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Proline Accumulation and Related Genes in Wheat Leaves under Salinity Stress

M. Tavakoli[1], K. Poustini [1*], and H. Alizadeh[1]

ABSTRACT

This study was carried out to evaluate the effects of salinity stress (16 dS m^{-1}) on proline accumulation and expression pattern of three genes involved in the proline synthesis (P5CS, P5CR) and degradation (PDH) in the fourth and the flag leaves. The experimental treatments consisted of two levels of salinity stress including 1.6 (control) and 16 dS m^{-1} and three wheat cultivars, namely, Hirmand and Chamran (as tolerant cultivars) and Atrak (as sensitive). These were arranged as factorial based on a Completely Randomized Design with three replications. There was an increase in Na^+ concentration and decrease in K^+ concentration in salinity stress compared to the control condition in the fourth and the flag leaves of all cultivars. However, the tolerant cultivars showed lower level of Na^+ concentration and higher level of K^+ concentration and K^+/ Na^+ ratio. Seemingly, the increase in proline accumulation was due to P5CS, P5CR up-regulation and PDH down-regulation. On the other hand, the higher proline degradation in the fourth leaf of the tolerant cultivars was probably due to the provision of a source for energy or nitrogen, which helped to enhance the yield of these cultivars under salinity stress.

Keywords: Proline, Salinity, Wheat, P5CS, P5CR, PDH.

INTRODUCTION

Salinity is one of the most important abiotic stresses and it limits the productivity and geographical distribution of plants. According to the FAO (2008) report, over 6% of the world's land (about 800 Mha) is affected by salinity. Most of this salinity-affected land has arisen from natural causes i.e. accumulation of salts over long periods of time in arid and semiarid zones, and also by human activities such as irrigation (Munns and Tester, 2008).

The adverse impacts imposed by salt stress are osmotic stress, ionic stress, nutrient imbalance, and the production of reactive oxygen species; then, the plants would display declining growth and photosynthesis rate, even death in the end (Wu *et al.*, 2014). Salinity affects many aspects of plant metabolism and the accumulation of various organic solutes that provide the turgor necessary for cell expansion. Among them, the accumulation of low molecular weight solutes and compatible osmolytes, such as proline and glycine betaine, function as osmoprotectants. In addition to osmotic role, it has been proposed that, in stress conditions, proline has different roles including a carbon and nitrogen storage component, ROS scavenger, buffer of cytosolic pH and cell redox balance status, stabilizer of proteins structure (Hare and Cress, 1997), and finally, signal of stress adaptive responses (Maggio *et al.*, 2002).

Proline accumulation has been demonstrated to be correlated with salinity tolerance in plants. Moreover, the increase of proline content in leaf is also significant in salt stressed sorghum (de Lacerda *et al.*, 2003), maize (Hajlaoui *et al.*, 2010), and

[1] Department of Agronomy and Plant Breeding, University College of Agriculture & Natural Resources, University of Tehran, Karaj, Islamic Republic of Iran
[*] Corresponding author: e-mail: kpostini@ut.ac.ir

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soybean (Çelik and Atak, 2012). Its concentration has been shown to be generally higher in stress-tolerant than in stress-sensitive plants (Ashraf and Foolad, 2007), but in wheat cultivars, Poustini *et al.* (2007) have shown a larger concentration of proline in salt sensitive cultivars than salt tolerant ones in salinity stress condition.

