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Isolation and Characterization of EgGST, a Glutathione S-transferase Protein Transcript in Oil Palm (*Elaeis guineensis* Jacq.)

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ABSTRACT

The formation of callus and somatic embryos remains one of the major bottlenecks in oil palm tissue culture. Unlike other crops, oil palm tissue culture is a very slow process. In the present study, EgGST (GenBank accession no. AIC33066.1), an oil palm gene coding for a putative glutathione S-transferase protein, has been characterized molecularly. The full length cDNA sequence of EgGST isolated from oil palm cultured leaf explants at the 6th week is 1002 bp in length, with an Open Reading Frame (ORF) of 651 bp. The deduced EgGST encodes a 216-amino-acid protein with a predicted molecular mass of 23.68 kD and a pI value of 6.16. Its protein sequence shares 63% identity with the glutathione s-transferase gstf2 from *Oryza sativa Indica* Group (GenBank accession no. ABR25713.1) and contains thioredoxin fold and chloride channel domain. Real-time RT-PCR results showed that the EgGST transcript was differentially expressed across a time series of fortnightly-cultured leaf explants and had a higher transcript levels in nodular callus (NC) compared to friable callus (FC) for oil palm ortet of clone 4178. EgGST was also found to be preferentially expressed in all tissue culture derived materials except for oil palm cell suspension culture (CSC), whereas there were almost negligible expressions in all the non-tissue culture derived materials, except for root. Hence, it can be suggested that EgGST transcript may possibly be regulated differently at different stages of tissue culture and various tissues. Interestingly, EgGST also displayed a tissue-specific expression pattern via RNA *in situ* hybridization. To our

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knowledge, this is the first reported study on the analysis of the localization of target mRNA transcript of EgGST in different oil palm tissues. We postulated that EgGST might play significant roles at different stages of oil palm callogenesis, and could potentially be a candidate marker for oil palm callogenesis.

Keywords: Oil palm, callogenesis, glutathione S-transferases, full length cDNA, real-time RT-PCR, RNA *in situ* hybridization

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is an unbranched monocotyledonous plant of the palm family (Arecaceae) originating from West Africa. The oil palm belongs to the family Palmaceae and the genus *Elaeis*. *Elaeis* is derived from the Greek work *elaion*, which means oil, while the specific name *guineensis* shows that Jacquin attributed its origin to the Guinea coast, West Africa (Corley & Tinker, 2003). At present, there is a potential demand for more than 100 million oil palm (*Elaeis guineensis* Jacq.) tissue cultured plantlets in the world (Corley, 2009; Sharifah & Abu, 2007). In terms of performance, clonal plantlets from selected ortets have out-yielded commercial DxP seedlings by 7%-34% in fresh fruit bunch (Kushairi *et al*., 2010; Sharma, 2006; Simon & Koh, 2005; Zamzuri *et al*., 2005; Khaw & Ng, 1997). The use of clonal palms has been predicted to improve oil production up to 30% (Low *et al*., 2008).

However, unlike other crops, oil palm tissue culture is a very slow process. The regeneration process through oil palm tissue culture takes 2 to 4 years, depending on the genotype. On average, at least 18 months are required to produce complete plants from callus derived from leaf explants, with a callusing rate of only about 20% for young leaf and root explants, and the rate of embryogenesis from proliferating callus culture too was only 3 - 6%, depending on the genotypes (Rajainadu *et al*., 2007; Rohani *et al.*, 2000; Wooi, 1995), making oil palm tissue culture rather inefficient. In an effort to gain insights into oil palm callogenesis, a previous study employed representational difference analysis (RDA) to a pair of cDNA populations from *E. guineensis*; one transcribed from the RNA of the 6th week oil palm cultured leaf explants (where callus initiation occurred) and the other from RNA of 0-day leaf explants, which led to the identification of the abundantly expressed partial glutathione S-transferases gene (Fatihah, 2010).

Plant glutathione S-transferases (GSTs) have been actively investigated during the past decades (Chronopoulou & Labrou, 2009; Basantani & Srivastava, 2007; Moons, 2005; Dixon *et al*., 2002; Edwards *et al*., 2000; Droog, 1997). All the GSTs are reported to be either soluble or loosely membrane-associated dimers with a monomeric size of 15 - 28 kDa, and together they comprise 1 - 3.5% of the total cellular protein (Pairoba & Walbot, 2003; Droog *et al*., 1995). GSTs are a superfamily of multifunctional enzymes in plants, subdivided into eight classes, seven of which (phi, tau, zeta, theta, lambda, dehydroascorbate reductase, and

tetrachlorohydroquinone dehalogenase) are soluble and one is microsomal (Dixon & Edwards, 2010; Lan *et al*., 2009; Basantani & Srivastava, 2007). Since their identification in plants in 1970, these enzymes have been well established as phase II detoxification enzymes that perform several other essential functions in plant growth and development. The GST enzymes have been associated with detoxification of xenobiotics, limiting oxidative damage and other stress responses in plants (Gong *et al*., 2005).

Currently, a large number of the GST genes have been identified or annotated from at least 17 plant species (Chronopoulou & Labrou, 2009; Conn *et al*., 2008; Basantani & Srivastava, 2007). The number of GST-like sequences found in different plant species ranges from 25 in soybean to 42, 53, 59 and 81 in maize, *Arabidopsis* (http://www.arabidopsis.org/browse/genefamily/gst.jsp), rice and *Populus trichocarpa*, respectively (Lan *et al*., 2009; Sappl *et al*., 2009; Sappl *et al*., 2004; Soranzo *et al*., 2004; Dixon *et al*., 2002; Wagner *et al*., 2002; McGonigle *et al*., 2000). Some of the GST genes have been patented (Chronopoulou & Labrou, 2009). Until now, no other data have been reported on the genome-wide identification of the GST family, although at least 20 plant genomes have been completely sequenced (http://www.genomesonline.org/gold.cgi) (Chi *et al*., 2011). To date, the whole family-based expression analyses have been carried out only in *Arabidopsis* (Sappl *et al*., 2009), *P. trichocarpa* (Lan *et al*., 2009) and rice (Jain *et al*., 2010). The transcript profiling of all family members for the other plants, including oil palm, as well as the functional divergence of the GST family, is still not available. Hence, it will be interesting to know the functional role of glutathione S-transferase genes (EgGSTs) in *E. guineensis*. To date, there is no reported full-length cDNA isolation of EgGST from oil palm. Thus, the aims of this study were to isolate a full-length EgGST cDNA from oil palm and perform molecular characterization.

