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Responses of phytochelatin and proline-related genes expression associated with heavy metal stress in *Solanum lycopersicum*

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Abstract – The expression of stress related-genes against adverse environmental conditions has essential importance for plants. This study, using RT-qPCR, determined the expression of *P5CS* and *PCS* genes to investigate their roles in the leaves of tomato plants grown under heavy metal conditions. The expression of the *PCS1* gene is significantly induced under such conditions. Transcript expression of *P5CS1*, a gene responsible for proline synthesis, changed depending on heavy metal doses; treatments of Cu (20 and 50 ppm), Cd and Pb (10 and 20 ppm) remarkably increased *P5CS1* expression. However, the *P5CS1* gene expression at 10 ppm dose of Cu and 50 ppm doses of Pb and Cd was not significantly different from that in control plants. The metal-chelating potency of the extract of tomato leaves exposed to Pb and Cd was higher than that of untreated plants. The proline content as assessed in the leaves of stressed plants was significantly increased by applications of 10 and 20 ppm of Cd and Pb, and high doses of Cu. In addition, the results showed that the proline content had a positive correlation with the *P5CS1* gene expression in tomato leaves under application of these tree heavy metals and that there was a positive relation between the *PCS1* gene expression and metal-chelating ability of Cd-stressed plants.

Keywords: gene expression, heavy metal, metal chelating activity, phytochelatin synthase gene (*PCS*), pyrroline-5-carboxylate synthetase gene

Introduction

Plants sustain their lives under varying external conditions and they respond to environmental stimuli by synthesizing amino acids and peptides such as proline, phytochelatins (PCs), and metallothioneins (MTs) (Chen et al. 1997). Heavy metals are major environmental pollutants for plants, and their accumulation of them in agricultural areas influences the growth, development, and productivity of the plants. Although plants need to some heavy metals such as Cu, Zn and Mn for various biochemical pathways, which aid in their growth and development, the excesses quantities of these metals and the presence of other non-essential heavy metals such as Cd and Pb reduce the plant life and degrade yield quality. Heavy metals can interact with thioyl and histidyl groups of proteins, induce the production of reactive oxygen species (ROS) production, which and eventually causes the toxicity in plants (Sharma and Dietz 2009, Nagajyoti et al. 2010). The accumulation of heavy metals changes the plant's molecular, biochemical and physiological responses and plants have developed advanced mechanisms to withstand the abiotic stress factors. Plant defense mechanisms in response to heavy metal toxicity include immobilization, exclusion, chelation, compartmentalization and synthesis of stress proteins (Toppi and Gabbrielli 1999, Lee et al. 2002, Rellán-Álvarez et al. 2006, Hossain and Komatsu 2012).

The overexpression of metal-binding proteins, such as PCs and MTs increases the metal-binding ability and tolerance in plants. These proteins are enzymatically derived and synthesized when plant exposed to metal ions (Mejáre and Bülow 2001). The potential toxicity of heavy metals requires rigorous control to maintain the cell homeostasis by chelation of metal ions with PCs which represent one of the general mechanisms and tolerance. PCs are post-translationally synthesized cytosol proteins. PCs are cysteine-rich and comprise the amino acids glutamine, cysteine, and glycine, and PCs are particularly synthesized in response to heavy metals, including Cd, Cu, Ni, Pb, and Zn. PCs have the general structure $(\gamma\text{-glutamylcysteinyl})_n$-glycine (n = 2–11) and are synthesized from reduced glutathione (GSH) by the enzymatic reaction of phytochelatin synthase (PCS) (Cazalé and Clemens 2001, Gupta et al. 2004, Shen et al. 2010, Shanmugaraj et al. 2013, Tripathi et al. 2013). Moreover, PCs biosynthetic pathway is regulated by a series of mechanisms one of which

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is the regulation of GSH biosynthesis and PCs biosynthesis has been correlated with the expression of GSH (Ben Ammar et al. 2008). Glutathione is synthesized from its constituent amino acids — cysteine, glycine, and glutamate — catalyzing *ɣ*-glutamylcysteine synthase (*ɣ*-ECS) and glutathione synthase (GS). The PC synthase (PCS) catalyzes the conversion of glutathione (GSH) into phytochelatin (Mejáre and Bülow 2001). Metal-chelating potency of plants indicates the ability of chelating compounds to bind the metal ions; this ability is dependent on the amount of phytochelatins and metallothioneins, unique phenolic structure of plants and location of the hydroxyl groups in the plant phenolic compounds (Wang et al. 2009, Flora and Pachauri 2010, Tito et al. 2011).

