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ISOLATION OF AN OIL PALM CONSTITUTIVE PROMOTER DERIVED FROM UBIQUITIN EXTENSION PROTEIN (*uep2*) GENE

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ABSTRACT

Constitutive promoters are essential component in genetic engineering. The promoters are usually derived from genes that are constitutively expressed or function as housekeeping genes. In this study, the ubiquitin extension protein (uep2) *gene was identified as a constitutively expressed gene in oil palm. The 5′ region of the oil palm* uep2 *gene was isolated and the promoter truncation analysis was performed. The full length of* uep2 (uep2a) *and its truncation derivatives* (uep2b *and* uep2c) *were linked to β-glucuronidase (gus) reporter gene. The strength and the activity of the* uep2 *promoter were transiently evaluated in various oil palm tissues and tobacco leaves. Histochemical GUS analysis showed that the activity of truncated* uep2b *promoter was higher than the full length promoter and* uep2c *derivative in most tissues tested. Result also showed that* uep2b *was capable of directing the expression of GUS in all tissues with the high activity detected in oil palm embryoid, green leaves, and tobacco leaves. The full length of* uep2 (uep2a) *promoter also exhibited the ability to direct the expression of GUS in all tissues with the high activity detected in the plantlet stem. By contrast, although the* uep2c *showed high activity in young leaves and embryoid, a significant decrease of GUS expression was seen in green tissues. These results indicated that the region between position -1096 and -3237 is essential for constitutive transcriptional regulation as deletion of the region resulted in a significant decrease in the promoter activity. Though maize ubi1 and CaMV35S promoters showed a relatively higher activity than* uep2, *the* uep2 *has the ability to induce the constitutive GUS expression in all tissues tested. These results showed that 5′ flanking region of* uep2 *gene is active in oil palm and tobacco, suggesting that the promoter could be used as an alternative promoter for driving constitutive expression of transgenes in oil palm and other plants.*

Keywords: oil palm, constitutive promoter, transient expression.

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INTRODUCTION

Oil palm has been known as a high-yielding source of vegetable oils and is widely used in food and oleochemical industries. Being a Malaysian major commodity crop, the oil palm industry has to remain competitive and sustainable to face major challenges such as unstable commodity price, limited acreage for plantation and labour shortage (Parveez *et al.*, 2015). Biotechnology offers vast opportunities for energising and revolutionising the oil palm industry, and one of the initiatives is through genetic engineering (Ravigadevi *et al.*, 2009). The technology provides novel opportunities to improve oil palm planting materials with the production of high value-added products. The main target of oil palm genetic engineering is to develop high oleate palm as feedstock for oleochemicals

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industry. Other targets are to produce oil palm with high content of stearic acid, palmitoleic acid, ricinoleic acid, lycopene and biodegradable plastics (Rasid *et al*., 2009; Parveez *et al*., 2015; Hanin *et al*., 2016; Masura *et al*., 2017; Zubaidah *et al*., 2018; Masani *et al*., 2018). The success to genetically engineer plants, including oil palm, relies on many factors, and one of them is the use of efficient promoters to drive the expression of transgenes (Potenza *et al*., 2004). The promoter sequence will determine when and where within the organism the transgene is expressed. Constitutive promoters will be a choice if continuous and ubiquitous expression of transgenes is desired throughout the plant cells (Douglas Des Moines, 2004).

Constitutive promoters are normally used to establish a reliable transformation system in plant genetic engineering by expressing reporter and selectable marker genes. The promoter is also useful to accumulate high levels of desired proteins for isolation and to produce compounds that require continuous activities all over the organism or developmental stages (Li *et al*., 2014). Constitutive promoters usually used in genetic transformation were from plant and pathogen origins. Plant derived constitutive promoters usually used in transgenic research are such as *Act1* (McElroy *et al*., 1990), maize *ubi1* (Christensen *et al*., 1992; Cornejo *et al*., 1993), pEmu (Last *et al*., 1991), RUBQ1 and RUBQ2 (Wang and Oard, 2003). These promoters are active for expressing gene of interest in monocots but their efficiencies are sometimes distinct in various ways. Besides, pathogen-origin derived promoters such as CaMV35S (Odell *et al*., 1985; Fang *et al*., 1989; Benfey and Chua, 1990) is numerously utilised in genetic engineering. High expression of transgene is usually produced by using this viral derived promoter in both monocots and dicots (Potenza *et al*., 2004). However, despite their advantages, the use of viral-derived promoters have several limitations. First concern is the perception of risk to human health disfavours its uses, especially for commercial purposes (Ho *et al*., 1999; Potenza *et al*., 2004). Another possible limitation is due to the cell ability to recognise these sequences as foreign and inactivate them (Elmayan and Vaucheret, 1996; Potenza *et al*., 2004), which then would lead to gene silencing. The stable shutdown of gene activity throughout the plant could occur due to gene silencing (Kloti *et al*., 2002), which could also affect the promoter efficiency. Gene silencing is unpredictable, but it may be less common when plant transformation is performed by using constitutive promoters derived from plant origin (Potenza *et al*., 2004).

