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CELLULAR AND MOLECULAR BIOLOGY

Characterization of trehalose-6-phosphate synthase and Na^+/H^+ antiporter genes in *Vuralia turcica* and expression analysis under salt and cadmium stresses

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Abstract: *Vuralia turcica* (Fabaceae; Papilionoideae) is a critically endangered endemic plant species in Turkey. This plant grows naturally in saline environments, although the photosynthesis and physiological functions of many plants are affected by salt stress. Molecular control mechanisms and identification of genes involved in these mechanisms constitute the critical field of study in plant science. Trehalose-6-phosphate synthase (*TPS*) is one of the essential enzyme genes involved in trehalose biosynthesis, which is protective against salt stress. Also, the vacuolar Na^+/H^+ antiporter gene (*NHX*) is known to be useful in salt tolerance. In this study, the *TPS* and *NHX*–like genes in *V. turcica* were partially sequenced using degenerate primers for the first time and submitted to the NCBI database (accession numbers MK120983 and MH757417, respectively). Also, the expression levels of the genes encoding *TPS* and *NHX* were investigated. The results indicate that the increase in both the level of applied salt and cadmium is coupled with the increase in the expression level of *NHX* and *TPS* genes. However, salt exposure significantly affected the expression level of the *NHX* gene. The findings suggest that the *NHX* gene might play a crucial role in the salt tolerance ability of *V. turcica*.

Key words: Gene profiling, *NHX*, salt tolerance, *TPS*.

INTRODUCTION

Salt stress is an often encountered problem in agriculture that decreases yield by enabling healthy plant growth (Zhu 2001). Trehalose-6-phosphate synthase (*TPS*) and vacuolar Na^+/H^+ antiporter (*NHX*) genes are known to be useful against abiotic stress conditions such as salinity and drought.

Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is one of the significant carbohydrate stores in not only plants also in a large variety of microorganisms such as yeast and animals. Various organisms, such as bacteria, fungi, plants, insects, and invertebrates, contain genes that enable them to produce trehalose endogenously (Elbein et al. 2003). Trehalose is a non-reducible disaccharide composed of two glucose units and provides rapid adaptations to an organism under various environmental conditions, and has a significant role in glucose uptake. It also functions as an osmoprotectant (Elbein 1974, Crowe et al. 1984). Trehalose is catalyzed by two enzymes: trehalose-6-phosphate synthase (*TPS*) and trehalose-6-phosphate phosphatase (*TPP*). Currently, there has been a great interest in trehalose metabolism to improve stress-tolerant plants. *TPS* gene was successfully transferred to tomato, potato, and tobacco plants to obtain stress-tolerant species, especially against drought (Jang et al. 2003, Avonce et al. 2004, Zhang et al. 2005, Kissoudis

et al. 2015). Also, there have been several studies conducted on the function of the *TPS* gene under salt and drought conditions and reported that overexpression of *TPS* was beneficial on the resistance mechanism (Kwon et al. 2004, Wu et al. 2006).

Sodium ions can cause salt stress in plants. Plants remove excess Na^+ ions by exclusion or compartmentation in saline soils. Na^+/H^+ antiporters, membrane proteins, play an essential role in Na^+ homeostasis and pH regulation in plant species. Na^+/H^+ antiporter genes are also found in animals, yeast, and bacteria (Shi & Zhu 2002, Fukuda et al. 2004). It is known that *NHX* is responsible for cadmium stress from previous studies (Cong et al. 2013). Cadmium is a non-essential heavy metal and toxic for plants, animals, and also humans due to causing many adverse effects such as oxidative stress and disturbance in metabolism (Adabnejad et al. 2015).

Although the molecular identification and the expression pattern of *TPS* and *NHX* genes are very important in terms of salt stress mechanism in *Vuralia turcica* (Tan et al. 1983) Uysal et al. (2014), to date, these genes have not been investigated. This study aims to identify *NHX* and *TPS* genes in *V. turcica* and to analyze gene expressions of these genes in response to cadmium and salt stresses.

MATERIALS AND METHODS

Plant materials

The leaves of salt (0, 1000, and 2000 ppm) and Cd (0, 5, 25, and 50 ppm) treated plants in the previous research (Tekdal & Cetiner 2018) were used for total RNA isolation. Leaf samples were obtained from the samples collected in the last experiment, treated with liquid nitrogen, and maintained at -80°C (Tekdal & Cetiner 2018).

