CHANGES IN WHEAT *P5CS* GENE EXPRESSION IN RESPONSE TO SALT STRESS IN WHEAT

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Drought and high salt are the most important adverse environmental factors that cause osmotic stress, negatively impacting plant growth and crop productivity. To maintain a stable intracellular environment in the presence of external environmental stresses, many plants increase their cellular osmotic potential through accumulation of intracellular organic osmolytes such as proline, glycine betaine, mannitol and trehalose (Wang *et al.*, 2007; Zhu, 2002). Proline is an osmoprotecting molecule that accumulates in many organisms, including bacteria, fungi, and plants, in response to low water stress and salinity (Claussen, 2005; Kumar *et al.*, 2003). Proline accumulation in plants not only increases cell potential but also stabilizes proteins, membranes, and subcellular structures (Verslues *et al.*, 2006). It also protects cells against oxidative damage by reactive oxygen species (Borsani *et al.*, 2005; Sharma and Dietz, 2006; Vendruscolo *et al.*, 2007).

In higher plants, proline is synthesized from glutamate or arginine/ ornithine. The pathway via arginine/ ornithine is not important for proline synthesis during osmotic stress (Hu *et al.*, 1992). The first two steps of proline biosynthesis from glutamate are known to be catalyzed by a bifunctional enzyme Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*), which is a rate-limiting enzyme in the pathway, triggering both γ-glutamyl kinase (γ-GK) and glutamic-γ-semialdehyde dehydrogenase (GSA-DH) activities (Yoshiba *et al.*, 1995).

In common with bacteria (Smith *et al.*, 1984), proline controls the γ-GK activity of P5CS by feedback in plants (Hong *et al.*, 2000). In higher plants, *P5CS* is encoded by a nuclear gene that was cloned from *Vigna aconitifolia* (Hu *et al.*, 1992), *Arabidopsis thaliana* (Strizhov *et al.*, 1997), *Medicaho truncatula* (Armengaud *et al.*, 2004), *Medicaho sativa* (Ginzberg *et al.*, 1998), and *Lycopersicon esculentum* (Fujita *et al.*, 1998) and other species. The expression patterns of different P5CS genes in plants have been studied under various stress conditions. For example, the gene *VaP5CS* was highly expressed in leaves and roots of salt stressed *V. aconitifolia* (Hu *et al.*, 1992). The *AtP5CS1* gene was expressed in most plant organs and tissues in Arabidopsis and was up-regulated by dehydration, high salinity, and abscisic acid (ABA) treatments, but was not expressed in dividing cell cultures

in the absence of stress .Expression of *AtP5CS2* in dividing cell cultures under stress induction was dependent on protein synthesis (Strizhov *et al.*, 1997). In rice (*Oryza sativa*), expression of *OsP5CS1* was induced by salt, drought, ABA, and cold, but not by heat treatment (Igarashi *et al.*, 1997). RT-PCR analysis showed that the *OsP5CS2* transcript was present in reproductive organs, especially in stamens (Hur *et al.*, 2004). In response to NaCl stress, mRNA of *tomPRO2* increased more than 3-fold, whereas the transcripts of *tomPRO1* were undetectable (Fujita *et al.*, 1998). Two P5CS genes of *M. truncatula* showed developmental and environment-specific features. *MtP5CS1* in different organs had steady-state transcript levels that were well correlated with proline levels, whereas *MtP5CS2* transcripts accumulated only in shoots of salt stressed plants (Armengaud *et al.*, 2004). It appeared that *MtP5CS1* acted as a developmental housekeeping enzyme responsible for the supply of proline to the reproductive organs, and MtP5CS2 acted as a shoot- specific osmoregulated isoform.

End-point PCR is a PCR system that it's whose results are recognized at the end of PCR steps on agarose gel like all different known PCR techniques. For example. randomly amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs) and inter simple sequence repeats (ISSRs) (Bered *et al.,* 2002; Weber and May, 1989; Nagaoka and Ogihara 1997), while the Real-time PCR is the technique of choice for nucleic acid quantification, because it offers the most time-ly, sensitive, and practical way of meeting new detection standards (Bustin, 2005; Engel *et al.*, 2006).

