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Original Scientific Paper

Expression of heavy metal ATPases (HMA1 and HMA3) in *Brassica nigra* and *B. juncea* grown at different Cu levels

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ABSTRACT:
Metal pollution is a major environmental problem which affects agriculture and human health. Turkey has significant Cu mining areas in Diyarbakır and its surrounding areas (Southeast Anatolia). Several crop plants cultivated in these areas are irrigated with water from the Tigris, and most agricultural lands are contaminated with Cu. *Brassica nigra* and *B. juncea* are well-known metal accumulator plant species which can hyperaccumulate metals, including copper, in their shoots. The purpose of this study is to evaluate their potential for the phytoremediation of Cu from these contaminated areas as an environmentally friendly and cost-effective means of reducing Cu-contamination. In this research, *B. nigra* and *B. juncea* plants were grown in soil at different Cu concentrations (0 to 1000 µM) and showed no toxicity symptoms while accumulating a significant amount of metal in their leaves. In the leaves of both species, the Cu content increased significantly with the increase in the Cu level in the media. HMA1 (Heavy Metal ATPase 1) in the leaves of both plant species gradually increased with increased Cu levels until 50 µM, then its expression slowly decreased with the further increase in Cu levels. The expression of HMA3 also increased with an increase in Cu in the leaves of both plant species. However, its expression pattern differed from that of HMA1. Our data showed that an increase in Cu levels in the leaves triggers the expression of both genes, suggesting that they play an active role in Cu detoxification. We propose that these plant species could be used for the decontamination of Cu from polluted soils. These data also indicate that Cu accumulation and tolerance in both plant species is probably a multi-genetic response, possibly involving several other transporter genes in the stress signal pathway. Hence, we also explored the expression of the other metal transporters, such as other HMAs (HMAs 5-8), Nramps (e.g., Nramp3), COPT proteins, and some Cu chaperons in these plant species.

Keywords:
metal toxicity, gene expression, metal transporters, phytoremediation

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INTRODUCTION

Copper (Cu) is one of the essential trace elements which plays a significant role in many plant functional processes. However, the excessive presence of Cu in plant tissue can cause damage to plants through different means. Some plants can cope with metal toxicity by relocating the metal in the cell at the subcellular level. For instance, the *Brassica nigra* (L.) W.D.J. Koch Diyarbakir ecotype collected from the Southeastern part of Turkey accumulated very high Cu concentrations (more than 700 ppm) in the shoots without showing any toxicity symptoms and was classified as a Cu accumulator (Memon & Zahirovic 2014). *Brassica juncea* (L.) Czern Tomcat is a well-known oilseed crop and is reported to accumulate several metals (e.g. Cu, Zn, Cd, Fe, Ni) in the leaves

(Mourato *et al.* 2015; Dalyan *et al.* 2017). It also accumulates toxic and deleterious metals/metalloids, including Ar, Cd, and Pb, in the roots and shoots (Salt *et al.* 1998). These species could be important in removing contaminants from metal-polluted soils (Hu *et al.* 2009; Memon & Schroder 2009; Shiyab *et al.* 2009; Memon *et al.* 2021; Kafle *et al.* 2022). In addition to the aforementioned *Brassica* species, as well as *B. napus* (Zn, Cd) and *B. oleracea* (Zn, Cd), many other plant species are also classified as hyperaccumulators: *Arabidopsis paniculata* (Cd, Pb), *Arabidopsis halleri* (Cd, Zn), *Noccaea caerulescens* (Cd, Zn), *Potentilla griffithii* (Cd, Zn), *Pteris vittata* (As), and *Sedum alfredii* (Zn, Cd), (Memon & Zahirovic 2014; Mourato *et al.* 2015; Reeves *et al.* 2018; Memon *et al.* 2021; Bortoloti & Baron 2022).

