EFFECT OF HEAVY METAL TREATMENTS ON METALLOTHIONEIN EXPRESSION PROFILES IN WHITE POPLAR (*Populus alba* L.) CELL SUSPENSION CULTURES

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Abstract. *Populus* species and hybrids are intensively cultivated as sources of woody biomass and are good candidates for phytoremediation because of their rapid growth rate, extensive root system and ease of propagation and transformation. To date, the molecular mechanisms that regulate heavy metal tolerance have not been fully investigated. In the present work, white poplar (*Populus alba* L.) cell suspension cultures were used as model system to investigate the response to heavy metal treatments. The *VFMT2* cDNA, encoding a type 2 metallothionein from *P. alba*, was isolated by RT-PCR approach. The expression profiles of the *VFMT2* gene were then investigated by Quantitative Real Time Polymerase Chain Reaction (QRT-PCR) under oxidative stress conditions. The latter were induced by exposing the cell suspension cultures to different doses of cadmium (75 and 150 μM $CdSO_4$), copper (50 and 100 μM $CuCl_2$) and zinc (1 and 2 mM $ZnSO_4$). Cell death was evidenced by Evans blue staining. The *VFMT2* gene was up-regulated in response to heavy metal treatments and the highest mRNA level (up to 5-fold) was observed 4 h following exposure to 100 μM $CuCl_2$.

Keywords: *Populus alba* L., cell suspension cultures, metallothionein, heavy metal stress, QRT-PCR

INTRODUCTION

Forest trees have a great economic and ecological value, as well as unique biological properties of basic scientific interest. The genus *Populus* spp. is regarded as model system for forest trees since it offers several advantages including rapid growth, high biomass production and prolific sexual reproduction, combined with a relatively small genome [4]. In addition, it is amenable to coppicing and short-rotation harvest, as well as *in vitro* propagation and genetic transformation [10]. Poplar is also regarded as a suitable candidate for use in phytoremediation of polluted soils due to relevant features such as the ability to withstand environmental stresses, the extensive root system and the high water uptake [3]. The white poplar (*Populus alba* L.) clone 'Villafranca', investigated in the present study, is a strong biomass producer with optimal performances in short rotation cultures, already used for several biotechnological applications [9].

Transition elements such as copper (Cu), zinc (Zn), or cadmium (Cd) which play several roles in cell metabolism, are absorbed by plants from the soil. However, these metals are potentially toxic when present at high concentrations and there is great evidence that exposure of plants to excess concentrations of heavy metal results in accumulation of genotoxic Reactive Oxygen Species (ROS) [20]. *P. tremula* plants treated with Cd exhibited growth inhibition and changes in gene expressions, in particular a decreased level of proteins involved in the regulation of oxidative stress responses [13]. Significant reduction in growth, and alteration of the photosynthetic parameters have been reported in *Populus x euramericana* after *in vivo* treatment with high Zn concentrations [11]. In *P. alba* cell suspensions cultures challenged with different copper doses, an increased nitric oxide (NO) production was observed, as well as an elevated ratio of programmed cell death (PCD) and necrosis [3].

