ISOLATION AND CHARACTERISATION OF AN ETHYLENE RECEPTOR (ERS-TYPE) FROM OIL PALM (*Elaeis guineensis* Jacq.) MESOCARP

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ABSTRACT

The oil palm (Elaeis guineensis *Jacq.*) *fruit is climacteric whereby ripening process of the fruits is accompanied by a burst of ethylene production. Hence, to understand the underlying molecular mechanisms and the role of ethylene in the ripening process of oil palm fruits, this study focused on the isolation and characterisation of the ethylene receptor gene, the first component in the ethylene signalling pathway. The full-length cDNA is 2225 kb long and encodes a polypeptide of 629 amino acid residues. Sequence analysis showed it has conserved domains and a protein structure similar to the ERS-type ethylene receptors found in ethylene receptor genes from other plant species. Northern and Southern analyses revealed that it is highly expressed in the mesocarp tissues and that this gene exists as multiple copies in the oil palm genome. These results provide evidence that oil palm fruit development is regulated by ethylene through the action of the EGER D3 ethylene receptor gene, which opens up the possibility of manipulating the ethylene receptor to control ethylene sensitivity during oil palm fruit development to help further improve oil palm yields.*

INTRODUCTION

Ethylene is widely known to regulate many aspects of plant growth and developmental processes. Among the prominent roles include seed germination, root and stem elongation, flower senescence, abscission, sex determination as well as fruit ripening especially in climacteric fruits (Abeles *et al*., 1992). The ripening process especially in fleshy fruits involves a wide range of biochemical, physiological and molecular changes that affect the appearance, colour, texture, flavour and aroma of the fruit and as a result, the ripe fruit becomes more attractive, desirable and palatable for consumption (Bouzayen *et al*., 2010). Apart from ethylene, multiple hormones including abscisic acid, auxin, gibberellin, cytokinin, as well as brassinosteroid are also known to be involved in the development and ripening process in fruits (McAtee *et al*., 2013).

Physiological and biochemical changes that accompany the development of oil palm fruits until they ripen have been widely reported and reviewed (Sambanthamurthi *et al*., 2000; Corley and Thinker, 2016; Nurniwalis, 2017). However, it is surprising that for an important crop such as the oil palm, fruit development especially hormonal control of fruit development, has not

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received much attention (Sambanthamurthi *et al.*, 2000; Corley and Thinker, 2016; Nurniwalis, 2017). Hormonal changes during early fruit development and maturation through to ripening of oil palm fruits have been recently described (Tranbarger *et al.*, 2011; Teh *et al.*, 2014). Five major phytohormones namely, auxin, gibberellin, cytokinins, abscisic acid and ethylene are involved in the development of oil palm fruits mesocarp (Tranbarger *et al.*, 2011; Teh *et al.*, 2014).

Several studies have demonstrated the role of ethylene in promoting the ripening process of oil palm fruit (Henderson and Osborne 1999; Tranbarger *et al.*, 2011; Nurniwalis, 2017). An autocatalytic burst of ethylene produced in mesocarp tissues was detected during the ripening stages which indicated the characteristics of a climacteric fruit. Oil palm research on ethylene has been limited but the possibility that ethylene may also be responsible for triggering oil production and other changes associated with fruit ripening has been examined but not in great detail (Sambanthamurthi *et al.*, 2000). Using molecular approaches such as expressed sequence tags (EST) and transcriptome data analyses, gene transcripts associated with ethylene synthesis and signalling pathway have been identified from the oil palm mesocarp (Nurniwalis *et al.*, 2008; Tranbarger *et al.*, 2011). These findings indicate an important role for ethylene in the development and ripening process of the oil palm fruits.

Hence, this study was conducted to further our understanding of the role of ethylene in oil palm fruit development at the molecular level, focusing on the ethylene signalling pathway and in particular the ethylene receptor genes. In this article, we report the isolation of a gene encoding an ethylene response sensor (ERS)-type ethylene receptor from oil palm. Gene characterisation via sequence analysis, Southern and expression analysis were conducted and its role and possible manipulation in oil palm fruit development are discussed.

MATERIALS AND METHODS

Plant Materials

All oil palm tissues from the commercial *tenera* planting material used in the study were obtained from MPOB-UKM Research Station, Bangi, Selangor, Malaysia. Inflorescences were tagged at various weeks after anthesis (WAA) and fresh fruit bunches were harvested for their mesocarp and kernel tissues. Spear leaves were acquired from unopened leaf fronds, roots from 2-year old seedlings and germinated seedlings were one- to two-week old.

Total RNA Isolation and cDNA Synthesis

Total ribonucleic acid (RNA) was extracted from various oil palm tissues as described by Nurniwalis *et al.* (2008; 2015). Reverse transcription of total RNA into single stranded cDNA was performed using Superscript II Kit (Gibco BRL Technology Inc., New York, USA) according to the manufacturer's protocol in a 20 µl reaction volume using 5 µg of total RNA.

Degenerate PCR

Degenerate polymerase chain reaction (PCR) amplification was performed using degenerate primer pair designed based on the conserved regions of ethylene receptor genes from dicot [papaya (Acc. No. AF311942), tomato (Acc. No.: T07794), peach (Acc. No.: AAM73756) and tobacco (Acc. No.: AAB97160)] and monocot [rice (Acc. No: AF013979) and maize (Acc. No.: BAB13718])] plants. PCR amplification was carried out in a 50 µl reaction mixture containing 50 ng 15 WAA cDNA, 1X PCR buffer (Perkin Elmer, USA), 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 2.5 µM MgCl2 (Perkin Elmer, USA), 0.6 µM each of the degenerate primers EF1 (5' 5' CHTACKATTTTGAAGACTAC 3') and ER1 (5` GATGAAAAACGGCTAATGC 3') and 0.05U/µl AmpliTaq DNA polymerase enzyme (Perkin Elmer,USA). The PCR conditions were as follows: one cycle of 94°C for 5 min, 40 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min and 30 s and a final extension of 72°C for 10 min.

Rapid Amplification of cDNA Ends (RACE)

Both 5' and 3' RACE were performed using SMART™ RACE cDNA Amplification and Advantage 2 PCR Kit (Clontech, USA). The first strand cDNA for both 5`and 3`RACE was synthesised from 1 µg of 17 WAA mesocarp total RNA following the manufacturer's instruction. For 3' RACE, PCR amplification was carried out in a 50 µl reaction mixture containing 1X Advantage 2 PCR buffer, 0.2 mM dNTP mix, 1X Advantage 2 Polymerase Mix, 1X universal primer A mix, 0.2 µM EF3 primer (5' CTGAACAACGATTGATGGTAGAAAC 3') and 100 ng of 3'-RACE-Ready cDNA with the following conditions: 94°C (5 s), 72°C (3 min) for five cycles; 94°C (5 s), 70°C (10 s), 72°C (3 min) for five cycles; 94°C (5 s), 68°C (10 s) and 72°C (3 min) for 25 cycles and a final extension at 72°C for 10 min.

For 5' RACE, PCR amplification was performed in a 50 µl reaction mixture containing 1X Advantage 2 PCR buffer, 0.2 mM dNTP mix, 1X Advantage 2 Polymerase Mix, 1X universal primer A mix, 0.2 µM EF4A primer (5' CCTTGCCAGTGGGCATGCGTGTGG 3') and

100 ng of 5'-RACE-Ready cDNA with the same conditions and procedures as described for 3' RACE.

Generation of the Full-length and 3`UTR Probe

The full-length cDNA was generated via long-distance PCR (LD PCR) using SMART™ RACE cDNA Amplification Kit. PCR amplification was carried out in a 50 µl reaction mixture containing 1X Advantage 2 PCR buffer, 1X Advantage 2 Polymerase Mix, 0.2 mM dNTP mix, 0.2 µM gene-specific primers EF11(5' GGAATGTAAGGGCAAGGTAGTAATTG 3`) and EGER9 (5` GCT TAA ACG TTC AAA TAT ATT ATA GCA AGC 3`), 100 ng of 3' RACE-Ready cDNA with the following conditions: 94°C (5 s), 72°C (3 min) for five cycles; 94°C (5 s), 70°C (10 s), 72°C (3 min) for five cycles; 94°C (5 s), 68°C (10 s) and 72°C (3 min) for 22 cycles and a final extension at 72°C for 7 min.