Metal accumulator plants have developed several detoxification mechanisms in their cells, such as metal chelation and sequestration, metal storage in the vacuolar compartment, and excreting metal in the trichomes of the leaves (Memon & Schroder 2009). A set of functional metal transporters involved in metal homeostasis have been reported and documented to play substantial roles in the phytoremediation process via the long-distance transportation of various metals between plant organs (Colangelo & Guerinot 2006). Several genes and proteins related to metal absorption and transportation have been identified and characterised in several accumulator plants. These metal transporters are subdivided into six main groups, including natural resistance macrophage protein (NRAMP), ZRT-like protein (ZIP), cation diffusion facilitator (CDF), yellow stripe-like (YSL), and heavy metal P1B-type ATPases (HMAs), etc. (Ma *et al.* 2021; Memon *et al.* 2021; Kumar & Srivastava 2022; Merakli *et al.* 2022). Details of the structural and functional analysis of HMAs in *Brassica* spp. are given in our previous work (Memon & Merakli 2022, 2023). For instance, heavy metal ATPases (HMAs), natural resistance-associated macrophage proteins (NRAMPs), metal tolerance proteins (MTPs), Zrt/IRT-like proteins (ZIPs), etc. are the major carriers in metal uptake and loading in plants and other eukaryotes (Williams & Mills 2005; Memon 2016). AtHMA1 resides in the chloroplast envelope and contributes to Cu transport into the chloroplast (Seigneurin-Berny *et al.* 2006; Moreno *et al.* 2008; Mikkelsen *et al.* 2012). HMA3 resides in the tonoplast membrane (Song *et al.* 2014) and is responsible for transporting Zn, and Cd ions into the vacuole (Morel *et al.* 2009; Li *et al.* 2018; Haque *et al.* 2022). However, the HMA3 role in Cu transport is not well defined.

In gaining a deeper understanding of the hyperaccumulation mechanism of Cu in these plant species, the expression patterns of HMA1 and HMA3 will provide useful information regarding the uptake, translocation, and accumulation of Cu from the contaminated soils. To this end, we analysed the expression patterns of these transporters in both plants grown at different Cu levels. Our data show that HMA1 and HMA3 represent key carriers in the accumulation and detoxification mechanism used for decreasing Cu stresses in *B. juncea* and *B. nigra*.

MATERIALS AND METHODS

Plant material, growth conditions, and Cu treatments. *Brassica nigra* ecotype seeds were collected from the Southeastern Region (N 38°18’04”, E 39°43’39”) of Anatolia, Turkey. *B. juncea* seeds were obtained from the Agricultural Research Institute, Tandojam, Sindh, Pakistan. The plant growth conditions and cultivation methods were similar to those previously reported (Merakli *et al.* 2022). Uniformly selected seeds were surface sterilised with commercial bleach (with 5% sodium hypochlorite containing Tween 20) for 15 minutes and then repeatedly washed with distilled water. Ten seeds were sown in different pots filled with soil and peat mixture (2:1). The pot experiments were conducted in a growth chamber with 25 ± 4°C day/night, 70 ± 5 relative humidity, and a 16 h light/8 h dark cycle photoperiod. Three-week-old plants were transferred to sterile plastic pots containing autoclaved soil and peat (2:1) (Merakli *et al.* 2022). The total properties of the soil are given in Table 1. The soil analysis methods were carried out according to those described by Soil and Plant Analysis Council Inc. (2017). The plants were grown with Hoagland solution for three weeks, and were then treated with different Cu treatments for two weeks (details are given in our previous reports, Memon & Zahirovic 2014; Cevher-Keskin *et al.* 2019; Merakli *et al.* 2022). 1/4th Hoagland solution was applied for three weeks (Hoagland & Arnon 1938). After two weeks of Cu treatment (0, 25, 50, 100, 200, 500, and 1000 µM), the plants were harvested, and the samples were either dried in the oven at 72°C for 48 h or frozen at -80°C for RNA isolation. Three replications were carried out for each experiment.

Determination of the Cu content. After harvest, soil samples were taken from each pot and were air-dried at room temperature for 72 h. The available Cu, Zn, and Fe in the soil were analysed with a solution containing 0.005 M DPTA (diethylenetriamine pentaacetic acid), 0.01 M $CaCl_2$, and 0.1 M TEA (triethanolamine) buffered at pH 7.3 (Norvell & Lindsay 1969). Metal extraction from the plant samples was done using the acid-wet digestion method (Memon *et al.* 1979). Cu analysis of the soil and plant samples was carried out by Atomic Absorption Spectrophotometer (PerkinElmer, AAnalyst 200) as previously described (Memon *et al.* 1979).

