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MESOCARP-SPECIFIC METALLOTHIONEIN-LIKE GENE PROMOTER FOR GENETIC ENGINEERING OF OIL PALM

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ABSTRACT

Primers from within the coding region were used to capture the 5′ regulatory sequence of the mesocarp-specific metallothionein-like gene, MT3-A*, via PCR-based genome walking. The amplified 1040 bp genomic fragment was cloned and sequenced. The sequence of the genomic clone showed total homology with the* MT3-A *cDNA sequence within their overlapping regions. Rapid amplification of 5′-cDNA ends (5′-RACE) was used to determine the full length cDNA sequence and the putative transcription site of the gene. The adenine residue at the 5′-end of the RACE product was chosen as the likely transcription start site. The 986 bp promoter region upstream of the adenine contains putative regulatory elements including a TATA box, an ethylene responsive element in reverse orientation and two I-boxes. Functional analysis of the* MT3-A *promoter was performed using a transient assay system. Transient expression of β-glucuronidase (GUS) examined using qualitative histochemical GUS assay can be detected in both oil palm mesocarp and leaf tissue slices bombarded with the pBI221 transformation vector which contains the GUS reporter gene under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. However, when the CaMV-35S promoter was replaced with* MT3-A *promoter in the transformation vector and used for bombardment, transient expression of GUS was detected in the oil palm mesocarp slices only and not in the leaf tissue. This suggests that the* MT3-A *promoter can be used to target specific gene expression into oil palm mesocarp tissues.*

Keywords: oil palm genetic engineering, mesocarp-specific promoter, metallothionein-like gene.

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INTRODUCTION

Myriad fatty acids are synthesized by plants but most in too small quantities to be of any economic use. Genetic engineering provides the means to unleash the potential by modifying the plants to produce more of the fatty acids of economic importance (reviewed by Napier, 2007). In addition, oil crops such as the oil palm can be engineered to produce high value novel products by metabolically engineering the proportions of intermediates and metabolites already being produced. In the Malaysia-MIT Biotechnology Partnership Programme (MMBPP), production of bioplastic (polyhydroxybutyrate, PHB) via expressing genes from the bacterium, *Alcaligenes eutrophus* (Madison and Huisman, 1999), is targeted to the oil palm mesocarp. The high acetyl-CoA pool in the mesocarp during oil synthesis period can be the initial substrate for PHB production (Reddy *et al*., 2003).

Genetic engineering relies heavily on reliable transformation techniques for achieving stable integration and regulatory sequences for controlling the expression of the introduced genes. There has been important progress in the development of a reliable transformation system using biolistic techniques for the oil palm (Parveez, 2000). Analysis

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of several plant promoters using transgenic plant systems showed that regions of several hundred to about one kilo base pairs can produce faithful expression patterns of reporter genes *in vivo* (Yoshida and Shinmyo, 2000). The availability of seed-specific promoters that drive gene expression over the entire period of oil deposition in oil-bearing crops like rapeseed (Voelker *et al*., 1996) and soyabean (Kinney, 1996) has been a major contribution to the success in altering oil composition by genetic engineering. These promoters have ensured that most of the effects on lipid metabolism are confined to storage lipids without greatly affecting lipid metabolism in the leaves or other tissues which may otherwise have had deleterious effects on the transgenic plants.

Similarly for the oil palm, efforts to modify the mesocarp oil composition would benefit greatly from the availability of temporally-regulated and tissue-targeted gene promoters. Such promoters would preferably be able to drive specific and high level expression of the introduced genes in the mesocarp during oil synthesis (16-20 weeks after anthesis). These promoters would also be applicable in metabolic engineering involving manipulation of the different biosynthetic pathways in this tissue. In the metabolic engineering endeavours to produce PHB in flax, for example, targeted expression into the stem using stem-specific promoter resulted in higher PHB production than that obtained using a constitutive CaMV 35S promoter (Wrobel *et al*., 2003).

