Cloning and expression of multiple metallothioneins from hybrid poplar

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Summary

• In an effort to understand processes that are related to heavy metal sequestration, we characterized six metallothionein genes (*PtdMTs*) in the hybrid cottonwood, *Populus trichocarpa* × *deltoides*.

• cDNA microarrays and reverse transcriptase-polymerase chain reaction were used to examine *PtdMT* expression in poplar tissues. They displayed differential gene expression patterns, which may be associated with the diverse roles and functions PtdMTs have in coping with particular developmental (e.g. root development and leaf senescence) and environmental cues.

• The heterologous expression in a cadmium (Cd)-hypersensitive yeast mutant showed the ability of *PtdMT* cDNAs to confer Cd tolerance. The concentration of *PtdMT* mRNAs were increased by zinc, but not by copper and Cd.

• Further studies will help to clarify the role of metallothionein genes in metal homeostasis and poplar development, and help to isolate poplar genotypes particularly tolerant to stress for use in experiments of phytoremediation.

Key words: functional complementation, heavy metal, metallothionein, *Populus trichocarpa* × *P. deltoides*, root development, senescence.

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Introduction

Transition elements such as iron (Fe), cobalt (Co), nickel (Ni), copper (Cu) and zinc (Zn) play a wide variety of roles in biology as enzyme cofactors and must be absorbed from the soil by plants. However, either naturally or as a result of human activity, these metals can be present at potentially toxic concentrations. A number of other metal ions with no known biological function, including cadmium (Cd), lead (Pb) and mercury (Hg), are also potentially highly toxic for plants. There are two basic strategies for decreasing the toxicity of metals: chelation or efflux from the cytosol, either into the apoplast, or by intracellular sequestration. Metallothioneins (MTs) are defined as low molecular mass cysteine-rich proteins that can bind heavy metals and may play a role in their intracellular sequestration. Since they were first purified from horse kidney (Margoshes & Vallee, 1957; Kägi & Vallee, 1960), MT genes and proteins have been discovered in many prokaryotic and eukaryotic organisms (Robinson *et al.*, 1993; Cobbett & Goldsbrough, 2002). In animals and fungi, MTs form complexes with heavy metals and transcription of *MT* genes is regulated by metals (Thiele, 1992). Metallothioneins play a role in detoxification of heavy metals and in homeostasis of intracellular metal ions (Cobbett & Goldsbrough, 2002). However, their exact function is still not completely understood.

Metallothionein proteins have been classified based on the arrangement of their cysteine (Cys) residues. Class I MTs are widespread in vertebrates, while class II MTs include all those from plants and fungi as well as from nonvertebrate animals. Based on their conserved Cys residues, plant MTs have been further divided into four types (Cobbett & Goldsbrough, 2002).

In plants, many genes and cDNAs encoding *MTs* have been isolated, but only one MT protein from wheat (Lane *et al.*, 1987) and two *Arabidopsis* MT proteins (Murphy *et al.*, 1997) have been purified from plant tissues to date. Most information about their putative function is derived from mRNA

expression studies. Existing data on the effect of metal ions and the role of plant MTs in metal detoxification and homeostasis is inconsistent. Copper, for example, induced the expression of type 1 *MTs* in *Arabidopsis* (Zhou & Goldsbrough, 1994), rice (Hsieh *et al.*, 1995) and tobacco (Choi *et al.*, 1996) but repressed the mRNA level of *MT*-like genes in *Mimulus guttatus* (de Miranda *et al.*, 1990). When expressed in MT-deficient strains of yeast (Zhou & Goldsbrough, 1994) and *Synechococcus* (Robinson *et al.*, 1996), *Arabidopsis* MTs were able to complement these mutations and to restore tolerance to Cu and Zn, respectively. The situation in plants is further complicated by the presence of the enzymatically synthesized phytochelatins (class III MTs) (Cobbett, 2000), which have been shown to play an important role in heavy metal detoxification (Cobbett & Goldsbrough, 2002).

Plant *MT* gene expression is regulated by many other factors, including different stresses such as wounding (Choi *et al.*, 1996), pathogen infection (Choi *et al.*, 1996; Butt *et al.*, 1998), symbiotic interaction (Laplaze *et al.*, 2002) and leaf senescence (Bhalerao *et al.*, 2003; Andersson *et al.*, 2004), suggesting that MTs may be expressed as part of a general stress response (Cobbett & Goldsbrough, 2002). It has further been argued that they function as antioxidants and play a role in plasma membrane repair (Hall, 2002). Plant *MT* genes are expressed in a tissue-specific manner. Expression of type 1 *MTs* tends to be higher in roots than in shoots, whereas the reverse is generally observed for type 2 *MTs* (Cobbett & Goldsbrough, 2002). Type 3 *MTs* are expressed at high levels in leaves and in ripening fruits, whereas the expression of type 4 *MTs* remains restricted to developing seeds (Cobbett & Goldsbrough, 2002).

In an effort to understand processes that are related to heavy metal sequestration in poplar and associated ectomycorrhizal fungi, we are characterizing the expression of genes coding for transporters (e.g. *PtdMTP1*; Blaudez *et al.*, 2003) and proteins involved in heavy metal binding, such as MTs. Various types of *Populus trichocarpa* × *Populus deltoides* MTs (*PtdMTs*) were identified in the PoplarDB EST database (http://mycor.nancy.inra.fr/poplardb/pub.html; Kohler *et al.*, 2003) and experiments were performed to analyse their functional properties and expression.

Materials and Methods

Plant growth conditions and treatments

All experiments were performed using rooted cuttings of the hybrid poplar *P. trichocarpa* × *P. deltoides* cv. Beaupré. This cultivar is one of the major hybrid poplars used in European large-scale plantations. For the construction of the three cDNA libraries (i.e. adventitious roots, water-stressed roots and expanding leaves), poplar cuttings were grown for 2 months in a glasshouse, as described by Kohler *et al.* (2003). For the dehydration treatment, 2-month-old plants were further grown for 19 d under restricted irrigation to reach 50% of the transpiration rate of fully watered plants (Kohler *et al.*, 2003). Leaves at different developmental stages (young, plastochron index (PI) 1; mature (PI 8) and senescent (PI 15) leaves) were harvested from 5-month-old poplar trees for RNA extraction.