RNA isolation, cDNA synthesis, and qRT-PCR conditions. Total RNA was extracted from the leaf tissue using the RNeasy Plant kit (ThermoFisher Scientific, GeneJet Plant R.N.A. Purification Mini Kit, #K0801) (Merak-

Table 1. The properties of the soil used in this experiment.

pH	Soil texture	Organic matter (%)	EC MicroS/cm	CaCO3 %	Available Water capacity(%)	P (ppm)	K (ppm)	Ca (ppm)	Na (ppm)	Cu (ppm)	Zn (ppm)	Fe (ppm)
7.5	loam	1.6	546	5.6	44	40	341	2500	558	2.0	0.6	5

Table 2. Primer sequences designed for the qRT-PCR analysis.

Genes	Primer Sequences (5'-3')
BR-HMA1	*F: 5'GGCAGCTTACTTGGCTTTCC3'
BR-HMA1	**R:5' AAGTCGAAAGTGGGTGGTGG 3'
BR-HMA3	F: 5' TTCTCCGTCATCGTCCCTTC 3'
BR-HMA3	R: 5' GCAGCTTCTGTGTAATCCTCC 3'
BJ-UBQ9	F: 5'GAAGACATGTTCATTGGCA 3'
BJ-UBQ9	R: 5'ACACCTTAGTCCTAAAAGCC 3'

*F, Forward; **R, Reverse primers

li *et al.* 2022). cDNA was synthesised from the isolated RNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific™, U.S.A., #K1641) following the manufacturer's instructions. The reaction was carried out as previously described (Cevher-Keskin *et al.* 2019; Merakli *et al.* 2022). qRT-PCR was carried out as previously described (Memon *et al.* 2019). Briefly, SYBR green master mix was used for qReal-Time PCR (BioRad, U.S.A.). Ubiquitin was selected as the reference gene (Memon *et al.* 2019). The qRT-PCR reaction contains 4 µl of synthesised cDNA, 1 µl each specific primer (10 pmol), 13 µl of Maxima SYBR Green qPCR Mastermix (Thermo Scientific, USA), and 6 µl of ddH_2O. The primer3 programme was used to design the primers, and the sequences for the qRT-PCR analysis are listed in Table 2.

Data evaluation and statistical analysis. To determine the ΔΔCT values for the target and standard control genes, the relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Cevher-Keskin *et al.* 2019). The statistical analysis was conducted using the IBM SPSS 25.0 programme. Data evaluation was performed using the Duncan test at the $p < 0.05$ significance level following one-way ANOVA analysis.

RESULTS AND DISCUSSION

In this research, three-week-old plants (*B. nigra* and *B. juncea*) were either treated with Hoagland solution containing different levels of Cu or with no Cu for two weeks. Both plant species grew well and showed no toxicity symptoms in response to the Cu treatment. The total properties of the soil used in this experiment are given in Table 1. The metal content in the soil prior to cultivation was analysed using AAS. The Fe, Zn, and Cu content in the soil before cultivation was 5.03 ± 0.03, 0.60 ± 0.03, and 2.00 ± 0.05 mg kg^{-1}, respectively.

After two weeks of Cu treatment, the Cu content in the leaf tissue of both plant species increased alongside an increase in Cu in the soil. A remarkable increase in Cu content (around 2 to 3 fold) in the leaves of *B. nigra* was observed compared to *B. juncea*. The treated plants accumulated significantly high Cu ($p < 0.05$) in the leaf tissue compared to the control (Fig. 1). The Cu content in the leaf tissue of *B. nigra* grown in 1000 µM Cu was approximately 1126 mg kg^{-1}, which was ~ 211-fold greater than the control plants. *B. juncea* also accumulated Cu in their leaf tissue, at an amount of ~ 70-fold compared to the control plants (Fig. 1A & B).

