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Expression pattern analysis of *TomPRO2* and *LaPA1* genes in tomato under *in vitro* salt stress by Semi-quantitative RT-PCR

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Abstract

The expression pattern of *TomPRO2* and *LaPA1* genes in two tomato (*Lycopersicon esculentum*) cultivars named as Isfahani and Shirazi under *in vitro* salt stress were investigated. Four to six weeks old *in vitro* grown seedlings were transferred on MS medium containing 0, 80 and 160 mM NaCl and untreated plants were used as control. RNA was extracted from root and leaf and then cDNA was synthesized. Semi-quantitative analysis of *TomPRO2* and *LaPA1* expression using specific primers showed that with increasing of NaCl concentration from 0 to 160 mM, the expression level of *TomPRO2* and *LaPA1* genes in roots of both tomato cultivars did not change significantly. In leaf TomPR2 gene expression decreased significantly at 160 mM NaCl. At the same concentration of NaCl (160 mM) *LaPA1* gene expression in leaf increased significantly. Based on the obtained results, *TomPRO2* and *LaPA1* genes have different expression patterns under *in vitro* salt stress in tomato root and leaf. However, both genes have a role in response to salt stress.

Keywords: Tomato; Salt stress; Semi quantitative RT PCR; Gene expression

Introduction

Rising human and animal populations and their needs for food have exerted tremendous pressure on environment. The continued increase in food production in order to keep population growth in the world is a serious problem for scientists. Approximately, half of the world's agricultural land lies in arid or semi-arid regions; consequently salinity and drought are the two major abiotic stresses that limit plant growth and productivity (Arcioni et al., 1990). Other agricultural regions have consistently low rain-fall and rely on irrigation to maintain yields. In both circumstances, crop plants which can make the most efficient use of water and maintain acceptable yields will be at an advantage. To access this approach, identification the gene(s) and its products which is responsible for salt and drought tolerance may lead to cope with this global problem. Tomato (*Lycopersicon*

esculentum Mill.), one of the important and widespread crops in the word, is sensitive to moderate levels of salt in the soil (Cuartero and Fernandez-Mun, 1999; Ramanjulu and Bartels, 2002). The response and adaptation of plants to such conditions are very complex and plants have developed various strategies to increase stress tolerance. These strategies include changes in metabolic processes, structural changes of membranes, expression of specific genes and production of some metabolites (Ramanjulu and Bartels, 2002; Shinozaki and Shinozaki-Yamaguchi, 1997).

In tomato, it has been reported that there is a proline loci in the nuclear genome; *TomPRO2* gene (Δ1-pyrroline-5- carboxylate synthetase, P5CS, from tomato) encodes a key enzyme for proline accumulation (Garcia et al., 1997). This gene has also been shown to be present in both Arabidopsis and alfalfa which is expressed in the most organs of Arabidopsis and induced rapidly by stress (Yoshiba et al., 1995; Zhang et al., 1995). The signals that mediate responses must be transmitted throughout the plant and may involve cell-to-cell signaling. Some of the most important signals include ethylene (O'Donnellet et al., 1996). ABA (Pena-Corte´s et al., 1989) and proline (Zhang and Verma, 1995).

It is known that, Aminopeptidases catalyze the hydrolysis of amino acids from the N terminus of peptides. Leucyl aminopeptidases (LAPs; EC 3.4.11.1) are members of the M17 family of peptidases (Barrett, 1998). Tomatoes express two forms of leucine aminopeptidase (LAP-A and LAP-N) and two LAP-like proteins. In tomato, *LapA1* (Leu aminopeptidase) transcripts, protein and activities increase locally and systemically in response to wounding (GU and Walling, 2000; Pautot et al., 2001) by the wound octadecanoid pathway (Chao et al., 1999). *LapA1* gene also responds to signals generated during water deficit and salinity stress (Ramanjulu and Bartels, 2002). For this reason, it was important to develop a comprehensive understanding of *LapA1* expression at the RNA level in response to salt stress. Using semi-quantitative method for evaluation of gene expression under stress condition has already been reported for some plants (Tozzini et al., 2000). The objective of this study is evaluation of expression level of *TomPRO2* and *LaPA1* transcripts as two important genes induced by salt stress using semiquantitative RT-PCR after *in vitro* salt treatments of Isfahani and Shirazi tomato cultivars.

