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THE USE OF *Arabidopsis thaliana* MODEL SYSTEM FOR TESTING OIL PALM PROMOTER: CASE STUDY ON OIL PALM *MT3-A* PROMOTER

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ABSTRACT

A previous study on transient expression of oil palm tissues has shown that the oil palm metallothionein-like type 3 (MT3-A) *gene promoter is specifically expressed in the mesocarp and not in other tissues. This study was conducted to determine whether or not* Arabidopsis *can be used as a model system to study oil palm promoter. Functional characterisation of the oil palm* MT3-A *promoter was performed using promoter::GUS fusion analysis in transgenic* Arabidopsis. *The localisation of β-glucuronidase (GUS) expression in several different tissues of transgenic* Arabidopsis *homozygous lines driven by the oil palm* MT3-A *promoter was determined. Histochemical GUS analysis in transgenic* Arabidopsis *revealed the highest expression in the cotyledon and hypocotyls as well as at the early stage of plant development and gradually decreased as the plant grew. Lower expression was detected in organs of mature plant and no expression was observed in reproductive tissues. This observation may suggest that* MT3-A *promoter might be involved during early stage of plant development. Since we use* Arabidopsis *which is neither a fruit nor mesocarp plant to check mesocarp-specific promoter it might not give a faithful pattern of expression but it may be suited to study oil palm specific promoter derived from other tissues.*

Keywords: oil palm, promoter analysis, model plant, β-glucuronidase.

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INTRODUCTION

One potential area that can be explored for improvement of oil palm profitability is by developing new and value-added products through genetic engineering. In order to genetically engineer oil palm, a number of tools have to be first made available. One of the absolute prerequisites is the availability of promoter sequences. The oil palm genetic engineering research at the Malaysian Palm Oil Board (MPOB) started with the aim to produce higher oleic acid content in the mesocarp. Subsequently, other products were targeted which include high contents of stearic, palmitoleic, ricinoleic, lycopene as well as biodegradable plastics (Parveez *et al.*, 2015). The promoter is responsible to initiate and regulate the transcription process of the transgene hence allowing gene expression (Billas *et al.*, 2016). Tissue-specific promoter allows the expression of the transgene to be directed to a particular tissue where the promoter is active. In oil palm, to genetically engineer the palm to modify the mesocarp oil composition, a mesocarp-specific promoter is required. This is to ensure that the transgene is expressed in the fruit mesocarp and to avoid accumulation of the targeted products in other parts of the plant that could cause detrimental effect to the plant growth.

A cDNA clone encoding a metallothionein-like protein designated as *MT3-A* was isolated from oil palm. Metallothionein (MT) are low molecular weight cysteine-rich protein (Rauser, 1999; Chatthai

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et al., 2004) and can be found in all organisms. The oil palm *MT3-A* is a type 3 MT-like gene and based on previous studies, Type 3 are mainly found in developing fruit (Reid and Ross 1997; Siti Nor Akmar *et al*., 2002; Endo *et al*., 2007). Northern blot analysis showed that the gene is expressed in the mesocarp throughout the ripening period between 12 to 20 weeks after anthesis (WAA) with maximum expression at 15 WAA (Siti Nor Akmar *et al*., 2002). Trace level of expression was detected in kernel at 15 WAA. The *MT3-A* expression was not detected in young leaves but was induced in senescence leaves.

MT3-A promoter (accession No: EU499363) was isolated using genome walking approach. Eleven putative regulatory motifs were detected in the promoter sequence and all the motifs are located at specific positions upstream of the transcription start site of *MT3-A* (Zubaidah and Siti Nor Akmar, 2010). Transient expression analysis revealed that the activity of the oil palm *MT3-A* promoter was only detected in the mesocarp tissue (Siti Nor Akmar and Zubaidah, 2007; 2008). Therefore, the *MT3-A* promoter may be potentially useful for targeting expression of introduced gene into oil palm tissues especially in the mesocarp. Promoter of type 3 MT genes is important due to its fruit-specific activity (Omidvar e*t al*., 2010). Several studies on the tissue-specificity of plant MT promoter were carried out previously (Fordham-Skelton *et al*., 1997; Chatthai *et al*., 2004). Analysis of *FeMT3* promoter from buckwheat in transgenic tobacco plant revealed strong activity in the root and pollen and characterisation of rice *OsMT2b* promoter in transgenic *Arabidopsis* has led to the identification of regulatory region that is responsible for differential expression in vegetative and reproduction organs (Ren and Zhao, 2009).