Metallothioneins are cysteine-rich metal binding proteins found in various plants and animals (Cobbett and Goldsbrough, 2002). The cDNA clones encoding for two differentially regulated metallothionein like genes, designated *MT3-A* and *MT3-B,* have been isolated from the oil palm. The *MT3-A* was shown to be specifically and abundantly expressed in the mesocarp tissues, while *MT3-B* is expressed (at a much lower level) in the mesocarp as well as the roots. The promoter sequence of *MT3-B* was isolated and found to contain several putative regulatory elements including a root-specfic element, ethylene-responsive elements and a metal-responsive element (Siti Nor Akmar *et al*., 2002). Isolation of the promoter sequence of *MT3-A* is of interest as a genetic engineering tool for targeting high level and specific expression of introduced genes into the oil palm mesocarp. This article reports on the isolation, sequence analysis and functional characterization of the oil palm *MT3-A* promoter.

MATERIALS AND METHODS

5′-RACE

Synthesis of first strand cDNA, addition of poly(dG) tail sequence and second strand cDNA synthesis were carried out according to Siti Nor Akmar *et al*. (2002). First strand cDNA was synthesized from 15 weeks after anthesis (WAA.) oil palm mesocarp RNA using the antisense 3′ sequence specific primer M1 (5′ CTA CCA ATA GCA ATC CAT TAA 3′) from 3′-UTR of *MT3-A* cDNA in a 20 µl reaction mixture containing 5 µg total RNA. Second strand cDNA synthesis was performed using the anchor primer KA1 (5′ CCT CCC CCC CCC CCC C 3′) and nested primer M32 (5′ CAC CAT GAC AGA AAC ATA TC 3′).

Promoter Isolation

Isolation of the mesocarp-specific promoter was carried out using the Universal GenomeWalker Kit (Clontech). Aliquots containing 2.5 µg DNA were digested with restriction enzymes *Dra* I, *Eco* RV, *Pvu* II and *Stu* I that produce blunt ends, and ligated to the GenomeWalker Adaptor creating the GenomeWalker libraries. Primary PCR was performed using 1 µl aliquots of each library with the antisense gene-specific primer GSP1 5′CCACACAAGCACAGCTAGCACCACACTTG3' from the 3′-terminal of the coding region of *MT3-A* and primer API provided with the Kit. The PCR product was diluted 50x and 1 µl used in the secondary PCR reaction with the antisense nested gene-specific primer GSP2 5′CTGGCTCTTGT CAGCACAATCGCAGTTGC3' from the 5'-terminal of the *MT3-A* coding region and primer AP2 from the Kit. PCR was carried out using the Advantage *Tth* Polymerase Mix from Clontech and a Perkin-Elmer 9600 thermal cycler following the cycle conditions recommended in the GenomeWalker Kit Manual. The secondary PCR product was analysed and purified from agarose gel using the gel extraction kit from Qiagen and cloned into PCRII-TOPO vector (Invitrogen). The recombinant clone designated pMT3A-P1a was sequenced using M 13 forward and reverse primers.

Sequence Analysis

Plasmid DNA for sequencing was extracted using the Qiagen plasmid mini kit. DNA sequencing was carried out from both directions using the ABI automated sequencer. The DNASIS Sequence Analysis Software was used for sequence analysis and search for similarity between nucleotide and amino acid sequences.

Cloning of the *MT3-A* Promoter into pBI221

Plasmid MT3AP-GUS was produced by replacing the CaMV 35S promoter in pBI221 with the oil palm *MT3-A* promoter. The CaMV 35S promoter was removed by digesting pBI221 with *Hind* III and *Xba* I. The *MT3-A* promoter was amplified using primers gC3 5' CCC AAG CTT AAA TTA CTG CCA TG 3'

which contains an introduced *Hind* III site and gC5. Primer gC5 5' TGC TCT AGA CAG GAA ACC AGA GAC 3' is an antisense primer 16 bases upstream of the translation start site with an *Xba* I site introduced.