MATERIALS AND METHODS

Plant Material

Tissue cultured materials of *Elaeis guineensis* Jacq. var. tenera were provided by Felda Agricultural Services Sdn. Bhd., Malaysia. For expression analysis, young leaf explants of clone 4178 cultured on Murashige and Skoog (MS) basal culture medium (Murashige & Skoog, 1962) supplemented with auxin, were collected every 2 weeks (day 0 to 26 weeks of culturing). Clone 4178 was selected as the plant material due to the high proliferation ability at the commercial laboratory of Felda Biotechnology Centre, Felda Agricultural Services Sdn. Bhd. (Fatihah, 2010). Meanwhile, the samples for tissue specificity study were provided by Malaysian Palm Oil Board (MPOB), Sime Darby Seeds and Agricultural Services Sdn. Bhd. (SDSAS) and Applied Agricultural Resources Sdn. Bhd. (AAR). The tissue samples were categorised into two groups: tissue culture derived materials from the leaf [embryogenic callus (EC), non-embryogenic callus (NEC), oil palm cell suspension culture (CSC) and the three

different morphologies of oil palm somatic embryos identified during the maturation stage (globular, haustorium and germinating embryo)] and non-tissue culture derived materials [female flower (FF), male flower (MF), apical meristem (M) and root (R)].

Total RNA Extraction

Each plant material (0.1 g) was ground in liquid nitrogen to fine powder using a mortar and pestle. Total RNA extraction was performed by using TRIzol® reagent (Molecular Research Centre, USA) according to the manufacturer's instructions. The RNA concentration was measured by absorbance at 260 nm (A_{260}) and the purity of the RNA sample was evaluated by A_{260}/A_{280} and A_{260}/A_{230} ratios using NanoPhotometer™ (Implen GmbH, Munich, Germany). The integrity of RNA obtained was determined by visualization on a 1.5% (w/v) formaldehyde agarose gel electrophoresis (Sambrook & Russel, 2001).

Full-Length cDNA Isolation of the EgGST

A partial-length cDNA sequence encoding putative glutathione S-transferase protein was previously identified by Fatihah (2010). This gene was designated as EgGST. EgGST had partial-length sequence lacking the 5'- and 3'-region. Two gene specific primers (5' GSP: 5'-GCATCGCAGA GGTCACCTTCTTGCACGC-3' and 3' GSP: 5'-CCGCATGTTAA GGCATGGTGGGAGG-3') were designed based on the partial-length of the cDNA sequence to isolate the 5' and 3'-region of the putative EgGST, respectively. The SMARTer™ RACE (Rapid Amplification of cDNA Ends) cDNA Amplification Kit (Clontech, USA) and the Advantage 2 Polymerase Mix (Clontech, USA), together with the gene specific primers, were used in the isolation of the 5' and 3'-regions of the gene. The sequences of all the partial-length, 5' and 3'-regions were assembled into contigs by the Contig Assembly Programme (CAP) using the BioEdit Sequence Alignment Editor Version 7.0.9.0 (Hall, 1999) to get the full-length sequence of the EgGST. Based on the full-length sequence, two gene specific primers (ORF forward:5'-AGACGATGGGGGTGAAGGTCTATG-3' and ORF reverse: 5'-ACGCAGATCCAG GCATCGCAGAG-3') were designed to amplify the Open Reading Frame (ORF) region. The ORF of the transcript was isolated by PCR amplification of the 5'-RACE-Ready cDNA template with the two gene specific primers. The PCR product was then cloned into the yT&A cloning vector (Yeastern Biotech, Taiwan) and sequenced at both directions (NHK Bioscience Solutions Sdn. Bhd., Malaysia).

Sequence Analysis

The 5'-RACE PCR product, 3'-RACE PCR product and ORF sequences of the putative EgGST were analyzed using the BLASTN, BLASTX and BLASTP programmes at the National Centre of Biotechnology Information (NCBI). These programmes were used to search for significant similarities between the isolated sequences with NCBI/Genbank databases. BLASTX and BLASTN were

used to search for significant identities in the non-redundant protein sequences (nr) and expressed sequence tags (ESTs) databases, respectively. The clustal W multiple alignment programme in the Bioedit software was used to align the amino acid sequence of the EgGST with homologous sequences from other plants. The acquired sequence data were also analyzed further using the Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences (PSORT) (http://psort.nibb.ac.jp) (Horton *et al*., 2007), plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) (Kuo-Chen & Hong-Bin, 2010), Biology Workbench Version 3.2 (http://workbench.sdsc.edu) (Subramaniam, 1998), SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen *et al*., 2011), Compute pI/Mw (Expasy) (http://br.expasy.org/tools/) and FEX Programme (http://www.molquest.com/molquest.phtml?group=index&topic=gfind) (Solovyev *et al*., 1994). The phylogenetic tree of EgGST was constructed using the Mega version 4 software (Tamura *et al*., 2007). In this study, the cut-off score and Expect (E) value were set at 50 and 10^{-5}, respectively; whereby all the matches with BLASTX scores equal to 50 or above and E value equal or less than 10^{-5} were considered significant.

Expression Study by using Real-Time RT-PCR (Reverse Transcriptase-PCR)

One microgram of the total RNA was used for reverse transcription into first-strand cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen, USA). Primers for the real-time RT-PCR analysis were designed using the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) based on the 3' un-translated regions (UTRs) of the full-length transcript sequence of the EgGST obtained. The suitability of the designed primers was checked by using the Oligonucleotide Properties Calculator software (http://www.basic.northwestern.edu/biotools/oligocalc.html). The Brilliant® SYBR Green QPCR Master Mix (Stratagene, USA) was used for real-time RT-PCR reaction. Each PCR mixture contained 1X master mix (comprising SYBR® green I dye, 2.5 mM $MgCl_2$, and dNTPs mixture), 100 nM of gene specific primers (Forward primer: 5'-ATCTGCGTGAGA GGTATCGGTTG-3' and Reverse primer: 5'-ATTACCCACCATCC CACCCTAGA-3'), 2 µL of 10-fold diluted first-strand cDNAs in a total volume of 20 µL. The amplification was performed in the iQ5 Real Time PCR Detection System (BioRad, USA) using the following program: 95°C for 10 minutes; 50 cycles of 95°C for 30 seconds, 60°C for 45 seconds and 72°C for 30 seconds. For each sample, three technical replicates were included. All the experiments contained a non-template control (negative control) and a calibrator. The quantity of the gene expression levels in every experimental tissue was expressed relative to the calibrator, i.e. 0-day leaf explants (clone 4178). Comparative C_T method was used to estimate the relative expression level of EgGST transcripts

