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Research Article

IDENTIFICATION OF GENES REGULATED IN RESPONSE TO Cu EXPOSURE IN *Brassica nigra* L.

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Abstract: Copper (Cu) is one of the essential trace metals required for plant growth. High amount of Cu in the media inhibits plant growth and is toxic to the plants. *Brassica nigra* L., a Cu accumulator, can tolerate a high amount of Cu and have specific mechanisms to relocate Cu within the cell compartments and keep the toxic amount of Cu away from the cytoplasm. This study aimed to evaluate the Cu-induced gene expression pattern of *B. nigra* Diyarbakir ecotype subjected to low Cu treatment. The Arabidopsis ATH1 genome array was used to determine the Cu-induced gene expression in the leaves of *B. nigra* grown at 25 µM Cu. Ninety-five genes were upregulated, and seventy-two genes were downregulated in the leaves of plants grown under 25 µM Cu. Cu responsive genes, such as glutathione S-transferase, glutathione reductase, heavy metal transporters, natural resistance-associated macrophage proteins, cytochrome p450, MYB-like transcription factor, copper/zinc, and Fe superoxide dismutases, and some protein kinases were highly expressed in the leaves of Cu-treated plants. The present work provides the global gene expression pattern in facultative metallophyte *B. nigra*, which could serve as a molecular tool for future phytoremediation studies.

Özet: Bakır (Cu), bitki büyümesi için gerekli olan temel eser metallerden biridir. Ancak, ortamdaki yüksek miktarda Cu, bitkide toksik etki göstererek büyümeyi olumsuz yönde etkilemektedir. Diğer bir yandan, Cu akümülatörü olan *Brassica nigra* L., sahip olduğu özel mekanizmalar ile yüksek Cu miktarlarını akümüle edebilmekte ve bu metali farklı hücre bölümlerine taşıyarak sitoplazmadan uzaklaştırabilmektedir. Bu çalışmada, *B. nigra'*nın düşük Cu seviyesindeki gen anlatım profilinin tespiti amaçlanmıştır. Düşük Cu seviyesinde yetiştirilen *B. nigra*'nın yapraktaki Cu ile indüklenen gen anlatım profili Arabidopsis ATH1 genom çipi kullanılarak tespit edilmiştir. Elde edilen sonuçlara göre, *B. nigra'*da 95 gen yukarı regüle ve 72 gen aşağı regüle olarak tanımlanmıştır. Glutatyon S-transferaz, glutatyon redüktaz, ağır metal taşıyıcılar, doğal dirençle ilişkili makrofaj proteinleri, sitokrom p450, MYB-gibi transkripsiyon faktörü, bakır/çinko ve Fe süperoksit dismutazlar gibi Cu ile ilişkili bazı genlerin ve bazı protein kinazların yüksek oranda anlatımının olduğu saptanmıştır. Bu çalışma, fakültatif metalofit olarak tespit edilen *B. nigra*' nın global gen anlatım profilini sunmakta olup, moleküler bir araç olarak ileriki fitoremediasyon çalışmaları için yardımcı olacaktır.

Introduction

Copper (Cu) is an essential element for plant growth and has important physiological and biochemical roles in metabolic processes, such as photosynthesis and respiration, cell wall metabolism, and hormone signaling (Marschner 1995, Nazir *et al.* 2019, Zhang *et al.* 2019). It also acts as an essential cofactor for several enzymes, such as copper/zinc superoxide dismutase, cytochrome c oxidase, amine oxidase, laccase, plastocyanin, and polyphenol oxidase, which are involved in the cellular biogenesis of plants (Yruela 2005, Burkhead *et al.* 2009, Puig 2014). Moreover, it affects many processes at a cellular level, including the transcription of genes in the signaling pathway, regulation of protein trafficking, mobilization of some essential ions such as iron and other metals required in oxidative phosphorylation (Meharg 1994, Prasad & Strzalka 1999, Pilon *et al.* 2006). However, Cu released into the environment from many agricultural pollutants, such as pesticides, fungicides, chemical fertilizer agents, sewage, and sludge from wastewater treatments are harmful for plant growth and

development (Maksymiec 1997, Ducic & Polle 2005, Yruela 2009, Guo *et al.* 2016).

The high Cu content in soil and water is highly toxic for plant growth and yield (Fernandes & Henriques 1991, Chiou & Hsu 2019). When Cu is present in excess, it can adversely affect many metabolic processes in plants, such as photosynthesis and respiration, nutrient distribution, synthesis of secondary metabolites, water usage and translational and transcriptional regulation (Kopsell & Kopsell 2007, Shahbaz *et al.* 2013). High Cu concentrations can also cause toxic symptoms, such as chlorosis, necrosis, decreased plant growth, and developments like root and shoot elongation (Marschner 1995, Murphy & Taiz 1995, Yruela 2009). The inhibition of primary metabolic processes in plants is closely related to the overproduction of reactive oxygen species (ROS) and oxidative stress triggered by Cu stress (Schutzendubel & Polle 2002, Küpper *et al.* 2003). For stress avoidance, the primary plant defense system (genes, antioxidants, and transporter genes) becomes active to scavenge ROS overproduction. For example, plants under metal stress significantly increase the production of ROS scavenger enzymes like glutathione S-transferase, glutathione reductase, catalase, superoxide dismutase, ascorbate peroxidase, and other secondary metabolites like phenolic and flavonoid compounds (Noctor & Foyer 1998, Asada 1999, Brahim & Mohamed 2011). The plants survive through a wide range of mechanisms, including the expression of Cu transporters, Cu chaperones, and Cu oxidases, and different transcription factors under Cu stress (Yruela 2009).

Cu accumulator plants have developed the ability to grow in contaminated soils with high Cu levels where non-accumulators cannot survive. These plants develop several detoxification mechanisms when they encounter high metal stress (Memon & Schröder 2009, Printz *et al*. 2016). However, much of the cellular Cu acquisition and intracellular Cu distribution mechanisms in plant cells have not been fully understood. In our previous studies, the Diyarbakir ecotype of *Brassica nigra* L. collected from the Southeastern part of Turkey (Diyarbakir district) was identified as a Cu hyperaccumulator (Memon & Zahirovic 2014, Cevher-Keskin *et al.* 2019). This accumulator Diyarbakir ecotype grown at 500 µM Cu showed several hundredfold gene expression related to glutathione pathway, metal ATPases, and ABC transporters compared to control (Memon & Zahirovic 2014).

Cevher-Keskin *et al.* (2019) also analyzed the Cu-induced gene expression pattern in leaves of non-accumulator *B. nigra* ecotype CGN06619 using Affymetrix Arabidopsis microarray. This non-accumulator ecotype showed toxic symptoms in its leaves (wilting of the leaves), even grown at a Cu concentration of 25 µM. Furthermore, when these non-accumulator plants were subjected to 25 µM Cu level, their shoot growth was retarded, and the expression level of several metal-related genes such as metallothionein 2B, metallothionein 3, metallothionein like protein 1C, Fe-superoxide dismutase 1, Cu/Zn superoxide dismutase 1, glutamine synthetase, and phytochelatin synthase was downregulated.