Proline accumulation is mediated by its increased synthesis and reduced oxidation. Some studies demonstrate that, in stress condition, most of the proline accumulated in plants is the result of enhanced synthesis from glutamate (Delauney and Verma, 1993; Hare and Cress, 1997). It is a two-step process that glutamate is reduced to Δ^1-pyrroline-5-carboxylate (P5C) by Δ^1-pyrroline-5-carboxylate synthetase (P5CS) and then it is followed by the reduction of P5C to proline, catalyzed by Δ^1-pyrroline-5-carboxylate reductase (P5CR). Proline degradation is also a two-step oxidation process that starts with the oxidation of proline to P5C by proline dehydrogenase (PDH) and subsequently the conversion of P5C to glutamate by pyrroline-5-carboxylate dehydrogenase (P5CDH) (Lehmann *et al.*, 2010). It has been reported that proline accumulation is regulated by transcriptional changes of proline biosynthesis and degradation (Mattioli *et al.*, 2009). Xue et al. (Xue *et al.*, 2009) have indicated that stress-induced accumulation of proline in rapeseed results from the activated biosynthesis and also the inhibited proline degradation. They have also shown that the expression of BnP5CS1 and BnP5CS2 were enhanced under salt stress, ABA treatment and dehydration, but the expression of BnPDH was inhibited. The objective of this research was to evaluate proline accumulation and its biosynthesis and degradation in transcriptional regulation under salinity stress in tolerant and sensitive wheat cultivars.

MATERIALS AND METHODS

This study was carried out in greenhouse pot culture from the middle of October 2010 to the middle of February 2011. The air temperature ranged from 22 to 28°C during the day and 14 to 17°C during the night. The experimental treatments were arranged as factorial based on a completely randomized design with three replications. Treatments consisted of two levels of salinity i.e. 1.6 (control) and 16 dS m^{-1} (salinity stress), and three wheat cultivars, namely, Hirmand and Chamran (as tolerance cultivars) and Atrak (as sensitive cultivar) (Tavakoli, 2011).

The pots contained 3.5 kg of soil (mixture of farm soil, sand, and farmyard manure in a 3:2:1 ratio). Ten grains were sown in each pot. They were daily irrigated with water until seedlings establishment (21days). Afterwards, the pots were watered with a solution of NaCl (150 mM) to reach the saline level of 16 dS m^{-1} gradually in 3 days by adjustment of the soil water content close to the field capacity. Plants were sampled at two stages: anthesis (Zadoks; 61) and harvesting (Zadoks; 92). The flag and the fourth leaves of plants were separated for measuring proline and ions concentration and analyzing genes expression. Leaves were dried at 60°C for 72 h for measuring the concentration of leaf Na^+ and K^+. The measurements were taken from the 2 N chloride acid extract of the samples(0.5 g) that had been burned at 600°C for 4 h, using a flame photometer (Poustini and Siosemardeh, 2004).

Proline content of the leaves and leaf discs was determined by homogenizing the samples in a mortar and pestle in 3% sulfosalicylic acid. After centrifugation at 13,000 rpm for 11 min, 2 mL supernatant was added to 2 mL glacial acetic acid and 2 mL ninhydrin solution (0.2 M). The mixture was kept at 95°C for 60 min, then, the reaction was stopped quickly by an ice bath. Toluene (3 mL) was added to the mixture and the organic phase was extracted and monitored at 520 nm by a spectrophotometer (Shimadzu, UV-160) (Bates *et al.*, 1973).

The mRNAs of the flag and fourth leaves were extracted using the Qiagen kit according to the manufacturer's protocol. Total cDNA was synthesized from mRNA

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(1 µg) by Fermentas kit according to the manufacturer's protocol. Quantitative real-time RT-PCR was carried out with wheat actin gene as the internal standard. The gene-specific primers were designed by PrimerQuest and evaluated by OligAnalyzer (Table 1). In the first step, amplification was started at 95°C for 10 min, followed by 40 cycles of PCRs: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s. A melting curve was run after PCR cycles. Detection of PCR products was done using the SYBR Green Real-time PCR Master Mix. Reactions were repeated 5 times for each sample and relative gene expression was calculated using the $2^{-\Delta\Delta C}T$ method (Livak and Schmittgen, 2001).

The analysis of variance of the data was done by SAS, 9.1 Software. The data mean comparisons were made following Least Significant Difference (LSD) test at 5% probability level.

RESULTS

Growth and Ionic Characteristics

Shoot biomass and grain weight were decreased by NaCl in all cultivars. This decrease was larger for Hirmand but it had the highest shoot biomass in the saline treatment (Table 2). Moreover, Chamran had the highest grain weight under salinity stress and it showed stability in grain weight. In contrast, the lowest value of grain weight was observed in Atrak with 76% decrease under salinity stress.