Oil palm native endogenous constitutive promoters derived from translationally control tumour protein (TCTP) (Masura *et al*., 2011) and ubiquitin extension protein (*uep1*) genes (Masura *et al*., 2010) have been isolated and characterised. These promoters are active constitutively and could be used for driving the expression of transgenes in oil palm or other plants. TCTP and *uep* genes belong to a multiple gene family. We have decided to work further on the uep gene by isolating and characterising another member of the gene family, namely *uep*2. UEP consists of a single ubiquitin monomer (76 amino acids) translationally fused to ribosomal protein. UEP exists in two forms in higher plants (Callis *et al*., 1990) as well as in other eukaryotic organisms (Monia *et al*., 1990; Ozkaynak *et al*., 1987). The single ubiquitin monomer is translationally fused to a coding region of either 76 to 81 amino acid or a 52 amino acid ribosome-associated polypeptide. As *uep1*, *uep2* is also the ubiquitin extension protein that contains 80 amino acid residues. The gene is involved in ribosome biogenesis, the most important component in cellular process of translation (Finley *et al*., 1989; Monia *et al*., 1990). Promoters derived from *uep* gene have been isolated from yeast (Ozkaynak *et al*.,1987) and several plants including tomato (Hoffman *et al*., 1991), barley (Gausing and Jensen, 1986), potato (Garbarino and Belknap, 1994), *Arabidopsis* (Callis *et al*., 1990) and *Jatropha curcas* (Toa *et al*., 2015).

The availability of oil palm *uep2* promoter will increase the range of promoters for the genetic engineering work. More endogenous monocot constitutive promoters are required for multiple transgene stacking and cisgenesis technologies (Chen *et al*., 2010; Holme *et al*., 2013). Utilisation of the different promoters for the different transgenes will possibly reduce the risk of homolog-dependent gene silencing in plant transformation system (Xiao *et al*., 2005). Thus, isolation and the use of more efficient promoters for efficient genetic transformation are essential as the success of genetic engineering technology also depends on their efficacious selection and use (Potenza *et al*., 2004).

MATERIALS AND METHODS

Plants Materials

The oil palm (*Elaeis guineensis* var *Tenera*) plant was used for isolating the gene promoter. Oil palm genomic DNA was prepared from oil palm green leaves using the method by Doyle and Doyle (1990). Total RNA from various oil palm tissues was extracted using method of Zeng and Yang (2002) with modifications.

Expression of Oil Palm Endogenous Gene

The expression pattern of *uep2* gene was analysed through Northern analysis. About 15 μg of total RNA from different oil palm tissues were heated for 10 min at 65°C in RNA loading buffer. The denatured

total RNA was loaded into the formaldehyde-containing agarose gel and electrophoresed in 1X MOPS buffer at 5 to 7.5 V cm^{-1}. Then the gel was stained in ethidium bromide (EtBr) solution (0.5 µg ml^{-1}) for 2 min and followed by destaining in sterile dH_2O for 90 min. By using the capillary transfer method, the total RNA was transferred onto a nylon membrane (Hybond N+ Amersham) for overnight. The membrane was fixed by UV cross-linking using XL-1500 UV Crosslinker (Spectronics Co.) set at 1.2 J cm^{-2}, for 40 s. The cDNA fragment was purified and labelled with [α^{32}P] dCTP radioisotope using Megaprime™ DNA Labelling System (Amersham Life Science). Pre-hybridisation, hybridisation and membrane washing were carried out following the standard technique of Sambrook *et al*. (1989). The membranes were exposed to Kodak XAR-5 film with an intensifying screen at -80°C for a week.

Promoter Isolation, Cloning and Sequence Analysis

A genomic sequence of *uep2* gene was obtained from oil palm genome database (http://genomsawit.mpob.gov.my). The targeted DNA was amplified in a reaction containing 1X High Fidelity PCR buffer, 0.2 mM of each dNTP, 200 nM of gene specific primers and 1 unit Platinum® *Taq* DNA High Fidelity Polymerase (Invitrogen™). The amplification was carried out by denaturing the mixture at 94°C for 2 min, then followed with denaturation at 94°C for 15 s, annealing at 60°C for 30 s and extension at 68°C for 1 min, for 35 cycles. The final extension was performed for 7 min at 72°C. The polymerase chain reaction (PCR) products were fractionated by agarose gel electrophoresis, purified and ligated into pGEMT-Easy vector. The ligation mixture was then transformed into ECOS™ (Yeastern Biotech) competent cells. The Qiaprep Spin Miniprep Kit (QIAGEN) was used to isolate the plasmid DNA. The digestion of recombinant clones with appropriate restriction enzymes was performed to confirm the DNA insertion. The promoter *in silico* analysis was performed using VectorNTI software (Invitrogen) and available databases online such as Softberry, PlantPan (Chang *et al*., 2008) and PlantCare (Lescort *et al*., 2002) to identify the presence of important *cis*-regulatory elements.

PCR amplification was carried out to generate 5′-truncations of *uep2* promoter from the full length construct. Two forward primers (uep2FA and uep2FB) and a reverse primer (uep2R) with additional restriction sites were used for the amplification (*Table 1*). The two truncated derivatives were ligated into pGEMT-Easy vector and transformed into ECOS™ (Yeastern Biotech) competent cells. Resulted colonies were screened for insert and the final confirmation was performed by DNA sequencing.

