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Analysis of Gene Expression Associated with Copper Toxicity in White Birch (*Betula papyrifera*) Populations from a Mining Region

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Authors' contributions

This work was carried out in collaboration between all authors. Author CLD conducted the experiments and analyzed the data. Authors GT and PM assisted with experimental design and implementation and data analysis. Author KKN designed and monitored the experiments and wrote the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

The Greater Sudbury Region (GSR) is one of the most ecologically disturbed regions of Canada. Recent studies have shown that *Betula papyrifera* accumulate metals in roots or leaves. The main objectives of the present study were to 1) determine the effects of copper treatment on *B. papyrifera* under controlled conditions and 2) assess the level of expression of genes associated with copper resistance in *B. papyrifera* populations from metal-contaminated and uncontaminated areas. Significant differences for damage rating were observed among copper dosages after eight days of treatment. There was also a trend of reduced plant growth as the dosage increased. RT-qPCR analysis showed 2x to 25x increase in leaves compared to roots of the expression of the gene for Multi-drug resistance associated protein (MRP4) belonging to the subfamily of ATP-binding cassette (ABC) transporters. A significant upregulation (ranging from 3x to 8 x increase) of

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the Metallothioneins (MT2B) gene in leaves compared to roots was also observed in three of the five sites studied. There were significant differences in expression of MRP4 and MT2 genes among sites, but no association between metal contamination and gene expression was identified. Likewise, no difference in expression of targeted genes was observed among the copper dosages used in growth chamber experiments.

Keywords: Copper toxicity; Betula papyrifera (white birch); gene expression; Northern Ontario.

1. INTRODUCTION

The Greater Sudbury Region (GSR) is one of the most ecologically disturbed regions of Canada. It has been documented that more than 100 million tonnes of sulfur dioxide and tens of thousands of tonnes of cobalt (Co), copper (Cu), and nickel (Ni) as well as iron ores were released into the Earth's atmosphere [1]. The average Cu and Ni content in organic horizons in distant reference sites normally range between 15 to 40 mg/kg and 20 to 30 mg/kg respectively, but it reached 9,700 mg/kg and 6,960 mg/kg for Cu and Ni, respectively in sites surrounding smelters [2]. Recent analysis showed that the amount of Cu within metal – contaminated sites varied between 650 mg/kg to 1, 330 and from 600 to 1,800 mg/kg for Ni [3-6]. Analysis of nickel toxicity and plant responses in *Betula papyrifera* has been reported [7-9]. Detailed information on effects of Cu on this species is lacking.

Copper (Cu) is an essential micronutrient required at very low concentrations for normal growth and development. On average, plants maintain Cu levels at 10 µg/g (DW) but this differs significantly between plant species [10]. Copper functions as a structural element in regulatory proteins, participates in photosynthetic electron transport, mitochondrial respiration, oxidative stress responses, cell wall metabolism and hormone signalling. It is also a cofactor in many enzymes including Cu/Zn superoxide dismutase (SOD), cytochrome c oxidase, amino oxidase, laccase, plastocyanin, and polyphenol oxidase. For this reason, Cu functions in signalling of transcription, protein trafficking machinery, oxidative phosphorylation and iron mobilization [11].

Uptake of Cu in plants is primarily achieved from the root system and like other metals, it moves to shoots via the xylem using complexing ligands. Although little is known about the specific uptake of Cu, there has been evidence that it is accomplished competitively with Fe and strongly associated with organic matter [12]. White birch (*Betula papyrifera* Marsh.) is a major component of the boreal forest of North America. It is considered a pioneer species because of its rapid growth and its intolerance for shade; therefore it is the first to rapidly colonise open areas after deforestation. Recent studies have shown that resistant *B. papyrifera* genotypes accumulate nickel in roots while susceptible genotypes translocate and store Ni in leaves [9,13].

The main objectives of the present study were to 1) determine the effects of copper treatment on *B. papyrifera* under controlled conditions and 2) assess the level of expression of genes associated with copper resistance in *B. papyrifera* populations from metal-contaminated and uncontaminated areas.

