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Isolation and characterization of an ethylene-responsive element binding protein (EgEREBP) from oil palm (*Elaeis guineensis*)

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Abstract

The AP2/ERF (APETALA2/ethylene-responsive factor) proteins play important regulatory roles in plant response to stress as well as plant growth and developmental processes. Using yeast one-hybrid system and three tandem repeats of the ERE (ethylene-responsive element) as a probe, an ethylene-responsive cDNA clone designated as *EgEREBP* (*Elaeis guineensis* ERE-binding protein) was isolated from oil palm fruits treated with ethylene hormone. The *EgEREBP*-encoded protein has a conserved AP2/EREBP DNA-binding domain (DNA-BD), a potential nuclear localization sequence, and a putative activation domain. EgEREBP protein was classified as a member of DREB/CBF (dehydration-responsive element binding protein/c-repeat binding factor) proteins based on the multiple sequence alignment and phylogenetic analysis. EgEREBP showed ERE-binding, transcriptional activation, and transactivation properties in yeast and *in vitro*. Electrophoretic mobility shift assay (EMSA) revealed that EgEREBP could bind to the DRE in addition to the ERE sequence. RT-PCR analysis revealed that the expression of *EgEREBP* gene was induced in oil palm fruits in response to a range of hormone treatments, including abscisic acid (ABA), methyl jasmonate (MJ), salicylic acid (SA) and abiotic stresses, including drought, cold and high-salinity. Our data demonstrated the regulatory functions of *EgEREBP* gene in stress response and ethylene signaling in oil palm which could be potentially useful for the production of stress-tolerant transgenic crops.

Keywords: Abiotic stress; AP2/ERF; Ethylene-responsive element; Transcription factor; Yeast one-hybrid.
Abbreviations: ABA-abscisic acid; ABRE-ABA-responsive element; AD-activation domain; AP2/ERF-APETALA2/ethylene-responsive factor; BD-binding domain; CRT-c-repeat; CBF-CRT binding factor; DRE-dehydration-responsive element; DREB-DRE-binding protein; EMSA-electrophoretic mobility shift assay; ERE-ethylene-responsive element; ERF-ethylene-responsive factor; MJ-methyl jasmonate; SA-salicylic acid.

Introduction

The AP2/ERF transcription factor (TF) family is involved in transcriptional regulation of gene expression in response to various biotic and environmental stresses (Yamaguchi and Shinozaki, 2006). They are also involved in different aspects of plant growth and development, such as flowering and fruit ripening. Genome sequencing projects have led to the identification of 147, 167, 132 and 200 AP2/ERF members in *Arabidopsis*, rice, grapevine and poplar genomes, respectively (Sakuma et al., 2002; Nakano et al., 2006; Riano et al., 2007; Guo et al., 2008; Zhuang et al., 2008). There are also 148, 85, and 117 AP2/ERF members identified in soybean, tomato, and wheat, respectively *via* expressed sequence tag analysis (Zhang et al., 2008; Sharma et al., 2010; Zhuang et al., 2011). The AP2/ERF proteins can be divided into five subfamilies based on their sequences similarities and the number of AP2/EREBP DNA-BDs: (i) ERF subfamily with a single AP2/EREBP domain, (ii) DREB/CBF subfamily containing one AP2/EREBP domain, (iii) AP2 (APETALA2) subfamily with two AP2/EREBP domains, and (iv) RAV subfamily containing a B3 domain besides a single AP2/EREBP domain (Nakano et al., 2006). The ERF and DREB/CBF subfamilies are of particular interest due to their involvement in plant response to various stresses. The ERFs constitute the largest group of the AP2/ERF family. Among the 147 identified AP2/ERFs from *Arabidopsis*, 66 belong to the ERF, 56 to the DREB/CBF, 18 to the AP2, and 6 to the RAV subfamilies. The ERFs play important roles in plant response to various hormones, pathogens and environmental cues, and display a diverse stress-regulated expression profile in response to disease-related stimuli, such as ethylene, jasmonic acid, salicylic acid, and pathogen attack (Lorenzo et al., 2003). Some ERFs, such as the maize ZmABI4 have been identified to have a regulatory role in ABA-dependent response in plants (Niu et al., 2002). Tomato LeERF1 and rice Sub1A/C proteins are involved in the regulation of plant developmental processes, such as fruit ripening and modulation of metabolic biosynthesis pathways, respectively (Fukao et al., 2006; Li et al., 2010). Two ERFs were isolated from ripening apple where their expression was found to be regulated through the

