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Selenium induced selenocysteine methyltransferase gene expression and antioxidant enzyme activities in *Astragalus chrysochlorus*

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Abstract – *Astragalus* sp. are used in folk medicine because of their biological activities and are known for the ability to accumulate high levels of selenium (Se). The purpose of this study was to explore gene expression of selenocysteine methyltransferase (SMT), responsible for forming MeSeCys, and activities of ascorbate peroxidase (APX), peroxidase (POX), catalase (CAT) and glutathione reductase (GR) enzymes in callus tissues of *Astragalus chrysochlorus* growing in different Se-containing media. Quantitative real-time polymerase chain reaction assay was done for quantification of SMT gene transcript and it was normalized to actin gene. It was found that transcript level of callus tissues grown at 5.2 µM and 26.4 µM Se-enriched media was lower than that of the control callus. In contrast, a high level of Se (132.3 µM) in the medium caused an approximately 4.26 times higher level of *SMT* transcript in callus than the control. APX, POX, CAT and GR enzymes were all effected by different Se concentrations. While POX and APX activities were higher then control, CAT and GR activities decreased. These results show that an increase of SMT gene expression led to a rise in APX and POX, but a suppression of CAT and GR enzymes activities in *Astragalus chrysochlorus*. This suggests that Se could be involved in the antioxidant metabolism in *Astragalus chrysochlorus*.

Keywords: antioxidant enzymes, callus, real time pcr, selenium, selenocysteine methyltransferase

Introduction

Antioxidant selenoproteins contain selenium (Se) which is a very important element for animals, microorganisms and some other eukaryotes (Birringer et al. 2002, Berken et al. 2002). These organisms contain the selenocysteine (SeCys) that may be incorporated in selenoproteins. In addition to nutritional needs for Se, the element also has health efficacy. It is also effective in reducing the incidence of some debilitating disorders (McKenzie et al. 2001, Foresta et al. 2002, Beck et al. 2003, Soriano-Garcia 2004) and there is some evidence that Se plays a role as a cancer preventive agent when given in certain amounts (Ip 1998, Combs and Gray 1998, Fleming et al. 2001, Whanger 2004).

Selenium is metabolized through the sulfur (S) assimilation pathway. Selenocysteine methyltransferase (SMT) is the key enzyme in Se metabolism and it was isolated from the Se hyperaccumulator *Astragalus bisulcatus* for the first time (Neuhierl et al. 1999). The SMT enzyme couples the methyl group with selenocysteine (SeCys) to produce Se-methylselenocysteine. *A. bisulcatus* SMT gene was overexpressed in *Arabidopsis thaliana* and *Brassica juncea* and it was shown that selenium accumulation and tolerance were enhanced in SMT transgenics (Ellis et al. 2004, LeDuc et al. 2004). In our previous work, *SMT* cDNA sequences were isolated from *Astragalus chrysochlorus* (Çakir and Ari 2013). *SMT* was expressed continuously in the tissues of the Se hyperaccumulator *Astragalus bisulcatus* (Pickering et al. 2003). While most plants cannot survive with high concentrations of Se, some of them can tolerate high concentrations and accumulate Se, like *A. bisulcatus*. Thus, the amount of Se is of vital importance for plants because it could be toxic at high concentrations. Se can change a plant's antioxidant capacity. This can be achieved on two ways; first, by changing the antioxidant activity of selenocompounds, and second, by Se inducing a plant's antioxidants (Hartikainen 2005). Enzymes of antioxidant metabolism such as ascorbate peroxidase (APX), peroxidase (POX), glutathione reductase (GR) and catalase (CAT) play an important role against reactive oxygen species (Apel and

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Hirt 2004, Habibi and Hajiboland 2012). Because of the biological activities like hepatoprotective, antioxidative, immunostimulative and antiviral properties, *Astragalus* species have been used in traditional medicine (Ionkova et al. 1997). The chemical contents of them have been broadly investigated because of their economic and medicinal importance (Calis et al. 1997, Bedir et al. 1998, Yahara et al. 2000). One of the Turkish endemics, *A. chrysochlorus*, has been determined as a secondary Se accumulator and a cDNA sequence of the SMT gene (accession number: GQ844862) named *AchSMT* was isolated (Ari et al. 2010). The *AchSMT* cDNA has an open reading frame (ORF) of 1020 bp and this ORF encodes 339 amino acid residues with a 36.94 kD molecular mass (Çakir and Ari 2013).