The adaptation of plants to abiotic stress is achieved by the synthesis of the compatible solutes, such as proline and osmotin. Proline accumulation is considered as an adaptive role in response to environmental stresses and this accumulation relies mainly upon the increased synthesis and reduced degradation (Verbruggen and Hermans 2008). Proline has important roles in protecting the cellular homeostasis from stress-induced damage (Li et al. 2015). Plants have two different precursors involved in proline biosynthesis - glutamate (Glu) and ornithine (Orn). The Glu pathway usually occurs in the cytosol and chloroplast under the abiotic stress, whereas the Orn pathway is associated with seedling development. Proline is synthesized from glutamate via GSA (glutamate-semialdehyde) and P5C (Δ^1-pyrroline-5-carboxylate); the first reaction is catalyzed to GSA by P5CS (Δ^1 -pyrroline-5- carboxylate synthetase) and it spontaneously converted to P5C. Then, P5C is reduced to proline by P5CR (Δ^1 -pyrroline-5-carboxylate reductase) (Verbruggen and Hermans 2008, Wang et al. 2015). The P5CS activity regulates the generation of proline in the biosynthetic pathway, which is controlled by the *P5CS* transcription. The *P5CS* gene has two copies: *P5CS1* and *P5CS2*. The *P5CS1* gene is believed to be ubiquitously expressed in both vegetative and reproductive organs and is induced by stress, whereas *PC5S2* is preferentially expressed in mature plants and in response to incompatible interactions (Turchetto-Zolet et al. 2009).

Environmental stress to plants is a global concern and leads to product loss by causing the injuries in plants. Although several molecular studies on have been done to study abiotic stress in plants, such as *Arabidopsis*, tomato, bean, rice, and chickpea, many studies are intensively focused on the biochemical and physiological parameters, particularly antioxidant enzymes and total phenolic content (Gupta et al. 2004, Mattioli et al. 2009, Tripathi et al. 2013, Chen et al. 2016, Singh et al. 2016). In the current study, to understand the molecular basis of *Solanum lycopersicum* under the heavy metals stress, the expression analysis of two selected genes (*PCS* and *P5CS*) encoding the key enzymes responsible for phytochelatin and proline biosynthesis respectively are investigated in the leaves of tomato exposed to different doses of Cd, Cu, and Pb. In addition, the proline content which is considered the main index under abiotic stress, and metal-chelating capacity depending on the hydroxyl groups in chelating compounds were determined in the leaves of tomato cultivated in heavy metal-polluted boxes.

Materials and methods

Plant material and growth conditions

The cultivars of tomato (*Solanum lycopersicum* cv. Çiko F1) provided by the Agricultural Faculty of Gaziosmanpasa University were cultivated in plastic boxes containing an 11-kg mixture of peat and garden soil (1:1) under unheated glasshouse conditions. The entire experiment was performed as a randomized plot design with 16:8 photoperiod, 25 ± 2 °C, and N (150 ppm), P (80 ppm), K (100 ppm) and B (20 ppm) were applied in sufficient quantities for growth and development. The characteristics of plant soil were pH 7.65, Mg = 6.86 ppm, Ca = 55.25 ppm, Fe = 10.83 ppb, Mn = 417.35 ppb, Zn = 79.26 ppb, Cu = 30.55 ppb, Pb = 12.13 ppb, and Cd = 0.95 ppb. After the plants had sufficiently grown in approximately 3 weeks, the exposure concentrations of Cd, Cu and Pb from $CdCl_2$, $CuSO_4$, and $Pb(NO_3)_2$ as sources were 10, 20 and 50 ppm (mg kg^{-1}). The application of heavy metals was performed three times with an interval of 2 days and this addition was done as a single dose to the pots containing soil, in the early hours of the day. The leaves of tomato were harvested for sampling two weeks after the treatments, and these samples of tomato were kept at –80 °C until RNA isolation and analysis of proline and metal-chelating capacity. Three biological replicates were used for analysis of these assays.