Material and Methods

Two tomato cultivars, Isfahani and Shirazi were obtained from Seed and Seedling Resources of Isfahan, Iran. In order to germinate, seeds were surface sterilized by soaking in 5% (v/v) sodium hypochlorite solution for 15 min followed by 3-4 times washes with sterile distilled water. *In vitro* germination was accomplished in 8 cm petri dishes containing sterile Water Agar medium. The pH of the medium was adjusted to 5.8 with NaOH before adding Agar (0.8%). Ten seeds were placed in each petri dish and incubated in the culture room under flourcent light (90 $molm^{-2}s^{-1}$), with 16-h light, at $25\pm2^{\circ C}$ for 6 days grown seedlings were then transferred to MS (Murashige and Skoog, 1962) medium supplemented with 0, 80 and 160 mM NaCl for 21 days and well grown root and leaf samples were used for RNA extraction.

RT-PCR

RT-PCR was preformed for salt treated root and leaf as well as untreated (control) plants using R$^{everse-i}$ T$^{TM\ One\ Step}$ system RT-PCR Kit (AB-0845) according to manufacture's instruction to detect RNA transcripts. RNA templates extracted from frozen 50-100mg root and leaf tissue using TRIZOL reagent (GIBCO BRL). Extracted RNA was treated with DNase-1 enzyme to remove DNA contamination. For cDNA synthesis one µl of RNA template (1 µg/ml) was mixed with 25 µl of 2X Reddy Mix^{TM} Master Mix, 1 µl primer (Table 1), 1 µl reverse transcriptase blend and DECP treated water up to 50 µl. Reverse transcription and PCR amplification was performed using the following thermal conditions: First strand cDNA synthesis at 47 °C for 30 min (1 cycle), reverse transcriptase inactivation and initial denaturation at 94 °C for 2 min (1 cycle), denaturation at 94 °C for 20 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 5 min (40 cycles) and final extension at 72 °C for 5 min (1 cycle).

Two-step RT-PCR conditions using Im Prom II reverse transcription kit for cDNA synthesis (Promega) with oligo dT primers were performed according to the instruction manual for increased sensitivity. The cDNA product was diluted 40 times and 1 µl was used as a template for PCR with a reaction mixture containing 5 mM of each primers, 2 mM dNTPs, 1X *Taq* buffer and 1 unit of *Taq* polymerase (BIOLINE) in a final volume of 20µl. The DNA fragment was amplified for 35 cycles using the following thermal conditions: denaturing DNA template 94 °C for 30 sec, primer annealing 5 °C below primer Tm for 15 sec, DNA synthesis 72 °C for 1 min. The primer sequence (designed by Oligo Porgram) is shown in Table 1.

Table 1. Primers used for RT PCR.

Name	Sequence
FW-TomPRO2	CAT CAT TAC TGA AGA TCA GGA AGT TGC TG
RV-TomPRO2	CCA TCA ACA ATT TGT CCA CTT CC
FW-LaPA1	CAA GGT GTT GAG AAG ATA ATT GAT CTG
RV-LaPA1	CCC GTG GCG TTT TTC TTT TC