The aim of this work was to investigate the expression pattern of the SMT gene and determine the alteration of activities of enzymes related to the antioxidant metabolism of *A. chrysochlorus* treated with Se. Here we report the activities of APX, POX, CAT and GR enzymes in callus tissues of *A. chrysochlorus* grown in different concentrations of Se and the expression of a crucial gene related to Se metabolism by real-time quantitative PCR.

Materials and methods

Plant material

Plants and seeds were collected from Sertavul, Karaman, Turkey and were identified by Prof. Dr. Tuna Ekim (Istanbul University, Faculty of Science, Department of Botany, ISTF no: 40006, Istanbul University, Faculty of Science Herbarium).

Germination of seeds and callus induction

Seeds were surface-sterilized in 70% alcohol for 1 min, then in 5% commercial bleach for 15 min, followed by three rinses for 15 min with sterile distilled water. Seeds were germinated aseptically in Petri dishes containing 25 ml of growth-regulator-free MS (Murashige and Skoog 1962) medium supplemented with 3% (w/v) sucrose and solidified with 0.8% agar (w/v). The pH of the MS medium was adjusted to 5.8 before sterilization by autoclaving at 121 °C at 105 kPa for 20 min. The pH of the MS medium was confirmed after sterilization. For callus induction, 30-day-old hypocotyl parts of seedlings were used. Callus proliferation was achieved with 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and was subcultured every three weeks. The calli used in this study were grown in MS medium supplemented with 5.2 µM, 26.4 µM and 132.3 µM sodium selenate (Sigma, S8295). Seed germination, callus induction and subculture were carried out in a growth chamber illuminated with fluorescent light (ca. 1400 µmol m^{-2} s^{-1}) over a 16/8 day and night at 25 ± 2 ºC.

Calli were treated with Se for three weeks, and after treatment, the calli were harvested, and their fresh weights were determined and samples were taken for various analyses.

Enzyme extractions and assays

Catalase (CAT, EC 1.11.1.6) activity was done according to Aebi (1984). Callus samples (0.5 g) were homogenized in 50 mM sodium phosphate buffer (pH 7.0) with prechilled mortar and pestle. The extract was centrifuged at 15000 g for 15 min at 4 °C, and the supernatant was used as enzyme extract. The reaction mixture contained 3% H_2O_2 and 0.1 mM EDTA in 50 mM sodium phosphate buffer. To measure the activity, absorbance was read at 240 nm, and it was calculated as µmol per minute.

Peroxidase (POX, EC 1.11.1.7) activity was determined according to Putter (1974), using the guaiacol oxidation method in a 3 mL reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), 20 mM guaiacol and 20 mM H_2O_2. The increase in absorbance was recorded at 470 nm within 20 s through 2 min after H_2O_2 was added. A unit of peroxidase activity was expressed as µmol H_2O_2 decomposed per minute.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to the method of Nakano and Asada (1981). 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2, and 0.1 mL enzyme extract in a final assay volume of 1 mL were the components of the reaction. Ascorbate oxidation was measured by reading absorbance at 290 nm. To calculate the oxidized ascorbate concentration, the extinction coefficient (ε = 2.8 mM^{-1} cm^{-1}) was used. 1 µmol ascorbate oxidized per minute was described as one unit APX.

Glutathione reductase (GR, E.C. 1.6.4.2) activity was determined according to the method of Foyer and Halliwell (1976). GR activity was determined spectrophotometrically at 340 nm in a reaction mixture containing 50 mM Tris-HCl buffer (pH 7.6), 1 mM GSSG, and 10 mM NADPH. 1 µmol NADPH oxidized per minute was used as one unit GR.