This study analyzed the gene expression pattern in the leaves of the accumulator *B. nigra* ecotype grown at 0 and 25 µM Cu levels. The gene expression pattern was compared to that of the non-accumulator ecotype reported in the Cevher-Keskin *et al.* (2019)'s. The results presented here show an overall view of gene expression in the leaves of the accumulator and non-accumulator ecotypes subjected to low and high Cu levels (Memon & Zahirovic 2014). For example, some critical metal stress-induced genes like glutathione S-transferase, glutathione reductase, cytochrome p450, ABA receptor 1, MYB-like transcription factor ETC1, Ras-related small GTP-binding proteins, and some protein kinases were differentially expressed in these plants subjected to low and high Cu levels (Memon & Zahirovic 2014, see result section). In addition, the accumulation pattern of glutathione, flavonoid, and phenolic compounds in the leaves varied considerably depending on the Cu level in the media.

Materials and Methods

Plant growth and Cu treatment

Brassica nigra (L.) W.D.J. Koch seeds were collected from Southeastern Region of Turkey (Diyarbakir Center ecotype) (38°18'04"N, 39°43'39"E). Uniformly selected seeds were surface sterilized with commercial bleach (with 5% sodium hypochlorite containing Tween20) 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). Pot experiments were conducted in a greenhouse having 25 ± 4°C day/night, 70 ± 5 relative humidity, and a 16 h light/8 h dark cycle photoperiod. After three weeks, healthy seedlings of the same height/physiological age were selected and were transferred to pots (one plant per pot) filled with a sterilized mixture of soil and peat (2:1 ratio). The plants were supplemented with $1/4^{th}$ Hoagland solution for three weeks (Hoagland & Arnon 1938). After three weeks, they were subjected to 0 µM and 25 µM $CuSO_4$ treatments and were harvested two weeks after the Cu exposure. Plants were separated into leaves and roots. After measuring their fresh weights, the samples were divided into two groups; one group was dried at 72°C in the oven for 48 h, and the other group was frozen at -80°C for RNA isolation. The experiments were replicated three times.

Metal analysis

After dried in the oven at 80°C, soil samples were extracted with 0.005 M DTPA + 0.01 M $CaCl_2$ + 0.1 M TEA (pH = 7.3) as reported by Norvell & Lindsay (1969). Soil solutions were shaken for one hour and filtered through Whatman No:42 filter paper. The Cu contents of the samples were determined by Atomic Absorption Spectrophotometry (PerkinElmer, AAnalyst 200). The harvested plant samples were dried in a 72°C oven for 72

h, and after drying, leaves were ground into a powder with mortar and pestle. After grinding, samples (0.5 g) were digested in 7 ml of an acid mixture containing $HNO_3/HClO_4$ (5:2) at 130°C on a hot plate, filtered through Whatman No:42 filter paper and then diluted with 10 ml deionized water (Memon *et al.* 1979). The Cu contents of the samples were analyzed by AAS, as previously reported by Memon *et al.* (1979).

RNA isolation and cDNA synthesis

The frozen plant leaves (0.2 g) were ground into powder in liquid nitrogen with mortar and pestle. Total RNA was isolated from the leaves of control and Cu-treated plants according to the manufacturer's protocol using the RNeasy Plant kit (ThermoFisher Scientific, GeneJet Plant RNA Purification Mini Kit, #K0801). RNA samples were treated with RNase-Free DNase I to eliminate genomic DNA. The extracted RNA concentration was quantified using NanoDrop Spectrophotometer (Thermo Scientific 8000), and its integrity was examined on formaldehyde agarose gel electrophoresis. The purity was verified using optical density (OD), the 260/280 nm absorbance ratio between 1.9 and 2.1. The cDNA was synthesized as previously described (Cevher-Keskin *et al.* 2019). Briefly, 100 ng of total RNA was used for cDNA synthesis using T7-oligo (dT) primer; the second strand was synthesized using DNA polymerase and RNaseH (Thermo Scientific Maxima cDNA synthesis kit for qRT-PCR, #K1641).

Microarray analysis

Affymetrix GeneChip Arabidopsis Genome Array (ATH1-121501 GeneChip, ATH1) and GeneChip 3'IVT Express Kit were used for the transcriptional profiling in response to 25 µM Cu grown plants. RNAs isolated from leaf tissues were hybridized with ATH1 chips. The hybridization process of the signals was scanned using the GeneChip Command Console software. The microarray analysis was carried out by Ay-Ka Co. Ltd. (www.ayka.ltd.com, Ankara, Turkey). The microarray data normalization was analyzed using GeneChip suite 4.0 (Affymetrix, USA). The GENESPRING 5.0 program was used to determine the fold changes of genes identified by microarray analysis. After the normalization of data, categorization of differentially expressed genes was carried out using ANOVA (95% confidence level $p<0.05$).

Glutathione (GSH) analysis

Glutathione was determined by the method of Newton & Fahey (1995). Briefly, the ground samples (0.5 g) were homogenized with 1 ml of 0.1 M (37%) hydrochloric acid (HCl) at 4°C and were centrifuged at 22.000 rpm for 10 minutes. The supernatant (120 µl) was mixed with 180 µl -(N-cyclohexyl amino) ethane sulphonic acid (0.2 M, pH 9.3) and 30 µl dithiothreitol (5 mM) and then stored for one h. Glutathione was determined with the RP18 column by HPLC (Agilent-1260). 99% potassium dihydrogen phosphate (H_2KO_4P), 85% phosphoric acid (H_3O_4P), and dH_2O (10:90 V/W) were used as mobile phase.

Data evaluation and statistical analysis

The results were expressed as mean values ± standard deviations of three replications. The statistical analysis was carried out by using IBM SPSS 25.0 for Windows statistical software package. The data were analyzed by Oneway variance (ANOVA) analysis, and the Duncan test was used to determine the statistical significance at 0.05 between treatments.

Results

Cu accumulation capacity of B. nigra Diyarbakir ecotype

Three-week-old plants were either supplied with 0 or 25 µM Cu for two weeks and then harvested for metal analysis and RNA isolation. During the two weeks of Cu treatment, plants were healthy without any deficiency symptoms (Fig. 1).

The iron (Fe), zinc (Zn) and Cu contents as micronutrients, in the soil used for the experiments were 5.03 mg/kg^{-1} DW, 0.60 mg/kg^{-1} DW and 2.00 mg/kg^{-1} DW, respectively. The Cu content in the leaf samples of *B. nigra* Diyarbakir grown at control and 25 µM Cu was 9.46 mg/kg^{-1} DW and 83.48 mg/kg^{-1} DW, respectively. The plant leaf growth was increased about nine times in 25 µM Cu compared to the control. It has been previously reported that these plants grew healthy both at normal and high Cu-treated soils and accumulated Cu with about 20,000 mg/kg^{-1} DW in their shoots without showing any toxicity effect when subjected to 500 µM Cu (Memon & Zahirovic 2014). Therefore, we classified this hyperaccumulator plant as a facultative metallophyte (Memon 2020).