Figure 1B shows that the Cu content in the roots is around 4–5 times lower than in the leaves. Our previous results with *B. nigra* showed about 90% accumulation of Cu in the shoots (stems and leaves) compared to the roots when the plants were grown in high Cu content in MS media (unpublished data).

The results of a previously conducted detailed microarray analysis of *B. nigra* Diyarbakir treated with and without Cu showed a 300 fold increase in the expression level of HMA6 (PAA1) in the leaf tissue of *B. nigra* treated with 500 µM Cu (Memon & Zahirovic 2014). The gene expression levels of HMA1 and HMA3 were evaluated two weeks after Cu treatment. The results demonstrated that the expression of HMA1 in the leaf tissue of both plant species gradually increased with Cu treatments, reaching a maximum level of 50 µM, and then slowly decreased with higher Cu treatments (Fig. 2A & B). The expression distinctly reduced in these plants at 1000 µM Cu.

Gene expression analysis revealed that HMA1 significantly increased in the leaves at low Cu concentrations with a positive correlation between Cu contents and HMA1 transcript levels in the leaf tissue. There was no significant change in the expression pattern of HMA1 following 50 µM Cu treatment (between 100 to 500 µM Cu^{2+}). Moreover, the qRT-PCR analysis showed that the HMA1 expression level in *B. juncea* grown under Cu stress was significantly higher than that of *B. nigra* (Fig. 2A & B).

The relative transcript level of HMA3 in the leaves of both plants grown under Cu^{2+} stress is shown in Fig. 3A & B. The expression pattern of HMA3 in the leaves of *B. nigra* differed to that of *B. juncea*. The HMA3 expression in the *B. nigra* leaves increased from the control to 25 µM treated plants and remained constant until 100 µM Cu, increasing significantly at 200 µM Cu (Fig. 3A). The expression of HMA3 in *B. juncea* increased con-

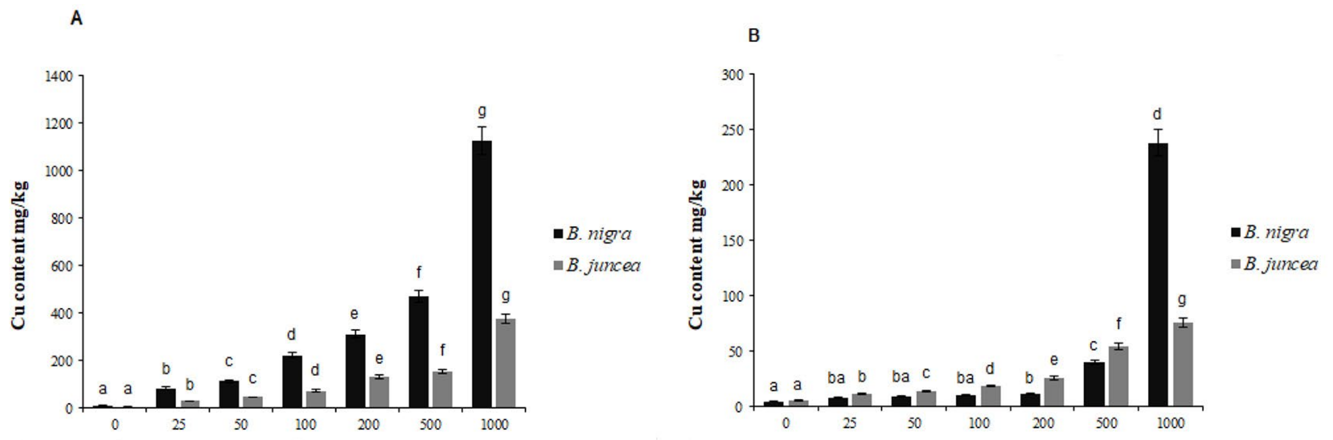

Fig. 1. Cu contents in the leaf tissues (A) and root tissues (B) of *B. nigra* and *B. juncea* exposed to different levels of Cu.