In This investigation we aimed to assess the changes in *P5CS* gene expression in response to salt stress in wheat plants on the transcriptional level using two different techniques; end-point (semi quantitative) reverse transcription RT-PCR and real-time PCR for the 1st strand of cDNA.

MATERIALS AND METHODS

Ten wheat cultivars were assessed for their salt tolerance according to the performance of some yield-related traits namely; Sakha94, Sakha61, Sakha93, Gimmeza10, Gimmeza9, Gimmeza7, Sids1, Sohag3, Giza168 and Sahel1. Grains were kindly provided by Wheat Research Dept., Field Crops Research Institute, Agricultural Research Center (ARC), Giza, Egypt. The ten wheat cultivars were grown in the greenhouse in ARC, Giza and were subjected to salt stress at 0 and 6000 ppm NaCl up to 105 days of planting. The measured traits were plant height (PH), youngest elongate blade leaf (YEB) length cm, shoot fresh weight (SFW), shoot dry weight (SDW), main pick length and main pick weight. The data were statistically analyzed using SAS program to determine the two contrasting cultivars in their salt tolerance.

RNA extraction

RNA was extracted from the leaves of salt treated plants and its control ones

of the most salt tolerant and the most salt sensitive cultivars according to Ashoub *et al*. (2006). RNA quality was examined on denatured agarose gel electrophoresis. The yield of the extracted RNA was quantified using spectrophotometer.

Reverse transcription-PCR

RNA of each of the four aforementioned samples was reverse transcribed (RT), to produce the first strand of cDNA in the presence of 5mM $MgCl_2$, 1X PCR Buffer, 1mM dNTPs, 25u MuLV Reverse Transcriptase, 4u RNA-guard Ribonuclease inhibitor and 2.5µl of 20 Pmol of *P5CS* reveres primer with the following sequence (GTT YAA RYT IGT NAG RGG IGC HTA) in a final reaction volume of 30µl - where R= A or G, Y= C or T, K= G or T, I = inosine, N= any base- . Reactions were carried out at 42°C for 30 min, followed by a 10 min step at 94°C to denature the enzyme, then cooling at 4°C.

End-point PCR to assess P5CS gene expression

Equal amounts of the produced cDNA (2 µg) of each sample was amplified using reaction mix containing of 1 unit *Taq* polymerase, 1x buffer, 1.5mM $MgCl_2$, 2.5 mM dNTPs and in the presence of the specific primers for dehydrin with the following sequences {P5CS (F) GTT YAA RYT IGT NAG RGG IGC HTA and P5CS (R) CTC RTA IGC ICK ICK IAR IAR RTA}.

Reactions were carried out in the following conditions, the first cycle for 4

minutes at 94°C, followed by 45 seconds at 94°C, 45 seconds at 55°C and 1 min at 72°C for 35 cycles. The conditions were chosen that none of the RNAs analyzed reached a plateau at the end of the amplification protocol, i.e., they were in the exponential phase of amplification, and that the two sets of primers used in each reaction did not compete with each other. Each set of reaction always included a negative control to rule out genomic DNA contamination.

The concentration of the products was measured using spectrophotometer on the wave length 280/260 and was used as detect the concentration of cDNA encoding for *P5CS* in the four samples under investigation (semi-quantitative RT-PCR).

Real-time quantitative PCR of P5CS cDNA

All PCR processes were performed using commercially available reagents that included a thermo-stable DNA polymerase, dNTPs, $MgCl_2$, and other salts and buffering agents necessary for optimum performance. One µl of cDNA of the four aforementioned samples was used as template in the reaction mix, in a final volume of 25 µl in all assays. Conventional PCR, using the aforementioned *P5CS* forward and reverse primers were employed to define the detection limit of the assay. Cycling was carried out in a Stratagene Mx-3000 Real-time PCR system which allows detection of most commercially available dyes including FAM,

SYBR® Green I, TET, HEX™, JOE™, VIC™, TAMRA™, TexasRed®, ROX™, Cy5™, Cy3™ and ALEXA Fluor® 350. The system supports 96-well plate format and can perform multiple sub-experiments up to four dyes in the same well. Bioron product, SYBR® Green I Real Time QPCR (cat No. 119205) master mix for (100 rcs) detection protocol was used in this investigation as described in the manufacturer's manual.