2. MATERIALS AND METHODS

2.1 Copper Toxicity Analysis

B. papyrifera seeds were harvested from trees growing at the Laurentian University research field site in the Region of Greater Sudbury (RGS), Ontario (Canada) and stored at 4℃ (Fig. 1). They were germinated at 27℃ on wet filter paper in Petawawa boxes. Seedlings were then transplanted into containers with a mixture of topsoil/peat moss. They were left to grow for four months at 27℃ being watered and fertilized as needed then transplanted into a 50:50 mix of quartz sand/peat moss.

To assess the toxicity of copper on *B. papyrifera*, plants averaging 45 cm in height were treated in growth chambers. Commercial salt of Cu (SO_4) was used for treatments. The trial was arranged in a completely randomized block design with 15 replications per treatment. The dosages included 9.16 mg and 1,312 mg of Cu per 1 kg of dry soil which are equivalent to bioavailable and total Cu in contaminated soils in GSR, respectively. Three times the amount of total Cu (3,936 mg/ kg) was also used as a treatment. Water treatment (0 mg Cu per 1 kg of dry soil) was used as a control. Potassium sulfate (K_2SO_4) treatment was used to control for the sulfate concentrations. The 9.16

mg/kg, 1,312 mg/kg, and 3,936 mg/kg of Cu corresponded to 0.063 mmol, 10 mmol, and 30 mmol, of Cu. The same molarity was used for K_2SO_4 salts. All the treatments were administrated in water. Damage ratings were measured using a 1 (no damage) to 9 (dead plants) scale. Details of the damage rating scale have been described in Theriault et al. [9]. Plant growth was taken as the difference in height between measurements on first and last day (8^{th} day after the treatment) of the experiments.

2.2 RNA Extraction and RT-qPCR

Total RNA was extracted from root and leaf samples using the methods previously described in Theriault et al. [7]. Root RNA was extracted from 15 individuals per site, and then quantified using the Qubit(R) RNA BR Assay kit by Life Technologies (Carlsbad, United States). The quality of the RNA was verified on a 1% agarose gel. One microgram of RNA per site from samples of the same treatment/site was pooled together and used for downstream processes.

RNA from three metal – contaminated sites (Kelly Lake, Laurentian, and Wahnapitae Hydro Dam) and uncontaminated areas (Capreol, St. Charles, and Onaping Falls) were used for gene expression analysis. The RNA was treated with DNase 1 (#EN0521) from Life Technologies. Several genes associated with copper resistance in model and non-model plants were targeted in

Fig. 1. Locations of *Betula papyrifera* sampling populations within the Greater Sudbury Region in Northern Ontario. 1: Daisy Lake; 2: Wahnapitae Hydro Dam; 3: Laurentian; 4: Kukagami; 5: Kingsway; 6: Falconbridge; 7: Capreol; 8: St-Charles; 9: Onaping Falls; 10: Airport

this study (Table 1). PCR primers were designed by matching gene sequences to the *B. nana* genome (dwarf birch) [14]. When possible, primers were designed to span the exon-exon border of the gene. Primers were then BLASTed using the *B. nana* genome. Primers were checked for hairpins, self, and hetero-dimers using the OligoAnalyzer 3.1 by IDT (https://www.idtdna.com/calc/analyzer). The cDNA was synthesised using the High-Capacity cDNA Reverse Transcription Kit by Life Technologies. Regular PCR was performed on both *B. papyrifera* DNA and cDNA. The size of the amplicons derived from amplification with the primer pairs was verified on agarose gels. Only primers that showed a reproducible single band of the appropriate size were used for RT-qPCR. This RT-qPCR was performed using the Dynamo HS SYBR Green qPCR Kit by Life Technologies and conducted according to the manufacturer's protocol. Each sample was amplified with the MJ Research PTC-200 Thermal Cycler in triplicates. The process includes (1) initial denaturing at 95℃ for 15 min; 2) denaturing at 94℃ for 30 s; 3) 30 s annealing; 4) elongation at 72℃ for 30 s ; 5) read; 6) repeat step 2 – 6 for 41 cycles; 7) final elongation at 72℃ for 7 min; 8) melting curve 72 – 95℃, every 1℃, hold for 10 s; and 9) final elongation at 72℃ for 3 min. This qPCR was run three separate times with each sample in triplicate. This resulted in a total of nine data points for each bulked sample.

