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Roles of catalase (CAT) and ascorbate peroxidase (APX) genes in stress response of eggplant (*Solanum melongena* L.) against Cu^{+2} and Zn^{+2} heavy metal stresses

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Abstract Eggplant (*Solanum melongena* L.) is a good source of minerals and vitamins and this feature makes its value comparable with tomato which is economically the most important vegetable worldwide. Due to its common usage as food and in medicines, eggplant cultivation has a growing reputation worldwide. But genetic yield potential of an eggplant variety is not always attained, and it is limited by some factors such as heavy metal contaminated soils in today's world. Today, one of the main objectives of plant stress biology and agricultural biotechnology areas is to find the genes involved in antioxidant stress response and engineering the key genes to improve the plant resistance mechanisms. In this regard, the current study was conducted to gain an idea on the roles of catalase (CAT) and ascorbate peroxidase (APX) genes in defense mechanism of eggplant (*S. melongena* L., Pala-49 (Turkish cultivar)) treated with different concentrations of Cu^{+2} and Zn^{+2}. For this aim, the steady-state messenger RNA (mRNA) levels of CAT and APX genes were determined by quantitative real-time PCR (qRT-PCR) in stressed eggplants. The results of the current study showed that different concentrations of Cu^{+2} and Zn^{+2} stresses altered the mRNA levels of CAT and APX genes in eggplants compared to the untreated control samples. When the mRNA levels of both genes were compared, it was observed that CAT gene was more active than APX gene in eggplant samples subjected to Cu^{+2} contamination. The current study highlights the importance of CAT and APX genes in response to Cu^{+2} and Zn^{+2} heavy metal stresses in eggplant and gives an important knowledge about this complex interaction.

Keywords CAT · APX · Eggplant · Cu^{+2} · Zn^{+2} · Heavy metal stress

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Introduction

Eggplant (*Solanum melongena* L.) which belongs to the *Solanaceae* family is an economically important vegetable crop widely cultivated in the tropics, subtropics, and warm temperate regions in the world (Sihachakr et al. 1994). It, also known as aubergine, is a good source of minerals and vitamins and this feature makes its value comparable with tomato which is economically the most important vegetable worldwide (Kalloo 1993; Clark et al. 2014). Eggplant is an important food component of the human diet, and it has been extensively used as traditional medicine for treatment of many diseases (Khan 1979; Kashyap et al. 2003). Due to its common usage in foods and medicines, eggplant

cultivation has a growing reputation worldwide under good care and management (Doganlar et al. 2002). But genetic yield potential of an eggplant variety is not always attained, and it is limited by some factors such as inappropriate growing season, pests outbreak and especially by abiotic stress factors in today's world. Heavy metal toxicity and non-availability of important nutrients, frost and cold injury, inappropriate atmospheric temperature, and soil salinity are some of the abiotic stresses (Soydam-Aydın et al. 2014). These abiotic stress conditions induce cellular damage and constitute some mechanical, metabolic, and oxidative effects on plants due to imbalance between generations of reactive oxygen species (ROS) varieties, superoxide radical (O_2^-), hydroxyl radical (OH.) and hydrogen peroxide (H_2O_2), and antioxidant scavenging system (Aydın et al. 2013; Bhattacharjee 2005; Kalefetoğlu and Ekmekçi 2005; Shao et al. 2008; Munns 2011; Soydam-Aydın et al. 2013a, b).

Plants have three interconnected aspects of activities to achieve heavy metal tolerance depending on the species, genotypes, the age, and the stage of development. These are preventing or alleviating the damage, reestablishing homeostatic conditions in stressful environment, and resuming to growth at a reduced rate (Zhu 2001). Antioxidant scavenging system plays an important role in preventing or alleviating the stress damage, and this system restricts and removes ROS damage and maintains ROS homeostasis in plant cells (Clark et al. 2014). The components of this system are non-enzymatic antioxidants, such as glutathione (GSH), proline, carotenoids, tocopherol, ascorbic acid (AsA), and enzymatic, such as monodehydro ascorbate reductase (MDHAR), dehydro ascorbate reductase (DHAR), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) (Mittler et al. 2004).

