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

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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 Goldsborough, 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 Goldsborough, 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; Narley 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 oxygen 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.
MATERIALS AND METHODS

Materials
Soybean cv Slamet was used as plant material. The primers of ActF (ATGGCAGATGCGAGGATAT) and ActR (CAGTGTTCGACACCTTGCA) 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 (TCGAGAAGAATCTTGGTATGG) and M7R (CTTGACATGCAAGTGAGAG) 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 H2O-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 Na₂-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) H2O-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 electrophoresed at 100 volt 30 minutes. After submerging in the EtBr solution (0.5 g mL⁻¹) for 30 minutes, and washing by H2O, 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 L RT buffer (5x), 20 pmol oligo(dT), 4 mM dNTP mix, 10 mM DTT, 1 U SuperScript III RTase (Invitrogen), and adjusted to 20 μL by the addition of H2O-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 L total cDNA, 1 μL Taq buffer (10x), 40 mM MgCl₂, 4 mM dNTP mix, 10 pmol ActF primer, 10 pmol ActR primer, 4% DMSO, 0.5 U Taq DNA polymerase (Fermentas) and H2O in the final volume of 10 L. 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 L total cDNA, 2 L 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 L by H2O. 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 L of PCR product, 1 L (10 ng) pGEM-T Easy, 0.5 U T4 DNA ligase (Promega), 1 L rapid ligation buffer (10x) and adjusted to 10 L by H2O. 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 L dH2O 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 EcoR1 (Fermentas) by mixing 200 ng plasmid DNA, 10 U EcoR1, 1x restriction buffer and dH2O in 20 Λ 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 OD260/OD280 ratio of total RNA was 1.88 indicating that isolated total RNA had a very good purity from the protein contaminant (Farrel, 1993).

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 specific primers for cDNA of exon1-exon2 of β-actin gene resulted one band DNA at 450 bp in size (Figure 2).

Results 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 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.

Figure 1. Total RNA isolated from root tips.
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 EcoR1 to excise insert GmMt2. Digestion of recombinant plasmid DNA by EcoR1 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.

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 BbvI,
Figure 6. Nucleotide and deduced amino acid sequences of GmMt2.

Figure 7. Orientation c

BsaBI, BtsCl, Tsel, FokI, ApoKI, CspCI, HpyCH4III, PshAI, BsrFI, SgrAI, Acul, BtgZI, MboII, SfaNI, Cac8I and Hpy188I 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 Goldsbridge, 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.

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REFERENCES