In plants exposed to toxic levels of heavy metals there is an increased production of metal-binding proteins such as metallothioneins (MTs) and phytochelatins. Differently from phytochelatins, which are enzymatically synthesized from glutathione, MTs are low-molecular-weight proteins which contain cysteine-rich domains at the amino- and carboxy-terminal regions [8]. Since they were first purified from horse kidney [15], MT genes and proteins have been discovered in many prokaryotic and eukaryotic organisms. Many genes encoding MTs have been isolated in plants and information about their putative function were obtained from gene expression studies. MT genes are part of a multigene family whose members are differentially regulated in relation to developmental stages and are implicated in the response to wounding, pathogen attack and oxidative stress [24]. Based on the arrangement of cysteine (Cys) residues, MTs are divided in two classes. Class I MTs contain 20 highly conserved Cys residues and are widespread in vertebrates. Class II MTs, lacking this typical organization of cysteine residues, are found in plants, fungi and non-vertebrates. Plant MTs can be further classified in four distinct classes. Type 1 and 2 MTs are expressed mainly in leaves and roots, Type 3 are expressed during fruit ripening and Type 4 are predominant in seeds [8]. It has been hypothesized that the cysteine residues found in MTs might be involved in ROS detoxification and maintenance of the redox level. In animals, MTs are known to function as ROS scavengers and to confer protection against oxidative DNA damage [17]. In plants, many genes and cDNAs encoding MTs have been isolated, with functions involved in the response to heavy metal-induced stress. In *Arabidopsis*, rice and tobacco the expression of Type 1 MT is induced by copper [8], while *Silene vulgaris* populations with copper tolerance are characterized by higher mRNA levels and gene copy number of a Type 2 MT gene [21]. MTs have been recently identified and characterized also in poplar. Six different genes are present in the hybrid *P. trichocarpa x P. deltoides* genome [14]. The occurrence of multiple MT genes in poplar might reflect their different roles in heavy metal sequestration and in other mechanisms

essential for plant growth. In a recent study, marker-free transgenic lines of *P. alba* cv. 'Villafranca' expressing a *Pisum sativum* Type 2 metallothionein (PsMT), produced using the MAT (Multi-Auto-Transformation) technology, showed increase tolerance to heavy metal toxicity [2].

Since overexpression of MTs has been generally recognized as an effective approach to improve stress tolerance *in planta*, further investigation are required to better define candidate *MT* genes suitable for biotechnological applications. Cell suspension cultures is a powerful tool for studying the effects of oxidative stress, since are homogenous populations which can be easily analyzed at biochemical and molecular levels [16]. In the present work, we report on the identification of the *VFMT2* gene, encoding a type 2 metallothionein in *P. alba* cv. 'Villafranca'. The expression profiles of the *VFMT2* gene were also analyzed in order to assess its possible involvement in the plant response to heavy metal stress conditions.

MATERIALS AND METHODS

Cell suspension cultures and treatments

The white poplar (*Populus alba* L.) cv. 'Villafranca' used in this study was kindly supplied by Dr. Stefano Bisoffi (C.R.A. - Research Unit for Wood Production outside Forest, Casale Monferrato, Alessandria, Italy). Cell suspension cultures of 'Villafranca' were obtained and maintained *in vitro* as previously described [22]. Exponentially growing (4-day old) cell suspension cultures were exposed to increasing concentrations of cadmium (75 and 150 µM $CdSO_4$; Sigma-Aldrich S.r.l., Milan, Italy), copper (50 and 100 µM $CuCl_2$; Sigma-Aldrich S.r.l., Milan, Italy), and zinc (1 and 2 mM $ZnSO_4$; Sigma-Aldrich S.r.l., Milan, Italy) and subsequently monitored at the indicated time points (4, 6 and 8 h).

Cell viability

Cell viability was evaluated by Evans Blue assay as described by Carimi *et al.* (2003) [6]. Cells were collected by centrifugation and incubated in 0.25% Evans Blue (dye content ≥ 75%, Sigma-Aldrich S.r.l., Milan, Italy) for 10 min and then washed extensively with distilled water to remove excess dye. The screenings for cell death was carried out using a ZEISS EM 900 electron microscope (Zeiss, Germany) equipped with a 30 µm objective aperture, operating at 80 kV. Three independent experiments were carried out and 50 cells were scored for each treatment combination.