Today, one of the main objectives of plant stress biology and agricultural biotechnology areas is to find the genes involved in antioxidant stress response and engineering the key genes to improve the plant response mechanisms (Koornneef and Peeters 1999; Pareek et al. 2010). Some of the genes have been well-characterized while some are suspicious in some plant species such as eggplant. Nevertheless, the analysis of these genes in many different plant species will increase overall knowledge of their exact roles in plant defense mechanism. In this regard, the current study was conducted to gain an idea on the roles of catalase (CAT) and ascorbate peroxidase (APX) genes in defense mechanism of eggplant (*S. melongena* L., Pala-49 (Turkish cultivar)) treated with different concentrations of Cu^{+2} and Zn^{+2}. For this aim, the steady-state messenger RNA (mRNA) levels of CAT and APX genes were determined by quantitative real-time PCR (qRT-PCR) in stressed eggplants. All results were evaluated statistically, and a probable correlation was demonstrated among stress type and concentration obtained from the study.

Materials and methods

Plant material, growth conditions, and stress treatment

Eggplant (*S. melongena* L., Pala-49 (Turkish cultivar)) seeds were germinated and grown hydroponically in pots containing 0.2 L modified 1/10 Hoagland's solution. Hoagland's solution includes macronutrients [K_2SO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $Ca(NO_3)_2 \cdot 4H_2O$, and KCl] and micronutrients (H_3BO_3, $MnSO_4$, $CuSO_4 \cdot 5H_2O$, NH_4Mo, and $ZnSO_4 \cdot 7H_2O$) with the following final concentrations of ions: 2 mM Ca, 1 μM Mn, 4 mM NO_3, 0.2 μM Cu, 1 mM Mg, 10 mM NH_4, 2 mM K, 1 μM Zn, 0.2 mM P, 0.1 mM Fe, and 1 μM B. Six plants were grown in each pot in a controlled environmental growth chamber, under light, with 250 mmol m^{-2} s^{-1} photosynthetic photon flux at 25 °C and 70 % relative humidity. Twenty-five-day-old plants grown in controlled media were used for the stress treatments. For the heavy metal application, Cu^{+2} and Zn^{+2} were added separately to the hydroponic solution for 24 h at concentrations of control (untreated samples), 80, 160, 320, 640, and 1280 μM.

Quantitative Real-time PCR and quantification of mRNA levels

Total RNA extraction was performed using Trizol protocol followed by RNeasy mini cleanup kit (Qiagen, Cat No. 74104). RNA quantity/quality was measured with a Nanodrop ND-Spectrophotometer 1000. Quality of RNA was also confirmed by gel electrophoresis which contains 1.5 % agarose. Complementary DNA (cDNA) synthesis which was based on reverse transcription reactions was performed with 2 μg of RNA and high fidelity cDNA synthesis kit (Roche) which contains 2.5 μM Anchored-oligo (dT)18, 1× transcriptor high fidelity reverse transcriptase reaction buffer, 20U

protector RNase inhibitor, 1 mM deoxynucleotide mix, 5 mM DTT, and 10U transcriptor high fidelity reverse transcriptase at final concentration. The following program was applied: 10 min at 65 °C, 30 min at 55 °C, and 5 min at 85 °C. cDNA quantity/quality was also measured with a Nanodrop ND-Spectrophotometer 1000 and results were shown in Table 1.

