1991). Adaptation of plants to osmotic stress brings about the development of a low osmotic potential similar to those in plant species from arid environments. As a single cell, few strategies can be conceivable to survive in such conditions. One of these mechanisms is the osmotic adjustment achieved by the storage of solutes within cells (Santos-Diaz and Ochoa-Alejo, 1994). Several breeding strategies, including marker-assisted selection, are being used to improve crop yields under water stress. These have been somewhat successful, but they are limited by the time and other resources required. To improve the efficiency of such strategies and to develop new options, a better understanding of the physiological and molecular bases of salinity tolerance in plants is required (Blum 1998; Trethowan et al. 2001). Expression changes of some genes involved in carbohydrate metabolism during salt stress have been reported in few studies (Bartels and Sunkar 2005; Cheeseman, 1988; Rathert, 1984; Zhu, 2002). The determination of expression pattern of sugar transporter proteins in response to salinity may leads to understanding more about mechanisms involved in such stresses tolerance. In the other hand, there are so many studies on alternation carbohydrate level under abiotic stress in physiological level (Tammam et al., 2008; Hasaneen et al., 2009) but molecular study on expression patterns of enzyme included in abiotic stress was so rare. In this study, we have investigated the changes of expression pattern of two sugar transporter genes in the growing seedlings under salinity stress in susceptible and tolerant plants. These genes were isolated from wheat genotypes under cold stress by SSH method and showed high homology sugar transporter genes in other plants like barley (Weschke et al., 2003) and rice (Buell et al., 2003).

Table 1. Sequence and some characterization of specific primers.

Name		Sequence	Tm	GC%	Product length
SuT4	For	5'-AGTGCCTTGACAACCTTGCT-3'	57.91	50	156
	Rev	5'-GGTTCCGACATCCAGAACAT-3'	59.79	50	
SuT5	For	5'-GAAGGCTGCAACAAAACCTC-3'	59.76	50	128
	Rev	5'- TTTGCCAAGGCTCTACTGTG-3'	59.07	50	
GAPDH	For	5'-TCACCACCGACTACATGACC-3'	60	50	121
	Rev	5'-ACAGCAACCTCCTTCTCACC-3'	60	55	

Material and method

Plant material and stress treatment

This study was carried out at the department of plant breeding and biotechnology, faculty of crop science, Gorgan University of Agricultural Science and Natural Resources, Iran. Salt stress was induced by NaCl. Six salt treatments with different osmotic potentials of -0.25, -0.5, -0.75, -1, -1.25 and -1.5 MPa were arranged as described by Coons et al., 1990. In this study distilled water served as a control. Wheat (*Triticum aestivum* L.) seeds of cv. Kavir (salinity-tolerant) and cv. Falat (salinity-sensitive) were sterilized with 5% detergent and wash several times with distilled water. Three replicates of 20 seeds of each cultivar were germinated in 2 rolled Whatman filter papers with 100 ml of respective test solutions (Rehman et al., 1996). In order to prevent evaporation, each rolled paper was put into a sealed plastic bag. Seeds were allowed to germinate at 20 ± 1 ^{o}C in the dark for 10 days. Germination percentage, shoot length, root length and seedling fresh weight were determined after 10 days.

Analysis of variance

The experimental design was two factorial, arranged in a completely randomized design with 3 replications and 20 seeds per replicate. The first factor was cultivars and the second was NaCl osmotic potentials (0, -0.25, -0.5, -0.75, -1, -1.25 and -1.5 MPa). Data given in percentages were subjected to arcsine transformation before statistical analysis. For all investigated parameters, analysis of variance was performed using the SAS version 9.1 (SAS institute Cary, NC). Significant differences among the mean values were compared by LSD test ($P < 0.05$ or 0.01).

RNA extraction and cDNA synthesis

All seedlings for each treatment sampled after 10 days and put in aluminum foil in different replications. The samples were immediately frozen in liquid nitrogen and stored at -80^{o}C until RNA extraction.
Lithium chloride (Merck, Germany) was used to extract total RNA using modified method as described by Naito et al., 1994. First strand cDNA was reverse transcribed from total RNA by Fermentase protocol using RevertAid^{TM} M-MuLV (Fermentase, Germany) as manufacture description and Ten-fold dilution was used as a template for real-time PCR.