Genomic DNA isolation

The MiniPrep DNA isolation method was used in gDNA isolation of *V. turcica* and was performed according to the protocol proposed by Edwards (1998). The purity of DNAs was achieved by spectrophotometric (NanoDrop ND 100, Wilmington, DE, USA) and electrophoresis (1% agarose) methods. Samples were adjusted with DNase and RNAse-free water at a concentration of 50 ng μl^{-1} for further analysis.

Detection of orthologs of *TPS* and *NHX* genes and degenerate primers design

Since the sequences of the *V. turcica TPS* and *NHX* genes are unknown, the degenerate primer design was first performed. To find the orthologs of *TPS* and *NHX* genes, the corresponding gene sequences of the legumes were first selected from the NCBI database (Table I). The known gene fragments (*TPS* and *NHX*) of the selected legumes were compared, and degenerate primers were designed for amplification of candidate orthologs from the genomic DNA of *V. turcica*. Degenerate primers for *TPS* and *NHX* were developed based on conserved sequence segments (Figures 1 and 2, respectively) identified using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al. 2011). The primers designed for *TPS* and *NHX* sequences and selected for qRT-PCR analysis are described in Table II. Primers were designed manually using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and synthesized by Sentebiolab (https://sentebiolab.com.tr/), Ankara, Turkey.

PCR reaction and agarose gel electrophoresis

To obtain the amplification product using the designed primers, gradient PCR was performed to determine the optimal temperatures at which the primers were bound to DNA. The best DNA binding temperature of each primer applied with

Table I. *TPS* and *NHX* gene homologs from NCBI GenBank used for *V. turcica* putative gene analysis and mRNA sequences comparison.

Family	mRNA	Species	Accession No
Trehalose-6-Phosphate Synthase (TPS)			
Fabaceae	*Alpha,alpha-trehalose-phosphate synthase [UDP-forming] 1-like (LOC101492749)*	*Cicer arietinum* L.	XM_004515548.2
Fabaceae	Trehalose-6-phosphate synthase gene	*Glycine max* (L.) Merr.	EU873088.1
Fabaceae	*Alpha-trehalose-phosphate synthase [UDP-forming] 6 (LOC106777636)*	*Vigna radiata* var. *radiata*	XM_014665298.2
Na^+/H^+ antiporter (NHX)			
Fabaceae	*Na^+/H^+ antiporter (NHX1)*	*Cicer arietinum* L.	HM602043.1
Fabaceae	vacuolar *sodium proton antiporter (nhx1)*	*Vigna radiate* (L.) R. Wilczek	JN656211.1
Fabaceae	*Na^+/H^+ antiporter (NHX1)*	*Galega orientalis* Lam.	EU340284.1
Fabaceae	*NHX1-like protein*	*Lotus tenuis* Waldst. & Kit.	EU727217.1

gradient PCR was determined according to the cleanest banding condition in 2% agarose gel. PCR amplifications were performed in a reaction volume of 25 µL reaction mixture containing 5 ng DNA, 0.8 µM of each primer (forward and reverse), 0.2 mM dNTP (Fermentas, #R0192), 0.125 unit Taq DNA Polymerase (Fermentas, #EP0402), 1X Taq DNA Polymerase buffer (Fermentas, 00061586), including 2.5 mM $MgCl_2$ (Fermentas, 00061590). The cycling parameters were as follows: 10 min at 95°C for initial denaturation and 35 cycles of 30 seconds each at 95°C (melting) and 30 seconds at a temperature specific for every primer pair - 48°C for *TPS* and 53°C for *NHX* primers – (annealing) and 1 min at 72°C (extension). These cycles were then followed by a final extension step at 72°C for 7 min. 10 µl of PCR products obtained from the designed degenerate primer analyzes were withdrawn, and 2 µl loading buffer (40% sucrose, 10 mM EDTA, 25% bromophenol blue) was added. The prepared mixture was studied in 2% agarose gel by adding 0.5x TBE (Trism Base, Boric Acid, EDTA) buffer. The gel was stained with 0.1% ethidium bromide solution. The presence of amplification products was determined by photographing under UV light (302 nm) on a Biorad Imager (Bio-Rad Laboratories, Segrate (Milan). The amplified and expected size PCR products were purified from the gel using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, USA) according to the protocol recommended by the manufacturer.