The PCR reaction mixture (50 µl) for amplifying the *MT3-A* promoter contained 5.0 µl 2 mM dNTP, 3.3 µl 15 µM gC3 primer, 3.3 µl 15 µM gC5 primer, 25 ng plasmid pMT3A-Pla, 5.0 µl 10x enzyme buffer containing 1.5 mM $MgC1_2$ and 2.6 U Expand High Fidelity Polymerase (Roche). The PCR conditions were as follows: 1 cycle 94°C for 3 min, 20 cycles 94°C for 1 min, 42°C for 1 min and 72°C for 90 s followed by 1 cycle 72°C for 10 min. The PCR product was purified using the QlAquick PCR purification kit (Qiagen). Ligation was performed using a 1:3 molar ratio of vector:insert in 15.0 µl reaction volume containing 1.5 µl 10x ligase buffer and 1.5 µl T4 DNA ligase (1U μl^{-1}) and incubation at 16°C O/N.

Two microlitres were used to transform competent *E. coli* host cells. The successful replacement of 35S CaMV promoter by the oil palm *MT3-A* promoter in pBI221 was verified by restriction analysis and sequencing. The recombinant vector was designated MT3AP-GUS.

Biolistic Method and Transient Expression for Promoter Analysis

Oil palm fruits (12 WAA) were sterilized by soaking in Tween 20 for 10 min followed by in 25% chlorox for 20 min. The fruits were then rinsed several times with sterile distilled water. The sterilized fruits were cut into small pieces (l cm x l cm), and the explants placed on Murashige and Skoog (Murashige and Skoog, 1962) medium. These cultures were kept at 28°C in the dark for 24-48 hr before bombardment.

Promoter analysis was carried out using β-glucuronidase (GUS) as the reporter gene using pBI221 (CaMV-GUS) and the constructed plasmid MT3AP-GUS. All the plasmids were isolated using the QlAgen Spin Miniprep Kit. The 30 µg DNA were added to 100 µl aliquot of gold. Then 100 µl 2.5 M $CaC1_2$ and 40 µl 0.1 M spermidine were added while vortexing. The mixture was centrifuged down at 10 000 rpm, the supernatant, removed and the microcarrier washed with 100% ethanol. These steps were repeated twice and, finally, the microcarrier resuspended in 60 µl ethanol and kept at -20°C until use.

The oil palm fruits and leaf tissues were bombarded with Biolistic GunHe/100 (Biorad) USA. Five to 10 microlitres of DNA-coated microcarrier were placed in the centre of the macrocarrier. The mesocarp tissues were bombarded with 1550 psi helium pressure at 9 cm distance between macrocarrier and target tissue, with 8 µl gold-coated DNA loaded per bombardment. The leaves (control tissues) were bombarded at 1100 psi helium pressure and 6 cm distance between macrocarrier and target tissue. The distance between the rupture disk and macrocarrier was fixed at 6 mm while the distance between the macrocarrier and stopping plate at 11 mm. The vacuum pressure was maintained at 27" Hg. The experiments were conducted in triplicate and repeated at least once for each tissue/plasmid combination.

Histochemical Assay for GUS

The substrate used for histochemical localization of β-glucuronidase activity was 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). This substrate works very well, giving a blue precipitate at the site of enzyme activity. The bombarded oil palm tissues were fixed for 5 min on ice in fixation solution containing 5% formaldehyde in sodium phosphate buffer pH 7.0. The histochemical assay was performed based on the method of Jefferson (1987). The fixed tissues were transferred to a GUS-staining solution (0.2 M Na_2HPO_4, 0.2 M $Na_2H_2PO_4$, 10% Triton X-100, 50 mg ml^{-1} X-Gluc, 0.25 M Na_2EDTA, 0.005 M KFerricyanide and 0.005 M KFerrocyanide, pH 7.0). The bombarded tissues were incubated at 37°C for several hours before analysis using a light microscope.