Results (Table 2) showed that K^+ concentration generally decreased in both leaves in all cultivars under salinity stress conditions, except in Chamran which showed the highest K^+ concentration in flag leaf under salinity. In the normal condition K^+ concentration of flag leaves was higher than 4th leaves in Chamran and Hirmand but Atrak showed a lower K^+ concentration in flag leaves. However K^+ concentration in flag leaves rather than flag leaves was: 1. higher in Chamran 2. similar in Atrak and 3. lower in Hirmand (Table 2).

Distribution of Na^+ in leaves changed significantly with their age and NaCl concentration. Dealing with all treatments of salinity, the greatest accumulation was observed in the 4th leaf. The concentration of Na^+ in the sensitive cultivars was 2 folds or more in both leaves compared to the tolerant cultivars. Atrak showed the highest level and change percent of Na^+ in flag and 4th leaves. While there wasn't different between all cultivar in the normal condition.

In leaves, K^+/ Na^+ ratio in all cultivars decreased significantly with the increase in salinity level as compared to the control treatment (Table 2). K^+/ Na^+ ratio was higher in the flag leaf than the 4th leaf and in the tolerant cultivars (Chamran and Hirmand) than the sensitive cultivar (Atrak).

Table 1. List of the primers used for RT-PCR analysis of the wheat candidate genes.

Gene	Accession No	Primer	Sequence	TM
Actin	AB181991.1	Forw	GCCACACTGTTCCAATCTATGA	60.2 ºC
		Reve	TGATGGAATTGTATGTCGCTTC	60.1 ºC
P5CS	JQ063079.1	Forw	TCGGTGCTGAGGTTGGCATAAG	59.5 ºC
		Reve	TTGTCACCATTCACCACTTGCCC	60.1 ºC
P5CR	AY880317.1	Forw	CGGGTAAACATCCAGGGCAGC	60.8 ºC
		Reve	TCGGCATCTTGTTGTGGCAGC	60.9 ºC
PDH	AK332189.1	Forw	CGGGATCCTCGACTACGGCATC	61.3 ºC
		Reve	TGATCTTGATACACACGCTCGCCG	61.1 ºC

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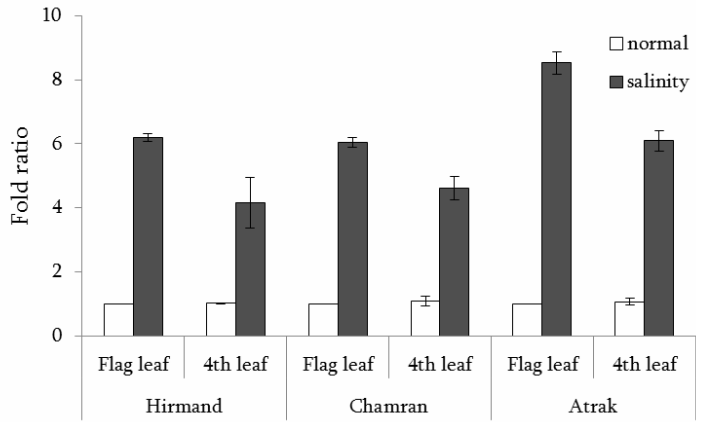
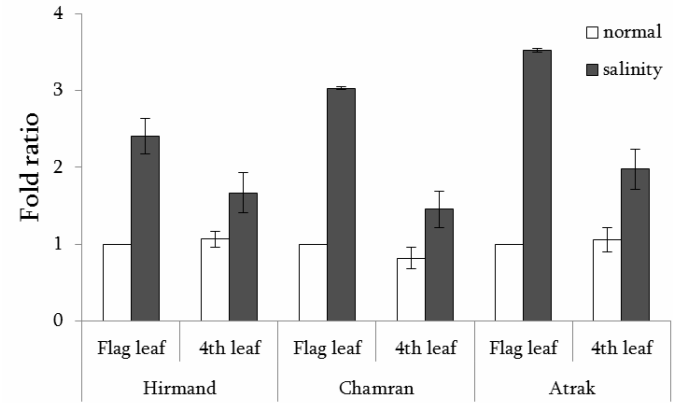
Table 2. Comparison of some traits in two leaves of three wheat cultivars under control and salt stress conditions.

