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# ISOLATION AND CLONING OF cDNA OF GENE ENCODING FOR METALLOTHIONEIN TYPE 2 FROM *MELASTOMA AFFINE*

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## ABSTRACT

Metallothionein is an important protein for detoxifying heavy metal ions. This research was conducted to isolate and clone cDNA of gene encoding for metallothionein type 2 from *Melastoma affine*. Total RNA was isolated from young leaves. Total cDNA was synthesized from the total RNA by reverse transcription. The *MaMt2* cDNA was successfully isolated by PCR technique. The *MaMt2* cDNA was inserted into pGEM-T Easy and the recombinant plasmid was successfully introduced into *Escherichia coli* DH5α. DNA sequencing analysis showed that this cDNA is full length consisting of 246 pb encoding 81 amino acid residues. This cDNA is identical to mRNA of *AtMt2* from *Arabidopsis thaliana*. It does not contain any restriction sites found in the cloning sites of pGEM-T Easy. The deduced protein of MaMT2 contains 14 cysteine residues distributed in the Cys-Cys, Cys-X-Cys, and Cys-X-X-Cys motifs.

**Key words:** cDNA, metallothionein, *Melastoma affine*, cloning, cysteine

## INTRODUCTION

Gene isolation has a very important role in the genetic improvement of crops. The genes can be isolated from genetic resources coming from microorganism, plants, or animals. *Melastoma malabathricum* L. or *M. affine* D. Don is an aluminum hyper-accumulator plant (Watanabe *et al.* 2005) and used as an indicator for acid soil. Since it is tolerant to acid and Al stresses, it is very important to use this plant as genetic resources for tolerance genes to acid and Al stresses. Tolerant plants to Al are very important to increase agricultural production in Indonesia because Indonesia has about 47.5 million hectares of pod soil yellow-red land which have low pH and high solubility of Al (CSAR 1997).

Metallothionein (MT) is a protein having low molecular weight, around 4-8 kDa (Vallee 1991). It is constituted of 45-48 amino acid residues containing 12-17 conserved cysteine residues (Kagi 1991). Cobbett and Goldsbrough (2002)

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have classified MT into four types based on distribution of cysteine residues in the C-terminal and N-terminal regions. Type 1 of MT is consisted of two domains with metal binding motif Cys-X-Cys. Type 2 is composed of two domains with metal binding motif in combination among Cys-Cys, Cys-X-X-Cys, and Cys-X-Cys. Type 3 is also called as a phytochelatin (PC) and has a structure of (γ-Glu-Cys)n-Gly, whereas type 4 contains rich in cysteine in repeated sequence.

MT can bind heavy metal, and has an important role in the detoxifying of some heavy metal ions (Clemens 2000, Hall 2002), like Zn (Nigel *et al.* 1996). The expression of BjMT2 in *A. thaliana* increased the seedling tolerance against Co and Cd (Zhigang *et al.* 2006).

The expression of *Mt2* gene is induced by Al stress in wheat (Snowden *et al.* 1995), by $H_2O_2$ and metal ion in rice (Zhou *et al.* 2005), by Cu and Co in *A. thaliana* (Zhou and Goldsbrough 1995), by drought stress in wild watermelon (Akashi *et al.* 2004) but it is down regulated by small GTPase OsRac I in rice (Wong *et al.* 2004). *Mt2* gene had been isolated from soybean (Kawashima *et al.* 1991), *Vicia faba* (Foley and Singh 1994), rice (Hsieh *et al.* 1995) and broccoli (Yang *et al.* 2000).

This research has the objective to isolate and to clone the cDNA of gene encoding for metallothionein type 2 from *M. affine* L. Since the expression of *Mt2* is induced by Al stress (Snowden *et al.* 1995), we suppose that the over-expression of this gene can increase the plant tolerance to Al. By this reason, this gene can be used to improve genetically the tolerance of important plant to Al by over-expressing the *Mt2* gene.

## MATERIALS AND METHODS

### Materials

Young leaves of *M. affine* were used as plant material. pGEM-T Easy (Promega) was used as cloning vector. *Escherichia coli* DH5α was used as a host for recombinant plasmid. Primers of *ActF* (ATGGCAGATGCCGAGGATAT), and *ActR* (CAGTTGTGCGACCACTTGCA) designed based on complete nucleotide sequence of a soybean actin gene (Shah *et al.* 1982, GenBank V00450) were used to amplify exon1-exon2 of β-actin as a control of cDNA. Primers of MF (TCGAGAAAAATGTCTTGCTGTG), and M7R (CTTCACTTGCAGGTGCA AGG) designed based on *Arabidopsis thaliana* MT2A mRNA (GenBank NM_1117733) were used to isolate *Mt2* cDNA of *M. affine*.

