OIL PALM MSP2 PROMOTER ISOLATION, IN SILICO ANALYSIS AND FUNCTIONAL CHARACTERISATION

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ABSTRACT

Arabidopsis was used as a stable transformation system to characterise the function of the oil palm mesocarp-specific promoter, MSP2. Five MSP2 promoter fragments ranging from 1558 bp to 3044 bp in size were successfully isolated. In silico sequence analysis showed various putative plant regulatory elements in the different MSP2 promoter regions. Two vector constructs containing MSP2-GLC and MSP2-GLG promoter fragments were attached to beta-glucuronidase (GUS) reporter gene and transformed into Arabidopsis thaliana. Histochemical GUS staining of the transgenic Arabidopsis showed that both promoter fragments were expressed in the flower, especially in petal and stigma, silique for MSP2-GLC and in anther and stamen for MSP2-GLG. The QELEMENTZMZM13 motif present only in the MSP2-GLG fragment is likely vital for the anther-specific expression. In MSP2-GLC transgenic Arabidopsis, GUS expression was enhanced under cold conditions, unlike MSP2-GLG transgenic Arabidopsis. A low-temperature response (LTR) motif present in MSP2-GLC may be important to enhance and drive the expression of a transgene under cold conditions. The deleted region in MSP2-GLG fragment caused the removal of the LTR motif, which most likely indicates that the size of the promoter is not necessarily important to drive gene expression, but the availability of a specific motif is key to determine its strength and specificity.

Keywords: Arabidopsis, Elaeis guineensis, heterologous system, mesocarp-specific promoter, model plant.

INTRODUCTION

Oil palm biotechnology, especially in genomics and genetic engineering, can be used to expedite crop improvement (Sambanthamurthi et al., 2009). At the Malaysian Palm Oil Board (MPOB), the oil palm genetic engineering programme mainly focuses on producing transgenic palms with higher oleic acid content (Cheah et al., 1995; Parveez et al., 2015). Oleic acid is monounsaturated and can be used for diversification as liquid and salad oils in the food industry. In addition, it can also be an attractive feedstock for further fatty acid modifications in the oleochemical industry in Malaysia (Cheah et al., 1995; Parveez et al., 2015).

To produce transgenic plants with the desired trait, the transgene carrying the trait of interest must be functionally expressed to produce a heterologous protein (Juven-Gershon and Kadonaga, 2010). The transgene expression is greatly dependent on the promoter. A promoter is responsible for initiating and regulating the transgene’s transcription process, allowing gene expression (Porto et al., 2013). There are various types of promoters used in plant genetic engineering. They are grouped as constitutive, tissue-/organ-/cell-specific, inducible, and synthetic; depending on their ability to control gene expression (Mithra et al., 2017). Tissue-specific promoters are normally used to target transgene expression to a target tissue where the promoter activity is high and specific (Zheng and Baum, 2008). Thus, the most important step towards isolating tissue-specific promoters is to isolate their corresponding tissue-specific genes (Nurniwalis et al., 2008; 2015).

In oil palm, to ensure the success of the genetic engineering program, choosing the correct promoter to direct transgene expression is essential. For oil modification and/or production of novel and value-
added products in the mesocarp, the availability of a mesocarp-specific promoter is vital. MSP1 is the first mesocarp-specific promoter isolated from oil palm (Siti Nor Akmar and Zubaidah, 2008). Several transformation vectors containing the MSP1 promoter were constructed to produce the desired traits such as high oleate content in oil palm mesocarp (Masani and Parveez, 2008). These constructs were transformed into oil palm embryogenic cultures where the transformed calli regenerated into plantlets, transformed palms were transferred to soil and grown in the biosafety nursery (Parveez et al., 2015). However, chimeric transgenic palms were also detected, suggesting the need to make further improvements to the developed transformation protocols (Nurfaithisza et al., 2014).

It is also notable that different promoters have a varying degree of strength to drive transgene expression (Que et al., 1997). Hence, the need for more oil palm mesocarp-specific promoters is crucial. This is also one of the approaches to avoid epigenetic silencing, resulting in a major drawback in transgenic technologies used for crop improvement (Rajeevkumar et al., 2015). Besides homology between transgene and promoter, the interaction between two homologous promoters can also cause undesirable gene silencing (Matzke et al., 2000). Thus, the dependence on a single promoter to drive transgene expression must be avoided, and the search for the next mesocarp-specific promoter is inevitable. MSP2, the second mesocarp-specific promoter, was isolated, and functional characterisation using mesocarp slices showed mesocarp-specific activity (Nurniwalis et al., 2015).