(Livak & Schmittgen, 2001). The relative expression were carried out across a time series of fortnightly-cultured leaf explants and in different oil palm tissues and analysed using the geNorm software (Primer-Design, UK) (Vandesompele *et al*., 2002). In the analysis, the relative quantity of the transcripts were normalized with the expression of three endogenous genes including unknown/hypothetical protein (EA 1332; GenBank accession no. EY406625.1), superoxide manganese dismutase (PD 569; GenBank accession no. EL682210.1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; GenBank accession no. DQ267444).

The sequences of the primers are as follow:

EA 1332 sense
5' – TTAAGAATGCTCGGGAAAGG – 3'
EA 1332 antisense
5' – CTACTTCTGTCTGCAATTTTGG – 3'
PD 569 sense
5' – ATCAACCACTCAATCTTCTGG – 3'
PD 569 antisense
5' – CTTCTGCGTTCATCTTTTGC – 3'
GAPDH sense
5' – GCCAGCTTTAACATCATTCCTAGC – 3'
GAPDH antisense
5' – AGCTTTCCATTTAAGGCAGGAAG – 3'

Expression Study by using RNA in situ Hybridisation

One morphology of oil palm somatic embryo identified during the maturation stage (germinating embryo), plus several oil palm *in vitro* cultured-derived samples such as leaf explants (LE), embryogenic callus (macroscopically nodular and friable cultures) and non-embryogenic callus (macroscopically not friable), were used to study the expression of EgGST transcripts. All the plant materials were provided by Felda Agricultural Services Sdn. Bhd., Malaysia. Sense and antisense riboprobes were synthesised by using Ampliscribe™ T3 High Yield and Ampliscribe™ T7 Flash™ Transcription Kit, respectively (Epicentre® Biotechnologies, USA) according to the manufacturer's instructions. Sense and antisense probes were generated by designing the gene specific primers containing the minimum T3 promoter sequence (5'-AATTAACCCTCACTAAAGG-3') and T7 promoter sequence (5'-TAATACGACTCACTATAGG-3'), respectively needed for efficient transcription (as bolded below). The primers were designed at the ORF and 3'UTR region of the EgGST sequence. The sequences of the primers are as follows:

T3F:
5'-**AATTAACCCTCACTAAAGG**GATGTGTGGTTGGAAGTGGAATC-3'
T7R:
5'-**TAATACGACTCACTATAGG**CTCAATAGACAGGGACTCACAGC-3'

In situ hybridisation (ISH) was performed according to the method described by Ooi *et al*. (2012). All the images were then viewed and photographed with a camera attached to the LEICA DM6000 B light microscope (Leica, Germany) and processed with the Progress Research Pro software (Leica, Germany).

RESULTS AND DISCUSSION

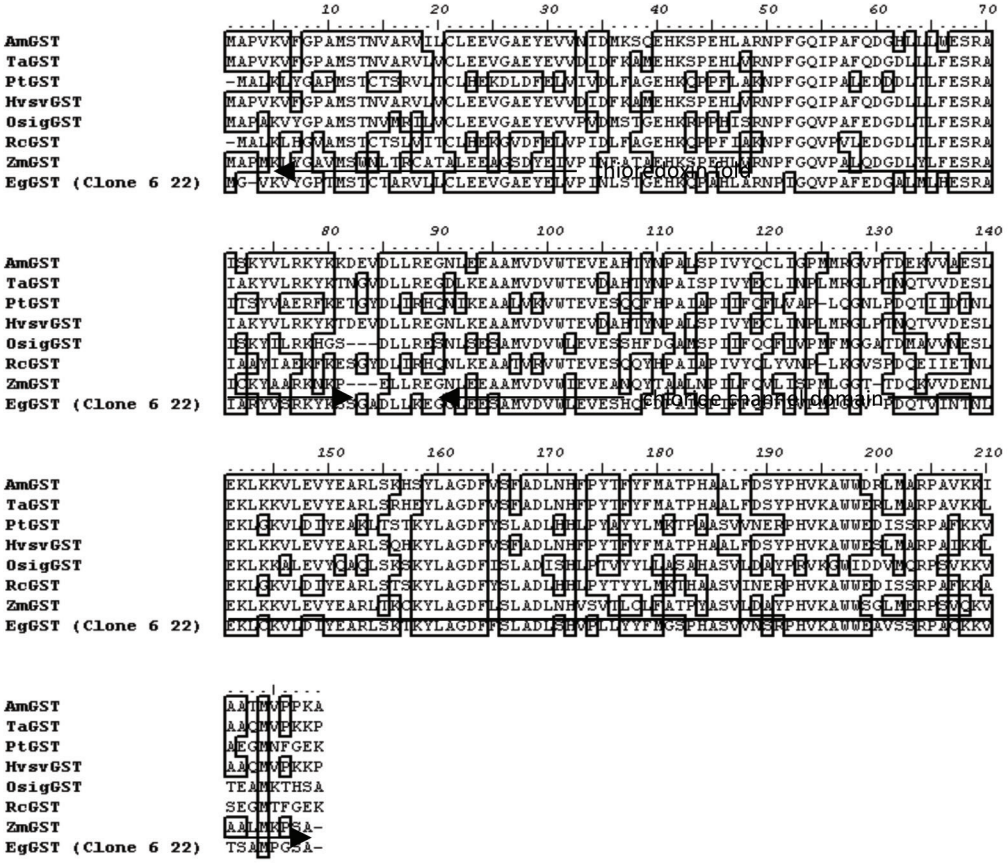
Sequence Analysis of the Full-Length cDNA of EgGST

