Cloning and Characterization of *PMET3a* from *Populus alba* × *Populus glandulosa*

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ABSTRACT

A type 3 metallothionein cDNA *(PMT3a)* from ozone-treated *Populus alba* × *Populus glandulosa* cDNA library has been isolated and characterized. *A PMT3a* cDNA is 459 nucleotides long and has an open reading frame of 201 bp with a deduced amino acid sequence of 66 residues (pI 4.94). The deduced amino acid sequence of PMT3a matched to the previously reported metallothionein genes. The deduced amino acid sequence of PMT3a showed the 86% identity with *P. balsamifera* × *P. deltoides*. Expression of *PMT3a* by the RT-PCR was increased 60 min than 30 min after drought treatment. The ozone treated poplar increased at 30 min in the early time and then decreased at 60 min.

Key words : Abiotic stress, *Populus alba* × *P. glandulosa*, RT-PCR, metallothionein

INTRODUCTION

Metallothioneins (MTs), small cysteine-rich proteins, are an extensive and diverse family that are found in all organisms. Their name derives from their high sulphur content and ability to bind metals in stable metal-thiolate clusters (Margoshes and Vallee, 1957). MTs are cysteine rich proteins with low molecular weight (Robinson *et al.*, 1993). They are grouped into class I, II and III. Class I and II polypeptides are direct gene products, class III MTs are non-translational cysteine-rich molecules named phytochelatins. Metal ions are sequestered by MTs through complexation with the -SH group of the Cys rich motifs. Lane *et al.* (1987) had provided the first direct evidence of the existence of MT in plants by isolating the Ec protein and genes (Kawashima *et al.*, 1992) from wheat.

In sight of their metal-binding capacity, it has been suggested that MTs may play a role in the homoeostasis of essential metal ions and the detoxification of heavy metals, such as Cd^{2+} or Hg (Hamer, 1986). However, MTs have now been implicated in a wide range of biological processes relating to normal development and both biotic and abiotic responses, some but not all of which obviously involve metal sequestration (Riordan and Vallee, 1991).

The expression of the reported MT-like genes from plants could be induced by heat stress and glucose

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starvation in rice (Hsieh *et al.*, 1995), leaf senescence in Brassica (Buchanan-Wollaston, 1994), wounding and viral infection in Nicotiana (Choi *et al.*, 1996), fruit development in kiwi (Ledger and Gardner, 1994) and tissue differentiation in maize (de Framond, 1991). The functions of the plant MT genes thus appear to be very diversified. Metal stressed expression of MT was reported in S. vulgaris (van Hoof *et al.*, 2001), Arabidopsis (Murphy and Taiz, 1995) and Brassica juncea (Haag-Kerwer *et al.*, 1999). The majority of the plant systems used for MT genes expression studies, however, were non-tolerant to heavy metals. The possible role for MTs in high-level metal tolerance remains elusive.

Poplar plants have been shown to be excellent candidates for phytoremediation purposes. But the poplar plants are damaged by the increasing air pollutants, such as sulfurous acid gas, acid rain and ozone. These air pollutants have been associated with the increased production of ROS. To increase the abiotic stress-tolerance by gene transformation in poplar, we analyzed 2,000 ESTs from a full length cDNA library constructed with ozone-treated poplar and isolated type 3 metallothionein gene (*PMT3a*). In this study, we characterized *PMT3a* gene and performed the expression analysis using quantitative RT-PCR.

MATERIALS AND METHODS

Plant materials

Poplar (*Populus alba* × *Populus glandulosa*) were grown in vitro on the Mccown woody plant medium (Lloyd and McCown, 1980) supplemented with 3% sucrose under the condition for 16 hr in the light irradiation and 8 hr in the dark. The poplar plants *in vitro* serially subcultured on the same medium with one-node culture attached a leaf per 5 weeks.