Primer design for RT-PCR

Primers were designed by Primer 3 (www.embnet.sk/cgi-bin/primer3_www.cgi) to obtain 18–21 bp length, 59 and 61 ^{o}C melting temperature and GC content between 55% and 65% avoiding hairpins and complementarity between primers. The primers designed based on two sugar transporter candidate genes namely, *SuT4* and *SuT5*. These genes were showed differentially expression under cold stress in wheat by SSH method and showed high homology with two different putative sugar transporter genes in barley and rice (accession no. AJ534445.1 and NM_197382.1, respectively). The characterization of genes and specific primers for *SuT4*, *SuT5 and* glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as reference gene (accession no. EF592180) was present in Table 1. The specifity of primers was checked by standard PCR and electrophoresis on agarose gel as shown in Fig. 1.

Quantitative real-time PCR

Real-time RT-PCR using SYBR Green I technology on iQ5 System (BioRad, USA) was performed. The SYBR Green PCR Master Mix (SYBR biopars, GUASNR, Iran) was used with a final concentration of 1x SYBR Green PCR Master Mix, in a total volume of 25 ul, 300 nM of each specific sense and anti-sense primers and 10 ng of cDNA were added. The following amplification program was used: 94ºC during 3 min, 35 cycles at 15s at 94ºC followed by 15s at 60ºC and 15s at 60ºC. All samples were amplified in triplicate from the same RNA preparation and the mean value was considered. The I was used as reference gene. Two biological replications were used for each plate. The real-time RT-PCR efficiency was determined for each gene with the slope of a linear regression model (Pfaffl, 2001). For this, bulks of each cDNA sample were used as PCR template in a range of 10 fold serial dilution. The corresponding real-time RT-PCR efficiencies were calculated according to the equation: $E = 10^{(-1/slope)}$ (Radonic et al., 2004).
Relative expression was computed using following formula which presented by Pfaffl, 2001, based on its real-time PCR efficiency (E) and the crossing point (CP) difference (Δ) of an treated sample (under stress) versus control ($\Delta CP_{control\text{-}sample}$) for both target and reference genes.

$$Ratio = \left(E_{target}\right)^{\Delta CP_{target(control-sample)}} \Big/ \left(E_{ref}\right)^{\Delta CP_{ref(control-sample)}}$$

The REST (Relative Expression Software tools) software (2008) used this formula to calculate ratio between the amount of target molecule and reference molecule *GAPDH* within the same sample. In this model the target gene expression was normalized by *GAPDH* expression which is a non-regulated reference gene. The normalized value was then used to compare differential gene expression in different samples.
A melt curve and agarose gel separation was used to detect whether the PCR reaction yields non-specific fragments or primer dimers. Because SYBR Green dye which used in this study is a non-specific dye and bind to all types of double strand DNA. To develop melt curve the real-time PCR products was heated at 94°C for 1 min followed by 110 cycles, 10 sec per cycle, staring at 40°C and increasing 0.5°C after each cycle.

Results

The effect of NaCl was significant ($P<0.01$, df=6) for all investigated traits except for germination percentage (Fig. 2).

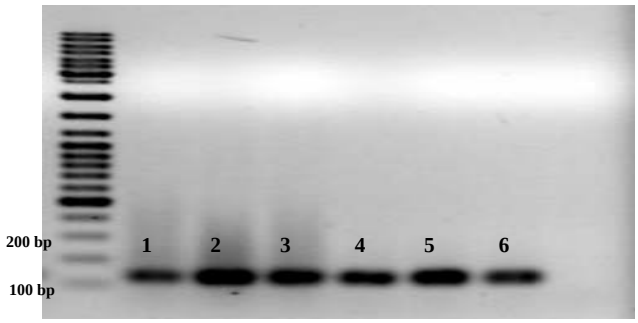

Fig 1. Agarose gel electrophoresis of specific primers. The lanes are specific amplified fragment on two cDNA sample by *SuT4* (1,2), *SuT5* (3,4) and *GAPDH* (5,6) primers.