The full-length cDNA sequence of EgGST (1002 bp) was predicted to encode a polypeptide of 216 amino acid residues with 78 bp of 5' UTR, 651 bp of open reading frame and 273 bp of 3' UTR including a poly-A tail (GenBank accession no. AIC33066.1). By using Compute pI/Mw (Expasy tools software), the EgGST has a predicted molecular mass of 23.68 kD, with a pI value of 6.16. The BLASTX analysis (NCBI) showed that the deduced amino acid sequence of EgGST was 63% identical to glutathione s-transferase gstf2 from *Oryza sativa Indica* Group and 62% identical to glutathione transferase from *Alopecurus myosuroides*. This was followed by phi class glutathione transferase GSTF3 from *Populus trichocarpa* (61%), glutathione transferase from *Hordeum vulgare subsp. vulgare* (61%), glutathione-S-transferase 19E50 from *Triticum aestivum* (61%), glutathione-s-transferase theta, gst, putative from *Ricinus communis* (60%), and glutathione S-transferase 1 from *Zea mays* (56%). The deduced amino acid sequence of EgGST has a thioredoxin fold domain located at the positions of 4 - 82 and a chloride channel domain located at the positions of 90 - 216 (see Fig.1).

Glutathione (GSH) is the tripeptide γ-glutamyl-cysteinyl-glycine and plays a central role in the processes of detoxification and redox buffering (Noctor & Foyer, 1998). GST proteins consist of two well-defined domains, the N-terminal domain that binds the primary substrate GSH and the C-terminal domain that binds the secondary substrate (Edwards *et al*., 2000). Plant GSTs acted by catalyzing nucleophilic conjugation of the reduced form of the tripeptide GSH to a wide variety of hydrophobic, electrophilic, and usually cytotoxic substrates. The toxic molecule, GSH conjugate can then be transported to the vacuole or apoplast and metabolised to a non-toxic compound such as peptide derivatives (Dixon & Edwards, 2010; Edwards *et al*., 2000).

Most GSTs are active as dimers, composed of either homogeneous (the most prevalent form) or heterogeneous subunits (Edwards *et al*., 2000). The thioredoxin-like N-terminal domain (4 - 82 amino acids), as shown in Figure 1, binds to GSH, and is conserved in all classes of GSTs (Dixon *et al*., 2002). By contrast, the C-terminal chloride channel domain (90 - 216 amino acids) (Figure 1) is the domain that provides structural elements for the recognition of xenobiotic substrates, which tends to exhibit much more diversity within and among classes of GSTs (Basantani & Srivastava, 2007; Edwards *et al*., 2000). In 2009, Lan *et al*. suggested that the C-terminal domain could lead to diversification in substrate selectivity and specificity among the members of *Populus trichocarpa* tau GSTs, while preserving the enzymes' primary function and thus, enhance the metabolism of substances encountered in the environment.

Plant GSTs are classified based on amino acid sequence identity and conservation of gene structure (i.e., exon/intron numbers),



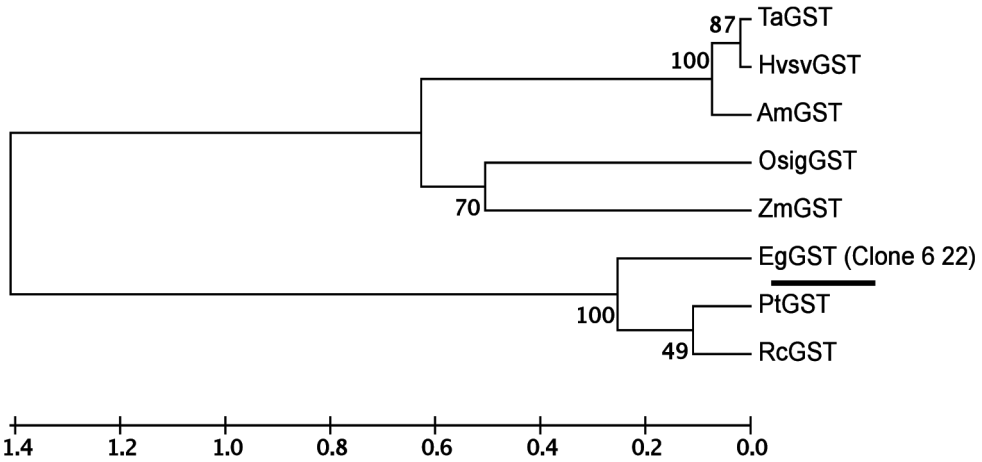
The identical amino acids are boxed. The sequences were downloaded from Genbank: AmGST, glutathione transferase of Alopecurus myosuroides (Acc. No. CAA09191.1); TaGST, glutathione-S-transferase 19E50 of Triticum aestivum (Acc. No. AAL47688.1); PtGST, phi class glutathione transferase GSTF3 of Populus trichocarpa (Acc. No. ADB11382.1); HvsvGST, glutathione transferase of Hordeum vulgare subsp. vulgare (Acc. No. AAL73394.1); OsigGST, glutathione s-transferase gstf2 of Oryza sativa Indica Group (Acc. No. ABR25713.1); RcGST, glutathione-s-transferase theta, gst, putative of Ricinus communis (Acc. No. XP_002531867.1); ZmGST, glutathione S-transferase 1 of Zea mays (Acc. No. NP_001105412.1). The thioredoxin fold domain (4 – 82 amino acids) and chloride channel domain (90 – 216 amino acids) are as shown above.

Fig.1: Alignment of Deduced Amino Acid Sequences of EgGST with *Alopecurus myosuroides, Triticum aestivum, Populus trichocarpa, Hordeum vulgare subsp. vulgare, Oryza sativa Indica Group, Ricinus communis* and *Zea mays* sequences.