Data analysis

Results from the Light Cycler, fluorescence thresholds were statistically analyzed using Microsoft Excel (Office 2000; Microsoft Corp.).

RESULTS AND DISCUSSIONS

The results of the six yield-related traits (Table 1) indicated that the highest average of plant height (PH) was recorded for cultivar Sakha93 (treatment was 139.05% of the control), while the lowest was obtained in Giza168 (treatment was 86.27% of the control). The highest average of the youngest elongate blade leaf (YEB) was recorded for cultivar Sakha93 (treatment was 166.41% of the control), while the lowest was in Giza168 (treatment was 69.05% of the control). The highest average of shoot fresh weight (SFW) was recorded for cultivar Sakha93 (treatment was 133.8% of the control), while the lowest was in Gimmeza 9 (treatment was 43.6% of the control). For shoot dry weight (SDW), the results indicated that the highest cultivar was Sakha93

(treatment was 137.58% of the control), while the lowest was obtained in Sids1 (treatment was 61.37% of the control). On the other hand, main pick length (M pick) indicated that the highest average was scored for cultivar Sohag3 (treatment was 175.16% of the control), while the lowest was obtained in cultivar Giza168 (treatment was 59% of the control). In addition, the main pick weight (M pick W) indicated that the highest was scored in cultivar Sakha93 (treatment was 149.37% of the control), while the lowest was scored in cultivar Gimmeza10 (treatment was 57.45% of the control). As shown in Table (1) also, the ten cultivars were arranged in descending order according to the average of their arrangement overall the six investigated traits and it could be concluded that Sakha93 was the highest cultivar in growth under salt stress (the most salt tolerant cultivar) while, Sakha61 was the lowest one in growth under salt stress (the most salt sensitive cultivar) as detected from the results. Hu *et al.* (2005) reported that salinity greatly reduces the leaf cross-sectional area of wheat during its development, which may lead to variation in the architectural properties of growing leaves that would result in a change in leaf physiological functions.

End-point RT-PCR to quantify P5CS gene expression

Quantification is rather accurate, but this technique requires a relatively large amount of cDNA and a large number of amplification reactions per sample and

moreover, requires intensive initial work when different RNAs have to be analyzed. Another interesting, recently devised method, is the single-tube PCR which permits reverse transcription and PCR, this is a very convenient method when a single RNA marker has to be analyzed in a large number of samples.

Plants grown under control condition and salt treated of the most salt tolerant cultivar (Sakha93) and the most salt sensitive one (Sakha61) were subjected to Semi quantitative RT-PCR analysis for *P5CS* gene expression using their cDNA as a template for PCR mix. The results in Table (2) and Fig. (1) showed the PCR products in the two cultivars under control and stress conditions but with different intensities. Higher intensity was observed in the salt stressed plants of cultivar Sakha 93 (the tolerant cultivar) as compared with the control ones of the same cultivar indicating that *P5CS* gene expression increased by 1.34 folds under salt stress. Equal volumes of PCR products were used for spectrophotometer assessment on the wave length 280/260. On the other hand, in the sensitive cultivar (Sakha 61), the intensity increased in the stressed plants as compared with the control ones but lower than that of the tolerant cultivar (1.08 folds of control plants). The results confirmed that the response for salt stress was greater in cultivar Sakha 93 than in cultivar Sakha 61 regarding *P5CS* gene expression as detected by semi quantitative reverse transcription-PCR.

Real-Time quantitative PCR of P5CS gene expression

The same cDNA of the aforementioned samples were used to perform the more accurate quantification technique (Real Time PCR) to analyze *P5CS* gene expression in control condition and salt treated plants of the tolerant and sensitive wheat cultivars. The results in Table (3) and Fig. (2) showed that the florescent dye increased from 23.00 in the tolerant control to 33.50 in the tolerant treated indicating the increase in the copy number of *P5CS* in the treated tolerant with 1.457 folds of the tolerant control. The increase in *P5CS* of the treated tolerant plants revealed an increase in the gene encoding for this enzyme which plays the key role in proline biosynthesis and accumulation in the plant as response for salt stress. On the other hand, in the sensitive cultivar (Sakha 61) the florescent dye increased from 27.50 in control plants to 30.50 in the stressed plants with 1.11 folds of the control ones. These results indicated that *P5CS* content increased in the salt stressed plants as a reflection of the increase in the encoding gene. The results of real time PCR also indicated that the increasing folds of *P5CS* were greater in the tolerant cultivar than that of the sensitive one under salt stress. Figure (3) showed a band with size of 380 bp produced using Real Time PCR for cDNA encoding P5CS in the two cultivars under investigation in both control and salt stress conditions. The differences in band intensities between control and salt stressed plants in the tol-

erant and sensitive cultivars confirmed the results of real time-PCR florescent dye.