Quantitative real-time PCR (qRT-PCR) was performed with Light Cycler® Nano System (Roche), thermal cycler. The experiment included three biological replicates, and three technical replicates, each for both stressed and non-stressed plant samples. The sequences of the primers used in the study are shown in Table 2. The primer sequences of the target genes catalase (CAT), ascorbate peroxidate (APX), and also actin (ACT) which is used for normalization were designed based on the sequences of eggplant genes available in the genebank (http://www.ncbi.nlm.nih.gov/). Amplifications of PCR product were monitored via SYBR Green I dye which is an intercalator-based method. After pre-denaturation, followed by 10 min at 95 °C, 45 cycles of 10 s at 95 °C, 30 s at 60 °C, 15 s at 72 °C were applied. Melt-curve analysis was performed to confirm the specificity of the chosen primers and absence of primer-dimers. Data collection for quantification was done during anneling period. Copy numbers of transcripts (CAT, APX, ACT) under stress treatments were determined by using amplification curves.

Statistical methods

The abundance of targeted gene transcripts was normalized to ACT and set relative to control plants (no stress exposure) according $2^{-\Delta\Delta CT}$ method (Livak et al. 2001).

Table 2 Primer sequences of CAT, APX, and ACT

Primer name	Sequences
CAT Fw	GAA GCT ATT AGA GTC GGA GGT
CAT Rev	GTT TGG CCC AAT ACG GT
APX Fw	AAG CTG AGC AAG CAC ATG G
APX Rev	CCA GGG TGA AAG GGA ACA
ACT Fw	TCT GTT TCC CGG TTT TGC TAT TAT
ACT Rev	TGC ATC AGG CAC CTC TCA AG

Changes in relative expression levels (REL) of the CAT and APX genes were checked for statistical significance according to one-way ANOVA. The results were considered statistically significant in the Dunnett test ($P<0.05$).

Results

In the current study, mRNA levels of CAT and APX genes were investigated in eggplant samples subjected to Cu^{+2} and Zn^{+2} heavy metal stresses. The abundance of the gene transcripts was compared in eggplant samples exposed to different concentrations of heavy metals (80, 160, 320, 640, and 1280 μM concentrations). Following 80, 160, 320, 640, and 1280 μM concentrations of Cu^{+2} and Zn^{+2} contaminations, mRNA levels of CAT and APX were analyzed by qRT-PCR and were shown in Figs. 1 and 2.

As seen in figures (Figs. 1 and 2), almost all concentrations of Cu^{+2} contamination stimulated mRNA levels of both CAT and APX genes in eggplant samples. The

Table 1 Quality and quantity of cDNA by ND 1000 Spectrophotometer

Sample ID	ng/μl	A_{260}	A_{280}	A_{260}/A_{280}	A_{260}/A_{230}
Control	2389.91	47.798	26.225	1.82	2.18
Cu 80 μM	2080.52	41.610	22.926	1.81	2.17
Cu 160 μM	1958.23	39.165	21.674	1.81	2.19
Cu 320 μM	2312.11	46.242	25.377	1.82	2.12
Cu 640 μM	2277.21	45.544	25.009	1.82	2.05
Cu 1280 μM	2299.05	45.981	25.355	1.81	2.14
Zn 80 μM	2158.95	43.179	23.822	1.81	2.22
Zn 160 μM	2171.64	43.433	23.797	1.83	2.21
Zn 320 μM	2261.98	45.240	24.872	1.82	2.20
Zn 640 μM	2302.20	46.044	25.401	1.81	2.02
Zn 1280 μM	2206.68	44.134	24.272	1.82	2.17

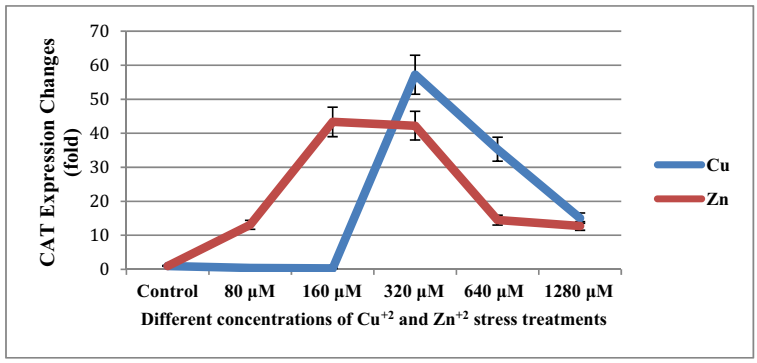

Fig. 1 CAT mRNA levels in the *Solanum melongena* L. seedlings exposed to different concentrations of Cu^{+2} and Zn^{+2}. All points represent the averages (n=6)