Protein concentration was determined according to Bradford (1976), using bovine serum albumin as a standard. The specific enzyme activity for all enzymes was expressed as units (U) per mg of proteins.

RNA isolation and cDNA synthesis

For RNA isolation 5.2 µM, 26.4 µM and 132.3 µM sodium selenate-treated calli and control without sodium selenate were used. At the end of third week of treatment, total RNA was extracted (TRIzol Reagent, Invitrogen, Carlsbad, CA) and reverse-transcribed (SuperScript First-Strand Synthesis System, Invitrogen, 11904-018) from *A. chrysochlorus*. RNA isolation and first strand cDNA synthesis were done according to the manufacturers instructions.

Real-time PCR conditions and analysis

Quantitative real time PCR (qPCR) was performed with the DyNAmo™ HS SYBR® Green qPCR Kit (Thermo Scientific) following the manufacturer's instructions. Primers are given in Table 1. The qPCR conditions were set as follows: 2 min at 50 °C (uracil-DNA glycosylase incubation), 5 min at 94 °C (pre-incubation), followed by 40 cycles of 30 s at 94 °C, 30 s at 64 °C and 30 s at 72 °C. To obtain melting curve data collection, a gradual temperature increase from 55 °C to 95 °C at the rate of 1 °C/10 s was added as a final step. A non-template control was run alongside this, and serial dilutions (1, 1:5, 1:25 and 1:125) of the reference (*actin*) and SMT gene were included with every assay.

Tab. 1. Primers used in quantitative real time PCR reactions. SMT – selenocysteine methyltransferase.

Target		Sequences (5′-3′)
SMT	Forward	ACGGAGCTTGAACGTCATGGTG
	Revers	ATCAAGATGCACCTGGCGAATGAG
Actin	Forward	TCAAGACGAAGGATG
	Revers	TTGGATTCTGGTGAT

Amplification specificity of each reaction was verified by melting curve analysis. *Actin* was used to normalize expression levels. MX3000 Stratagene software (Agilent Technologies, Inc., Santa Clara, USA) was used to determine relative gene expression. All qPCR experiments were done in triplicate experiments for each sample.

Statistical analysis

All tests were done in triplicates. Analysis of variance was performed using Graphpad prism ver. 5.0 trial version. The data were presented as the means for each treatment. Data were compared using the one-way ANOVA test with post Tukey test at the 5% probability level.

Results

Establishment of callus cultures

The hypocotyl parts of 30 day old seedlings produced friable and green callus on MS medium supplemented with 0.5 mg L^{-1} 2,4-D after a 15-day culture period (Fig. 1A). After induction of callus, 2,4-D was used for proliferation purpose and *A. chrysochlorus* callus was maintained for over ten years by subculture at three week intervals on MS medium supplemented with 0.5 mg L^{-1} 2,4-D. The calli were then taken and grown in media containing 5.2 µM, 26.4 µM and 132.3 µM sodium selenate for 21 days (Fig. 1B, C, D). The color red was observed when the concentration of selenium was 132.3 µM (Fig. 1D).

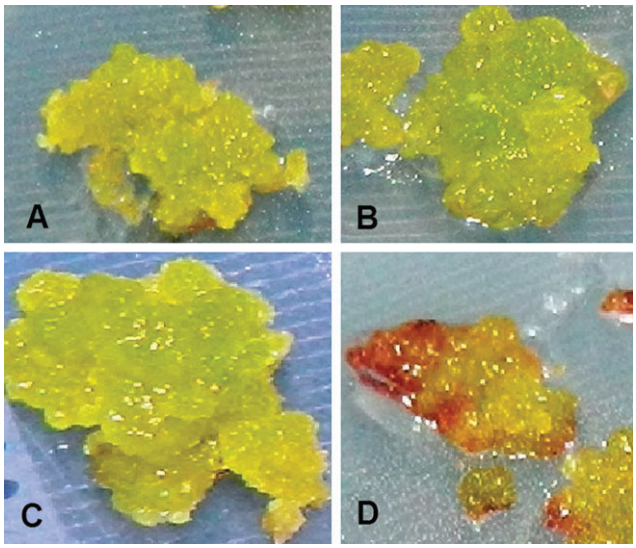

Fig. 1. *Astragalus chrysochlorus* callus: untreated control (A), and selenium-treated tissue: 5.2 µM (B), 26.4 µM (C), and 132.3 µM (D).