It might be concluded that both the assessment techniques, semi quantitative RT-PCR and Real Time-PCR, indicated the increase in the content of cDNA encoding P5CS with more than one fold in tolerant cultivars than sensitive cultivars. Also the results indicated the efficiency of semi quantitative RT-PCR in gene expression assessment in plants similar to that of Real Time-PCR technique, moreover, end-point RT-PCR (semi quantitative RT-PCR) is cheaper and easier technique.

Real-time PCR was used to quantify changes in relative mRNA abundance for 333 sorghum genes that responded to NaCl or osmotic stress. Buchanan *et al*. (2004) reported that osmotic stress inducible sorghum genes were identified for the first time included a beta-expansin expressed in shoots, actin depolymerization factor, inositol-3-phosphate synthase and oleosin. Analysis of response profiles demonstrated the existence of a complex gene regulatory network that differentially modulates gene expression in a tissue and kinetic-specific manner in response to high salinity and water deficit. Modulation of genes involved in signal transduction, chromatin structure, transcription, translation and RNA metabolism contributes to sorghum's overlapping but nonetheless distinct responses to high salinity and osmotic stress.

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Table (1): The increase or decrease as % of the six measured traits under salt stress (6000 ppm) as compared to control conditions in the ten wheat cultivars and the order of these cultivars for each trait.

Cultivar		PH (cm)	YEB (cm)	SFW (g)	SDW (g)	M PICK Length (cm)	M PICK Weight (g)	Order
Sakha 94	%	107.59	104.75	50.15	64.06	65.63	73.03	
	rank	3	3	7	7	9	6	6
Gimmeza 10	%	105.31	110.75	93	81.93	104.17	57.45	
	rank	5	2	4	6	2	10	4
Sakha 61	%	90.83	72.78	45.63	62.24	85.39	75.48	
	rank	9	9	9	9	6	5	10
Gimmeza 9	%	104.86	81.2	43.6	62.66	103.51	92	
	rank	6	7	10	8	3	3	7
Sids 1	%	107.15	91.5	49.03	61.37	70.37	66.89	
	rank	4	6	8	10	8	8	9
Sohag 3	%	103	75.676	81.14	122.5	175.16	99.1	
	rank	7	8	6	4	1	2	2
Gimmeza 7	%	116.2	97.95	102.97	108.75	71.1	65.49	
	rank	2	5	3	5	7	9	5
Sakha 93	%	139.05	166.41	133.8	137.58	99.1	149.37	
	rank	1	1	1	1	4	1	1
Giza 168	%	86.27	69.05	93	135.7	59	81.56	
	rank	10	10	4	2	10	4	8
Sahel 1	%	98.19	98.2	106.4	123.3	88.61	71.43	
	rank	8	4	2	3	5	7	3

PH = plant height, **YEB = youngest elongate blade leaf length cm,** **SFW = shoot fresh weight,**
SDW = shoot dry weight, **M pick length= main pick length and** **M pick weight= main pick weight.**

Table (2): Concentrations of cDNA encoding for *P5CS* as detected by spectrophotometer for the two cultivars under control and salt stress as assessed using semi quantitative RT-PCR protocol.