The 3' UTR probe was generated via PCR in a 50 µl reaction mixture containing 1X BD Advantage buffer, 1X dNTP mix, 0.2 mM each of gene-specific primers EF12 (5' CGG TTT GAA TGT CTG GTT AGC AAT ACC 3') and EGER9, 1X BD Advantage 2 Polymerase and 0.5 µg of the plasmid DNA (*EGER D3*) with the following conditions: 95°C (1 min), 95°C (30 s), 60°C (3 min) for 30 cycles; a final extension at 60°C for 3 s.

Cloning and Sequencing Analyses

PCR products purified using QIAquick Gel Extraction Kit (Qiagen, Germany), were individually cloned into pCR2.1® vector and transformed into One Shot® Chemically Competent *Escherichia coli* TOP10 following the manufacturer's instruction (Invitrogen, USA). Plasmid DNA was isolated using QIAgen Plasmid Mini Kit (Qiagen, Germany) and the inserts were verified by *EcoRI* digestion. Sequence analysis was performed using DNASIS (Hitachi Software Package, 1997) and Biology Workbench Version 3.2 (http://workbench.sdsc. edu.). NCBI-BLAST (http://blast.ncbi.nlm.nih. gov/Blast.cgi) was used to search for similarities in the GenBank database. Prediction of transmembrane segments was performed using TMAP from Biology Workbench. Protein motifs were analysed using conserved domain search (Marchler-Bauer *et al.*, 2017) and Prosite (http://prosite.expasy.org/ scanprosite/).

Expression Analyses

For dot blot analysis, blotting, preparation and fixation of RNA to a nylon charged membrane (Hybond-N+, Amersham, USA) was carried out as described by Sambrook and Russell (2001). The blotting manifold set up and preparation of the RNA (10 µg) in RNA denaturation containing 66% (v/v) deionised formamide, 21% (v/v) formaldehyde and 1.3X MPOS buffer) solution was performed as described by Nurniwalis (2006).

For northern hybridisation, 15 µg of total RNA was denatured at 55°C for 15 min in a GFP mixture containing 78% (v/v) deionised formamide, 16% (v/v) deionised glyoxal and 10 mM sodium phosphate buffer. The denatured total RNA was electrophoresed on 1.2% (w/v) agarose gel and transferred to nylon charged membrane (Hybond-N+, Amersham, USA) overnight by capillary blotting as described by Nurniwalis (2006).

The blot was probed with the degenerate PCR fragment, full-length and 3' UTR fragments of the *EGER D3* ethylene receptor gene. Hybridisation of the membrane with the α-^{32}P labelled probe was performed in a buffer containing 5X SSC, 5X Denhardt, 0.5% (w/v) sodium dodecyl sulphate (SDS), 50% formamide at 42°C overnight. Washing of the membrane was performed using 2X SSC, 0.1% (w/v) SDS for 10 min at 60°C, 1X SSC, 0.1% SDS (w/v) for 15 min at 60°C and 0.5X SSC, 0.1% SDS (w/v) for 30 min at 60°C. Membrane exposure to X-ray film was performed for three days at -80°C with an intensifying screen.

Southern Analysis

Genomic DNA from oil palm leaf was extracted according to the modified Cetylatrimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1990). A total of 30 µg of DNA was digested individually with *BamHI*, *EcoRI* and *XbaI*. The digested DNA was electrophoresed on 1% (w/v) agarose and transferred onto nylon charged membrane via capillary blotting. Hybridisation was performed using the full-length and 3' UTR probes. Hybridisation, washing and generation of blotting signal was performed similarly as described for dot blot and northern analyses with a few modifications on the temperature and hybridisation buffer. The hybridisation buffer consists of 6X SSC, 5X Denhardt, 0.5% (w/v) SDS and the hybridisation reaction was carried out at 55°C. Washing of the blot was carried out in 4X SSC for 5 min at 55°C and 2X SSC, 0.1% SDS (w/v) for 30 min at 55°C.

RESULTS AND DISCUSSION

Degenerate PCR, 5' and 3' RACE Amplification

Degenerate PCR resulted in the amplification of a single PCR product from 17 WAA mesocarp cDNA and the success in cloning of the PCR product is shown in *Figure 1*. The partial cDNA contains 726 nucleotides and the nucleotide sequence analysis provided the necessary information to design gene-specific primers in the efforts to obtain the complete cDNA.
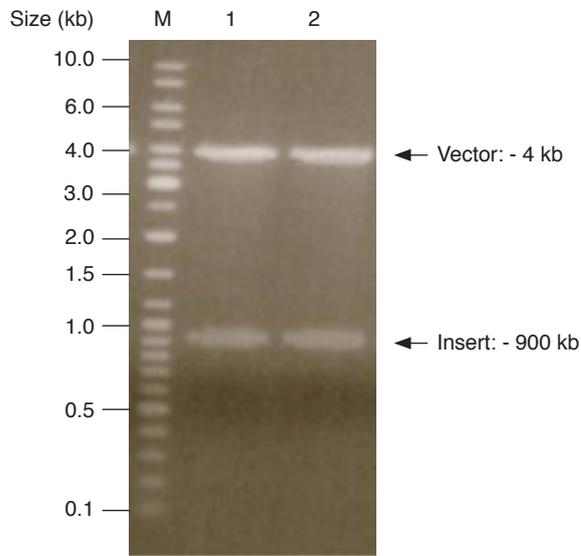
Figure 1. EcoRI digestion analysis of the plasmids carrying the degenerate polymerase chain reaction (PCR) products on 1% (w/v) agarose gel electrophoresis. Lanes 1 and 2 represent the digested plasmids carrying the insert. M is Gene Ruler DNA Ladder Mix (Fermentas).

Amplification of the 5′ and 3′ regions of the cDNA via 5′ and 3′ RACE resulted in the amplification of singular PCR products of about 900 bp (*Figure 2*) and 1.1 kb (*Figure 2*) in size, respectively. DNA sequence analyses showed that the 5′ RACE cDNA is 747 bp whereas the 3′ RACE cDNA contains 1053 nucleotides including a 28 bp poly (A)⁺ tail. Through the assembly of the degenerate PCR, 5 and 3′ RACE cDNA sequences, an overlap of 225 and 74 nucleotides at the 5′ and 3′ regions of the degenerate PCR cDNA was observed. The 5′ RACE PCR product is 100% identical to the degenerate PCR cDNA sequence while the 3′ RACE PCR product has 99% identity, both of which are within the overlapping regions at the nucleotide level.

Generation and Sequence Analysis of *EGER D3*

Reconstitution of all three sequences (degenerate PCR, 5′ and 3′ RACE) resulted in the successful amplification of the full-length cDNA encoding for an ethylene receptor gene designated as *EGER D3*. *EGER D3* is 2197 bp in length [excluding the poly (A)⁺ tail] and contains a 1890 bp open reading frame (ORF) (*Figure 3*). The ORF is flanked by 42 bp of 5′ untranslated region (UTR), 265 bp of 3′ UTR and encodes a polypeptide of 629 amino acid residues. The predicted polypeptide has a deduced molecular mass of 70.013 kDa. The predicted value is similar to the ethylene receptors ERS1 (68 kDA) from Arabidopsis (Hall *et al*., 2000), Cm-ERS1 (75 kDA) from melon (Takahashi *et al*., 2002) and Nt-ERS1 (70 kDA) from tobacco (Terajima *et al*., 2001).