Microarray analysis and gene expression pattern in leaf tissues of B. nigra Diyarbakir ecotype

We carried out a detailed comparative transcriptome analysis to determine the expression of Cu-induced genes in the leaves of *B. nigra* Diyarbakir grown at 0 and 25 µM Cu. Gene expression analysis was carried out using the Affymetrix GeneChip Arabidopsis Genome Array (ATH1-121501 GeneChip) chip (Cevher-Keskin *et al.* 2019). The statistical relationship of differentially expressed (upregulated and downregulated) genes in the control and 25 µM Cu-treated plants was analyzed by Pearson's correlation analysis (Fig. 2).

The microarray results demonstrated that low Cu concentration highly influenced the gene expression pattern in *B. nigra* Diyarbakir ecotype (Table 1). Genomic array analysis showed that 95 genes were upregulated and 72 genes were downregulated in the leaf tissues of *B. nigra* Diyarbakir ecotype grown at 25 µM Cu (Fig. 3).

Some specific genes were upregulated several-fold among the expressed genes compared to the control plants (Table 1). Table 1 shows the list of the genes that were significantly ($p<0.05$) upregulated in *B. nigra* Diyarbakir after two weeks of Cu exposure. These upregulated genes were classified into seven groups according to the

B. nigra Diyarbakır 0µM Cu Control R1	*B. nigra* Diyarbakır 25µM Cu R1	*B. nigra* Diyarbakır 50µM Cu R1	*B. nigra* Diyarbakır 100µM Cu R1	*B. nigra* Diyarbakır 200µM Cu R1	*B. nigra* Diyarbakır 500µM Cu R1	*B. nigra* Diyarbakır 1000µM Cu R1

Fig. 1. *B. nigra* Diyarbakir ecotype was grown at different Cu concentrations.

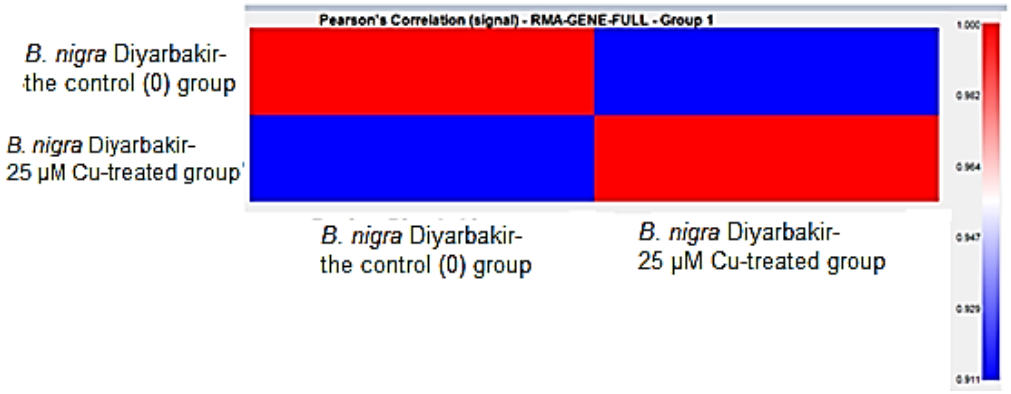

Fig. 2. Pearson's Correlation analysis was carried out after the normalization of the signals from leaf tissues of 0 and 25 µM Cu-treated *B. nigra* Diyarbakir ecotype.

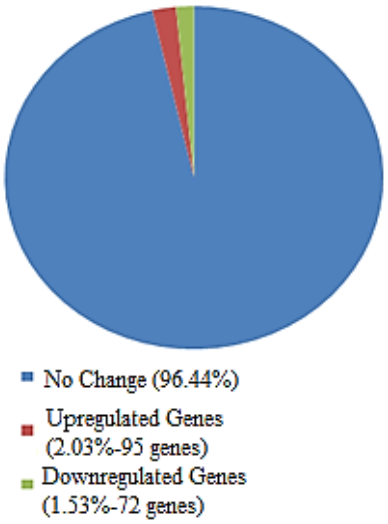

Fig. 3. Percentage (%) distribution of upregulated and downregulated genes in the leaf tissues of *B. nigra* Diyarbakir ecotype grown at 25 µM Cu.

biological processes and molecular function of the genes at cellular level (Table 1). These groups contain Cu-induced genes involved in biological processes such as metal transport, metal homeostasis, biosynthesis, signal transduction, and metabolism.

As shown in Table 1, many genes related to metal transport were highly expressed in the Cu-treated plants. Among them, Cu transporting ATPases [AT5G44790-HMA7 (RAN1); AT1G63440-HMA5; AT5G21930-HMA8 (PAA2)]; natural resistance-associated macrophage protein (NRAMPs) [(AT1G80830-Metal ion transport-NRAMP1; AT1G47240-Cellular metal ion homeostasis-NRAMP2; AT5G67330-Metal ion transport-NRAMP4)], Cu transporter proteins (COPTs) [(AT5G59030-COPT1; AT5G20650-COPT5)], zinc transporters (ZIPs) (AT3G12750- ZIP1; AT2G04032-ZIP7) were upregulated in Cu-treated plants compared to the control.

Some chaperone genes such as metal-nicotianamine transporters (YSLs) (AT4G24120-YSL1; AT5G53550-YSL3; AT1G65730-YSL7), Cu chaperone (AT3G56240-CCH), Cu transport protein (AT1G66240-ATX1), $Fe^{(2+)}$ transport proteins (IRTs) (AT4G19690-IRT1; AT4G19680-IRT2; AT1G60960-IRT3), metallothionein 2B (AT5G02380-MT2B), phytochelatin synthase 2 (AT1G03980-PCS2) were also highly expressed in the leaves of Cu treated plants ($p<0.05$). Furthermore, the expression levels of three cytochrome c oxidase (COXs) genes, COX1 (ATMG01360), COX2 (ATMG00160), and COX17 (AT3G15352), increased with about 1.38, 1.72 and 1.10 fold, respectively, in the leaves of Cu-treated plants.