ethylene signaling pathway (Wang et al., 2007). A large number of DREBs/CBFs have been identified with regulatory functions in abiotic stress response in plants, such as DREB1A/2A from *Arabidopsis* (Liu et al., 2002), OsDREBs and RCBF2 from rice (Dubouzet et al., 2003; Liu et al., 2007), HvDREB1 and HvCBF from barley (Xu et al., 2009), and TaDREB1 from wheat (Shen et al., 2003). The AP2 subfamily proteins are involved in the regulation of plant developmental processes, such as embryo development, flowering and fruit ripening control (Boutilier et al., 2002), and have been largely characterized from *Arabidopsis*, including APETALA2 (AP2), AINTEGUMENTA (ANT), PLETHORA1/2 (PLT1/2), BABY BOOM (BBM), TINY, and PUCHI (Bowman et al., 1991; Elliott et al., 1996; Boutilier et al., 2002; Aida et al., 2004; Sun et al., 2008; Karim et al., 2009). Identification of AP2/ERFs from oil palm is crucial for better understanding of the ethylene-dependent gene expression mechanism and the regulatory function of these proteins in plant response to stress and different aspects of plant growth and development. In this study, an ethylene-responsive cDNA clone (designated as *EgEREBP*) was isolated from oil palm fruits using yeast one-hybrid system. The DNA-binding, transcription activation ability and transcriptional regulation of the encoded protein in response to various hormones and abiotic stresses were further investigated.

Results

Isolation and characterization of EgEREBP cDNA

To isolate ERE-binding proteins from oil palm, yeast one-hybrid assay was carried out using oil palm cDNA library and three tandem repeats of the ERE as a bait. Approximately 3 millions of yeast transformed clones were screened on the selective SD/-His/-Leu/-Trp/+50 mM 3-AT media. Two hundred clones were subjected to PCR analysis, and 10 were selected based on the size of cDNA insert and subjected to sequence analysis. A full length cDNA was identified and designated as *EgEREBP* (GenBank accession no. JX023463). An open reading frame of 636 bp was identified within the *EgEREBP* cDNA sequence, which encodes a 212 amino acid protein with a predicted molecular weight of 23.6 kDa. Analysis of the amino acid sequence of the *EgEREBP*-encoded protein revealed the presence of a conserved AP2/EREBP DNA-BD with two functional amino acids (valine and glutamic acid) at its 14th and 19th residues. EgEREBP protein contains a basic region at its N-terminal region that may act as a nuclear localization sequence. The LWSY conserved region was identified at the C-terminal region of this protein which may function as a transcriptional activation domain. The AP2/EREBP domain of this protein was made of 58 amino acid residues, which was predicted to fold into a spatial structure made up of one α-helix and three *β*-sheets using the Swiss-Model analysis (http://swissmodel.expasy.org//SWISS-MODEL.html). This structure may be responsible for the DNA-binding activity (Li et al., 2010). A homology search of the GenBank database revealed strong similarities between the EgEREBP and DREBs/CBFs, which are involved in the abiotic stress response in plants. EgEREBP showed 90% homology to the CBF-like TFs from *Elaeis oleifera* and 85% to *Dypsis lutescens* throughout the sequences. Amino acid sequence alignment of EgEREBP with the other related AP2/ERFs using the ClustalW program showed strong conservation within the AP2/EREBP domain of these proteins (Fig. 1). Phylogenetic analysis showed that EgEREBP is evolutionarily close to the CBF-like TF from *E. olifera*, as they have been clustered in a same group (Fig. 2).

ERE-binding and transactivation activity of EgEREBP protein in yeast

EgEREBP protein was expressed as a fusion to the GAL4 AD using the yeast pGADT7-Rec2 expression vector in order to investigate its ERE-specific binding activity. Upon recognition of the ERE-binding site by the EgEREBP-GAL AD fusion protein, the HIS reporter gene was activated and enabled the cells to grow on a His deficient SD media. As shown in Fig. 3A, transformed yeast cells carrying the pHIS2.1/3x ERE + pGADT7-Rec2/EgEREBP formed colonies on SD/-His/-Trp/-Leu/+50 mM 3-AT, while transformed yeast cells carrying the pHIS2.1/3x mERE + pGADT7-Rec2/EgEREBP did not grow on the same selective media. This observation confirmed the ERE-binding activity and transcriptional activation ability of EgEREBP protein in yeast. The full coding region of *EgEREBP* gene was fused downstream of the GAL4 DNA-BD in the pGBKT7 expression vector in order to test the ability of the encoded EgEREBP protein to transactivate the expression of the yeast *LacZ* reporter gene. The recombinant construct was transformed into the yeast strain Y187 harboring the GAL4-binding sites fused upstream of the *LacZ* reporter gene. As illustrated in Fig. 3B, yeast cells harboring the pGBKT7/EgEREBP were able to grow on a selective SD/-Trp media and developed blue color after the *β*-galactosidase assay. In the control experiment, whereby the yeast cells were transformed with the pGBKT7 vector alone, the cells were not able to produce any colonies on SD/-Trp media, since the TRP reporter gene was not activated due to the absence of the GAL4-AD in the pGBKT7 vector. These results indicated the transactivation ability of EgEREBP protein in yeast.