One of the widely used approaches to analyse promoter activity is via transient gene expression assay using reporter genes. It allows fast expression of the reporter gene and is more cost-effective (Baum et al., 1997; Jelly et al., 2014). This assay requires the promoter to be fused to a reporter gene and then delivered into target tissues via various transformation methods. Different transformation methods have been optimised and developed for transient promoter studies in oil palm (Masani et al., 2013; Parveez, 1998; Shariza Hanim et al., 2018; Zubaidah and Siti Nor Akmar, 2003). The transient gene expression assay was successful in determining the activity of oil palm promoters in a tissue-specific, constitutive and inducible manner (Zubaidah et al., 2018).

Nevertheless, the best way to functionally determine the role of oil palm promoters is to perform in vivo oil palm transformation. However, due to the long breeding cycle, the characterisation of the promoter in oil palm would require an extensive amount of time. Another limiting factor would be the low transformation efficiency in oil palm, hence will dampen the work for functional analysis of the promoters. As an alternative, Arabidopsis, a model plant widely used in plant molecular biology and plant biotechnology research, has many important features to offer. It has a short generation time, compact size that requires less space for growth facilities, efficient, rapid, and high-throughput transformation system, and a complete sequenced genome (The Arabidopsis Genome Initiative, 2000). These advantages have enabled researchers to functionally validate the heterologous genes and promoters from valuable oil crops such as oil palm in Arabidopsis (Hanin et al., 2016; Parveez et al., 2010; Zubaidah et al., 2017).

The availability of an established Arabidopsis laboratory facility in MPOB (Zubaidah, 2005) also paved the way to functionally characterise the MSP2 promoter in Arabidopsis as a model system. Thus, this work focuses on the analysis and characterisation of the MSP2 promoter and its regulatory region to provide additional support and/or produce a more accurate promoter expression.

MATERIALS AND METHODS

Plant Material, Growth Conditions and Stress Treatment

Oil palm Elaeis guineensis variety tenera (dura x pisifera) spear leaf tissue from MPOB-UKM Research Station, Bangi, Selangor was used for DNA extraction. Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used for functional characterization. Cultivation of Arabidopsis plants followed the method of Zubaidah et al. (2017). The seeds were sown on Murashige and Skoog media plate, incubated at 4°C for 48 hr and transferred into an environmentally controlled growth chamber with photoperiods of 16 hr light/8 hr dark at 22°C with 70% relative humidity. At the four to six rosette leaf stage, the seedlings were transferred into flowerpots containing a thoroughly wetted soil mixture. Cultivated plants were grown in an environmentally controlled growth chamber with similar conditions as described earlier. The untransformed wild-type Arabidopsis plants were used as a negative control, whereas Arabidopsis transformed with pBI221 carrying 35S promoter and β-glucuronidase (GUS) as the reporter gene was used as the positive control.

For cold stress treatment, transgenic Arabidopsis seeds and two-week-old seedlings carrying the respective MSP2 promoter-fragment constructs were used. The incubation/vernalisation of the seeds at 4°C was performed for an additional one to three days. For seedlings at four to six rosette leaf stage, the seedlings were transferred to a pot and left at 4°C for one to three days. After cold treatments, the seedlings continued to be grown in an environmentally controlled growth
chamber with parameters as described previously. For control plants, the transgenic seeds and two-week-old seedlings carrying the respective MSP2 promoter-fragment constructs were not exposed to cold treatment and grown as described earlier.

**PCR Amplification and Purification**

Genomic DNA used as a DNA template for PCR amplification was extracted from oil palm spear leaf using DNeasy® Extraction (Qiagen, Germany). The MSP2 promoter and its regulatory region or MSP2 fragments were individually PCR amplified in a 50 μL reaction mixture containing 50 ng oil palm genomic DNA, 1x Advantage 2 PCR buffer (Takara Bio USA, Inc., USA), 0.2 mM dNTP mix (Takara Bio USA, Inc., USA), 0.1 μM each of reverse and forward gene-specific primers and 0.1x Advantage 2 Polymerase Mix (Takara Bio USA, Inc., USA). PCR amplification was performed with denaturation at 94°C for 1 min, amplification at 94°C for 15 s, 58°C for 15 s and 72°C for 3 min for 30 cycles. The final extension was performed at 72°C for 7 min. Some adjustments (± 5°C) were made to the annealing temperatures and time for non-specific amplification. For the MSP2-GLG fragment, a two-step PCR condition was performed with denaturation at 95°C (1 min); amplification at 95°C (30 s), 65°C (3 min) for 30 cycles and a final extension at 65°C for 5 min.