Construction of Transformation Vector

Constructions of transformation vectors for *uep2* and its truncation fragments were carried out using pBI221 based vector by removing CaMV*35S* promoter region. The fragments were digested from the intermediate cloning vectors using suitable restriction enzymes. The digestion was carried out by adding 5 µg DNA, 1X restriction buffer and 1 unit of each restriction enzyme in 50 µl reaction mixtures and incubated at 37°C for overnight. The DNA fragments were purified using QIAquick Gel Extraction Kit (QIAGEN) after being fractionated in agarose gel electrophoresis. The DNA promoter fragment was ligated into the digested pBI221 vector in reaction mixture consist of 10 ng of vector plasmid, 50 ng of the purified DNA insert, 1X T4 ligation buffer and 1 unit of T4 ligase. The ligation mixture was transformed into ECOS™ (Yeastern Biotech) competent cells according to manufacturer procedure. The recombinant clones were screened using restriction analysis and sequenced.

Plant Materials and Particle Bombardment

The activity of *uep2* promoter was evaluated in different oil palm tissues and green leaves of tobacco. The young leaflet from a mature palm, embryoid, green leaves, root and stem of oil palm plantlets and tobacco green leaves were cultured on EC medium [MS salts (Murashige and Skoog, 1962), Y_3 vitamins (Eeuwans, 1976), 1 mg $litre^{-1}$ napthaleneacetic acid (NAA), 30 g $litre^{-1}$ sucrose and 0.8% agar]. The explants were cut into small pieces before placing onto the medium. The tissues were then pre-cultured in dark at 28°C, one day prior to bombardment. The precipitation of plasmid DNA and the particle bombardment of oil palm tissues

TABLE 1. PRIMER SEQUENCES FOR *uep2* PROMOTER ANALYSIS

Primers	Restriction site	Sequences
uep2pFA	*Sph*1	5′ GCA TGC CAG TTT GAG AAT CAG AA AAA3′
uep2pR	*Xba*1	5′ TCT AGA CTT GGC AGC GGC AAC GGC GCT 3′
uep2pFB	*Pst*1	5′ CTG CAG GAC CCA AGC CCA TTC AAA AAT TT 3′
uep2pFC	*Pst*1	5′ CTG CAG TGC AGG GGT TTC TTT CAA ATA ATC 3′

Note: *uep2* - ubiquitin extension protein.

was carried out using Bio-Rad PDS-1000 (Bio-Rad, Hercules, CA, USA) delivery system as described by Parveez *et al*. (2000). All tissues were bombarded by using 1100 psi rapture discs and at 6 cm distance between stopping plate to target tissues, except for green leaf, which was bombarded using 1350 psi rapture disc and at 4.5 cm distance between stopping plate to target tissues. The bombarded tissues were incubated at 28°C in the dark for two days before the GUS histochemical assay was performed.

GUS Histochemical Assay

The β-glucuronidase (GUS) histochemical assay was performed according to Klein *et al*. (1987) and Parveez *et al*. (2000). The bombarded tissues were stained in the histochemical reagent containing 0.1 M $NaPO_4$ buffer pH7.0, 0.5 mM K-ferrocyanide, 0.5 mM K-ferricyanide, 0.01 M EDTA, 1 mg ml^{-1} X-gluc (5-Bromo-4-Chloro-3-Indolyl-β-D-glucuronide), 1 µl ml^{-1} Triton-X and 20% methanol (v/v) at 37°C for overnight (16 hr). The tissues were fixed in the FAA (5% formaldehyde, 5% acetic acid and 37.5% ethanol) solution to stop the GUS reaction. The green tissues were soaked in 70% ethanol to clear the chlorophyll content. The Nikon UFX-DX microscope system was used to score the GUS blue spots optically.

RESULTS AND DISCUSSION

Identification of *uep2* as an Oil Palm Constitutively Expressed Gene

The present work describes the identification of an oil palm endogenous constitutively expressed gene and the isolation of its promoter for plant genetic transformation. Detailed analysis was carried out by comparing the *uep1* (Accession number: XM_010923047) and *uep2* (Accession number: XM_019854938) sequences. By using pairwise sequence alignment tool (Emboss Needle), results showed that the two genes shared 92.4% similarity of coding region, and 61.1% and 52.2% similarity of 5′ and 3′ non-coding regions, respectively.

Northern analysis was performed to validate the constitutive expression of *uep2*. Results indicated that *uep2* transcript was detected in total RNA of various oil palm tissues including mesocarp at 6, 8, 12, 17 weeks after anthesis (WAA), kernel, green leaves, flower, root, and embryoid (*Figure 1*). A high level of *uep2* expression was detected at the early stages of mesocarp development, namely at the 5 to 12 WAA before it was slightly decreased at later stages. The high level of transcript was also detected in the root and embryoid. Results indicated that the *uep2* expression pattern was similar to the expression pattern of oil palm *uep1* (Masura *et al*., 2010). The expression was high in young tissues and in the tissues containing meristematic cells. The expression profile of *uep2* coincides with its role in ribosome biogenesis. Ribosome is the most important component in the cellular process of translation. The high expression of *uep2* in these tissues is essential for an active protein synthesis. This pattern of expression were observed in tomato (Hoffman *et al*., 1991), *Arabidopsis* (Callis *et al*., 1990), barley (Gausing and Jensen, 1986) and potato (Garbarino and Belknap, 1994). Although the expression level varied among the tissues, *uep2* was ubiquitously expressed throughout the different development stages of oil palm.