Abiotic stress treatments

To investigate the response of poplar against oxidative stresses, such as hydrogen peroxide (H_2O_2), drought and ozone, we used the leaf of poplar cultured in vitro for 4 weeks. Poplar leaves attached in the middle region cutted with a scalpel. Cutted poplar leaves soaked in 1 mM H_2O_2 for the oxidative stress and left on the dried filter paper (Whatman, England) for the drought during 30 min and 60 min, respectively. Poplars grown during 4 weeks in vitro opened the stopper of glass bottle and then transferred in the incubator. Ozone was fumigated with 300 ppb (Ozonature, Korea) into the incubator during 30 min and 60 min. After the stress treatments, poplar leaves were immediately frozen in liquid nitrogen and stored at -80°C.

Purification of RNA and construction of cDNA library

Total RNA was isolated from the stress treated poplar leaves using the method of guanidine isothiocyanate (TRIzol, Gibco BRL). The tissue was frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle. Extraction reagent was added 1 ml and ground more than 3 min. The mixture was centrifuged for 5 min at 4°C. The supernatant was removed to a new tube, added 0.2 ml chloroform, and vigorously mixed. Following centrifugation, total RNA was precipitated with isopropanol. The pellet was washed once with 75% ethanol, dried in vacuum for 3 min and dissolved in DEPC treated DDW. The RNA solution was stored at -80°C for the RT-PCR analysis.

Nucleotide sequencing and sequence analysis

pTriplEx phagemids were excised from the Uni-ZAP XR library and used as templates for sequence analysis. The 5' ends of randomly selected cDNA inserts were sequenced using the 5' sequencing primer by an automatic DNA sequencer (ABI prism 3700).

Nucleotide and amino acid sequence analyses were performed using DNASIS program (Hitachi). Comparison of sequences to DNA and protein databases at NCBI was performed using the blast algorithm of Altschul *et al.* (1990).

Quantitative RT-PCR analysis

For the expression analysis of the *PMET3a* against the oxidative stresses, we employed the quantitative RT-PCR. A pair of gene-specific primers of the *PMET3a* gene was designed and used for RT-PCR analysis. Specific primers included the following: (*PMET3a*-forward) 5'-AGG ATC CAT GTC TAG CAC CTG CGA C-3'; (*PMET3a*-reverse) 5'-TGG ATC CTT AAT GAC CGC ATG TGC AGG TAG-3'. As a control, we used a pair of the specific primers to poplar actin gene (*PAct*), 5'-CTT TCT GGT GGT GCA ACC ACC TTG A-3' (forward) and 5'-CAC CAT TGG TGC TGA GCG ATT CCG T-3' (reverse). Ten microgram of total RNA was used for the RT-PCR analysis, according to the method of Takakura *et al.* (2000). The PCR cycles for quantitative RT-PCR numbered 30 for the *PMET3a* and the *PAct* genes. RT-PCR products were run on 1% (W/V) agarose gel in 0.5 × TAE buffer and then photograped for the expression analysis.

RESULTS AND DISCUSSION

A full length cDNA library prepared with cDNA from ozone-treated poplar was used for EST (expressed sequence tags) analysis. Phagemids were excised from the Uni-ZAP XR library and used as templates for sequence analysis. The 5' ends of randomly selected cDNA inserts were sequenced using the 5' sequencing primer and then comparison of sequences to DNA and protein databases at NCBI was performed using the blast algorithm (Altschul *et al.*, 1990). Type 3 metallothionein homologs of analyzed EST clones were isolated and full sequenced with 5' and 3' sequencing primers and named with *PMT3*.

The PMT3a characterized in this work was 459 nucleotides long and possess an open reading frame of 201 bp (Fig. 1). BLASTX database searches with the PMT3a sequence gave metallothioneins and metallothionein-like proteins from various organism as the top 50 best matches. The PMT3a clone had 86% identity with the sequence from *P. balsamifera* × *P. deltoides* (AY594299.1). The encoded protein of PMT3a has the typical structure of plant MT-like proteins with two cysteine-rich (Cys-rich) terminal domains separated by a cysteine-free (Cys-free) central domain (Riordan and Vallee, 1991)(Fig. 2). The various types (types 1, 2 and 3) of plant MT-like genes are differentiated by characteristic arrangements of cysteine residues in their encoded polypeptides. The PMT3a sqeuence has the same numbers and arrangement of cysteine residues in a specific and conserved pattern in two domains at the N- and C- terminals, as the other type 3 MT-like genes from different plant species.