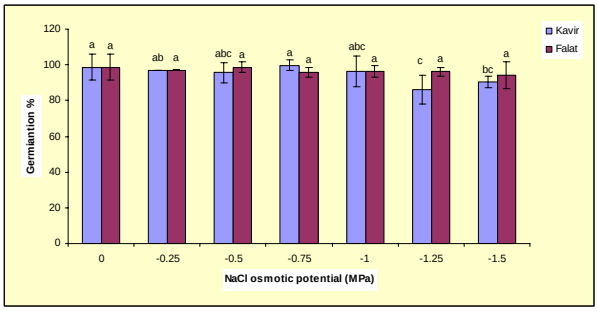

Fig 2. Changes in germination percentage of wheat cultivars at different osmotic potentials of NaCl. Values show the real germination percentages but variance analysis was performed using arcsine transformed values. Bars represent one standard deviation. Means followed by the same letter(s) are not significantly different at P = 0.01. There are no significant differences between different treatments in germination percentage.

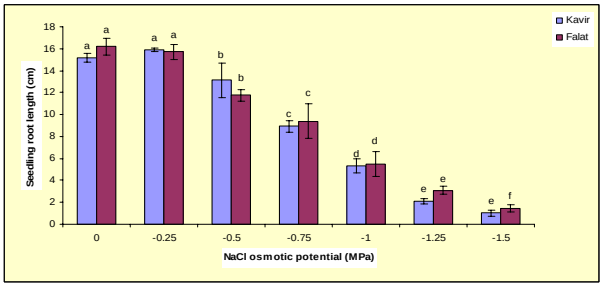

Fig 3. Root length of wheat cultivars at different osmotic potentials of NaCl. Means followed by the same letter(s) are not significantly different at P = 0.01. Bars represent one standard deviation. Root length was decrease significantly in all NaCl treatments.

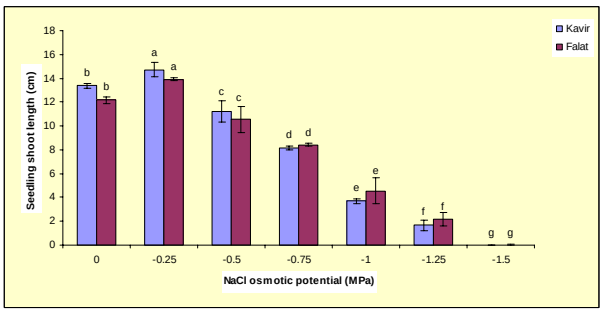

Fig 4. Shoot length of wheat cultivars at different osmotic potentials of NaCl. Means followed by the same letter(s) are not significantly different at P = 0.01. Bars represent one standard deviation. NaCl treatments had significant effect to decrease Shoot length.