The sequence flanking the start codon site matched the canonical initiation motif, A[A/C/T/G][A/C/T/G]ATGG as described by Kozak (1999). At the 3′ UTR region, two putative polyadenylation sites were present at nucleotide position 1968 to 1973 and 2177 to 2182. It contained 4-5 of a six
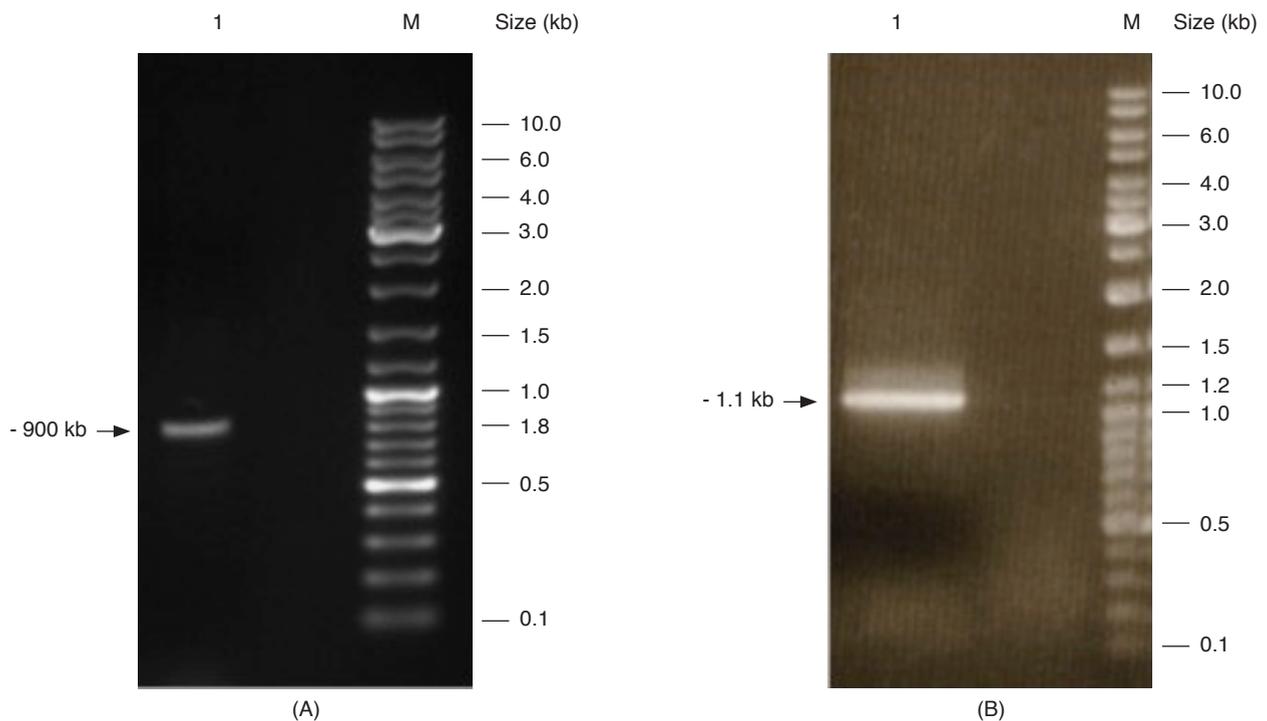

Figure 2. Analysis of the RACE PCR products by electrophoresis on 1% (w/v) agarose gel. A and B represents the 5′ and 3′ RACE PCR products. Lane 1 = the amplified 5′ RACE product, lane 2 = the amplified 3′ RACE product and M = Gene Ruler DNA Ladder Mix (Fermentas).

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                EF11                                    M   E   G   C   D   C      6
    1   ggaatgtaagggcaaggtagtaattgcagtggttgctggaagatggagggttgtgactgc    60

        F   E   P   Q   W   P   A   E   E   L   L   I   K   Y   Q   Y   I   S   D   F     26
                                                          I
   61   tttgagccacaatggcctgctgaagaacttctaattaaatatcaatacatatcagatttc   120

        F   I   A   L   A   Y   F   S   I   P   L   E   L   I   Y   F   V   K   K   S     46
  121   ttcatagcccttgcttatttctctattccacttgagctcatttattttgtgaagaagtct   180

        S   F   F   P   Y   R   W   V   L   V   Q   F   G   A   F   I   I   L   C   G     66
                       II
  181   tccttctttccatatagatgggttctggttcagtttggtgcatttatcatcctttgtgga   240

        A   T   H   L   I   N   L   W   T   F   T   V   H   S   K   T   V   A   I   V     86
                                                          III
  241   gcaacccatctcataaacttgtggactttcacagttcactcaaaaactgttgccattgtt   300

        M   T   V   A   K   I   S   T   A   A   V   S   C   A   T   A   L   M   L   V    106
  301   atgactgttgcaaaaatttcaactgccgctgtgtcctgtgcaacagctctaatgcttgtt   360

        H   I   I   P   D   L   L   S   V   K   T   R   E   L   F   L   K   S   K   A    126
  361   catatcattcctgatttgctgagtgtaaagacaagagagttgttttttgaagagtaaagct   420

        E   E   L   D   R   E   M   G   L   I   R   T   Q   E   E   T   G   R   H   V    146
  421   gaagaactggatagagaaatgggcttgatacggacacaggaagaaacaggaagacatgtt   480

        R   M   L   T   H   E   I   R   S   T   L   D   R   H   T   I   L   K   T   T    166
                                             GAF
  481   agaatgcttacgcatgaaatcagaagcacacttgatagacatactatattaaagactacc   540

        L   V   E   L   G   R   T   L   G   L   E   E   C   A   L   W   M   P   S   R    186
  541   cttgtggagttggggaggactttgggtttggaggaatgtgcactctggatgccatcacgt   600

        S   G   S   S   L   Q   L   S   H   T   L   R   H   Q   I   T   V   G   S   T    206
  601   agtggttcaagtcttcagctttctcataccctgcggcaccagattacagttgggtcaact   660

        V   P   I   N   H   S   I   V   N   Q   V   F   S   S   S   H   A   I   I   I    226
  661   gtaccaattaatcattctatagtcaatcaagtgtttagtagcagtcatgcaataataatt   720

        P   H   A   C   P   L   A   R   I   R   P   L   A   G   R   Y   V   P   P   E    246
  721   ccacacgcatgcccactggcaaggattcggcctcttgcaggaagatatgtcccaccagag   780

        V   A   A   V   R   V   P   L   L   H   L   S   N   F   Q   I   N   D   W   P    266
  781   gtggctgcagtgagagttcctctttttgcatctttcaaatttccaaattaatgattggcca   840

        E   L   S   A   K   S   Y   A   V   M   V   L   M   L   P   S   D   S   A   R    286
  841   gagctttctgcaaaaagttatgctgtgatggttttgatgctcccatcagatagtgcaaga   900

        K   W   H   I   H   E   L   E   L   V   E   V   V   A   D   Q   V   A   V   A    306
  901   aaatggcacattcatgaactggagcttgttgaggtggttgctgatcaggtagcggtagca   960
                                                                  HK
        L   S   H   A   A   I   L   E   E   S   M   R   A   R   D   L   L   M   E   Q    326
  961   ctttcacatgctgctattctggaagaatctatgcgggcacgtgatttactcatggaacaa  1020