For the analysis of adventitious root development, tissue-specific expression, and the metal treatment, dormant poplar cuttings (25 cm) were rooted in plastic boxes containing the following growth liquid medium: 7.91 mM KNO_3, 7.96 mM $Ca(NO_3)_2{\cdot}4H_2O$, 2.69 mM $NaH_2PO_4{\cdot}2H_2O$, 3 mM $MgSO_4{\cdot}7H_2O$ and micronutrients (Kanieltra 6Fe; Hydro Azote, Ambès, France). The Cu and Zn concentrations were 2 μM and 7 μM, respectively. The aeration (250 l h^{-1}) was provided by aquarium pumps and plantlets were kept in a controlled environment growth chamber (17 h photoperiod at 200 μmol m^{-2} s^{-1} at 23°C). Developing adventitious roots of cuttings were harvested at six different stages (I, dormant bark tissues; II, root primordia; III, root calli; IV, emerging roots; V, primary adventitious roots, and VI, lateral roots) for RNA isolation. Preformed root initials lie dormant in cuttings and develop when cuttings were placed in the growth medium. The first macroscopic evidence of root initiation was the appearance of the root primordium, as a stem outgrowth, 3–4 d after the beginning of the culture. A callus was clearly seen after 4–5 d and root tip emerged from the callus at 6 d. Primary adventitious roots then elongated for 2 d before lateral root tips formed at 10 d. The first cell divisions, which occurred in the stem parenchyma, started at *c.* 24 h of culture. Photographs of the different developmental stages are available at http://mycor.nancy.inra.fr/poplardb/. For metal treatments, $CuSO_4$ (50 or 150 μM), $ZnSO_4$ (1 mM) or $CdSO_4$ (25 or 100 μM) were added to freshly replaced liquid medium of 3-wk-old rooted cuttings. Roots for RNA extraction were harvested at 6, 18, 30 and 48 h after addition of heavy metals, snap frozen in liquid nitrogen and stored at −70°C pending analysis.

cDNA library construction, DNA sequencing, sequence processing and analysis

Three cDNA libraries were constructed as described by Kohler *et al.* (2003) using the SMART cDNA synthesis kit in λTriplEx2 (Clontech, Palo Alto, CA, USA). Library R was made from the adventitious root system of 2-month-old rooted cuttings. For the library RSH, the root system of 2-month-old plants that were drought-stressed for 19 d was used. Library F was constructed from expanding leaves of 2-month-old poplar plants. DNA sequencing, sequence processing and analysis were as previously described (Kohler *et al.*, 2003). The program MULTALIN (Corpet, 1988) was used for multiple alignment of the *PtdMT* nucleotide sequences and for the alignment of deduced PtdMT amino acid sequences

with other plant MT amino acid sequences retrieved from the National Centre for Biotechnology Information (NCBI) database.

The *MT* genes were identified using the six different *PtdMT* cDNAs of *P. Trichocarpa* × *deltoides* as a query in a BLAST (Altschul *et al.*, 1990) search against the raw genomic sequence database of *P. trichocarpa* cv. Nisqualy available at the Joint Genome Institute (JGI) (http://genome.jgi-psf.org/poplar0/poplar0.home.html). Assembly of the retrieved genomic sequences into groups of tentative gene sequences was performed using the contig routine (85% identity over 40 nucleotide (nt) length) of SEQUENCHER (version 4.2) (Gene Codes Corporation, Ann Arbor, MI, USA) program for Macintosh. This will be available on the PoplarDB web site: http://mycor.nancy.inra.fr/poplardb/pub.html. Analysis of the intergenic regions for putative transfactor binding sites was done using the PLACE database (Higo *et al.*, 1999).

RNA extraction

Total RNA extraction was performed with the RNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France) from 100 mg of frozen (−80°C) material. For the RNA extraction from root tissues, 20 mg ml^{-1} of polyethylene glycol (PEG) 8000 (Sigma, St Quentin Fallavier, France) was added to the Qiagen homogenization buffer containing guanidine hydrochloride. DNase I (Qiagen) treatment was included in the extraction procedure according to the manufacturer's instructions to eliminate traces of genomic DNA.

Generation and analysis of cDNA arrays

A Unigene set was selected from the expressed sequence tags (ESTs) from adventitious roots (library R) and leaves (Library F) (Kohler *et al.*, 2003) and 4608 polymerase chain reaction (PCR)-amplified cDNA inserts were spotted onto nylon membranes by using the *Bio*Grid arrayer (*Bio*Robotics, Cambridge, UK) according to the manufacturer's instruction (Eurogentec, Saraing, Belgium). The transcript populations of leaves, roots and developing adventitious root tissues were separately amplified, ^{33}P-labelled and hybridized to nylon microarrays as described previously (Lacourt *et al.*, 2002). Owing to the high hybridization stringency used here, cross-hybridization was only observed between the highly similar *PtdMT1a* and *PtdMT1b* sequences. Air-dried filters were then wrapped in plastic foils and exposed to a phosphor screen (Eastman Kodak Company, Rochester, NY, USA) for varying periods (12 h to 3 d), after which the imaging plate was scanned by a Personal Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA, USA) at a resolution of 50 µm per pixel. Two series of two biological repeats were carried out for analysing the root development using different array generations. Only one series of representative data (i.e. two biological repeats) is shown. These biological repeats were performed with different filters and independent cDNA probes derived from plant material corresponding to pools of at least 12 rooted cuttings for each time point, thus minimizing variation between individual samples, filters or probes.

The raw image data obtained with the phosphorimager imaging system were imported into the XDotsReader program (version 2.0; Cose, Paris, France) and quantified. Detection and quantification of the signals were performed using the 'volume quantification' method. Each spot was defined by automatic grid positioning over the array image and average pixel intensity of each spot was determined. Net signal was determined by subtraction of the local surrounding background from the intensity for each spot. The data table generated by XDotsReader, containing the intensity of each spot, was then exported to the EXCEL X:mac worksheet program (Microsoft, Redmond, WA, USA) for further manipulation. Spots that had an intensity of less than twofold that of the background were flagged as undetectable and had their intensity raised to a minimum threshold value of 0.1 to avoid spurious expression level ratios at the bottom of the spot intensity range. To take account of experimental variations in specific activity of the cDNA probe preparations or exposure time that might alter the signal intensity, the raw data obtained from different hybridizations were normalized (scaled) by dividing the intensity for each spot by the average of the intensities of all the spots present on the filter, to obtain a centered, normalized value (Eisen *et al.*, 1998). A Microsoft EXCEL spreadsheet with the full data set of MT transcripts will be available on the PoplarDB web site (http://mycor.nancy.inra.fr/poplardb/pub.html).

Data quality assessment was performed using a Bayesian statistical framework implemented in the Cyber–T web interface (http://www.igb.uci.edu/servers/cybert/) (Long *et al.*, 2001).

Reverse transcriptase (RT)-PCR

First strand cDNA was synthesized from 200 to 300 ng DNase-treated total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Marnes-la-Coquette, France) in a total volume of 20 µl according to the manufacturer's instructions. One microliter of RT products were amplified by PCR under the following conditions: 94°C for 2 min followed by 25–30 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 45 s, and a final extension at 72°C for 2 min (PtdMT1a, PtdMT1b, PtdMT2b, PtdMT3a, PtdMT3b and Ubiquitin) or 94°C for 2 min followed by 25–30 cycles at 94°C for 30 s, 68°C for 30 s and 72°C for 45 s, and a final extension at 72°C for 2 min (PtdMT2a). The specific primers listed in Supplementary Material Table 1 (see Supplementary Material section for details) were used for PCR. Aliquots of 5–10 µl of the PCR products were run on 2% agarose gels and stained with ethidium bromide. Gels were visualized under UV-light and

Table 1 Frequency of metallothioneins in cDNA libraries from *Populus trichocarpa* × *Populus deltoides* cv. Beaupré

	cDNA library R (roots)	cDNA library RSH (drought-stressed roots)	cDNA library F (leaves)
PtdMT1a	97	4	0
PtdMT1b	163	12	0
PtdMT2a	13	0	0
PtdMT2b	20	5	1
PtdMT3a	4	0	0
PtdMT3b	1	0	3
Total *MTs* (%)	298 (6.4)	21 (2.0)	4 (0.7)
Total number of ESTs	4655	1026	549

EST, expressed sequences tag.

images were taken using a gel documentation system (Vilber Lourmant, Marne-la-Vallée, France). Images in TIFF format were uploaded in Adobe Photoshop and a negative version of the images is shown in the figures. All PCRs were replicated at least twice using first strand cDNA from two independent RNA preparations (i.e. biological repeats).