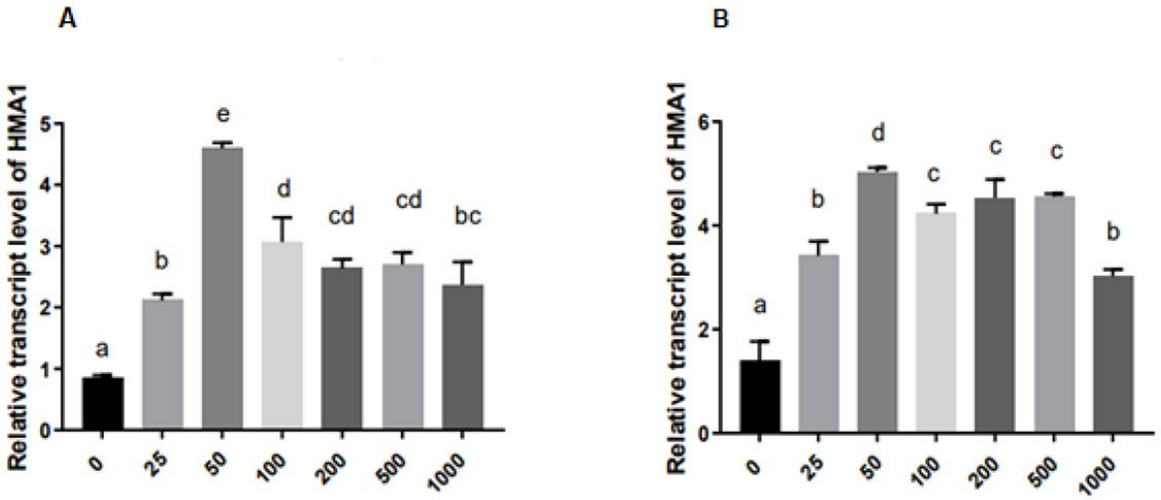

Fig. 2 Expression profile of the HMA1 gene in *B. nigra* (A) and *B. juncea* (B) plants exposed to Cu^{2+} stress from qRT-PCR analysis. Three-week-old plants were treated for two weeks with varying concentrations of Cu^{2+}. RNA from the leaf samples was extracted, and after cDNA synthesis, qRT-PCR was carried out similarly to our previous report (Cevher-Keskin *et al.* 2019). Ubiquitin was used as a reference gene. All the values represent the mean of three replicates. Different letters show the significant differences in the HMA1 gene after Cu^{2+} stress ($p < 0.05$).

comitantly from 0 to 200 µM. Its expression gradually declined at 500 and 1000 µM Cu treatments (Fig. 3B). The highest concentration of Cu (1000 µM) reduced the HMA3 expression level in both plant species compared to the other treatments.

Cu homeostasis in plant cells is controlled by several metal transporters, including P_{1B}-type ATPases (Lee *et al.* 2007; Takahashi *et al.* 2012; Memon *et al.* 2021; Merakli *et al.* 2022). Plant metal transporters are essential for metal homeostasis. Cu acquisition, intracellular translocation and compartmentalisation mechanisms in crop plants, including *B. nigra* and *B. juncea,* are not well defined. The gene expression analysis in certain model plants, such as *Arabidopsis thaliana* (L.) Heynh *and Noccocea caerulescens* (J. Presl & C. Presl), reveal the critical role of HMA proteins in carrying several transition metals such as Cu, Zn, and Fe (Lwalaba *et al.* 2020).

The biological function of these proteins (HMA1 and HMA3) involved in Cu transport remains to be identified in crop plants (Seigneurin-Berny *et al.* 2006; Burkhead *et al.* 2009). Boutigny *et al.* (2014) reported that HMA1 and PAA1/HMA6 play a crucial role in Cu import in the chloroplast of *A. thaliana*. The results suggested that HMA1 and PAA1 are part of the distinct pathway for copper import and chloroplast targeting. In our previous study, the *B. nigra* Diyarbakir ecotype showed a several hundredfold increase in the expression level of PAA1 when subjected to 500 µM Cu (Memon & Zahirovic 2014; Cevher-Keskin *et al.* 2019). HMA1 is reported to be the main metal ion pump which transports Cu in the roots and translocates it into the shoots