Effect of selenium treatments on antioxidant enzyme activities

The activity of CAT decreased by 13%, 22% and 27% in the 5.2 µM, 26.4 µM and 132.3 µM Se-treated calli of *A. chrysochlorus*, respectively (Fig. 2A). In all calli, the activity of this enzyme was not modified and the decrease was not significant statistically according to treatment with Se. The POX activity increased 4, 3 and 2 fold when compared to the control calli, respectively, after treatment with Se (Fig. 2B). Lower doses of Se (5.2 µM and 26.4 µM) have increased the POX activity more than 132.3 µM Se. The increase in the POX activity was statistically significant in 5.2 µM and 26.4 µM Se-treated calli. The APX activity was significantly increased in 5.2 µM and 26.4 µM Se-treated calli (Fig. 2C). The activity of this enzyme was higher in 132.3 µM Se treated callus tissue than in control tissues but the increase was not significant. Treatment with Se resulted in an increase of GR activity in 5.2 µM-treated callus tissues and a decrease in 26.4 µM and 132.3 µM-treated calli tissues, respectively (Fig. 2D). However, in the 5.2 µM Se-treated callus tissues, this enzyme activity did not significantly change.

Isolation and quality detection of total RNA

Electrophoresis of isolated RNA on 1% agarose gel stained with EtBr showed distinct 28S and 18S rRNA bands, indicating a good quality of total RNA (On-line Suppl. Fig. 1). The A_{260}/A_{230} absorbance ratio is usually calculated to check polysaccharide or polyphenolic contamination, and the A_{260}/A_{280} ratio for protein contamination. We determined that A_{260}/A_{230} ratios were greater than 2, and the A_{260}/A_{280} ratios between 1.9 and 2.1. This result showed that the isolated RNAs were intact and not contaminated.

Expression analysis of SMT gene

To evaluate the expression of the transcript, the reverse-transcribed cDNAs of samples (control, and treated tissues with 5.2 µM, 26.4 µM and 132.3 µM of Se) were used as templates. *SMT* was amplified by the method for an SYBR Green qPCR assay. We can conclude that PCR amplification curves show that amplification has good reproducibility in each sample.

The Se metabolism pathway was studied extensively, and the conservation level of this pathway is high in plants. The expression of *SMT* were observed by qPCR in all samples of selenium-treated *Astragalus* plant (Fig. 3). From the results obtained, we conclude that *SMT* transcript levels for 5.2 µM and 26.4 µM selenium-treated callus were lower than for the control callus tissues. In contrast, the *SMT* expression level of 132.3 µM in selenium-treated callus was 4.26 times higher than in the control callus.

Discussion

Selenium hyperaccumulation is involved with SMT enzymatic activity and accumulation of S-methylcysteine (MeCys) and MeSeCys (Sors et al. 2005). In this study, our