Yeast complementation assays

The full-length open reading frames (ORFs) of *PtdMTs* were amplified with *Pfu* polymerase (Promega, Madison, WI, USA) and pTriplEx2 plasmids containing the corresponding MT cDNAs as template and using the specific primers listed in Supplementary Material Table 2 (see Supplementary Material section for details). These primers introduced a *Bam*HI site and a *Hin*dIII site at the 5′- and 3′-end of the fragment, respectively. The *Bam*HI–*Hin*dIII fragments were digested and introduced into the *Bam*HI–*Hin*dIII-digested pYES2 plasmid (Invitrogen, Paisley, UK). pYES2-MT constructs and the empty pYES2 vector were used to transform the *yap-1*Δ yeast mutant (Kuge & Jones, 1994). Yeast cells were transformed as described by Gietz *et al.* (1992). Transformants were selected on synthetic defined (SD) medium (2% glucose) lacking uracil. Metal tolerance tests were performed on SD plates (2% galactose) containing different concentrations of Cd.

Results

The poplar genome contains at least six *MT* genes

We searched the EST database of *P. trichocarpa* × *deltoides* roots (Kohler *et al.*, 2003) for sequence homologies to known *Arabidopsis* MT types. Expressed sequence tags coding for members of the *MT* gene family were abundant in the adventitious root library (Table 1). Multiple alignment of these ESTs revealed the existence of six different *MT* cDNAs distributed in three subgroups (Fig. 1). We named the poplar MTs, PtdMTs (*P. trichocarpa* × *deltoides* metallothioneins), in line with the suggestion in a recent review (Cobbett & Goldsbrough, 2002). This classification system recognizes four MT categories based on the amino acid sequence and the distribution of conserved cysteine residues. *PtdMT1* cDNAs (Accession nos AY594295 and AY594296) contain 219-bp ORFs encoding 73 amino acids with predicted molecular mass of 7594 Da for PtdMT1a and 7640 Da for PtdMT1b. The deduced amino acid sequences of PtdMT1a and PtdMT1b were 90.41% identical to each other. Six Cys-Xaa-Cys (where Xaa represents another amino acid) motives are distributed equally among two domains, separated by a spacer of 40 amino acids, as found in other plant type 1 MTs (Cobbett & Goldsbrough, 2002). PtdMT1a and PtdMT1b sequences were closer to type 1 MTs from alfalfa and rice than to *Arabidopsis* MT1, where the internal spacer is missing.

The *PtdMT2a* cDNA (Accession no. AY594297) contains a 237-bp ORF encoding 79 amino acids with a predicted molecular mass of 7792 Da. The *PtdMT2b* cDNA (Accession no. AY594298) contains a 234-bp ORF encoding 78 amino acids with a predicted molecular mass of 7901 Da. The deduced amino acid sequences of PtdMT2a and PtdMT2b were 70.9% identical to each other and they showed 57% (PtdMT2a) and 74% (PtdMT2b) identity to PtdMT1a. PtdMT2a and PtdMT2b belong to the type 2 MTs, characterized by a Cys-Cys motif in amino acid positions 3 and 4, an overall highly conserved *N*-terminal domain and six Cys residues in the *C*-terminal domain arranged as Cys-Xaa-Cys motifs (Cobbett & Goldsbrough, 2002).

The two *PtdMT3* cDNAs (Accession nos AY594299 and AY594300) contain 198-bp ORFs encoding 66 amino acids with predicted molecular mass of 6966 Da for PtdMT3a and 6857 Da for PtdMT3b. The deduced amino acid sequences of PtdMT3a and PtdMT3b were 83% identical to each other, but they each showed 27% and 26% identity to PtdMT1a. PtdMT3a and PtdMT3b are type 3 MTs characterized by only four Cys residues in the *N*-terminal domain (Cobbett & Goldsbrough, 2002).

The deduced amino acid sequences of the six PtdMTs were aligned with other plant MTs (Fig. 1b). Conserved cysteine signatures characteristic for the MTs were identified in all six poplar MT polypeptides (Fig. 1b). It is of interest to note that the 5′-untranslated and the 3′-noncoding regions of each cDNA are large (See Fig. 1 in the Supplementary Material, http://mycor.nancy-inra.fr/poplardb/pub.html).

(a)

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PtdMT3a  ATGTCTAGCACCTGCGACAA------CTGCGACTGCGCTGACAAGACCCAGTGTGTCAAGAAGGGAAGCAGCTACACTGCTGACATCGTTGAGACTGAGAAGAGCCATGTCTACACTGGA
PtdMT3b  ATGTCTAGCACCTGCGACAC------CTGCGACTGCGCTGACAAGACCCAGTGTGTCAAGAAGGGAAGCAGCTACACTGCTGGCATCGTCGAGACTGAGAAGAACTATGTCTCCGCCGTA
PtdMT1a  ATGTCTGGCTGTAG------------CTGTGGCTCTGACTGCAAGTGTGGCAGTGGCTGCAA---ATGTGGCATGTACCCTGACTTAGGTTTCTCAGAGAACACCACAACTGAGACAATC
PtdMT1b  ATGTCTGGTTGTAG------------CTGTGGCTCTGACTGCAAGTGCGGCAGTGACTGCAA---ATGCGGCATGTACCCTGACTTGGGTTTCTCAGAGAGCACCACAACCGAGACAATC
PtdMT2b  ATGTCTTGCTGTGGAGGAAACTGTGGCTGCGGCTCTGGATGCAAGTGCGGCAGTGGCTGCAATGGATGCAGCATGTACCCAGACTTGAGTTTCTCCGAGACCACCACAAGTCAGACAATC
PtdMT2a  ATGTCTTGCTGTGGAGGAAACTGTGGGTGTGGCTCTGGCTGCAAGTGCGGCAGCGGCTGTGGAGGATGCAAGATGTACCCTGACATGAGCTCCTCGGAGACGATCACCAAAGAAACTCTG