Isolation of total RNA and RT-qPCR analysis

Tomato leaves were ground into fine powder in liquid N_2; the tissue powder obtained was used for RNA extraction. The total RNA was extracted by using the Plant RNA Mini-Preps Kit with the EZ-10 spin column according to the manufacturer's protocols (Bio Basic, Ontario, Canada). The obtained RNA was dissolved in RNA-free water, and RNA concentration was then quantified by using a μDrop plate (Thermo Scientific, Forssa, Finland). The first-strand cDNA was synthesized from the isolated total RNA; the relative expression levels of the selected genes of tomato were detected by quantitative real-time PCR (RT-qPCR) with the one-step QuantiTect SYBR Green RT-PCR kit (Qiagen) according to the manufacturers' protocols. The capillary tubes comprised the master mix 10 μL, primers 2 × 2 μL, RT mix 0.3 μL, and template RNA 3 μL; the reaction volume was brought up to 20 μL with RNase-free water. The QuantiTect SYBR Green RT-PCR master mix was included in pre-optimized ROX as the passive reference dye for fluorescence normalization. The genes sequences for *PCS* and *P5CS* were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/nucleotide) databank and they also were verified by the SOL Genomics Network (http://solgenomics.net/feature/17933775/details) using previous studies (Ouziad et al. 2005; Sangu et al. 2015). Gene-specific primers were designed by using the Primer3 software (version 4.0) based on the NCBI GenBank and the % GC and Tm values of primers were checked with an oligonucleotide properties calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html). The following primers were used: *PCS1* for 5' – TGCTAGCATTTGTTGCCAAG –3'

(forward), 5'– ACGTAGGGACCAGAACATCG -3' (reverse); *P5CS1*: 5' CTGTTGTGGCTCGAGCTGAT –3' (forward), 5'– GACGACCAACACCTACAGCA -3'(reverse) and *Actin* 5'– GGGATGGAGAAGTTTGGTGGTGG-3' (forward), 5'– CTTCGACCAAGGGATGGTGTAGC-3'(reverse). These nucleotide sequences have been deposited in the EMBL nucleotide sequence database under accession numbers AW154892 and, AY897574 for *PCS1* and *P5CS1*, respectively.

The quantitative real-time PCR (RT-qPCR) was performed in triplicate on a Light-Cycler 1.5 PCR machine (Roche) under the following conditions of the Qiagen protocols with minor modifications: the reverse transcription step 20 min at 50 °C, the PCR initial activation step 15 min at 95 °C, followed by 50 cycles, denaturation 15 s at 94 °C, annealing 30 s at 54 °C, extension 30 s at 72 °C, cooling 20 s at 40 °C. The fold expression of target genes was normalized with *actin* as an internal control. The relative transcript expression levels of the selected genes were quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Determination of proline content

The free proline content was estimated using with the ninhydrin reaction (Bates et al. 1973). The tomato leaves were crushed into liquid N_2, homogenized with 4 mL 3% (w/v) sulfosalicylic acid, and the solution was then filtered with filter paper. The filtrate (1 mL) was reacted with 1 mL of acid-ninhydrin and 1 mL of glacial acetic acid in the test tubes for 1 h at 100 °C, and the reaction was stopped by using ice bath. The reaction mixture was extracted with toluene (2 mL), and the absorbance of the chromophore was read at 520 nm. The proline content was calculated using the calibration curve obtained from pure proline and expressed as mg g^{-1} FW.