Isolation of Promoter Region and Sequence Analysis

The detailed analysis of *uep2* sequence was carried out using the oil palm genome and transcriptome databases. The advances in oil palm genome sequencing and the availability of public access databases have accelerated the discovery of oil palm novel promoters and their important *cis*-acting elements. The *uep2* gene was found to contain 471 nucleotide residues which code for 157 amino acids with the calculated molecular mass of 17.43 kDa. It also contains 113 bp of 5′ end and 312 bp of 3′ end untranslated regions including its poly A^+ tail. Upstream of *uep2* start codon, only a total of 3336 bp fragment that has the sequence information. The sequences of region beyond that are ambiguous. Since this nucleotide bases reads may contain regulatory elements of *uep2*, the region was isolated for promoter analysis. The location of putative TSS was predicted at nucleotide A (Adenine) which is 99 bp upstream of the start codon. A potential TATA (TATAAAA) box sequence was identified at about -30 bp upstream of transcription start site (TSS). A CAAT-box sequence (CAAT) was present at 27 bp upstream of the TATA-box sequence, at position -64. Usually, 50-100 bp sequences adjacent to the TSS is the location of core promoter region, while the upstream promoter regions of 1-2 kb or

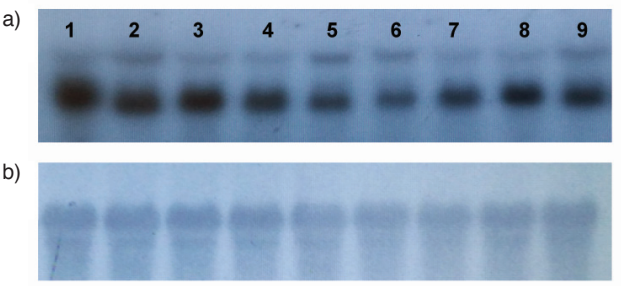

Figure 1. Northern analysis of ubiquitin extension protein (uep2) *gene (a). Each lane contained 15 µg of total RNA prepared from different tissues of oil palm. Lane 1-4: mesocarp 6, 8, 12, 17 weeks after anthesis (WAA), lane 5: kernel 12 WAA, lane 6: green leaves, lane 7: flower, lane 8: root, lane 9: embryoid. Equal loading of RNA was verified with 28S ribosomal DNA (b).*

more contains several *cis*-regulatory elements or DNA motifs that serve as the binding sites for gene specific regulators (Wu *et al*., 2001). The analysis of the *uep2* promoter sequence has revealed a number of conserved DNA motifs upstream of TSS. The putative *cis*-elements were found with different copy numbers and varying distribution pattern (*Figure 2*). These were the potential binding sites for transcription factors. The sequence of uep2 promoter and the predicted putative *cis*-regulatory elements were shown in *Figure 2* and summarised in *Table 2*.

Figure 2. The nucleotide sequence of oil palm ubiquitin extension protein (uep2) *promoter. The position of putative transcription start site (Adenin) is indicated with large and bold font and underlined is numbered as +1. Putative regulatory elements on both strands are shadowed. Arrows indicated the location of* uep2pFA, uep2pFB, uep2pFC *and* uep2pR *primers.*

In summary, the results showed that *uep2* promoter contains multiple *cis*-acting elements which may contribute to its constitutive activity. The oil palm constitutive *uep1* and TCTP (Masura *et al*., 2010; 2011), and other constitutive promoters from *Jatropha curcas* (*JcUEP*) (Toa *et al*., 2015), *Brassica rapa* (beta tubulin) (Mubeen *et al*., 2015) and tomato (sucrose synthase) (Bacha *et al*., 2015), were also shown to contain multiple putative *cis*-elements that are associated with light regulated, hormones responsiveness, abiotic and biotic stresses, and tissue specificity, which may contribute to the total activity of the promoters. The constitutive promoters such CaMV*35S* also contain a string of separate elements, each contributing to the high constitutive expression seen in plants (Fang *et al*., 1989). The individual elements that are classified as 'Domain A' and 'Domain B' in *35S* have been found to be involved in tissue-specific expression (Benfey *et al*., 1990). For *uep2*, the functions of these putative regulatory elements are yet to be determined. Results of some studies have suggested that constitutive promoters of higher plants may not behave as their viral counterparts (Odell *et al*., 1985; Benfey and Chua, 1990). They may not be a collection of numerous tissue specific (motifs) (Odell *et al*., 1985; Benfey and Chua, 1990), but may have non-specific elements that are well conserved and simply more efficient at protein recruitment of transcription (Hernandez-Garcia and Finer, 2014). For example, G box like