in which the number of exons is different for each class (Licciardello *et al*., 2014; Mohsenzadeh *et al*., 2011). For example, phi class of GST genes contains three exons, tau class contains two exons and zeta class contains ten exons in their genes. In the FEX Program (Prediction of internal, 5'- and 3'- exons) analysis, EgGST was predicted to have five potential exons. Conserved Domain Database (CDD) analysis (NCBI) on the deduced amino acid sequence of EgGST (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) (Marchler-Bauer *et al*., 2013) showed that EgGST belongs

to phi class GSTs. However, this is only a prediction by bioinformatics data and the exact classification of EgGST can only be confirmed after resolving the crystal structures of EgGST through X-ray crystallography. The tau and phi class GSTs are the most represented ones, plant-specific and chiefly involved in xenobiotic metabolism (Basantani & Srivastava, 2007; Moons, 2005). Genome-wide analysis of biochemical characteristics of *Arabidopsis thaliana* and *Populus trichocarpa* tau and phi GSTs found that these two classes of GSTs have broad substrate specificities (Dixon *et al*., 2009; Lan *et al*., 2009), which may be related to the high tolerance to abiotic stresses, especially to a broad spectrum of xenobiotics such as herbicides, salt and UV stressors (Jha *et al*., 2011). Zeta- and theta-class GSTs have very restricted activities towards xenobiotics. Theta-class GSTs are glutathione peroxidases and involved in oxidative-stress metabolism, whereas zeta-class GSTs act as glutathione-dependent isomerases and catalyse the glutathione-dependent conversion of maleylacetoacetate to fumarylacetoacetate. Zeta-class GSTs participate in tyrosine catabolism. Dehydroascorbate reductase- and lambda-class GSTs function as thioltransferases (Basantani & Srivastava, 2007). Dehydroascorbate reductase is also a key enzyme in the ascorbate-glutathione cycle that maintains reduced pools of ascorbic acid, which serves as an important antioxidant (Moons, 2005). Microsomal-class GSTs are members of the MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) superfamily (Basantani & Srivastava, 2007).

In the WoLF PSORT and Plant-mPLoc analysis, EgGST was predicted to be located in the cytosol. The GSTs reported so far are mostly soluble cytosolic enzymes, and have been classified in classes ranging from mammals, plants, insects, parasites, fungus, to bacteria (Mohsenzadeh *et al*., 2011; Wongsantichon & Ketterman, 2005). By using SignalP, EgGST was predicted to not having any signal peptide, implying that it is located in the cytosol. A sequence comparison between the deduced EgGST with GST protein of other plants revealed that GST proteins are indeed well conserved across monocot and eudicot plants (Figure 1). The similarities are almost evenly distributed throughout the sequence, in the thioredoxin fold domain (4 - 82 amino acids) and the chloride channel domain (90 - 216 amino acids). A phylogenetic tree was plotted to estimate the relationship between the sequences of EgGST with other sequences of plant glutathione transferase proteins (Figure 2). The resulting phylogenetic tree (Figure 2) is organized into two clades. Interestingly, EgGST that encodes a putative glutathione transferase protein appeared to be belonging to clade 2, together with the putative PtGST sequence from *Populus trichocarpa* and RcGST sequence from *Ricinus communis*. Hence, EgGST might belong to either phi- or theta-class GSTs. Therefore, the comparison of monocot and eudicot GST proteins in this study revealed that during evolution eudicot, members of the GST family have



An unrooted neighbour-joining tree generated from the multiple alignment of EgGST protein (underlined) with GST proteins of other plant's proteins, with repeat verification for 5000 times by Bootstrap. The number on the branch means the percentage of repeat verification credibility. Bootstrap values are indicated for branches supported by more than 50% of 5000 replicates. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The length of the branches is proportional to the number of amino acid substitutions per residue. MEGA (Molecular Evolutionary Genetic Analysis version 4) was used to construct the phylogenetic tree based on neighbour-joining method.

Fig.2: Phylogenetic Relationship of EgGST and Different Plant Species Based on the Deduced Amino Acid Sequences

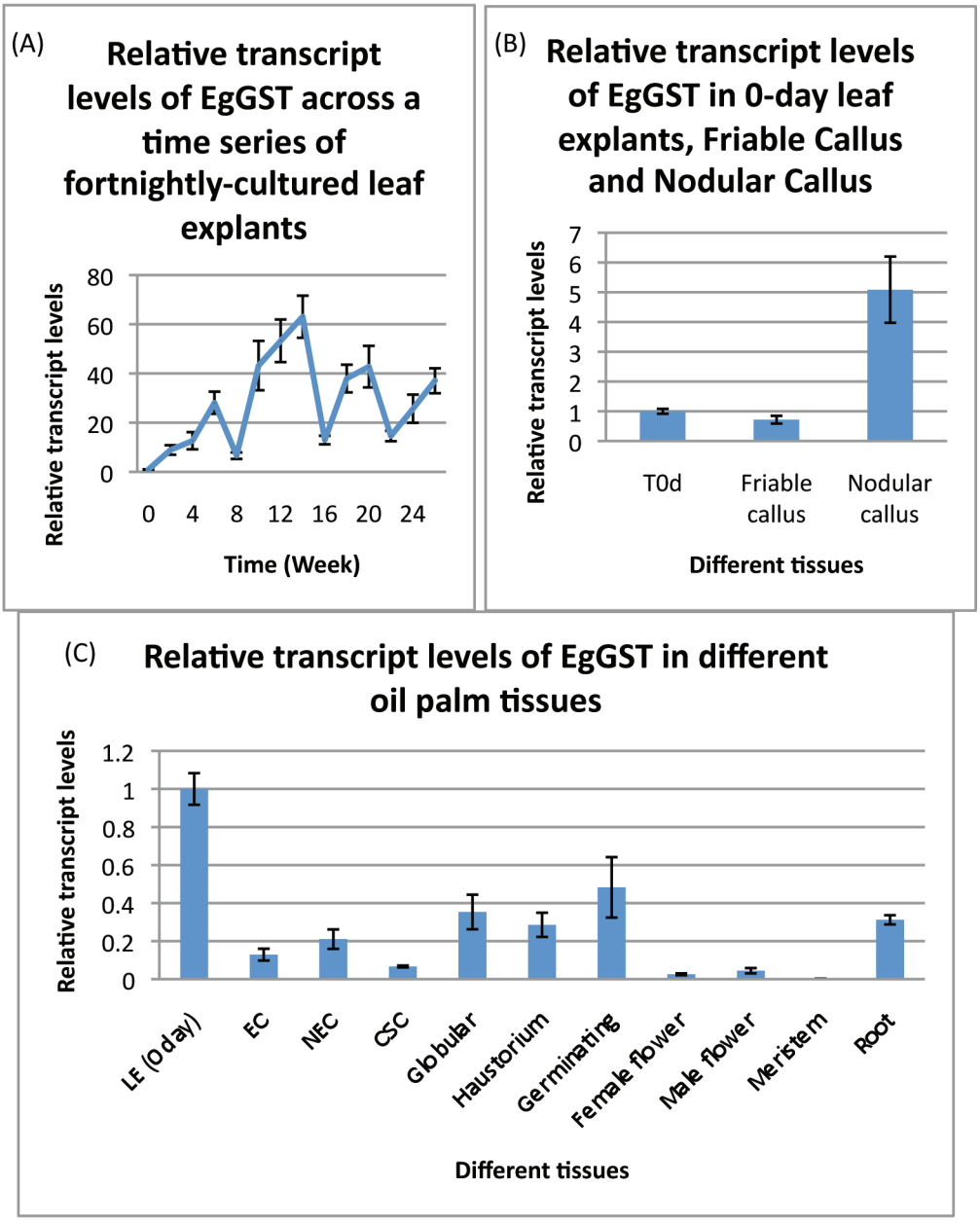
developed along two different directions. Hence, understanding the genomic and functional evolution of gene families is essential for understanding the phenotypic diversification of organisms and their genetic systems. Lan *et al*. (2009) revealed the complex history of genome duplications and chromosomal rearrangements in *Populus* through the course of genome evolution, which is thought to occur in order to supply raw genetic material, allowing functional divergence and rapid biological evolution. The genome duplications and chromosomal rearrangements process could have probably been taking place in oil palm too through the course of evolution as GSTs are evolutionarily ancient proteins. Hence, EgGST might also be descendant from gene duplication. However, to date, limited information is available on the patterns of functional diversification governing the evolution of most classes of gene families in the plant kingdom (Lan *et al*., 2009). Previous phylogenetic analysis had suggested that Theta, Zeta and Omega GSTs as the most ancestral classes in plants (Chi *et al*., 2011; da Fonseca *et al*., 2010). GSTs were also thought to have evolved from a thioredoxin-like ancestor in response to the development of oxidative stress (Martin, 1995; Koonin *et al*., 1994).