Metal-chelating ability assay

The metal-chelating capacity of the leaf extract was determined by the previously described ferrous ion chelating assay (Hsu et al. 2003). Tomato leaves (2 g) were grounded into liquid N_2, extracted with 10 mL of methanol-chloroform (4:1), and the obtained homogenates were sonicated in the ultrasonic bath for 30 min at ambient temperature. The filtered extract (120 µL) was dissolved in methanol and mixed with 50 µL of 2 mM $FeCl_2$, and 200 µL of 5 mM ferrozine. After the vortexing, the mixture was incubated for 10 min at room temperature and then the absorbance was measured at 562 nm. The metal-chelating capacity was calculated by using the following equation with EDTA as the control:

$$\text{Chelating capacity (\%)} = [1 - (A_{562\ nm,\ sample}/A_{562\ nm,\ control})] \times 100.$$

Statistical analysis

Data analysis was conducted with one-way analysis variance, followed by Duncan multiple tests (SPSS 20.0) to detected the differences between control and treated groups. Standard deviation was calculated by using the results of the $2^{-\Delta\Delta CT}$ method. The results are presented as mean ± SD. Differences were considered significant at $p < 0.05$. The relations among the proline content, *P5CS1* expression, *PCS1* expression and metal-chelating activity in the leaves of tomato in response to heavy metal stress was analyzed with bivariate (Pearson's) correlation using the individual values of the results.

Results

The effect of heavy metals on the *PCS1* gene expression in the leaves of tomato plants exposed to the different concentrations of Cu, Cd, and Pb is shown in Fig. 1. Compared with control plants, the leaves of treated plants showed significantly higher accumulation of *PCS1* transcript due to the heavy metal treatment ($p < 0.05$). The most evident expressions were observed in plants given the high doses of heavy metals, with the highest expression observed in the plant exposed to 50 ppm of Pb. Moreover, Cd and Pb which are not essential for plant development, induced the expression of *PCS1* more than Cu did.

The transcript level of *P5CS1* in the leaves of tomato subjected to Cu, Cd, and Pb varied with depending on the heavy metals and their doses (Fig. 2). The application of Cu to the plants through soil growth medium increased the expression of *P5CS1* at the 20 and 50 ppm; however, the expression was not significantly different from that in controls at the low dose of Cu (10 ppm) ($p < 0.05$). The expression level of the *P5CS1* gene significantly increased with the applica-

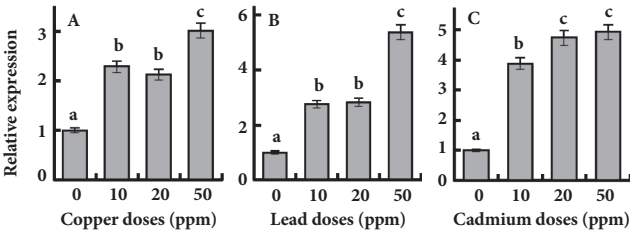

Fig. 1. The expression level of *PCS1* in leaves of tomato subjected to 0, 10, 20, 50 ppm of $CuSO_4$ (A), $Pb(NO_3)_2$ (B) and $CdCl_2$ (C). The gene expression was normalized with the housekeeping actin transcript. Bars represent means values ± SD from independent experiments (n = 3). Values with the different letters indicate significant difference between means at $p \leq 0.05$.

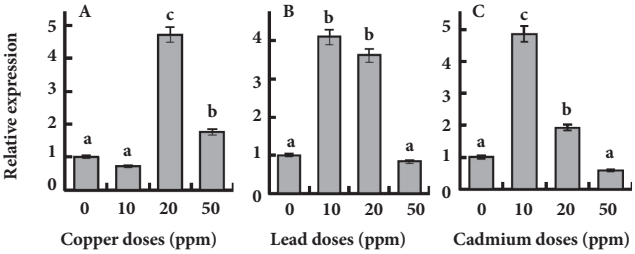

Fig. 2. The expression level of *P5CS1* in leaves of tomato subjected to 0, 10, 20, 50 ppm of $CuSO_4$ (A), $Pb(NO_3)_2$ (B) and $CdCl_2$ (C). The gene expression was normalized with the housekeeping actin transcript. Bars represent means values ± SD from independent experiments (n = 3). Values with the different letters indicate significant difference between means at $p \leq 0.05$.

tion of Pb and Cd except for the expression at 50 ppm of Pb and Cd, which showed expression not significantly different from that in the controls.