From the cDNA sequence, the amino acid sequence of PMT3a was deduced and compared to other related metallothionein gene products. The *PMT3a* cDNA encodes a protein of 66 amino acids with pI 4.94 (Fig. 1). Fig. 2 shows the alignment of the putative protein of PMT3a with MT Type 3 reported for other plants. PMT3a protein sequence shared the highest homology (86%) with PtdMT3a of *P. balsamifera* × *P. deltoides*. The other MT3 protein shared a homology of 76% with A. *hypogaea*, 75% with C. *papaya*, 67% with *V. vinifera*, 59% with *B. juncea*, 64% with *M. acuminata* and 61% with *R. nigrum*. In the phylogenetic analysis, The PMT3a was closer with MT3 of *P. balsamifera* × *P. deltoides* and *B. juncea*(Fig. 3).

We performed quantitative RT-PCR analysis to examine the expression of *PMT3a* gene related with abiotic stresses, such as H_2O_2, drought and ozone. Identification of the PCR product amplified with *PMT3a* specific primers was confirmed by DNA

sequence analysis. Expression of the Poplar actin gene, *PActin*, served as an internal control for quantitative RT-PCR analysis.

The PMT3a gene in poplar was detected both stem and leaf tissues, but the expression at the leaf tissue observed about 5 times higher than stem. H_2O_2 is a signaling molecule which triggers, among other responses, secondary pathways and programmed cell death. We tested whether exposure to H_2O_2 had effects on the induction of *PMT3a*. When the poplar leaves soaked in 1 mM H_2O_2, the gene expression was decreased at 60 min after treatment. But drought induced the strong expression of *PMT3a* gene at 60 min after treatment.

Ozone (O_3) is the most important phytototoxic air pollutant (US EPA, 1996). causes reductions in plant growth and productivity (Heagle *et al.*, 1998), and changes in crop quality (Schenone *et al.*, 1992). The symptoms of ozone injury include bleaching of mesophyll cells, chlorotic motting, changes in pigmentation, and necrosis. Acute short-term exposure to high ozone concentrations (>200 ppb) generally results in visible damage (Heagle, 1989). In this work, we treated 300 ppb ozone to poplar leaf for expression analysis of *PMT3a* gene. Acute ozone treatment induced the strong expression at the early 30 min after treatment and then decreased as the control level.

We describe here the isolation and characterization of type 3 MT-like gene from *Populus alba* × *P. glandulosa*, *PMT3a*. Many plant systems used for MT genes expression studies were non-tolerant to heavy metals. The possible role for MTs in high-level metal tolerance and oxidative stress remains elusive. Therefore, we will continuously study the relations between PMT3a and abiotic stress and then produce the abiotic stress-tolerant transformants by re-introduction of *PMT3a* into *Populus alba* × *P. glandulosa*.

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ATACAAGCTAAGCAAAGAACAACTTCGTATTTAGTTCATCCATCTGCTTCATCAATCAAT   60

CACCATGTCTAGCACCTGCGACAACTGCGACTGCGCTGACAAGACCCAGTGTGTCAAGAA  120
    M  S  S  T  C  D  N  C  D  C  A  D  K  T  Q  C  V  K  K    19

GGGAAGCAGCTACACTGCTGGCATCGTTGAGACTGAGAAGAGCTATGTCTCCACTGGAGC  180
 G  S  S  Y  T  A  G  I  V  E  T  E  K  S  Y  V  S  T  G  A    39

CATGGAGGTTCCAGCAACCGAGAACGATGGCAAGTGCAAGTGCGGCGCTAACTGCACTTG  240
 M  E  V  P  A  T  E  N  D  G  K  C  K  C  G  A  N  C  T  C    59