Real-Time RT-PCR Analysis of EgGST

The EgGST transcript was shown to be differentially expressed across a time

series of fortnightly-cultured leaf explants. The expression showed an increase from 0-day leaf explants to 6th week cultured leaf explant and dropped at 8th week but started to peak again from 10th week until 14th week cultured leaf explants (see Figure 3A). The expression profile of the EgGST transcript was similar to that reported previously by Che *et al*. (2006) who observed that the frequency of stress response genes increased with time during callus development in *Arabidopsis* tissue culture. However, it dropped again at 16th week, followed by a rhythmic pattern of expression at different time points, peaking at 20th week with a subsequent decline at 22nd week, and a slight increase from 24th week to 26th week cultured leaf explants. The result, as illustrated in Figure 3A, is also in agreement with the previous finding by Fatihah (2010), where the relative transcript level of EgGST was found higher in the cultured leaf explants at 6th week in comparison to the 0-day leaf explants. FC is embryogenic callus and has the capacity to produce somatic embryo of oil palm but the frequently produced callus is NC, which is non-embryogenic callus. An expression comparison was done in both stages of the oil palm callus development, which might help to enhance our understanding on the embryogenic callus production. EgGST had higher transcript levels in nodular callus (NC) compared to friable callus (FC) for oil palm ortet of clone 4178 (Figure 3B), with 5.08764 and 0.71569 folds, respectively, compared to that of the expression level in 0-day leaf explants. The expression level of EgGST transcript in 0-day leaf explants was used as a reference point (calibrator). The result shown in Figure 3B is in agreement with that of Low *et al*. (2008). GST was reported to be up-regulated in non-embryogenic callus of oil palm compared to embryogenic callus and embryoid via northern blot analysis, but was to some extent genotype-dependent (Low *et al*., 2008). Nevertheless, Legrand *et al*. (2007) had also reported that two GSTs were preferentially expressed in the cultured explants from a non-embryogenic genotype of *Cichorium intybus* L. via in silico EST data analysis and real-time RT-PCR experiments.

Evidence showed that the transcript of plant GST genes was regulated by various abiotic and biotic stresses, as well as hormones including xenobiotic-type stresses such as herbicide application (Edwards *et al*., 2000), chilling (Seppänen *et al*., 2000), dehydration (Bianchi *et al*., 2002; Kiyosue *et al*., 1993), hypoxic stress (Moons, 2003), wounding (Vollenweider *et al*., 2000), pathogen attack (Mauch & Dudler, 1993), ethylene (Zhou & Goldsbrough, 1993), auxin (Chen & Singh, 1999), 2,4,6-trinitrotoluene (TNT) (Brentner *et al*., 2008), hydrogen peroxide (H_2O_2) and the defence signal salicylic acid (SA) (Chen *et al*., 1996). Callus is often induced in or upon contact of the wounded part of the explants with the media. During tissue culture, mechanical wounding, osmotic shock, hormonal imbalances and environmental cues such as exogenous auxin induction and cutting may cause significant stress effects that can trigger the somatic cells to



Relative amounts of EgGST transcripts were normalized to the geometric mean of the three endogenous references (EA 1332, PD 569 and GAPDH) by using the Comparative C_T method and were then rescaled to the expression values in 0-day leaf explants (clone 4178). The error bars represent mean ± SD of three technical replicates. LE, leaf explant; EC, embryogenic callus; NEC, non-embryogenic callus; CSC, cell suspension culture.

Fig.3: Expression Profiles of EgGST. (A) Relative transcript levels of EgGST in fortnightly-cultured leaf explants, (B) Relative transcript levels of EgGST in 0-day leaf explants, Friable Callus and Nodular Callus, (C) Relative transcript levels of EgGST in different oil palm tissues.