### Total RNA isolation

Young leaves (1 g) were ground with pestle in the mortar in the presence of 0.3 g sands and 10 ml warm extraction buffer (2% CTAB, 2% PVP 25000, 100 mM Tris-HCl pH8, 20 mM EDTA, 1.4 M NaCl, 1% β-mercaptoethanol) previously heated at 65°C. This suspension was poured into 20 ml centrifuge tube, and incubated at 65°C for 10 minutes, and then added by 10 ml of chloroform:isoamylalcohol (24:1). After vortexing to mix this suspension, the tube was centrifuged at 42 000 xg (rotor SW11, Sorvall Ultra Pro 80), 4°C for 10 mins. The upper liquid phase were collected and added by 0.25 volumes of 10 M LiCl. After incubation at -32°C for 2.5 hours, this suspension was centrifuged at 42 000 xg (rotor SW11, Sorvall Ultra Pro 80), 4°C for

10 mins. The pellet containing total RNA was suspended by adding 500 ml TE (10 mM Tris-HCl pH 7.4, 10 mM EDTA). This total RNA suspension was extracted by adding 1x volume of phenol pH 9, then vortexing and centrifuging at 14000 rpm (Jouan BR4i) at 20 °C for 10 mins. The total RNA suspension was recovered from the upper phase, and then extracted by 1x volume of phenol:chloroform:isoamylalcohol (25:24:1), then centrifuged at 15 000 rpm, 20°C for 10 min. The total RNA contained in upper phase was precipited by adding 0.25 volume of 10 M LiCl, and incubated at -32°C overnight. This total RNA suspension was centrifuged at 15 000 rpm, 4°C, 10 min. Total RNA pellet was washed by 500 ml ethanol 70% and centrifuged at 15 000 rpm, 4°C, 10 min. After drying with vacuum dryer total RNA was added by DEPC treated $H_2O$ to make a suspension.

**Synthesis of total cDNA**

Total cDNA was synthesized by mixing 5 µg total RNA, 4 µl 5x buffer SuperScript III Reverse Transcriptase (Invitrogen), 20 pmol oligo(dT), 4 mM dNTP, 10 mM DTT, 1 U enzyme SuperScript™III RTase dan DEPC treated $H_2O$ in the final volume of 20 µl. Reverse transcription to synthesize total cDNA was carried out at 52°C for 50 mins by using PCR machine (MJ Research TM 100). The purity of total cDNA was verified by using PCR with specific primers of for cDNA of exon1-exon2 of β-actin. The composition of PCR was 1 µl total cDNA, 1x taq buffer, 40 mM $MgCl_2$, 4 mM dNTP mix, 20 pmol *ActF* primer, 20 pmol *ActR* primer, 4% DMSO, 0.75 U taq DNA polymerase (Toyobo) and $H_2O$ in the final volume of 20 µl. The condition of PCR was pre-PCR 95°C, 5 mins, denaturizing at 94°C, 30 seconds, annealing at 57°C, 30 seconds, extension at 72°C, 1.5 min, and post-PCR at 72°C, 5 min. The PCR was conducted for 35 cycles.

**Isolation of *MaMt2* cDNA by PCR**

The composition of PCR was 1 µl total cDNA, 2 µl 10x buffer taq, 4 mM dNTP, 20 pmol MF primer, 20 pmol M7R primer, 4% DMSO, 0.75 U taq DNA polymerase (Toyobo) and $H_2O$ in the final volume of 20 µl. The PCR was conducted for 35 cycles at 95°C, 5 min. for pre-PCR, 94°C, 30 seconds for denaturising, 60°C, 30 seconds for annealing, 72°C, 1.5 min. for extension and 72°C, 5 min. for post-PCR.

**Cloning *MaMt2* cDNA into pGEM-T Easy**

*MaMt2* cDNA of PCR product (3 ml) was mixed with 10 ng pGEM-T Easy (Promega), 3 U T4 DNA ligase (Promega), 1x ligation buffer, and $H_2O$ in the final volume of 10 µl. The reaction was carried out at 4°C overnight. The ligation product was introduced into *E. coli* DH5α as described by Suharsono (2002).