Cloning procedure and sequence analysis

RNA isolation was carried out using the NucleoSpin RNA II extraction kit (Macherey-Nagel, M-Medical S.r.l., Cornaredo, Italy), according to manufacturer's instructions. cDNAs were obtained using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems International, Monza, Italy) according to manufacturer's suggestions. The degenerated oligonucleotide primer pair: MT2-fw (5'-ATGTAYCCAGAYCTTGGTTTYTCWG-3') and MT2-rev (5'-CCRTTWGAACCRCAYTTRCAATC−3') was used to amplify the *VFMT2* sequence. Amplification was obtained using the following PCR conditions: 94˚C 50 s, 45˚C 50 s, 72˚C 1 min (5 cycles), 94˚C 50 s, 50˚C 50 s, 72˚C 1 min (30 cycles) in a T-Gradient PCR apparatus (Biometra GmbH, Goettingen, Germany), using the *Taq* Polymerase (Celbio, Milan, Italy). PCR products were purified from agarose gel (Duchefa Biochemicals) using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences GmbH, Milan, Italy) and subsequently cloned into the pTZ57R vector, using the InsT/Aclone™ PCR Product Cloning Kit (Fermentas, M-Medical S.r.l., Cornaredo, Italy). The recombinant plasmid pTZ57R-VFMT2 was then purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega, Milano, Italy). Sequence analysis was performed with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit, using the ABI PRISM R310 Genetic Analyzer (Applied Biosystems, Monza, Italy).

Quantitative Real Time Polymerase Chain Reaction (QRT-PCR)

The expression profiles of *VFMT2* gene was analyzed by QRT-PCR. The reactions were carried out in 25 µl, using the QuantiTect SYBR Green PCR kit (Qiagen), according to manufacturer's instruction, in a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Australia). An ubiquitin gene (GenBank Accession No. DQ056362) was used as standard control in the QRT-PCR reactions. The oligonucleotide primer sets were designed using the Real-Time PCR Primer Design program from GenScript (Table 1). An amplicon size of ~ 100 bp was targeted in order to obtain efficient amplification. Conditions were as follows: denaturation at 95°C for 15 min, cycling at 94°C 15 sec, 60°C 30 sec, 72°C 30 sec (40 cycles). For each primer set, a no-template water control was used. The QRT-PCR results were interpreted using the LinRegPCR computer software [19]. The Logarithm of Relative Fluorescence Unit (LogRFU) was used for graphic representation.

Table 1. Sequences of oligonucleotide primes utilized in QRT-PCR.

OLIGONUCLEOTIDE	SEQUENCE	AMPLICON SIZE	EFFICIENCY*
qPmt2f	5'-CACCACAAGTCACACGATCA-3'	105 bp	1.72
qPmt2r	5'-CCACATTTGCAACCATTCTC-3'		
qPubyf	5'-TTTGACTGGGAAGACCATCA-3'	99 bp	1.86
qPubyr	5'-CTCTGTTGGTCTGGAGGGAT-3'		

* Efficiency of the primer pair in QRT-PCR

Bioinformatic and statistical analysis

Comparison of amino acid sequences was obtained using ClustalW multiple sequence alignment program (http://www.ebi.ac.uk/Tools/clustalw/index.html accessed in sept. 2010). The Motif Scan tool (http://myhits.isb-sib.ch/cgi-bin/motif_scan accessed in sept. 2010) was used to identify specific protein motives. Oligonucleotide pairs for QRT-PCR analysis were designed using the Real-Time PCR Primer Design program from GenScript (https://www.genscript.com/ssl-bin/app/primer accessed in sept. 2010). The QRT-PCR results were subjected to Analysis of Variance (ANOVA) and the means were compared by the Tukey's test.

RESULTS

Cell viability in white poplar cell suspension cultures exposed to heavy metals

Cell death caused by exposure to heavy metals was evidenced by staining with Evans blue. Viable cells were colourless, with an intact protoplast with dense cytosol (Fig. 1A), while blue coloration and protoplast shrinkage were evidenced at high frequency when cells were challenged with heavy metals (Fig. 1B).

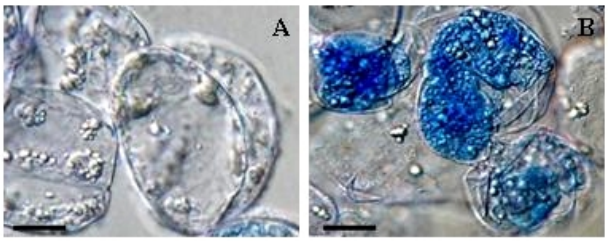

Figure 1. Cell death evidenced by Evans blue staining. (**A**) Untreated viable cells. (**B**) Cell death in response to copper (100 µM $CuCl_2$) 6 h following treatment. Bar: 15 µm.