Table 1. Candidate genes involved in Cu resistance in model and non-model plant species

Gene	Species	Reference
RAN1	*Arabidopsis thaliana*	Kobayashi et al. [29]
MRP4	*Betula pendula*	Keinänen et al. [30]
COPT 1	*Arabidopsis thaliana*	Sancenon et al. [31]
MT2B	*Arabidopsis thaliana*	Guo et al. [32]

The data were analyzed using the MJ Opticon Monitor 3.1 by BioRad and C(t) values were determined. C(t) values were only normalized to the housekeeping genes. Housekeeping genes led to the same results therefore only one (α-tubulin) was used for simplicity.

2.3 Statistical Analysis

The data for damage ratings, plant growth, and gene expression levels were analyzed using SPSS 20 for Windows, with all data being $\log_{10}$ transformed to achieve a normal distribution. ANOVA, then Tukey's HSD multiple comparison analyses, were performed to determine significant differences among means. The Student-T test was performed for field experiment to compare means between roots and leaves ($p \leq 0.05$).

3. RESULTS AND DISCUSSION

3.1 Copper Toxicity

Significant differences for damage rating and plant growth were observed among copper dosages after eight days of treatment based on Tukey mean comparison tests. Overtime plant reactions to Cu treatments are summarized in Table 2. There was no significant damage caused by Cu at 9.16 mg/kg dosage (amount of bioavailable Cu in the natural site) throughout the experiment (Table 1). Significant differences in plant damage ratings were found between the 9.16 mg/kg and 1,312 mg/kg dosages. The highest dose of 3,929 mg/kg resulted in severe damage on plants two days after the treatment and almost all the plants were dead within four days of Cu application. There was a trend of reduced plant growth as the dosage increased. No significant difference in plant growth was observed between 9.16 mg/kg and the control and 1,312 mg/kg and 3,936 mg/kg dosages (Fig. 2).

Table 2. Damage rating of *Betula papyrifera* treated with different doses of copper

Treatment	Soil concentration (mg/kg)	Damage rating			
		Days after treatment			
		Day 2	Day 4	Day 6	Day 8
Copper	9.16	1.0 ± 0.0 **a**	1.0 ± 0.0 **a**	1.0 ± 0.0 **a**	1.0 ± 0.0 **a**
	1,312	2.6 ± 1.5 **a**	6.2 ± 2.2 **b**	7.3 ± 2.4 **b**	7.8 ± 2.1 **b**
	3,936	6.8 ± 2.1 **b**	8.1 ± 2.2 **b**	8.5 ± 1.6 **b**	8.5 ± 1.6 **b**

ANOVA, followed by Tukey's HSD multiple comparison analysis, were performed to determine significant differences among means. Means followed by different letters are significantly different (P< 0.05; n = 15)

According to the Ministry of the Environment Ontario (Canada), the background concentrations of copper in Ontario soils are on average less than 25 mg/kg with exceptional circumstances at 85 mg/kg [15]. The average copper concentration in Canadian soil is estimated to be 20 mg/kg with a range between 2 and 100 mg/kg (British Columbia Ministry of the Environment, Lands and Parks 1992). European Union (EU) recommends warning legislative limit of copper concentration in soil is 50 mg/kg (Council Directive 86/278/EC, 1986) [15]. Hence, the total level of copper in GSR soil used in the present study (1,312 mg /kg) was 15 x to 50 x higher than the concentration in other regions in Canada.

