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Characterization of a type 3 metallothionein isolated from *Porteresia coarctata*

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Abstract

Metallothioneins are involved in detoxification of heavy metals. A cDNA encoding type 3 metallothionein (*PcMT3*) was isolated from the salt stressed leaf cDNA library of *Porteresia coarctata* (Roxb.) Tateoka (wild rice) that grows well in the heavy metal laden estuarine soils. The *PcMT3* cDNA (581 bp) encodes a protein of 64 amino acids. PcMT3 is highly homologous (82 %) to OsMT-I-3a of rice, but is unique from other type 3 plant MTs due to the presence of an additional glycine residue in the C-terminal domain. Analysis of the 5′ upstream region of *PcMT3* showed the presence of *cis*-acting elements like the CG box and STRE previously reported to be involved in gene expression under heavy metal stress. Southern analysis suggested the presence of more than one copy of *PcMT3*-like sequences in the *P. coarctata* genome. Analysis of genomic clone of *PcMT3* revealed the presence of two introns. A comparison of the genomic sequence of *PcMT3* with closely similar type 3 MTs from rice and mangrove species revealed conservation in the number and position of introns. Transcript profiling for *PcMT3* in *P. coarctata* leaves in the presence of Cd, Cu and Zn showed an increase in transcript accumulation.

Additional key words: *cis*-acting elements, heavy metals, salt stress, wild rice.

Introduction

Plants acquire heavy metal tolerance through various mechanisms like compartmentalization, sequestration, chelation or exclusion. Of all the detoxification mechanisms, chelation of heavy metals is most effective and is achieved using metallothioneins (MTs; Zhou and Goldsbrough 1994, Murphy *et al.* 1997). MTs are low molecular mass, cysteine (Cys) rich proteins identified in animals, fungi, cyanobacteria and plants (Robinson *et al.* 1993). Plant MTs have been classified based on the arrangement of Cys residues into classes I, II and III. Most of the plant MTs are Class I proteins containing two Cys-rich clusters separated by a spacer region, and can be further categorized into several types (types 1 to 4) based on the distribution pattern of Cys residues in the amino (N)- and carboxy (C)-terminals (Robinson *et al.* 1993). In Class II MTs, Cys residues are distributed in a scattered manner in the entire protein sequence. Class III MTs are non-gene encoded polypeptides, composed of poly-(γ-glutamylcysteinyl) glycine, also called phytochelatins (Robinson *et al.* 1993). Multiple MT isoforms have been reported in plants like rice, hybrid poplar, oil palm and lichens (Abdullah *et al.* 2002, Kohler *et al.* 2004, Zhou *et al.* 2006, Bačkor and Loppi 2009) and they exhibit a differential pattern of expression.

Amongst the Class I MTs, type 1 MTs are expressed abundantly in roots as observed in *Vicia faba*, hybrid poplar and *Arabidopsis* (Foley *et al.* 1997, Kohler *et al.* 2004, Zhou and Goldsbrough 1995). Rice MT1 is an exception and is highly expressed in leaves and shoots (Zhou *et al.* 2006). Type 2 MT transcripts have been detected primarily in leaves and shoots of most plants (Zhou *et al.* 2006, Garcia-Hernandez *et al.* 1998), while type 3 MT genes are expressed in leaves and ripening fruits (Cobbett and Goldsbrough 2002). Type 4 MT sequences are normally expressed in developing seeds (Guo *et al.* 2003). Differential tissue-specific expression of MTs (roots, shoots, seeds, trichomes and fruits) suggests that these proteins, in addition to controlling metal homeostasis, have other important roles during plant development (Zhou *et al.* 2005), senescence

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Abbreviations: cys - cystein; MTs - metallothioneins; TAIL-PCR - thermal asymmetric interlaced PCR; DEPC - diethyl-pyrocarbonate.
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(Garcia-Hernandez *et al.* 1998, Yu *et al.* 1998, Navabpour *et al.* 2003), and protection against oxidative stress (Akashi *et al.* 2004, Gisela *et al.* 2004, Wong *et al.* 2004). Plant MTs show a differential response to different heavy metals. In most plant species, type 2 MTs are constitutively expressed with heavy metal treatments (Roosens *et al.* 2005, Gonzalez-Mendoza *et al.* 2007, Usha *et al.* 2007) while type 1 and type 3 MTs exhibit increased expression upon exposure to heavy metals (Lee *et al.* 2004, Usha *et al.* 2009).