Cloning using TOPO TA Cloning™ Dual Promoter Kit (Invitrogen, USA) and plasmid isolation using Qiagen Plasmid Mini Kit (Qiagen, Germany) were performed following the protocol as recommended by the manufacturer. Digestion of the plasmid was performed in a 20 μL reaction mixture containing 1 μg plasmid DNA, 1x CutSmart® Buffer and 0.5 U EcoRI-HF® (10 U/μL) (New England Biolabs, UK) at 37°C for 2 hr.

**Sequence Analyses**

Nucleotide sequences were analysed using Bioedit tools (http:// www.mbio.ncsu.edu/BioEdit/ bioedit.html). Regulatory motifs in the promoter sequences were identified using PlantCARE (http:// bioinformatics.psb.ugent.be/webtools/plantcare/html/), PLACE (www.dna.affrc.go.jp/PLACE/), Softberry (www.softberry.com) and TransFAC (www.genome.jp).

**Multiple Promoter-vector Constructions**

The Gateway® Technology cloning system (Invitrogen, USA) was used to move the MSP2 promoter fragments of varying sizes into multiple vector systems before *Arabidopsis* transformation. The various MSP2 promoter fragments were initially cloned into an entry vector, pCR®8/GW/TOPO® to generate entry clones. Subsequently, an LR® recombination reaction was carried out to subclone the promoters from the entry clones into destination vector pGWB3 (Nakagawa et al., 2009) to generate the expression constructs. All protocols were carried out as recommended by the manufacturer.

**Transformation of Arabidopsis Plants**

Transformation of the constructed expression vectors into *Agrobacterium tumefaciens* strain C58 was carried out by electroporation (Zubaidah et al., 2017). Subsequently, the expression constructs were transformed into *Arabidopsis* via the floral dip method (Clough and Bent, 1998). Screening of the putative transformants was carried out following Mendel’s law to generate T1, T2 and finally T3 homozygous transgenic lines. Three independent T3 homozygous transgenic lines carrying MSP2-GLC (Lines B, F and P) and MSP2-GLG (Lines B, E and J) expression constructs were used in these experiments.

**Transformant Verification and Histochemical GUS Assay**

Genomic DNA from putative transgenic *Arabidopsis* leaf tissues was extracted using DNeasy® Extraction (Qiagen, Germany). PCR reaction was carried out in a 25 μL reaction mixture containing 2x Advantage 2 PCR Buffer (Clontech), 2x Advantage 2 Polymerase mix (2.5 units mL⁻¹) (Clontech), 0.2 mM dNTP mix (Clontech), 0.2 mM of forward and reverse gene-specific primers and 50 ng DNA template. For the MSP2-GLC promoter, the gene-specific primer pairs used were GLF3 & GUSR1 and GLF4 & GUSR1 for the MSP2-GLG promoter. PCR conditions used are as described earlier. Histochemical GUS assay was carried out as described by Zubaidah et al. (2017). Blue spots on the analysed tissues were detected using a stereoscopic microscope (Nikon SMZ800, Japan).

**RESULTS AND DISCUSSION**

**Isolation of Longer MSP2 Promoter and In Silico Analysis**

Our previous studies have demonstrated that the oil palm mesocarp-specific promoter, MSP2 has mesocarp-specific activity based on transient expression assay using the GUS reporter gene bombarded into oil palm mesocarp slices (Nurniwalis et al., 2015; Nurniwalis, 2017). As the size of the MSP2 promoter was less than 1 kb, we wanted to determine whether the size of the promoter was sufficient to drive gene expression to the target
tissue or if a longer promoter is needed to drive a stronger gene expression. Therefore, in the present study, PCR reaction based on information from the oil palm genome (Singh et al., 2013) resulted in the amplification of five fragments that correspond to and are longer than the MSP2 promoter. The five promoter fragments designated as MSP2-GLC, MSP2-GLG, MSP2-GLN, MSP2-GLP and MSP2-GLI contain various sizes ranging from ~1.6–3.0 kb. MSP2-GLC and MSP2-GLI are located at 1558 bp and 3044 bp upstream of the transcription start site (TSS), respectively. MSP2-GLG and MSP2-GLP and MSP2-GLN are located at 887 bp and 2373 bp upstream of the initial MSP2 region. In comparison, MSP2-GLC, MSP2-GLP and MSP2-GLN are located at 1676 bp, 2373 bp and 2662 bp upstream of MSP2 and contain an internal deletion of 323 bp of the initial MSP2 promoter.