PtdMT3a  GTCATGGAGGTTCCAGCAACCGAGAACGATGGCAAG-----------------------------------TGCAAGTGCGGCGCTAACTGCACTTGCACTACCTGCACATGCGGTCATTAA
PtdMT3b  GTCATGGAGGTGCCTGCAGATGAGAACGATGGCAAG-----------------------------------TGCAACTGCGGTACTGGCTGCACTTGCACTACCTGCACATGCGGTCATTAA
PtdMT1a  ATTGCAGGTGTTGCACCAGTTAAGATGTTCTATGAGAGGTCTG---AGATGGACTTCGGTGCTGAGAATGGCTGCAAGTGTGGATCAAACTGCACCTGTGATCCATGCTCCTGCAAATGA---
PtdMT1b  ATTGCAGGTTTTGCACCAGTTCAGATGTTCTACGAGAGGTCTG---AGATGAACTCTGGTGCTGAGAATGGCTGCAAGTGTGGATCAAACTGCACCTGCGATCCATGCACTTGCAAATGA---
PtdMT2b  ATTGCTGGTGTTGCTCCAGTTAGGATGTTCTACGAGAGCTCTG---AGACGAACTTTGGTGCTGAGAATGGCTGCAAATGTGGATCAAACTGTACCTGTGATCCATGCTCCTGCAAATGA---
PtdMT2a  GTTCTTGGTGTGGCACCAGAGAAGGGTCACTTTGAGGGAGCTGCTGAGACGGTCATGGGAGCCGAGAGTGGCTGCAAGTGTGGAGCCAACTGTACCTGCGATCCCTGCACTTGTAAATGA---
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(b)

Type 1

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PtdMT1a   --MSGCSCGSDCKCGSGCKCGMYPD-LGFSENTTTETIIAGVAPVKMFYERSEMDFGAENG--CKCGSNCTCDP-CSCK
PtdMT1b   --MSGCSCGSDCKCGSDCKCGMYPD-LGFSESTTTETIIAGFAPVQMFYERSEMNSGAENG--CKCGSNCTCDP-CTCK
MsMT1     --MSGCNCGSSCNCGDNCKCNSRSSGLGYLEGETTETVILGVGPAKIHFEGAEMGVAAEDG-GCKCGDSCTCDP-CNCK
OsMT1     ---MSCSCGSSCSCGSNCSCGKKYPDLEEKSSSTKATVVLGVAPEKKAQQFEAAAESGETAHGCSCGSSCRCNP-CNC-
AtMT1c    MAGSNCGCGSSCKCGDSCSCEKNYN---------------------------------KECDNCSCGSNCSCGSSCNC-
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Type 2

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PtdMT2b   MSCCGGNCGCGSGCKCGSGCNGCSMYPDLSFS-ETTTSQTIIAGVAPV-RMFYE-SSETNFGAENGCKCGSNCTCDPCSCK-
PtdMT2a   MSCCGGNCGCGSGCKCGSGCGGCKMYPDMSSS-ETITKETLVLGVAPE-KGHFEGAAETVMGAESGCKCGANCTCDPCTCK-
PhMT2     MSCCGGNCGCGSGCKCGNGCGGCKMYPDFSYT-ESTTTETLILGVGPE-KTSF-GSMEMGSPAENGCKCGSDCKCDPCTCSK
AtMT2a    MSCCGGNCGCGSGCKCGNGCGGCKMYPDLGFSGETTTTETFVLGVAPAMKNQYEASGESNNAENDACKCGSDCKCDPCTCK-
AtMT2b    MSCCGGSCGCGSACKCGNGCGGCKRYPDL----ENTATETLVLGVAPAMNSQYEASGETFVAENDACKCGSDCKCNPCTCK-
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Type 3

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AtMT3     MSSNCGSCDCADKTQCVKKGTSYTFDIVETQESYKEAMIMDVGAEENNANCKCKCGSCSCVNCTCCPN
PtdMT3a   MSSTCDNCDCADKTQCVKKGSSYTADIVETEKSHVYTGVMEVPATENDGKCKCGAN-CTCTTCTCGH-
PtdMT3b   MSSTCDTCDCADKTQCVKKGSSYTAGIVETEKNYVSAVVMEVPADENDGKCNCGTG-CTCTTCTCGH-
AdMT3     MSDKCGNCDCADSSQCVKKGNS--IDIVETDKSYIEDVVMGVPAAESGGKCKCGTS-CPCVNCTCD--
OsMT3     MSDKCGNCDCADKSQCVKKGTSYGVVIVEAEKSHFE----EVAAGEENGGCKCGTS-CSCTDCKCGK-
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Fig. 1 Multiple alignment of the *PtdMT* nucleotide sequences (a) and alignment of the deduced PtdMT amino acid sequences with other plant MT amino acid sequences (b). Conserved cysteine residues in each of the six MTs are marked gray and START and STOP codons are in bold. Primer sequences are underlined. The protein sequences are predicted from cDNA sequences of *Populus trichocarpa* × *Populus deltoides* (Ptd), *Arabidopsis* (At), alfalfa (lucerne) (Ms), rice (Os), petunia (Ph), and kiwifruit (Ad).

Genomic sequences of PtdMTs

The six poplar *MT* genes corresponding to *MT* cDNAs were identified in the *P. trichocarpa* cv. Nisqually genomic sequence (http://genome.jgi-psf.org/poplar0/poplar0.home.html). Sequence alignment of the cDNAs and genomic sequences showed that *MT* genes contained one to three introns (see Fig. 1 in the Supplementary Material, http://mycor.nancy.inra.fr/poplardb/pub.html). The intron sizes range from 71 to 275 bp. In *PtdMT1a*, *PtdMT1b*, *PtdMT2a*, and *PtdMT2b*, the first intron (82–258 bp) is inserted immediately upstream of the ATG start codon. We analysed regions 5′-upstream of the start codon for potential transacting factor-binding sites in an effort to gain insight into how expression of the various *MT* genes may be regulated. All of the *MT* genes contained putative TATA and CAAT boxes in a region between −8 to −635 bp, relative to the predicted start codon (data not shown). A variety of other regulatory elements were also found to be in common with several of the *MT* genes. For example, *PtdMT1a* and *PtdMT1b* both contained MYC- and MYB-binding sites involved in drought- and abscisic acid-regulated gene expression. Both *PtdMT2a* and *PtdMT2b* contained ABRE-like and MYB-binding sequences required for stress and senescence, gibberellin-responsive element and G-box (data not shown).

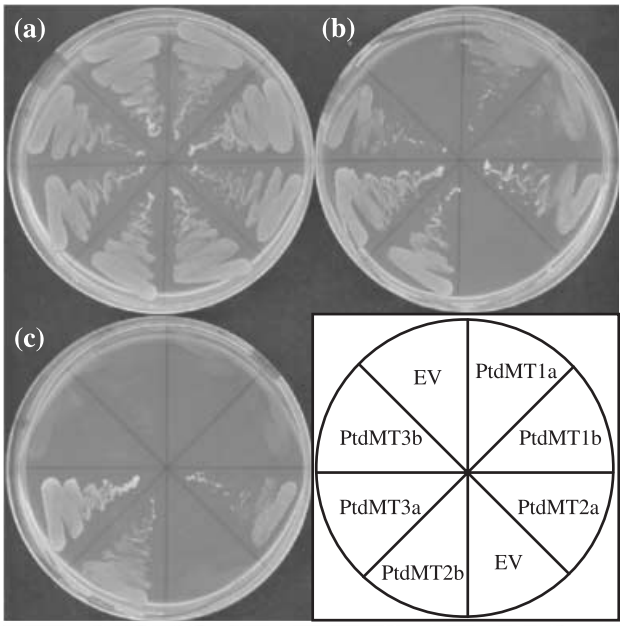

Fig. 2 Growth of *yap-1*Δ yeast cells expressing PtdMTs under various cadmium (Cd) concentrations. The Cd-sensitive *yap-1*Δ strain was transformed either with the pYES2 empty vector (EV) or with the different pYES2–*PtdMT* constructs. Cells were grown on normal SD medium (a) or supplemented with 75 μM (b) or 125 μM (c) Cd. Plates were incubated for 5 d at 30°C. Equivalent data were obtained in two other replicate experiments with independent transformants.