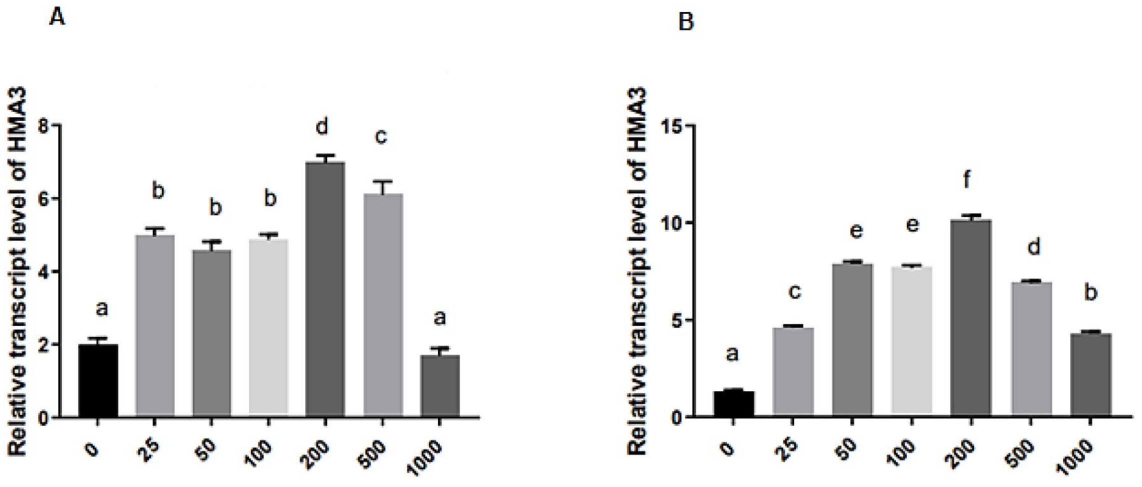

Fig. 3 Expression profile of the HMA3 gene in *B. nigra* (A) and *B. juncea* (B) plants exposed to Cu^{2+} stress from qRT-PCR analysis. Three-week-old plants were treated for two weeks with varying concentrations of Cu^{2+} (details of the experimental procedure are given in Fig. 2).

in *A. thaliana.* Its overexpression significantly enhances the metal tolerance of plants (Shikanai *et al.* 2003; del Pozo *et al.* 2010, Ma *et al.* 2021; Tang *et al.* 2023). Metal tolerance employing HMA1 has also been shown in other organisms (Seigneurin-Berny *et al.* 2006; del Pozo *et al.* 2010, Tang *et al.* 2023). del Pozo *et al.* (2010) demonstrated that the expression of HMA1 confers Cu tolerance to *Saccharomyces cerevisiae* and restores the Cu tolerance of various yeast mutants. Furthermore, the Cu-induced expression of HMA1 in *Populus* (*Populus trichocarpa* Torr. & A.Gray ex Hook.), *M. truncatula* Gaetrn., and other legume species improve Cu tolerance in the leaves and roots of these plants (Li *et al.* 2015; Ma *et al.* 2021).

The expression of HMA3, a tonoplast membrane transporter involved in Zn, Cd, and Co transport in the vacuolar compartments of the plant cells, has also been investigated (Morel *et al.* 2009; Song *et al.* 2014). HMA3 plays a key role in the molecular mechanism of plant stress response to heavy metals at the transcriptional level in plants (see Fig. 3A & B) (Gravot *et al.* 2004; Miyadate *et al.* 2011; Liu *et al.* 2017; Ma *et al.* 2021; Tang *et al.* 2023). However, its function in regulating Cu homeostasis remain unclear. In addition to its role in Zn and Cd homeostasis, our results showed that it also plays an essential role in the transport of Cu in *B. nigra* and *B. juncea* (see Fig. 3A & B).

This study investigated gene expression responses in Cu hyperaccumulator plant *B. nigra* Diyarbakir and metal tolerant plant *B. juncea* grown under low, moderate, and high levels of Cu. The expression profiles of HMA1 and HMA3 were examined using qRT-PCR to assess whether the Cu contents in the leaves could produce a transcriptional response in *B. nigra* Diyarbakir and *B. juncea* Tomcat. Our data revealed a significant increase in HMA1 and HMA3 expression in the leaves of these plants grown under moderately high concentrations.