Promoter fused to reporter systems is frequently used for monitoring genetic activity in plant cells. Amongst the most popular reporter systems include the firefly luciferase, β-glucuronidase (GUS) and green fluorescent protein (GFP). Transient expression assay of reporter gene was widely used to study spatial and temporal expression of plant promoters as described by several previous reports (Takahashi *et al*., 1992; Moriwaki *et al*., 1999; Crone *et al*., 2001; Wu *et al*., 2014; Jelly *et al*., 2014).

Since the establishment of the *in vivo* floral dip method for transforming *Arabidopsis* plants (Clough and Bent, 1998) and the completion of its genome sequence, *Arabidopsis* has become a very popular heterologous system for studying the function of plant genes and promoters for economically important crops such as oil palm (Zubaidah and Siti Nor Akmar, 2010; Parveez *et al*., 2010; 2015; Hanin *et al*., 2016; Siew-Eng, *et al*., 2016). This little plant has many advantages over other plants with its small size and simple growth habit that allows easy propagation under laboratory conditions (Anderson and Roberts, 1998). In contrast to *Arabidopsis*, oil palm is a parennial oil crop with a long life cycle; therefore to characterise its genes and promoters *in vivo* requires substantial space and time. As an alternative, it would be useful and advantageous to use model plants, for example, *Arabidopsis* to characterise the oil palm *MT3-A* promoter.

This article reports on functional characterisation of oil palm *MT3-A* promoter using *Arabidopsis thaliana* as a heterologous system. In this study, the expression profile of the *MT3-A* promoter was evaluated in stably transformed *Arabidopsis* plants as stronger evidence than the expression pattern observed from the previous transient analysis (Siti Nor Akmar and Zubaidah, 2008).

MATERIALS AND METHODS

Construction of the Plasmids

In this study, *Hind* III and *Xba* I restriction sites were introduced by polymerase chain reaction (PCR) to flank the *MT3-A* promoter using mspfor and msprev DNA primers (*Table 1*). The amplified promoter was then cloned into the promoterless pBI101 vector at the same restriction sites to yield the plasmid MSP1pro::GUS carrying GUS as the reporter gene. For comparison purposes, we also cloned the constitutive (CaMV 35S) promoter into the binary promoterless (pBI101) plasmid. The ligation procedure was performed with the Roche Cloning Kit according to the manufacturer's instructions (Roche, USA) with a few modifications (Zubaidah and Siti Nor Akmar, 2010). The resultant clones were confirmed by DNA sequencing analysis.

Preparation of the *Arabidopsis thaliana* for Transformation

Plants of *Arabidopsis thaliana* (Columbia ecotype) were grown in a mixture of two parts of Steven Dutch potting mix (Holland) and one part of vermiculite in a 9 cm pot and placed in the growth chamber. The plants were maintained under controlled environment as described by Zubaidah (2009), until flower buds appeared. When the first bolts appeared (four to five weeks after planting), they were removed to enhance the proliferation of many secondary bolts.

Preparation of the *Agrobacterium* Culture

The MSP1pro::GUS plasmid was transformed into *Agrobacterium tumefaciens* C58 using

electroporation methods (Zubaidah and Siti Nor Akmar, 2010). *Agrobacterium tumefaciens* harbouring the desired plasmid construct was grown overnight in 20 ml of LB broth containing kanamycin (50 mg litre^{-1}) and rifampicin (100 mg litre^{-1}). *Agrobacterium* cultures were prepared prior to *in planta* transformation via floral dip method (Zubaidah, 2009).

In-planta Transformation

Arabidopsis plants were dipped in *Agrobacterium* culture containing 1 μl ml^{-1} silwet for 5-10 s (Zubaidah, 2009). The *Arabidopsis* plants were enclosed in a plastic bag and the bag was clipped at the top with a paper clip for one to two days. The plants were maintained under control environment until seed set.

Selection of the Transformants

The putative transgenic seeds were sterilised according to method as described by Zubaidah and Siti Nor Akmar (2010) and cultured on MS selection media containing kanamycin antibiotic (50 mg litre^{-1}) (Murashige and Skoog, 1962). Healthy surviving plants were transferred individually to soil to select homozygous lines for further analysis. The status of the transgenic *Arabidopsis* plants was further verified using PCR (as described in the section verification of the transformants).