In vitro binding activity of EgEREBP protein

The ERE-specific binding activity of EgEREBP protein was examined *in vitro* using EMSA. Nuclear protein extracts from the transformed yeast Y187 carrying the pGADT7-Rec2/EgEREBP and non-transformed control yeast Y187 (pGADT7-Rec2 alone) were challenged with the labeled ERE probe (Fig. 4). The retarded band in lane 4 showed a strong interaction between the EgEREBP protein and labeled ERE probe, while no interactions were observed when either the mERE or endogenous nuclear protein extract from the control yeast was used (Lanes 1, 2, and 3). The specificity of the ERE/EgEREBP interaction was further investigated by challenging the labeled ERE probe with 200-fold molar excess of the unlabeled ERE probe in a competitive EMSA (Fig. 4, Lane 5). As it is shown, the band shift due to the ERE/EgEREBP complex could be inhibited by competition from excess amount of the unlabeled specific probe. This observation confirmed the ERE-specific binding of EgEREBP protein.

EgEREBP protein binds to both ERE and DRE sequences

Some ERFs have been characterized with dual binding activity to both ERE and DRE sequences (Zhang et al., 2004; Lee et al., 2005). To test if the EgEREBP protein could bind to the DRE *in vitro*, EMSA was carried out using the DRE sequence (TACCGACAT) as a bait. As shown in Fig. 5, the retarded band in lane 4 corresponded to the interaction of the EgEREBP protein with the DRE probe, while no interaction

