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Metal inducible activity of the oil palm metallothionein-like gene promoter (*MT3-A*) in prokaryotes

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Reporter gene activity under the regulation of the oil palm metallothionein-like gene, *MT3-A* promoter was assessed in prokaryotes. Vector constructs containing *MT3-A* promoter with (*W1MT3-A*) and without (*W2MT3-A*) five prime untranslated region (5′-UTR) fused to ß-glucuronidase (GUS) gene in pCAMBIA 1304 vector were produced. 5′-rapid amplification of cDNA ends (RACE) using mRNA isolated from *Escherichia coli* and *Agrobacterium tumefaciens* harboring *W1MT3-A* confirmed that fusion transcripts of *MT3-A* 5′-UTR-GUS were successfully produced in both bacteria. Competitive PCR and GUS fluorometric assay showed changes in the level of GUS gene transcripts and enzyme activity in response to increasing concentrations of Cu^{2+} and Zn^{2+}. The application of Cu^{2+} increased GUS activity and GUS mRNA level in both *bacteria*. In *E. coli*, a high level of GUS activity driven by *W1MT3-A* and *W2MT3-A* was observed in treatment with 25 µM Cu^{2+} resulting in an increase in the GUS mRNA level to 7.2 and 7.5×10^{-4} pmol/µl respectively, compared to the control (5.1×10^{-4} pmol/µl). The lowest GUS activity and GUS mRNA level were obtained for *W1MT3-A* and *W2MT3-A* in the presence of 100 µM Cu^{2+} in both bacteria compared to the control (without Cu^{2+}). The application of different Zn^{2+} concentrations resulted in a strong decrease in the GUS activity and GUS mRNA level in *E. coli* and *A. tumefaciens*. These findings showed that the oil palm *MT3-A* promoter is functional in prokaryotes and produced detectable GUS transcripts and enzyme activities. This promoter may potentially be used in prokaryotic systems which require metal inducible gene expression.

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[**Key words:** Metallothionein gene promoter; Competitive PCR; *Elaeis guineensis*; *Escherichia coli*; *Agrobacterium tumefaciens*]

There are numerous differences between the transcription machinery of eukaryotes and prokaryotes. In prokaryotes, RNA polymerase consists of five different proteins, 2α, 1ß, 1ß′ subunits and one of a variety of regulatory σ subunits (1). The σ protein in the polymerase enables it to interact with sequences at −10 and −35 for initiation of transcription. Unlike prokaryotes, the process of transcription in eukaryotes is much more complex. The sigma factor homologue does not exist in eukaryotes. Instead, six general transcription factors, TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH are important for proper initiation of transcription (2). Also, the task of transcription is shared by three different RNA polymerases; RNA polymerase I, II and III. In addition, a complex consisting of RNA polymerase II and the general transcription factors, catalyzes the transcription of all protein coding genes in eukaryotes. Due to these substantial differences, it is usually considered that an eukaryotic promoter transferred into prokaryotes will not produce an efficient transcriptional activity (3).

Information on the activities of eukaryotic promoters in prokaryotes is limited. Eight different mammalian promoters fused to the coding region of the chloramphenicol acetyl transferase (CAT) reporter gene were tested for their activities *in vivo* in *E. coli* (4). It was demonstrated that the activities of the different promoters vary in strength and the transcription initiation occurred at the sites expected for eukaryotes rather than prokaryotes suggesting that it is the DNA sequence and not RNA polymerase which determines the site of transcription initiation.

Metallothionein genes (*MT*) are found in a wide range of organisms, including prokaryotes, yeast, plants, invertebrates and vertebrates (5,6). In bacteria, *MTs* are only found in cyanobacteria (7). However, Robinson (8) reported a cryptic copper MT in the bacterium that causes tuberculosis. MTs are involved in homeostasis of essential metal ions such as Zn^{2+}, Fe^{2+} and Cu^{2+} and also in the detoxification of toxic metals like cadmium, copper, silver and mercury (9,10). The expression of several type 1 and type 2 plant MT-like genes has been shown to be regulated by heavy metals. The expression of rice and wheat MT-like genes was found to be induced by a wide range of metals such as Cd^{2+}, Cu^{2+}, Zn^{2+} and Fe^{2+} (11).