TABLE 2. PUTATIVE *CIS*-ACTING ELEMENTS IN *uep2* PROMOTER

Cis-regulatory elements	Matrix sequences	Function	References
E2FANTRNR	TTTCCCGC	Involved in upregulation of the promoter at G1/S transition	Sozzani *et al*. (2006)
E2F1OSPNA	GCGGGAAA	Involved in transcriptional activation in actively dividing cells and tissue	Chabouté *et al*. (2002)
OSE1ROOTNODULE	AAAGAT	One of the consensus sequence motifs of organ-specific elements	Stougaard *et al*. (1990)
OSE2ROOTNODULE	CTCTT	(OSE) characteristic of the promoters activated in infected cells of root nodules	
AACACOREOSGLUB1	AACAAAC	Involved in endosperm-specific expression	Wu *et al*. (2000);
ACGTOSGLUB1	GTACGTG	Involved in endosperm-specific expression	Thomas (1993)
PROLAMINBOXOSGLUB1	TGCAAAG	Involved in endosperm-specific expression	
GATA	GATA	Involved in light responsiveness	Arguello-Astorga and Herrera-Estella (1998)
GT-1	GRWAAW	Involved in light responsiveness	Villain *et al*. (1996);
G box	CACGTG	Involved in light responsiveness	Manzara *et al*. (1991);
		Involved in light responsiveness	Sawant *et al*. (2001)
ABREAZMRAB28	TCCACGTCTC	ABA-responsive element (ABRE B)	Busk and Pagès (1998)
GAREAT	TAACAAR	Gibberellin responsive elements	Ogawa *et al*. (2003);
GARE1OSREP1	TAACAGA	Gibberellin responsive elements	Sutoh and Yamaghuci
GARE2OSREP1	TAACGTA	Gibberellin responsive elements	(2003)
AUXRETGA1GMGH	TGACGTAA	Putative auxin-resonsive element	Liu *et al*. (1994)
LTRE	CCGAA	Low temperature responsive elements	Dunn *et al*.(1998)
MYBCONSENSUSAT	YAACKG	Involved in dehydration and ABA-inducible expression	Ma *et al*. (2009);
MTB1AT	WAACCA	Involved in dehydration and ABA-inducible expression	Abe *et al*. (2003)
MYBARRD22	CTAACCA	Involved in dehydration and ABA-inducible expression	
SURECOREATSULTR11	GAGAA	Core of sulphur-responsive element	Maruyama-Nakashita *et al*. (2005)
TC-rich repeats	ATTTTCTCAA	Involved in defense and stress responses	Zhao *et al*. (2012)

Note: *uep2* - ubiquitin extension protein.

motif is well conserved within the polyubiquitin, including rice RUBQ2, *rubi3*, soyabean *Gmubi3*, *Gmubi7*, sunflower UbB1, maize *Zmubi1*, and potato *Ubi7*, suggesting that the motif contributes considerably to the strong expression mediated by polyubiquitin promoter (Hernandez-Garcia and Finer, 2014). Interestingly, a G box like motif is also present in *uep2* promoter at position -892 (*Figure 2*).

Evaluation of Promoter Activity in Oil Palm Tissues

In order to determine the activity of different regions of the promoter, promoter truncation analysis was performed. The PCR products that contain *uep2* truncation promoter regions with size of 2255 bp and 1194 bp were amplified. The size of amplicon was determined basically based on the presence of putative INTRONLOWER motif (TGCAGg) predicted at position -1095, which is 1194 at length from the start codon. Several studies indicated that the presence of intron at the 5′ untranslated region (5′UTR) could significantly impact gene expression. For region with size of 2255 bp, the fragment consists of the 1194 bp region and spans with about 1000 bp sequence immediately upstream of INTRONLOWER motif. The reporter systems were generated by cloning the full length of *uep2* promoter and the truncation fragments into non-binary vector pBI221, replacing the CaMV*35S* promoter to drive the *gus* reporter gene coding sequence. The expression vectors were designated as pUEP2A (3336 bp), pUEP2B (2255 bp) and pUEP2C (1194 bp) (*Figure 3*).

Histochemical staining for GUS activity was conducted to assess the activity of full length *uep2* promoter and its 5′ deletion constructs. In the present study, the relative strength and expression pattern of the *uep2* promoter were compared with the constitutive *ubi1* and CaMV*35S* promoters. The constructs were bombarded into oil palm tissues and tobacco leaves. Each blue spot detected as a results of GUS activity, whether a single or in a group of cells, was considered as one expression unit (Klein *et al*., 1987). The GUS activity was determined by scoring the