Notably, peroxidases, catalases, glutathione-related genes and superoxide dismutases that play a key role in response to metal-related oxidative stress were also upregulated following Cu stress. For instance, several

genes, such as L-ascorbate peroxidase 1 (AT1G07890-APX1), glutathione peroxidase (AT2G31570-GPx), glutathione reductase (AT5G45020-GR), putative glutathione S-transferase (AT1G65820-GSH), catalase 2 (AT4G35090-CAT2), catalase 3 (AT1G20620-CAT3), superoxide dismutase [Fe] (AT4G25100-FSD1), iron/manganese superoxide dismutase family protein (AT3G56350), copper/zinc superoxide dismutase 3 (AT5G18100-CSD3) related to antioxidant activities were upregulated in response to 25 µM Cu. In addition, several other genes, such as alanine: glyoxylate aminotransferase 2 (AT4G39660-AGT2), omega-3 fatty acid desaturase (AT2G29980-FAD3), patatin-related phospholipase A (AT4G37070-PLP1), bifunctional sn-glycerol-3-phosphate 2-O-acyltransferase/phosphatase (AT4G00400-GPAT8), putative galactinol-sucrose galactosyltransferase (AT3G57520-SIP2), proline-rich protein 3 (AT3G62680-PRP3) related to metabolism were also upregulated in the leaves of 25 µM Cu grown plants. The upregulated genes listed in Table 1 have essential roles in growth and development processes such as metabolism, transcription, signal transduction, metal homeostasis, oxidative stress and antioxidant activity (Yang *et al.* 2005, Shahid *et al.* 2015, Cevher-Keskin *et al.* 2019).

Table 1. Cu-induced changes in gene expression in the leaves of *B. nigra* Diyarbakir ecotype grown at 25 µM Cu compared to control (0 µM Cu) conditions (plants were grown in low Cu soil supplied with Hoagland solution without Cu). The expression pattern was obtained with three biological replicates by using the Affymetrix GeneChip Arabidopsis Genome Array (ATH1-121501 GeneChip) (see Material and Methods for details).

Gene (Upregulated genes)	Control 0 µM Cu	25 µM Cu Exposure	Fold change compared to control	Accession # (nucleotide blast)
Metal transporters				
Copper-transporting ATPase RAN1 (HMA7)	0.79	1.05	1.33	AT5G44790
Putative copper-transporting ATPase HMA5	1.37	1.52	1.11	AT1G63440
P-type ATPase HMA8	0.78	0.87	1.12	AT5G21930
Zinc transporter 1 (ZIP1)	0.68	0.68	1.00	AT3G12750
Zinc transporter 2 (ZIP2)	0.86	0.86	1.00	AT5G59520
Zinc transporter 3 (ZIP3)	0.37	0.37	1.00	AT2G32270
Zinc transporter 4 precursor	0.65	0.72	1.10	AT1G10970
Zinc transporter 5 (ZIP5)	0.72	0.84	1.16	AT1G05300
Zinc transporter 7 (ZIP7)	1.09	1.34	1.23	AT2G04032
Fe(II) transporter isolog family protein (ZIP9)	1.24	1.24	1.00	AT4G33020
Putative zinc transporter ZIP2 -like protein (ZIP11)	0.86	0.94	1.09	AT1G55910
Fe(2+) transport protein 1 (IRT1)	0.54	0.54	1.00	AT4G19690
Fe(2+) transport protein 2 (IRT2)	0.72	0.76	1.06	AT4G19680
Fe(2+) transport protein 3 (IRT3)	0.58	0.58	1.00	AT1G60960
Copper transporter 1 (COPT1)	0.98	1.34	1.37	AT5G59030
Copper transporter 3 (COPT3)	0.44	0.48	1.09	AT5G59040
Copper transporter 4 (COPT4)	1.15	1.41	1.23	AT2G37925
Copper transporter 5 (COPT5)	0.67	0.70	1.04	AT5G20650
Copper transporter 6 (COPT6)	0.54	0.54	1.00	AT2G26975
Metal transporter NRAMP1	1.19	1.80	1.51	AT1G80830
Metal transporter NRAMP2	0.77	1.01	1.31	AT1G47240
Metal transporter NRAMP3	0.44	0.44	1.00	AT2G23150
Metal transporter NRAMP4	0.74	0.96	1.30	AT5G67330
Metal transporter NRAMP5	0.63	0.63	1.00	AT4G18790
Metal transporter NRAMP6	0.99	1.05	1.06	AT1G15960
Putative copper transport protein	0.63	0.73	1.16	AT5G52670
Copper binding protein CutA	1.08	1.21	1.12	AT2G33740
ABC transporter family protein	0.60	0.60	1.00	AT1G54350
Putative phospholipid-transporting ATPase 5	0.94	1.25	1.33	AT1G72700
Metal-related chaperones				
Cytochrome c oxidase 1 (COX1)	10.92	15.07	1.38	ATMG01360
Cytochrome c oxidase 2 (COX2)	8.12	13.97	1.72	ATMG00160
Cytochrome c oxidase 17 (COX17)	0.86	0.95	1.10	AT3G15352
Metal-nicotianamine transporter (YSL1)	1.14	1.78	1.55	AT4G24120
Metal-nicotianamine transporter (YSL3)	0.71	0.77	1.09	AT5G53550
Protein Ferric Reductase Defectıve 3 (FRD3)	0.98	0.98	1.00	AT3G08040
Copper chaperone (CCH)	0.60	0.60	1.00	AT3G56240
Copper transport protein ATX1	0.90	0.95	1.05	AT1G66240
Farnesylated protein 6 (FP6)	0.77	0.85	1.10	AT4G38580
Cytochrome c biogenesis protein CCS1	0.71	0.81	1.14	AT1G49380
Phytochelatin synthase 2 (PCS2)	0.73	0.80	1.10	AT1G03980
Metallothionein 2B (MT2B)	0.63	0.74	1.17	AT5G02380

Table1. Continued.