Functional expression of MTs in yeast

To confirm the ability of *PtdMTs* to confer heavy metal tolerance, we expressed their cDNAs in a yeast system. In yeast, various genes involved in heavy metal tolerance are under the positive control of the transcription factor YAP-1 (Kuge & Jones, 1994). Although yeast *MT* genes are not direct targets of YAP-1, *yap-1*Δ mutants are particularly sensitive to Cd and thus can be used to highlight tolerant phenotypes deriving from the expression of exogenous cDNAs (Wu *et al.*, 1993; Lanfranco *et al.*, 2002). We therefore expressed *Populus MT* cDNAs in *yap-1*Δ mutants and growth was monitored under control or Cd-supplemented medium. Figure 2 shows that cells transformed with the empty vector were unable to grow at 75 μM Cd, whereas cells expressing the six different PtdMTs could grow. The strongest tolerant phenotype (growth at 125 μM Cd) was obtained with cells transformed with PtdMT2a, PtdMT2b and PtdMT3a.

Occurrence of poplar MTs in EST databases

PtdMTs represented 6.4, 2.0 and 0.7% of the total ESTs in adventitious root, drought-stressed root, and leaf cDNA libraries (Table 1). *PtdMT1a* and *PtdMT1b* were the most abundant *MTs* in roots, whereas *PtdMT3b* was the most highly expressed in leaves. The percentage of *PtdMT2b* ESTs strikingly increased in drought-stressed roots. Microarray gene profiling confirmed that the expression levels of *PtdMT1a* and *PtdMT1b* were repressed two- to three-fold in drought-stressed roots compared with the expression levels in control roots, whereas the transcript level of *PtdMT2b* was slightly elevated in drought-stressed roots (data not shown).

To date, 154 509 *Populus* ESTs have been deposited in the NCBI EST database (dbEST release 030504); they have been sequenced from different poplar species (e.g. *P. trichocarpa* and *P. tremula*) and from a wide range of tissues (e.g. root, leaf, flower). To obtain information about similar *MTs* expressed in other poplar species and in other tissues, we searched for *PtdMT* homologs in the NCBI *Populus* EST database. The abundance of *MT* ESTs in the respective libraries and their tissues of origin are shown in Fig. 3. In order to more reliably reflect the abundance of *MT* transcripts in the tissues, cDNA libraries with < 500 sequenced ESTs were not considered. *PtdMT* homologs were found in cDNA libraries from *P. trichocarpa* (Sterky *et al.*, 1998), *P. tremula* (Sterky *et al.*, 1998; Bhalerao *et al.*, 2003; Andersson *et al.*, 2004), *P. tremula* × *tremuloides* (Sterky *et al.*, 1998; Bhalerao *et al.*, 2003; Andersson *et al.*, 2004; Nehls *et al.* unpublished), *Populus alba* × *tremula* (Déjardin *et al.*, 2004; J. S. Lee *et al.*, unpubl. data) and *P. tremuloides* (P. Ranjan *et al.*, unpubl. data) and in a wide range of tissues, such as flowers, wood, leaves and seeds (Fig. 3). Type 1 *MTs* (i.e. *PtdMT1a* and *PtdMT1b*) were preferentially expressed in roots, while *PtdMt3b* was found in high amounts in cDNA libraries from leaves. Type 2 *MTs* (i.e. *PtdMT2a* and

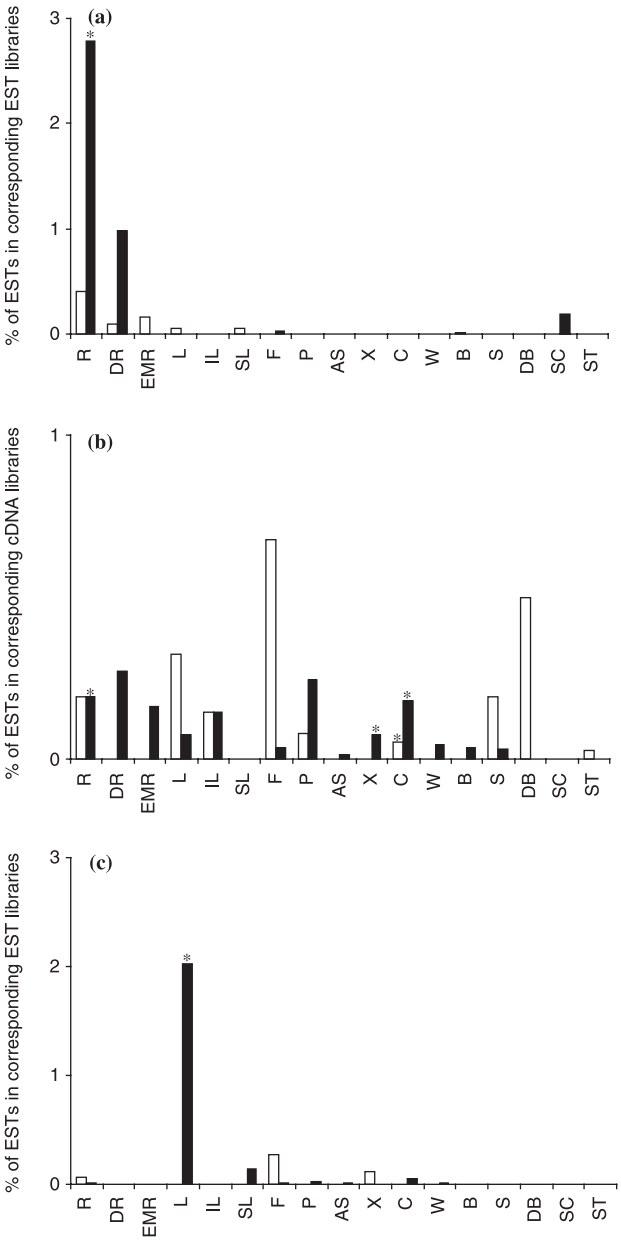

Fig. 3 Occurrence of *PtdMTs* homologs in the National Center for Biotechnology Information (NCBI) expressed sequences tag (EST) database. *MT* ESTs were identified using the six *PtdMT* sequences of *Populus trichocarpa* × *Populus deltoides* as a query in a BLASTX search against the 154 509 *Populus* ESTs in the NCBI dbEST database (release 030504). The abundance of ESTs with an *e*-value of 0.0 in the cDNA libraries in which they were found is given. Data is shown for *PtdMT1a/b* (a), *PtdMT2a/b* (b) and *PtdMT3a/b* (c), respectively (open columns, *PtdMT1a*, *PtdMT2a* and *PtdMT3a*; closed columns, *PtdMT1b*, *PtdMT2b* and *PtdMT3b*). R, roots; DR, drought-stressed roots; EMR, ectomycorrhizal roots; L, leaves; IL, infected leaves; SL, senescing leaves; F, flowers; P, petioles; AS, apical shoot; X, xylem; C, cambium; W, wood; B, bark; S, seeds; DB, dormant buds; SC, suspension cells, and ST, stem. ESTs derived from more than one cDNA library are indicated by a star.