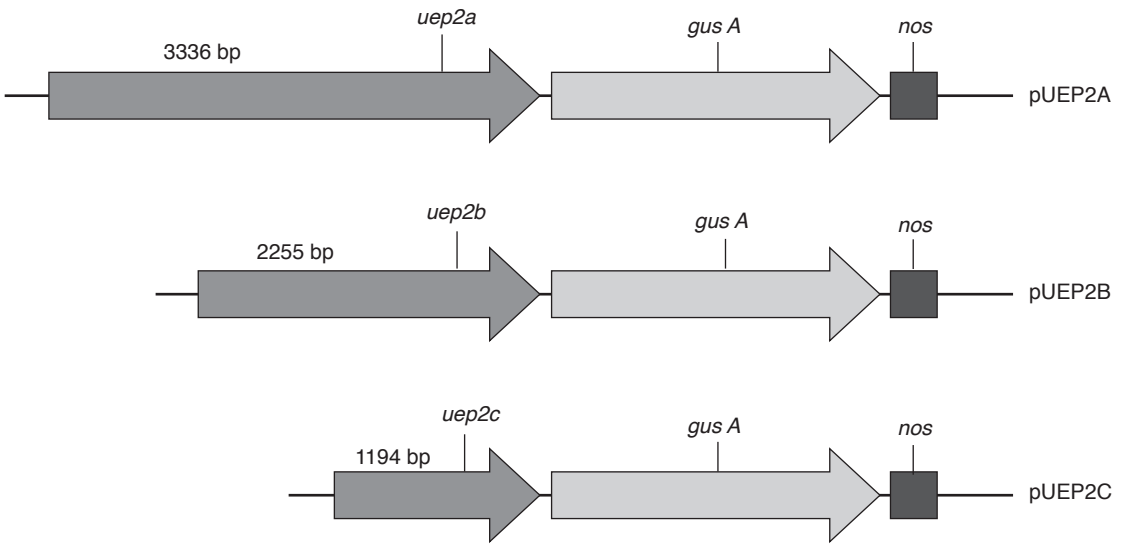

Figure 3. Schematic diagram of the β-glucuronidase (GUS) constructs containing the ubiquitin extension protein (uep2) *promoter. Three* uep2 *promoter fragments (3336, 2255 and 1194 with respect to the ATG) were amplified by polymerase chain reaction (PCR) and fused to gus gene in the pBI221 plasmid, replacing CaMV*35S.

number of GUS positive spots optically. The data collected were then summarised in the form of mean comparison and standard deviation of five replicates. The GUS histochemical data for each of the constructs are shown in *Table 3*.

The activity of *uep2* was evaluated in oil palm cabbage or also defined as young leaflet from mature palm (YLMP). The tissue is routinely used as an explant for callus in vitro propagation (Tarmizi *et al*., 2011). Results indicated that all *uep2* constructs were able to direct the expression of GUS in YLMP tissue (*Figure 4*). The highest activity was shown by *uep2c*, followed by *uep2a* and u*ep2b*. Besides, the GUS histochemical staining was also evaluated in oil palm embryoid (*Figure 4*). The *uep2b* was shown to produce the highest activity, followed by *uep2c* and *uep2a*. The results obtained for *uep2c* was unexpected. GUS should not be expressed in oil palm tissues bombarded with pUEP2C construct since uep2C was predicted as a splice intron region due to the presence of INTRONLOWER motif based on promoter in silico analysis performed using PLACE. Nevertheless, the result could indicate that the prediction might not be accurate as the PLACE database was developed based on *cis*-regulatory elements found in other vascular plants such as rice and *Arabidopsis* (Higo *et al*., 1999).

The activity of *uep2* constructs was also evaluated in oil palm plantlets. The constructs were bombarded into the green leaves, stem and roots of plantlets (*Figure 4*). All *uep2* constructs were able to direct the expression of GUS in green leaves and stem. In green leaves, the activity of *uep2a* was higher than *uep2b* and *uep2c*, but the activity was lower compared to *35S*. In stem, *uep2a* and *uep2b* showed a higher expression of GUS than *35S* and the activity of *uep2c* was comparable to *35S*. In plantlet roots, the expression of GUS driven by all promoters was very low. The low expression of GUS in root could be due to the inappropriate

TABLE 3. COMPARISON OF PROMOTER STRENGTH BY TRANSIENT *gus* GENE EXPRESSION AND GUS ACTIVITY IN OIL PALM TISSUES TWO DAYS AFTER BOMBARDMENT

Promoter/construct	Means (standard error) of GUS foci					
	EM	GL	PL ST	T	YMLP	R
Ubi1/pAHC25	998±101.73	91.67±11.67	120.67±12.33	8±1.73	1 783±58.77	7±0.7
CaMV35S/pBI221	665.33±22.92	50.67±4.055	60.11±11.45	81±20.74	933±116.75	11±2.5
uep2a/pUEP2A	293.33±2.73	28.67±2.60	107.33± 12.33	11±2.08	331±63.43	5±1.2
uep2b/pUE2B	534.00±5.73	30.33±4.63	85.00±2.89	30.67±6.98	370.67±3.93	5±0.5
uep2c/pUEP2C	522.99±60.61	5.33±0.88	50.33±1.07	9.33±1.76	460±37.00	0

Note: *EM - embryoid. GL - green leaves. PL S - plantlet stem. T - tobacco leaves.
YLMP - young leaflet from mature palm. R - plantlet root. *gus* - β-glucuronidase.