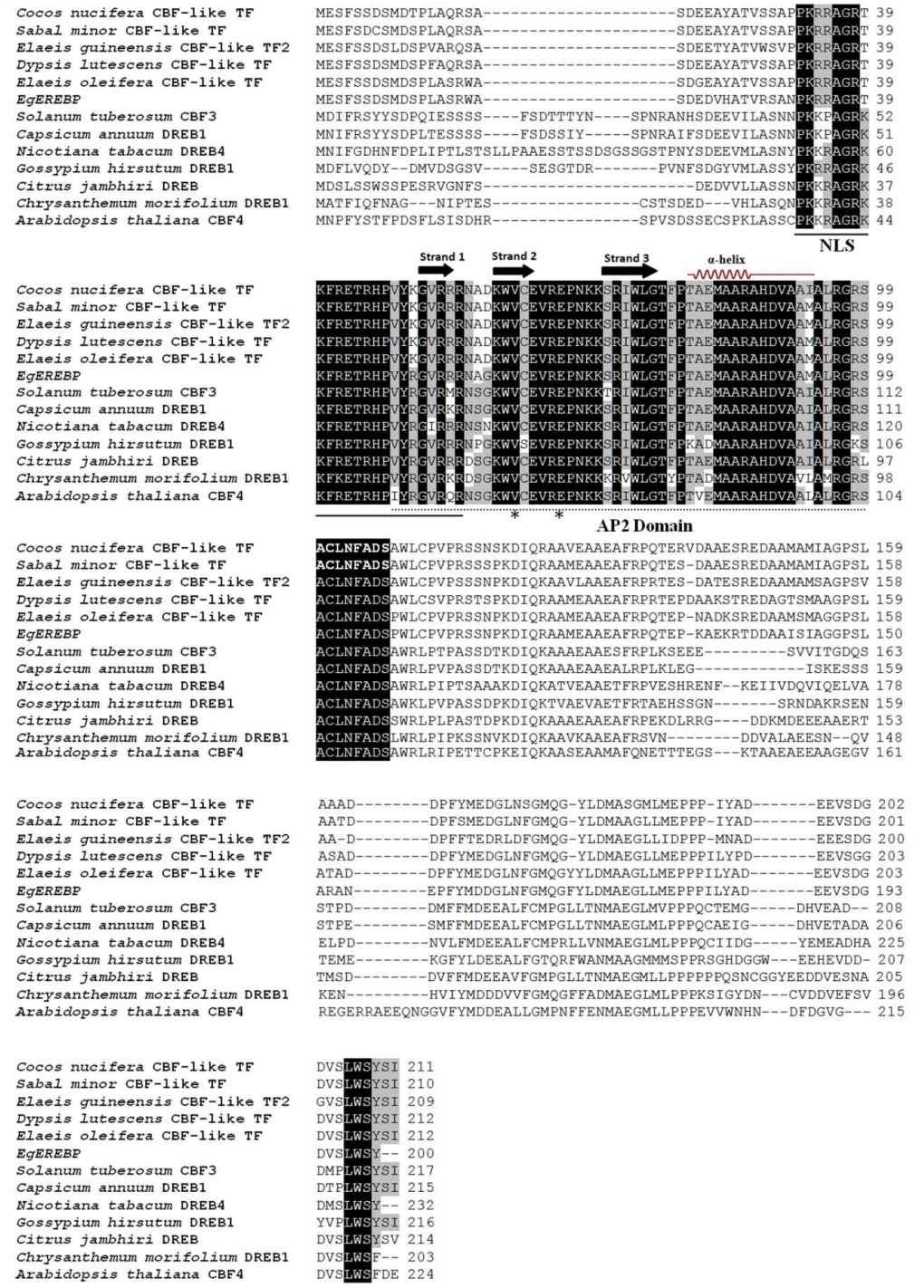

Fig 1. Comparison of the deduced amino acid sequences of EgEREBP protein with the other related AP2/ERFs. The shading indicates identities; the conserved AP2/EREBP domain and putative nuclear localization sequence (NLS) are underlined. The stars indicate the conserved valine (V) and glutamic acid (E) at the 14th and 19th positions of the AP2/EREBP domain, respectively. The predicted α-helix and *β*-sheets are also indicated. The deduced amino acid sequences are CBF-like TF from *Cocos nucifera* (GenBank accession no. ABF59745), CBF-like TF from *Sabal minor* (GenBank accession no. ABF59737), CBF-like TF2 from *Elaeis guineensis* (GenBank accession no. ABF59741), CBF-like TF from *Dypsis lutescens* (GenBank accession no. ABF59744), CBF-like TF from *Elaeis oleifera* (GenBank accession no. ABF59740), CBF3 from *Solanum tuberosum* (GenBank accession no. ACB45095), DREB1 from *Capsicum annuum* (GenBank accession no. AAR88363), DREB4 from *Nicotiana tabacum* (GenBank accession no. ACE73696), DREB1 from *Gossypium hirsutum* (GenBank accession no. ABD65473), DREB from *Citrus jambhiri* (GenBank accession no. ACZ37079), DREB1 from *Chrysanthemum morifolium* (GenBank accession no. ACU00263), and CBF4 from *Arabidopsis thaliana* (GenBank accession no. NP_200012).

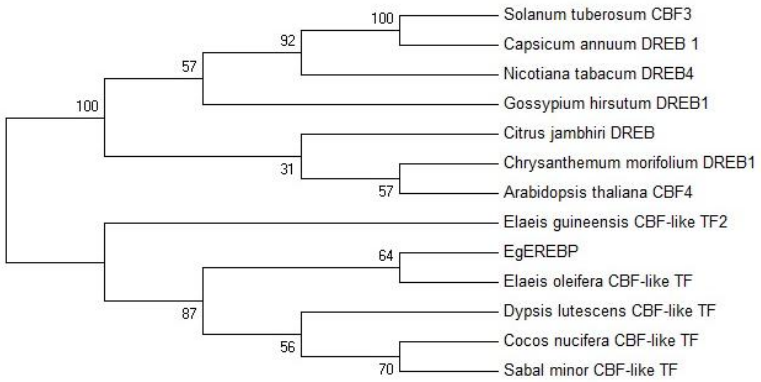

Fig 2. Phylogenetic analysis of the EgEREBP protein and other related AP2/ERFs. Phylogenetic tree was constructed using the MEGA software version 4.0 *via* the neighbor-joining method. Bootstrap analysis was performed with 1,000 replicates and bootstrap values are shown as percentages.

was observed when either the mDRE (CGCTAATGT) or endogenous nuclear protein extract from the control yeast was used (Lanes 1, 2, and 3). This result showed that EgEREBP protein could bind to both ERE and DRE sequences and might be involved in both ERE- and DRE-mediated signaling pathways.

Transcriptional analysis of EgEREBP gene

The expression of *EgEREBP* gene was analyzed in oil palm fruits at different developmental stages, leaves and roots and in response to different hormones and abiotic stresses using a semi-quantitative RT-PCR approach. The oil palm actin housekeeping gene was used as an internal control. *EgEREBP* gene had a weak expression in intact fruits, leaves, and roots, while in response to ethylene its expression was constitutively up-regulated in all the three tissues (Fig. 6A). To further study the effect of various stresses on the expression of *EgEREBP* gene, the fruits of 17 W.A.A were subjected to ethylene, MJ, SA, ABA, drought, cold, and salt treatments. In response to ethylene, the expression of *EgEREBP* gene increased after 2 h of treatment and reached a maximum level after 24 h (Fig. 6B). This observation indicated the positive regulatory function of this gene in the ethylene signaling pathway. In response to ABA, *EgEREBP* gene transcripts increased after 2 h, peaked at 12 h, and then decreased (Fig. 6C). *EgEREBP* expression was induced in response to MJ after 2 h, reached a maximum level at 8 h, and then slightly decreased (Fig. 6D). SA treatment induced the expression of *EgEREBP* gene after 2 h which reached a maximum level at 12 h. In response to drought, the expression of *EgEREBP* gene increased slightly after 4 h, reached its peak at 8 h, and then declined. In cold condition, the expression of *EgEREBP* gene slightly increased after 2-4 h and gradually increased up to 12 h, and then declined. In response to high-salt concentration, the expression of *EgEREBP* gene was induced and reached its highest level after 4 h.