DNA sequencing and BLAST analysis

The sequencing service was commercially provided by Medsantek Ankara, Turkey (http://www.medsantek.com.tr/). The raw sequence data transmitted from Medsantek were manually checked, and the forward and reverse complemented sequences were aligned using the Pairwise Sequence Alignment (Nucleotide) (http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html) constructed by EMBL-EBI. The resulting consensus sequence was searched via the GenBank database at the National Center for Biotechnology Information (NCBI), Bethesda, USA, using the BLAST (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi) search program (Altschul et al. 1990) to determine similar sequences. The identified *V. turcica TPS* and *NHX* partial sequences were recorded in the NCBI GenBank using the Sequin program (https://www.ncbi.nlm.nih.gov/Sequin/).

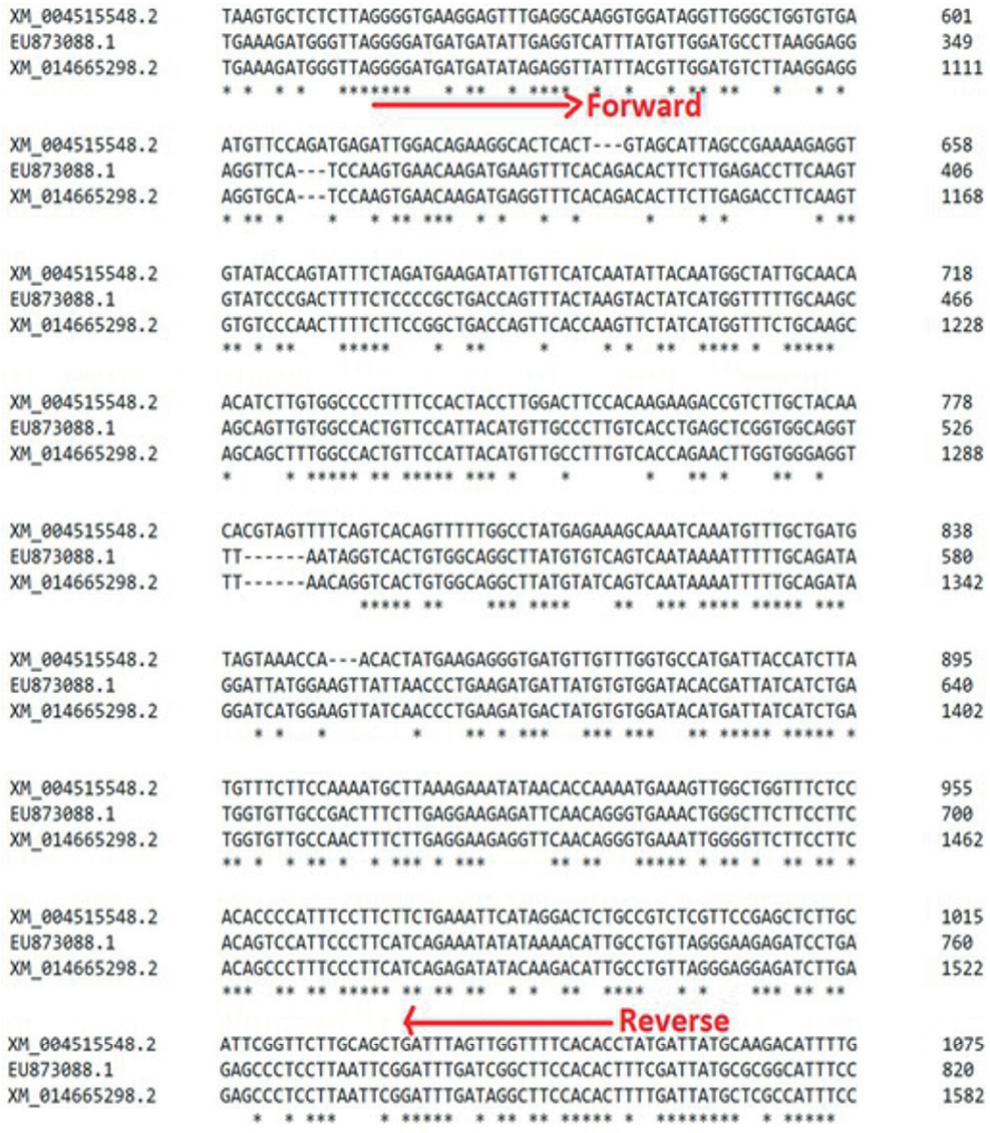

Figure 1. Multiple alignment of *TPS* orthologs in relative legume species of *V. turcica* (*Cicer arietinum* L., *Glycine max* (L.) Merr., and *Vigna radiata* (L.) R. Wilczek; Table I) and designing of degenerate primers based on mostly conserved sequences; arrows, selected forward and reverse primers; '*', Identical amino acid residues; ':' Conserved substitutions; '.' Semi-conserved substitutions.

RNA extraction and cDNA synthesis

Total RNAs were isolated from frozen leaf samples (~500 mg) by peqGOLD TriFast™ reagent (VWR International, LLC), following the manufacturer's instructions. Isolated RNAs were quantified by measuring the absorbance of samples at 260 nm using a NanoDrop spectrophotometer (ND-1000) and were checked on 2% (w/v) agarose gel in terms of the integrity. RNA samples were kept at -80°C until the usage. cDNA was synthesized by reverse transcription from 2 µg RNA with a commercial kit and oligo-dT primers (SensiFast™ cDNA Synthesis Kit (Bioline, A Meridian Life Science), following the manufacturer's instructions.

The quantitative real-time PCR analysis

Quantitative Real-Time (qRT) PCR was performed on 30 ng of cDNA using SensiFAST™ SYBR® No-ROX Kit (Bioline) and analyzed by a LightCycler® 480 Instrument (Roche Diagnostics, Mannheim, Germany). The results were then normalized to the expression of the *18S* rRNA of Fabaceae (Song 2005) as a housekeeping gene.