Gene (Upregulated genes)	Control 0 µM Cu	25 µM Cu Exposure	Fold change compared to control	Accession # (nucleotide blast)
Metallothionein 1C (MT1C)	0.66	0.66	1.00	AT1G07610
Ferric reduction oxidase 1 (FRO1)	0.50	0.50	1.00	AT1G01590
Ferric reduction oxidase 4 (FRO4)	0.56	0.68	1.21	AT5G23980
Ferric reduction oxidase 6 (FRO6)	0.71	0.80	1.12	AT5G49730
Ferric reduction oxidase 7 (FRO7)	0.94	0.81	1.10	AT5G49740
Ferric reduction oxidase 8 (FRO8)	0.83	1.03	1.26	AT5G50160
Oxidative stress-related antioxidants				
Glutathione S-transferase TAU 8	0.50	0.50	1.00	AT3G09270
Putative glutathione S-transferase	0.79	1.04	1.31	AT5G45020
L-ascorbate peroxidase 1 (APX1)	0.87	1.16	1.33	AT1G07890
L-ascorbate peroxidase 2 (APX2)	0.78	0.96	1.23	AT3G09640
L-ascorbate peroxidase S (SAPX)	0.80	0.88	1.10	AT4G08390
Peroxidase 71	0.84	1.08	1.28	AT5G64120
Peroxidase 37	0.90	1.35	1.50	AT4G08770
Thioredoxin superfamily protein	0.89	1.23	1.38	AT1G20225
Thioredoxin-like 1-3	0.69	0.83	1.20	AT2G33270
Glutathione peroxidase GPx (GPX2)	1.03	1.15	1.12	AT2G31570
Alcohol dehydrogenase (ADH2)	0.82	1.01	1.23	AT1G09490
ATP sulfurylase 1 (APS1)	1.12	1.28	1.14	AT3G22890
Cysteine synthase A (OASA1)	0.54	0.54	1.00	AT4G14880
Peptide methionine sulfoxide reductase A1 (PMSR1)	0.66	0.66	1.00	AT5G61640
Peptide methionine sulfoxide reductase A4 (PMSR4)	0.85	1.04	1.22	AT4G25130
Glutathione S-transferase THETA 2 (GSTT2)	0.63	0.69	1.10	AT5G41240
Glutathione S-transferase THETA 3 (GSTT3)	0.77	1.02	1.31	AT5G41220
1-Cys peroxiredoxin PER1	1.45	2.07	1.43	AT1G48130
2-Cys peroxiredoxin BAS1	0.86	1.13	1.31	AT3G11630
2-cysteine peroxiredoxin B	0.75	0.92	1.23	AT5G06290
Glutathione reductase (GR)	0.86	0.86	1.00	AT3G54660
Glutamine synthetase 1;4 (GLN1;4)	1.67	2.15	1.29	AT5G16570
Glutamine synthetase 2 (GS2)	0.73	0.73	1.00	AT5G35630
Peroxidase family protein	1.02	1.34	1.31	AT3G32980
Catalase 1 (CAT1)	0.87	0.97	1.12	AT1G20630
Catalase 2 (CAT2)	1.01	1.36	1.35	AT4G35090
Catalase 3 (CAT3)	1.25	1.56	1.25	AT1G20620
Iron/manganese superoxide dismutase family protein	1.34	1.55	1.16	AT3G56350
Superoxide dismutase [Cu-Zn] (CSD1)	0.91	0.91	1.00	AT1G08830
Copper/zinc superoxide dismutase 2 (CSD2)	0.82	0.90	1.10	AT2G28190
Copper/zinc superoxide dismutase 3 (CSD3)	1.18	1.45	1.23	AT5G18100
NADH-ubiquinone/plastoquinone (complex I) protein	5.14	12.80	2.49	AT2G07689
Plastocyanin-like domain-containing protein	1.19	1.84	1.55	AT3G28958
Superoxide dismutase [Fe] (FSD1)	0.91	1.16	1.28	AT4G25100
Superoxide dismutase [Fe] (FSD2)	1.11	1.30	1.17	AT5G51100
Superoxide dismutase [Fe] (FSD3)	1.12	1.28	1.14	AT5G23310
Proline-rich protein 3	0.57	0.60	1.05	AT3G62680
Signal transduction-related genes				
Mitogen-activated protein kinase kinase kinase ANP1	0.81	0.81	1.00	AT1G09000
MAP kinase kinase 7	0.54	0.54	1.00	AT1G18350
CDPK-related kinase	0.81	0.65	1.12	AT3G50530
GSK3/Shaggy-like protein kinase 1	0.96	0.91	1.10	AT1G06390
Receptor-like kinase (RLK)	1.55	1.74	1.12	AT1G48480
Wall-associated receptor kinase-like 2 (WALK)	1	1.22	1.22	AT1G16130
Wall-associated receptor kinase 1 (WAK)	0.52	0.61	1.17	AT1G21250
Ethylene receptor 2 (ETR2)	0.92	1.01	1.09	AT3G23150
Ethylene response sensor 1 (ERS1)	1.21	1.34	1.11	AT2G40940
Histidine kinase 3 (HK3)	0.96	1.23	1.28	AT1G27320
SNF1-Related protein kinase 2.5	0.76	0.95	1.25	AT5G63650
Metabolism-related proteins				
Bifunctional sn-glycerol-3-phosphate 2-O-acyltransferase/phosphatase (GPAT8)	0.50	0.55	1.11	AT4G00400
Putative galactinol--sucrose galactosyltransferase 2	1.10	1.46	1.33	AT3G57520

Table1. Continued.

Gene (Upregulated genes)	Control 0 µM Cu	25 µM Cu Exposure	Fold change compared to control	Accession # (nucleotide blast)
Proline-rich protein 3	0.57	0.60	1.05	AT3G62680
Alanine:glyoxylate aminotransferase 2 (AGT2)	0.87	1.08	1.24	AT4G39660
Omega-3 fatty acid desaturase (FAD3)	0.78	0.98	1.25	AT2G29980
Patatin-related phospholipase A (PLP1)	0.64	0.81	1.26	AT4G37070
Phenolic glucoside malonyltransferase 1	1.27	1.96	1.54	AT5G39050
Flavonoid 3-monooxygenase	1.04	1.04	1.00	AT5G07990
Abiotic stress-related transcription factors				
Dehydration-responsive element-binding protein 1A (DREB1A)	0.42	0.42	1.00	AT4G25480
Dehydration-responsive element-binding protein 1B (DREB1B)	0.79	1.05	1.33	AT4G25490
Ethylene-responsive transcription factor 1B (ERF1)	0.32	0.32	1.00	AT3G23240
Peptide chain release factor eRF subunit 1 (ERF1-1)	1.30	1.86	1.43	AT5G47880
Ethylene-responsive transcription factor 8 (ERF8)	1.01	1.40	1.39	AT1G53170
Ethylene-responsive transcription factor RAP2-3 (EBP)	0.88	0.88	1.00	AT3G16770
Aquaporin TIP1-1 (GAMMA-TIP)	0.67	0.76	1.14	AT2G36830
Aquaporin TIP2-1 (DELTA-TIP)	0.97	1.43	1.47	AT3G16240
Tonoplast intrinsic protein 2;2 (TIP2;2)	0.68	0.75	1.10	AT4G17340
Aquaporin TIP4-1	0.90	0.90	1.00	AT2G25810
Aquaporin PIP1-1(PIP1A)	0.80	1.06	1.33	AT3G61430
Aquaporin PIP2-1 (PIP2A)	0.39	0.44	1.12	AT3G53420
Putative aquaporin NIP5-1 (NIP5;1)	1.05	1.05	1.00	AT4G10380
Putative aquaporin NIP7-1 (NIP7;1)	0.85	0.97	1.14	AT3G06100
Aquaporin SIP1-1 (SIP1A)	0.66	0.66	1.00	AT3G04090
Transcription factors				
Nitrate-inducible, GARP-type transcriptional repressor 1-like protein (HHO_2)	0.51	0.56	1.10	AT1G68670
GRAS family transcription factor	1.03	1.29	1.25	AT5G66770
HMG-box (high mobility group) DNA-binding family protein	0.65	0.74	1.13	AT5G05330
WRKY family transcription factor	0.80	1.01	1.26	AT3G32090
MYB transcription factor (MYB96)	0.94	1.25	1.33	AT5G62470
bZIP transcription factor	0.82	0.97	1.18	AT1G58110
Basic helix-loop-helix (bHLH) DNA-binding superfamily protein	0.79	0.79	1.00	AT1G49830
Ethylene-responsive transcription factor-like protein (ERF)	1.39	1.53	1.10	AT4G13040
ARID/BRIGHT DNA-binding	0.75	0.92	1.23	AT4G11400
Putative AT-hook DNA-binding family protein	1.19	1.80	1.51	AT4G17800