Discussion

The AP2/ERFs play important roles in plant response to various stresses as well as in plant growth and development, but none has been isolated so far from oil palm. To isolate these regulatory proteins, the ERE was used as a bait in yeast one-hybrid assay. A full length cDNA sequence designated as *EgEREBP* was isolated from the oil palm fruits treated with ethylene. A conserved AP2/EREBP domain, a putative nuclear localization sequence, and an activation domain were identified within the EgEREBP amino acid sequence. Homology search and phylogenetic analysis suggested that EgEREBP belongs to the DREB/CBF subfamily of the AP2/ERFs, which are generally involved in the DRE/CRT-mediated regulation of gene expression; however, the encoded EgEREBP protein in this study was isolated based on its interaction with the ERE sequence. The EgEREBP and other related DREBs/CBFs shared a high homology within the AP2/EREBP domain. EgEREBP was found to be evolutionary close to the CBF-like TF from *E. olifera*. Biochemical analysis revealed that EgEREBP protein could bind to both ERE and DRE sequences *in vitro*, which was very similar to the characteristics of some ERFs, such as TINY, TERF1, JcERF, Tsi1, and Pti4 (Gu et al., 2000; Park et al., 2001; Tang et al., 2007; Sun et al., 2008; Zhang et al., 2008). This observation indicated that EgEREBP protein not only takes part in ERE-mediated regulation of the gene expression, but probably also regulates the DRE-mediated gene expression. Transactivation assay showed that EgEREBP protein could act as a transcriptional activator of the *lac*Z reporter gene in yeast. *EgEREBP* gene was induced in response to hormone treatments, including ethylene, JA, SA, ABA and abiotic stresses, including drought, cold, and high salinity. The ABA phytohormone is involved in abiotic stress signaling, whereas hormones such as ethylene, JA, and SA are part of the biotic stress response (Fujita et al., 2005). Our data suggests an extensive involvement of *EgEREBP* gene in both biotic and abiotic stress signaling pathways, and indicates the possible regulatory role of this gene as a connector among different stress signal transduction pathways. Many reports indicated the connection between ethylene and ABA signaling pathways in plant's response to abiotic stresses (Zhang et al., 2008). Several ERFs have been reported to be induced by ABA, such as the maize ERF protein ZmABI4, a homolog of the *Arabidopsis* ABI4, which interacts with the ABRE coupled element 1-like (CE1-like, CACCG) and enhances sensitivity to ABA during the seed germination (Niu et al., 2002), and the barley HvDRF1, which is involved in the ABA-mediated gene expression by interacting with a CT-rich element [T(T/A)ACCGCCTT] (Xue and Loveridge, 2004). Kizis and Pages (2002) reported that the activity of maize ERF protein DBF1/2 is regulated by ABA. The DREBs/CBFs are mainly involved in response to abiotic stresses, such as drought, cold, salt, and they are usually less responsive to phytohormones, such as ethylene, ABA, JA and SA (Sakuma et al., 2002; Liu et al., 2010). However as a member of this group, *EgEREBP* gene was also highly responsive to hormonal stimuli, which indicates that the DREBs/CBFs could modulate multiple aspects of