Figure 2. Multiple alignment of *NHX* orthologs in relative legume species of *V. turcica* (*Cicer arietinum* L., *Vigna radiata* (L.) R. Wilczek, *Galega orientalis* Lam., and *Lotus tenuis* Waldst. & Kit.; Table I) and designing of degenerate primers based on mostly conserved sequences; arrows, selected forward and reverse primers; '*', Identical amino acid residues; ':' Conserved substitutions; '.' Semi-conserved substitutions.

Target gene expression was analyzed with a mathematical method proposed by Pfaffl (2001).

RESULTS

The salt and Cd stress tests on *V. turcica* were established by applying different concentrations of NaCl (0, 1000, and 2000 ppm) and Cd (0, 5, 25, and 50 ppm) (Tekdal & Cetiner 2018). In this study, leaf samples of piyan plants, which were applied to salt and Cd stresses (target groups) and not applied to any stress (control group), were obtained from the study implemented by Tekdal & Cetiner (2018). Genomic DNAs that were needed for *TPS* and *NHX* gene identification were isolated from the leaves of NaCl- and Cd-treated *V. turcica* (target) and non-treated *V. turcica* (control) successfully. The quality of isolated gDNAs and RNAs was in good quality, with a 260/280 ratio of 1.8-2.0, and their integrity was determined using agarose gel electrophoresis (Figure S1 - Supplementary Material).

To determine the effect of salt and Cd on the expression pattern of the *TPS* and *NHX* genes, total RNAs from leaves of NaCl- and Cd-treated plants were extracted and analyzed using agarose gel electrophoresis (Figure S2).

In this study, gDNAs were used as the template for PCR-based amplification using degenerate primers designed to *TPS* and *NHX* genes using conserved regions of *TPS* and *NHX* mRNAs available in the GenBank database (Table I). As a result of PCR analysis using degenerate primers, multiple bands were produced when used designed *TPS* primers, whereas single

Table II. The list of the designed degenerate primers, control and housekeeping gene primers employed in this research (F, forward, R, reverse).