RT-PCR was performed to measure the relative expression level of *TomPRO2* (gene ID, 544281, mRNA accession, U60267.1) and *LaPA1* genes (gene ID, 544017, mRNA accession, U50151.1). After amplification of cDNA using specific primers (Table 1), the PCR products (5 µl) were electrophoresed on 1.4% agarose gels in TBE buffer (89 mM Tris base pH 7.6, 89 mM boric acid, 2 mM EDTA). The gels were stained with ethidium bromide [10 µg/ml] and photographed on top of a 280 nm UV light box. The gel images were digitally captured with a CCD camera. For quantification, gels were scanned, and the pixel intensity for each band was determined using ImageJ program (NIH Image, Bethesda, MD) and all data were normalized (using Sigma STAT program). Mean (± standard deviation) of three replicates of RT-PCR values were presented as a relative value (relative intensity) in each treatment based on intensity of surface area of each DNA band. Quantification of transcripts was further confirmed by repeated PCR in which different mRNA samples were used (data not shown). The amount of PCR product increased exponentially in early cycles of the reaction. On the basis of these experiments, all

quantitative RT-PCR analyses were conducted at 35 cycles. To avoid the effect of genomic DNA-derived PCR products, we performed PCR with genomic DNA as templates, and compared the sizes of products derived from genomic DNA and expected size of cDNA fragments (data not shown).

Results

Expression of TomPRO2 gene

Expression level of *TomPRO2* transcripts in two tomato cultivars (Isfahani and Shirazi) was determined by semi quantitative RT PCR. The expression level of *TomPRO2* mRNA in roots of Isfahani and Shirazi tomato cultivars did not change significantly when the concentration of NaCl increased from 0 to 160 mM. However, the expression level of *TomPRO2* mRNA was decreased significantly at 160 mM NaCl concentration in leaves of Isfahani and Shirazi tomato cultivars (Figure 1 and 2).

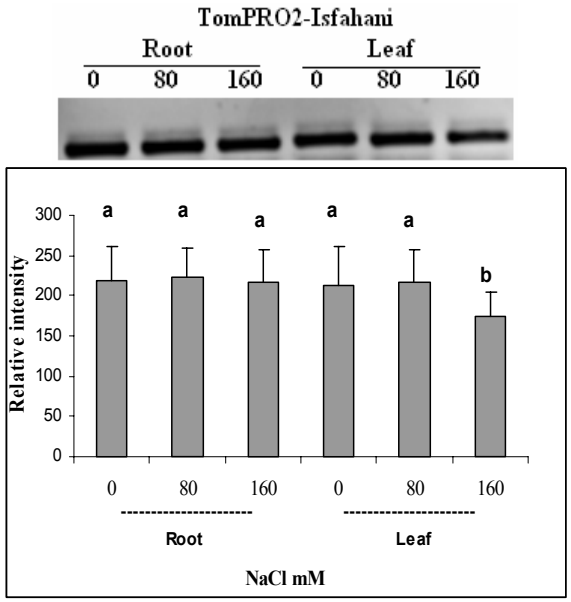

Figure 1. Relative expression of *TomPRO2* gene under *in vitro* salt treatment in cultivar Isfahani. (Uncommon letters are significant $P<0.05$ based on Tukey test).

Expression of LaPA1 gene

The result of relative expression level of *LaPA1* mRNA was shown in Figure 3 and 4. The general patterns of expression level of this gene under salt treatment was similar in two tomato cultivars (Isfahani and Shirazi). In both cultivars *LaPA1* was expressed in roots much higher than leaf. In root, increasing of salt concentration increased the level of

transcripts a little but the difference between the NaCl concentration of 0 and 80, 160 mM was not significant. In contrast, salt treated leaf increased the mRNA level for *LaPA1* gene at 160 mM NaCl significantly in two tomato cultivars.