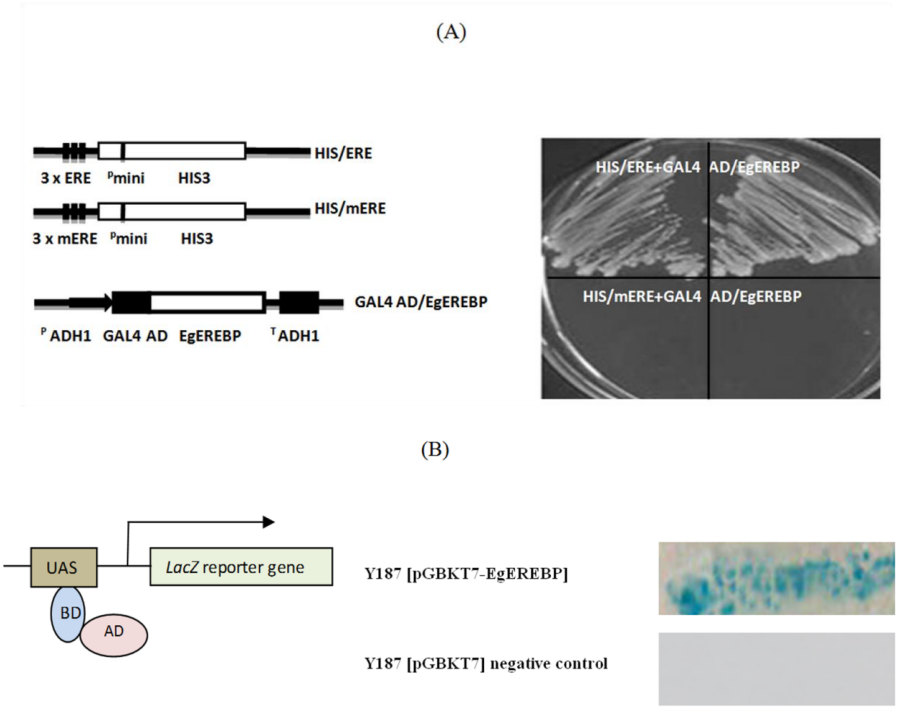

Fig 3. ERE-binding and transactivation activity of EgEREBP protein. (A) ERE-binding activity of EgEREBP in yeast. Sketch maps on the left show the vector constructs used in this experiment. Photograph on the right shows the yeast transformants on a SD/-His/-Trp/-Leu/+50 mM 3-AT plate. (B) Transactivation analysis of EgEREBP protein. The protein is expressed as a fusion to the GAL4 DNA-BD in the pGBKT7 vector. Transformed yeast cells with the pGBKT7 vector alone were used as a control.

plant responses to stress by interacting with one or more *cis*-acting elements or other transcription factors (Buttner and Singh, 1997). The existing cross-link between different stress signaling pathways could provide a potential regulatory mean for triggering multiple defense and stress-response mechanisms coordinately (Chinnusamy et al., 2004). Some ERFs, such as Tsi and TINY bind to both DRE/CRT and ERE elements with similar affinity and activate the expression of DRE- and ERE-driven genes (Sun et al., 2008). These intervening transcription factors are thought to be involved in a cross-talk between abiotic and ethylene-dependent biotic stress signaling pathways (Park et al., 2001). The AP2/ERFs have been reported to play key roles in plant growth and development (Sharma et al., 2010). ERFs, such as the tomato LeERF1, SI-ERF2 and apple MdERF1/2 have been reported to be differentially expressed during fruit ripening (Li et al., 2007; Wang et al., 2007). In order to explore the possible regulatory involvement of *EgEREBP* gene in growth and developmental processes, its expression pattern was investigated in intact oil palm fruits at different developmental stages, leaves and roots; however it was found to be very weak in all tissues and not being differentially regulated at different stages of the fruits. In summary, herein we report the isolation of an ethylene-responsive regulatory protein from oil palm and characterizing its regulatory role in the ERE- and DRE-mediated downstream gene expression using yeast one-hybrid assay. Our results indicated that the *EgEREBP* is a novel stress-responsive transcription factor gene from oil which has a possible regulatory role in multiple stress signaling pathways as an interconnector, and furthermore suggest the application of this gene for the production of transgenic plants with a higher tolerance to environmental stresses. Transformation of *EgEREBP* gene into plants and further analysis should reveal its regulatory function in plant response to stresses.

Materials and methods

Preparation of plant materials and stress treatments

Plant tissues were prepared following the procedure described by Agius et al. (2005). The oil palm (*Elaeis guineensis*, D×P) fruits at 8, 12, 15, 17, 20 weeks after anthesis (W.A.A), leaves and roots were collected from the Malaysian Palm Oil Board. Stress treatments were carried out by excising the mesocarp section of the fruits and placing them in a MS media containing either 100 µM ABA or 100 µM MJ or 100 µM SA or 20% PEG or 300 mM NaCl for 4 h. Ethylene treatment was performed on the tissues for 6 h using 2 ml of 40% (w/v) ethephon, and 59.52 mM sodium bicarbonate (Ethylene gas is liberated by ethephon under such conditions) (Zhang et al., 2004). Cold treatment was performed by incubating the tissues at 4°C for 4 h. The leaves and roots were also subjected to the same treatments. The chemicals and hormones were purchased from Sigma-Aldrich (Sigma-Aldrich Sdn Bhd, Malaysia)