Cultivars	Leaf	Potassium concentration [a]			Sodium concentration [a]			K^+/Na^+			Proline concentration [b]			Shoot biomass [c]			Grain weight		
		C[d]	S	Ch	C	S	Ch	C	S	Ch	C	S	Ch	C	S	Ch	C	S	Ch
Atrak	flag	23.2 ±0.9[g]	20.8±0.6	-10.6	0.6 ±0.0	2.4 ±0.3	300	39.2 ±0.9	8.6 ±0.3	-78.2	0.51±0.1	2.02±0.20	295.4	2.01± 0.8	1.51± 0.2	-24.9	0.75± 0.35	0.25± 0.12	-76
	4th	30.7 ±0.8	20.6±0.9	-32.8	3.5 ±0.8	23.6 ±2	582.6	8.9 ±0.4	0.9 ±0.2	-90.4	0.18±0.01	1.65±0.01	798.2						
Chamran	flag	21.9 ±1.2	30±0.8	37.3	0.6 ±0.1	1.6 ±0.1	158	35 ±1.8	19.7 ±0.5	-43.9	0.29±0.03	1.81±0.08	515.9	2.48± 0.9	1.97± 0.3	-20.6	1.04± 0.46	0.74± 0.2	-28.8
	4th	20.6±1	14.8±1	-28.4	3.1 ±0.3	12.7 ±1.1	3116.4	6.8 ±0.1	1.2 ±0.3	-83	0.4±0.08	1.3±0.09	223.1						
Hirmand	flag	32.1 ±0.7	22±0.8	-31.6	0.4 ±0.0	1.1 ±0.0	163	75.7±2.1	20.9 ±0.9	-72.4	0.16±0.02	1.9±0.10	1087.5	3.31± 0.9	2.06± 0.3	-37.8	1.09± 0.91	0.67± 0.12	-37.7
	4th	29.3 ±1.3	25.2±0.2	-14.2	4.7 ±0.2	6.9 ±0.2	48.4	6.3 ±0.8	3.7 ±0.2	-42.1	0.31±0.02	1.1±0.05	258.7						

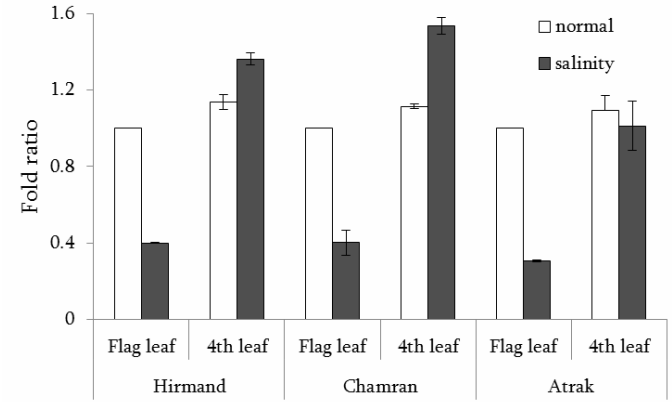
[a] mg g^{-1} dry matter. mg g^{-1}, [b] fresh weight. [c] g $plant^{-1}$. [d] C; control, S: salinity and Ch: changes made by salinity in percentage, [d] Values are mean ± standard error (n=3).



(a)



(b)



(c)

Figure1. Effect of salinity on (a) P5CS (b) P5CR (c)PDH expression in the flag and 4th leaves of wheat cultivars.

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Proline Concentration and Genes Expression Pattern

All three cultivars showed increase in the level of proline in their tissue upon salinity treatment. Proline concentration in flag leaves was larger than the 4th leaves and it also was higher in the sensitive cultivar (Atrak) compared to the tolerant ones (Hirmand and Chamran).