Mangrove forests, considered most productive systems in the coastal regions, are constantly polluted by heavy metals from industries, mines, shipping industries and agricultural fields (*e.g.* Tam and Wong 1993). *Porteresia coarctata* is a mangrove associate and a wild relative of rice, known to thrive on heavy metal polluted coastal regions (Jagtap *et al.* 2006). In this study, we have characterized a type 3 metallothionein from *P. coarctata* (*PcMT3)*. The present communication analyses the transcript profile of *PcMT3* in different tissues as well as under different heavy metal stresses. 5′ upstream region of PcMT3 (putative promoter) was isolated and analyzed for the presence of metal responsive elements.

Materials and methods

Porteresia coarctata (Roxb.) Tateoka (syn. *Oryza coarctata*) growing in high saline conditions was collected from the estuary located in Pichavaram, Tamil Nadu, India. The plants were grown in pots with clay soil in greenhouse under natural light (12-h photoperiod) and temperature 30 ± 2 °C. For heavy metal treatments, the plants were acclimated in half strength MS liquid medium for two days and then transferred to fresh medium containing $CdSO_4$, $CuSO_4$ and $ZnSO_4$ (0, 50, 100, 250, 500 and 1000 μM) to identify the sub-lethal concentration. Plants were then subjected to the sub-lethal doses of heavy metals (0.5 mM Cd, 1 mM Cu and 1 mM Zn) and leaves from the stressed plants were collected at regular intervals of 0 (control), 24, 48 and 72 h and frozen in liquid nitrogen and stored at -80 °C until use. For analyzing tissue specific expression, leaf and root tissues were collected from unstressed *P. coarctata* plants. Total RNA was isolated from frozen samples according to Usha *et al.* (2009). About 10 μg of total RNA was electrophoresed in 1.3 % formaldehyde-agarose gel, transferred to nylon membrane (*Hybond N*$^+$, *GE Healthcare,* UK) and probed with α^{32}P-dCTP labeled 3′ UTR region of *PcMT3* cDNA. Hybridization was carried out as per Sambrook *et al.* (1989).

Genomic DNA was isolated from 2 g of *P. coarctata* leaf tissue by the cetyltrimethylammonium bromide (CTAB) method (Dellaporta *et al.* 1983). 20 μg of genomic DNA was digested with different restriction enzymes (*Hind* III, *Sac* I, *Eco* RV, *Dra* I, *Pvu* II), electrophoresed in 0.8 % agarose gel and transferred onto a nylon membrane (*Hybond N*$^+$). The hybridization was carried out as per Sambrook *et al.* (1989) with *PcMT3* cDNA 3′ UTR region as probe.

Genomic sequence of *PcMT3* cDNA was amplified from *P. coarctata* genome using 5′ and 3′ UTR specific forward (5′ GCGAAAGCAGCAGCTAGCAG 3′) and reverse primers (5′ CACACAAATACACGCTGCATTA 3′). PCR conditions: 94 °C for 45 s, 59 °C for 45 s and 72 °C for 90 s for 30 cycles. The amplified product was cloned into a pTZ57R/T cloning vector (*MBI Fermentas*, St. Leon-Rot, Germany) and sequenced.