Cevher-Keskin *et al.* (2019) analyzed the Cu-induced gene expression pattern in the leaves of non-accumulator *B. nigra* ecotype CGN06619 by using Affymetrix Arabidopsis microarray. This non-accumulator ecotype showed toxicity-related symptoms on its leaves (necrosis and wilting of the leaves) when grown at 25 µM Cu. The growth of this non-accumulator ecotype was retarded at 25 µM Cu treatment, and several metal-related genes, for example, metallothionein 2B, metallothionein 3, metallothionein like protein 1C, Fe-superoxide dismutase 1, Cu/Zn superoxide dismutase 1, glutamine synthetase, and phytochelatin synthase were downregulated (Cevher-Keskin *et al.* 2019). On the contrary, these genes were upregulated in the Cu-accumulator Diyarbakir ecotype when subjected to 25 µM Cu (Table 1). Table 2 shows the gene expression levels in both Cu accumulator (Diyarbakir ecotype) and non-accumulator ecotype (CGN06619) of *B. nigra* grown at 25 µM Cu. Several known genes were associated with metal-oxidative stress; in particular, the genes encoding the enzymes involved in ROS-scavenging were differentially expressed in both plants (Table 2).

As shown in Table 2, the expression levels of superoxide dismutase [Fe] (FSD1) and glutathione S-transferase F3 (GSTF3) genes were upregulated in the accumulator ecotype ($p<0.05$), but they were downregulated in non-accumulator (CGN06619) ($p<0.05$). A similar gene expression pattern was also observed for Arabidopsis NAC domain-containing protein; ARALYDRAFT, *Arabidopsis thaliana* phosphate transporter 2 (ATPT2), alanine-glyoxylate aminotransferase (AGT), *Arabidopsis thaliana* cytochrome P450, family 706, subfamily A, polypeptide 2 (CYP706A2), and MAP3K epsilon protein kinase 1 (MAPKKK7) in the leaves of both accumulator and non-accumulator ecotypes at 25 µM Cu. Interestingly, the expressions of early responsive genes to dehydration, chaperone protein ClpD (ERD1), nitrate excretion transporter 1 (AXT1), PRP4 (proline-rich protein 4) were significantly downregulated in both ecotypes at 25 µM Cu (Table 2). A distinct change in the expression levels of MAPKKK7, zinc finger protein 2 (ZFP2), and lipid transfer protein 4 (LTP4) were observed in both ecotypes under Cu stress. However, the expression level was high in the non-accumulator ecotype (CGN06619) compared to the accumulator.

Table 2. Upregulated and downregulated (-) gene expression levels in the Cu accumulator (Diyarbakir ecotype) and non-accumulator ecotype (CGN06619) of *B. nigra* grown at 25 µM Cu (control plants were grown in low Cu soil supplied with Hoagland solution without Cu) (see Material and Methods).

TAIR code	Best blast hit	Fold change compared to control in *B. nigra* Diyarbakir ecotype	Fold change compared to control in *B. nigra* CGN06619 ecotype	Biological Process	Molecular Function	Cellular component	Lenght-Molecular weight
AT4G25100	Superoxide dismutase [Fe](FSD1)	1.28	-3.12	Response to copper ion	Copper ion binding	Chloroplast Thylakoid Mitochondrion	212 aa-23790.7
AT2G02930	Glutathione S-transferase F3 (GSTF3)	1.05	-2.46	Glutathione methabolic process	Glutathione binding	Plasma membrane, chloroplast	212 aa-24120.4
AT1G08830	Copper/zinc superoxide dismutase 1 (CSD1)	1.00	2.07	Copper ion binding superoxide dismutase activity	Response to oxidative stress	Cytoplasm, cytosol	152 aa-15097.6
AT5G51070	Early responsive to dehydration1; chaperone protein ClpD (ERD1)	-1.08	-2.09	Response to water deprivation	Nucleotide binding, ATP binding	Chloroplast stroma, chloroplast	945 aa-103233.7
AT3G45650	Nitrate excretion transporter1 (NAXT1)	-1.08	-2.55	Nitrate assimilation, nitrate transport	Transmembrane transporter activity	Plasma membrane, Nucleus	584 aa-64074.0
AT2G38940	Arabidopsis thaliana phosphate transporter 2 (ATPT2)	1.00	-2.14	Transmembrane transporter	Protein binding	Golgi apparatus	534 aa-58598.7
AT4G22710	Arabidopsis thaliana cytochrome P450, family 706, subfamily A,polypeptide 2 (CYP706A2)	1.04	-1.96	Oxidation reduction	Electron carrier activity, oxygen binding	Plasma membrane, chloroplast	526 aa-59425.3
AT3G13530	MAP3K epsilon protein kinase 1 (MAPKKK7)	1.00	1.93	Protein phosphorylation	Kinase activity, ATP activity	Plasma membrane	1368 aa-151175.3
AT5G57520	Zinc finger protein 2 (ZFP2)	1.14	1.94	Regulation of transcription	Zinc binding ion, nucleic acid binding	Nucleus	150 aa-16955.5
AT5G59310	Lipid transfer protein 4 (LTP4)	-1.19	2.39	Lipid transport	Lipid binding, response to water deprivation	Extracellular region	112 aa-11405.3
AT2G29980	Omega-3 fatty acid desaturase (FAD3)	1.25	-2.25	Unsatured fatty acid biosynthetic process	Omega-3 fatty acid desature activity	Chloroplast	336 aa-44076.4
AT2G13360	Alanine-glyoxylate aminotrans. (AGT)	1.04	-2.32	Photorespiration	Alanine-glyoxylate transaminase activity	Chloroplast, Mitochondrion	401 aa-44207.7
AT4G38770	Proline-rich protein 4; (PRP4)	-1.03	-2.04	Development and abiotic stress tolerance	micRNA biogenesis	Cell wall	448 aa-49136.0
AT5G66300	Arabidopsis NAC domain containing prot.; ARALYDRAFT	1.12	-2.39	Regulation of transcript	DNA-binding transcription factor activity	Nucleus	292 aa-34320.0
AT2G36390	1,4-alpha-glucan-branching enzyme 2-1, Chloroplastic/Amyloplastic;SBE2.1	-1.25	-1.94	Carbohydrate metabolic process	Starch metabolic process	Chloroplast	858 aa-97659.0
AT1G12920	Eukaryotic peptide chain release factor subunit 1-2; ERF 1-2	-1.37	-1.98	Cytoplasmic translational termination	Translation release factor activity	Cytoplasm	434 aa-48940.9
AT4G33630	Protein executer 1, chloroplastic; EX1	-1.15	-2.06	Response to singlet oxygen	Response to singlet oxygen	Chloroplast	684 aa-76534.0
AT4G36410	Probable ubiquitin-conjugating enzyme E217; UBC17	-1.07	2.40	Protein polyubiquitination	Ubiquitin conjugating enzyme activity	Nucleus	161 aa-18674.1