Cultivar	spectrophotometer reading	Folds
Sakha 93 (Tolerant) control	143.17	1.34 times control
Sakha 93 (Tolerant) treated	192.14	
Sakha 61 (sensitive) control	99.87	1.08 times control
Sakha 61 (sensitive) treated	107.63	

Table (3): Concentrations of cDNA encoding for *P5CS* as detected by florescent dye for the two cultivars under control and salt stress as assessed using Real Time-PCR protocol

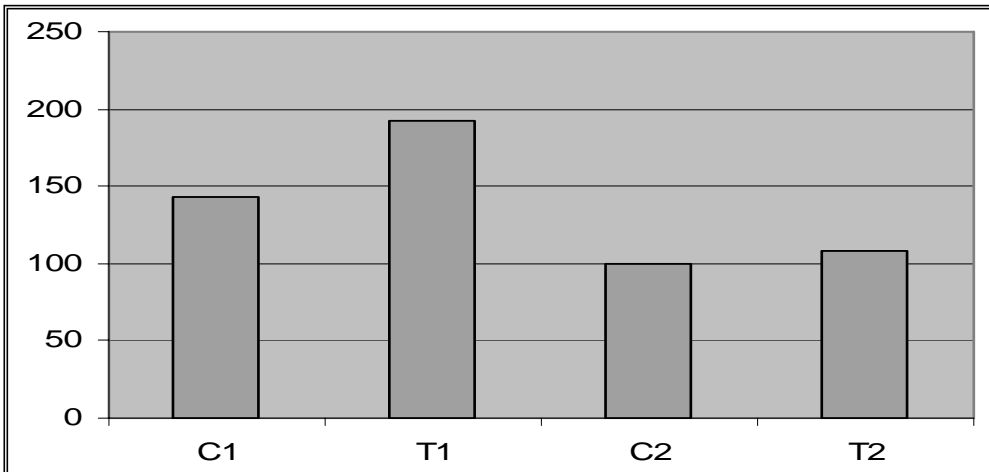| Cultivar	Real Time-PCR reading	Folds
Sakha 93 (Tolerant) control	23.0	1.457 times control
Sakha 93 (Tolerant) treated	33.5	
Sakha 61 (sensitive) control	27.5	1.11 times control
Sakha 61 (sensitive) treated	30.5	

Fig. (1): Diagram showing the concentrations of cDNA encoding for *P5CS* as detected by spectrophotometer for the two cultivars under control and salt stress as assessed using semi quantitative RT-PCR protocol, where C1 = Sakha 93 (Tolerant) control, C2 = Sakha 61 (sensitive) control, T1 = Sakha 93 (Tolerant) treated and T2= Sakha 61 (sensitive) treated.
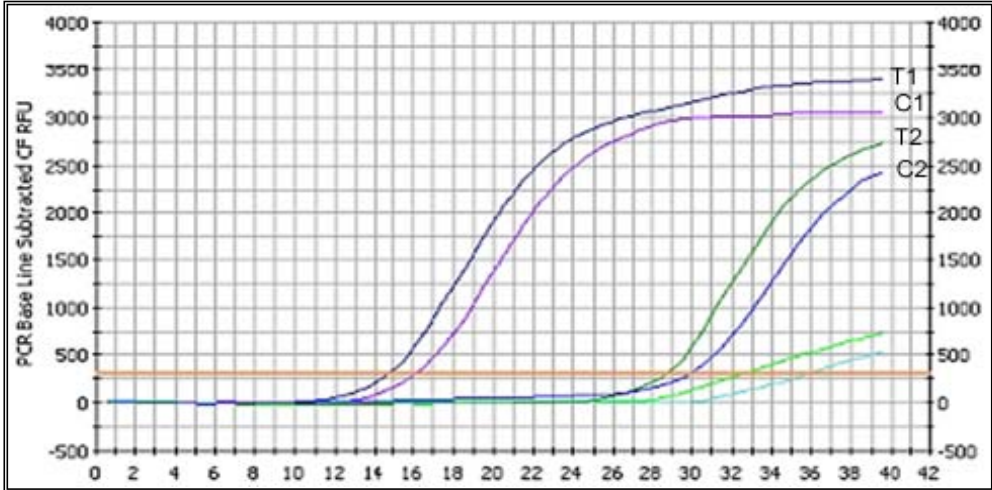
Fig. (2): Chart for the concentrations of cDNA encoding for *P5CS* as detected by fluorescent dye for the two cultivars under control and salt stress as assessed using Real Time-PCR protocol. Where C1 = Sakha 93 (Tolerant) control, C2 = Sakha 61 (sensitive) control, T1 = Sakha 93 (Tolerant) treated and T2 = Sakha 61 (sensitive) treated.

Fig. (3): Agarose gel electrophoresis for Real time-PCR of cDNA encoding for *P5CS* (380 bp) for the two wheat cultivars under control and salt stress.