B I O D I V E R S I T A S
Volume 10, Number 3, July 2009
Pages: 109-114

ISSN: 1412-033X (printed edition)
ISSN: 2085-4722 (electronic)
DOI: 10.13057/biodiv/d100301

Isolation and Cloning of cDNA of Gene Encoding for Metallothionein Type 2 from Soybean [*Glycine max* (L.) (Merrill)] cv. Slamet

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Received: 10th January 2009. Accepted: 26th May 2009.

ABSTRACT

Metallothionein has an important role in the detoxification of metal ions. It has a low molecular weight and contains cysteine-rich residue. The objective of this research is to isolate and clone the cDNA of gene encoding for metallothionein from soybean [*Glycine max* (L.) (Merrill)] cv Slamet (*GmMt2*). We had successfully isolated total RNA by reverse transcription and synthesized total cDNA from total RNA as template. cDNA of *GmMt2* had been isolated from total cDNA by PCR. It was successfully inserted into pGEM-T Easy plasmid, and the recombinant plasmids were introduced into *Escherichia coli* strain DH5α. Sequence analysis by using T7 and SP6 primers showed that the length of PCR-isolated fragment was 257 bp containing 246 bp completed sequence of *Mt2* cDNA encoding for 81 amino acids. Enzyme restriction analysis showed that *GmMt2* did not contain any restriction sites found in the multi cloning sites of pGEM-T easy. Nucleotide and amino acid alignment analysis using BLAST program showed that *GmMt2* was similar with completed cDNA of *AtMt2A* from *Arabidopsis thaliana* (L.) Heynh. Amino acid sequence analysis showed that the motifs of Cys sequence of GmMT2 are Cys-Cys, Cys-X-Cys, and Cys-X-X-Cys.

Key words: soybean, metallothionein, cDNA, isolation, cloning, cysteine.

INTRODUCTION

Metallothionein (MT) is low molecular weight proteins (4-8 kDa), containing rich of cysteine, and capable to bind heavy metal ions. Based on the pattern of cysteine residues distribution along the MT sequence, MT proteins of several species of plants are divided into two types, i.e. type 1 and type 2. Type 1 has a motif Cys-X-Cys, whereas type 2 has Cys-Cys, Cys-X-Cys, and Cys-X-X-Cys motifs where X is an amino acid other than Cys. MTs are present in a vast range of organisms including plants, mammals, fungi and procaryotic organisms (Valle, 1991; Cobbett and Goldsbrough, 2002; Coyle et al., 2002).

In the animals and the plants, MTs not only have an important role in the homeostatic mechanism and detoxification heavy metal ions (Cobbett and Goldsbrough, 2002; Hall, 2002), but they also involve in the process of physiology, regulation of cell growth, proliferation, the activities of metalloenzymes, transcription factor (Haq et al., 2003; Akashi et al., 2004; Wong et al., 2004), and the infection and cell death (Vasak and Hasler, 2000). In addition, the presence of MTs in the nuclear can protect the DNA from the damage induced by oxidative stress (Chubatsu and Meneghini, 1993; Cai et al., 1995). The synthesis of MTs is induced by several metals, growth signals and hormones (Templeton et al., 1985; Andrews et al., 1987; Nartey et al., 1987). In wheat, aluminum stress caused the increase of expression of gene encoding for MT (Snowden and Gardner, 1993). Pilon-Smith and Pilon (2002) showed that the detoxification of Al was due to the binding of Al by cysteine-rich containing proteins as MTs, glutathione (GSH) and phytochelatin. MT type 2 (MT2) is one of MT family protein.

Soybean [*Glycine max* (L.) (Merrill)] cv Slamet is a local cultivar tolerant to acid soil and Al stress. Al can cause oxidative stress by creating radical oxygene species (ROS) (Panda et al., 2003). Since MTs involve in the detoxification of ROS, we suppose that MTs involve also in the detoxification of Al. Therefore the isolation and cloning of cDNA of gene encoding of MT2 from soybean is important. This research has an objective to isolate and clone cDNA of gene encoding for MT2 of soybean cv Slamet.