In silico analysis of the promoter sequences using the PlantCARE database detected 34 cis-acting regulatory elements in the longer MSP2 promoter regions (Table 1). Sixteen of the cis-acting regulatory elements are present in the initial 671 bp MSP2 promoter (Nurniwalis et al., 2015; Nurniwalis, 2017). Eighteen new additional cis-acting regulatory elements were detected in the longer MSP2 promoter fragments. They contain mainly cis-acting regulatory motifs that are related to stress responses. This includes additional motifs that are involved in light response-associated elements, hormones as well as low-temperature responses. The presence of these additional putative regulatory elements suggests that the longer MSP2 promoter regions may respond to a variety of environmental signals including abiotic stress such as cold and light responses.

One low temperature response element (LTR) was found in the 671 bp MSP2 promoter using PlantCARE and is not detected in the longer promoter regions. In addition, in silico analysis using PLACE detected two low temperature response motifs, i.e., LTRECOREATCOR15 and LTRE1HVBLT49 in both forward and reverse orientations within the various promoter fragments (Table 2). One LTRE1HVBLT49 motif was detected in MSP2-GLC and MSP2-GLI fragments, and the sequence motif is the same as the LTR motif detected by PlantCARE (Tables 1 and 2). On the other hand, LTRECOREATCOR15 has a different core sequence motif and was detected in three locations within all five promoter fragments (Table 1). Both motifs present in the promoter regions of various plants have been shown to be involved in cold responses either solely or in combination with other responsive elements (Baker et al., 1994; Catala et al., 2011; Dunn et al., 1998).

Another possible cis element related to low-temperature stress responses identified in the promoter fragments is as-1 motif (TGACG) (Table 1). The motif is present in a single copy and in the reverse orientation in both promoter fragments. In rice seedlings, the as-1 motif is involved with the early responses to chilling stress (Cheng et al., 2007). Promoter deletion/mutation analyses also showed that the as-1 motif can repress or induce the cold responsiveness of the promoter (Liu et al., 2016).

Generation of the Transformation Promoter: Reporter-vector Constructs

Two recombination reactions were performed to generate the entry and expression clones. The success in generating entry and expression constructs containing the longer MSP2 promoters was confirmed via restriction digest and sequence analyses. Five entry clones were generated and subsequently, four of the expression constructs were transformed into Agrobacterium tumefaciens (C58) by electroporation and the success of the transformation procedure was verified via PCR analysis. The constructs designated as promMSP2-GLC::GUS, promMSP2-GLG::GUS, promMSP2-GLGec::GUS and promMSP2-GLIec::GUS carried the longer MSP2 promoter fragments with the expected sizes of ~ 1.6, ~ 2 and ~ 3 kb, respectively.

Screening and Validation of Transformants in Transgenic Arabidopsis

To further characterise the promoters in a stable plant transformation system, A. thaliana was used as the model plant species. Two fusion vectors containing MSP2-GLC and MSP2-GLG promoter fragments and the GUS gene were transformed into Arabidopsis. Both promoters differ in size where MSP2-GLG is 462 bp longer than MSP2-GLC. Six putative transformants were selected on selection media and screening of the transformants was validated via PCR analysis in all including in T1, T2 and T3 homozygous transgenic lines. Figure 1 represents the verification of the transformants carrying the promMSP2-GLC::GUS and promMSP2-GLGec::GUS constructs, indicating the presence of the promoters in transgenic Arabidopsis.

GUS Gene Expression in Transgenic Arabidopsis

GUS histochemical analysis in various transgenic Arabidopsis homozygous line tissues showed both MSP2-GLC and MSP2-GLG promoter fragments could regulate GUS gene expression but at different strengths and tissues (Figure 2). MSP2-GLC can direct GUS reporter gene expression in the reproductive organs, especially in the flower petals and stigma and in the elongating siliques. No visible GUS expression was observed in the other examined tissues. A close-up view of the flowers (unopened and open) showed that GUS expression was detected in the petals, especially in the unopened flower buds (Figure 3a). In addition, GUS expression can also be
**TABLE 1. PREDICTION OF THE PUTATIVE CIS-REGULATORY ELEMENTS USING PLANT CIS-ACTING REGULATORY DNA ELEMENTS (PLANTCARE) DATABASE**