        N   I   A   L   D   L   A   R   R   E   A   E   M   A   I   R   A   R   N   D    346
 1021   aatattgccttagatttggctcggcgggaggcagaaatggccatacgtgctcgcaatgat  1080
                                      H
        F   L   A   V   M   N   H   E   M   R   T   P   M   H   A   I   I   A   L   S    366
 1081   tttctagctgtcatgaaccatgaaatgcggactccaatgcatgcaattattgctctttct  1140
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367    S   L   L   L   E   T   E   L   T   P   E   Q   R   L   M   V   E   T   I   L    386
1141   tccctgcttctcgaaactgagctcactcctgaacaacgattgatggtagaaaccatattg  1200
387    K   S   S   N   L   L   A   T   L   I   N   D   V   L   D   L   S   K   L   E    406
1201   aagagtagcaaccttctagctacgcttatcaatgatgtttttagatctgtctaaactcgag  1260
407    D   G   S   L   E   L   E   I   G   P   F   N   L   H   A   V   F   R   E   V    426
1261   gatggaagccttgagttagagattggacctttcaatcttcatgcagttttttagagaggtc  1320
427    M   N   L   I   K   P   I   A   A   V   K   K   L   S   V   S   V   M   L   A    446
1321   atgaatttgataaagccaattgcagctgtgaagaaattgtcagtgtcagtgatgttggcg   1380
                                                                           N
447    P   D   L   P   L   C   A   I   G   D   E   K   R  [L   M   Q   T   I   L   N]   466
1381   ccagacttgccattgtgtgccattggtgatgaaaagcggctcatgcagactattttaaat  1440
467   [I   A   G   N   A]  V   K   F   T   K   E   G   R   I   S   L   T   A   S   V    486
1441   attgctggtaatgctgttaaatttacaaaggagggccgcatttcacttacagcttcagtt  1500
487    A   K   P   E   Y   L   R   D   I   P   D   F   C   P   V   P   S   D   R   H    506
1501   gccaagccagaatatttgagggacattcctgacttctgtccggttcccagtgatcggcat  1560
                                      G1
507    F   Y   L   K   V   Q  [V   K   D   T   G   C   G   I   S]  P   Q   D   K   A    526
1561   ttttacctgaaagttcaggttaaggatactggttgcggaatcagtccccaagacaaagca  1620
              F                                                          G2
527    H  [L   F   T   K   F]  A   Q   A   Q   S   G   K   N   Q   G   Y   S  [G   S]   546
1621   catttatttaccaaatttgcacaggctcaaagtggaaaaaaccaaggatatagcggcagt  1680
547   [G   L   G   L]  A   I   C   K   R   F   V   S   L   M   E   G   H   I   W   L    566
1681   ggacttgggcttgccatttgcaagagatttgtgagtcttatggaaggacacatctggctt  1740
567    E   S   E   G   A   G   K   G   C   T   A   T   F   I   V   K   L   G   T   C    586
1741   gaaagtgaaggtgctggtaagggttgtacagccacgttcatcgtgaagcttgggacgtgt  1800
587    E   N   P   I   G   F   Q   Q   Q   V   V   P   K   A   R   P   S   H   K   E    606
1801   gaaaatccaattggttttcagcaacaagttgtgcctaaagcaaggccaagtcataaagag  1860
607    A   D   L   S   G   P   R   A   L   S   K   D   E   K   G   L   A   R   Y   Q    626
1861   gcagatctttcgggcccaagagcattgtcaaaagacgagaaaggactggctcgataccaa  1920
627    K   S   V   *    EF12  ──────────────────────────────►         630
1921   aaaagtgtatagacggtttgaatgtctggttagcaataccaattttgataaaattcaatg  1980
1981   tgtgatatgtggaaatccatgattctgatgctttcagctagcacattttgttgcacaaat  2040
2041   gagaattgagcagctcttgcaatagtaaaggtagtactgtagaaatgctggttccaacac  2100
2101   tgacttgatggtagtatctattatttgaagcactgtatatcaacatccttctatcggctt  2160
2161   gattgaagcttgctataatatatttgaacgtttaagc◄──────── EGER9  aaaaaaaaaaaaaaaaaaaaaaaaa  2220
2221   aaaaa  2225
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Figure 3. Nucleotide and deduced amino acid sequences of the full-length EGER D3 cDNA. The nucleotide sequence is shown in lower case whereas the amino acid sequence is capitalised. Numerals at both ends of the nucleotide rows indicate nucleotide numbers. The start codon, (atg) and the stop codon, (tag) are in bold and marked with an asterisk, respectively. The putative polyadenylation signal is italicised and double underlined. Arrows represent specific (EF11, EF12) and complementary (EGER9) primer sequences designed for LD PCR and generation of 3' UTR gene-specific probe. The triple transmembrane domains I, II and III are shaded in blue boxes, the GAF domain from amino acid 158 to 309 are italicised whereas the histidine kinase (HK) domain from amino acid 326 to 585 is underlined. The five histidine kinase conserved motifs (H, N, D, F, G) are boxed and the amino acid residues important for ethylene receptor function are in red and bold.

nucleotide match of the conserved sequence found in most plants (Hunt, 2011). These polyadenylation sites provide alternative poly (A) site choice that has the potential to affect plant gene expression (Hunt, 2011).

Result from blastx homology search showed that the nucleotide sequence of EGER D3 exhibited a high sequence identity to predicted ethylene receptors from a wide range of plant species. The top three highest identity of 99%, 98% and 94% was found matching to a predicted probable ethylene response sensor 1 isoform X1 (XP_010921594.1) and isoform X2 (XP_010921597.1) from oil palm and a probable ethylene response sensor 1 from date palm (XP_008775899.1).

Comparison at the amino acid level with several established members of the ethylene receptor family from other plants such as rice (Yau *et al.*, 2004), tomato (Klee and Tieman, 2002) and Arabidopsis (Chang *et al.*, 1993; Hua *et al.*, 1998) showed that the deduced EGER D3 protein is closely related to ERS-type ethylene receptors (*Table 1*). Multiple sequence alignment of amino acids between *EGER D3* and other ERS-type ethylene receptors from other plants showed that the EGER D3 deduced protein has two structural domains: a sensor (transmembrane) and a histidine kinase (transmitter) domain; and just like any other ERS-type ethylene receptors, it lacks the receiver domain (*Figure 4*).

The transmembrane domain located at the amino (N)-terminal was predicted to contain three potential transmembrane segments specifically located at amino acid positions 20 to 42, 50 to 70 and 82 to 110 (*Figure 3*). In Arabidopsis, the transmembrane domain is located within the endoplasmic reticulum membrane and is essential

TABLE 1. COMPARISON OF AMINO ACID SEQUENCE SIMILARITY (%) BETWEEN EGER D3 AND THE ESTABLISHED MEMBERS OF THE ETHYLENE RECEPTOR FAMILIES OF ARABIDOPSIS (At), TOMATO (Le) AND RICE (Os) (BL2SEQ, Biology Workbench, Version 3.2, University of California, San Diego)

Ethylene receptors	EGER D3 (%)
At-ETR1	75
At-ETR2	39
At-ERS1	75
At-ERS2	35
At-EIN4	37
Le-ETR1	74
Le-ETR2	72
Le-Nr	71
Le-ETR4	42
Le-ETR5	41
Le-ETR6	38
Os-ETR2	41
Os-ETR3	40
Os-ETR4	34
Os-ERS1	84
Os-ERS2	72

for ethylene-binding (Schaller and Bleeker, 1995). Several conserved residues that are important to bind ethylene are also present in EGER D3. These include alanine at positions 31 and 102, isoleucine at position 62 and cysteine at position 65 (*Figure 3*). Site-directed mutagenesis of any of these amino acid residues has been shown to cause dominant insensitivity to ethylene (Chang *et al.*, 1993; Schaller and Bleeker, 1995).

Two cysteine residues (Cys_4 and Cys_6) important for the formation of disulfide-linked dimer at the transmembrane domain were also found at the N-terminal of the deduced EGER D3 protein. In addition, both Cys_{65} and His_{69} as found in EGER D3 have also been shown to serve as ligands for the copper ion to bind to ethylene. Ethylene receptors have been shown to form disulfide-linked dimers, whereby each dimer bind a single ethylene with the assistance of a copper ion cofactor (Schaller and Bleeker, 1995; Rodríguez *et al.*, 1999) through the action of the copper transporter RAN1 to balance the ethylene receptors and copper homeostasis (Binder *et al.*, 2010).

Following the transmembrane domain, a sub-domain called GAF was detected. The GAF domain was located at amino acid positions 158 to 308 (*Figure 3*). Earlier studies have suggested that the GAF domain is known to bind small molecules such as cyclic guanosine monophosphate (cGMP) (Aravind and Ponting, 1997). A decade later, Gao *et al.* (2008) demonstrated protein-protein interactions between the GAF domains using yeast two-hybrid analysis of the Arabidopsis ethylene receptor family, suggesting that it plays an important role in mediating signalling between the ethylene receptors.