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MATERIALS AND METHODS

Materials

Soybean cv Slamet was used as plant material. The primers of ActF (ATGGCAGATGCCGAGGATAT) and ActR (CAGTTGTGCGACCACTTGCA) designed based on cDNA of β-actin of soybean (Shah et al., 1982) were used to amplify exon1-exon2 of cDNA of β-actin as a control for the purity of total cDNA. Primers of MF (TCGAGAAAAATGTCTTGCTGTG) and M7R (CTTGCACCTGCAAGTGAAGG) designed based on cDNA of MT2 of *Arabidopsis thaliana* (L.) Heynh (Acc: NM_1117733) were used to isolate the cDNA of MT2 of soybean (*GmMt2*). pGEM-T Easy plasmid (Promega) was used as a cloning vector. *Escherichia coli* strain DH5α was used as a host of recombinant plasmids.

Isolation of total RNA

The isolation of total RNA was carried out by using Trizol Kit (Invitrogen) as described by Mashuda (2006). For this purpose, 1 g of root tips was ground in the mortar in the presence of liquid nitrogen to become the fine powder. These powder were mixed into suspension with 800 µL Trizol in the 1.5 mL micro tubes. This suspension was then incubated in the room temperature for 5 minutes, and after that added with 200 µL chloroform and vortexed thoroughly. After incubation for 3 minutes in the room temperature, the suspension was centrifuged at 10,000 rpm (Jouan BR4i) at 4°C for 15 minutes. The supernatant was collected and added by 500 µL isopropyl alcohol, then incubated at the room temperature for 10 minutes. After the centrifugation at 10,000 rpm, at 4°C for 10 minutes, the liquid was discarded and the pellet was added by 500 µL ethanol 75%, then the tubes were centrifuged at the same condition as previous step. The liquid was discarded, and the pellet was vacuum-dried. The pellet was suspended in 30 µL H_2O-DEPC. The quantity and quality of total RNA were determined by spectrophotometer UV-VIS (Cecil CE 2020).

The integrity of total RNAs was determined by electrophoresis in the 1% agarose gel (FMC, USA) in the MOPS buffer (4.2 g L^{-1} MOPS, 0.41 g L^{-1} Na-acetate, 0.37 g L^{-1} Na_2-EDTA). For electrophoresis, 1 µL total RNA was mixed with 12 µL premix solution (MOPS, 50% (v/v) formamide, 17.5% (v/v) formaldehyde and 27.5% (v/v) H_2O-DEPC). This suspension was heated at 65°C, 10 minutes, and immediately cooled in the ice for 5 minutes, and added by 1/6 volume loading dye (0.25% blue bromophenol, 0.25% xylene cyanol FF, 30% glycerol). This suspension containing total RNAs was electrophorezed at 100 volt 30 minutes. After submerging in the EtBr solution (0.5 µg mL^{-1}) for 30 minutes, and washing by H_2O, the total RNAs in the gel were visualized on the UV GelDoc transilluminator (Labquip) and recorded by digital camera.

Synthesis of total cDNA

Total cDNA was synthesized from total RNA by using Superscript III Reverse Transcriptase enzyme (Invitrogen). The composition of the synthesis of total cDNA was 5 µg total RNA, 4 µL RT buffer (5x), 20 pmol oligo(dT), 4 mM dNTP mix, 10 mM DTT, 1 U SuperScript ^{TM}III RTase (Invitrogen), and ajusted to 20 µL by the addition of H_2O-DEPC. The synthesis of total cDNA was carried out at 45°C for 50 minutes. The successful of synthesis of total cDNA and the purity of total cDNA from genomic DNA contaminant were determined by PCR using specific actF and actR primers for exon1-exon2 of β-actin. The composition to amplify cDNA of β-actin was 1 µL total cDNA, 1 µL Taq buffer (10x), 40 mM $MgCl_2$, 4 mM dNTP mix, 10 pmol ActF primer, 10 pmol ActR primer, 4% DMSO, 0.5 U Taq DNA polymerase (Fermentas) and H_2O in the final volume of 10 µL. The condition of PCR was pre-PCR at 95°C, 5 minutes, denaturation at 94°C, 30 seconds, primer annealing at 55°C, 30 seconds, and extension at 72°C, 1.5 minutes, with 35 cycles, and post-PCR at 72°C, 5 minutes followed by 15°C, 5 minutes.