The free proline content in tomato leaves varied with the application of heavy metals, and these results are shown in Fig. 3. The proline content in the leaf extract significantly increased with the addition of 10 and 20 ppm of Pb and Cd, but significantly decreased at high doses (50 ppm) ($p < 0.05$). The maximum increase in proline content relative to controls was seen with 10 ppm doses of Pb. However, compared to control plants, 20 ppm and 50 ppm of Cu increased and 10 ppm of Cu decreased the proline content in the leaves of tomato.

The metal-chelating potency of tomato leaves is shown in Fig. 4. The metal-chelating activity was higher in plants cultivated in Pb and Cd-containing soils than in control plants ($p < 0.05$). However, the leaf extracts of tomato grown in Cu-containing medium showed different chelating potency; the plants grown in soil containing 10 ppm of Cu showed lower chelating ability than control plants; however, those grown in soils containing 50 ppm of Cu showed higher activity than control plants.

The proline content is indicated to have a positive correlation with *P5CS1* transcript expression when the tomato plant is subjected to the three heavy metals (Tab. 1). The metal-chelating activity has a positive correlation with the *PCS1* transcript expression under the Cd application. Moreover, the metal-chelating activity has a positive correlation with proline content under Cu and Pb treatments. In addition, *P5CS1* transcript expression and the metal-chelating potency were positively correlated in the leaves of tomato plants grown in the Pb containing soil (Tab. 1).

Tab. 1. Correlation coefficients (R values) among the proline, transcript levels of *P5CS1* and *PCS1* genes, and metal chelating activity in the leaves of tomato under treatments with Cu, Pb, and Cd. Correlation is significant at the 0.01 (**) and 0.05 (*) level (2-tailed).

Applications		*P5CS1*	*PCS1*	Proline	Metal-chelating
Copper	*P5CS1*	1			
	PCS1	0.161	1		
	Proline	0.689*	–0.250	1	
	Metal-chelating	0.378	0.187	0.884**	1
Lead	*P5CS1*	1			
	PCS1	–0.169	1		
	Proline	0.854**	–0.338	1	
	Metal-chelating	0.733**	0.400	0.717**	1
Cadmium	*P5CS1*	1			
	PCS1	0.137	1		
	Proline	0.645*	–0.132	1	
	Metal-chelating	0.201	0.976**	0.064	1

Discussion

Plants have been exposed to the different biotic and abiotic stresses because of changing of environmental conditions, and they respond to these situations with several complex physiological, biochemical and molecular mechanisms for survival, growth, and productivity. The responses of the plants to environmental stresses are quite sophisticated, and they are known to respond to stress by synthesizing various molecules and compounds. The synthesis of proline and PCs are considered as a metabolic response of plants when subjected to the heavy metal stress, and several genes responsible for their synthesis are induced by environmental stimuli. The genes associated with a the plant's response to stress may lead to a series of biochemical and physiological changes (Zhang et al. 2005, Goel et al. 2010, Patade et al. 2013).