| Element name  | Promoter | Sequence & Strand | Function description |
|---------------|----------|-------------------|----------------------|
| AAGAA-motif   | GLP, GLI, GLG, GLC | GAAAGAA (-) | Involved in abscisic acid responsiveness |
| ABRE          | GLP, GLI, GLN, GLG | ACGTGA (-) | Involved in light responsiveness |
| ACE           | GLI, GLC | GCCACGTACC (-) | Involved in light responsiveness |
| AE-box        | GLP, GLI, GLN, GLG, GLC | AGAAACTT (+) | Part of module light response |
| ARE           | GLP, GLI, GLN, GLG | AAACCA (-/+)| Essential for the anaerobic induction |
| AT1-motif     | GLI, GLC | AATTATTTTTATT (+) | Part of a light responsive module |
| ATCT-motif    | GLP, GLI, GLN | AATCTAATCC (-/+)| Part of a conserved DNA module involved in light responsiveness |
| As-1          | GLP, GLI, GLN, GLG, GLC | TGACG (-) | Involved in shoot-specific expression and light responsiveness |
| Box4          | GLI, GLC | ATTAAT (+) | Part of a conserved DNA module involved in light responsiveness |
| CAAT box      | GLP, GLI, GLN, GLG, GLC | CAAT (+/-)| Common cis-acting element in promoter and enhancer regions |
| CAT box       | GLP, GLI, GLN, GLG, GLC | GCCACT (-) | Cis-acting regulatory element related to meristem expression |
| CGTCA-motif   | GLP, GLI, GLN, GLG, GLC | CGTCA (+) | Involved in the MeJA-responsiveness |
| chs-CMA1a     | GLP, GLI, GLN | TTACTTAA (+) | Part of a light responsive element |
| chs-CMA2a     | GLI, GLC | TCACTTGA (-) | Part of light responsive element |
| ERE           | GLP, GLI, GLN | ATTTTAAA (+/-) | Ethylene responsive element |
| G-box         | GLP, GLI, GLN, GLG, GLC | CACGGIT (+); TCCACATGCA (+); CACGGAC (+) | Involved in light responsiveness |
| GATA-motif    | GLP, GLI, GLN, GLG, GLC | AAGATAAGATT (-); AAGGATAAGG (+); AAGATAAAGATT (-) | Part of a light responsive element |
| GCN4_motif    | GLP, GLI, GLN | TGAGTCGA (-) | Involved in endosperm expression |
| GTGANTG10     | GLG, GLC | GTGA (+/-) | Involved in pollen-specific expression |
| I-box         | GLP, GLI, GLN, GLG, GLC | CCATATCCAAT (+); GGATAAGG (+); CCGTATCT (+) | Part of a light responsive element |
| LTRE          | GLI, GLC | CCGAAA (-) | Involved in low-temperature responsiveness |
| MYB           | GLP, GLI, GLN | CAACCA (-) | Involved in abiotic stress responses and secondary wall deposition |
| MYC           | GLP, GLI, GLN, GLG, GLC | CATTTG (-/+); CATGTG (+/-); CAATGG | Involved in zein metabolism regulation |
| O2-site       | GLP, GLI, GLN, GLG, GLC | GGTGACGTGA | Gibberellin-responsive element |
| P-box         | GLP, GLI, GLN | CCTTTTG (-) | Involved in pollen-specific expression |
| POLLENILELAT52| GLG, GLC | AGAAA (+/-) | Stress response element |
| STRE          | GLP, GLI, GLN, GLG | AGGGG (-) | Involved in salicylic acid responsiveness |
| TCA-element   | GLP, GLI, GLN, GLG, GLC | CATCTTTTTT (-) | Part of a light responsive element |
| TCT-motif     | GLP, GLI, GLN, GLG, GLC | TCTTAC (+) | Involved in the MeJA-responsiveness |
| TGACG-motif   | GLP, GLI, GLN, GLG, GLC | TGACG (-) | Stress response element |
| Unnamed_1     | GLP, GLI, GLN, GLG | CGTGG (+) | Negative regulators of abiotic stress response/tolerance |
| Unnamed_4     | GLP, GLI, GLN, GLG, GLC | CTCC (+/-) | Stress response element |
| W box         | GLP, GLI, GLN, GLG | TGAGCC (-) | Stress response element |
| WRE3          | GLP, GLI, GLN, GLG, GLC | CCACCT (-); AAATATCTT (-) | Stress response elements |

* TATA BOX – A core promoter element around -30 of transcription start is not included in this table.
seen in the stigma as the flower matures and the petal starts to open. In the silique, GUS expression was observed in young silique especially in the peduncle and in the silique apex. On the contrary, GUS expression was not detected in the mature silique (Figure 3a). These results indicate that the MSP-GLC promoter can drive gene expression in reproductive tissues during different developmental stages and may play a role in modulating floral organ maturity.