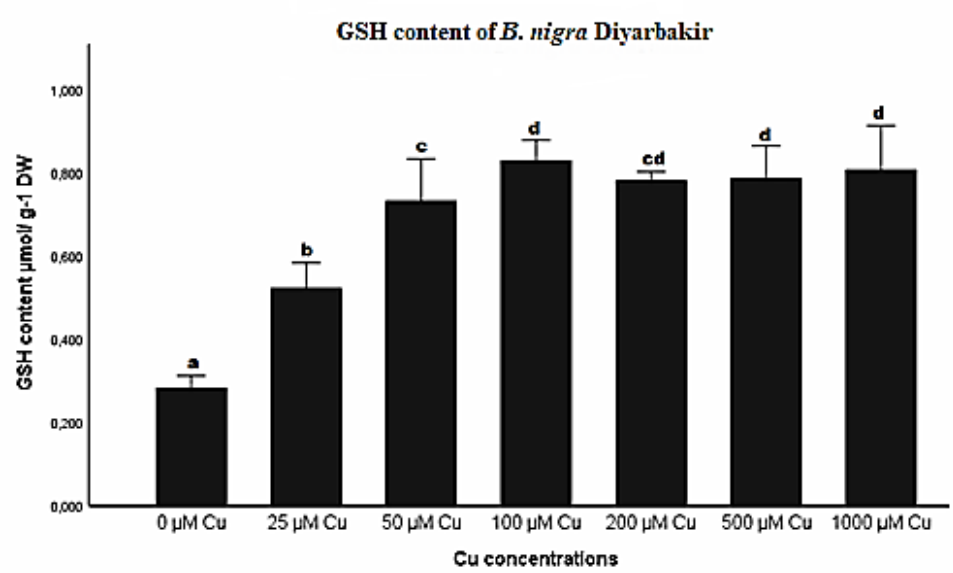

Fig. 4. Reduced Glutathione (GSH) content in the leaves of *B. nigra* Diyarbakir ecoype plants grown at different Cu levels. Bars represent the standard deviation of three replicates. Different letters (a, b, c, d) show significant differences between groups ($p<0.05$) based on the Duncan test.

Changes in Reduced Glutathione (GSH) in B. nigra Diyarbakir ecotype under Cu stress

The expression level of GSH obtained from microarray analysis was verified by analyzing the reduced glutathione content in the leaves of accumulator plants by HPLC. The content of GSH in the leaves of *B. nigra* Diyabakir ecotype grown at 25 µM Cu is shown in Fig. 4. The Cu-treated plants resulted in a significant increase in GSH content compared to control plants ($p<0.05$). GSH was gradually increased with an increase in Cu levels till 100 µM; then plateaued and remained constant at higher Cu levels (Fig. 4).

Discussion

Cu is an essential micronutrient for plant growth and development and is required in a small quantity to activate several enzymes in plant metabolism. For example, it is a cofactor for several enzymes like cytochrome c oxidase, ascorbate oxidase, laccase, polyphenol oxidase, and Cu-Zn superoxide dismutase (Liu *et al.* 2015, Ahanger *et al.* 2016, Printz *et al.* 2016) and plays a crucial role in photosynthesis, especially chlorophyll formation (Mohanty *et al.* 1989, Schröder *et al.* 1994, Patsikka *et al.* 2001, Patsikka *et al.* 2002). Excess Cu in the growth media is toxic to the plant and retard the root growth (Dresler *et al.* 2014, Liu *et al.* 2015) and competes with the uptake of other micronutrients, e.g., Fe, Zn, and Mo (Bosnić *et al.* 2019). High levels of Cu in media reduce the plant biomass and show chlorotic and necrosis symptoms in the leaves (Yruela 2005). The toxic level of Cu in the environment, especially in the soils and water, occurs due to the fertilizers, fungicides high in Cu, metalliferous mining, metal processing, and waste disposal activities (Kabata-Pendias & Pendias 2001, Pilon-Smits & Pilon 2002). High Cu concentration (concentration above optimal growth) is toxic to most plants except the plant species that can hyper accumulate Cu, such as *Brassica nigra*, *B. juncea* L., *Arabidopsis halleri* L. and *Noccaea caerulescens* (J. & C. Presl) F. K. Mey (Mourato *et al.* 2015, Memon 2016).

Memon *et al.* 2008 showed that the *B. nigra* Diyarbakir ecotype, collected from the Southeastern part of Turkey (Diyarbakir district), accumulates several thousandfold Cu in its leaves compared to the non-accumulator ecotype. Microarray data with Diyarbakir ecotype grown at 0 and 500 µM Cu concentrations showed that some of the genes were highly expressed (several hundred/thousandfold) in the leaves when grown at 500 µM Cu. The majority of these genes, like the genes in the glutathione pathway, metal ATPase and ABC transporters, were involved in metal tolerances in this ecotype (Memon & Zahirovic 2014). In this study, we were interested in analyzing the behavior of this facultative metal accumulator plant, especially the growth and gene expression pattern when grown at low Cu concentration (e.g., 25 µM Cu). In addition, this gene expression pattern was compared to our previous reported gene expression data of the non-accumulator *B. nigra* ecotype CGN06619 grown under 25 µM Cu (Cevher-Keskin *et al.* 2019).

The treatment of the Diyarbakir ecotype of *B. nigra* (a Cu accumulator) with 25 µM Cu for two weeks increased Cu content in the leaves by nine-fold compared to the control. These plants were healthy and had remarkable growth even at high Cu treatments (Fig. 1). Our previous results with the non-accumulator ecotype showed necrosis and wilting of the leaves when treated with 25 µM Cu (Cevher-Keskin *et al.* 2019). On the other hand, the

Diyarbakir ecotype showed no toxicity effect at high Cu presence (500 µM Cu) (Memon and Zahirovic 2014, Merakli & Memon 2019). Thus, these facultative accumulator plants can survive and grow well at low or high metal contents (Weber *et al.* 2006, Pollard *et al.* 2014, Memon 2020).