Target	Name	Sequence (5´-3´)	Definition	Product size (bp)
NHX	NHX-F NHX-R	GGTGTTGTGAATGATGCTAC GAACTCTSAGTCACATTATG	NHX-F/sense primer NHX-R/antisens primer	300
TPS	TPS-F TPS-R	GGGGWKRAKGAKWTWGAGG GTGTGRAARCCDAYYAAATC	TPS-F/sense primer TPS-R/antisens primer	471
18S rRNA	18S-F 18S-R	TACCGTCCTAGTCTCAACCATAA CAGAAGTGAACCTTTTCTTC	18S-F/sense primer 18S-R/antisens primer	Reference Song (2005)
TPS-1 (sequence specific primer)	TPS-1-F TPS-1-R	CAGATACTTCTTGAGTCC AGCTTGCCACAGAGACCG	TPS-1-F/sense primer TPS-1-R/antisens primer	155
NHX-1 (sequence specific primer)	NHX-1-F NHX-1-R	GCTTAATTCCAGGCACTC GAGACATAACAATCCCAC	NHX-1-F/sense primer NHX-1-R/antisense primer	204

bands were obtained when used designed *NHX* primers (Figure 3). Multiple bands production is possibly related to amplifying unrelated sequences. The bands were expected sizes were extracted from the gel and purified for sequencing.

To identify the putative orthologues of *TPS* and *NHX* in *V. turcica*, isolated and purified PCR products were directly sequenced by the Sanger method. The sequences obtained through PCR were verified to be 230 bp in length for the *NHX* gene and 237 bp in length for the *TPS* gene and were deposited in the NCBI GenBank database with the following accession numbers: MH757417.1 and MK120983.1, respectively. The result of the BLAST search against the GenBank nucleotide database indicated that *V. turcica* and *Arachis ipaensis* Krapov. & W.C. Greg. shared the maximum identity (92%) at the nucleotide level in terms of the *TPS* gene, whereas and the identity between *V. turcica* and *Cajanus cajan* (L.) Millsp. was very high in terms of the *NHX* gene (Table III). Conceptual translation of the ORFs of the identified *TPS* sequence of *V. turcica* yielded an amino acid sequence of 79 aa (Figure 4), and the *NHX* sequence produced an amino acid sequence of 69 aa (Figure 5).

To understand whether Cd exposure influences salt tolerance ability, *NHX* and *TPS* expressions in Cd-treated plants were examined. In low Cd concentration (5 ppm), the transcript level of *TPS* gradually increased, and the expression level of *TPS* increased by >5-fold in medium Cd concentration (25 ppm). In a high concentration of NaCl (2000 ppm) and Cd (50 ppm), *NHX* expression level and salt tolerance ability increased.

DISCUSSION

Abiotic stress factors affect crop production negatively in Turkey as well as all over the world. Plants activate stress-related genes against such stress conditions. Therefore, elucidating the molecular control mechanisms developed by plants against stress conditions will contribute

Table III. Sequence similarities according to The BLAST search against NCBI database based on identified partial sequences in *V. turcica*.

mRNA	Species	Accession No	Similarity (%)
TPS			
alpha,alpha-trehalose-phosphate synthase [UDP-forming]	*Arachis ipaensis* Krapov. & W.C. Greg.	AYW34350.1	92
alpha,alpha-trehalose-phosphate synthase [UDP-forming]	*Prunus avium* (L.) L.	XP_021821062.1	90
trehalose-6-phosphate synthase	*Ricinus communis* L.	EEF34829.1	87
NHX			
sodium/hydrogen exchanger 1-like	*Cajanus cajan* (L.) Millsp.	XP_020226542.1	81
Sodium/hydrogen exchanger 1	*Cajanus cajan* (L.) Millsp.	KYP74393.1	81
Sodium/hydrogen exchanger 2, partial	*Mucuna pruriens* (L.) DC.	RDX62800.1	78