Similarly, the MSP2-GLG promoter does not drive the GUS gene expression in almost all of the tested tissues except flowers (Figure 2). However, the GUS expression of MSP2-GLG promoter in flowers appeared to be much lower based on the strength of the blue-stained colour of the tissues. A close-up view of both young and mature flowers showed that GUS expression was only observed within the anthers of unopen flower buds (Figure 5a). GUS gene expression was not detected in the negative control plants but all the tested tissues in the positive control plants (Figure 2).

The MSP2 promoter was isolated from an oil palm lipase class 3 gene, FLL1 (Nurniwalis et al., 2015; Nurniwalis, 2017). In Arabidopsis, the most closely related orthologue gene to oil palm FLL1 is AtOBL1, where the transcript is expressed in many tissues, including flower petals and stamen (Klepikova et al., 2016). The enzymatic assay also revealed that AtOBL1 is localised to lipid droplets, storing TAGs in the pollen tube (Müller and Ischebeck, 2018). Based on the expression of the reporter gene, we presumed that there might be motifs contributing to flower-specific expression in the MSP2 promoter fragments. Sequence analysis using the PLACE database showed an E-box motif (CANNTG) or MYC recognition site (Table 1). The cis-acting regulatory element was detected in flower-specific promoter in Arabidopsis where in combination with
MYB elements can drive the expression of the chs gene promoter stronger (Hartmann et al., 2005). Both MSP2-GLC and MSP2-GLG promoter fragments also contain several copies of cis-acting elements involved in anther/pollen-specific expression (Chen et al., 2010; Geng et al., 2009; Rogers et al., 2001). This includes motifs like 'GTGA' or GTGANTG10 and POLLEN1LEL1AT52 or 'AGAAA' (Table 1). Both motifs are distributed evenly among both promoter fragments. However, using PLACE database, a QELEMENTZMZM13 (AGGTCA) motif was only obtained in the MSP2-GLG fragment and none in MSP2-GLC. The QELEMENTZMZM13 motif has been reported to enhance pollen-specific activity (Hamilton et al., 2001). Considering the results obtained, the presence of three copies of the putative QELEMENTZMZM13 motif is likely important for anther-specific expression of the MSP2-GLG promoter in transgenic Arabidopsis.

**Promoter Activity in Response to Cold Treatment**

As both MSP2-GLC and MSP2-GLG promoter fragments contain several putative stress-responsive elements, further work was performed to determine if the promoters are affected by low temperature or cold stress conditions. MSP2-GLC contains a predicted LTR or LTR1HVBLT49 motif (CCGAAA(-)) absent in the MSP2-GLG promoter. Therefore, we tested the effect of cold treatment on the seeds before seed germination and during seedling growth. For promMSP2-GLC::GUS gene construct, GUS reporter gene in transgenic Arabidopsis germinated from seeds given longer cold treatments were expressed in the same tissues as the transgenic control plant (Figure 3). GUS gene expression was detected in the flowers and silique and none in the rest of the tested tissues. In the young and mature flowers, the GUS expression was detected in the petals. In the open and mature flowers, the GUS expression was detected in the petals and stigmas. However, unlike the untreated control seedling, a one-day cold treatment in transgenic Arabidopsis seedlings also resulted in detecting GUS activity in the stamen filament of the mature and open flower (Figures 4b and 4c). After three-days of low-temperature exposure, a GUS signal was also detected in the anther of the cold-treated seedlings (Figure 4c). GUS gene expression was also detected in young siliques and the expression is stronger at the silique apex and peduncle and appears to be slightly stronger in the plants germinated from cold-treated seeds than in the control plant (Figure 3). However, the mature siliques GUS expression was detected in the silique apex and peduncle after an additional day of vernalisation treatment but at a lower level than the young siliques (Figure 3b). In addition, GUS expression was also detected in the septum after an additional two-day seed exposure to low temperature (Figures 3c and 3d).