Isolation of cDNA of GmMt2

The cDNA of *GmMt2* was isolated from total cDNA by PCR. The composition of PCR was 2 µL total cDNA, 2 µL Taq buffer (10x), 4 mM dNTP mix, 20 pmol MF primer, 20 pmol M7R primer, 4% DMSO, 1 U *Taq* DNA polymerase (RBC) and completed to 20 µL by H_2O. The condition of PCR was pre-PCR at 95°C, 5 minutes, denaturation at 94°C 30 seconds, primer annealing at 58°C, 30 seconds and extension at 72°C, 1.5 minutes, with 35 cycles, post-PCR at 72°C, 5 minutes followed by 15°C, 5 minutes.

Cloning of cDNA of GmMt2

The cDNA of *GmMt2* resulted from PCR was ligated to pGEM–T Easy (Promega) by mixing 3 µL of PCR product, 1 µL (10 ng) pGEM-T Easy, 0.5 U T4 DNA ligase (Promega), 1 µL rapid ligation buffer (10x) and adjusted to 10 µL by H_2O. This reaction mixture was incubated at 4°C for overnight. The ligation product was introduced into *E. coli* DH5α as described by Suharsono (2002).

Selection of **E. coli** *containing recombinant plasmid*

E. coli DH5α containing recombinant plasmid was selected by using ampicillin and blue-white selection in the solid LB media (10 g L^{-1} bacto-trypton, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, pH7.5, 1.5% bacto agar) containing 100 mg L^{-1} ampicillin, 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 50 mg L^{-1} X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). White colony grown in the selection media was used as source of template for PCR to detect the presence of *GmMt2* in the transformed bacteria. For this purpose, one white colony was picked up by using toothpick, then suspended in 6.5 µL dH_2O and heated at 95°C in the waterbath for 10 minutes and

immediately cooled in the ice for 5 minutes. This suspension was used as the template to amplify the insert *GmMt2* with the same composition and condition of PCR as described for isolation of cDNA of *GmMt2*.

Isolation and analysis of recombinant plasmid containing GmMt2

The isolation of recombinant pGEM-T Easy plasmid DNA contained in *E. coli* DH5α was carried out as described by Suharsono (2002). To excised the insert *GmMt2* cDNA, recombinant plasmid DNA was cut by *Eco*R1 (Fermentas) by mixing 200 ng plasmid DNA, 10 U *Eco*R1, 1x restriction buffer and dH_2O in 20 µL solution. The solution was incubated at 37°C for 2 hours.

Sequencing and sequence analysis of GmMt2

Sequencing of *GmMt2* cDNA was performed by using DNA sequencer ABI Model 3100/3130 MERCIAN. Local alignment analysis of *GmMt2* was carried out by using BLAST (Basic Local Alignment Search Tools) (http://www.ncbi.nlm.nih.gov/BLAST/) program. Amino acid sequence was deduced by using translation program of EXPASY (http://www.expasy.ch/tools/dna.htm). The analysis of open reading frame (ORF) was carried out by using BESTORF program (http://www.softberry/bestorf/htm). Analysis of restriction sites in the *GmMt2* cDNA was performed by using NEBCutter program (http://www.firstmarket.com/cutter/cut2.html).

RESULTS AND DISCUSSIONS

Isolation of total RNA

Total RNA of root tips of soybean cv Slamet has been successfully isolated. Quantification of total RNA by using spectrophotometer showed that the efficacy of total RNA isolation was 187 µg of total RNA per g root tips. This result was enough for the synthesis of total cDNA. The OD_{260}/OD_{280} ratio of total RNA was 1.88 indicating that isolated total RNA had a very good purity from the protein contaminant (Farrel, 1993).

