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# 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.

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## 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 Kadonagaa, 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-

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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 (Nurfahisza *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 damper 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 characterisation. 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 95ºC (1 min); amplification at 95ºC (30 s), 65ºC (3 min) for 30 cycles and a final extension at 65ºC 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 30 min to 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-GLC and MSP2-GLI promoter fragments contain the initial 671 bp MSP2 promoter (Nurniwalis *et al*., 2015) and are located at 887 bp and 2373 bp upstream of the initial MSP2 region. In comparison, MSP2-GLG, 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 *A. tumefaciens* (C58) by electroporation and the success of the transformation procedure was verified via PCR analysis. The constructs designated as promMSP2-GLCec::GUS, promMSP2-GLGec::GUS, promMSP2-GLPec::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-GLCec::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 (unopen 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 and strand | Function description |
|---|---|---|---|
| AAGAA-motif | GLP, GLI, GLG, GLC | GAAAGAA (-) | |
| ABRE | GLP, GLI, GLN, GLG | ACGTG (-) | Involved in abscisic acid responsiveness |
| ACE | GLI, GLC | GCGACGTACC (-) | 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 | AATTATTTTTTATT (+) | 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 | CACGTT (+); TCCACATGGCA (+); CACGAC (+) | Involved in light responsiveness |
| GATA-motif | GLP, GLI, GLN, GLG, GLC | AAGATAAGATT (-); AAGGATAAGG (+); AAGATAAGATT (-) | Part of a light responsive element |
| GCN4_motif | GLP, GLI, GLN | TGAGTCA (-) | Involved in endosperm expression |
| GTGANTG10 | GLG, GLC | GTGA (+/-) | Involved in pollen-specific expression |
| I-box | GLP, GLI, GLN, GLG, GLC | CCATATCCAAT (-); GGATAAGGTG (+); CCTTATCCT (-) | 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 (+/-); CAATTG | |
| O2-site | GLP, GLI, GLN, GLG, GLC | GTTGACGTGA | Involved in zein metabolism regulation |
| P-box | GLP, GLI, GLN | CCTTTTG (-) | Gibberellin-responsive element |
| POLLEN1LELAT52 | GLG, GLC | AGAAA (+/-) | Involved in pollen-specific expression |
| STRE | GLP, GLI, GLN, GLG | AGGGG (-) | Stress response element |
| TCA-element | GLP, GLI, GLN, GLG, GLC | CCATCTTTTT (-) | Involved in salicylic acid responsiveness |
| TCT-motif | GLP, GLI, GLN, GLG, GLC | TCTTAC (+) | Part of a light responsive element |
| TGACG-motif | GLP, GLI, GLN, GLG, GLC | TGACG (-) | Involved in the MeJA-responsiveness |
| Unnamed_1 | GLP, GLI, GLN, GLG | CGTGG (-) | |
| Unnamed_4 | GLP, GLI, GLN, GLG, GLC | CTCC (+/-) | |
| W box | GLP, GLI, GLN, GLG | TTGACC (-) | Negative regulators of abiotic stress response/tolerance |
| WRE3 | GLP, GLI, GLN, GLG, GLC | CCACCT (-) | |
| WUN-motif | GLP, GLI, GLN, GLG, GLC | CAATTACAT (-); AAATTTCTT (-) | Stress response elements |

Note: * TATA BOX – A core promoter element around -30 of transcription start is not included in this table.

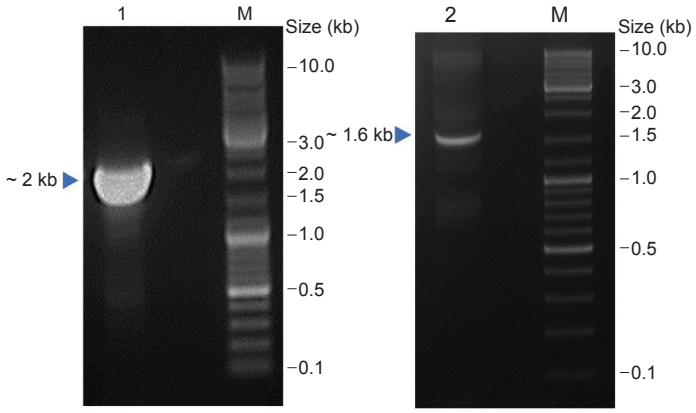


*Figure 1. Validation of the promoter-vector expression constructs in transgenic* Arabidopsis *via PCR using electrophoresis on 1% (w/v) agarose gel. Lane 1 = promMSP2-GLGec::GUS, Lane 2 = promMSP2-GLCec::GUS, M = Gene Ruler DNA Ladder Mix (Fermentas).*