The cell death rates were determined using a spectrophotometric assay (Fig. 2). In the absence of heavy metals (NT), the percentage of dead cells in the population was about 5%. In contrast, the rate of cell death reached 27% (1SD 2.8), 28% (1SD 4.6) and 21% (1SD 1.5) after 6 h of incubation with the highest concentration of cadmium, copper and zinc, respectively. The Analysis of Variance revealed significant differences in the rate of cell death for all the treatments in relation with the untreated sample (P<0.001). An even more significant increase in the percentage of dead cells was observed after 24 h of inoculation (data not shown).

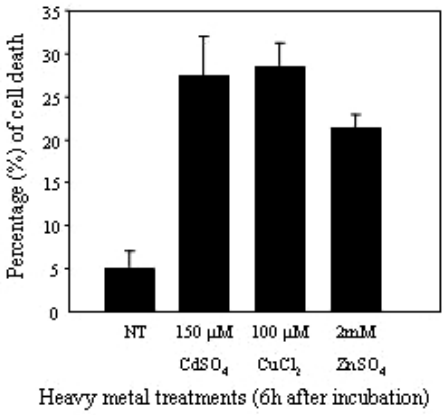

Figure 2. Percentage of cell death induced by heavy metals at 6 h following treatments.

Cloning and characterization of the white poplar *VFMT2* gene

The *Populus alba VFMT2* cDNA (220 bp) (Fig. 3) was amplified using degenerated primers, designed starting from the metallothionein sequences of *P. trichocarpa*, available on database.

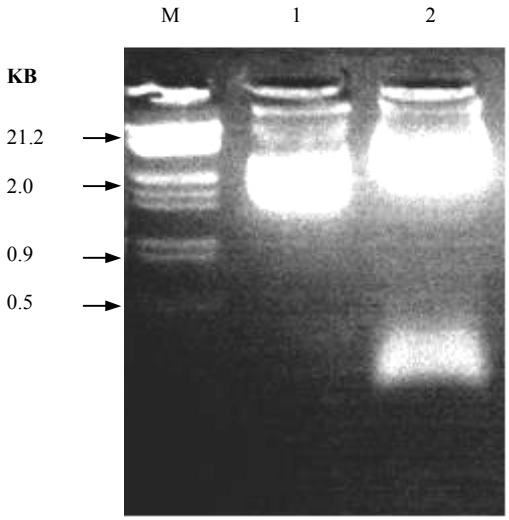

Figure 3. Restriction analysis of VFMT2 clone. 1 – undigested pTZ57R-VFMT2 plasmid; 2 – plasmid digested with *Bam*HI and *Eco*RI; M – molecular marker Lambda DNA/*Eco*RI+*Hind*III.

The *VFMT2* gene contains an open reading frame (ORF) of 220 bp encoding a protein of 73 amino acids (aa) with a putative molecular mass of 7.2 kDa. The VFMT2 protein contains two Cys-rich domains separated by a spacer of about 40 aa. A Cys-Cys motif is located at positions 8 and 10 within the N-terminal domain. Overall, the sequence of the N-terminal domain is highly conserved among type 2 metallothioneins. A Cys-Gly-Gly-Cys motif is also present at the end of the C-terminal Cys-rich domain (Fig. 4). The search for other motifs performed with MotifScan Tool revealed the presence of three putative phosphorylation sites and six N-myristoylation sites, which are known to play a key role in signal transduction in plants in response to environmental

stress (data not shown). The VFMT2 amino acid sequence shows a 41% identity with the hybrid poplar *P. trichocarpa x P. deltoides* MT2a sequence (GenBank Accession No. AAT02524), a 38% identity with the *A. thaliana* MT2b sequence (GenBank Accession No. NP195858) and a 31% identity with the *Oryza sativa* MT2 sequence (GenBank Accession No. ABR25595). The overall similarity between the type 2 metallothionein proteins in these species is around 25% (Fig. 4).