Next to the GAF domain towards the carboxyl termini is a histidine kinase (HK) domain located at amino acid positions 350 to 585 (*Figure 3*). The HK domain comprises of His kinase A (His KA) and a histidine kinase-like ATPase (HATPase-c) and is similar to the bacterial two-component HK signal system, which is commonly found in prokaryotes but not in eukaryotes (Chang, 2016). The deduced EGER D3 protein contained all five HK conserved motifs found in bacteria (H, N, D, F and G) which are essential for HK activity (Mascher *et al.*, 2006). The motifs are arranged in the same order and spacing as in ERS-type ethylene receptor polypeptides of other plants (*Figure 4*). Comparison with the ERS-type ethylene receptors revealed that all five HK motifs are well conserved with one another (*Figure 4*). The F and G motifs in EGER D3 is 100% identical to that of the bacterial two-component system whereas motifs H, N and D have sequence similarities of 78%, 83% and 78% identity, respectively. In bacteria, a stimulus is sensed by a HK and is then transmitted to a response regulator (RR), which results in signalling (Mascher *et al.*, 2006). The HK catalyses

```
                                      I                                    II
Ps-ERS1   MMESCECIDTPYPPDELLVKYQYISDVLIALAYFSIPVELIYFVQKSAFFPYRWVLMQFG
Cp-ER     -MESCDCIDTPCPGEELLVKYQYISDVLIALAYFSIPLELIYFVHKSACFPYRWVLMQFG
Cm-ERS1   MMESCDCIDAQWPPDELLVKYQYISDFLIALAYFSIPLELIYFVQKSAFFPYRWVLMQFG
At-ERS1   -MESCDCFETHVNQDDLLVKYQYISDALIALAYFSIPLELIYFVKKSSFFPYRWVLIQFG
Le-NR     -MESCDCIEALLPTGDLLVKYQYISDFFIALAYFSIPLELIYFVHKSACFPYRWVLMQFG
Dl-ERS1   -MDSCACIETQWPADELLVKYQYISDFFIALAYFSIPLELIYFVQKSAFFPYRWVLMQFG
Os-ERS1   -MDGCDCIEPLWPTDELLIKYQYISDFFIALAYFSIPLELIYFVKKSSFFPYRWVLIQFG
MSP2      -MEGCDCFEPQWPAEELLIKYQYISDFFIALAYFSIPLELIYFVKKSSFFPYRWVLVQFG
Ma-ERS1   -MDGCDCIEPQWPADELLIKYQYISDFFIALAYFSIPLELIYFVKKSSFFPYRWVLIQFG
          *:.* *::.    :**:****:*: :**:*****:*****:**: ***:***:***

                                  III
Ps-ERS1   AFIVLCGATHFINLWTFSAHSKAVAVVMTIAKVSCAIVSCATALMLVHIIPDLLSVKTRE
Cp-ER     AFIVLCGATHFINLWTFSMHSKAVAVVMTIAKVACAAVSCVTALMLVHIIPDLLSVKTRE
Cm-ERS1   AFIVLCGATHFINLWTFSMHSKAVAVVMTVAKVACAIVSCATALMLVHIIPDLLSVKTRE
At-ERS1   AFIILCGATHFINLWMFFMHSKAVAIVMTIAKVSCAVVSCATALMLVHIIPDLLSVKNRE
Le-NR     AFIVLCGATHFISLWTFFMHSKTVAVVMTISKMLTAAVSCITALMLVHIIPDLLSVKTRE
Dl-ERS1   AFIVLCGATHLINLWTFSMHSKTVAMVMTIAKISTAVVSCATALMLVHIIPDLLSVKTRE
Os-ERS1   AFIVLCGATHLINLWTFTTHTKTVAMVMTVAKVSTAVVSCATALMLVHIIPDLLSVKTRE
MSP2      AFIILCGATHLINLWTFTVHSKTVAIVMTVAKISTAAVSCATALMLVHIIPDLLSVKTRE
Ma-ERS1   AFIVLCGATHLINLWTFALHSRTLAIVMTVAKVSTAVVSCATALMLVHIIPDLLSVKTRE
          ***:*******:*.** *   *:::*:***::*:  * *** ***************.**

                                            GAF
Ps-ERS1   LFLKNKAEELDREMGLILTQEETGRHVRMLTHEIRSTLDRHTILKTTLVELGRTLGLEEC
Cp-ER     LFLKNKAEELDREMGLILTQEETGRHVRMLTHEIRSTIDRHTILKTTLVELGRTLGLEEC
Cm-ERS1   LILKNKAEQLDREMGLILTQEETGRHVRMLTHEIRSTLDRDTILKTTLVELGKTLGLEEC
At-ERS1   LFLKKKADELDREMGLILTQEETGRHVRMLTHGIRRTLDRHTILRTTLVELGKTLCLEEC
Le-NR     LFLKTRAEELDKEMGLIIRQEETGRHVRMLTHEIRSTLDRHTILKTTLVELGRTLDLAEC
Dl-ERS1   LFLKNKAEELDREMGLIRTQEETGRHVRMLTHEIRSTLDRHTILKTTLVELGRTLGLEEC
Os-ERS1   LFLKNKAEQLDREMGLIRTQEETGRHVRMLTHEIRSTLDRHTILKTTLVELGGTLGLEEC
MSP2      LFLKSKAEELDREMGLIRTQEETGRHVRMLTHEIRSTLDRHTILKTTLVELGRTLGLEEC
Ma-ERS1   LFLKNKAEELDREMGLIRTQEETEGNVRMLTHEIRSTLDRDTVLKTTLVELGRTLDLKDC
          *:**.:*::**:*****  ****  :******** ** *:**.*:*:******* ** * :*

Ps-ERS1   ALWMPSRSGLNLQLSHTLTYHVQVGSTVPTNLPIVNEVFNSPRATRIPHTCPLARIRPLV
Cp-ER     ALWMPSRSGLNMQLSHTLNYHIQVGSTLPTNLPIVNEVFNNARAMRIPYTCPLARIRPLV
Cm-ERS1   ALWMPSRNGLSLQLSHALNYQIPVGTNIPINLPVVNDVFNSNRAICVPYTCQLARVRTPV
At-ERS1   ALWMPSQSGLYLQLSHTLSHKIQVGSSVPINLPIINELFNSAQAMHIPHSCPLAKIGPPV
Le-NR     ALWMPCQGGLTLQLSHNLNNLIPLGSTVPINLPIINEIFSSPEAIQIPHTNPLARMRNTV
Dl-ERS1   ALWMPSRTGMNLQLSHTLRHQIPVGSTVSTNLPIVTQVFNSNQAIKIPHTCPLARIGPTA
Os-ERS1   ALWMPSRSGSSLQLSHTLRHQITVGSTVSINLPVVNQVFSSNRAIIIPHTSPLARIRPLA
MSP2      ALWMPSRSGSSLQLSHTLRHQITVGSTVPINHSIVNQVFSSSHAIIIPHACPLARIRPLA
Ma-ERS1   MLWMPSHSGTSLQLSHTLHHQIPTGSAVPINLPVVNQIFSSNHAMIIPHTCRLARIWPLS
          ****.:  *  :**** *    :  *:  .  * .::::**..  *   :*:: **::

Ps-ERS1   G-RYVPPEVVAVRVPLLHLSNFQINDWPDLSAKSFAIMVLILPTDSARKWRDHELELVDV
Cp-ER     G-RYVPPEVVAVRVPLLHLSNFQINDWPDHSAKSYAIMVLILPTDSARKWRDHELELVEV
Cm-ERS1   GGRYLPPEVVAVRVPLLNLSNFQMNNWPDGSSRSYAIMVLILPTDSARKWRDHELELVDV
At-ERS1   G-RYSPPEVVSVRVPLLHLSNFQGSDWSDLSGKGYAIMVLILPTDGARKWRDHELELVEN
Le-NR     G-RYIPPEVVAVRVPLLHLSNFT-NDWAELSTRSYAVMVLVLPMNGLRKWREHELELVQV
Dl-ERS1   G-RYVPPEVVAVRVPLLHLSNFQINDWPELSAKSYAVMVLMLPTDSARKWHDHELDLIEV
Os-ERS1   G-RYVPPEVAAVRVPLLHLSNFQINDWPELSAKSYAIMVLMLPSDSARKWHVHELELVEV
MSP2      G-RYVPPEVAAVRVPLLHLSNFQINDWPELSAKSYAVMVLMLPSDSARKWHIHELELVEV
Ma-ERS1_  V-RHVPPEVAAVRVPLLHLSNFQISDWSELSTKSYAVMVLILPSDSGRKWHVHELELVEV
          *: **** .:****** ****  .:*.:  *  *.::*:***:**  .  ***: ***:*::

                                            HK                  ◇◆HE◇◆◇PL
Ps-ERS1   VADQVAVALSHAAILEESMRARDQLMEQNVALDLARREAEMAIHARNDFLAVMNHEMRTP
Cp-ER     VADQVAVALSHAAILEESMRARDQLMEQNVALNLARQEAEMAIHARNDFLAVMNHEMRTP
Cm-ERS1   VADQVAVALSHAAILEESMRARDQLVDQNVALDLARREAETAIHARNDFLAVMNHEMRTP
At-ERS1   VADQVAVALSHAAILEESMHARDQLMEQNFALDKARQEAEMAVHARNDFLAVMNHEMRTP
Le-NR     VADQVAVALSHAAILEDSMRAHDQLMEQNIALDVARQEAEMAIRARNDFLAVMNHEMRTP
Dl-ERS1   VADQVAVALSHAAILEESMRARDMLMEQNIALDMARREAEMAIRARNDFLAVMNHEMRTP
Os-ERS1   VADQVAVALSHAAILEESMRARDLLMEQNVALDLARREAEMAIRARNDFLAVMNHEMRTP
MSP2      VADQVAVALSHAAILEESMRARDLLMEQNIALDLARREAEMAIRARNDFLAVMNHEMRTP
Ma-ERS1   VADQVAVALSHAAILAESMRARDLLTEQNVALYLARQEAELAIRARNDFLAVMNHEMQTP
          ***************:*:*:.:* * *:**.** **:*** *::*************:.**
```

```
                                                                  ◊•Q◊◊◊N◊◊•NA
Ps-ERS1    MHAIIALSSLLLETELTPEQRVMIETVLKSSNVLAALINDVLDLSRLEDGSLELEMGKIN
Cp-ER      MHAIIALSSLLLETELTPEQRVMIETVLKSSNLLATLINDVLDLSRLEDGSLKLDSETFN
Cm-ERS1    MHAIIALSSLLLETELTPEQRVMIETILKSSNLLATLINDVLDLSRLEDGSLVLDMGSFN
At-ERS1    MHAIISLSSLLLETELSPEQRVMIETILKSSNLVATLISDVLDLSRLEDGSLLLENEPFS
Le-NR      MHAVIALCSLLLETDLTPEQRVMIETILKSSNLLATLINDVLDLSRLEDGILELENGTFN
DI-ERS1    MKAVIALSSLLLEIELTPEQRVMIETVLKNSNLLAALINDVLELSRLEDGSLELDIRTFN
Os-ERS1    MNAIIALSSLLLETELTPEQRLMVETVLKSSNLLATLINDVLDLSKLEDGSLELEIKAFN
MSP2       MHAIIALSSLLLETELTPEQRLMVETILKSSNLLATLINDVLDLSKLEDGSLELEIGPFN
Ma-ERS1    LHATITLSSLLLETELSPEQRSMVETVLKSSSLLATLINDILDLSRLEDGRLELEIGDFN
           ::* *:*.***** :*:**** *:**:**.*.::*:**.*:*:**:****  *  *:    ..
```

```
                                                      ◊•Q◊◊◊N◊◊•NA
Ps-ERS1    LHGVLEEVIELIKPIASVKKLPITLILAPDLPDNAIGDVKRLMQTLLNVVGNAVKYTKEG
Cp-ER      LQGIFREVINLIKPITSVKKLSMTLIMAPDLPACAVGDEKRLMQAILNVVGNAVKFTKEG
Cm-ERS1    LHAIFKEALDLIKPIASVKKLSMALILASDLPICAVGDEKRLMQIILNIVGNGVKFTKEG
At-ERS1    LQAIFEEVISLIKPIASVKKLSTNLILSADLPTYAIGDEKRLMQTILNIMGNAVKFTKEG
Le-NR      LHGILREAVNLIKPIASLKKLSITLALALDLPILAVGDAKRLIQTLLNVAGNAVKFTKEG
DI-ERS1    LHGIFREVLNMIKPITAVKKLSLTLMLSPDLPVVAVGDEKRLMQIMLNVVGNAVKFTKEG
Os-ERS1    LHAVFKEVMSFIKPIAAIKRLSVSVMLAPDLPLCAIGDEKRLMQTILNISGNAVKFTKEG
MSP2       LHAVFREVMNLIKPIAAVKKLSVSVMLAPDLPLCAIGDEKRLMQTILNIAGNAVKFTKEG
Ma-ERS1    LHSVFREVMNFIKPIRAVKKLQVSLMLSPDLPLYAIGDEKRLMQIMLNVAANAVRFTKEG
           *:.::.*.:..:****.::*:*  : :: ***  *:.** ***:*  :**:. *.*:.****
```

```
                                   ◊•D◊G◆G◊◆                    ◊F◊PF
Ps-ERS1    YVSIRASVAKPESLQDWRPPEFYPASSDGHFYIRVQVKDSGCGILPQDIPHLFTKFTQSQ
Cp-ER      YVTIVASVAKPDASRDW--PECYPVSSEGHFHLCVQVKDSGCGIHPQDIPHLFTKFAQPR
Cm-ERS1    HVSIIASIAKLDSLRDWRPTEFYPMQSDGQFYLRVQVKDSGCGIPPQDIPHLFTRETQLQ
At-ERS1    YISIIASIMKPESLQELPSPEFFPVLSDSHFYLCVQVKDTGCGIHTQDIPLLFTKFVQPR
Le-NR      HISIEASVAKPEYARDCHPPEMFPMPSDGQFYLRVQVRDTGCGISPQDIPLVFTKFAESR
DI-ERS1    YVSVIASVAKAESLREWQAPEIFMVSSDSHFHLCVQVKDSGCGISPQEIPHLFTKFTQLR
Os-ERS1    HITLVASVVKADSLREFRTPDFHPTASDDNFYLKVQIKDTGCGISPQDLPQVFTKFPQSQ
MSP2       RISLTASVAKPEYLRDI--PDFCPVPSDRHFYLKVQVKDTGCGISPQDKAHLFTKFAQAQ
Ma-ERS1    DISLTASVARTDYLREFRALDFYPVASDGHFYLRVTIKDTGCGISHQDLPNLFTKFSQSQ
           :::  **:. : :    ::        *: :*::  *  ::*:**** *:  :**:*  : :
```

```
                G◆GLGL
Ps-ERS1    SGAGRSSSGSGLGLAICKRFVNLMGGHIWMESEGPDKGSTATFVVKLGICGNPDSSDHQA
Cp-ER      SGSSRKNGSAGLGLAICKRFVNLMGGHIWIESEGADKGCTATFIVKLGICNSPTDPSERK
Cm-ERS1    TRSNKTNSGVGLGLALCKRFINLMGGHIWIESEGPDKGTTAMFIVKLGICNANPNDLSVK
At-ERS1    TGTQRNHSGGGLGLALCKRFVGLMGGYMWIESEGLEKGCTASFIIRLGICNGPSSSSGSM
Le-NR      PTSNRSTGGEGLGLAICRRFIQLMKGNIWIESEGPGKGTTVTFVVKLGICHHPNALPLLP
DI-ERS1    SGQNRSNDGAGLGLALCKRFLNLMEGHIWVDSDGTDKGCTVTIIVKLGICEGQPEPSNLQ
Os-ERS1    PGGNRGYSGSGLGLAICKRFVTLMGGHIWLDSEGTGRGCTVTFVIQLGICDNTNAYQQKL
MSP2       SGKNQGYSGSGLGLAICKRFVSLMEGHIWLESEGAGKGCTATFIVKLGTCENPIGFQQQV
Ma-ERS1    NVSSQGYSGNGLGLAICERYVSLMQGHIWVESDGIGKGCTATFVVRLRLCENPDGSQQKI
           :  .. *****:*.*:: ** * :*:::*:*  :* *. ::::*  *
```

```
Ps-ERS1    TTRGQAYSGSGGLARFKHLVNDGDIGFSNRRNQRSF
Cp-ER_     VGTKGQTTHGSGDLTRHKPLYRDNNGSTSSNPRYQRSF
Cm-ERS1    QVAPIVNHRSADLHGQRPIFRETGQVAFSNSRYQRSL
At-ERS1    ALHLAAKSQTRPWNW---------
Le-NR      MPPRGRLNKGSDDLFRYRQFR-GDDGGMSVNAQRYQRSL
DI-ERS1    VVSRPRLS----------------
Os-ERS1    IPLVWPSSGDADFVGPVPNAPNEEKGQASLKSRYQRSI
MSP2       VPKARPSHKEADLSGPRALS-KDEKGLARYQKSV
Ma-ERS1    LPLPWKKQT-QKFSSSRGMPTDPGGLFD---
```