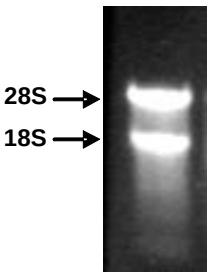

Figure 1. Total RNA isolated from root tips.

Electrophoresis of total RNA in denaturated agarose gel containing formaldehyde resulted two dominant bands. These two bands were 18S and 28S ribosomal RNA (rRNA) (Figure 1). This result indicated that isolated total RNA was in a very good quality. Therefore mRNA contained in the total RNA was also in a very good quality for the integrity. The integrity of mRNA is very important in the synthesis of cDNA.

Synthesis of total cDNA.

Total cDNA had been successfully synthesized from total RNA as template by reverse transcription method. By using oligo(dT) primer, only mRNA can be used as template for cDNA synthesis because it contains poly-A tail. This poly-A tail can form a complementary pair with oligo(dT) primer. rRNA and tRNA do not have poly-A tail, so they can not be used as template for cDNA synthesis. PCR by using total cDNA as template and spesific primers for cDNA of exon1-exon2 of β-actin gene resulted one band DNA at 450 bp in size (Figure 2).

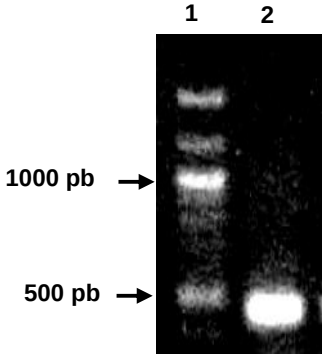

Figure 2. cDNA of exon1-exon2 of β-actin resulted from PCR by using total cDNA as template. 1= 1 kb ladder DNA, 2= exon1-exon2 of β-actin cDNA.

This result showed that the amplified region was cDNA of exon1-exon2 and was not genomic DNA of β-actin. Amplication of exon1-exon2 region of genomic DNA resulted about 550 bp DNA fragment containing intron1. This intron1 was spliced during the synthesis of mRNA. The size of intron1 of actin of soybean is around 90 bp (Shah et al., 1982). The absence of 550 bp DNA band in the agarose gel (Figure 2) showed that the synthesized total cDNA was pure and free from genomic DNA contaminant. Beside showing the purity of cDNA from genomic DNA contaminant, this result showed also that the isolated total RNA had a very good quality.

Isolation and cloning of GmMt2 cDNA

The isolation of *GmMt2* cDNA by PCR by using total cDNA as a template and specific primers for *Mt2* resulted cDNA fragment of about 250 bp (Figure 3). This fragment was then called *GmMt2* (*Glycine max Mt2*) fragment. It has the same size as *Mt2* cDNA of *A. thaliana* (*AtMt2A*).

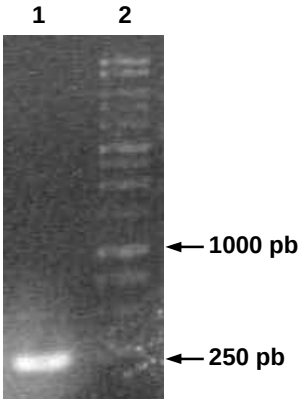

Figure 3. *GmMt2* fragment resulted from PCR using total cDNA as template. 1= *GmMt2*, 2= 1 kb marker DNA.

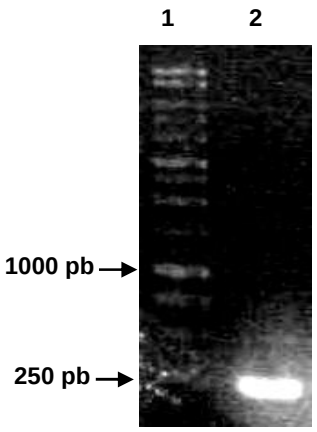

Figure 4. The result of PCR using white colony as the source of template. 1= 1 kb DNA marker, 2= *GmMt2*.