In this study, we examined the changes in the gene expression in the leaf tissues of the Diyarbakir ecotype exposed to 25 µM Cu. Microarray analysis showed the differential expression of the genes involved in metal stress, metal transport, oxidative stress, metal chaperons, signal transduction, and transcription factors for metal and other abiotic stress-related genes. Interestingly, the expression level of several transporter genes, for example, metal ATPases like HMA5, HMA7, HMA8, several ZIPs, and NRAMP, was significantly increased in leaf tissues under 25 µM Cu compared to 0 µM Cu (Table 1). Our data showed that some common genes in the abiotic stress-related pathway were upregulated in this ecotype when treated with 500 µM Cu (Memon & Zahirovic 2014). For example, glutathione S-transferases (GSTs), some transcription factors like MYB, and P-type metal ATPase were highly expressed in this ecotype at high Cu treatment (Memon & Zahirovic 2014). Several transporters located at the plasma membrane, tonoplast, chloroplast, and other organelle membranes play active roles in Cu uptake and transport in roots and shoots of the plants (Hall & Williams 2003). These proteins are essential to maintain the metal homeostasis in plant cells and keep the proper metal ion concentration in the cytosol (Hall & Williams 2003, Puig *et al.* 2007, Printz *et al.* 2016). HMAs, NRAMPs, COPT, and ZIPs function in the metal uptake, delivery or utilization and maintain cellular metal homeostasis by sequestering and detoxifying metal ions in plants at the subcellular level (Williams *et al.* 2000, Krämer *et al.* 2007, Andresen *et al.* 2018).

The present results (Table 1) showed that these metal transporters were indeed highly expressed when plants (Diyarbakir ecotype) were subjected to 25 µM Cu. For example, most of the metal detoxification-related genes, including three P-type ATPases family transporters (HMA5, HMA7 (RAN1), HMA8 (PAA2)), five COPTs family transporters (COPT1, COPT3, COPT4, COPT5, COPT6), six NRAMPs family transporters (NRAMP1, NRAMP2, NRAMP3, NRAMP4, NRAMP5, NRAMP6), IREG family transporter (IREG2), 11 ZIPs family transporters (ZRT/IRT like proteins) (ZIP1, ZIP2, ZIP3, ZIP4, ZIP5, ZIP7, ZIP9, ZIP11, IRT1, IRT2, IRT3), two CDF family transporters (MTPB1, MTPA2), and five ABC family transporters (MRP3, MRP4, MRP7, MRP10, MRP15) were upregulated. AtHMA5 is involved in Cu translocation from root-to-shoot and is possibly located in the plasma membrane of xylem (Colangelo & Guerinot 2006, Andrés-Colás *et al.* 2006, Deng *et al.* 2013) and is closely associated with HMA7 (RAN1) (Andrés-Colás *et al.* 2006, Puig *et al.* 2007). Based on our microarray results, the expression of both ATPases, HMA5, and HMA7 (RAN1), were upregulated in the leaves of this accumulator ecotype when grown at 25 µM Cu (Table 1). HMA7 (RAN1) interacts with a Cu chaperone (CCH) and COPT1 and is involved in the intracellular delivery of Cu in plants (Williams *et al.* 2000, Puig *et al.* 2007). Similarly, COPT1, ZIP2 transporters, FRO3 metal reductase, CCH Cu chaperon and chloroplast FeSOD are expressed in plants grown at low Cu levels (Sancenon *et al.* 2003, Wintz *et al.* 2003, Mukherjee *et al.* 2006). Several Cu chaperons (Cu chaperone (CCH), Cu chaperone SCO1-like protein HCC1), and Cu stress-related transcription factors were upregulated in the Diyarbakir ecotype at 25 µM Cu (Tables 1 and 2). In plants, these proteins serve an essential role in forming metal-ligand complexation in response to metal stress and enhancing the metal tolerance (Wintz & Vulpe 2002, Hossain *et al.* 2012).

The results also showed that the DREB transcript factors expressed in drought stress and ERFs (Ethylene-responsive transcription factors) were also upregulated in Cu-stress in the Diyarbakir Merkez ecotype (Table 1). These transcription factors are involved in both biotic and abiotic stresses in plants (Hossain *et al.* 2012, Dey & Corino Vlot 2015). Several aquaporin genes, which play a central role in regulating plant water homeostasis, were also upregulated in Cu-treated plants (Hachez *et al.* 2006, Chaumont & Tyerman 2014, Afzal *et al.* 2016).

Plant cells produce antioxidant enzymes like superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) at alleviated metal stress. These enzymes help the plants in reducing the toxic effect of the metal through scavenging the toxic ROS produced due to the high metal stress (Maksymiec & Krupa 2006, Ghori *et al.* 2019). The genes encoding these antioxidants were upregulated in the leaf tissues of *B. nigra* Diyarbakir grown at 25 µM Cu but were downregulated in the non-accumulator ecotype (CGN06619) (Table 2) (Cevher-Keskin *et al.* 2019). In addition, the plant cells subjected to metal stress produce a series of ROS-scavenging nonenzymatic antioxidants such as ascorbic acid, phenolic and flavonoid compounds, carotenoids, and sulfur-containing antioxidants including glutathione (GSH), metallothionein (MTs), phytochelatin (PCs), as well as SOD, (PODCAT) and GPX (Štolfa *et al.* 2015). Similarly, we observed that antioxidants such as GSH, ascorbic acid, phenolic and flavonoid compounds were upregulated when the Diyarbakir ecotype encountered metal stress (see Tables 1 and 2). Interestingly, several genes involved in ROS regulation were shown to be downregulated in Cu-sensitive ecotype (CGN06619) at 25 µM Cu (Table 2). For example, Fe-superoxide dismutase, GST, P450 family cytochrome, proline-rich protein 4, and NAC domain-containing proteins were downregulated in Cu-sensitive ecotype at 25 µM Cu.

In consistence with our microarray data where several genes of glutathione pathway were highly expressed in the Cu treated plants, the metabolites of glutathione pathway,

such as glutathione, also increased in the leaves with the intermittent increase in Cu level. HPLC analysis with accumulator plants showed that GSH was consistently increased in the plants grown at different Cu levels (Fig. 4). GSH plays a vital role in the glutathione pathway and is responsible for metal detoxification in plants (Xiang & Oliver 1998). Our results give a holistic picture of metal tolerance in hyperaccumulator plants and the genes involved in metal regulation and detoxification in *Brassica* model accumulator plant *B. nigra*. The present findings will help in understanding of the metal tolerance and detoxification mechanisms in other crop plants in the genus *Brassica*.

Conclusion

Our microarray data showed differential gene expression patterns in the Cu accumulator and non-accumulator *Brassica nigra* ecotype when subjected to 25 µM Cu treatment. In conclusion, a series of genes, such as HMA5, HMA7, COPT1, COPT5, NRAMP1, NRAMP2, ZIP1, and ZIP7, etc. related to metal transport and delivery were shown to be upregulated in the Cu hyperaccumulator ecotype of *B. nigra* when subjected to Cu stress. Several other genes encoding antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), laccase, superoxide dismutase [Fe], as well as genes encoding nonenzymatic antioxidants such as ascorbic acid, phenolic and flavonoid compounds, carotenoids, and sulfur-containing antioxidants including GSH, metallothioneins, phytochelatins were also upregulated in the accumulator ecotype. The mechanism of Cu tolerance in the Cu accumulator and non-accumulator plants is discussed in detail. Further detailed validation experiments could be carried out by qRT-PCR. In the future, CRISPR/cas9 technology could be used to overexpress or knockout these genes to understand their roles in metal regulation and detoxification in plants.

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