There are some evidences that *MT* promoters are inducible by metal ions in eukaryotes. *MT* promoter from Ciliate (protozoa) showed a strong copper-inducible activity (12). Trout *MT-A* promoter and mouse *MT-I* promoter revealed greater activities in fish cells after exposure to zinc (13). Binding of transcription factor MTF-1 to metal-responsive element increased in chicken *MT* promoter in response to

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metal ions and oxidative stress in transgenic mice (14). *MT-1* gene promoter of mouse showed differential expression in response to various concentrations of metal ions in the environment (15). Analysis of *Arabidopsis thaliana MT2-A* promoter sequence showed the presence of a Cu-responsive *cis*-acting element, which is responsible for the Cu-induced expression in cotyledons, leaves, and lateral root tips (Bundithya, W., Ph.D. thesis, Purdue University., Purdue, 1999).

MT-like gene promoter (*MT3-A*) has been isolated from the oil palm *Elaeis guineensis* (Siti Nor Akmar, A., and Zubaidah, A.R., US patent 7,173,120 B2, 2007). A Cu-responsive element (GTAC) was identified in the promoter sequence. In this study, activities of the oil palm *MT3-A* promoter as influenced by prokaryotic transcription machinery were determined in two bacterial models, *E. coli* and *A. tumefaciens* using GUS reporter gene assay. We present evidence that the oil palm *MT3-A* promoter is functional in *E. coli* and *A. tumefaciens* and its activity is influenced by metal ions in both bacteria.

MATERIALS AND METHODS

Preparation of vector constructs Genomic DNA was extracted from leaves of oil palm *Elaeis guineensis* and used as a template to amplify genomic fragments containing *MT3-A* promoter in a PCR reaction. Three primers were designed and used for the amplification of the *MT3-A* promoter. The *Hind* III and *Bgl* II restriction sites were introduced to one forward primer (5′-ccc aag ctt aaa tta ctg cca tgg-3′) and two reverse primers (5′-gaa gat cta cca tgt ttg agt ttc g-3′ and 5′-gaa gat cta cca tgg ttg ttg agg aa-3′), respectively to facilitate cloning into pCAMBIA 1304. Furthermore based on CaMV 35S sequence two primers 5′-cat gcc atg ggg cac tgg ccg-3′ (forward) and 5′-ccc aag ctt gtc aag agt ccc-3′ (reverse) were designed and used for amplification of CaMV 35S promoter. The *Nco* I and *Hind* III restriction sites were introduced to the forward and reverse primers, respectively to change the direction of the CaMV 35S promoter which is considered as a promoterless vector (16). The PCR was performed using a high fidelity platinum Taq DNA polymerase (Invitrogen Corporation, Carlsbad, California. USA) with 30 s pre-denaturation at 94°C and subsequent 35 cycles at 94°C for 30 s, followed by 54°C for 30 s, 68°C for 1 min and a final extension step at 68°C for 5 min. Three DNA fragments containing *W1MT3-A* (1053 bp), *W2MT-A* (990 bp) from genomic the DNA of oil palm and one DNA fragment containing CaMV 35S (700 bp) were amplified from plasmid 1304. The PCR products were separated on 1% agarose gel and purified using a gel purification kit (QIAGEN Inc. Valencia, CA, USA). Binary vector pCAMBIA 1304 (12.36 kb) was constructed by replacing the CaMV 35S promoter with *W1MT3-A*, *W2MT3-A* and reverse direction of CaMV 35S (RCaMV), separately (Fig. 1). Ligation of digested vector and inserts were carried out by using T4 DNA ligase according to the manufacturer's instruction (Fermentas Inc, Hanover, USA).

Bacterial transformation The newly prepared plasmid constructs and intact plasmid 1304 (as a positive control) were introduced into competent cells of *E. coli* strain DH5-α and then spread on LB agar plate containing 50 μg/ml of kanamycin and 40 μl of 0.02% X-gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside). White colonies were grown in LB broth medium and lysed by an alkaline lysis method, followed by purification of the plasmid DNA using QIAGEN kit. Fusion of the inserts and pCAMBIA 1304 vector was confirmed by double digestion and sequencing. Transformation of *Agrobacterium tumefaciens* LBA4404 was carried out using the same protocol as *E. coli* (as described above). Antibiotic selection of the bacterial cells was done on LB agar plate containing 50 μg/ml rifampicin, 30 μg/ml streptomycin and 50 μg/ml kanamycin. Transformed colonies appeared after 2–3 days of incubation at 28°C.