**Selection of *E. coli* containing *MaMt2***

*E. coli* containing *MaMt2* was selected based on the resistance to ampicillin and blue-white selection. The insert of *MaMt2* in the white ampicillin resistant colony was confirmed by PCR-colony. The colony was picked up by tooth-picker, then suspended in 5 ml $H_2O$, and heated at 95°C, 10 mins and cooled at 15°C for 5 mins. The suspension was added by 1.5 µl 10x buffer taq, 3 mM dNTP, 15 pmol

*MF* primer, 15 pmol M7R primer, 4% DMSO, 0.75 U enzim taq DNA polymerase (Toyobo). PCR was conducted in the same condition as for *MaMt2* cDNA isolation. The insert of *MaMt2* cDNA was also confirmed by plasmid DNA isolation as described by Suharsono (2002). The plasmid DNA was digested by *Eco*RI at 37°C for 2 hours.

**Analysis of cDNA**

DNA was sequenced by using automated DNA sequencer ABI Prism 3100 version 3.7. Local alignment analysis of cDNA was carried out by using BLAST2 program (http://www.ebi.ac.uk/blast2) (Mount 2001). Restriction sites in the cDNA of *MaMt2* was analyzed by NEB Cutter program (http://www.firstmarket.com/cutter/cut2.html).

## RESULTS AND DISCUSSIONS

**Total RNA isolation**

Total RNA of *M. affine* had been successfully isolated from young leaves and the efficiency of isolation was 118-213 μg RNA/g leaves. The absorbent ratio of λ260/λ280 was 1.4-1.6. It showed that these total RNAs were probably contaminated by remaining protein and/or phenol. The purity of total RNA is high if the λ260/λ280 absorbent ratio is around 1.8-2.0 (Saunders and Parker 1999).

Electrophoresis analysis in formaldehyde denaturized agarose gel in the MOPS buffer showed that the total RNA contained two prominent bands. These two bands were corresponded to the integrated 28s and 18s ribosomal RNA (Figure 1). Since the total RNA contained integrated 28s and 18s rRNA, the integrity of total RNA was also high, and could be used as template for total cDNA synthesis. The integrity of mRNA is very important to isolate the full length of coding sequence of DNA. To use the gene for genetic improvement by over-expression, the full length of gene is indispensible.

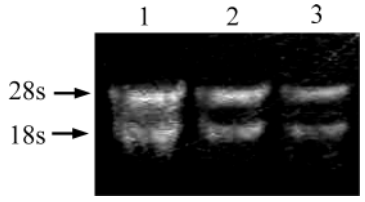


Figure 1. Electrophoresis of total RNA of *M. affine* isolated in different time from young leaves. 1=10 o'clock, 2=12 o'clock, and 3=14 o'clock.

**Total cDNA synthesis**

By using total cDNA as template and specific primer for cDNA of β-actin, amplification by PCR resulted to 450 bp band corresponding to cDNA of exon1-exon2 of β-actin (Figure 2). The result showed that the total cDNA had been successfully synthesized and this total cDNA was free from the contamination by genomic DNA. If genomic DNA contaminated the total cDNA, the amplification of β-actin would result to two bands, 450 bp and 550 bp, corresponding to amplified cDNA and

genomic DNA respectively. The 550 bp DNA was resulted from the amplification of exon1-intron-exon2 of β-actin, and the size of intron beetwen exon1 and exon2 is around 100 bp (Shah *et al.* 1982). This intron is spliced during the mRNA synthesis, so cDNA derived from mRNA does not contain this 100 bp intron.

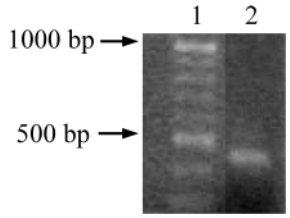


Figure 2. Amplification of exon1-exon2 cDNA of β-actin. 1= 100 bp DNA marker, 2= exon1-exon2 cDNA of β-actin

cDNA is very important for genetic engineering of eucaryotic organisms. This DNA represents the coding region of gene. The size of cDNA is equal or smaller than the corresponding DNA of gene. Since the size is smaller, the expression of gene by using cDNA is more efficient than by using DNA of gene. Therefore, the cDNA of gene is more preferable than DNA although the isolation of gene by using cDNA is more difficult than by using DNA in eucaryotic organisms.