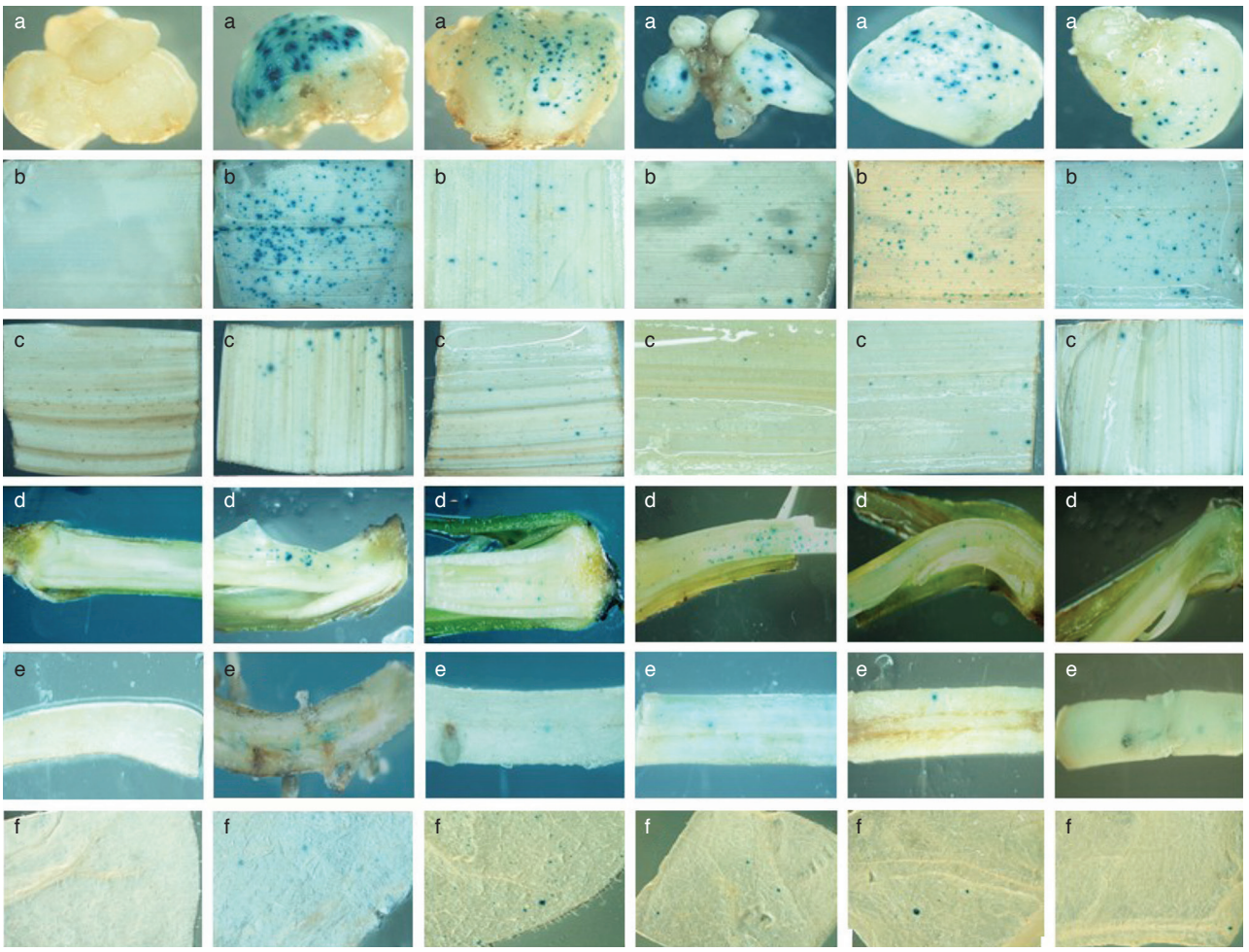

Figure 4. Transient histochemical β-glucuronidase (GUS) assay in oil palm tissues and tobacco leaves. The tissues were bombarded with constructs carrying ubi1, *CaMV*35S, uep2A, uep2B *and* uep2C. *a) Embryoid, b) young matured leaflet palm, c) plantlet green leaf, d) plantlet stem, e) plantlet root, and f) tobacco leaf.*

parameters of particle bombardment that were used for delivering the plasmid DNA into the tissue. As optimisation of bombardment physical parameters for root has not yet been carried out, delivery of the plasmids into this tissue was only performed using the parameters optimised by Parveez *et al*. (1997). The optimised parameters were for oil palm transformation using embryogenic calli. The highest activity was observed in the root tissue bombarded with *35S*, followed by *ubi1*, *uep2a* and *uep2b*. The activity of *uep2a* was similar to *uep2b*. However, no expression of GUS was observed for *uep2c*.

Although *uep2* is active in oil palm, its activity is relatively lower than maize *ubi1* in most tissues. *Ubi1* is a strong constitutive promoter that has been extensively used to express chimeric genes in monocot transformation studies (Streatfield *et al*., 2004). The promoter was also shown to drive a strong expression of GUS in oil palm tissues as reported by Chowdhury *et al*. (1997). The *Ubi1* activity was also higher than oil palm *uep1* and TCTP promoters (Masura *et al*., 2011; 2010). The use of strong constitutive promoters such as those derived from polyubiquitin or *Act1* that are capable of driving high expression of transgenes is certainly desired. However, the use of very strong promoters have shown to have some drawbacks. The excessive promoter activity might lead to the undesirable phenotypes such as growth retardation or abnormalities (Takeda *et al*., 2003; Potenza *et al*., 2004). This could possibly be due to the overexpression of a specific transgene at the wrong time in development, particularly in tissues where it is not normally expressed, or expressed at a very high level (Potenza *et al*., 2004). Moreover, strong plant promoters can also be easily silenced by methylation (Li *et al*., 2014). Methylation can occur in the exon/intron region of promoter complexes. Methylation of the exon/intron in the *ubi1* promoter complex, and condensation of the chromatin in regions containing transgenes was reported in barley (Meng *et al*., 2003).