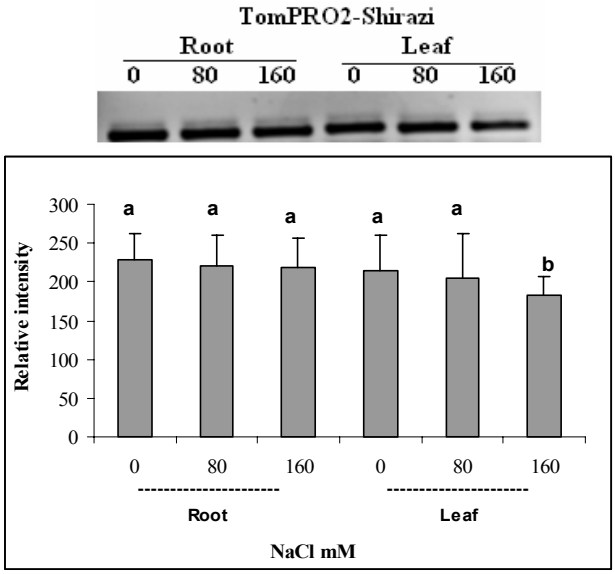

Figure 2. Relative expression of *TomPRO2* gene under *in vitro* salt treatment of Shirazi cultivar. (Uncommon letters are significant $P<0.05$ based on Tukey test).

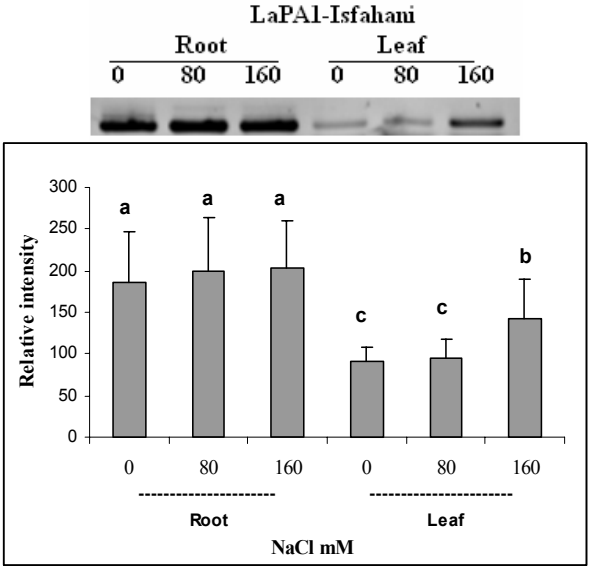

Figure 3. Relative expression of *LaPA1* gene after *in vitro* salt treatment of Isfahani cultivar (Uncommon letters are significant $P<0.05$ based on Tukey test).

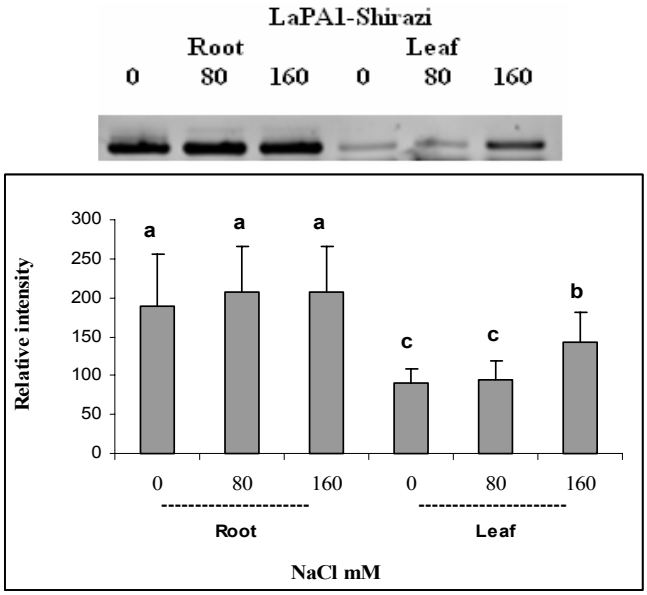

Figure 4. Relative expression of *LaPA1* gene after *in vitro* salt treatment of Shiraz cultivar. (Uncommon letters are significant $P<0.05$ based on Tukey test).