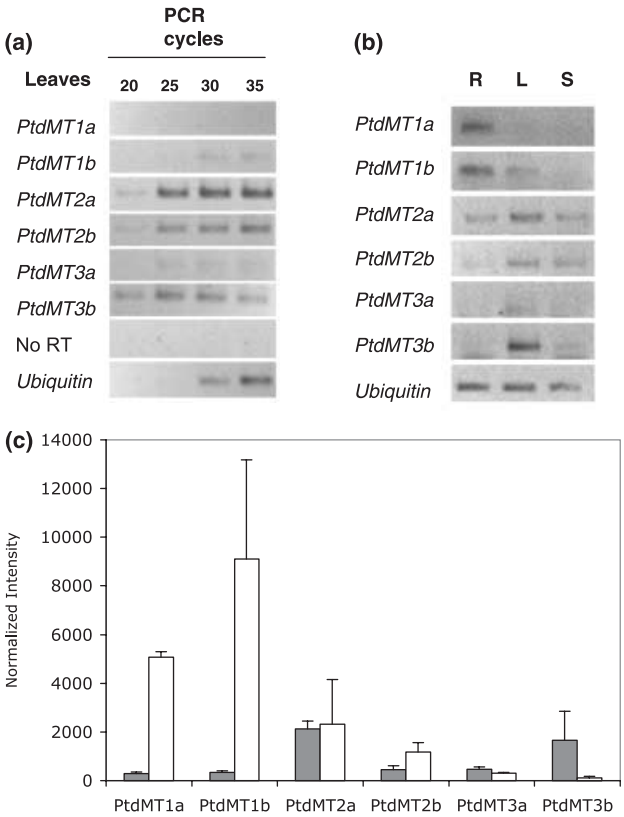

Fig. 4 Transcript levels of *PtdMTs* in poplar roots, stem and leaves. (a) Total RNA of leaves from 3-wk-old, hydroponically grown, rooted cuttings was isolated and aliquots of 300 ng were used for first strand cDNA synthesis. Polymerase chain reaction (PCR) was performed with 1 μl of first strand cDNA using *PtdMT*- and *ubiquitin*-specific primers, and 20, 25, 30 or 35 cycles. A control with no reverse transcriptase in the first strand cDNA synthesis reaction mix was included to control for the lack of genomic DNA. *Ubiquitin* (clone R55D06, GenBank Accession no. CA825222) was used to check for equal loading of RNA. (b) Total RNA from roots (R), leaves (L) and twigs/young stems (S) of 3-wk-old, hydroponically grown, rooted cuttings was isolated. Aliquots of 300 ng from each RNA preparation were used for first strand cDNA synthesis, followed by PCR (30 cycles) with *PtdMT*- and *ubiquitin*-specific primers. (c) Total RNA from roots and leaves of 2-month-old rooted cuttings, grown in a glasshouse, was isolated. Probes for hybridization with nylon cDNA arrays were prepared from 500 ng total RNA. Array data from two biological replications was normalized (see the Materials and Methods section). The mean intensity values and the standard deviation are shown for the *PtdMTs* in the different tissues. Shaded columns, leaves; open columns, roots.

PtdMT2b), however, were more ubiquitous and occurred in cDNA libraries from roots, leaves, flowers, xylem, cambium and seeds. *PtdMT3a* was found in cDNA libraries from flowers, xylem and roots.

MT expression patterns

We determined the transcript levels of *MTs* in various tissues from hybrid poplar by semiquantitative RT-PCR analysis (Fig. 4a,b) and cDNA array analysis (Fig. 4c). The RT-PCR

analysis employed *MT* cDNA-specific primers (Table S1, see Supplementary Material section for details) able to distinguish different *MT* cDNAs used as templates (data not shown). The PCR cycle number was optimized for each gene to ensure that amplification endpoints were in the logarithmic phase (e.g. expression in leaves, Fig. 4a). These RT-PCR analyses of *MT* transcript levels showed that *MT* transcripts were present in all tissues with differential expression of the various types in roots, twigs/stems, and expanding and mature leaves when normalized to ubiquitin amplification (Fig. 4). The concentration of this ubiquitin transcript was not altered in the various stages of development of poplar roots and shoots, during growth on various media and during the interactions with rust and mycorrhizal fungi (data not shown). It should be noted, however, that ubiquitin concentration generally increased during senescence (Andersson *et al.*, 2004). *PtdMT1a* and *PtdMT1b* were preferentially expressed in roots, whereas *PtdMT3b* transcript concentration was greater in leaves than in roots, as previously documented by the digital EST analyses (Table 1, Fig. 3). By contrast, *PtdMT2a* and *PtdMT2b* were expressed in roots (Fig. 4b,c), leaves (Fig. 4b,c) and twigs/stems (Fig. 4b). The *PtdMT3a* transcript was barely detectable in the tissues analysed (Fig. 4b,c).

Differential expression of PtdMT mRNA during adventitious root development

The mRNA accumulation patterns of poplar *MTs* during adventitious root development were examined by cDNA array analysis. *PtdMT* transcripts were ubiquitously present in all tissues (dormant bark cortical tissues, root primordia, calli, emerging roots, adventitious and lateral roots) with differential expression of the various *MT* types at the different developmental stages (Fig. 5). In the bark tissues of dormant cuttings, all six *PtdMTs* were expressed at very high levels. At the beginning of the root primordium formation, the concentration of *PtdMT* transcripts was strongly decreased (sixfold for *PtdMT2b* to 35-fold for *PtdMT3a*). The level of *PtdMT1a*, *PtdMT1b* and *PtdMT2b* transcripts increased slightly in the emerging roots and reached high values in the primary adventitious and lateral roots. *PtdMT2a* expression was also higher in the dormant bark tissues than in the root primordium, but further on, although expressed in all developmental stages, its expression seemed to be less effected by the root development. As observed for the other *PtdMTs*, the expression of *PtdMT3a* and *PtdMT3b* was decreased in the root primordium, compared with the one in dormant bark tissues, but it remained at a low level during the root formation. In the primary adventitious and lateral roots, both *PtdMT3a* and *PtdMT3b* expression was very low, which coincided with the low transcript levels that were found for fully developed root systems by RT-PCR and microarray analysis (Fig. 4).