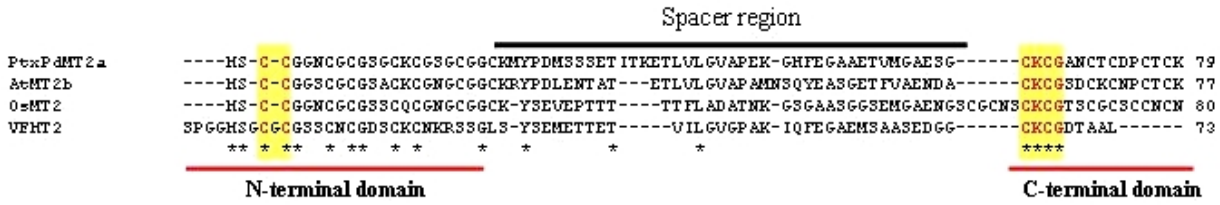

Figure 4. Comparison of the VFMT2 amino acid sequence with the MT2 amino acid sequences from *P.trichocarpa x P. deltoides* (PtxPdMT2a), *A. thaliana* (AtMT2b) and *O. sativa* (OsMT2). The cysteine-rich domains at the C-terminal and N-terminal domains are shown in red and yellow boxes. The spacer region is also underlined. The conserved amino acids are marked by asterisks ("*").

Expression profiles of the *VFMT2* gene in relation to heavy metal-induced stress

The expression profiles of the *VFMT2* gene in white poplar cell suspension cultures challenged with heavy metals were evaluated by QRT-PCR (Fig. 5). The 'Villafranca' cell suspension cultures were treated with increasing concentrations of cadmium (75 and 150 µM $CdSO_4$), copper (50 and 100 µM $CuCl_2$) and zinc (1 and 2 mM $ZnSO_4$) and cells were then collected and analyzed at different time points (0, 4, 6 and 8 h). Significant changes in the *VFMT2* gene expression were observed in white poplar cells treated with cadmium. At the lowest concentration (75 µM $CdSO_4$), the Analysis of Variance showed a significant increase in the amount of *VFMT2* mRNA ($P<0.05$) compared with the untreated control at 4 and 6 h after treatment (with a peak of 3-fold increase at 6 h), while no differences were observed after 8 h (Fig. 5 $CdSO_4$). With the highest dose (150 µM $CdSO_4$), the *VFMT2* gene was up-regulated (2-fold) at 6 h after treatment, while down-regulation (0.5-fold) was observed at 8 h. According to Tukey's test, the average level of *VFMT2* mRNA was significantly enhanced ($P<0.001$) at 6 h and decreased ($P<0.05$) at 8 h. Also when the cell suspension cultures were treated with copper, a significant up-regulation was observed in the *VFMT2* mRNA levels ($P<0.05$). In this case, the highest expression was evidenced at 4 h after treatment for both doses (2-fold and 5-fold, respectively). It is worth noting that the treatment with 100 µM $CuCl_2$, resulted into a much higher expression of the *VFMT2* gene than the lowest dose (Fig. 5 $CuCl_2$). As for the zinc treatments, results from the QRT-PCR analysis show that at the lowest dose (1 mM $ZnSO_4$) the *VFMT2* mRNA level was slightly increased at 4, 6 and 8 h after treatment in comparison with the untreated sample. However, when a higher dose was used (2 mM $ZnSO_4$) the *VFMT2* mRNA levels were significantly ($P<0.05$) enhanced at 6 h after incubation and subsequently decreased at 8 h (Fig. 5 $ZnSO_4$).