differentiate into embryogenic competent cells (Singla *et al*., 2007; Fehér *et al*., 2003; Dixon *et al*., 2002; Pfeiffer & Höftberger, 2001). The stress effect may produce reactive oxygen species (ROS) such as H_2O_2 that may lead to lipid peroxidation, biological macromolecule deterioration, membrane dismantling, ion leakage, and DNA-strand cleavage and finally death of plants (Rascio & Navari-Izzo, 2011; Hossain *et al*., 2010; Romero-Puertas *et al*., 2002). H_2O_2 is a small, diffusible molecule that is widely considered to be a signal molecule in the regulation of the defense system (Neill *et al*., 2002). H_2O_2 plays a dual role in plants: at low concentration, it acts as a signal that induces the expression of numerous defense genes encoding cellular protectants such as glutathione S-transferase and glutathione peroxidase, and activates multiple defense responses to abiotic stresses, while excessive accumulation leads to cellular oxidative damage and even programmed cell death (Levine *et al*., 1994; Prasad *et al*., 1994). The ability of a plant to express stress-response genes to endure stress and regulate ROS levels can inevitably help the proliferation of culture lines into embryoids. This was supported by Lin *et al*. (2009), who reported that most of the disease- and defense-related ESTs isolated during oil palm somatic embryogenesis code for GST. This is also in line with the finding of a previous study by Fatihah (2010), which showed that EgGST was up-regulated in leaf explants at 6th week as compared to 0-day, which could be stress responsive effect or to initiate callus formation. Besides its plausible role in the initiation of meristematic cells that led to callus formation in 6th week cultured leaf explants, GST was probably involved in the morphogenesis of NC. Stressful environment can also induce morphogenic events *in vitro* (Gong *et al*., 2005). Stress-induced growth is related to the production of ROS that might trigger the expression of GST (Gong *et al*., 2005; Fehér *et al*., 2003; Dixon *et al*., 2002).

In contrast, there are also reports that GSTs were expressed in cultured leaves of *Cichorium* undergoing somatic embryogenesis (Galland *et al*., 2001) and thus, have been linked with somatic embryo formation in carrot (Kitamiya *et al*., 2000). Consistently, GST accumulation has been reported in somatic embryos of *Cyclamen persicum* (Winkelmann *et al*., 2006), *Vitis vinifera* (Marsoni *et al*., 2008), and embryogenic cells of *Medicago truncatula* (Imin *et al*., 2004). This is not surprising as GSTs are represented by a large and diverse gene family in plants which can be divided on the basis of sequence identity into phi, tau, theta, zeta and lambda classes (Dixon *et al*., 2002). This can also be explained by the compensatory potential of other members of the GST family. Hence, it can be suggested that EgGST transcript might possibly be regulated differently at different stages of tissue culture, FC and NC. However, this can only be verified by performing validation tests using a much larger numbers of samples and a wider range of genotypes.

Over all in oil palm, EgGST was found to be preferentially expressed in all tissue

culture derived materials from leaf except for CSC, whereas there were almost negligible expression in all the non-tissue culture derived materials except for root (Figure 3C). In 2005, Gong *et al*. had reported that *BjGSTF2*, a gene homologous to the phi class GSTs, accumulated differentially in mustard organs, where the transcript was most abundant in root. In addition to that, a plant GST from *Arabidopsis thaliana, AtGSTU17* had been reported to be involved in seedling development and root elongation, whereby the loss-of-function mutant of *AtGSTU17* resulted in a reduced biomass of seedlings and number of lateral roots in the presence of auxin (Jiang *et al*., 2010). Since the GST enzymes have long been associated with detoxification of xenobiotics, limiting oxidative damage and other stress responses in plants (Gong *et al*., 2005), a higher expression of EgGST transcript in root is probably to exclude the processes from the sensitive metabolism in shoot. The relative transcript levels of EgGST were fluctuating during the embryoid transition from globular to germinating stages which occurred at the late stages of somatic embryogenesis. During the transition period, EgGST was found to be highly expressed in germinating embryoid followed by globular and haustorium, with 0.48258, 0.35309 and 0.28537 fold, respectively, compared to that of the expression level in 0-day leaf explants. Since the expression of EgGST transcripts were detected in all tissue culture derived materials from leaf (except for CSC) and in root, it can be concluded that EgGST may have a broader roles in oil palm growth and development, in addition to having functions in various stresses as supported by Gong *et al*. (2005) and Moons (2005). Besides, there are also a few other reported roles of GSTs in endogenous plant developmental processes; in the vacuolar sequestration of anthocyanins in maize, petunia and *Arabidopsis* (Kitamura *et al*., 2004; Alfenito *et al*., 1998; Marrs *et al*., 1995), as binding proteins by binding to various hormones including auxin (Smith *et al*., 2003) and cytokinin (Gonneau *et al*., 2001), as well as porphyrin compounds (Lederer & Böger, 2003) to regulate their activities. Recently, *Arabidopsis* GSTF2 was found to selectively bind the indole-derived phytoalexin camalexin as well as the flavonol quercetin-3-O-rhamnoside, suggesting a role in regulating the binding and transport of defense-related compounds in plants (Dixon *et al*., 2011). The fact that plant GSTs can be induced by a wide variety of phytohormones, including ethylene, auxin, methyl jasmonate, salicylic acid, and abscisic acid (ABA) (Moons, 2003; Smith *et al*., 2003; Wagner *et al*., 2002) and that all these hormones regulate many aspects of plant development also supports that plant GSTs may play vital roles in plant growth and development as well. However, evidence to substantiate this role is still limited.