Figure 4. Amino acid sequence alignment of EGER D3 with ethylene receptors ERS1 from other plants. '' = single, fully conserved residue, ':' = conservation of strong groups, '.' = conservation of weak groups whereas those without any symbol = no consensus (CLUSTWALW, Biology Workbench Version 3.2, University of California). The triple transmembrane domain region at the amino terminal are labelled I, II, III and are boxed in red. The GAF domain is boxed in black. The histidine kinase domain is boxed in blue. The five conserved motifs (H, N, D, F and G) in the histidine kinase domain found in bacteria protein kinase are shaded and symbols are labelled on top of the sequences according to Masher* et al. *(2006). '◊' = non-polar residues (I, L, M, V), '◆' = polar residues (A, G, P, S, T), '+' = basic residues (H, K, R), '-' = amidic or acidic residues (D, E , N, Q) whereas '.' = wild card position with less than 50% conservation in bacterial histidine kinases. The ERS1 amino acid sequences were obtained from GenBank:* Arabidopsis thaliana *At-ERS1 (Acc. No. U21952),* Oryza sativa *Os-ERS1 (Acc. No. AF013979),* Pisum sativum *Ps-ERS1 (Acc. No. AJ005829),* Carica papaya *Cp-ER (Acc. No. AF311942),* Cucumis melo *Cm-ERS1 (Acc. No. BAB13735),* Lycopersicon esculentum *Le-NR (Acc. No.U38666),* Delphinium *spp. L DI-ERS1 (Acc. No. AB106891) and* Musa acuminata *Ma-ERS1 (Acc. No. AF113748).*