mRNA levels of both genes continued to increase up to 320 µM concentration of Cu^{+2} contamination and started to decrease above this concentration. Three hundred twenty micrometers concentration led to a maximum increase, approximately 57-fold, in CAT gene expression level compared to the untreated control sample. When the mRNA levels of both genes were compared, a more pronounced CAT gene stimulation was observed compared to APX gene in eggplant samples subjected to Cu^{+2} contamination.

Eggplant samples subjected to Zn^{+2} contamination revealed increased mRNA levels in both CAT and APX genes at all concentrations. The mRNA level of CAT gene continued to increase up to 160 µM concentration of Zn^{+2} contamination while the maximum level was observed at 640 µM of Zn^{+2} concentration. CAT gene was stimulated more than the APX gene in eggplant samples at almost whole concentrations of Zn^{+2} contamination.

Discussion

Principally, all plants have a basal tolerance mechanism against heavy metals. Some key elements such as uptake/efflux and transport/sequestration play an important role in homeostatis of essential metal micronutrients by removing of toxic ions from cellular loci. The plant kingdom is divided into two groups: (hyper) accumulating and non-accumulating plants, based on the heavy metal accumulation capacities of the plant species (Viehweger 2014). Most of the plants including eggplant are found in non-accumulator plants group. But they have also take heavy metals from soil for nutrition purposes. Hence, they have to have a finely tuned mechanism for living in soils with excessive heavy metals (Viehweger 2014; Soydam-Aydın et al. 2013a).

Abiotic stresses including heavy metal stress lead to increased reactive oxygen species (ROS) which are highly reactive and toxic for the plant cells. Plants have

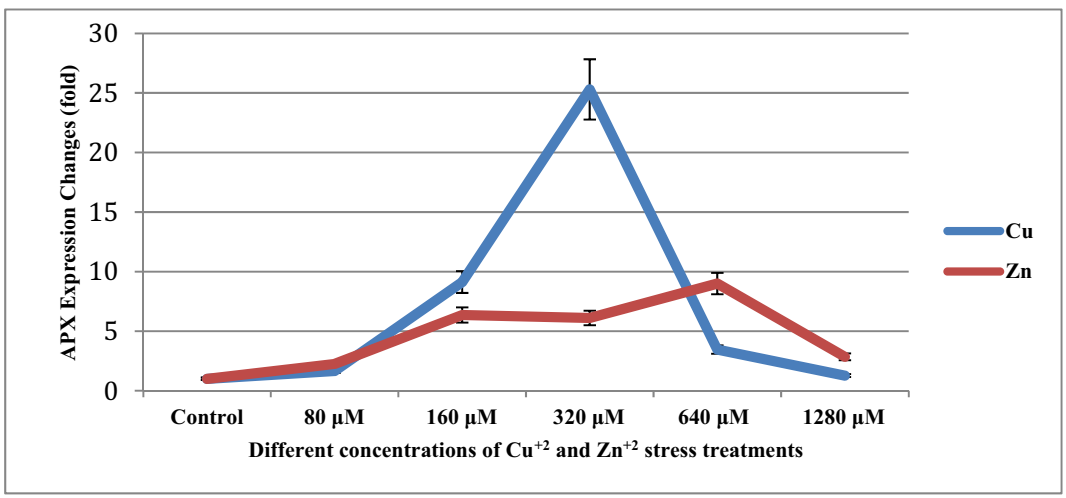

Fig. 2 APX mRNA levels in the *Solanum melongena* L. seedlings exposed to different concentrations of Cu^{+2} and Zn^{+2}. All points represent the averages (n=6)

antioxidant systems which protect them against oxidative damage mainly caused by overproduction of ROS under abiotic stress conditions. Catalase (CAT) and ascorbate peroxidase (APX) are important members of antioxidant defense system which protect plant cells by scavenging of ROS (Gill and Tuteja 2010; Viehweger 2014).