RESULTS AND DISCUSSION

Aliquots from the different oil palm genome walker libraries were used as templates in PCR reactions using a gene-specific primer (GSP1) and primer AP1 from the Adaptor sequence. The GSP1 sequence falls within a fairly variable part of the coding region where three out of eight amino acid residues encoded by *MT3-A* are different from *MT3-B*. The sizes of the bands from this primary PCR reaction using the four different genome walker libraries are shown in *Figure 1a*. The product of the *Dra* I library of 1.2 kb, which was the biggest, was selected for a second round PCR using a nested gene-specific primer (GSP2). This secondary PCR reaction should specifically amplify the fragments containing the *MT3-A* sequence excluding fragments produced in the primary PCR reaction due to non-specific binding of the primers. The band obtained, which was slightly smaller than the primary PCR product as expected (*Figure 2b*), was cloned and sequenced.

Total RNA from 15 WAA oil palm mesocarp was used as template in 5′ RACE reactions in order to obtain the full length cDNA sequence of *MT3-A* and to determine the putative transcription start site of this gene. First strand cDNA synthesis was carried out using a gene-specific primer, M1, based on the sequence at the 3′ end of the *MT3-A* cDNA just prior to the poly(A) tail. Primers from the coding region were not used to prevent amplification of the *MT3-B*

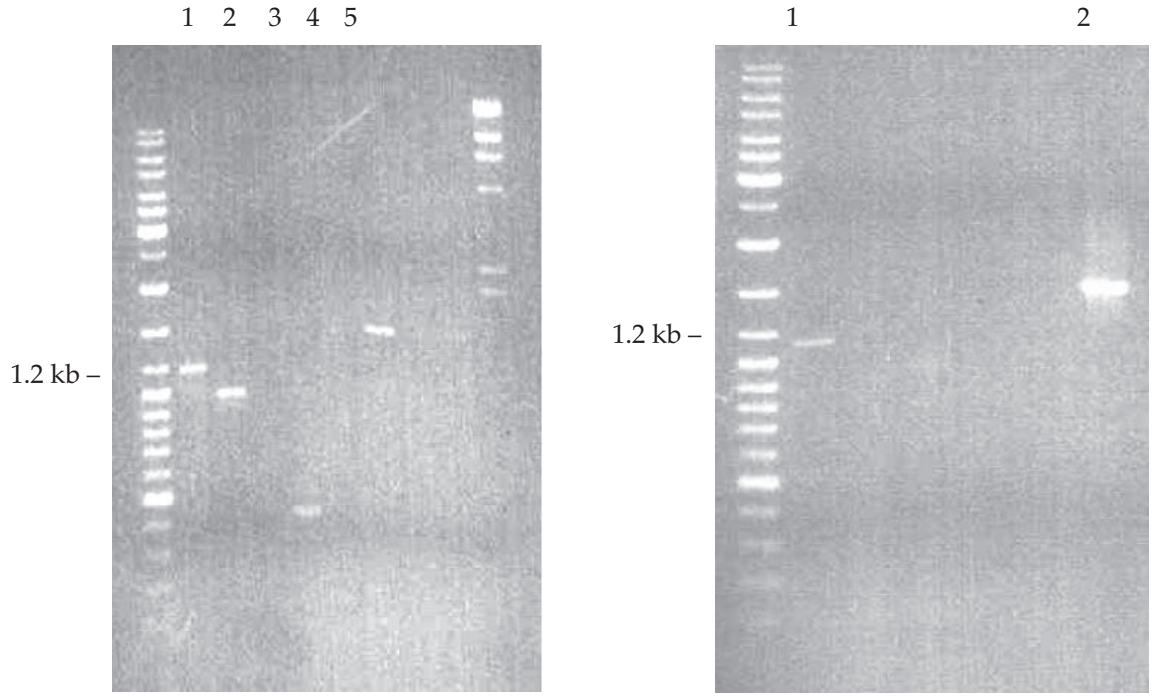

Figure 1. a. Amplification of a genomic fragment containing the MT3-A *promoter from oil palm genome walker libraries. The picture shows the products from the primary PCR reaction using the gene-specific primer 1 (GSPI) and AP1. Lanes 1, 2, 3 and 4 are the products obtained using 1 µl aliquots of the* Dra *I,* EcoR *V* Pvu *II and* Stu *I GenomeWalker libraries, respectively. Lane 5 is the product from the control library provided with the GenomeWalker Kit.*