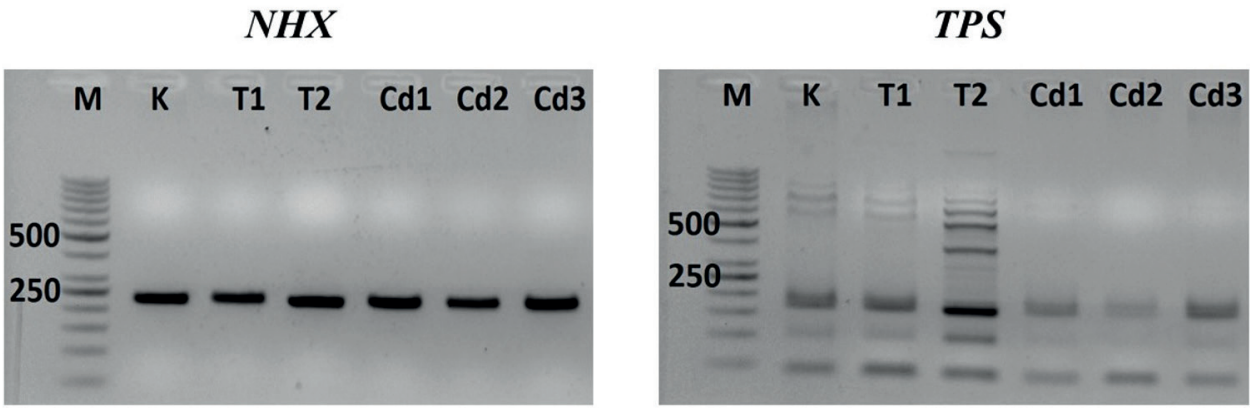

Figure 3. 2% agarose gel electrophoresis results of PCR analysis of isolated leaf samples of *V. turcica* using degenerate *NHX* (left) and *TPS* (right) primers; K: control; T1: 1000 ppm NaCl treated leaves; T2: 2000 ppm NaCl treated leaves; Cd1: 5 ppm Cd treated leaves; Cd2: 25 ppm Cd treated leaves; Cd3: 50 ppm Cd treated leaves, M: 50 bp DNA ladder.

to plant development. Salt stress is one of the abiotic stress factors and has a significant effect on product yield. Therefore, it is crucial to elucidate the molecular control steps of tolerance mechanisms in plants that can quickly develop in soils with high salt content. *V. turcica* (piyan) is an essential source of a gene that is in danger of extinction. As a result of the studies carried out with this plant, it was determined that the plant could develop in soils with high salt content.

As a result of studying with plants exposed to high temperature and drought, trehalose, a nonreducible disaccharide, was found to be effective in salt tolerance (Garg et al. 2002). *TPS* is the most well-known enzyme gene that plays a role in the biosynthesis of trehalose, which is protective against salt stress (Carcia et al. 1997). Many studies indicate an increase in the expression of the *TPS* gene in stressed plants (Almeida et al. 2005, Zhang et al. 2005, Kosmas et al. 2006, Lunn 2007). The vacuolar Na^+/H^+ antiporter gene has been reported to be effective in salt tolerance in many plants (*Arabidopsis thaliana* (L.) Heynh. (Apse et al. 1999); *Lycopersicon esculentum* Mill. (Zhang &

gag gtt att tat gtt gga tgc ctt aag gaa gaa gtg cat cca aat gaa caa gat gag gtt
E V I Y V G C L K E E V H P N E Q D E V
tca cag ata ctt ctt gag tcc ttt aaa tgt atc cca act ttt ctc cca gat gat atg ttt
S Q I L L E S F K C I P T F L P D D M F
act agg tac tat cat ggg ttt tgc aaa caa caa ttg tgg cca ctg ttt cat tac atg ttg

Figure 4. Translation of the ORFs of the identified *TPS* sequence of *V. turcica.*

attt ttt gct taa ttc cag gca ctc tac aga tcg tgg gtg ttg tga atg atg cta atg gca
F F A - F Q A L Y R S W V L - M M L M A
tac ctc tcc tac atg ctg gct gaa gta agt tct aaa gac gtt tag taa tgt ttt ctg gag
Y L S Y M L A E V S S K D V - - C F L E
gct gta tga tgc tga tgt atc tac ctt ttc tgc agt tat gct atc tga gtg gca ttc taa

Figure 5. Translation of the ORFs of the identified *NHX* sequence of *V. turcica.*