GmMt2 fragment had been inserted in the middle of *lacZ* of pGEM-T Easy plasmid and this ligation had been successfully introduced into *E. coli* strain DH5α. The successful of insertion of *GmMt2* into pGEM-T Easy plasmid and introduction of recombinant pGEM-T Easy plasmid into *E. coli* was demonstrated by the presence of white colony grown in the selection LB media containing ampicillin, IPTG and X-gal. Only *E. coli* strain DH5α containing plasmid can survive in this selection media, and only the colony containing recombinant plasmid had a white color. The blue colonies survived in this selection media contained non-recombinant plasmid. The development of blue color is due to the conversion of uncolored X-gal substrate into blue color by β–galactosidase encoded by *lacZ* gene. The cloning sites (CS) are located in the middle of *lacZ* gene. The expression of *lacZ* is induced by IPTG. The blue color of colonies is developed when in the middle of *lacZ* does not have an insertion of DNA. If *GmMt2* fragment inserts in the *lacZ* gene, β-galactosidase is not synthesized and the *E. coli* colonies develop in white color.

The presence of *GmMt2* inserted in the recombinant plasmid contained in the white colonies of *E. coli* was confirmed by colony-PCR. Colony-PCR using white colony as source of template resulted 250 bp DNA fragment showing that this white colony contained *GmMt2* fragment (Figure 4).

To reconfirm that *GmMt2* had been inserted in the pGEM-T Easy, recombinant plasmid DNA had been isolated from white colony, and cut with *Eco*R1 to excise insert *GmMt2*. Digestion of recombinant plasmid DNA by *Eco*R1 resulted two DNA fragments, one was 3,000 bp fragment DNA corresponding to pGEM-T Easy vector, and the other was 250 bp DNA fragment being similar in size with *GmMt2* fragment (Figure 5). This result showed that the *GmMt2* fragment had been inserted into pGEM-T Easy plasmid.

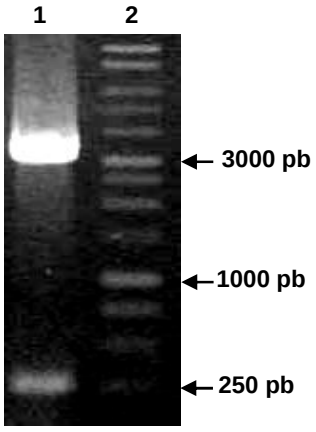

Figure 5. Digestion of recombinant plasmid by *Eco*R1. 1= recombinant plasmid cut by *Eco*R1, 2= 1 kb DNA marker.

Analysis of GmMt2 fragment

DNA sequencing of insert *GmMt2* fragment resulted 257 nucleotides containing 246 bp *ORF* (*open reading frame*). This ORF encodes 81 amino acids with 14 cysteine residues. Local alignment analysis with BLASTn showed that *GmMt2* had similarity 100% with *AtMt2A* of *A. thaliana*.

Since the nucleotide sequence is the same, so the deduced amino acid sequence of *GmMt2* is also the same as *AtMt2A*. Therefore, GmMT2 apparently has a similar role as AtMT2A in the binding and detoxifying metal ions and avoiding oxidative damage (Zhou and Goldsbrough, 1995). Nucleotide analysis showed that *GmMt2* contains start (ATG) and stop (TGA) codons, therefore this isolated *GmMt2* is a full length of cDNA of *Mt2* (Figure 6).

Based on restriction site analysis, *GmMt2* fragment does not contain restriction sites located in the CS of pGEM-T Easy, therefore all restriction sites in the CS can be used to excise the insert *GmMt2* from pGEM-T Easy. *GmMt2* fragment contains *Bbv*I,