Yeast one-hybrid assay

Three tandem repeats of the core sequence of ERE (GCC box: AGCCGCC) and its mutant mERE (GACTACT) were cloned upstream of the HIS3 minimal promoter in the yeast pHIS2.1 cloning reporter vector according to the protocol described by Clontech (Clontech, Mountain View, CA, USA). The double-stranded (ds) cDNAs were constructed using the Matchmaker™ Library Construction & Screening Kits (Clontech) and fused to the downstream region of the GAL4 activation domain (AD) of the yeast pGADT7-Rec2 expression vector *via* homologous recombination during yeast co-transformation process. The pHIS2.1-ERE, pGADT7-Rec2 and ds cDNAs were co-transformed into the yeast strain Y187. The putative transformants were screened

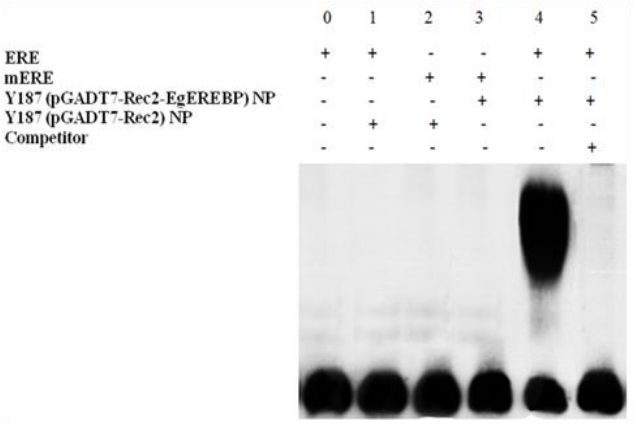

Fig 4. EMSA showing interaction of the ERE probe with nuclear protein extract from the yeast strain Y187 expressing EgEREBP protein. Lane 0: position of the free ERE probe. Lanes 1 and 2 (controls): interaction of the ERE and mERE probes with nuclear protein extract from untransformed yeast, respectively. Lane 3 (control): interaction of the mERE probe with EgEREBP protein. Lane 4: interaction of the ERE probe with EgEREBP protein. Lane 5: competition of the ERE probe with 200-fold molar excess of the competitor (unlabeled probe).

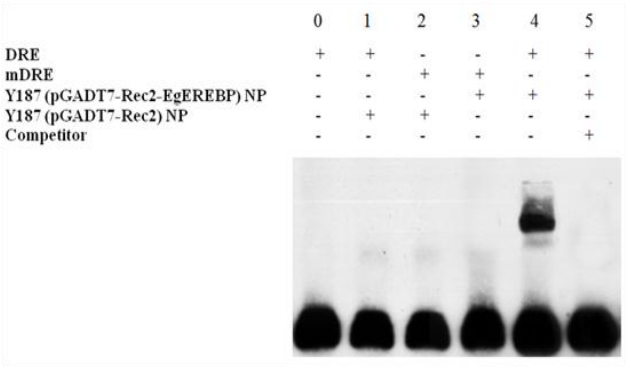

Fig 5. EMSA showing interaction of the DRE probe with nuclear protein extract from the yeast strain Y187 expressing EgEREBP protein. Lane 0: position of the free DRE probe. Lanes 1 and 2 (controls): interaction of the DRE and mDRE probes with nuclear protein extract from untransformed yeast, respectively. Lane 3 (control): interaction of the mDRE probe with EgEREBP protein. Lane 4: interaction of the DRE probe with EgEREBP protein. Lane 5: competition of the DRE probe with 200-fold molar excess of the competitor (unlabeled probe).

on SD media minus His/Leu/Trp plus 50 mM 3-amino-1,2,4-triazole (3-AT) (The 3-AT is a competitive inhibitor of the HIS3 gene product) and subjected to the Yeast Colony PCR and sequence analysis according to the protocol described by Clontech. ClustalW programme was used to perform multiple protein alignments, and phylogenetic trees were constructed using the MEGA software version 4.0 *via* the neighbor-joining method (Tamura et al., 2007). A bootstrap analysis was performed with 1,000 replicates and the values were shown as percentages.