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the autophosphorylation of a conserved His residue and transfer the phosphate group to a conserved aspartate residue in the RR domain (Mascher *et al.*, 2006). EGER D3 has a histidine (His354) residue in the histidine motif that may function as a phosphorylation site just like in the bacterial two-component signalling system, however, it does not contain the RR.

Histidine and/or serine/threonine kinase activity *in vitro* has been demonstrated in ethylene receptors from Arabidopsis (Gamble *et al.*, 1998) and tobacco (Xie *et al.*, 2003; Zhang *et al.*, 2004). The different kinase activities however, do not show their dependence on the conserved His residue to transmit the signal (Xie *et al.*, 2003; Zhang *et al.*, 2004). There are also other strong evidence that show interactions between ethylene receptors, constitutive triple response 1 (CTR1) and ethylene-insensitive 2 (EIN2) are the primary components mediating ethylene signalling as compared to the HK (Chang, 2016). In the absence of ethylene, the ethylene receptors activate the kinases in CTR1, which results in phosphorylation and inhibition of EIN2 function. On the other hand, when ethylene is detected, the receptor-CTR1 signalling is deactivated causing EIN2 to become dephosphorylated and releases the EIN2 C-ends via proteolytic cleavage, hence allowing the downstream ethylene signalling pathway to proceed and evoke ethylene responses in plants (Chang, 2016).

Determination of Gene Copy Number

Ethylene signalling is mediated by a family of receptors (Klee and Tieman, 2002; Yau *et al.*, 2004; Chang, 2016). Therefore, to determine the organisation of the ethylene receptor family in the oil palm genome, Southern analysis was performed. Southern hybridisation using 3′ UTR as the gene specific probe showed that at low-stringency conditions, the probe hybridised to a single fragment in the digested oil palm genomic DNA, indicating that there is probably one copy of the EGER D3 gene present in the oil palm genome (*Figure 5*). Using a gene specific probe containing the full-length sequence of *EGER D3*, Southern hybridisation at low stringency washing conditions detected the presence of multiple bands in both *BamHI* and *EcoRI* digested genomic DNA (*Figure 5*). The presence of multiple bands suggests that in oil palm, EGER D3 is a member of a multigene family. This is in agreement with ethylene receptors found in many dicot as well as monocot plants species such as Arabidopsis (Chang *et al.*, 1993; Hua *et al.*, 1998), tomato (Klee and Tieman, 2002), rice (Yau *et al.*, 2004), peach (Rasori *et al.*, 2002), melon (Sato-Nara *et al.*, 1999), and tobacco (Xie *et al.*, 2003). To date, there is at least one member of the ethylene receptor family identified in orchids (Thongkum *et al.*, 2009) and a maximum of nine members in apple (Ireland *et al.*, 2012).
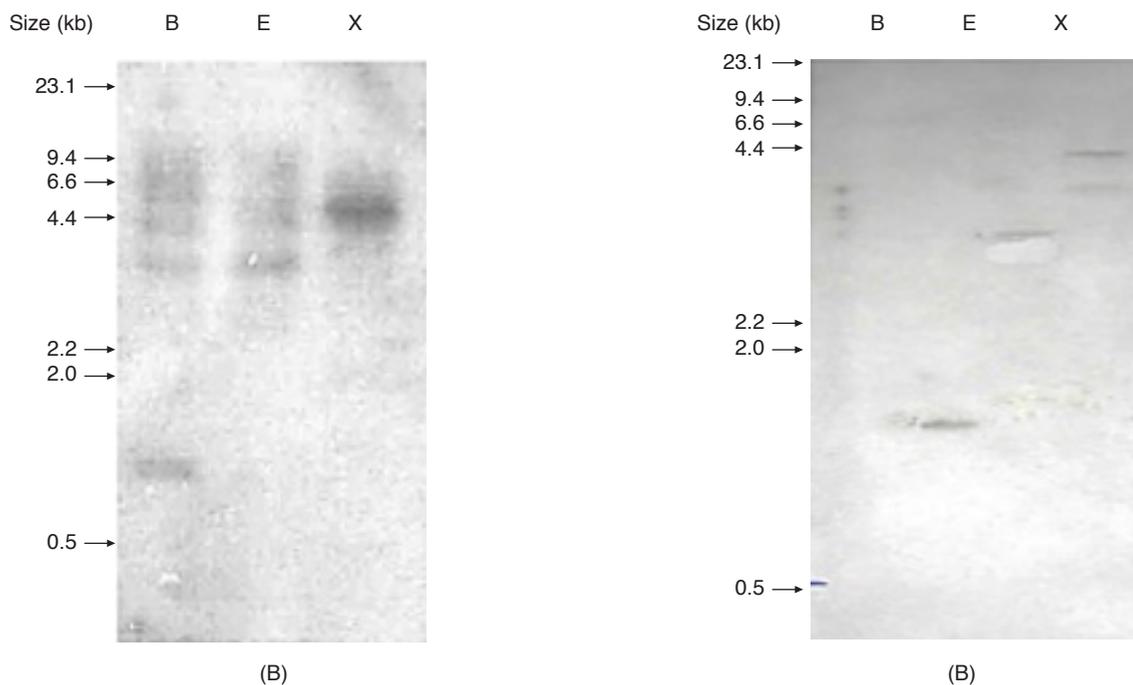

Figure 5. Southern blot hybridised with the 3' UTR (A) and full-length (B) EGER D3 probes. Each lane represents 30 µg of oil palm genomic DNA digested with different restriction enzymes. Lanes B, E, X represents DNA digested with Bam HI, EcoR I and Xba I, respectively.