In cold-treated transgenic Arabidopsis seedlings carrying promMSP2-GLC::GUS gene construct, the GUS reporter gene was expressed in the same tissue as the transgenic control plant (Figure 4). GUS gene expression was detected in the flowers and silique and none in the rest of the tested tissues. In the young and unopen flower buds, GUS expression was detected in the petals. In the open and mature flowers, the GUS expression was detected in the petals and stigmas. However, unlike the untreated control seedling, a one-day cold treatment in transgenic Arabidopsis seedlings also resulted in detecting GUS activity in the stamen filament of the mature and open flower (Figures 4b and 4c). After three-days of low-temperature exposure, a GUS signal was also detected in the anther of the cold-treated seedlings (Figure 4c). GUS gene expression was also detected in young siliques and the expression is stronger at the silique apex and peduncle and appears to be slightly stronger in the cold-treated seedlings than in the control plant (Figure 4). In the mature siliques, a low level of GUS gene expression was also observed in the silique apex and peduncle in cold-treated seedlings (Figure 4). In addition, GUS expression was also observed in the septum region of the mature siliques from seedlings exposed longer to low-temperature treatment (Figure 4).

In this work, despite whichever plant development stage was tested, observation in both transgenic seeds and seedlings carrying promMSP2-GLC::GUS gene construct exposed to cold temperature showed similar GUS expression.

### Table 2. Comparison of the Putative Low Temperature Response Motifs Identified Using Plant cis-Acting Regulatory DNA Elements (PlantCARE) and a Database of Plant cis-Acting Regulatory DNA Elements (PLACE)

| Database | Element name | Promoter fragment | Sequence and Strand | Function description |
|----------|--------------|-------------------|---------------------|----------------------|
| PlantCARE | LTR | GLI, GLC | CCGAAA (-) | Cis-acting element involved in low-temperature responsiveness |
| PLACE | LTRE1HVBLT49 | GLI, GLC | CCGAAA (-); | Cis-acting element involved in low-temperature responsiveness |
| LTRECOREATCOR15 | GLI, GLC, GLG, GLP, GLN | CCGAC (-/+) | |

Source: Lescot et al., (2002) and Higo et al. (1999)
patterns. GUS expression was targeted to the flowers and siliques and increased especially within specific regions in these tissues with prolonged exposure to cold.

For MSP2-GLG promoter, vernalisation treatment before seed germination showed GUS expression in transgenic Arabidopsis tissues under cold treatment prior to seed germination (a – d). a = two days seed vernalisation at 4°C (control), b = three days seed vernalisation treatment, c = four days seed vernalisation treatment and d = five days seed vernalisation treatment.

Figure 3. Comparison of GUS histochemical assay of promMSP2-GLC::GUS constructs in transgenic Arabidopsis tissues under cold treatment prior to seed germination (a – d). a = two days seed vernalisation at 4°C (control), b = three days seed vernalisation treatment, c = four days seed vernalisation treatment and d = five days seed vernalisation treatment.

For MSP2-GLG promoter, vernalisation treatment before seed germination showed GUS expression in transgenic Arabidopsis remained in the flower buds specifically in the anther (Figure 5). However, there is a slight change in GUS gene expression compared to the transgenic Arabidopsis control tissues (Figure 5). Visual observation based on the strength of the blue-stained colour of the anther showed that GUS expression was found to decrease slightly in transgenic Arabidopsis germinated from seeds exposed longer to low-temperature treatment. The most prolonged vernalisation treatment (additional three days) showed the lowest GUS expression (Figure 5d). In the silique of transgenic Arabidopsis without cold treatment, a = transgenic Arabidopsis plants with one day cold treatment and c = transgenic Arabidopsis plant with three days exposure to cold treatment.

Figure 4. Comparison of GUS histochemical assay of promMSP2-GLC::GUS construct in transgenic Arabidopsis tissues under cold treatment at 4°C during plant growth (a – c). a = control transgenic Arabidopsis, b = transgenic Arabidopsis plants with one day cold treatment and c = transgenic Arabidopsis plant with three days exposure to cold treatment.

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Integration of the transgene at different locations of a genome has been shown to affect gene expression levels in many organisms (Betts et al., 2019; Chen and Zhang, 2016). Position effects on expression level can also vary with the promoter used (Chen and Zhang, 2016) and/or promoter elements (Wilson, 1990).

For transgenic plants carrying promMSP2-GLG::GUS gene construct, GUS expression in the young flower buds from transgenic Arabidopsis seedlings exposed to cold treatment is similar to transgenic Arabidopsis germinated from seeds exposed to cold treatment, especially in the flower buds. Visual observation of the blue-stained tissue showed GUS signal was detected only in the anther of young and unopened flowers and the expression decreased as cold treatment was applied (Figure 6). The three day cold-treated seedlings showed the weakest GUS expression while the control plant showed the highest expression (Figure 6). No GUS expression was observed in the open and mature flowers, young and mature siliques as cold treatment was applied (Figure 6). Even with prolonged cold exposure at both seeds and seedling growth development stages, GUS expression in the tested tissues from the two different developmental stages remained similar.