Selection for Transgenic Homozygous Lines

Sixteen plants from each line (T2 generation) were planted individually in 9 cm pots until seed set. The seeds (T3 generation) from each individual lines were collected and screened for kanamycin resistance. Plates containing all plants that survived were considered homozygous lines. All homozygous lines were selected for further analysis.

Verification of the Transformed Plant

PCR method was used to confirm the introduction of the oil palm *MT3-A* promoter into the *Arabidopsis* genome with the correct orientation. In this study, DNA was extracted from leaf tissues of each individual transgenic line using Plant DNAeasy Kit (Qiagen, USA) and PCR was performed using gene specific primers (mspfor and msprev) and mspfor and GUSrev that have been designed based on *MT3-A* promoter and GUS sequences. These primers are listed in *Table 1*.

TABLE 1. LIST OF PRIMERS USED FOR AMPLIFICATION AND VERIFICATION OF THE *MT3-A* PROMOTER

Primer name	Sequence
Mspfor	5'-CTG CCA TGG AGG GTC ACA ATG ATG -3'
Msprev	5'-GAC GAA TAA TCC GGA AGG AAT CTG-3'
GUSrev	5'-TCA CGG GTT GGG GTT TCT AC-3'

Expression Studies via GUS Assay

GUS activity was determined using histochemical staining as described by Jefferson *et al*. (1987) with minor modifications (Zubaidah and Siti Nor Akmar 2010). The treated tissues were observed under a light microscope (Nikon SMZ800, Japan).

RESULTS AND DISCUSSION

Generation of the Transformation Vector Containing the Oil Palm *MT3-A* Promoter

The *Hind* III and *Xba* 1 restriction sites were introduced by PCR to flank the *MT3-A* promoter region. The PCR reaction generated a 950 bp product as expected. This product was then ligated into the promoterless pBI101 plasmid at the same restriction sites. The resulting clone was designated as pBMS::GUS construct which contained the *MT3-A* promoter. The recombinant was digested with *Hind* III and *Xba* I restriction enzymes to confirm the presence of the insert. Restriction digestion analysis confirmed the presence of the expected insert size (lanes 2-5) in the clone containing the *MT3-A* promoter as shown in the *Figure 1*. Further verification of the pBMS::GUS clone was performed by DNA sequencing analysis.

Agrobacterium*-mediated Transformation of *Arabidopsis

In this study, we determined that factors such as the age, health and vigour of the plants used as well as the developmental stage of the flower buds have affected the transformation efficiency (Weigel and Glazebrook, 2002). The healthy five-week-old plants with density of 8 to 10 plants per pot (*Figure 2a*) with unopened flowers (*Figure 2b*) and prior to the emergence of siliques are the best parameters to enhance the transformation efficiency. In addition, higher transformation rate was achieved when the first batch of flower buds was removed because it would increase the density of the flower buds per plant.

Selection and Verification of the Transformants

The transformed seeds were germinated and produced healthy growing plants on selection media (*Figure 3*) while the untransformed

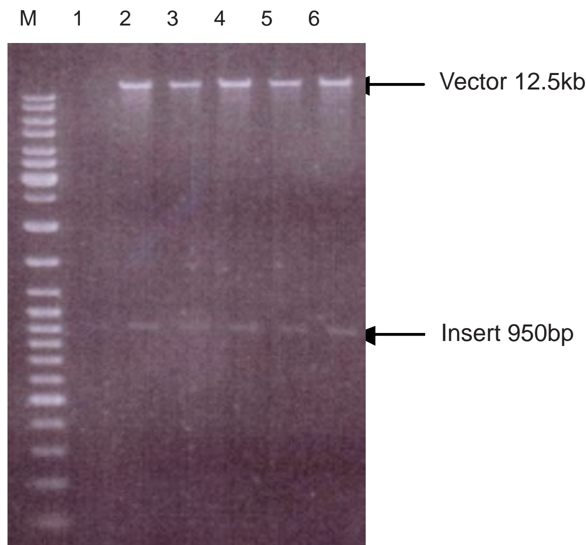


Figure 1. Restriction digestion analysis of the constructed plasmid (pBMS:GUS). Lane M is the DNA ladder mix marker. Lane 1 is the negative control and lanes 2-6 constructed plasmid with the expected insert size (950 bp) and the vector (12.5 kb).