Sequence corresponding to the 5′ upstream region of *PcMT3* was amplified from genomic DNA using thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) with modifications according to Liu *et al.* (1995) and Sessions *et al.* (2002). Three nested gene specific reverse primers (NP1-5′ CGAGAGAGGTACGGTAC TTACAC 3′, NP2-5′ GCACTTGTCCGACATGATCG AAG 3′, NP3-5′ GAAGCGAGCGATGAATTCCTTGTG 3′) and one of four arbitrary degenerate primers (AD1, 5′ NGTCGASWGANAWGAA 3′; AD2, 5′ TGWGNAG SANCASAGA 3′; AD3, 5′ WGTGNAGWANCANAGA 3′ and AD4, 5′ STTGNTASTNCTNTGC 3′) were used in successive rounds of TAIL-PCR cycling. The primary PCR product was diluted 40-fold and used in the secondary reaction while the latter was diluted 10-fold for the tertiary reaction. The products of the primary, secondary and tertiary reactions were analyzed on a 0.8 % agarose gel. Fragments exhibiting a difference in size consistent with nested gene specific-primer positions were cloned and sequenced completely. The putative *cis*-acting DNA elements in the specific isolated 5′ region were identified using the *PLACE* database (Higo *et al.* 1999).

Results and discussion

An EST clone showing high sequence similarity to plant MTs was identified by large scale sequencing of randomly selected EST clones from a *P. coarctata* leaf cDNA library (Senthilkumar *et al.* 2005). The full length cDNA (581 bp) on *BLASTX* (Altschul *et al.* 1990) analysis showed high homology to putative plant type 3 metallothioneins (*PcMT3* - GenBank accession no. AF257465). The longest open reading frame is 192 bp and codes for a 64 amino acid protein with an approximate molecular mass 6.5 kDa. A multiple sequence analysis (Thompson *et al.* 1994) of PcMT3 with other plant type 3 MTs showed maximum homology to

type 3 MTs from *Oryza sativa* (82 % identity to OsMT-I-3a and 66 % identity to OsMT-I-3b). The analysis also revealed the conservation of ten Cys residues, four at the N-terminal end and six at the C-terminus among the proteins analyzed.

The distribution of cysteine residues at the N-terminus is conserved (CXXCXCXXXXXC) as seen in other plant type 3 MTs (Cobbett and Goldsbrough 2002). The C-terminal domain with six Cys residues (CKCGSSCSC GTDCKC), shows two CKC motifs on either side of a central CSC motif. The C-terminal region of PcMT3 is different from the conserved CXCXXXCXCXXCXC domain, as it contains an additional glycine residue after the CSC motif, which is absent in other plant type 3 MTs. The N- and C-terminal cysteine domains are separated by a spacer region of 30 amino acids, which lack cysteine residues and is characteristic of Class I metallothioneins.

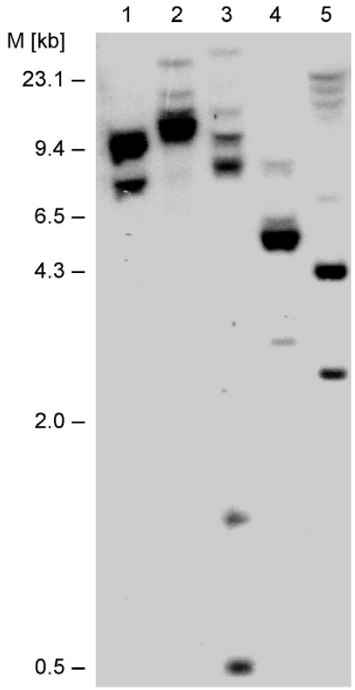

Fig. 1. Southern blot analysis of *PcMT3* in *P. coarctata.* Genomic DNA was digested with *Hind* III (1), *Sac* I (2), *Eco* RV (3), *Dra* I (4), *Pvu* II (5) and hybridized with radio-labeled *PcMT3* cDNA. Molecular size markers (M) are indicated on the left.