In this current study, the transcript expression patterns of *PCS1* and *P5CS1* genes, proline content, and metal chelating potency were investigated in leaves of tomato plants when grown under heavy metal-containing soil. The results of the present study indicated that the transcript expression of *PCS1* is obviously stimulated in the leaves of tomato cultivated in the Cu, Cd and Pb-containing soils. The increases varied with heavy metals and their doses; Pb treatment at 50 ppm resulted in a strong increase in the transcript expression of *PCS1* compared with control plants. Treatment with Cu,

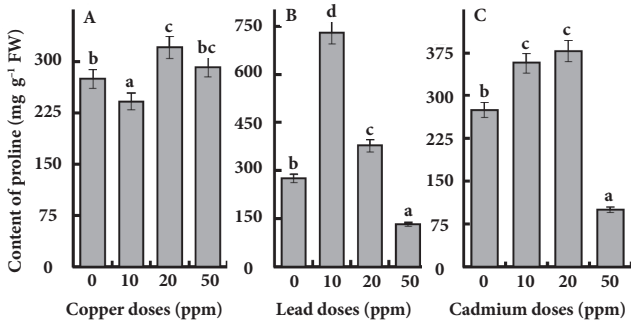

Fig. 3. The free proline content in leaves of tomato subjected to 0, 10, 20, 50 ppm of $CuSO_4$ (A), $Pb(NO_3)_2$ (B) and $CdCl_2$ (C). Bars represent means values ± SD from independent experiments (n = 3). Values with the different letters indicate significant difference between means at $p \leq 0.05$.

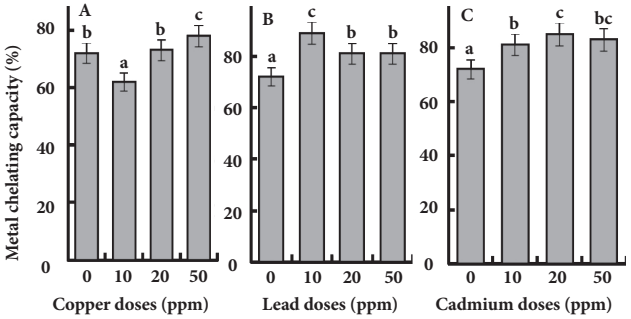

Fig. 4. The metal chelating activity of the leaves extract of tomato subjected to 0, 10, 20, 50 ppm of $CuSO_4$ (A), $Pb(NO_3)_2$ (B) and $CdCl_2$ (C). Bars represent means values ± SD from independent experiments (n = 3). Values with the different letters indicate significant difference between means at $p \leq 0.05$.

an essential element for plant growth and development, induced the transcript expression of *PCS1*, albeit only marginally compared with the other two non-essential elements. It was observed that the leaf extract of tomato plants grown in Pb and Cd-containing soils exhibited the higher metal chelating ability than control plants; however, the Cu influence on the chelating potency of leaves was dose-specific, with highest activity observed at 50 ppm of Cu compared to plants grown unpolluted soils. A study on *Lycopersicon esculentum* reported that after the tomato plants were exposed to different concentrations of $CdCl_2$, *PCS1* transcript expression was improved in both leaves and roots, and mRNA accumulation of *PCS1* was significantly higher in roots than in leaves. However, no expression changed was observed in leaves and roots under any concentration of $CuSO_4$ treatment (Hui et al. 2010). It was stated that in the leaves of tomato, the content of PCs increased on treatment of 1, 5 and 10 μM Cd, but it decreased at higher concentrations. In addition, the level of PCs increased in the roots of tomato, and the production of PCs was higher in roots than leaves (Ben Ammar et al. 2008). The content of PCs in the leaves of *Solanum nigrum* linearly enhanced with the increasing Cd concentrations (10, 25, 50 and 100 μg g^{-1}), whereas the content of PCs in the leaves of *S. melongena* increased under the Cd exposures of 25 μg g^{-1} and evidently declined obviously at higher concentrations (Sun et al. 2007). The *PCS1* transcript expression in rice cultivars in response to arsenite showed that compared to the control plants, the transcript expression was significantly increased in the roots of the tolerant cultivar but not in the sensitive cultivar. PCS activity showed an important positive correlation with total PC, and PCS activity PC_2, PC_3, PC_4 increased on treatment with arsenite (Tripathi et al. 2013, Begum et al. 2016). *PCS* transcript expression was reportedly induced with increasing doses of $CdCl_2$ in *Brassica* cultivars (Shanmugaraj et al. 2013). A study performed on the roots of *Allium sativum* L. stated that *PCS1* transcript expression was significantly induced by Cd, As, and heat shock not by Cu and salt stress. However, in a study Cu induced evident accumulation of mRNA transcript of *PCS1* in the leaves of garlic seedlings (Zhang et al. 2005). In addition, RT-qPCR analysis showed no significant change in *PCS1* transcript expression in roots of Alfalfa in respond to $CdCl_2$ and K_2SiO_3 stresses (Kabir et al. 2016). Another study on metal stress noted that Cd and As rapidly increased the formation of PCs in the roots, but other metals, such as Cu, Zn, Co, and Ni, did not induce any such change. However, none of these metals initiated any evident production of PCs in the leaves of chickpea (Gupta et al. 2004). PCs are the primary binding peptides involved in chelating heavy metal ions in plants (Guo et al. 2008). In the current study, the transcriptional profile of *PCS1* was investigated in the leaves of tomato subjected to Cu, Cd, and Pb, and the expression of *PCS1* was induced by these heavy metals. It is considered that plants increase their HM-binding capacity through chelators to avoid the harmful effect of HM by synthesizing various metal-binding peptides/proteins to withstand heavy metal stress. It has been suggested that one of the possible detoxification and tolerance mechanism is tight control of metal ions, and the expression of the *PCS* gene and the accumulation of PCs may play a general role in metal homeostasis (Cazalé and Clemens 2001, Begum et al. 2016).