GUS assay GUS analysis was carried out based on the protocol of Verne (17) with the following modifications. Transformed *E. coli* and *A. tumefaciens* were incubated overnight in 5 ml of LB broth with 0.2 g/l $MgSO_4$ (to reduce clumping) at 37°C and 28°C, respectively with 200 rpm shaking. Overnight cultures of untransformed *E. coli* and *A. tumefaciens* were used as negative controls. The overnight grown (100 μl) cultures were subcultured into 10 ml of the same medium using the same condition until they reached $OD_{600} = 0.5$. Then 1 ml of medium from each culture was mixed with 100 μl of GUS assay buffer [50 mM Na_2HPO_4 pH 7.0, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 10 mM Na_2EDTA pH 8.0, 0.1% Triton X-100, and 1 mM 5-bromo-4-chloro-3-indolyl-3-D-glucuronic acid (X-gluc) in Dimethylformamide] and finally incubated at 37°C.

Treatment of bacterial culture with copper and zinc and RNA Isolation Bacterial cultures (500 μl) containing, *W1MT3-A*, *W2MT3-A*, RCaMV construct and CaMV 35S with $OD_{600} = 0.5$ (as described above) were subcultured into 5 ml of LB broth supplemented with each of $CuSO_4$ and $ZnSO_4$ at different concentrations (0, 25, 50, 75 and 100 μM). The *E. coli* and *A. tumefaciens* cultures were incubated at 37°C and 28°C for 4 h on a shaker, respectively. One ml of each culture was centrifuged for 2 min at 14,000 rpm, the supernatant was removed and the pellet was frozen at −80°C until used (18). Two volumes of RNA protect bacterial reagent (QIAGEN) were added to the rest of the cultures. The RNA lysozyme lysates were used for isolation of RNA according to the manufacturer's instructions of RNeasy Mini kit (QIAGEN). The integrity and purity of the total RNA was determined by 1% agarose gel electrophoresis analysis and spectrophotometry.

Reverse transcription polymerase chain reaction (RT-PCR) Reverse transcription was performed based on the protocol of Jones (19) with some modifications. For first strand cDNA synthesis, 0.2× First-Strand Buffer, 0.4 mM Dithiothreitol (DTT), 20 U RNase inhibitor, 0.2 mM dNTP mix and 0.2× Random Primer Mix were annealed to 1 μg of each total RNA as determined by spectrophotometry and extended using Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase (Clontech) for 1.5 h at 42°C and 70°C for 10 min. The products were diluted (10×) with Tricine–EDTA buffer and then stored at −20°C until used.

5′-Rapid Amplification of cDNA End (5′-RACE) in prokaryotes A Switching Mechanism at 5′ ends of RNA Transcript (SMARTer™) technology (Clontech

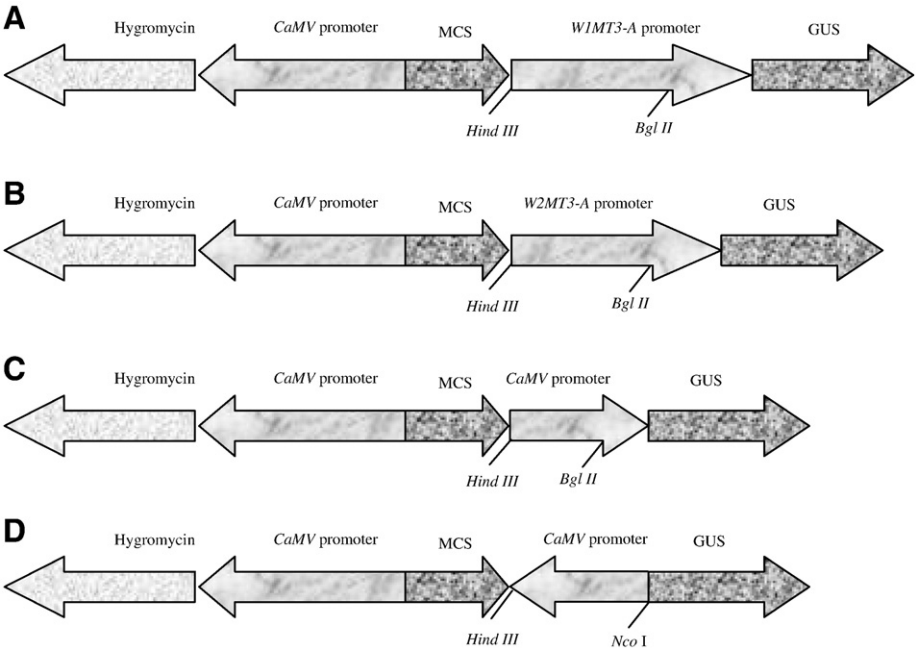

FIG. 1. Schematic representation of gene constructs within the T-DNA region containing A) *W1MT3-A*; B) *W2MT3-A*; C) CaMV 35S and D) RCaMV promoter.

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