The activity of *uep2* promoter was also investigated in dicotyledonous plant using tobacco leaves as a target tissue. The use of tobacco plant could indicate the potential of *uep2* promoter for

genetic engineering work of dicots. In addition, it is a suitable model plant to evaluate the efficiency of *uep2* promoter for stable expression as oil palm has a long regeneration cycle. Results indicated that all *uep2* constructs were able to direct the expression of GUS, with the highest activity produced by *uep2b*, followed by *uep2a* and *uep2c* (*Figure 4*). However, the activity of *35S* promoter was higher than *uep2b*. This was expected as the activity of *35S* was reported to be more effective in dicots than monocots (Battraw and Hall, 1990; Benfey and Chua, 1990). Nevertheless, the results still indicated the potential application of *uep2* promoter in dicots.

In general, the truncated *uep2b* promoter showed the highest expression of GUS in most tissues as compared to other *uep2* derivatives. The *uep2b* was able to direct the expression of GUS in all tissues tested with the high activity detected in oil palm embryoid, green leaves, and tobacco leaves. The full length of *uep2* (*uep2a*) promoter also has the ability to direct the expression of GUS in all tissues with the high activity detected in the plantlet stem. The strength of *uep2a* and *uep2b* was similar in the plantlet roots. By contrast, the *uep2c* promoter deletion derivative was shown not effective in driving GUS expression in all tissues tested. Although *uep2c* showed a high activity in YLMP and embryoid, a significant drop in GUS expression occurred in green leaves, plantlet stem and tobacco leaves, whereas no expression was observed in plantlet root. These results indicated that the 5′ flanking region of *uep2* gene was active in oil palm and tobacco leaves. Deletion of the 2141 bp region between position -1096 and -3237 produced a significant decrease in GUS expression, denoting the presence of important *cis*-acting regulatory elements or enhancer that are related to the constitutive transcriptional regulation of *uep2* gene.

Regulation of gene expression at the promoter level is mainly controlled by the *cis*-acting elements localised upstream of the transcriptional initiation sites (Hernandez-Garcia and Finer, 2014). Its mechanism involves complex processes of DNA-protein and protein-protein interactions that are diverse in response to environmental conditions and developmental cues (Manzara *et al*., 1991). The results so far indicated that the *uep2* promoter activities were influenced by the deletion sections of the 5′ upstream sequence. The different sizes of promoter deletions contain the different copy number of *cis*-elements and have different distribution pattern in promoter region, which may affect the gene expression. For example, GATA box was highly distributed in *uep2a* (19 repeats) and *uep2b* (eight repeats) as compared to *uep2c* (five repeats) (*Figure 2*). The GATA elements have been found in many promoters of light-responsive genes such as the RBCS, CAB and GAP (Argüello-Astorga and Herrera-Estella 1998; Jeong and Shih, 2003) and are essential for their high expression levels (Gidoni *et al*.,1989). Deletion or mutation of GATA elements in both green tissue specific *(Lhcb2)* and constitutive (CsVMV) promoters have strongly reduced the promoters activity in photosynthetic tissues (Kehoe *et al*., 1994; Verdaguer *et al*., 1998). Results indicated that the deletion did not interfere the constitutive expression of *gus* gene under *uep2b*. For *uep2c*, even though the promoter was active in YLMP and embryoid, its activity decreased particularly in green tissues. This indicated that *uep2c* promoter fragment may contain all of necessary regulatory elements but needed strong enhancers to be functional. In *uep2b* region, the detailed analysis reveals the presence of a 23BPZM27KDAZEIN motif (aatgttggtttcctttagACGCC) at position -1221. The motif confers a high level of transcriptional activity in an orientation-dependent manner (Ueda *et al*., 1994). The region also has a TA-rich region at –2096 that functions as an enhancer for transcription (Sarmast, 2017). The results showed that the *cis*-regulatory elements that may be necessary for high transcriptional activity were distributed at location -1096 and -3237. The elements are fully present in *uep2a* and *uep2b*.

In general, as *uep1* (Masura *et al*., 2010), *uep2* promoter strength was also relatively moderate compared to the strong *ubi1* and *35S* promoters. However, constitutive promoters with a moderate activity level might sometimes be more desirable for genetic engineering application to reduce energy loss and to avoid phenotypic changes (Li *et al*., 2014). It is also useful for the expression of transgenes that are toxic or otherwise inhibitory in abundance, for which a very strong promoter would not be suitable (Park *et al*., 2010). Toa *et al*. (2015) reported that the strength of ubiquitin extension protein (*JcUEP*) promoter from *Jatropha* was lower than the CaMV*35S* in most tissues. Nevertheless, the promoter was constitutively active in the transgenic *Jatropha* and *Arabidopsis*, and its activity was also maintained under stress condition such as in low temperature, high salts, dehydration and exogenous ABA treatments (Toa *et al*., 2015). Our results also showed that oil palm *uep2* has the ability to drive the expression of *gus* reporter gene constitutively and in certain tissues, such as plantlet and embryoid, the *uep2* activity was comparable to the *35S* promoter.

CONCLUSION

We have successfully isolated a promoter from ubiquitin extension protein gene *(uep2)*. The activity of *uep2* promoter and its 5′ deletion derivatives was characterised using transient *in vitro* analysis. Although transient system may not provide definitive information on gene expression

pattern, the system has served as an indicator that showed the potential of *uep2* as an ideal alternative constitutive promoter for genetic engineering work. Our novel oil palm promoter may also be useful for transgene expression in other monocots and dicots as well as other important crops for beneficial traits.

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