Discussion

Several salt-stress responding genes have been isolated from plants exposed to different kinds of stresses, such as drought and low-temperature (Winicov, 1994). Some genes induced by abiotic stresses other than salt, such as drought, ABA, or osmotic shock, can be related to responses to NaCl (Shinozaki and Shinozaki-Yamaguchi, 1997). To determine whether salt stress is involved in the expression level of *TomPRO2* and *LaPA1* genes quantification of transcripts of these genes under performed by semi-quantitative RT-PCR analysis to find the changes in transcript level of these genes under *in vitro* salt stress. After amplification of tomato *TomPRO2* and *LaPA1* cDNAs from Isfahani and Shirazi cultivars equal amounts of cDNAs were used for each RT-PCR analysis. Salt responsive genes in plants under salt stress are often expressed in different parts of the plants (Winicov, 1994). In the present study, *TomPRO2* and *LaPA1* genes in tomato showed different expression patterns in root and leaf.

TomPRO2 gene (tomato P5CS) expressed in yeast, allow enhanced accumulation of proline under salt stress. Furthermore, it has also been observed that the intracellular proline accumulation in yeast and plant is directly correlated with increased vacuolization. (Bone et al., 1998; Chang et al., 1996). This finding suggests that proline acts as signal/regulatory molecule able to activate multiple stress responses. In our previous investigations we found that proline accumulation in alfalfa callus (Ehsanpour and Fatahian, 2002) and tomato (Amini and Ehsanpour, 2005) increase salt tolerance. Since, *TomPRO2* is a key gene in proline biosynthesis pathway; it may increases proline content in response to salt stress. In

the present study, unchanged level of *TomPRO2* and *LaPA1* mRNA in roots of both tomato cultivars indicated that tomato roots may less affected by salt stress. In contrast, decreasing level of *TomPRO2* mRNA in tomato showed that, leaf is more sensitive to salt stress than root. Similar results have also been reported by Fujita et al., (1998). One possibility of decreasing level of *TomPRO2* gene in leaf indicates that accumulation of proline is might be regulated by feedback inhibition process (Zhang et al., 1995; Zhang et al., 1997) and consequently this system decreases the expression level of *TomPRO2* gene in tomato plant at 160 mM NaCl

Tomato *LaPA1* is a member of the Lap (Lucin aminopeptidase) gene family. It has been reported that, Arabidopsis *LaP* gene is constitutively expressed (Pena-Corte´s et al., 1989) for example; potato Lap mRNAs do not accumulate systemically after wounding stress in roots (Herbers et al., 1994; Hildmann et al., 1992). In our study, similar pattern of *LaPA1* expression was observed in tomato roots under salt stress. In contrast, *LaPA* transcripts and proteins are abundant in leaves after wounding, pathogen invasion, and insect infestation, the same pattern of expression for *LaPA1* in tomato leaf was observed. Increasing level of *LaPA1* mRNA in leaf in the present study may play an important role in the tomato defense response under salt stress (Pautot et al., 2001). Comparisons of *LaPA1* gene expression patterns relative to patterns of expression for the wound-response gene pin2, the ABA-response gene le4, and three PR protein genes (PR-1, PR-4, and GluB [basic β-1,3-glucanase]) in ABA-deficient or ABA-producing lines demonstrated that each gene responded to abiotic stress signals in a distinct manner. In our study, we found that in leaves of both tomato cultivars (Isfahani and Shirazi) when NaCl concentration increased, the level of *LaPA1* expression increased significantly at 160 mM NaCl. Increasing the expression of *LaPA* gene in respond to salt stress has also been reported by other study (Chao et al., 1999). Similar data has indicated that at least few numbers of signaling pathways are required to modulate salt stress gene expression in tomato plants. Also few investigation have shown that, *LAPA* enzyme preferentially hydrolyzes substrates with N-terminal Leu, Arg, and Met and does not cleave substrates with N-terminal Asp, Glu, or Gly (Gu and Walling, 2000, 2002), however, the precise role of this gene is remained to be identified in plant growth and development under biotic and abiotic stress.

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