The control (0 MPa) did not show any differences among the cultivars for all traits. A non-significant 2-way interaction (variety×stress) was found for germination percentage. Increasing NaCl resulted in decrease root length in all variety. No cultivar was able to grow roots at -1.5 MPa of NaCl (Fig. 3). The first level of NaCl (-0.25) did not decrease root length significantly and it declined considerably in osmotic potential more than -0.75. NaCl had a stimulating effect on the root growth of Kavir from 0 to -0.25 MPa of osmotic potential. Root length did not change significantly between salinity tolerant (Kavir) and susceptible (Falat) variety. NaCl showed stimulating effect on shoot growth in both varieties at -0.25 MPa (Fig. 4). Shoot length did not show significantly differences between salt tolerant and susceptible varieties. 2-way interaction (variety×stress) was non-significant. Decreasing water potential by NaCl caused reduction in seedling fresh weight (Fig. 5). Differences among the varieties were significant. Decreasing in seedling fresh weight by NaCl started from -0.5 MPa and NaCl has stimulating effect on Kavir seedling fresh weight from 0 to -0.25 MPa. NaCl influenced seedling fresh weight of varieties in different ways. The sugar level in the salt-stressed leaves is dependent on the transportation activity of sugar transporters. For this, we monitored the Sugar Transporter (*SuT*) expression patterns of two genes with 96–98% nucleotide identity to the Rice sugar transporters genes (*SuT4* and *SuT5*). This is the first report which studied these genes under salt stress. The *SuT* genes were originally isolated by SSH from a library constructed from low-temperature-treated wheat crown tissue (unpublished data), because accumulation of carbohydrates occur in all water potential stress including cold, drought and salinity. Analysis of variance for *SuT4* and *SuT5* transcript accumulation in seedling showed highly significant effects of variety, osmotic potential and variety×osmotic potential. There was a sharp increase in *SuT4* transcripts in salt tolerant variety (Kavir) by increasing osmotic potential to -0.25 Mpa followed by a high steady state to -0.75 Mpa of NaCl osmotic potential (Fig. 6). Its transcripts decreased 70% at -1 Mpa and increase 50% in -1.5 Mpa in Kavir. In salt susceptible variety, *SuT4* transcripts gradually decline to -1 Mpa followed by 15% increase from -1 to -1.5 Mpa. The *e*xpression pattern of *SuT5* was almost the same in salt tolerant and susceptible variety at the first NaCl osmotic potential but its transcript level is almost higher in tolerant cultivar than in susceptible cultivar (Fig. 7). It is down-regulated about (25–30%) in both cultivar at -0.25 Mpa of NaCl but it can be recover 100% of its transcripts level in -0.75 Mpa in Kavir and 80% in Falat. It sharply down regulated in salt susceptible in -1 Mpa and its transcripts decreased 75%, followed by a sudden increase at -1.25 Mpa and keep a steady-state levels of transcript to -1.5. Kavir showed a decrease in *SuT5* expression from to -0.75 Mpa to -1.25 Mpa followed by a sudden increase in transcripts level at -1.5 Mpa.

Discussion

NaCl adversely affected seedling growth of wheat. Root and shoot length, and seedling fresh weight were decrease by increasing NaCl concentrations. Our results are consistent to studies which evaluated effects of NaCl on the germination and seedling growth of pea by Murillo-Amador et al., (2002), cowpea by Okcu et al., (2005) and soybean by Khajeh-Hosseini et al. (2003). However, our study showed that NaCl had greater inhibitory effects on seedling growth than germination because no significant decrease in germination in the two cultivars was observed. Seeds always germinated

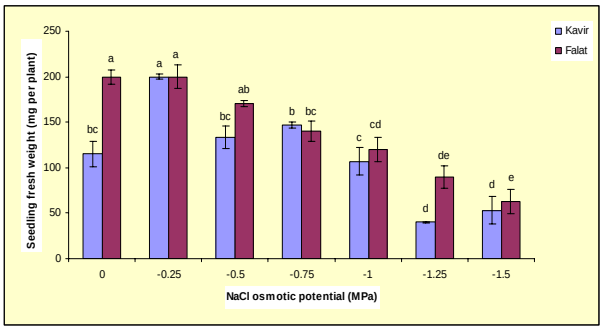

Fig 5. Seedling fresh weight of wheat cultivars at different osmotic potentials of NaCl. Means followed by the same letter(s) are not significantly different at P = 0.01. Bars represent one standard deviation. Seedling fresh weight was decreased significantly in high NaCl concentrations.

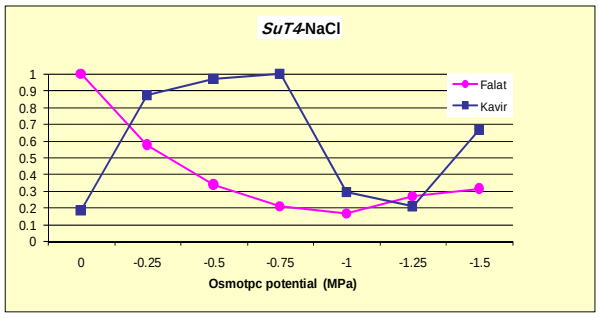

Fig 6. Quantitative expression patterns of *SuT4* gene in seedling of Kavir and Falat in different NaCl osmotic potential. The highest expression level of this gene was in middle NaCl treatments (-0.5 to -0.75 Mpa) in Kavir as tolerant cultivar. The expression level of this gene was reduced in susceptible cultivar (Falat).