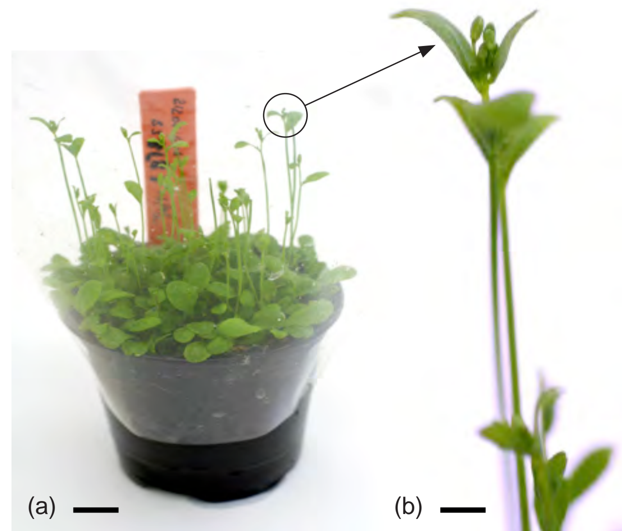


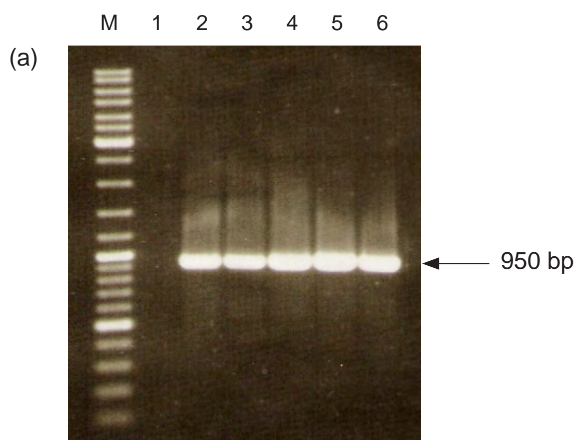
Figure 2. The best developmental stage of Arabidopsis thaliana *plant for* Agrobacterium*-mediated transformation. (a) Bar = 1 cm; (b) bar = 1 mm.*

Figure 3. Healthy growing transformed Arabidopsis *plant (left) survived on MS medium containing 50 mg litre^{-1} kanamycin and the yellowish untransformed plant (right). Bar = 1 mm.*

seeds remained as yellowish plants and would not survive on selection media. Surviving plants were transferred individually to soil to select for homozygous lines prior to further analysis. The status of the transgenic *Arabidopsis* plants was further verified using PCR. DNA was extracted from leaf tissues of the transgenic *Arabidopsis* and PCR reactions were conducted using two different sets of primers. Positive results were obtained from all the plant tested (*Figures 4a* and *4b*).

Spatial and Temporal Expression Pattern of the Oil Palm *MT3-A* Promoter

In this study, an *Arabidopsis* plant was used as the model system for analysing the strength and specificity of the oil palm mesocarp-specific (*MT3-A*) promoter. Several transgenic homozygous



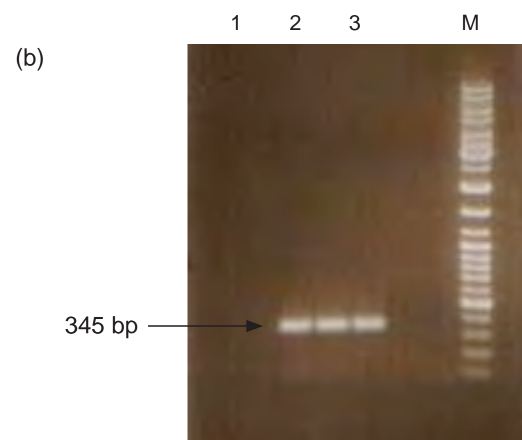


Figure 4. (a) Example of polymerase chain reaction (PCR) analysis of PCR analysis of ProMSP1::GUS transgenic plant with the amplification of about 1 kb expected band using MT3-A *specific primers. Lane M is the 1 kb marker; lane 1 is the negative control and lanes 2 to 6 are the transgenic* Arabidopsis *carrying* MT3-A *promoter. (b) PCR analysis of the transgenic plant carrying the same construct with* MT3-A *specifc primer and β-glucuronidase (GUS) primer (lane 1 – 3), lane M = marker 1 kb ladder.*