Uptake of contaminants by plants and their mechanisms have been studied by several researchers [13,16-18]. Plants can act as both accumulators and excluders. The accumulators are able to survive despite the high concentrations of metals that are contained in their aerial shoots. They biotransform the contaminants into inert forms in their tissue. The excluders limit contaminant uptake into their biomass. Plants have developed specific mechanisms to translocate and store micronutrients. These mechanisms are involved in the uptake, translocation and storage of toxic elements [16]. Recent field studies have shown that *B. papyrifera* growing in the GSR doesn't accumulate metal in leaves and the translocation factor for copper from roots to leaves was only 0.076 [13].

The sensibility or resistance of plants to metals depends on physiological and molecular mechanisms that include uptake and accumulation of metals usually by binding to extracellular exudates and cell walls complexation of ions inside the cells. Some plants prevent metal ions from entering the cytosol through the action of plasma membrane [18-20]. The present study shows that the 9.16 mg / kg of Cu corresponding to bioavailable amount in the soil within the GSR is not toxic to *B. papyrifera*. At higher dose of 1,913 mg/kg (equivalent to total amount of Cu in soils from GSR), there was a range of reactions in *B. papyrifera* genotypes tested from moderately resistant (damage rating of 4 to 6) to susceptible (7 to 9). Analysis of Cu tolerance in *Mimulus guttatus* revealed a single major dominant gene inheritance [21,22], but our recent study indicated that Cu tolerance in *B. papyrifera* might be controlled by single recessive genes [9]. Overall our understanding of relationship between metal toxicity and plant genetic response is limited because of the scarcity of the limited knowledge on mechanisms of Cu homeostasis, uptake, transport, and accumulation in *B. papyrifera*.

3.2 Copper Gene Expression

Primers that were used to amplify target genes are listed in Tables 3 and 4. Two of the seven primer pairs targeting housekeeping genes generated strong bands. They include α tubulin 1 and cyclophilin 2. When all the samples from the five sites (Kelly Lake, Kingsway, Onaping, Capreol, and Killarney) were tested, MRP4 and MT2B gave a consistent and repeatable band. For MRP4 there was 2 x to 25 x increase of expression in leaves compared to roots in all the sites (Fig. 3). The highest levels of expression in leaves were found in Onaping Falls (reference site) and Kingsway (metal-contaminated site) and the lowest level in Killarney. For root, samples Kelly Lake (metal – contaminated) showed the highest level of expression while the other four were statistically similar. For MT2B gene, there was significant upregulation (ranging from 3x to 8 x increase) of this gene in leaves compared to roots in three of the five sites (Fig. 4). The highest increase of this gene expression in leaves was found in samples from Onaping Falls followed by Kingsway and Capreol. Expression of MT2B in roots was the highest in samples from Kelly Lake while the expressions in other sites were statistically similar. No association between metal contamination and gene expression was observed. The lack of amplification with primer pairs targeting RAN 1 and COPT 1 genes could have been due to the absence of primer binding sites or weak primer bindings.

There was no significant difference for MRP4 and MT2B expression when samples from *B. papyrifera* genotypes treated with different doses were compared. This suggests that these genes are not affected by Cu toxicity in *B. papyrifera*.

Toxic levels of Cu in plants are well known to cause oxidative stress from increased production of oxygen free radicals. More specifically, Cu catalyzes the formation of hydroxyl radicals (OH^-) inducing changes in antioxidative pathways including ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR),

glutathione reductase (GR), superoxide dismutase (SOD), and guaiacol peroxidase. These antioxidant responses are dependent on Cu concentrations and exposure time. The main target of Cu transport has been discovered to be the photosystem II (PSII). Both the acceptor and the donor side are highly sensitive to the toxic action of Cu and this action is highly dependent on Cu concentrations [23]. Several families of HM transporters have been identified that are involved in Cu transportation including 1) P-type ATPase Cu-transporters, which use ATP to pump a variety of charged substrates including Cu^{2+} across biological membranes; 2) Cu chaperones, belonging to a family of cytosolic, soluble, low-molecular-weight metal-receptor proteins named metallochaperones, involved in intracellular transportation of Cu [23].