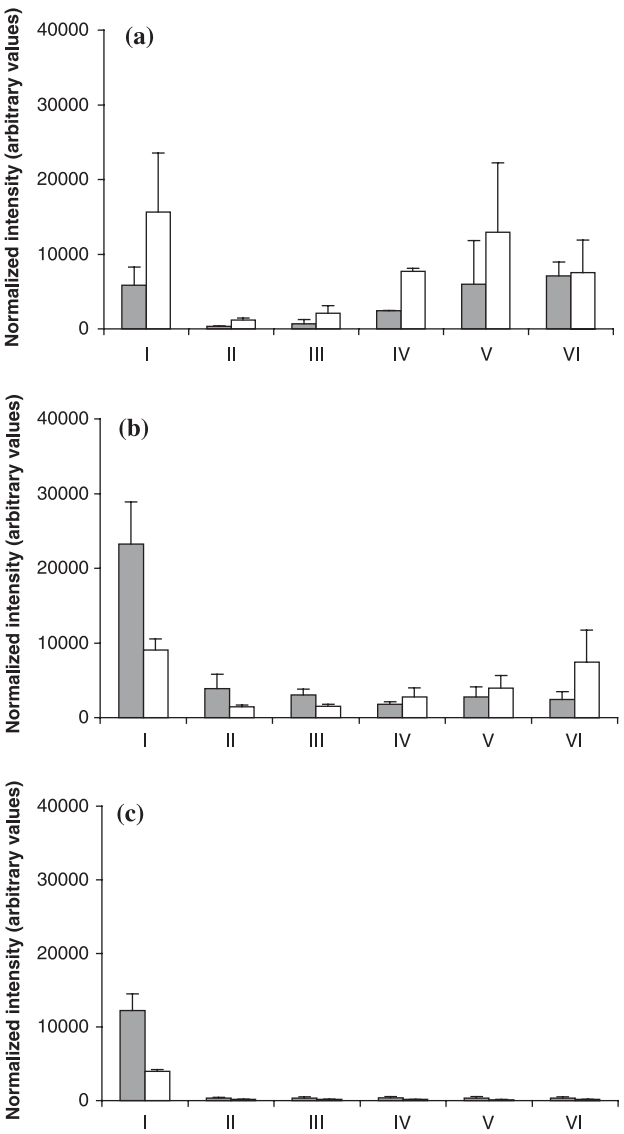

Fig. 5 Transcript profiling of PtdMTs during adventitious root development. Total RNA from six root developmental stages of hydroponically grown poplar cuttings (I, dormant bark tissues, day 0; II, root primordia, days 3–4; III, root calli, days 4–5; IV, emerging roots, day 6; V, primary adventitious roots, day 8; VI, lateral roots, day 10) was isolated. Probes for hybridization were prepared from 500 ng of total RNA. Array data from two biological replications were normalized. The mean intensity values for the expression of *PtdMT1a/b* (a), *PtdMT2a/b* (b) and *PtdMT3a/b* (c), respectively, and the standard deviation are shown (shaded columns, *PtdMT1a*, *PtdMT2a* and *PtdMT3a*; open columns, *PtdMT1b*, *PtdMT2b* and *PtdMT3b*).

Expression profiles during leaf senescence

The most abundant class of ESTs in autumn leaves of aspen (*P. tremula*) encoded MTs (Bhalerao *et al.*, 2003; Andersson *et al.*, 2004). *PtdMT* expression was thus examined by RT-PCR during leaf senescence in hybrid poplar (Fig. 6). Leaves at different developmental stages (PI 1–15) were collected

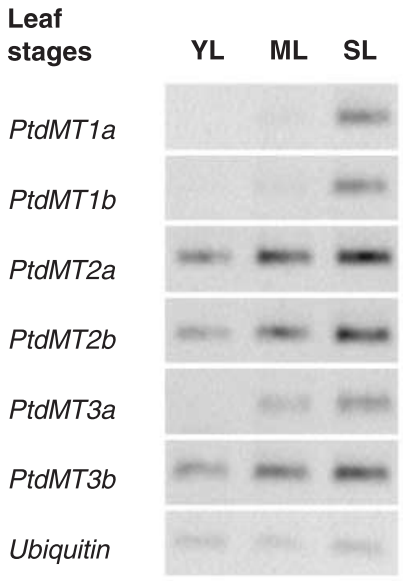

Fig. 6 Transcript profiling of *PtdMTs* during leaf senescence. Total RNA was isolated from young (PI1), mature (PI8) and senescent (PI15) leaves of 5-month-old poplar trees. Aliquots of 200 ng were used for first strand cDNA synthesis, followed by polymerase chain reaction amplification (25 cycles) using *PtdMT*- and *ubiquitin*-specific primers.

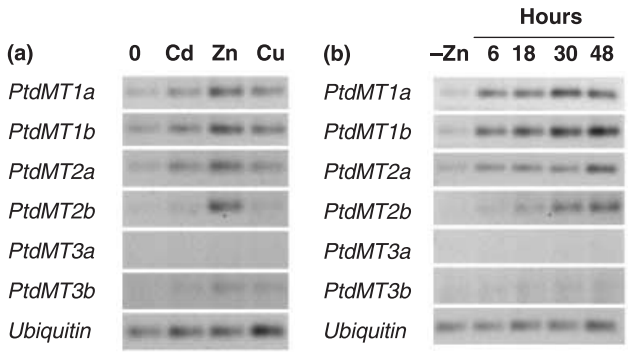

Fig. 7 Regulation of *PtdMT* mRNA expression by metals. (a) Effect of cadmium (Cd), zinc (Zn), and copper (Cu) on *PdtMT* transcript levels. Total RNA was isolated from roots of 3-wk-old, hydroponically grown, rooted cuttings treated for 18 h with 25 μM $CdSO_4$, 1 mM $ZnSO_4$ or 150 μM $CuSO_4$, respectively. (b) Time-course of $ZnSO_4$-induced *PtdMT* mRNA expression. Total RNA was isolated at different time-points (6, 18, 30 and 48 h) from roots of 3-wk-old, hydroponically grown, rooted cuttings treated with 1 mM $ZnSO_4$. For both experiments, 200 ng from each RNA preparation was used for first strand cDNA synthesis, followed by a polymerase chain reaction amplification (25 cycles) with *PtdMT*- and *ubiquitin*-specific primers.

from 5-month-old rooted cuttings grown in the glasshouse. In young leaves (PI 1) only *PtdMT2a*, *PtdMT2b* and *PtdMT3b* were expressed a significant level. Their expression increased in mature leaves (PI 8) and it was further slightly induced during leaf senescence. In senescent leaves (PI 15), all *PtdMTs* were expressed at a higher level than in young leaves.