lines containing the oil palm *MT3-A* promoter were generated. Expression of the GUS gene from T3 homozygous transgenic lines were obtained from six independent lines. Analysis of the transgenic homozygous lines revealed that GUS expression was highest in the cotyledon and hypocotyls especially at the aerial part of the seedlings (*Figures 5a* to *5d*). As the plant grew to maturity, lower GUS expression was observed in leaf and no expression detected in stem tissues. GUS expression was also absent in reproductive tissues such as flower, silique and pollen (*Figures 5e* to *5g*). The overall expression pattern showed that *MT3-A* expression was only found in actively dividing hypocotyls, cotyledon and leaf tissues suggesting that this gene might be involved in cell division and plant development. *Figure 6a* shows the GUS expression pattern driven by the oil palm *MT3-A* promoter observed in transgenic *Arabidopsis*. GUS expression was mainly observed in aerial tissues compared to transgenic *Arabidopsis* plants carrying the constitutive *CaMV 35S* promoter (*Figure 6b*) where the GUS expression was constitutively expressed in all tissues. A previous study on MT gene of *Brassica napus* L. also showed similar pattern of expression with highest MT expression levels in cotyledon tissues while mature organs exhibited lower levels and no expression was observed in flower (Dąbrowska *et al*., 2013).

MT-3 type gene expression was also apparent in young, extensively growing seedling as opposed to organs of mature plant (Dąbrowska *et al*., 2012). However, based on transient expression pattern of oil palm tissue slices using GUS as reporter gene (Siti Nor Akmar and Zubaidah, 2008) indicated that GUS expression was only observed in mesocarp tissue but absent in matured leaf. However, *MT-3* type gene expression occurs predominantly in fleshy fruit but can be observed in other organ of plants lacking such kind of fruit, such as leaf tissues in *Arabidopsis thaliana* (Gou *et al*., 2003). Another possibility is that the size of the isolated oil palm promoter (*MT3-A*) of about 1 kb might not drive faithful expression in the *Arabidopsis* system. In addition, *Arabidopsis* may not be the best system to study a fruit or mesocarp-specific promoter in contrast to the study on oil palm leaf-specific promoter (Hanin *et al*., 2016).

In this study, the use of model plant species clearly does not provide data that would be as robust as that obtained by using expression in oil