Comparison of the 861 bp genomic sequence of *PcMT3* (Genbank accession no. EU121847) with that of its cDNA revealed the presence of two introns (between bases 120 to 314 and 362 to 507) with canonical donor (5′-GT) and acceptor (AG-3′) sites that divided the coding region into three exons. The position of the first intron relative to the coding region in the *PcMT3* genomic sequence is found to be conserved when compared with rice (*OsMT-I-3a*) and mangrove (*Avicennia marina* - *AmMT3*) MTs. However the length of the introns was found to vary among the closely related species. Southern blot analysis using *PcMT3* cDNA 3′ UTR region as probe revealed multiple hybridization signals (Fig. 1), suggesting that *PcMT3* may be part of a multigene family in *P. coarctata.*

A 953 bp fragment upstream of the translational initiation site (TIS) was isolated by TAIL-PCR. Putative *cis*-acting elements like the TATA box, CAAT box, GC Box, sequences required for root specific expression, ABARE (ABA responsive element) were identified in this sequence. A CGCG box (A/C/G)CGCG(G/T/C), found in promoters of genes responsive to ethylene, abscisic acid and H_2O_2 signaling (Yang and Poovaiah 2002), was present in this putative promoter. A stress-responsive element (STRE), characterized by the core sequence AGGGG (Ruis and Koller 1997), was found 702 bp upstream of the TIS. The putative transcription initiation site **(**YYYAYYA) was located 77 bp upstream of the ATG start codon. Unlike animal and most plant Class I MTs, the 5′ upstream region of *PcMT3* lacks metal responsive element (MRE). The important *cis*-regulatory elements in the 5′ upstream region of *PcMT3* are highlighted in Fig. 2.

Tissue-specific expression of *PcMT3* was examined in leaves and roots of *P. coarctata* under normal conditions. *PcMT3* expression was higher (~ 3 fold) in roots than that observed in leaves (Fig. 3*A*). To demonstrate the expression pattern of *PcMT3* under heavy metal stress, *P. coarctata* plants were subjected to cadmium (1 mM), copper (0.5 mM) or zinc (1 mM) for a time period of 72 h. A gradual increase of *PcMT3* transcript was observed (from 0, 24, 48 and 72 h) with maximum level at 72 h upon Cd, Zn and Cu application (Fig. 3*B*).

P. coarctata is one of the pioneering species during ecological succession in mangrove regions (Latha 2000). Mangroves are constantly subjected to increased heavy metal pollution due to urbanization and industrialization. Several mangrove plants possess great tolerance to high heavy metal pollution. To develop further insights into the molecular mechanisms behind ability to tolerate heavy metals, we have isolated and characterized a type 3 metallothionein from *P. coarctata*.

The cDNA encoding metallothionein-like gene was grouped into type 3 MTs based on the highly conserved CGNCDC and QCVKKG sequence present at the N-terminus and three CXC motifs in the C-terminus. PcMT3 is highly homologous to type 3 MT of rice, except for an additional glycine residue present in the conserved C-terminal CXCXXXCXCXXCXC domain. Presence of glycine can increase the flexibility of proteins, thereby directing the cysteine residues to metal binding pocket (Roosens *et al.* 2005). Similar modifications have been observed in the conserved domains of MT-like proteins of various plants. For instance, in *Thlaspi caerulescens*, a type 1 MT lacks threeCys residues in the N-terminal domain (Roosens *et al.* 2005) and a type 2 MT from sweet potato contains additional amino acids in the conserved sequence at the C-terminal domain (Chen *et al.* 2003).