**Isolation and cloning of *MaMt2* cDNA**

PCR by using total cDNA as template and MF and M7R as primers of resulted around 250 bp DNA (Figure 3). This result was the same as predicted because the size of *Mt2* in several plant species is around 250 bp as found in soybean (Kawashima *et al*. 1991), wheat (Snowden and Gardner 1993), *A. thaliana* (Zhou and Goldsbrough 1994), and rice (Zhou *et al*. 2006).

This cDNA was then inserted in pGEM-T Easy, and the recombinant plasmid DNA was successfully introduced into *E. coli* DH5α shown by the white colonies grown in the selective media containing ampicillin, X-gal and IPTG. pGEM-T Easy plasmid contains *lacZ* in its cloning site (CS). Only *E. coli* containing plasmid can survive in the media containing ampicillin and only *E. coli* containing recombinant plasmid can grow in white colony in the presence of X-gal and IPTG in the media. The insertion of cDNA in the *lacZ* found in the cloning site caused the inactivation of *lacZ* to be expressed. On the other hand, if there are no insertions in the *lacZ*, this gene is expressed to produce β-galactosidase and this enzyme will convert uncolored X-gal to become blue color. As a result, the color of colonies which do not contain the recombinant plasmid is blue.

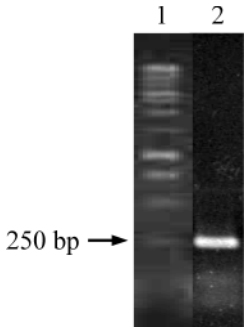


Figure 3. cDNA of *MaMt2* resulted by PCR

To confirm the inserted cDNA of *MaMt2*, the white colony was picked up and used as template for PCR-colony. The PCR-colony resulted 250 bp DNA (Figure 4). The plasmid DNA was successfully isolated from this white colony. The digestion of this plasmid DNA with *Eco*RI resulted two fragments of DNA, one is 250 bp and the other is 3 kb corresponding to the cDNA of *MaMt2* and pGEM-T Easy cloning vector, respectively (Figure 4). This result showed that the white colony contained *MaMt2* cDNA found in the pGEM-T Easy.

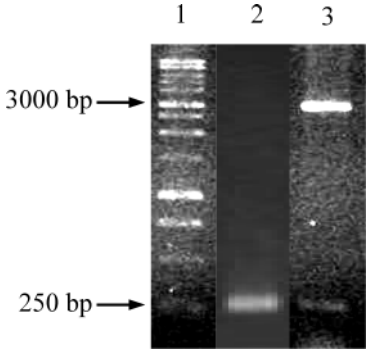


Figure 4. Analysis of cDNA insert in pGEMT-Easy recombinant by PCR-colony (2) and *Eco*RI digestion (3). 1= 1 kb plus DNA marker.

**Analysis of cDNA of *MaMt2***

Based on sequencing from SP6 primer, the recombinant pGEM-T Easy contained insert DNA of 257 bp consisted of 246 bp open reading frame encoding for 81 amino acid residues (Figure 5). Alignment analysis by BLASTn showed that this cDNA had similarity of 100% with mRNA of *AtMt2A* of *A. thaliana* (Accession: NM_111773.3), 90% with mRNA of *BjMt2* of *Brassica juncea* (Y10850.1), 89% with mRNA of *BrMt* of *B. rapa* (D78498.1), 88% with mRNA of *BcMt* of *B. camprestris* (L31940.1), and 88% with mRNA of *BoMt2* of *B. oleracea* (AF200712.1). This alignment analysis showed that the cDNA isolated in this research is a full length of *MaMt2* of *M. affine* containing start and stop codons.

```
1    TCG AGA AAA ATG TCT TGC TGT GGA GGA AAC TGC GGA TGT GGA  42
1                Met Ser Cys Cys Gly Gly Asn Cys Gly Cys Gly  11

43   TCT GGC TGC AAG TGC GGC AAC GGT TGT GGA GGT TGC AAA ATG  84
12   Ser Gly Cys Lys Cys Gly Asn Gly Cys Gly Gly Cys Lys Met  25

85   TAC CCT GAC TTG GGA TTC TCC GGC GAG ACA ACC ACA ACT GAG 126
26   Tyr Pro Asp Leu Gly Phe Ser Gly Glu Thr Thr Thr Thr Glu  39

127  ACT TTT GTC TTG GGC GTT GCA CCG GCG ATG AAG AAT CAG TAC 168
40   Thr Phe Val Leu Gly Val Ala Pro Ala Met Lys Asn Gln Tyr  53

169  GAG GCT TCA GGG GAG AGT AAC AAC GCT GAG AAC GAT GCT TGC 210
54   Glu Ala Ser Gly Glu Ser Asn Asn Ala Glu Asn Asp Ala Cys  67

211  AAG TGT GGA TCT GAC TGC AAG TGT GAT CCT TGC ACC TGC AAG 252
68   Lys Cys Gly Ser Asp Cys Lys Cys Asp Pro Cys Thr Cys Lys  81

253  TGA AG                                                 257
82   End
```