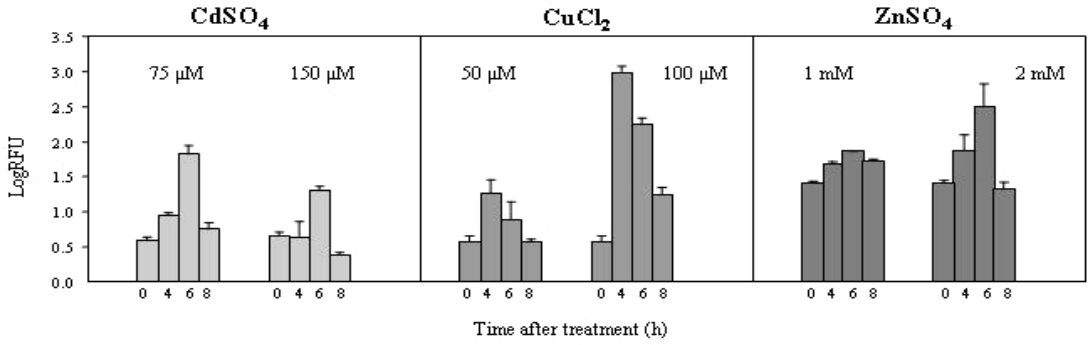

Figure 5. Results from QRT-PCR analysis carried out on *P. alba* cv. 'Villafranca' cell suspension cultures challenged with heavy metals.

DISCUSSION

Several studies have so far demonstrated that *Populus* species represent useful tools for phytoremediation and that it is important to understand the basal aspects of cellular and molecular mechanisms which are responsible for heavy metal tolerance in trees. The clonal cultivar 'Villafranca', investigated in this study, is currently used in field trials on polluted areas and it was also genetically modified to obtain clones with enhanced antioxidant defenses [2, 22].

In the present work, the response of 'Villafranca' cell suspension cultures to different concentrations of Cd, Cu and Zn, was evaluated using cellular and molecular approaches.

The cell morphology of white poplar suspension cultures exposed to heavy metals and stained with Evans blue was screened to assess the rate of cell death in the cell population. The reported data showed that at 6 h following heavy metal treatment, the cell death percentage significantly increased (approximately 6-fold) in comparison with the untreated cells. This finding is in agreement with previous reports [1, 3]

describing the cell death response of white poplar cells exposed to heavy metals. In these studies, changes in the PCD/necrosis ratio in relation to the heavy metal concentration were also highlighted.

The expression profiles of *VFMT2* gene from *P. alba* were analyzed by QRT-PCR, an innovative, precise and rapid technique for mRNA detection and quantification [5]. The *VFMT2* gene is differently expressed in response to heavy metal treatments. The highest up-regulation was observed with 100 µM $CuCl_2$ at 4 h after inoculation, while Cd-induced treatments showed a peak of expression at 6 h. On the other hand, a lower induction of *VFMT2* gene expression was observed when cells were exposed to Zn treatments. Thus, the *VFMT2* gene is responsive to heavy metal stress as demonstrated for other plant *MT* genes. A number of reports provide evidence of a correlation between *MT* gene expression and heavy metals. In *Arabidopsis* seedlings, the *MT2* transcript was induced by Cu, Cd and Zn treatments [23]. In tomato (*Lycopersicon esculentum*) root tips, the *LEMT2* gene expression was strongly induced by Zn, while Cu had the opposite effect [12]. White poplar plantlets exposed to high Cu concentrations did not show induction of *MT* genes expression, while in Zn-treated shoots the expression of *MT* genes was highly up-regulated [7]. These results suggest that different plant species and experimental systems might respond differently, in terms of *MT* gene expression, to the same heavy metal. Our results also show that the induction of *VFMT2* gene expression was not always dose-dependent and it did not increase linearly with exposure time. Furthermore, it is worth noting that at 8 h after treatments, the *VFMT2* mRNA level dramatically decreased, and this is in agreement with the results obtained in hybrid poplar (*P. trichocarpa x P. deltoides*), where the Cu-induced *PtdMT2a/b* gene expression was stimulated at 18 h and inhibited after 24 h [14]. This might be due to the fact that the heavy metal could be detoxified and, therefore, the *MT* transcripts return to control levels, or that the amount of *MT* mRNA is reduced due to excessive cell damage.

In conclusions, the results reported in the present paper show that the *VFMT2* gene is differently regulated in response to heavy metal treatments. The experimental work carried out on *P. alba* cv 'Villafranca' expands the information concerning the cellular responses to heavy metals in forest tree species, strengthening the applicability of cell suspension cultures as a model system.

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