Under unstressed conditions, the P5CS gene was not different between cultivars, but, under salinity condition, both the flag and 4th leaves showed a dramatic increase (Figure 1). Sensitive cultivar (Atrak) showed a larger P5CS gene expression than salinity stress tolerant cultivars (Chamran and Hirmand). Expression of P5CS in the 4th leaf of Atrak was 1.5 fold more than the other cultivars and it was the same as the flag leaf expression under salinity stress (Figure 1).

Expression of P5CR increased in all cultivars under salinity stress and its pattern was similar to P5CS, but in terms of intensity they were different i.e. the maximum expression of P5CR was 3.5 fold and in P5CS it was 8.5 fold more than the control (Figure 2).

In contrast, expression pattern of PDH decreased in flag leaves of all cultivars and also increased in the 4th leaves of Chamran and Hirmand under salinity stress, but Atrak's 4th leaf did not show any change in PDH expression by NaCl (Figure 3). Proline content and level of P5CS and PDH expression in Atrak's 4th leaves was higher than other cultivar's 4th leaves. But level of P5CR expression in Atrak's 4th leaves didn't show any significant difference with other cultivar's 4th leaves.

DISCUSSION

Salt tolerance reflects the mechanisms associated with tissues tolerance to the accumulation of Na^+ as well as the ability of the plants to exclude Na^+ (Benderradji *et al.*, 2011). Based on the results obtained, Chamran showed a relative salt tolerance in grain yield and biomass production, and Hirmand showed to be an absolute salt tolerant cultivar. Chamran showed superior shoot biomass and yield (less reduction), Hirmand showed only less reduction in yield, but higher reduction in shoot biomass compared to Atrak. Therefore, maybe it is proper to say that Hirmand is salt tolerant during reproductive period, but not during vegetative growth stage.

The difference between the sensitive and tolerant groups can be related to their difference in response to high available Na^+ Under salinity stress conditions, the relatively salt tolerant cultivars try to exclude Na^+ and keep high K^+/ Na^+ ratio, especially in the flag leaf (Husain *et al.*, 2003). Atrak was considered salt sensitive cultivar because it showed high loss of yield and biomass, and low ability in Na^+ exclusion, and low K^+/ Na^+ ratio in flag leaf.

Under salinity, the K^+/Na^+ ratio falls dramatically, therefore, one of the key features of salt tolerant plants is the ability of cells to maintain optimal K^+/Na^+ ratio in the cytosol (Tester and Davenport, 2003). This occurs as the result of both excessive Na^+ accumulation in the cytosol and also enhanced K^+ leakage from the cell, the latter resulting from NaCl-induced membrane depolarization under saline conditions (Chen *et al.*, 2005). Therefore, K^+/Na^+ ratio in plant tissues has often been suggested as a potential screening tool for plant breeders (Asch *et al.*, 2000; Asch *et al.*, 1999; Poustini and Siosemardeh, 2004). (Asch *et al.*, 2000) indicated a highly significant correlation between leaves K^+/Na^+ and salinity-induced grain yield reduction. They showed that the most sensitive cultivars had the lowest leaves K^+/Na^+ and the largest yield reductions. The accumulation of free proline under salinity stress is reported in Pisum sativum (Najafi *et al.*, 2007), Brassica juncea (Rais *et al.*, 2013) and Triticum aestivum (Ashfaque *et al.*, 2014) as an osmoprotectant that reduce adverse effects of salinity by regulating redox potentials, scavenging oxygen radicals,

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reducing the acidity in the cell and acts as storage compound and nitrogen source. It seems that the increase in proline accumulation was due to P5CS, P5CR up-regulation and PDH down-regulation. Verslues and Sharma (2010) also proposed a model for proline metabolism in Arabidopsis under salinity stress that attributed proline accumulation to PDH and P5CDH down-regulation and P5CS up-regulation. Also, Xue *et al.* (2009) reported that the expression of BnP5CS1and BnP5CS2 was induced, while the expression of BnPDH was inhibited under salt stress, ABA treatment, and dehydration, prior to proline accumulation in *Brassica napus*. PDH inhibition by knockout or antisense repression resulted in no or only marginal increases in Pro levels under normal conditions. However, during stress, Pro levels were several-fold higher as in the wild type(Mani *et al.*, 2002; Nanjo *et al.*, 2003). Exceptions to the standard model of up-regulated Pro synthesis and down-regulated catabolism leading to Pro accumulation have been reported. For example, Kaplan *et al.* (2007) saw increased expression of PDH1 in longer term (24–96 h) cold treatments even while Pro contents were high and increasing. Stines *et al.* (1999) found high levels of Pro despite low P5CS1 expression in grape (*Vitis vinifera*) berries.