The results of proline content and gene expression of *P5CS1,* a gene responsible for proline synthesis in the present study indicated comparable induction by the application of all heavy metals, except for the induction obtained with 10 ppm doses of Cu and 50 ppm doses of Cd; however, the level of increase did not occur likewise in the leaves of tomato. The application of Pb and Cd at 10 and 20 ppm doses resulted in clear increases of *P5CS1* transcript expression, and Cu at 20 and 50 ppm increased the expression of *P5CS1* gene in leaves. Further, compared with untreated plants, no significant change was observed in plants treated with a high dose (50 ppm) of Pb and Cd. Compared with the control plants, the free proline content was increased in treated plants by adding 10 ppm and 20 ppm concentrations of Pb and Cd in plant growth medium; the application of 20 ppm and 50 ppm doses of Cu moderately increased free proline content in leaf samples. Heavy metal applications, other than mentioned above, decreased the content of proline content when compared with control plants. A previous study showed that Cd-induced stress increased the proline content in leaves of tomato treated with 0.2 and 1 mM $CdCl_2$ (Gratao et al. 2012). The concentration of free proline in *S. melongena* reportedly reached the highest level when plants were treated with 10, 25 and 50 μg g^{-1} Cd and declined to the level of control when plants were exposed to the highest Cd doses (100 μg g^{-1}) (Sun et al. 2007). A previous study on *Lycopersicon esculentum* reported that the expression of *P5CS* gene significantly increased on 2 h of cold exposure in transgenic tomato; however, in response to a 24-h exposure, the expression level of *P5CS* decreased to significantly lower levels than in the control plants. However, in wild type tomato plants subjected to 2 or 24 h of cold stress, the expression of *P5CS* was significantly reduced as compared to that in the untreated control plants. In addition, the proline content in the same work reduced significantly in the wild type plant on 2 and 24-h cold exposure, while the proline accumulation was significantly higher in transgenic tomato plants exposed to cold for 2 h than untreated plants or those exposed for 24 h (Patade et al. 2013). The transcript pattern of *P5CS1* of barley genotypes was mainly up-regulated in response to drought stress at all exposures (Guo et al. 2009). It was stated that the *P5CS* gene was up-regulated in the heat tolerant tomato genotypes CLN1621L (at the mature stage) and CLN5915-93D (at the meiosis stage) whereas it was down-regulated in the heat-sensitive genotypes CA4 and CLN2498E (Sangu et al. 2015). In addition, it was indicated that the proline content increased in tomato exposed to different stress conditions, such as NaCl stress, cold stress and nutrient deficit (Claussen 2005, Zhao et al. 2009, Goel et al. 2010, Singh et al. 2016). The transcript expression of the *P5CS1* gene was slightly increased under the low salt stress (100 mM) and it was significantly induced by higher salt doses (200 and 300 mM); however, the gene expression reduced again slightly at 400 mM NaCl in the