Expression Analysis

Preliminary analysis on the expression pattern of the ethylene receptor gene was performed using dot blot analysis. The entire fragment amplified from the degenerate PCR amplification was used to probe the nylon charged membranes containing total RNA from the mesocarp at five different development stages, two developmental stages of kernel and spear leaf tissues. It was found that the labelled probe hybridised to all five mesocarp tissues with strong signal intensities. On the other hand, very faint signals were detected in kernel tissues at 12 and 13 WAA, as well as in leaves (*Figure 6*).

Northern blot hybridisation was then performed to confirm the expression pattern and determine the transcript size of the ethylene receptor gene in mesocarp tissues. An additional mesocarp tissue at 10 WAA was also included to represent oil palm fruit developmental stages from young until ripening. The full-length insert of *EGER D3* and the 3' UTR probes hybridised to a single transcript of about 2.3 kb, similar to the size of *EGER D3* (*Figure 6*). The expression of *EGER D3* appeared to be strong and it was differentially expressed in the mesocarp during oil palm fruit development. The expression in mesocarp tissue gradually increased from the early fruit developmental stages from 10 WAA up to 17 WAA and decreased slightly when the fruit has fully ripened. This result is in agreement with that found

from the dot blot analysis. A similar expression pattern with the period when oil synthesis starts in the mesocarp was also observed based on the high expression of *EGER D3* at 15 WAA. Similarly to *EGER D3*, increase in transcript levels of ERS-type ethylene receptors have also been observed during the ripening process in fruits such as melon (Sato-Nara *et al.*, 1999) and peach (Rasori *et al.*, 2002).

Fruit ripening is normally associated with changes in fruit texture, colour and softening of the flesh with an increase in the sweetness, flavour and aroma (McAtee *et al.*, 2013). Unlike other fleshy fruits, the main changes in the ripening of oil palm fruits are basically associated with the accumulation and increase of storage oil in the form of oil bodies in both storage tissues, *i.e.* the mesocarp and kernel (Sambanthamurthi *et al.*, 2000). Being classified as a climacteric fruit (Tranbarger *et al.*, 2011; Nurniwalis, 2017), the ripening-related gene expression of *EGER D3* is likely the consequence of increased ethylene production in the ripening mesocarp tissues, indicating the important role of *EGER D3* in oil palm fruit ripening. In addition, the expression of *EGER D3*, similar to that of oil synthesis in the mesocarp, suggests the possible involvement of ethylene in the accumulation of storage oils in mesocarp tissues.

In fruits like tomato, the ripening-related evidence to show that ethylene is required for climacteric fruit ripening was through the identification of an ethylene receptor *nr* mutant
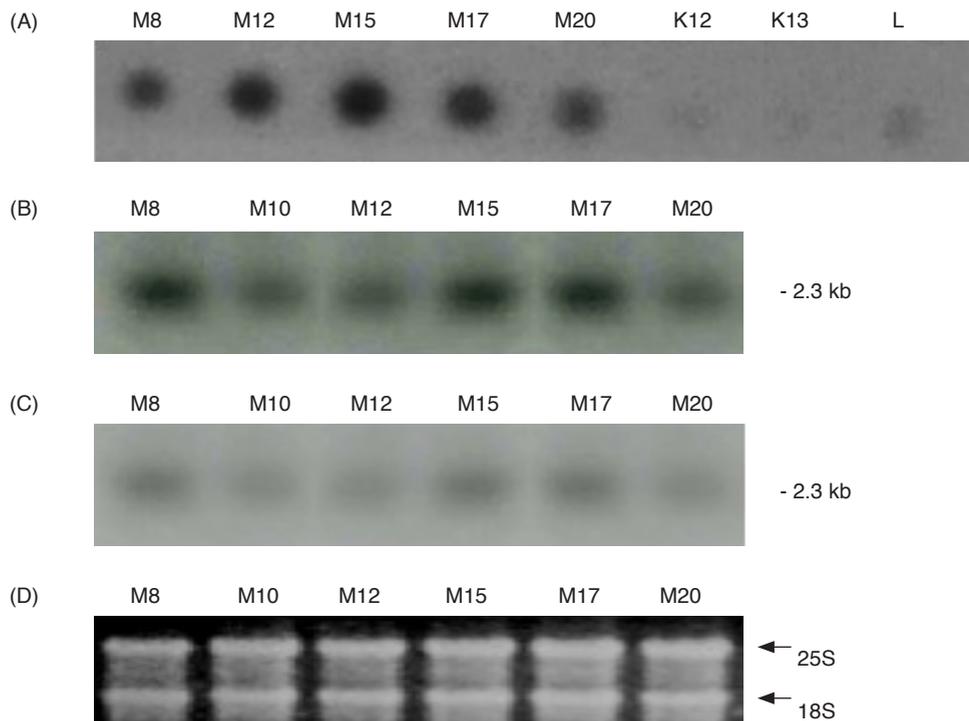

Figure 6. Gene expression analyses of the partial (A), full-length (B), and 3`UTR (C) EGER D3 ethylene receptor via Dot blot (A) and northern blot (B and C) analyses. D = ethidium bromide stained gel to show approximate loading of total RNA. M = mesocarp, K = kernel, L = spear leaves. Numbers represent fruit developmental stages at weeks after anthesis (WAA).

(Wilkinson *et al.*, 1995). The *nr* mutant has a specific point mutation within the ethylene-binding domain, eliminating its ability to bind ethylene which resulted in a tomato plant that has dominant insensitivity to ethylene and produced non-ripening fruits (Wilkinson *et al.*, 1995). Characterisation of the role of ethylene receptors in many plants especially Arabidopsis and tomato have also demonstrated how plants regulate their ethylene sensitivity (Agarwal *et al.*, 2012; Chang, 2016). Thus, this information have allowed the use of biotechnology tools to alter ethylene action in plants for crop improvement (Agarwal *et al.*, 2012). For example, overexpression and suppression of ethylene receptor genes have produced transgenic crops with altered phenotypes, senescence and ripening (Agarwal *et al.*, 2012). Antisense suppression of the tomato ethylene receptor NR gene in tomato *nr* mutants restores normal ethylene sensitivity and produced fruits that ripen normally (Hackett *et al.*, 2000; Tieman *et al.*, 2000). On the other hand, when ethylene receptor LeETR4 was inserted into tomato via antisense and RNAi, down regulation of LeETR4 produced transgenic tomatoes that are extremely sensitive to ethylene which results in early fruit ripening (Tieman *et al.*, 2000; Kevany *et al.*, 2008). The possibility of manipulating ethylene receptors to control ethylene sensitivity in oil palm can therefore be looked into as a mean to understand processes related to oil palm fruit ripening such as the unsynchronised ripening of oil palm fruits within the fruit bunch, which may be of value to help further improve oil palm yields.

CONCLUSION

In conclusion, we have successfully isolated and characterised the first member of the ethylene receptor gene family from oil palm. EGER D3 contains conserved amino acid residues important to bind ethylene indicating the possibility of EGER D3 to function just like other normal ethylene receptors. Gene expression studies showed that the expression of *EGER D3* was regulated throughout oil palm fruit development until ripening. The findings from this work clearly paves the way to explore further on the roles of ethylene in regulating the many developmental processes in oil palm, especially on fruit development and ripening processes.

ACKNOWLEDGEMENT

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