Table 3. Sequences of *Betula papyrifera* primers, designed from housekeeping genes, screened for RT-qPCR

Target	Melting temp (°C)	Primer	Expected amplicon (bp)	PCR product in DNA (bp)	PCR product in cDNA (bp)
α-tubulin 1	F: 65.10	F: TGTTGACTGGTGCCCACCG	187	187	187
	R: 65.16	R: CACAAAGGCGCGCTTGGCAT			
Cyclophilin 2	F: 64.78	F: TGGGCGGATCGTGATGGAGC	370	370	370
	R: 65.10	R: CACGACCTGGCCGAACACCA			

Table 4. Sequences of designed primer pairs targeting Cu resistance genes using the dwarf birch (*Betula nana)* genome

Target	Melting temp (°C)	Primer	Expected amplicon (bp)	PCR product in DNA (bp)	PCR product in cDNA (bp)
RAN1	F: 63.73	F: CCTTGTGCTTTGGGTCTGGC	337	None	
	R: 62.51	R: GCTATTGTTATCGGCATCCTTGG			
MRP4	F: 63.46	F: GCTTGATCCTCTGCCTTTCTACTTG	380	380	380
	R: 63.67	R: CCACTTCCTGTTCGACCAACAAC			
COPT1	F: 68.34	F: GCACATGACCTTCTTCTGGGGCA	303	None	
	R: 69.30	R: AACCCAACGGCGTGGCCAG			
MT2B	F: 65.83	F: CTTGTGGAGTTCAAAGGCGGAAAG	387	387	387
	R: 65.02	R: GGCAGCCAAGCTGACAGTATGAAC			

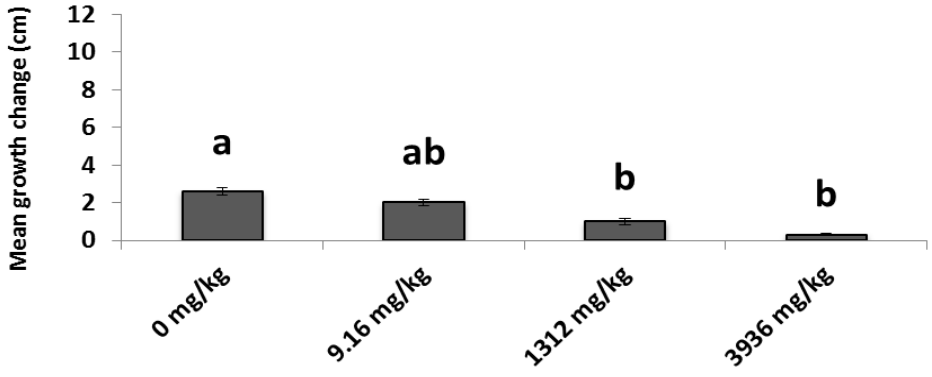

Fig. 2. Mean growth of *Betula papyrifera* plants treated with different doses of copper eight days after treatments in growth chamber screening tests. ANOVA, followed by Tukey's HSD multiple comparison analysis, were performed to determine significant differences among means (P< 0.05). Bar represents standard errors. The mean growth is the difference between height measures at day 1 and day 8 after treatment

Means followed by different letters are significantly different (P< 0.05; n = 15)