In the current study, our strategy was to determine the mRNA expression levels of CAT and APX genes in stress response in eggplants subjected to Cu^{+2} and Zn^{+2} heavy metals. The results of the current study clearly showed that different concentrations of Cu^{+2} and Zn^{+2} stresses altered the mRNA levels of CAT and APX genes in eggplants compared to the untreated control samples. We observed higher levels of CAT gene expression compared to APX gene, suggesting a more crucial role for CAT gene rather than APX in eggplant (Figs. 1 and 2). The current study gives an important information on comparative analysis of both genes in eggplant subjected to Cu^{+2} and Zn^{+2} heavy metals. Although few studies on eggplant have been reported, studies mainly focused on the importance of overexpression of CAT and APX genes in enhanced tolerance to ROS-induced stress in other crop species such as tomato, pea, rice, and bean (Roxas et al. 2000; Shinozaki and Yamaguchi-Shinozaki 2000; Payton et al. 2001; Pekker et al. 2002; Romero-Puertas et al. 2007; Dai et al. 2007; Goupil et al. 2009; Rosa et al. 2010; Fortunato et al. 2010; Soydam-Aydın et al. 2013a).

Recent studies carried out show similarities with our study. Ibrahim and Bafeel (2009) analyzed the mRNA levels of CAT gene in *Lepidium sativum* plants subjected to lead toxicity and found that CAT gene expression is a valuable stress marker in ecophysiological studies (Ibrahim and Bafeel 2009). In an another study conducted in our laboratory, we got similar results for CAT in tomato plants subjected to Cu^{2+} and Zn^{2+} heavy metal stresses. Three hundred twenty micrometers concentration of Cu^{2+} and Zn^{2+} contaminations was found as the critical point that leads to highest mRNA levels of CAT gene in tomato plants, similar to the value found in eggplant in the current study (Unpublished data, 2014). All the results obtained pointed the importance of CAT gene in stress response in eggplant as the mRNA level has increased up to 57-fold upon contamination. It can also be inferred from the results that defense capability of this gene is restricted above 320 μM concentration of Cu^{2+} and Zn^{2+} contaminations.

Lin et al. (2007) showed that gene expression levels of APX in eggplant might be related with hydrogen peroxide detoxification under stress conditions induced by flooding (Lin et al. 2007). Our results also indicated that APX can be responsible for stress scavenging in eggplant subjected to Cu^{+2} and Zn^{+2} heavy metal. In another study, Rosa et al. (2010) observed that the transcript levels of almost all OsAPX genes were significantly increased after 8 h of 20 ppm aluminum exposure (Rosa et al. 2010). In bean plants, Pekker et al. (2002) found that APX was induced at mRNA levels in leaves of plants response to iron overload (Pekker et al. 2002). In a study by Lee et al. (2007), transgenic tall fescue plants (expressing the CuZnSOD and APX genes in chloroplasts) were subjected to Cu^{+2} and Cd^{+2} treatment and increased APX activities were observed in plants due to metal stress (Lee et al. 2007). All the previous and current results emphasize the important role of APX gene in plants under stress.

To overcome the limitations for plant productivity and to improve crop yields under heavy metal stresses, it is important to perform gene expression studies to understand the molecular mechanisms of stress responses in higher plants. Current study highlights importance of CAT and APX genes in response to Cu^{+2} and Zn^{+2} heavy metal stresses in eggplant and gives important information about this complex interaction. In the light of these results, advanced genetic manipulations using CAT and APX genes can be applied in order to enhance stress tolerance in the upcoming years. The current study also emphasizes once more the crucial effects of heavy metal pollution on plants and how plants developed mechanisms to cope with these environmental stresses.

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