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                                                                    nuk aa
atg tct tgc tgt gga gga aac tgc gga tgt gga tct ggc tgc aag tgc      48
 M   S   C   C   G   G   N   C   G   C   G   S   G   C   K   C        16
ggc aac ggt tgt gga ggt tgc aaa atg tac cct gac ttg gga ttc tcc      96
 G   N   G   C   G   G   C   K   M   Y   P   D   L   G   F   S        32
ggc gag aca acc aca act gag act ttt gtc ttg ggc gtt gca ccg gcg     144
 G   E   T   T   T   T   E   T   F   V   L   G   V   A   P   A        48
atg aag aat cag tac gag gct tca ggg gag agt aac aac gct gag aac     192
 M   K   N   Q   Y   E   A   S   G   E   S   N   N   A   E   N        64
gat gct tgc aag tgt gga tct gac tgc aag tgt gat cct tgc acc tgc     240
 D   A   C   K   C   G   S   D   C   K   C   D   P   C   T   C        80
aag tga                                                             246
 K   -                                                                81
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Figure 6. Nucleotide and deduced amino acid sequences of *GmMt2.*

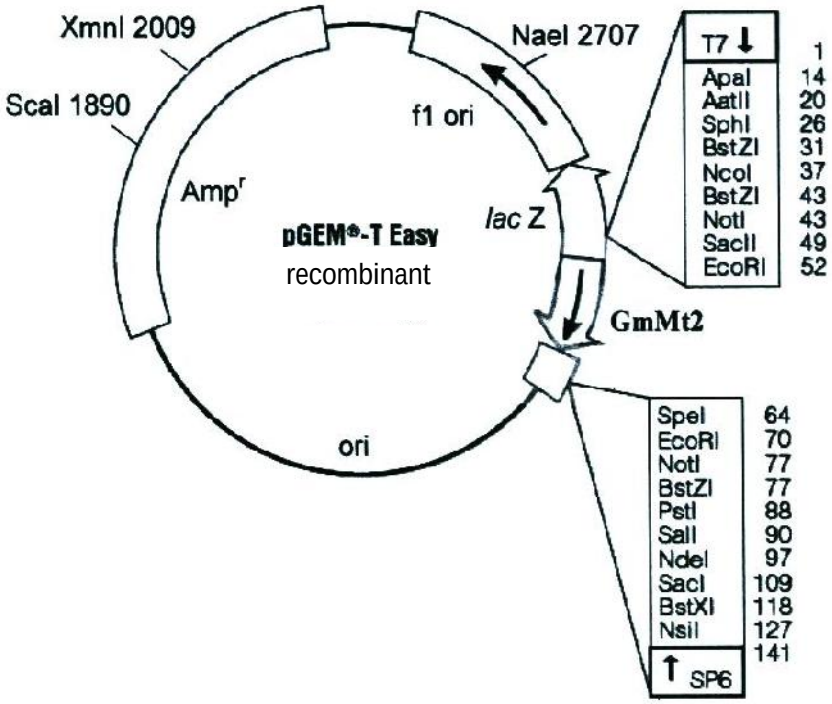

Figure 7. Orientation c

*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, so these sites can not be used to clone this gene because they will cut it into two fragments or more. The restriction site analysis of the DNA fragment is very important for the genetic engineering.

The analysis of *GmMt2* orientation in the CS based on nucleotide sequence showed that *GmMt2* fragment was located in the downstream of T7 primer inserted in the *lacZ* and in the opposite orientation of the *lacZ* gene (Figure 7). The orientation of gene is very important for the gene expression.

The analysis of amino acid sequence deduced from nucleotide sequence showed that the motifs of Cys amino acid sequence of GmMT2 are 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). These motifs of Cys sequence of GmMT2 are specific for MT protein type 2 of plant (Robinson et al., 1993; Cobbett and Goldsbrough, 2002).

CONCLUSIONS

Full length of cDNA encoding for metallothionein of soybean cv Slamet (*GmMt2*) had been isolated and cloned into pGEM-T Easy plasmid. This cDNA has 246 nucleotides encoding for 81 amino acids. *GmMt2* has the same sequence as *AtMt2* of *A. thaliana*. The motifs of Cys amino acid sequence of GmMT2 were Cys-Cys, Cys-X-Cys, and Cys-X-X-Cys.

AKNOWLEDGMENT

This research was financially supported by Competence Research Grant (Hibah Kompetensi), Directorate Research and Community Services, Directorate General of Higher Education, Ministry of National Education, Republic of Indonesia, by Contract SPK 008/HIKOM/DP2M/2008.

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