b. Amplified DNA fragment from the secondary PCR reaction using nested primers GSP2 and AP2. Only the biggest primary PCR product obtained using Dra *I library was used in the reaction. The band was subsequently purified from the agarose gel and cloned into the PCR II TOPO vector for sequencing. Lanes 1 and 2 are the secondary PCR products from the* Dra *I library and control library, respectively.*

sequence which is strongly homologous to *MT3-A*. The product from the secondary PCR reaction using nested primers was cloned and sequenced. The sequence obtained (*Figure 2*) showed further 5′ extension of the published cDNA sequence of *MT3-A* (Siti Nor Akmar *et al*., 2002), thus providing the complete 5′-untranslated region (5′-UTR) sequence.

The complete sequence of the genomic clone from the secondary PCR using the *Dra* I genome walker library is given in *Figure 2*. When the sequence of the genomic clone was aligned with the cDNA sequence of the 5′ RACE product, it was found that 113 bp of the 3' terminal region of the genomic clone overlaps with the 5' terminal sequence of the 5′ RACE product. Within the overlapping region, the two sequences are 100% identical. The most likely transcription start site is an adenine at the 5′-end of the 5′-RACE product, located 26 bp downstream of the TATA box (*Figure 2*), which is consistent with the expected distance of 32 ± 7 (Joshi, 1987).

The promoter sequence of *MT3-A* has no significant homology with the reported promoter sequence of *MT3-B* (Siti Nor Akmar *et al*., 2002). An ethylene-responsive element in reverse orientation (ERE-reverse) and two I boxes were identified at positions -317, -603 and -943, respectively, in the *MT3-A* promoter sequence. The ERE is similar to the ERE, AATTCAAA, of the ripening-specific *E4* gene of tomato (Montgomery *et al*, 1993) and the ERE, ATTTCAAA, associated with the regulation of carnation glutathione-S-transferase gene (*GST*) in senescing tissues. The ERE in the *GST1* gene operated in an orientation independent manner (Itzhaki *et al*., 1994). An I-box was also found in the fruit-specific promoter of strawberry (Agius *et al*., 2005) and other light-regulated genes (Terzaghi and Cashmore, 1995). However, *MT3-A* promoter does not have the putative metal-responsive and root-specific elements found in the *MT3-B* promoter. The differences in the promoter sequences of *MT3-A* and *MT3-B* together with having different regulatory elements, may be responsible for the distinctly different expression profiles of these two closely related genes.

The approximately 850 bp CaMV 35S promoter was removed from pBI221 by digesting with *Hind* III and *Xba* I. The entire *MT3-A* promoter region until