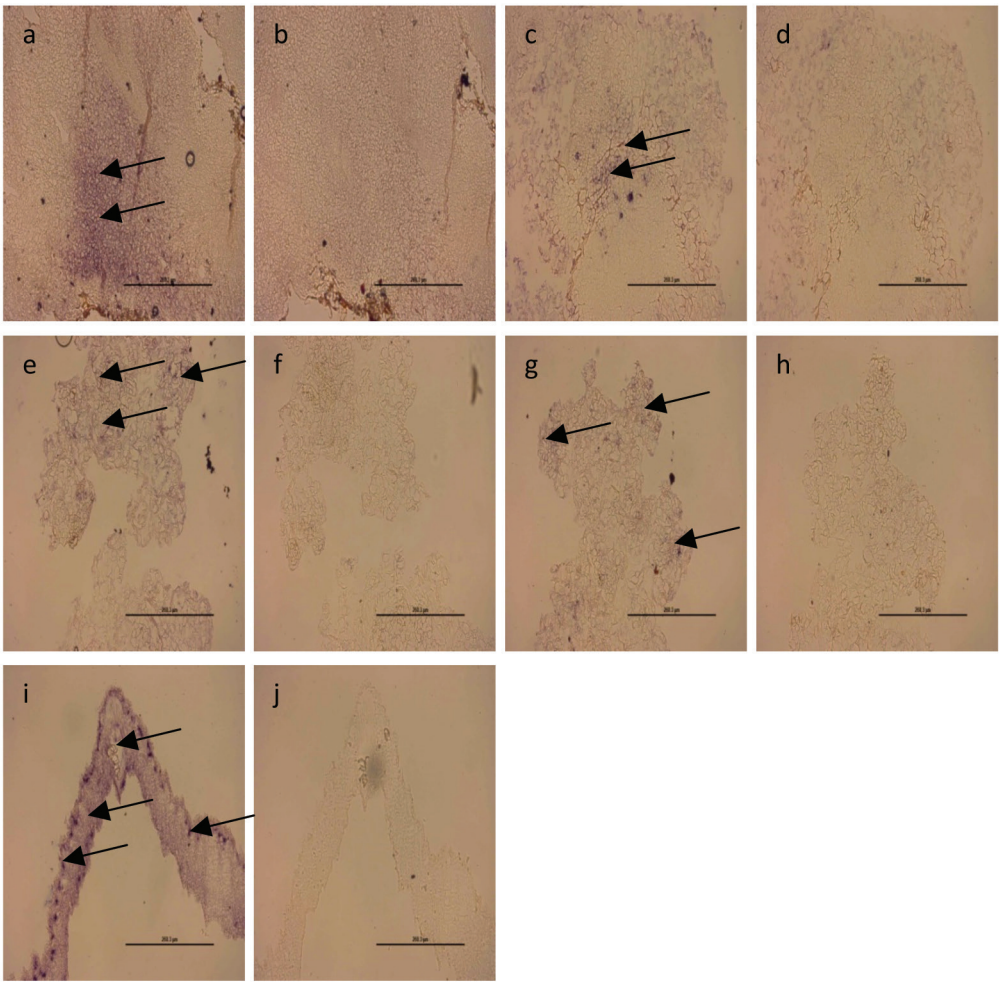
RNA in situ Hybridization Analysis of EgGST

The analysis of the localization of target mRNA transcript of EgGST was performed in germinating embryo, EC, NEC and LE, as the transcripts were found to be preferentially expressed in the respective

tissues of oil palm (Figure 3C). RNA *in situ* hybridization is a widely used method that allows one to analyse the localization of target mRNAs in a preserved tissue section (Bayer *et al*., 2009). The main objective of this part of study is to examine the spatial expression pattern of EgGST in various oil palm tissues at cellular level and to clarify the signals detected in real-time RT-PCR. In addition, eukaryotic cells are highly compartmentalized, and the correct localization of proteins is essential for their function (Boruc *et al*., 2010).

In both germinating embryo and EC hybridized with antisense and sense of riboprobe each, positive signal was detected in the middle of the developing germinating embryo and EC (Figure 4a, 4b, 4c, 4d). No expression was observed in the rest of the germinating embryo and EC tissue. Meanwhile, in NEC hybridized with antisense and sense of riboprobe each, a weak signal was detected at the corner of the developing NEC (Figures 4g & 4h). No expression was observed in the rest of the NEC tissue. When NEC was hybridised with the elongation factor *ELF* antisense probe (positive control), a clear signal was observed that was exclusively localized to the actively-dividing cell layer at the surrounding of NEC (Figures 4e & 4f). Besides its plausible role in the initiation of meristematic cells that led to callus formation in 6th week cultured leaf explants, EgGST is probably involved in the morphogenesis of nodular callus (Fatihah, 2010). Moreover, Galland *et al*. (2001) suggested that the GST transcript accumulation is not only caused by abiotic and biotic stress but might also be involved in cellular proliferation activity. They also reviewed that GST was expressed during transition of G to S phase of mitosis of tobacco mesophyll protoplast and *Arabidopsis* zygotic embryogenesis. During tissue culture, mechanical wounding, osmotic shock, hormonal imbalances and environmental cues such as exogenous auxin induction and cutting may cause significant stress effects that can trigger the somatic cells to differentiate into embryogenic competent cells (Singla *et al*., 2007; Féher *et al*., 2003; Dixon *et al*., 2002; Pfeiffer & Höftberger, 2001). This indicated the role of GST in the initiation of meristematic activity of differentiated cells (Vernoux *et al*., 2000; Takahashi & Nagata, 1992).

On the other hand, strong positive signals were observed in the oil palm leaf explants when hybridised with antisense and sense of EgGST riboprobe each (Figures 4i & 4j). This is not surprising as during tissue culture, mechanical wounding and cutting of oil palm leaf explants may cause significant stress effects that induce the expression of GST. To date, this has been the first reported study on the analysis of the localisation of target mRNA transcript of EgGST in different oil palm tissues. Hence, we proposed that EgGST might play significant roles at different stages of oil palm callogenesis and could potentially be a candidate marker for oil palm callogenesis. As such, EgGST can be used for screening explants with high callusing rates, thus enabling reductions in time and costs in the micropropagation process.



a, b: germinating embryo hybridized with EgGST; c, d: embryogenic callus (EC) hybridized with EgGST; e, f: non-embryogenic callus (NEC) hybridized with elongation factor (*ELF,* positive control); g, h: non-embryogenic callus (NEC) hybridized with EgGST; i, j: oil palm leaf explants (LE) hybridized with EgGST. The a, c, e, g, i are antisense and b, d, f, h, j are sense hybridizations. The presence of purple stain or deposit is regarded as positive signal (black arrows). Scale bar = 260.3 µm.

Fig.4: Localization of mRNA transcript of selective probes (EgGST or *ELF*) on various oil palm tissues.

CONCLUSION

The full length cDNA sequence of EgGST (GenBank accession no. AIC33066.1) isolated from oil palm cultured leaf explants at the 6th week is 1002 bp in length with an Open Reading Frame (ORF) of 651 bp. The deduced EgGST encodes a 216-amino-acid protein and contains thioredoxin fold and chloride channel domain. Based on the real-time RT-PCR results obtained, it can be suggested that EgGST transcript might possibly be regulated differently at different stages of tissue culture and various tissues. EgGST also displayed a tissue-specific expression pattern via RNA

in situ hybridisation. Hence, we postulated that EgGST might play significant roles at different stages of oil palm callogenesis and could potentially be a candidate marker for oil palm callogenesis. It may be interesting to further explore the expression profiles of EgGST across a wider range of oil palm genotypes in order to confirm the suitability as putative marker.

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