Figure 5. Nucleotide and deduced amino acid sequences of *MaMt2*. Underline sequences indicate the forward and complementary reverse primers of used to isolate *MaMt2*, bold letters indicate start and stop codons respectively, italic letters indicate the cys amino acids.

Restriction site analysis showed that *MaMt2* contains *Bbv*I, *Bsa*BI, *Bts*CI, *Tse*I, *Fok*I, *Ape*KI, *Csp*CI, *Hpy*CH4III, *Psh*AI, *Bsr*FI, *Sgr*AI, *Acu*I, *Btg*ZI, *Mbo*II, *Sfa*NI, *Cac*8I and *Hpy*188I sites (Figure 6). These sites cannot be used to clone and engineer *MaMt2* because they can cut *MaMt2* in two or more fragments. Fortunately, *MaMt2* does not contain any restriction site found in CS of pGEM-T Easy. It means that, all restriction sites found in CS can be used to isolate the *MaMt2* cloned into pGEM-T Easy.

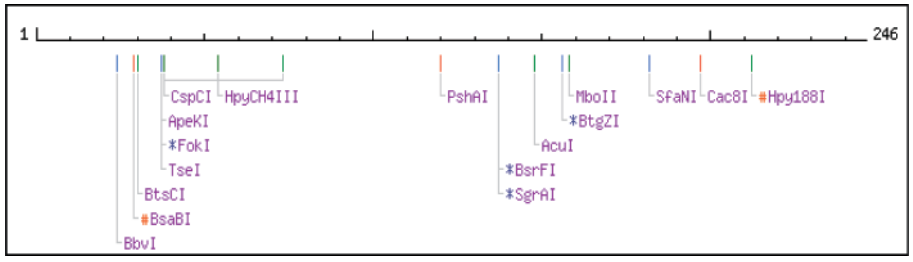


Figure 6. Restriction site map of *MaMt2*.

To perform genetic engineering, the information of the nucleotide sequence and the restriction sites of the gene are indispensable. The nucleotide sequence information can be used to modify the sequence by *in vitro* directed mutagenesis. The information of restriction sites of the gene is very important to decide the restriction enzymes which will be used to cut off the gene and then to fuse with other genes or expression elements as promoter and terminator.

Deduced amino acid residues demonstrate that MaMT2 contains 81 amino acid residues with 14 cysteine residues. This result is in accordance with the number of amino acid residues and cysteine residues of MT type 2 from several plants (Kojima 1991). Analysis of cysteine sequence motif showed that MaMT2 has a motif Cys-Cys (3rd-4th residues), Cys-X-Cys (8-10, 14-16, 67-69, 73-75, 78-80) and Cys-X-X-Cys (20-23) (Figure 5). This specific Cys sequence demonstrates that MaMT2 has the similar motif of Cys distribution with MT type 2 of several plants (Robinson *et al*. 1993; Cobbett and Goldsbrough 2002).

The full length of *MaMt2* cDNA isolated in this report is the first MT family genes isolated from *M. affine* as the indigenous plant in tropical rain forest like Indonesia. Since this plant is very tolerant to Al, the *MaMt2* can be used to improve genetically the important plant for Indonesia as soybean. The genetic improvement can be done by over-expressing *MaMt2*. We are now targeting the over-expression of *MaMt2* in soybean to improve the resistance to Al by using strong promoter. In the near future, we also would like to over-express this *MaMt2* cDNA in *Jatropha curcas*.

## CONCLUSIONS

The total RNA of *M. affine* had been isolated and converted into total cDNA. The full length of cDNA of *MaMt2* gene had been isolated from this total cDNA and cloned into pGEM-T Easy. This *MaMt2* cDNA contains 246 bp and encodes 81 amino acids. MaMT2 contains 14 Cys amino acids distributing in Cys-Cys, Cys-X-Cys, and Cys-X-X-Cys motifs. This full length of *MaMt2* cDNA is the first cDNA of *Mt* family gene isolated from *M. affine*. It can be applied to improve genetically the Al tolerance of plant.

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