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                                                  I-box
AAATTACTGCCATGGAGGGTCACAATGATGCAACTAAACATGATAATCTC -936
ATCACCTCCATCTACATCATCTTTAATCCTACATGAATCATTCCATATCA -886
AATACCTCCCAACTAAAATCGAACCCCATCTTCTTGTCCAAGTGACAAAG -836
AATGATACCATGGAGGCAAGCATTTAACCAGAACTAGAAGTCTGCCAAGG -786
AGTAGTTGACAGAATCGAACCCCATCTTATTGTCCACCATTCTTGACACA -736
AAAATAACCCGTGCAGATAGACTATAGTCAATCGTTATCCAAGTCCACAT -686
GAACTCTGCCAAGGAGTAGTTGGTTTTACTTACAAATTTTGGTTATCTTT -636
                                  I-box
ATAAACATTTTGTAAGGTGTAGGATTTGTGTGATAACATTTTGATTAGTT -586
AGGCTAAAAATTGTATGTGCTGAGCTACGATACCTTCTTTCTGGTAGAAA -536
GCTACGATACCTTTAACCAGAAAAGGATCCAAAGAAACGAACGAAACCAA -486
ACTAGGGGCTAACTCCAAAGTAGGCGAAGCTAGAAAAGACTCGACTTCTC -436
TGTACCCCAGAAGATTGCAAGGCAATTTCTCTCCACAAAAACAAAAGAAC -386
CACGAAGCAAACAAAGCAAAACCAAGCAAATTTCCTTCCAATTTGCGTCA -336
                ERE-reverse
CCGGGTTATCTCCACGCATTAGAATTTTGAATCGATCTCCATCAAACGTC -286
ACGACAGACAAAGAAGACGACTTGACTAGCACTGTACCAAGAATGTGTGA -236
CGTGGCAGCTTGCGTGTGCCAGCGGTATGGTCTTCAGCCAGGAAGAAAAA -186
GAGGGAGATATGACAAGAGAGGCTTGTGGGAAATCACACACCCATTATTG -136
AGATTCCTTCCGGATTATTCGTCTAGAGGCGTGTGCACGGCGCTGAGAAG  -86
CGTGTGGGCTCTGCGAATTACCAAGCTAGCCTCTCATGGCCAAGTAAATG  -36
  TATA-box                Transcription start
CCTATAAATGCCCATCGCCTCCGCCCTTCCTCAACAACGTATTGGGTGAT   15
CGAATCTATCAGAGTTACAAACATTGTCTCTGGTTTCCTGCTTCGAAACT   65
Translation start
CAAAACATGTCGACCTGCGGCAACTGCGATTGTGCTGACAAGAGCCA     114
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Figure 2. Nucleotide sequence of the genomic fragment containing the promoter sequence of MT3-A. *The putative ethylene-responsive element (ERE-reverse), I-boxes, TATA box, adenine at the 5′ end of the 5′ RACE product (likely transcription start site) and ATG codon for the start of translation are in bold and underlined. The sequence of the 5′-extension in the 5′-UTR obtained from 5′-RACE is in italics.*

16 bp upstream of the translation start was flanked with *Hind* III and *Xba* I sites and ligated to the digested vector and cloned into an *E. coli* host. Restriction analysis of the cloned product (*Figure 3*) followed by further verification by sequencing showed the successful cloning of the *MT3-A* promoter upstream of the GUS coding sequence. The recombinant vector produced was designated MT3AP-GUS.

Application of the transient assay system is a useful alternative for the analysis of specificity and strength of plant promoters (Yamakawa *et al*., 2004; Agius *et al*., 2005) as a similar analysis if carried out in homologous transgenic system will take a long time. For the production of transgenic oil palm bearing fruit bunches will require at least four years from introduction of gene construct in culture. A transient assay using the biolistic method on oil palm tissue slices for analysing the strength and confirming the specificity of oil palm gene promoters has been developed (Zubaidah and Siti Nor Akmar, 2003). In this method, regions of the promoter are ligated upstream to sequence encoding reporter genes, such as GUS and the green fluorescence protein (GFP). Tissue slices bombarded with the promoter: reporter gene construct can be assayed for transient expression of the reporter gene.

The pBI221 is designed to express GUS from the strong constitutive CaMV 35S promoter. In order to compare the expression of GUS under the control of the CaMV 35S promoter and the oil palm *MT3-A* promoter, pBI221 and MT3AP-GUS were used for bombarding oil palm mesocarp slices at 12 WAA. and leaf tissues. Qualitative histochemical assay of GUS activity was performed on the bombarded tissues. The GUS expression was observed on both mesocarp slices and leaf tissues bombarded with pBI221 (*Figure 4*). Expression of GUS was only detected on mesocarp slices bombarded with MT3AP-GUS and no expression observed on the leaf tissues bombarded with this construct. This clearly indicates that the activity of the oil palm *MT3-A* promoter is specific to the mesocarp tissues. A patent for *MT3-A* promoter has been filed for in a few countries (Siti Nor Akmar and Zubaidah, 2003) and has recently been granted for the United States of America. This promoter is currently being used in the oil palm genetic engineering programme to modify the mesocarp oil composition and for poduction of novel products in the mesocarp.