PcMT3 full length sequence amplified from the

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GGCCTATATCTGTTTTGATTCACAACACCACACATTTCTGCTCCAGCGAAACCGTTCACA -954

CATTCTCACTGAATTTGAATTCGGTTTTTGGATATGTTACTTCGTGTCGGGATCAAACGT -834

AAAGGAGAATTCAGATCTGGAGTATCTGAGCATATACGCATTTTGGATTCATCTGTCAAT -774

TTGAAAATTCTCACCGATTGGTGGATCGAGTCGGACGGAGATGGTCGACGACGGAGACCT -714
      STRE                                  CAAT BOX
GACGTGAGGGGGGAGAACACTGTAAAACATTGACAAGACGCCAATGGCGATAAGGTCGCC -654

GATATATACATCCACAATAACATTCTTCCTTGCCCTGGCTCGCTCTTGAGAATTTTTATC -594

GACCAAGTAAAAATTTGAGATCATTGCGCCAGCTCGCCATGAGTCTGTTTGGATCAAACA -534

CGGGGTGTTACCATAAAATATAAGCGCTCCTACCTATTATTTAAATTCTATACCAAAATA -474

CAAACACTCGTAACGCACTACTTCTCTCATTTAATTTTTTTTAATCTAATTTTATCGTCA -414

ACCGCTGTCCTACTCGTCAACCTTAGTTTTTGCTCTAGAAAAAAAACTTGAAGCGTTTAT -354
ROOT MOTIF                                  ABARE
ATTTTGGAACGGCTGAGTAGTTCCTCGCCTAATACCCCTGCGAAGTTACGTGCTCCGAAA -294
                                                    CG BOX
ACAATCGAAAGATTGCTACGTTCACGAGACCTAAAGCTTGGCGCTAGCCGCCGCGGCGGA -234
            CG BOX
TGGCGCCTTTGGAGCGCGTGCCTCTCGATGGGACGGAGACGTCATGGCGTGTGGCCACCA -174
                    GC-BOX                                  TATA BOX
GACAGCAGCACTGCCCCTGTCTCCACTTGATTCTTCTTCTCCCCGTATCCGGCCGCTATA -114
                          TRANSCRIPTION START
TACACCACCCGAAGCGGCAGCCATTGCTCCTCACCAGAGCTAAGCTTAAGCGAAAGCAGC -54
                                                        M   S
AGCTAGCAGCACAAGGAATTCATCGCTCGCTTCAGCTAATCTCTTCTTCGATCATG TCG
 D   K   C   G   N
GAC AAG TGC GGC AAC
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Fig. 2. 5′ flanking region of *PcMT3*. The sequence is numbered relative to the TIS. Putative *cis*-acting elements including the TATA box, CAAT box, GC Box, ABARE, Root motif, CG box, STRE and a putative transcription initiation site are marked. Nucleotide sequences of these elements are underlined and shown in bold.

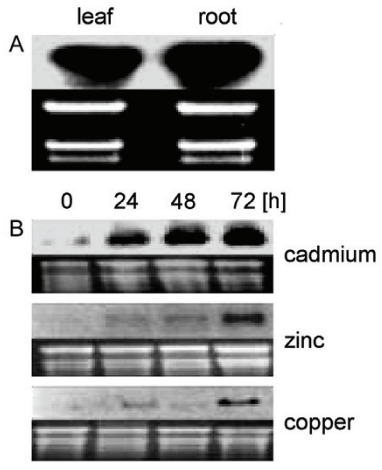

Fig. 3. Northern analysis of *PcMT3* expression. *A* - Tissue specific expression of *PcMT3* in leaf and root tissues of *P. coarctata*. *B* - Northern analysis of *PcMT3* expression in leaves under heavy metal treatments; $CdSO_4$ (1.0 mM), $ZnSO_4$ (1.0 mM) and $CuSO_4$ (0.5 mM).

genomic DNA consists two introns in the coding region. Number of introns is highly conserved in type 3 MTs across various plant species, for instance, in rice (Zhou *et al.* 2006), *Avicennia marina* (Usha *et al.* 2007), *Arabidopsis* (www.arabidopsis.org) and oil palm (Abdullah *et al.* 2002). All type 3 MTs contain two introns that disrupt the coding region into three exons with the first intron positioned at around 50 to 60 bases from the translation start site (ATG). These parameters may also be considered for identification and classification of MTs in addition to distribution and arrangement of Cys residues. Enhanced intron splicing and thus increased mRNA expression may be influenced by intron position (Snowden *et al.* 1996). In rice, the expression of GUS was enhanced by the presence of the rgMT (OsMT-I-1a) intron (Hsieh and Huang 1998). Enhanced *PcMT3* expression under all heavy metal treatments may be attributed to the position of introns.