The results suggest that Cu triggers the expression levels of both HMA1 and HMA3 whereby they rise linearly with the increase in Cu and then slowly decrease at higher concentrations, indicating a significant correlation between these transporters and Cu transport (see Figs. 2 & 3). The expression level of HMA3 is severely inhibited in both species at particularly high Cu levels (at 1000 µM), indicating a reduction in vacuolar transport. Additional transporters could be involved in Cu homeostasis in the cell. For example, NRAMPs and ZIPs contributed to heavy metal transport and accumulation and increased the growth and development ability of the plant to survive in soils with toxic levels of contamination (Memon 2016; Cevher-Keskin *et al.* 2019; Merakli *et al.* 2022). Similar results have been observed with other plant species (Seigneurin-Berny *et al.* 2006; Lee *et al.* 2007; Mikkelsen *et al.* 2012; Zhang *et al.* 2021), suggesting the importance of these transporters in metal hypertolerance and hyperaccumulation in accumulator and tolerant plant species. However, the accumulation mechanism of various heavy metals is still poorly understood (He *et al.* 2020). Our data indicate that HMA1 and HMA3 possibly transport metals in the shoots and compartmentalise Cu at the subcellular level, especially in chloroplast and vacuolar compartments.

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REZIME

Ekspresija ATPaza teških metala (HMA1 i HMA3) kod *Brassica nigra* i *B. juncea* uzgajanim na različitim nivoima Cu

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Zagađenje metalima je veliki ekološki problem koji utiče na poljoprivredu i zdravlje ljudi. Turska poseduje značajne značajne površine u Dijarbakiru i okolnim područjima (jugoistočna Anadolija) na kojima se rudari Cu. Nekoliko poljoprivrednih biljaka koje se uzgajaju u ovim oblastima se navodnjavaju vodom Tigra, a većina poljoprivrednih površina je kontaminirana Cu. Fitoremedijacija je ekološki prihvatljivo i isplativo sredstvo za smanjenje kontaminacije Cu u ovim zemljištima. *Brassica nigra* i *B. juncea* su dobro poznate metalofite koje u svojim nadzemnim delovima mogu hiperakumulirati metale, uključujući bakar. Svrha ove studije je da se proceni njihov potencijal za fitoremedijaciju Cu iz ovih kontaminiranih područja, kao ekološki prihvatljivog i isplativog sredstva. U ovom istraživanju *B. nigra* i *B. juncea* su uzgajane u zemljištu sa različitim koncentracijama Cu (0 do 1000 μM). Biljke nisu pokazivale simptome toksičnosti i akumulirale su značajnu količinu metala u svojim listovima. U listovima obe vrste, sadržaj Cu se značajno povećao sa povećanjem njegovog nivoa u podlozi. Sadržaj HMA1 u listovima obe biljne vrste postepeno se povećavao sa povećanjem nivoa Cu do 50 mM, a zatim se njegova ekspresija polako smanjivala sa povećanjem nivoa Cu. Ekspresija HMA3 se takođe povećavala sa povećanjem Cu u listovima obe biljne vrste, s tim da se njen obrazac ekspresije razlikovao od HMA1. Naši podaci su pokazali da povećanje nivoa Cu u listovima pokreće ekspresiju oba gena, što sugeriše da oni igraju aktivnu ulogu u detoksikaciji Cu. Dobijeni podaci ukazuju na to da se ove biljne vrste mogu koristiti za dekontaminaciju Cu sa zagađenog zemljišta, kao i da je akumulacija i tolerancija Cu u obe biljne vrste verovatno multi-genetski odgovor, koji verovatno uključuje nekoliko drugih gena transportera u putu signala stresa. S tim u vezi, istražuje se i ekspresiju drugih metalnih transportera, kao što su drugi HMA (HMAs 5-8), Nramps (npr. Nramp3), COPT proteini i neki Cu šaperoni u ovim biljnim vrstama.

Ključne reči: toksičnost metala, ekspresija gena, transporteri metala, fitoremedijacija