CACTACCTGCACATGCGGTCATTAAGCACATGAACCGTCATGTGTGTGGCGTAGGGAGTC  300
 T  T  C  T  C  G  H  *

GACTAATAATGTAATTTGTGTCCTTCTGCTATAGTACTTGTGGAAGGAAAAAAAGAAGGT  360
GATAGTGAGTGTCTTATGGTCGTGTCTTCTTCTTCTTCAATGTTATGTAAGGGCCATGTC  420
CATGGCCTTGTGTGTGGTTTAATGGAATATTATGGATGT                       460
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Fig. 1. **Nucleotide and deduced amino acid sequence with the open reading frame from 65 to 265.** The positions of nucleotides are shown on the left and the positions of amino acids under the below. Asterisk shows the termination codon. The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequencing Database under the accession number AB211224.

P. alba × *glandulosa*	MSSTCDNCDCADKTQCVKKGSSYTAGIVETEKSYVSTGAMEVPATENDGKCKCGANCTCT	60
P. balsamifera × *deltoides*	MSSTCDTCDCADKTQCVKKGSSYTAGIVETEKNYVSAVVMEVPADENDGKCNCGTGCTCT	60
C. papaya	MSNTCGNCDCADKTQCVK-GNKYGVDIVETEKRMVETVVMEVPAGENDGKCKCGANCSCT	59
A. hypogaea	MSDTCGNCDCADKTQCVKKGSSYTADIIETEKS-IMTVVMDAPAAENDGKCKCGPSCSCT	59
V. vinifera	MS-TCGNCDCADKSQCVKKGNSYGIDIVETEKSYVATVVMEVPAAQHEGSCKCGDSCACI	59
B. juncea	MS-TCGNCDCVDKSQCVKKGNSYGIDIVETEKSYVDEVIVAAEAAEHDGKCKCGAACACT	59
M. acuminata	MS-SCGNCDCADKTNCPKKGNSYGFDIIETQKSYDDVVVMDVQAAENDGKCKCGPSCSCV	59
R. nigrum	MSDKCGSCDCADKTQCVKKGTSYTFDIVETQESYKEAMFMDVGAEENGCQCKCGSTCSCV	60
	** * *** ** * * * * * ** * * ** * *	

P. alba × *glandulosa*	TCTCGH-	66
P. balsamifera × *deltoides*	TCTCGH-	66
C. papaya	NCTCGH-	65
A. hypogaea	NCTCGH-	65
V. vinifera	DCTCGQ-	65
B. juncea	DCKCGN-	65
M. acuminata	GCSCGH-	65
R. nigrum	NCTCCPN	67
	* *	

Fig. 2. **Comparison of the putative amino acids sequence of PMT3a with those of metallothionein genes from other plants;** *P. alba* × *glandulosa* (AB211224), *P. balsamifera* × *P. deltoides* (AY594299.1), *C. papaya* (CAA69624.1), *A. hypogaea* (AAO92264.1), *V. vinifera* (CAB85630.1), *B. juncea* (BAB85601.1), *M. acuminata* (AF268393.1), *R. nigrum* (AJ007577.1). Sequence data was obtained from GeneBank listed and aligned using DDBJ ClustalW (Thompson *et al.*, 1994 and 1997) and GeneDoc (Nicholas *et al.*, 1997).