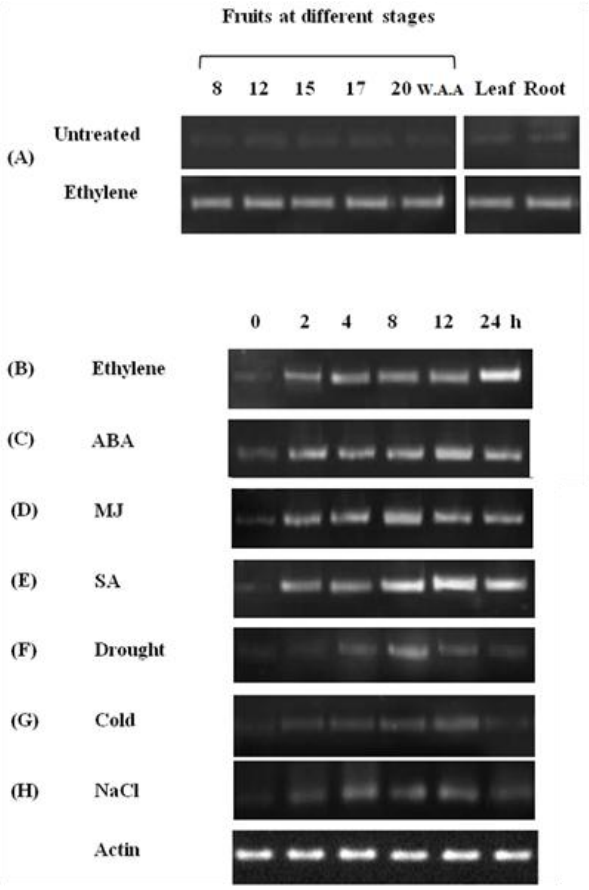

Fig 6. Transcriptional analysis of *EgEREBP* gene at different developmental stages of oil palm fruits, leaves and roots and under various plant hormones and abiotic stresses. (A): Expression pattern of *EgEREBP* gene in fruits of different ages (8-20 W.A.A), leaves and roots before (untreated) and after treatment with ethylene. (B), (C), (D), (E), (F), (G), (H): Expression pattern of *EgEREBP* in fruits in response to ethylene, ABA, MJ, SA, drought, cold, and NaCl, respectively. The time intervals for all treatments were the same and RT-PCR was carried out at 0, 2, 4, 8, 12, and 24 h after each treatment. The actin housekeeping gene was used as an internal control RNA. The expression pattern of the actin gene was similar in all the tissues and all the treatments.

ERE-binding and transactivation assay of EgEREBP protein in yeast

To confirm the ERE-binding activity of EgEREBP protein, the recombinant pGADT7-Rec2 expression vector containing the GAL4 AD/*EgEREBP* cDNA and the pHIS2.1 vector containing three tandem repeats of the ERE or the mERE were co-transformed into the yeast strain Y187. The transformed cells were analyzed on SD/-His/-Leu/-Trp/+50 mM 3-AT media to test the expression of the HIS reporter gene. Transactivation assay was performed by cloning the entire coding region of the *EgEREBP* cDNA downstream of the GAL4 DNA-BD in the pGBKT7 vector which carries the TRP reporter gene (Clontech). The recombinant plasmid was transformed into the yeast strain Y187 harboring the *Lac*Z reporter gene. The transformed cells growing on selective SD/-Trp media were subjected to β-galactosidase assay as described in the yeast protocols handbook (Clontech).

In vitro binding assay

In order to examine the ERE-binding activity of EgEREBP protein *in vitro*, EMSA was carried out using the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA). Three tandem repeats of the ERE and mERE sequences were synthesized and labeled using the Biotin 3´ End DNA Labeling Kit (Pierce). Total protein was extracted from the transformed yeast Y187 (pGADT7-Rec2-EgEREBP) and control yeast Y187 (pGADT7-Rec2 alone) according to the procedure described in the yeast protocols handbook (Clontech). Binding of the ERE probe with yeast protein extract was carried out in a 20 µl binding reaction containing 1x binding buffer, 2.5% (v/v) glycerol, 5 mM $MgCl_2$, 50 ng/µl of poly(dI-dC), 0.05% (v/v) NP-40, 3 µl of nuclear protein extract, and 20 fmoles of the biotin-labeled probe. Four pmoles of the unlabeled probe was used for competition with the labeled probe in EMSA. The binding reactions were separated on a 6% native polyacrylamide gel and blotted onto a nylon membrane. DNA-protein interactions were detected using the Chemiluminescent Nucleic Acid Detection Module (Pierce).

RT-PCR

Total RNA was extracted from the stress-treated tissues using the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands). Gene-specific primers were designed for *EgEREBP* gene as well as the oil palm housekeeping actin gene (internal control). RT-PCR was carried out using the QIAGEN® OneStep RT-PCR Kit in a 50 µl reaction containing 1x QIAGEN OneStep RT-PCR Buffer, 1mM dNTP mix, 1 mM forward and reverse primers, 10 units of QIAGEN OneStep RT-PCR Enzyme Mix, and 200 ng of total RNA. RT-PCR was performed by reverse transcription at 50˚C for 30 min, initial PCR activation at 95˚C for 15 min, followed by 30 cycles of 94˚C for 1 min, 55˚C for 1 min and 72˚C for 1 min. A final extension was programmed at 72˚C for 10 min. Electrophoresis analysis was carried out on a 1.2% EtBr/agarose gel.

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