As observed previously for other type 3 MTs from *Arabidopsis* (Lee *et al.* 2004), *A. marina* (Usha *et al.*

2007), *Prosopis juliflora* (Usha *et al.* 2009) and sorghum (Shanker *et al.* 2004), *PcMT3* also showed an enhanced expression on exposure to heavy metals like Cd, Zn and Cu. Thus *PcMT3* may participate in homeostasis of metal ions and hence protection against the damage that heavy metals can cause by the production of reactive oxygen species (Cu, Fe, Hg, Ag) or by blocking the function of essential biomolecules (Cd, Zn, Ni, Pb). *PcMT3* expression was three fold higher in roots as compared to leaves suggesting a possible mechanism to arrest the translocation of the metals from roots to leaves. Higher expression of type 3 MT in roots has also been reported in oil palm and hybrid poplar (Abdullah *et al.* 2002, Kohler *et al.* 2004). In rice, *OsMT-I-3b* mRNA was noticeably higher in leaves, but was barely detected in the roots while *OsMT-I-3a* was abundant in roots (Zhou *et al.* 2006). Spatial expression pattern showed that *PcMT3* isoform may be similar to *OsMT-I-3a* of rice which is also supported by a high homology (82 %) between *PcMT3* and *OsMT-I-3a.* As in rice, *PcMT3* may also exist in different isoforms as shown in Southern blot analysis.

MTs are induced by various chemical and physical agents acting directly or indirectly on multiple *cis*-acting elements in the regulatory regions of MT genes (Haq *et al.* 2003). Analysis of multiple *cis*-acting motifs in the regulatory regions of MT genes may give an overall view of various biotic and abiotic factors regulating basal MT gene transcription and induction /repression of MT gene activity. We have identified several putative regulatory elements in the promoter region of *PcMT3* gene. A putative root-specific element was identified similar to that found in pea (Fordham-Skelton *et al.* 1997) and maize MT-like genes (Elmayan and Tepfer 1995). A high level of expression in roots as shown by the Northern analysis may be due to the presence of root specific element in the putative promoter region of *PcMT3*. MREs are essential for binding transcription factors that regulate the expression of MTs and they are well characterized in animals (Coyle *et al.* 2002). MRE-like elements have been reported in the upstream sequences of a few plants like pea (Evans *et al.* 1990), tomato (Whitelaw *et al.* 1997) and oil palm (Abdullah *et al.* 2002). Analysis of the *PcMT3* promoter revealed the lack of MREs. Screening of type 3 MT isoforms in rice also revealed the lack of MRE core sequences in the promoter regions (Zhou *et al.* 2006). Other metal regulatory elements like copper responsive elements (CRE) are also absent in the *PcMT3* promoter. This suggests that there may be unique and unidentified MREs present in the *PcMT3* promoter or other regulatory elements such as the CGCG box, STRE (stress responsive element) that may be involved in the indirect regulation of *PcMT3* gene under heavy metal stress.

This study involves the characterization of a type 3 metallothionein from a mangrove associate *P. coarctata*. Increased expression of *PcMT3* in root tissues suggests a possible role of *PcMT3* in sequestering heavy metals in roots. High levels of *PcMT3* transcript was also observed in the leaf tissues of plants exposed to heavy metals which suggests that *PcMT3* may be involved in maintaining the redox balance either by sequestering heavy metals or by directly scavenging deleterious oxygen radicals. A further investigation on different types of metallothioneins will help understand their role in heavy metal tolerance of *Porteresia* and other mangrove associates in the mangrove ecosystem.

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