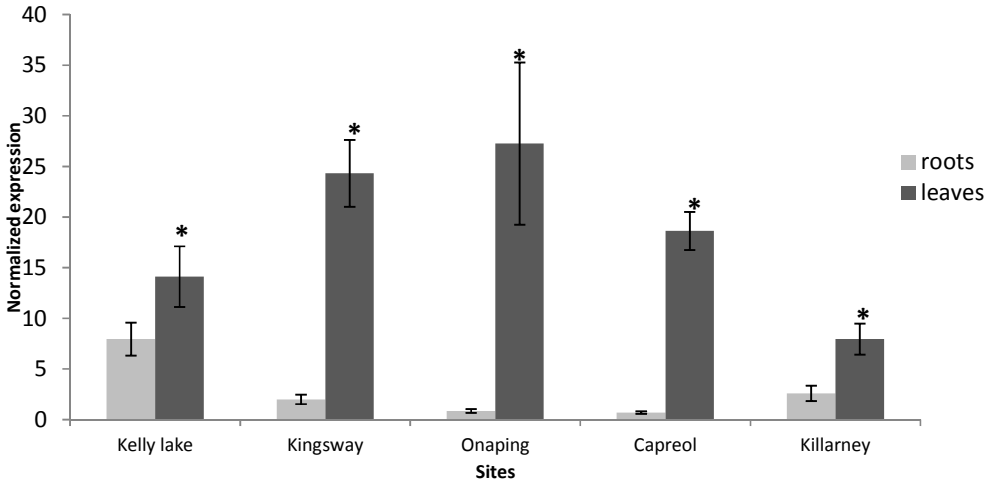

Fig. 3. Expression of MRP4 (Multi drug resistance associated protein 4) in white birch (*Betula papyrifera)* for field samples from metal contaminated and reference root and leaves. Expression of MRP4 was standardized based on the housekeeping gene α-tubulin 1. Significant differences were found between root and leaf data using student-T *test* (P < 0.05)
** indicates significant difference between root and leaf expression levels for each site*

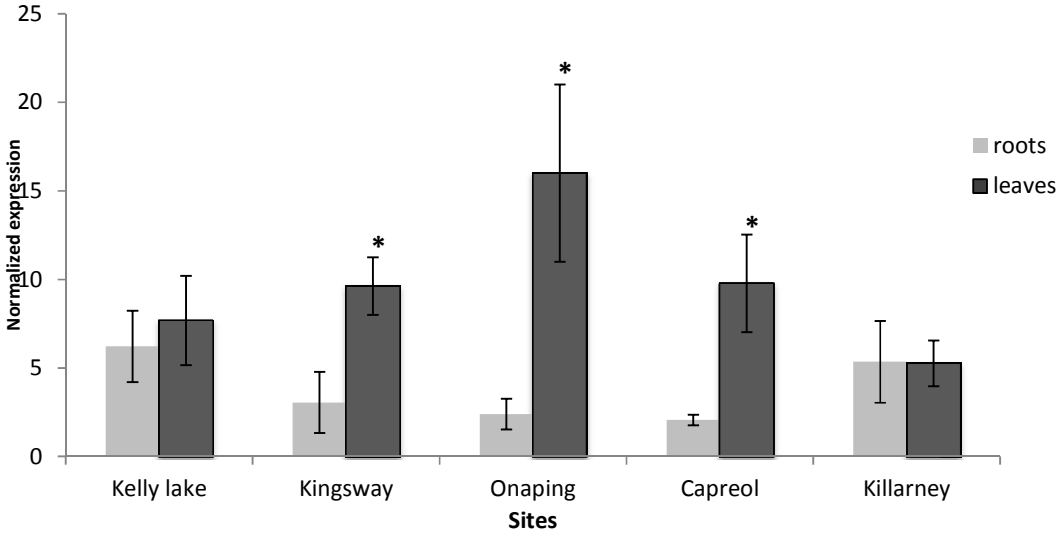

Fig. 4. Expression of MT2b (metallothionein 2b) in white birch (*Betula papyrifera)* for field samples from metal contaminated and reference roots and leaves. Expression of MT2b was standardized based on the housekeeping gene α-tubulin 1. Significant differences were found between root and leaf data using student-T *test* (P < 0.05)