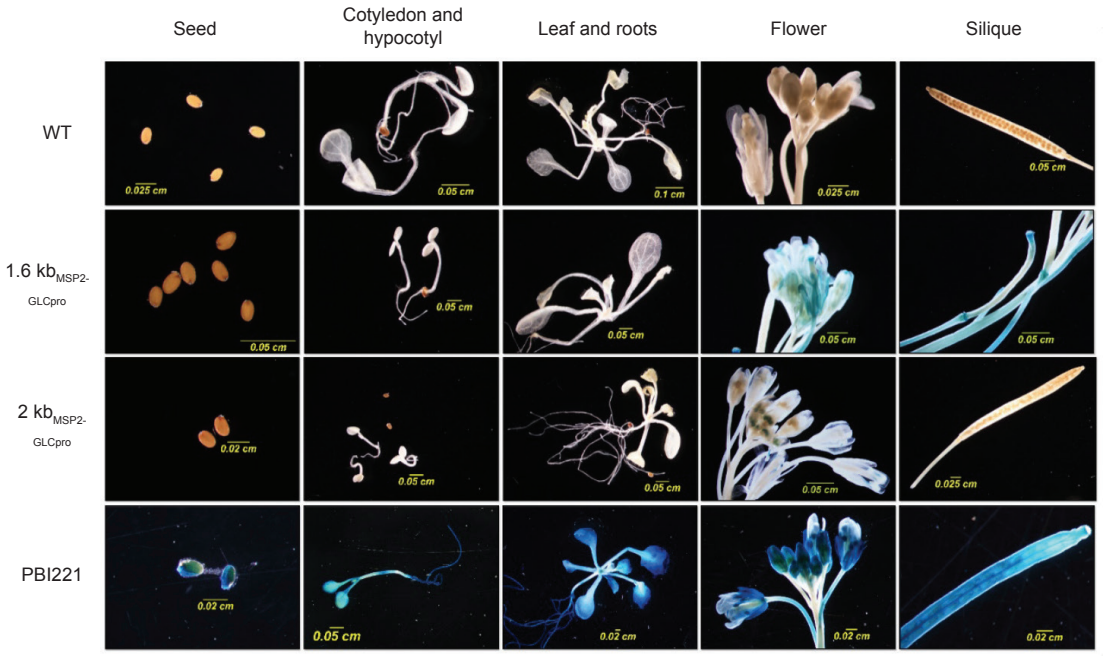


*Figure 2. Histochemical GUS assay of the GLC and GLG promoters in various transgenic* Arabidopsis *tissues. WT = wild-type* Arabidopsis*, 1.6kb$_{MSP2\text{-}GLCpro}$ = transgenic* Arabidopsis *driven by MSP2-GLC promoter, 2kb$_{MSP2\text{-}GLGpro}$ = transgenic* Arabidopsis *driven by MSP2-GLG promoter.*

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

**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).

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 POLLEN1LELAT52 or 'AGAAA' (*Table 1*). Both motifs are distributed evenly among both promoter fragments. However, using PLACE database, a QELEMENTZMZM13 (AGGTCA) motif was only detected in MSP2-GLG fragment and none in MSP2-GLC. The QELEMENTZMZM13 motif has been reported to enhance pollen-specific activity (Hamilton *et al.*, 1998). 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 contained 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 LTRE1HVBLT49 motif (CCGAAA) motif (*Table 2*) 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, GUS gene expression in the petals did not show a noticeable change even after the seeds were exposed up to three days longer to cold treatment (*Figure 3*). GUS gene expression in the stigma of open and mature flowers also remained similar to the control plant. However, GUS signal was also detected in the stamen filament after additional two days of vernalisation treatment (*Figures 3c* and *3d*). In the young developing silique, visual observation of GUS expression in the silique apex and peduncle appears to be slightly stronger in the plants germinated from cold-treated seeds than in the control plant (*Figure 3*). However, the mature silique 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 silique (*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 silique 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 silique, 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 silique 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


*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.*


*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 *without cold treatment, b = transgenic* Arabidopsis *plants with one day cold treatment and c = transgenic* Arabidopsis *plant with three days exposure to cold treatment.*

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* 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* germinated from cold-treated seeds, a low GUS expression level was occasionally found in the septum of the mature silique. Still, the observation in the silique was not consistent between all independent homozygous lines studied. The observed variation in GUS gene expression could be attributed to the position effect since the transformants were generated by random *Agrobacterium* transformation.


*Figure 5. Comparison of GUS histochemical assay of promMSP2-GLG::GUS construct 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.*

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 silique as cold treatment was applied to the treated and untreated seedlings. 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 *FLLI* 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*.,


*Figure 6. Comparison of GUS histochemical assay of promMSP2-GLG::GUS construct in transgenic* Arabidopsis *tissues under cold treatment at 4°C during plant growth (a – c). a = control plant without cold treatment, b = plants with 1-day cold treatment at 4°C and c = plant with 3 days exposure to cold treatment.*

1991; 1995) 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, *Eg*DREB1, 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 analysing 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 (Hernandez-Garcia and Finer, 2015). However, despite the absence of a fleshy fruit form in *Arabidopsis*, promoter functional characterisation 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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