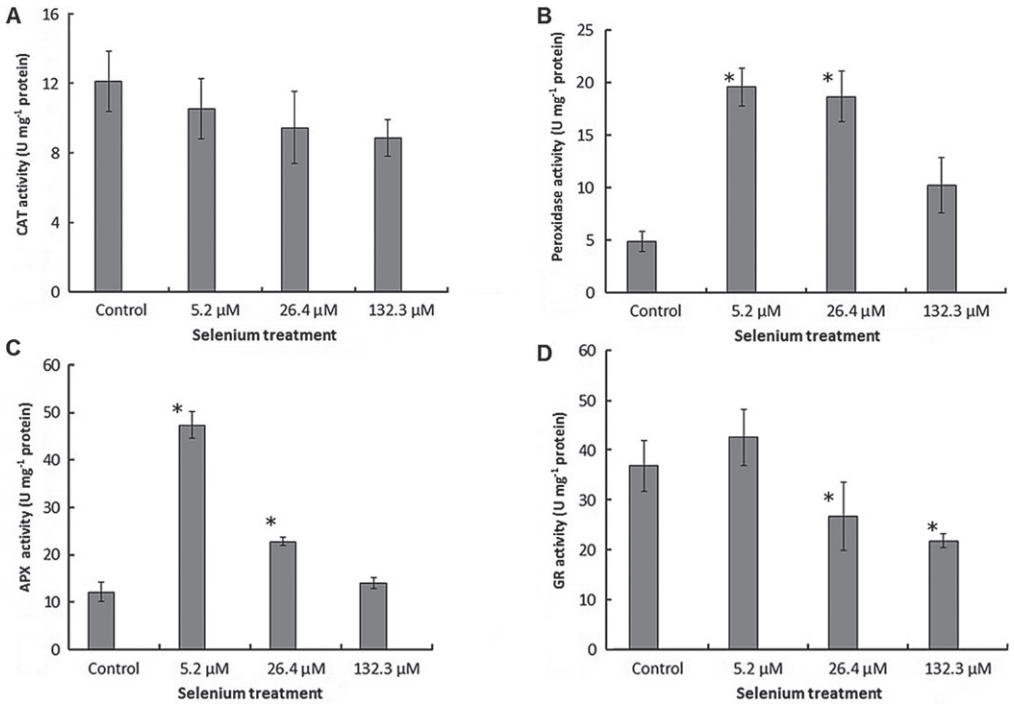

Fig. 2. Antioxidant enzyme activities in callus tissue of *Astragalus chrysochlorus* treated with different concentrations of selenium: A) catalase (CAT), B) peroxidase C), ascorbate peroxidase (APX), D) glutathione reductase (GR). Asterisk (*) means statistically significant ($p < 0.05$); bars indicate ± standard deviation.

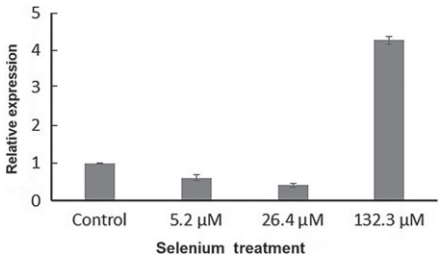

Fig. 3. Relative expression levels of selenocysteine methyltransferase gene in callus tissue of *Astragalus chrysochlorus* treated with different concentrations of selenium in comparison to untreated control. The ratio of the relative quantity of the target gene and the relative quantities of reference genes (actin) of each treatment was normalized against the untreated control (value = 1) and plotted using arbitrary units. Each data point represents the mean ± SE, n = 3.

main object was to investigate the expression profile of the *SMT* gene (AchSMT, Accession number:GQ844862) involved in selenium metabolism in *A. chrysochlorus* callus under increasing concentrations of selenium. We used qPCR to measure *SMT* gene expression. As a result, *SMT* expressions in 5.2 µM and 26.4 µM selenium-treated callus were slightly lower than in the control callus. In contrast, the *SMT* expression level of 132.3 µM selenium-treated callus was found to be higher and the fold change was 4.26. In our previous work (Çakir and Ari 2013), *A. chrysochlorus* plantlets were treated with 0, 5.2 µM, 26.4 µM and 132.3 µM sodium selenate and analysed with semi-quantitative reverse transcription PCR. According to the results, differentially, *SMT* expression sodium selenate treated plantlets were constitutively expressed like *AbSMT* from hyperaccumulator *A. bisulcatus* in all selenate treatments.