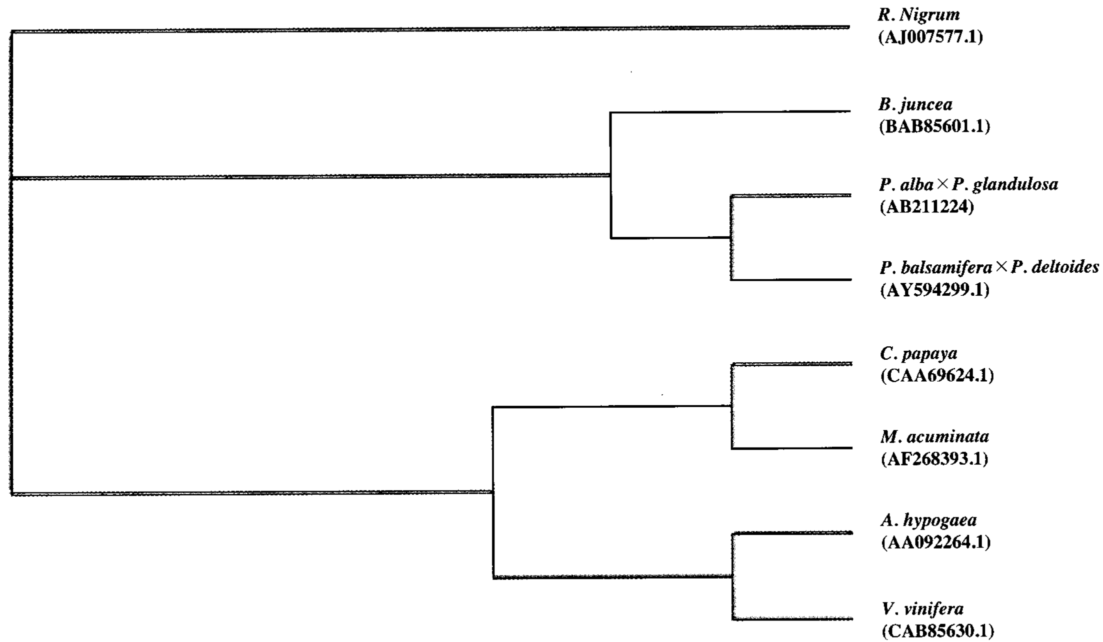

Fig. 3. **Phylogeny of the metallothionein protein family from *P. alba* × *P. glandulosa* and other plants.** Phylogenetic analysis is based on the deduced amino acid sequences of metallothionein genes from various plant species. The branch lengths are proportional to divergence, with the scale of 0.1 representing 10% change.

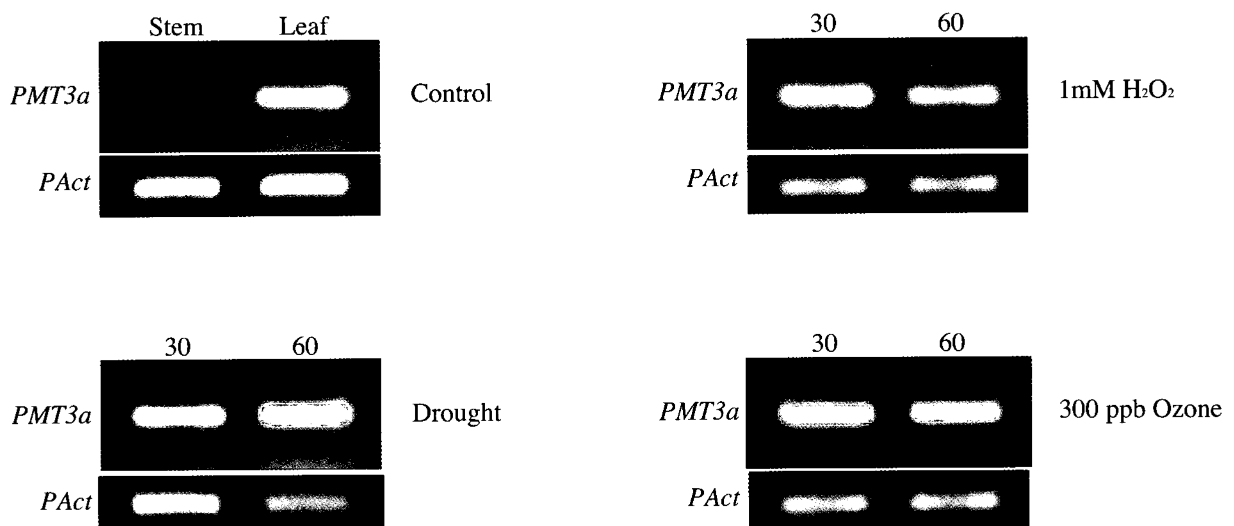

Fig. 4. Expression pattern of *PMT3a* by various stress. Total RNA from stress-treated samples served as templates for quantitative RT-PCR with gene-specific primers. *PAct* gene was used for PCR control.

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