Metal induction of *PtdMT* mRNA expression in roots

To examine the effect of heavy metal treatment on the expression of *PtdMTs*, 3-wk-old hydroponically grown poplars were treated with Cu, Cd or Zn. The transcript levels of *PdtMTs* were measured after 18 h in roots (Fig. 7a). Incubation in the presence of 1 mM $ZnSO_4$ clearly induced an increase in *PtdMT1a*, *PtdMT1b*, *PtdMT2a* and *PtdMT2b* transcripts. The expression of these transcripts was already slightly induced after 6 h and further increased up to 48 h (Fig. 7b). By contrast, the expression levels of *PtdMT3a* and *PtdMT3b* were only slightly altered by the Zn treatment. Incubation with 25 μM Cd for 18 h had only a small effect on the expression of *PtdMT1a*, *PtdMT1b* and *PtdMT2a* (Fig. 7a). In 100 μM Cd, the transcript levels of *PtdMTs* were reduced even in treated roots compared with their levels in control roots (data not shown). A similar result was obtained in Cu-incubated roots. Treatment with 50 μM $CuSO_4$ only slightly stimulated the expression of *PtdMT1a*, *PtdMT1b*, *PtdMT2a* and *PtdMT2b* (data not shown). In 150 μM Cu, the expression of the last two *PtdMTs* was weakly altered after 18 h (Fig. 7a), but after 48 h incubation the transcript levels were also reduced in Cu-treated roots compared with the levels in control roots (data not shown).

Discussion

Multiple isoforms of MTs in poplar

Metallothionein is encoded by a small gene family in hybrid poplar. We have identified six different *MT* sequences from root and leaf cDNA libraries of *P. trichocarpa* × *deltoides* (Fig. 1a). They were further divided into three subgroups, corresponding to the previously described type 1, type 2 and type 3 MTs from other plants (Cobbett & Goldsbrough, 2002). Type 4 *MT* sequences were not found in our cDNA libraries and the NCBI poplar ESTs, but their expression seems to be clearly restricted to developing seeds (Guo *et al.*, 2003). In an autumn leaf library from aspen, *P. tremula*, Bhalerao *et al.* (2003) also identified six MTs. For each of the *PtdMT* cDNA type, we have identified the corresponding genomic ORF in the JGI *P. trichocarpa* genome sequence (http://genome.jgi-psf.org/poplar0/poplar0.home.html), confirming the existence of at least six *MT* genes in poplar.

As observed previously for other type 1 *MT* genes (Guo *et al.*, 2003), *PtdMT1a* and *PtdMT1b* were both predominantly expressed in roots. *PtdMT2a* and *PtdMT2b* were expressed in roots, leaves and twigs/stems, indicating a less restricted tissue-specific expression. This is not in accord with what has been generally found for type 2 plant *MTs* which show a higher expression in shoots than in roots (Cobbett & Goldsbrough, 2002). For *PtdMT3b*, we found a preferential expression in leaves, which was confirmed by the distribution of ESTs in *Populus* cDNA libraries at the NCBI. *PtdMT3a*

transcripts were barely detectable or present in very low amount in roots, leaves, and growing stems. By contrast, we found high transcript concentrations of this MT in bark tissues.

A role of poplar MTs in heavy metal tolerance

Using the yeast *yap-1*Δ mutant (Wu *et al.*, 1993), which is particularly sensitive to Cd, we showed that PtdMTs were capable of restoring Cd tolerance (Fig. 2). We further investigated whether *PtdMT* gene expression was regulated by heavy metals. In poplar roots, only treatment with Zn had a stimulating effect on type 1 and type 2 *MT* gene expression (Fig. 7). *PtdMT3a* gene expression was not effected by the Zn treatment, whereas *PtdMT3b* expression was weakly affected. By contrast, Cu (50 μM) and Cd (25 μM) only slightly enhanced the transcript levels of *PtdMTs* compared with the transcript levels in untreated roots and they repressed *MT* expression at higher concentrations. Our results suggest a role for poplar PtdMTs mainly in Zn metabolism/detoxification rather than in Cd or Cu metabolism/detoxification, but further experiments will be necessary to establish the exact function of PtdMTs. The occurrence of multiple *MTs* in poplar (and other plants) may reflect the diversity of their functions in heavy metal sequestration, but also other mechanisms essential for plant growth and development.

MT expression is regulated by root development

The expression patterns of the various types of *PtdMTs* are dramatically altered by the adventitious root development (Fig. 5). As stressed above, all *PtdMTs* transcripts were very abundant in the bark tissues of dormant cuttings. When root development initiated, the expression of all *PtdMTs* dropped down. In poplar roots incubated in the presence of auxins, a decreased expression of type 1 and type 2 *MT* genes was also observed (A. Jambois, UMR IaM, INRA, Nancy, France, unpubl. data), suggesting that *PtdMT* expression in developing roots was regulated by auxins. When the root emerged from the bark tissues, the transcript level for type 1 *PtdMTs* were already increasing and it further increased during root development, which is in agreement with the finding that type 1 *PtdMTs* are the major *MTs* in roots.

MTs in senescing leaves

Previous studies demonstrated, that *MT* expression was induced during leaf senescence (Buchanan-Wollaston, 1994; Butt *et al.*, 1998; Garcia-Hernandez *et al.*, 1998; Bhalerao *et al.*, 2003; Guo *et al.*, 2003; Andersson *et al.*, 2004). It has been argued that during senescence metal ions are transported from leaves to other parts of the plant to be recycled and that MTs are involved in the cellular protection against elevated metal concentration during this process (Guo *et al.*, 2003). In hybrid poplar, all *PtdMTs* were expressed at higher levels in senescing leaves than in young leaves (Fig. 6). However, a different expression pattern was obtained during autumn senescence in aspen leaves (Andersson *et al.*, 2004). The mRNA of aspen *PMt4*, which shares a high homology with *PtdMT2b*, decreased in expression when senescence proceeded. Perhaps the discrepancy between expression patterns results from the fact that Andersson *et al.* (2004) used leaves from an adult aspen tree (> 30 yr old), while we harvested leaves from a 5-month-old glasshouse-grown poplar.

Conclusions

The experiments reported in this paper showed that *MT* genes are present in the hybrid poplar genome, where they form a multigene family. Our data showed that six different *MT* transcripts are present constitutively in poplar and are increased by different stimuli, supporting the hypothesis that MTs participate to processes of metal homeostasis and possibly tolerance. To further elucidate the role of PtdMTs, we plan to construct reporter genes and to investigate the transcriptional regulation and expression of *PtdMTs* in transgenic plants. Such studies will help to clarify the role of these genes in metal homeostasis and root development, and to isolate poplar genotypes particularly tolerant to stress, to be used in experiments of phytoremediation (van Hoof *et al.*, 2001; Di Baccio *et al.*, 2003).

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Supplementary Material

The following material is available as Supplementary material at http://www.blackwellpublishing.com/products/journals/suppmat/NPH/NPH1168/NPH1168sm.htm

Table S1 Specific primers used for RT-PCR of *PtdMTs*

Table S2 Primers used for yeast complementation

The following material is available as Supplementary material at http://mycor.nancy.inra.fr/poplardb/pub.html
Figure S1 Multiple alignment of genomic, cDNA and coding nucleotide sequences of the metallothioneins PtdMT1ab, PtdMT2ab, and PtdMT3ab from the hybrid poplar, *Populus trichocarpa* × *P. deltoides* cv. Beaupre. Alignment was carried out using MULTALIN.

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