In this work, we found that the transgenic Arabidopsis plants react to cold treatment including prolonged cold exposure similarly despite exposure at the different stages of plant development via the GUS reporter gene. Comparison of GUS expression between MSP2-GLC and MSP2-GLG promoter fragments in transgenic Arabidopsis showed a differential expression pattern in the tissues tested. In MSP2-GLG transgenic Arabidopsis under low-temperature treatments, GUS activity specifically in the anther was not affected or slightly reduced when exposed longer to low temperature during seed incubation as well as seedling growth stages. In contrast, GUS activity in MSP2-GLC transgenic plants specifically in flowers and siliques appeared to be enhanced by low-temperature, especially when exposed longer to cold treatments. The MSP2-GLC promoter fragment contains the LTR motif known to be implicated in cold-regulated gene expression. However, the LTR motif is absent in MSP2-GLG. Deleting 323 bp from the initial MSP2 promoter has resulted in the removal of the LTR motif in MSP2-GLG promoter fragment. In barley, both LTR and LTREs are present in the promoter region of the blt4.9 gene however, only the LTR motif is required for low-temperature responses (Dunn et al., 1998). Therefore, the absence of the LTR motif is likely causing the inability of the MSP2-GLG promoter to drive gene expression under low-temperature influences, despite having a longer promoter size than MSP2-GLC. The LTR element in MSP2-GLC promoter fragment suggests that it may drive the expression of its corresponding gene at a low temperature.

Semi-quantitative RT-PCR demonstrated that the oil palm FLL1 gene is highly expressed in the mesocarp tissues, especially in the mature and ripening fruits. FLL1 is also induced under cold treatment (Nurniwalis et al., 2015; Nurniwalis, 2017). The result of the MSP2-GLC promoter analysis in transgenic Arabidopsis is very much consistent with native FLL1 the gene expression analysis (Nurniwalis et al., 2015; Nurniwalis, 2017) as well as enzymatic assay (Cadena et al., 2013; Sambanthamurthi et al.,...
where oil palm lipase is activated by cold temperature. The low temperature effect on oil palm fruits can cause tissue damage, allowing the lipases access to their substrates. For oil palm crop that grows in a tropical country, the effect of low temperature on fruit tissue damages is doubtful. However, oil palm is exposed to physiological plant stresses, which include drought (Roslan et al., 2011). Farooq et al. (2009) showed that cold and drought stresses are inter-related, where cold stress causes plant osmotic balance destruction, leading to dehydration stress. Characterisation of a dehydration responsive element binding (DREB) transcription factor, EgDREB1, from oil palm also has demonstrated that it plays a role in enhancing tolerance to drought and cold stresses (Azzreena et al., 2017). In addition, a combination of cold and light-responsive elements are also integrated into Arabidopsis for adaption to the changes in the environment (Catala et al., 2011).

In this work, Arabidopsis may not be the most suitable heterologous system for analyzing the tissue-specificity of a mesocarp-specific promoter due to the difference in the anatomy structure. Other reasons such as differences in factors that regulate gene expression and differential performance of dicot and monocot plants have also been raised (Hernández-Garcia and Finer, 2015). However, despite the absence of a fleshy fruit form in Arabidopsis, promoter functional characterization of the oil palm mesocarp-specific promoters has shown a possible role of the promoter. The MSP1 promoter in Arabidopsis has mimicked the expression of its corresponding MT-3 gene in Brassica napus (Zubaidah et al., 2017). Similarly, promoter analysis of the oil palm MSP2 promoter fragments in Arabidopsis has shown a resemblance to its most closely related orthologue gene, AtOBL1 (Klepikova et al., 2016).

CONCLUSION

We have successfully used Arabidopsis to study the function of the MSP2 promoter fragments under low temperature conditions. Deletion of the LTR motif showed that the 1558 bp fragment of MSP2-GLC promoter is required to drive the expression of a transgene under cold-treatment based on the presence of the LTR motif. The size of the promoter is not necessarily important to drive gene expression, but the availability of a specific motif is key to determine its strength and specificity. Information obtained from this study showed that the MSP2 promoter fragments can function in a heterologous system like Arabidopsis.

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