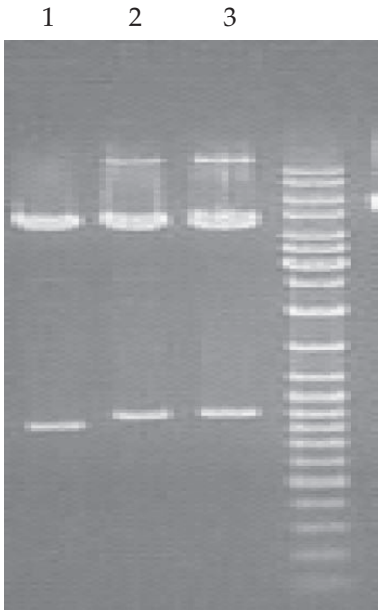

Figure 3. Result of restriction analysis of the chimeric transformation vectors MT3AP-GUS containing a 986 bp MT3-A *promoter sequence and GUS reporter gene. Digestion was performed with* Hind *III and* Xba *I. Lane 1 shows the digested products of pBI121. Lanes 2 and 3 show the digested products of MT3AP-GUS.*

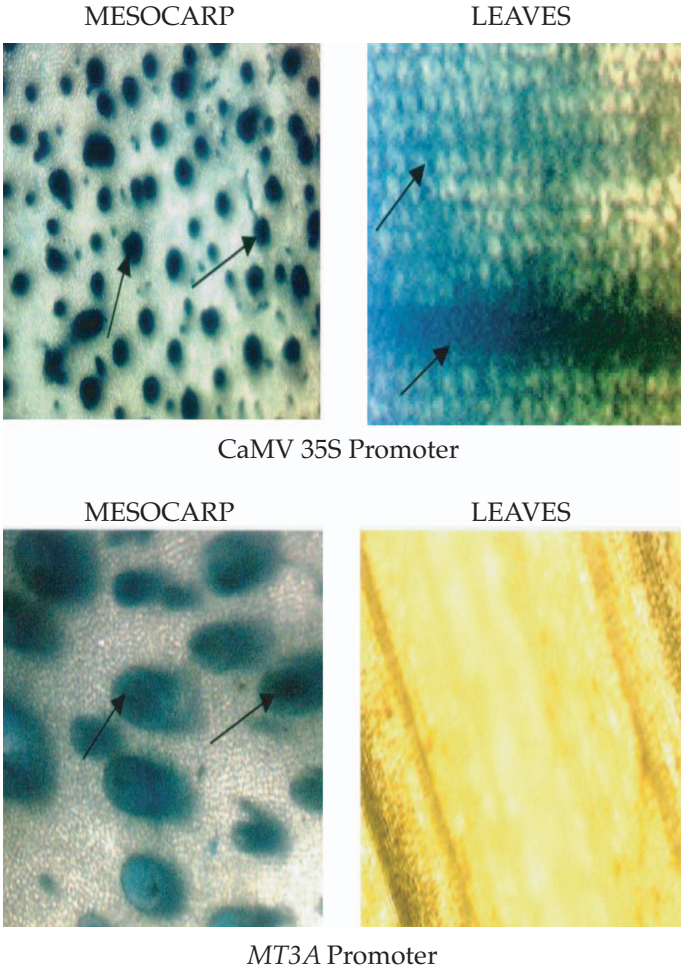

Figure 4. Results of histochemical assay to compare expression of GUS between mesocarp and leaf tissues bombarded with the plasmid pBI221 (containing the constitutive CaMV 35S promoter) and tissues bombarded with MT3AP-GUS (cotaining the 986 bp oil palm MT3-A promoter sequence). The arrows point to examples of GUS spots on the bombarded tissues.

CONCLUSION

The promoter sequence of the abundantly expressed mesocarp-specific metallothionein-like gene (*MT3-A*) of 986 bp was succesfully isolated from the oil palm. The putative regulatory elements found on the promoter include a TATA box, an ethylene-responsive element and two I-boxes. The promoter activity was analysed via qualitative transient assay on bombarded oil palm tissue slices employing GUS as the reporter gene. The activity of *MT3-A* promoter was only detected in mesocarp tissue whereas the activity of the constitutive CaMV 35S promoter was observed in both mesocarp and leaves. This suggests that the *MT3-A* promoter can direct mesocarp-specific gene expression.

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