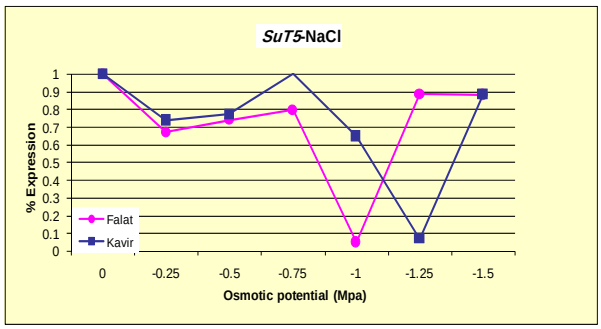

Fig 7. Quantitative expression patterns of *SuT5* gene in seedling of Kavir and Falat in different NaCl osmotic potentials. The highest transcript level of this gene was accumulated in middle level of NaCl (-0.75 Mpa) in tolerant cultivar (Kavir) like as *SuT4* gene.

properly in NaCl compared to other abiotic stress in line with earlier observations made for soybean by Khajeh-Hosseini et al., (2003). This may be due to the uptake of Na^+ and Cl^- ions by the seed, maintaining a water potential gradient allowing water uptake during seed germination. In conclusion, early seedling growth of wheat was reduced as water potentials increased. Kavir was more tolerant to salt concentration while Falat appeared susceptible. However seedling growth was more sensitive to salt stress than germination. Compatible solutes, low-molecular weight organic molecules such as the previously discussed glucose, sucrose, fructose, Put and many others, are known to accumulate under stress conditions (Guy et al., 2008). They are considered to stabilize proteins and membranes and contribute to cell osmotic pressure (Kaplan et al., 2004). The present work focuses on gene expression upon NaCl osmotic potential in wheat seedling. Through QRT-PCR study we showed a differential expression for sugar transporter genes involved in carbohydrate accumulation under salinity stress. Although we found cold stress can share signal transduction pathway to induce responding gene to water deficit stress (unpublished data). Salinity stress is known to reduce photosynthesis rates and to induce the accumulation of water soluble carbohydrates. These changes were accompanied by alteration in expression levels of a large number of carbohydrate metabolic genes. With comprehensive metabolic-pathway-based expression analysis using accurate and quantitative RT-PCR, we demonstrated significant alterations in the expression levels of two previously unknown salinity responsive genes involved in the major carbohydrate metabolic pathways in salt stressed seedling. Expression pattern of *SuT4* and *SuT5* genes was changed under salt stress, which means product of these genes are important in signal transduction pathway under osmotic stress caused by different NaCl concentrations. Both genes significantly up regulated in -1.5 Mpa of NaCl osmotic potential. In this osmotic potential there was no shoot growth but root grew very low.

This means the carbohydrate necessary to growth shoot was stored in root but some inhibitory effect likely ion toxic effect inhibit growing shoot. In our study, the increase of sugar transporter transcripts in seedling of Kavir was higher than Falat, therefore it seems that the accumulation of sugars was necessarily correlated with accumulation of sugar transporter genes transcripts and salinity tolerance. Increasing in sugar content along with increasing NaCl concentration was reported by Cachorro et al., 1993 in *Phaseolus vulgaris*. In another study, sorghum grown in highly saline soil showed 28% increase in soluble sugar content in leaves (Chavan and Karadje, 1986). Sugar content of tomato leaf sap was affected differently by sodium chloride stress (Bezerra, 1992). A complex essential role of soluble sugars in plant metabolism is well known as products of hydrolytic processes, substrates in biosynthesis processes, energy production but also in a sugar sensing and signaling systems. Recently it has been claimed that even sugar flux may be a signal for metabolic regulation (Gibson, 2005).

Protection against dehydration by the former sugars was correlated with the increase in shoots and roots. Soluble sugars may also function as a typical osmoprotectant, stabilizing cellular membranes and maintaining turgor. Under stress conditions, accumulation of sugar transporter transcripts may be to counter the osmotic stress.

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