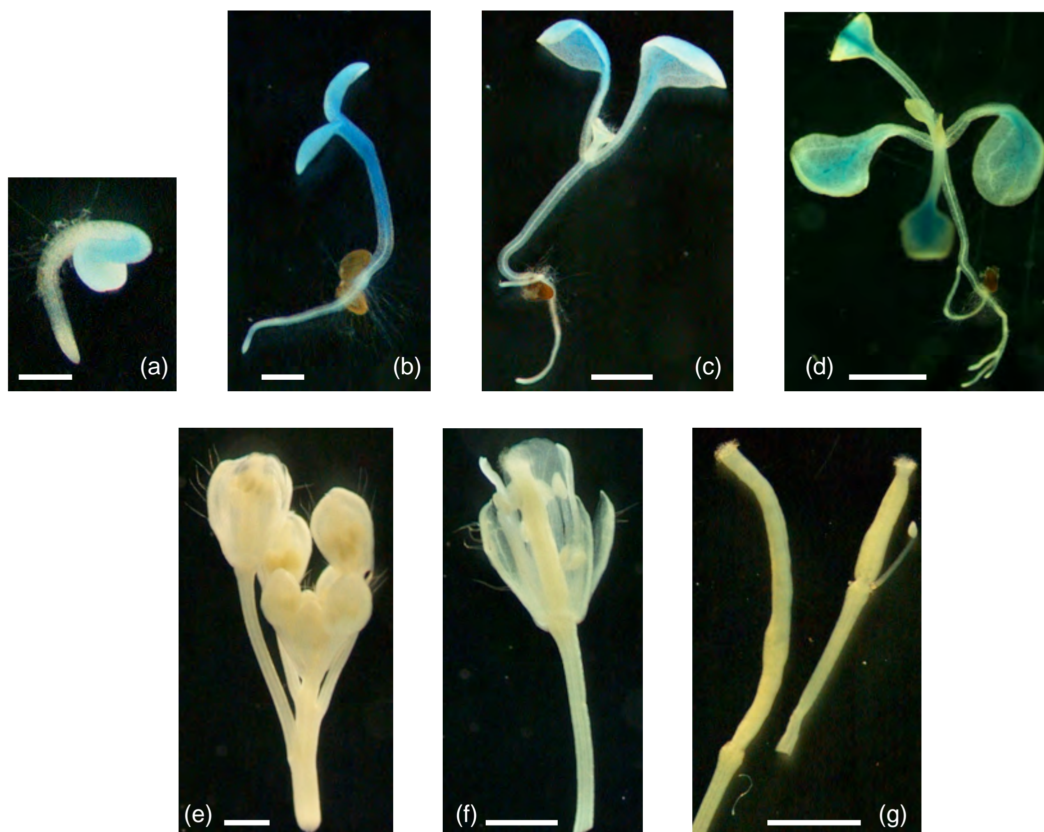


Figure 5. The β-glucuronidase (GUS) staining patterns in the aerial part of the seedling. (a) GUS expression in the plumule of four days old germinated seedling; (b) Expression in hypocotyl and cotyledon; (c) Expression observed in seedling at two leaf rosette stage; (d) GUS expression in the transgenic seedling at four leaf stage. No GUS expression was observed in other tissues: (e) flower buds; (f) open flower and (g) siliques. a and b, bar=250 µm; c,e and f bar=1 mm ; d and g, bar=1 cm.

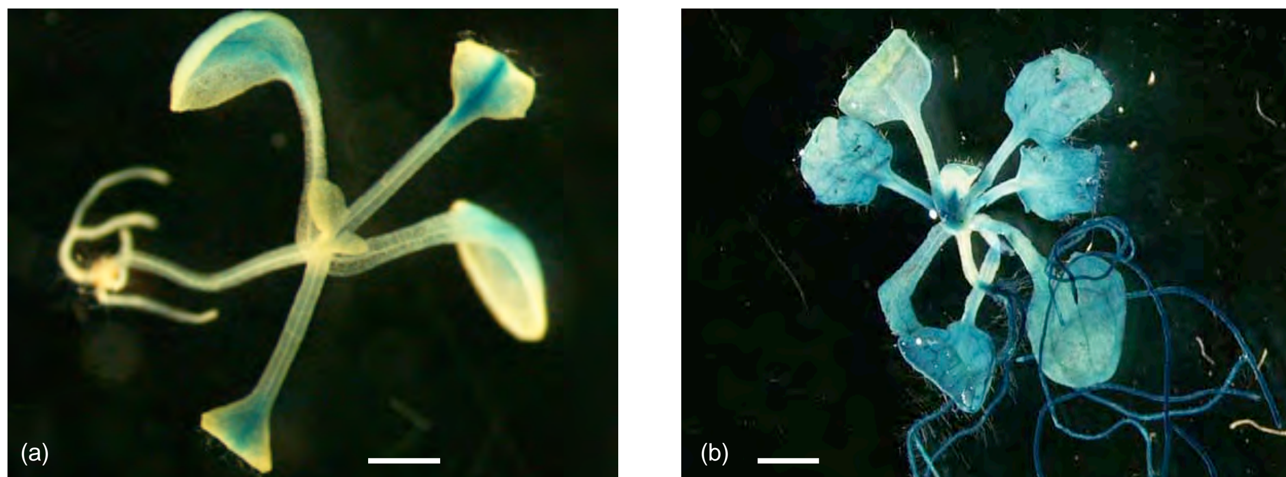


Figure 6. Comparison of β-glucuronidase (GUS) expression driven by the oil palm MT3-A *promoter (a) and constitutive CaMV* 35S *promoter (b). Bar=1 cm.*

palm itself. Moreover, a monocot promoter might not provide faithful representation of expression in a dicot model species especially in certain tissues. Currently, systems to transform oil palm are somewhat limited and it can take one to two years from transformation to obtain transgenic seedlings (Parveez *et al*., 2015). The duration and efficiency of the process will no doubt improve over the coming years and it is hoped that GUS analysis in oil palm transgenic plant may become more routine in future.

CONCLUSION

This study demonstrated that *Arabidopsis thaliana* provides some opportunities for studying oil palm gene expression. However, it is also evident that there may be limitations using *Arabidopsis* for studying oil palm genes and promoters. Therefore, careful interpretation and extrapolation of observations to the oil palm crop is needed. Use of a longer promoter may provide additional support or generate a more faithful expression of the gene. The use of other model systems for testing oil palm promoters such as the fleshy fruit, for example tomato, can be explored in future.

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