Copper resistance mechanisms include avoidance, tolerance and hyperaccumulation. Avoidance mechanisms of Cu toxicity in plants involve the binding of organic acids excreted by the plant by certain mycorrhizal species, inhibiting metal uptake [24]. Different uptake mechanisms in plants are directly related to different mechanisms of metal tolerance at the whole plant level [25]. If Cu successfully enters root cells, metal-binding proteins allow their transportation from roots-to-shoots. In excess, Cu is often transported to the vacuole, apoplast or to specialized cells (epidermal cells, trichomes) for storage to avoid toxicity [23]. A second tolerance mechanism of Cu in plants involves the accumulation of organic acids in the xylem, facilitating metal complexing. This would enhance both the mobility of Cu in the xylem and

the chemical gradient for metal ions [23]. Genes that have been hypothesized to play a role in Cu tolerance include copper-transporting ATPase (RAN1), multi-drug resistance associated protein (MRP4), copper transporter protein (COPT1), and metallothionein (MT2B).

Response to antagonist 1 (RAN1) belongs to the P-type ATPase Cu-transporters and is classified as the subtype 1B ATPase. RAN1 is involved in intracellular transportation of Cu to chloroplasts and is localized at the post-Golgi compartment [23]. RAN1 is also involved in the delivery of Cu to ethylene receptors in *A. thaliana,* a hormone which plays a crucial role in plant growth [11].

Multi-drug resistance associated protein (MRP4) belongs to the subfamily of ATP-binding cassette (ABC) transporters, which are ATP-dependent and an integral part of plant detoxification. MRP4 has been demonstrated to be a multi-purpose membrane protein, suggested to be involved in vacuolar sequestration of potentially toxic metabolites and highly up-regulated in roots and shoots of Cu-tolerant *B. pendula* [26].

Metallothioneins (MT2B) are cysteine-rich proteins that bind metals including Cd, Cu and Zn. MT's protect plants from oxidative stress by detoxifying hydroxyl radicals. MT2B is proposed to be involved in the distribution of Cu via the phloem and have a role in Cu tolerance, homeostasis and long distance transport of Cu in *A. thaliana* [27].

Copper transporter protein (COPT1) belongs to a family of putative *Arabidopsis* copper transporters (COPT1-COPT5). COPT1 is involved in the uptake and accumulation of Cu via the root apical zone in *A. thaliana*. It is expressed in root tips and pollen grains and functions in root elongation and pollen development. In leaves, COPT1 is expressed exclusively in trichomes and stomatal guard cells, suggesting a role in Cu detoxification [28].

It has been established that some plants prevent metal ions from entering the cytosol through the action of plasma membrane [17,18,20]. The lack of association between MRP4 and MT2B gene expression and copper contamination suggested that the regulation mechanism of selected copper resistance genes is different from species to species. Overall the scarcity of knowledge on mechanisms of Cu homeostasis, uptake, transport, and accumulation in *B. papyrifera* limit our understanding of relationship between metal toxicity and plant genetic response.

4. CONCLUSION

The analysis of copper toxicity under field and growth chamber conditions has been performed. We also investigated whether genes known to play a role in copper resistance in other species are involved in *B. papyrifera* responses to soil copper contamination. Significant differences for damage rating were observed among copper dosages after eight days of treatment. There was also a trend of reduced plant growth as the dosage increased. RT- qPCR analysis showed 2x to 25x increase in leaves compared to roots of the expression of the gene for Multi-drug resistance associated protein (MRP4) belonging to the subfamily of ATP-binding cassette (ABC) transporters. A significant upregulation (ranging from 3x to 8 x increase) of the Metallothioneins (MT2B) gene in leaves compared to roots was also observed in three of the five sites studied. There were significant differences in expression of MRP4 and MT2 genes among sites, but no association between metal contamination and gene expression was identified. Likewise, no difference in expression of targeted genes was observed among the copper dosages used in the growth chamber experiments. Transcriptome analysis of *B. papyrifera* genotypes treated with different copper dosages is being completed to identify genes that are actually expressed under copper contamination.

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COMPETING INTRESTS

Authors have declared that no competing interests exist.

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