Results of this study showed that the salt sensitive cultivar had higher proline accumulation than the salt tolerant ones. Higher proline accumulation as well as Na^+ in the sensitive cultivar than in the tolerant ones has been indicated by previous studies (Aziz *et al.*, 1998; Poustini *et al.*, 2007). Rout and Shaw (1998) attributed the higher proline levels to higher injury which indictes more Na^+ accumulation in sensitive wheat cultivars. Although there was a positive correlation between proline and Na^+ in each leaf, proline in flag leaves was more than in the 4^{th} leaves, which had higher Na^+ concentration. Also, proline accumulation in flag leaf was more in tolerant cultivars than in salt sensitive one, while it is the opposite for 4^{th} leaf. This makes sense since flag leaf the young organ that needs more protection during stress and tolerant cultivars play this role perfectly.

Proline homeostasis is important for actively dividing cells as it helps to maintain sustainable growth under long-term stress. It also underpins the importance of the expansion of the proline sink during the transition from vegetative to reproductive growth and the initiation of seed development (Kishor *et al.*, 2014). Because of these two reasons, it seems that proline catabolism is a very important mechanism of tolerance in the 4^{th} leaf. This leaf has little energy due to senescence and stress. When proline is degraded, NAD(P)H will be released and cell energy will be higher. This leaf also is the source of nitrogen for grain and other young parts of plant. In this situation, proline degradation along with the increase of glutamate, a plant transportable nitrogen, will help to increase nitrogen source strength. According to this hypnosis, the expression pattern of PDH increased in the 4^{th} leaves of Chamran and Hirmand under salinity stress, but Atrak's 4^{th} leaf did not show any change in PDH expression in salinity treatment. It seems that the tolerant cultivars show higher degradation in the 4^{th} leaf to increase cell energy and transportable nitrogen level.

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تجمع پرولین و ژنهای مرتبط با آن در برگ‌های گندم تحت تنش شوری

م. توکلی، ک. پوستینی، و ه. علیزاده

چکیده

این مطالعه به منظور ارزیابی اثر تنش شوری(۱۶ دسی زیمنس بر متر) بر تجمع پرولین و الگوی بیان سه ژن دخیل در سنتز(P5CS و P5CR) و تجزیه (PDH) پرولین در برگ‌های چهارم و پرچم گندم صورت پذیرفت. تیمارهای آزمایش شامل دو سطح شوری ۱.۶ و ۱۶ دسی زیمنس بر متر و سه رقم گندم هیرمند و چمران (متحمل) و اترک (حساس) که به صورت فاکتوریل در قالب طرح کاملا تصادفی با سه تکرار

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آرایش یافتند. تیمار شوری موجب افزایش غلظت سدیم و کاهش غلظت پتاسیم در برگ‌های چهارم و پرچم همه ارقام نسبت به تیمار شاهد شد. اگرچه ارقام مقاوم غلظت سدیم کمتری را نسبت به رقم حساس نشان دادند، غلظت پتاسیم و نسبت پتاسیم به سدیم در این ارقام بیشتر بود. چنین به نظر می‌رسد که تجمع پرولین از طریق افزایش بیان دو ژن P5CS و P5CR و کاهش بیان ژن PDH بوده است. از طرفی تجزیه پرولین بالای برگ‌های چهارم در ارقام مقاوم احتمالا به دلیل مهیا کردن منبعی از انرژی و نیتروژن است که می‌تواند به افزایش عملکرد در این ارقام تحت تنش شوری کمک کند.

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