SMT gene has been isolated from a Se hyperaccumulator *A. bisulcatus* (Neuhierl et al. 1999, Neuhierl and Bock 1996). It was thought that SMT is the key player in the accumulation of selenium and protection of the plants from the toxic effects (Neuhierl et al. 1999). Pickering et al. (2003) concluded that SMT gene expression was continuous in tissues of *A. bisulcatus*. In contrast, Lyi et al. (2005) reported that broccoli SMT (*BoSMT*) expression is up-regulated by selenate treatment. Its expression in leaf has been determined to be involved in development. Tao et al. (2012) reported that in young leaves of selenium-treated tea plant, the expression level of SMT gene was similar to mature leaves, and it was the highest in roots. These findings suggested that selenite is immediately converted to organic Se and remains in the root tissues. In the present study, we determined that *SMT* expression level was differentially affected upon treatements with increasing concentrations of Se. In low concentrations (5.2 µM and 26.4 µM) *SMT* expression was lower than in control tissues (0.397 and 0.597 times, respectively). Our results revealed that the transcriptional levels of SMT gene fluctuated after Se treatment. The changes or variations in expression levels of SMT gene after treatment with different concentrations of Se may be due to the delayed response of Se metabolism. Se assimilation and hyperaccumulation pathway are a very complicated metabolic process. One of the important approaches is to investigate *SMT* gene expression. Also further research into *SMT* at genetic translation level and the content of related amino acids in different parts of the *A. chrysochlorus* plant is needed to validate *SMT* status in the process of selenium metabolism.

Selenium is an important micronutrient for many organisms. *A. chrysochlorus* has been found to be secondary Se

accumulator in our previous study (Ari et al. 2010). Se is very important for antioxidant metabolism and response. In this study the activities of APX, POX, CAT and GR enzymes were also investigated. Antioxidant responses of *A. chrysochlorus* callus tissues varied in a concentration-dependent manner. CAT levels decreased gradually in increasing concentrations of Se, nevertheless this decrease was not significant when compared to control. POX and APX showed similar activity patterns. At 5.2 μM level, there was a significant increase, on the other hand at 26.4 μM and 132.3 μM levels the activities were decreased compared to the 5.2 μM level. GR activity was increased at 5.2 μM and decreased significantly at higher concentrations. Under the concentration of 132.3 μM, Se stimulated antioxidant metabolism. As shown in figure 2, despite the decrease in CAT and GR activity, POX and APX activities were increased significantly ($p < 0.05$). It is thought that the activities of POX and APX are more convenient to reduce the oxidative stress caused by Se. The results indicate that the increase in POX and APX activities were sufficient to protect callus cells. At the higher concentration of 132.3 μM, growth rate of callus cultures was decreased and pigmentation started. It is thought that this pigmentation is related to biosynthesis of secondary metabolites and also it is an environmental stress response of *A. chrysochlorus*. However, POX activity was still higher than in control callus cultures. In our previous studies, crude ethanol and ethyl acetate extracts of this plant exhibited cytotoxic activity and apoptosis-inducing properties (Karagoz et al. 2007) and also antioxidant effects (Hasancebi, unpublished data). Besides the biotechnological importance of *Astragalus* species, in callus tissues of the secondary Se accumulator *A. chrysochlorus* it was found that antioxidant enzymes and *SMT* gene expression were affected by Se treatment in callus tissues. Therefore, it is thought that it could be related and important for accumulation and tolerance to Se of these species.

In conclusion, Se triggered significant transcriptional responses in *A. chrysochlorus*. The *SMT* gene was repressed by low concentrations of Se. Results of a cross comparison showed that the SMT gene was up-regulated by 132.3 μM Se in *A. chrysochlorus*. The results obtained provide novel information for understanding the effects of different Se concentrations on plant gene expression and will help to reveal the Se metabolism of plants.

Acknowledgements

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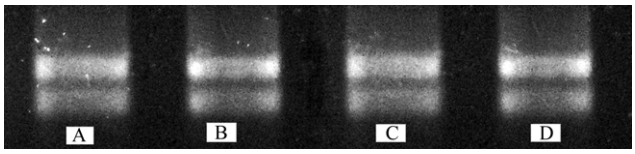

On-line Suppl. Fig. 1. Representative RNA isolated from *Astragalus chrysochlorus* callus and analyzed by gel electrophoresis: A) untreated control, and selenium-treated tissue in the following concentrations: B) 5.2 μM, C) 26.4 μM, D) 132.3 μM.