leaves of *Kosteletzkya virginica*. The proline content remarkably increased in roots, leaves and stems after the NaCl stress (Wang et al. 2015). Another study reported that compared to control plants, in leaves of *Nicotiana benthamiana*, water deficit and $CuSO_4$ increased the gene expression of *P5CS* and proline content; however, the water deficit was more responsible than Cu for the increases in *P5CS* gene expression and proline accumulation (Ku et al. 2012). The accumulation of stress metabolites, such as proline, is induced by adverse environmental conditions, and proline content varies with the plant species and varieties (Verbruggen and Hermans 2008). In the current study, *P5CS1* transcript expression and the free proline content were examined, and levels of *P5CS1* and proline fluctuated according to heavy metal concentrations. However, it can be interpreted from the results that *P5CS1* transcript expression and the proline content generally increased except for when the plants were grown under high doses of heavy metals. The gene expression analysis of *P5CS*1 revealed differential transcript regulation in the leaves of tomato on heavy metal exposures. Proline has been considered an important osmo-protectant, and it can play a critical role in adapting to abiotic stress by increasing the osmotic potential of cells. Plants alter the transcriptional pattern of stress-responsive genes, such as the *P5CS* gen involved in the synthesis of functional amino acid and peptides, such as proline (Chen et al. 2010).

Proline accumulates in various plants in response to environmental stress, and its accumulation can affect stress tolerance in multiple ways. Proline has various roles, such as that of a protective molecule as an osmolyte or a molecular chaperone as well as an antioxidant molecule working as ROS scavenger; it supports to the maintenance of redox balance by contributing to the reductions of free radicals. Although proline accumulation is usually considered as a response to stress conditions, it is assumed that the accumulation can change depending on the plant species (Szabados and Savoure 2009). The production of metal-PC complexes is the evidence of plant metal tolerance, and PC synthesis is considered as a protective mechanism used by plants to protects heavy metal-sensitive enzymes (Citterio et al. 2003). In this study, the expression of genes associated with proline and PC was examined, and it is indicated that the expression of *P5CS1* has positive correlation with free proline content under heavy metal stress. In addition, there was a positive correlation between the data of *PCS1* gene and metal-chelating activity in the leaves of tomato exposed to Cd. This study also revealed a strong correlation between metal-chelating potency and proline content in Cu and Pb applications. It has been previously found that free proline chelates Cd ions in plant tissues and converts them into non-toxic Cd-proline complexes (Sharma et al. 1998). The lack of correlation between *PCS1* and metal-chelating activity for Pb and Cu applications could be compensated by proline, since metal-chelating potency and proline showed a strong correlation for these treatments. Besides the upregulation of *PCS1* in all treatments, proline could play a role as a potential metal chelator for plants growing under to Cu and Pb presence, thus protecting them from deleterious effects.

Plants have responded to biotic and abiotic stress factors with a series of mechanisms through the transcriptional regulation of stress responsive, regulatory, and effector genes (Patade et al. 2013). By this study, the gene expressions of *PCS1* and *P5CS1* related with the PCs and proline content, respectively were investigated in the leaves of tomato under heavy metal stress. To further define and elucidate the responses of plants under stress conditions, the expression of genes and their corresponding enzymes associated with PCs and proline metabolisms, such as *P5CR* (pyrroline-5-carboxylate reductase), *P5CDH* (P5C dehydrogenase), *PDH* (Pro dehydrogenase), *POX* (proline oxidase), *ProT* (proline transporter), *ɣ- ECS* (*ɣ*-glutamylcysteine synthetase), and PC synthase, should be studied together to reveal the depth of responses in tomato subjected to abiotic stresses, such as heavy metals.

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