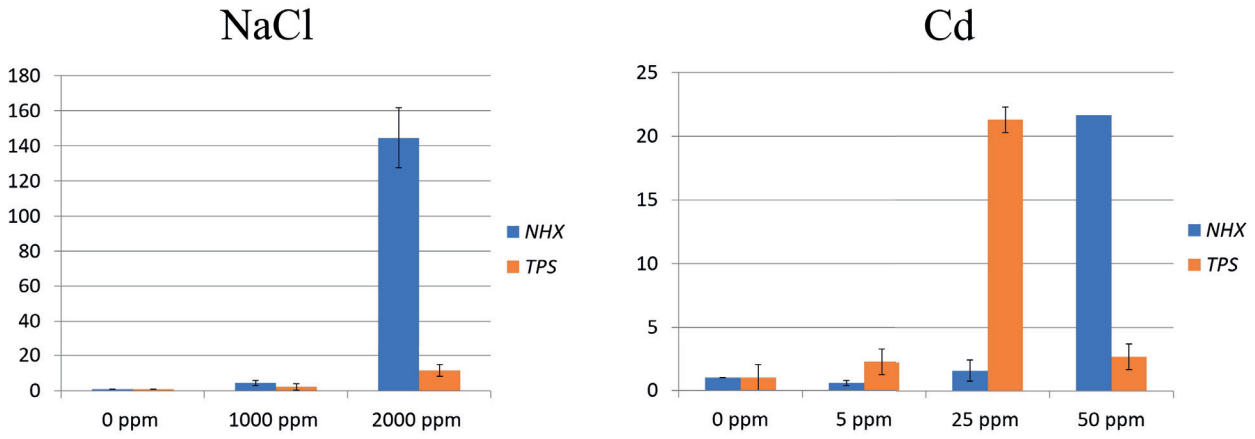

Figure 6. Effect of NaCl and Cd on *TPS* and *NHX* expression in *V. turcica* leaves. Data normalized to *18S* rRNA are expressed as mean relative Ct(Cp) values of three independent experiments. Gene expression was quantified by qRT-PCR in *V. turcica* leaves treated for 1 month with NaCl (1000 and 2000 ppm) and Cd (5, 25, and 50 ppm). Untreated leaves were taken as controls.

Blumwald 2001); *Brassica napus* L. (Zhang et al. 2001); *Oryza sativa* L. (Fukuda et al. 2011)). In salt stress study with *V. turcica*, it was determined that the salt tolerance of the species was found as a result of phenological and physiological studies (Tekdal & Cetiner 2018). Within the scope of the present study, the partial sequences of the *TPS* and *NHX* genes were characterized. Salt tolerance ability of plants can be affected by increasing the osmolyte production or stress proteins (Zhu 2001). In a previous study, the *NHX* gene of *A. thaliana* enhanced salt tolerance ability of wheat and tomato (Zhang et al. 2001, Xue et al. 2004). Expression levels of *NHX* and *TPS* in response to NaCl and Cd were evaluated by qRT-PCR (Figure 6). Likewise, as a result of PCR study with primers designed for *TPS* and *NHX* genes and cDNAs of samples subjected to salt stress,

the expression level of the *NHX* gene was higher than that of the *TPS* gene. Under salt stress, *NHX* and *TPS* gene expressions were examined in *V. turcica* leaf tissues, and there was no significant change in *TPS* gene expression. It was thought that the difference in the expression of the *TPS* gene could be seen by prolonging the stress period applied or by examining the root tissues. According to the literature, it was found that the *TPS* gene was overexpressed in transgenic plants resistant to salt stress; *Ginkgo biloba* L. (Wu et al. 2006), rice (Garg et al. 2002, Jang et al. 2003), tobacco (Almeida et al. 2005, Zhang et al. 2005). The expression of *NHX* was high by applying 50 ppm Cd. It is, therefore, likely that the *NHX* gene was most active in *V. turcica* exposed to high Cd concentration (50 ppm).

CONCLUSION

Under salt and cadmium stress conditions, plants tend to change their developmental physiology mostly to overcome stress. *NHX* and *TPS* genes are essential for homeostasis in plants. Since there are no studies in the literature on the determination of *V. turcica NHX* and *TPS* genes and the elucidation of expression levels of these genes, it is thought that the present study findings will fill an essential gap in the literature. As a result of this study, partial sequences of *TPS* and *NHX* genes, which are thought to be useful in the mechanism of tolerance to salt stress in *V. turcica,* were determined for the first time and introduced into the literature.

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SUPPLEMENTARY MATERIAL

Figures S1 and S2.

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