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EgHOX1, a HD-Zip II gene, is highly expressed during early oil palm (*Elaeis guineensis* Jacq.) somatic embryogenesis

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ABSTRACT

The embryogenesis rate in oil palm tissue culture still averages at a low 6%. Hence, a better understanding of the regulatory mechanisms during somatic embryogenesis and identification of molecular markers may help to improve the oil palm cloning process. *EgHOX1*, a previously isolated cold plaque clone, was further characterized and identified to be a putative HD-Zip II gene. High levels of *EgHOX1* transcripts were detected in early tissue culture stages involving embryogenesis induction. Transcripts accumulated preferentially in meristematic centers of embryogenic callus and then in the provascular regions of developing somatic embryos. *EgHOX1* was modestly induced by exogenous auxin and highly induced by dehydration and osmotic stresses. However, its expression was down-regulated by light. Based on its expression patterns, this novel oil palm HD-Zip II gene may be involved during the acquisition of embryogenic competency in early somatic embryogenesis and in vascular development later on, similar to the roles of some HD-Zip genes in other plants.

1. Introduction

The oil palm, *Elaeis guineensis* Jacq., is the highest yielding oil crop in the world. Vegetative propagation of elite high yielding palms would generate large numbers of good planting material. As the biological characteristics of oil palm do not allow its vegetative propagation by conventional means, the best way to propagate elite oil palms is through somatic embryogenesis (Rajanaidu et al., 1997). However, one of the problems hampering tissue culture is the cloning efficiency, particularly the low embryogenesis rate (Soh et al., 2011).

The oil palm tissue culture process has not varied much after three decades and involves indirect somatic embryogenesis with a callogenesis transition phase. Successive subcultures of callus cultures will sporadically give rise to embryogenic calli. Auxins, normally required for callus induction and proliferation (Nwankwo and Krikorian, 1983), is the primary phytohormone used in oil palm tissue culture. The suspension culture process which is gaining popularity, utilizes friable embryogenic calli to initiate suspensions (De Touchet et al., 1991; Roowi et al., 2010). Embryos are later obtained by spreading the proliferating embryonic nodules on hormone-free medium.

Previous molecular studies on oil palm embryogenesis have focused partly on discovering potential molecular markers that can be used to assist tissue culturists in selection of potentially embryogenic tissues at an early stage. Some efforts were targeted on the isolation of regulatory genes, for example transcription factors. Of particular interest are the homeodomain proteins, many of which play important roles during embryogenesis in the animal kingdom as well as in plants. The plant specific homeodomain leucine-zipper (HD-Zip) family of proteins has been associated with developmental functions as well as in the plant's responses to external environmental conditions.

The HD-Zip proteins are divided into four classes, HD-Zip I to IV. Different members within each class as well as the different classes play a wide variety of functions, ranging from possible roles in embryogenesis and organ development to responding to environmental and stress conditions (Baima et al., 2001; Ingram et al., 2000, Ingram et al., 1999; Ito et al., 2002; Masucci and Schiefelbein, 1996; Soderman et al., 1999). Moreover, HD-Zip proteins can homodimerize or heterodimerize due to their leucine-zipper domain (Lee and Chun, 1998).

To understand the molecular mechanisms involved in somatic embryogenesis of the oil palm, insights into the roles of regulatory genes such as transcription factors would be useful. In this study, molecular characterization was conducted on a novel HD-Zip II gene from oil palm, designated as *EgHOX1*, which was previously isolated from embryogenic suspension cultures through cold plaque screening (Ho et al., 2007). Gene expression patterns of *EgHOX1* were investigated during oil palm tissue culture and in response to exogenous auxin and abiotic stresses.

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2. Materials and methods

2.1. Plant materials

Most tissue culture materials including oil palm embryogenic suspension cultures (various clones/lines), non-embryogenic calli, leaf explants with attached embryogenic and non-embryogenic calli, white and green embryoids for RNA expression studies were kindly provided by Applied Agriecological Research Sdn. Bhd., Malaysia. Embryogenic suspensions, derived from various *dura x pisifera* palms, SC291-90, SC269-21, SC295-75, SC295-112, SC282-1, SC283-1 and SC283-20A were cultured in auxin supplemented MS-basal medium, while MS282-1A was cultured in hormone-free MS-basal medium. Zygotic embryos were excised from oil palm fruits at 15 weeks after anthesis (WAA) and 16 WAA, which were kindly provided by Sime Darby Bhd., Malaysia. Young leaf tissues and embryogenic suspensions E107 and E90 were obtained from Malaysian Palm Oil Board.

2.2. Cloning of the EgHOX1 gene and promoter

Genomic DNA was extracted from *dura* × *pisifera* young leaf tissues (Dellaporta et al., 1983). *EgHOX1* gene was amplified from genomic DNA by standard PCR methods. PCR with specific primers, 5′-CACATCTAGCATGGTTGAAGAGG-3′ and 5′-GAAACAACTCTCATGAAGT CTGC-3′ was carried out at 95 °C, 2 min; 35 cycles of 95 °C, 30 s, 65 °C, 30 s, 72 °C, 2 min; with a final extension at 72 °C, 7 min. The putative promoter of *EgHOX1* was previously isolated by standard Inverse PCR methods (Ooi et al., 2010). The promoter sequence was analyzed using SignalScan program to the plant *cis*-acting regulatory elements PLACE database (Higo et al., 1999; Prestridge, 1991). For nucleotide sequence analysis and further manipulations, PCR products were cloned into pCR2.1-TOPO® vector (Invitrogen, Inc.). Nucleotide sequences were used in searches to GenBank databases using the BLAST algorithms (Altschul et al., 1997). The neighbor-joining tree was constructed using CLUSTALW (Biology Workbench, San Diego Supercomputing Centre) and MEGA (Molecular Evolutionary Genetic Analysis (MEGA), Version 2.1 (2001)) tools.

2.3. Southern hybridization

A specific probe spanning the 3′-UTR region of *EgHOX1* was generated by PCR with primers 5′-CGGGTGCAGCATCCAAGACTACGC-3′ and 5′-GAAACAACTCTCATGAAGTCTGC-3′. This 240 bp fragment was cloned into pCR-II TOPO® vector (Invitrogen Inc.), verified by sequencing and used for the generation of α-[^{32}P]dCTP-radiolabelled probes with High Prime reaction mix (Roche Diagnostics). Southern hybridization was carried out on restriction-digested genomic DNA from oil palm young leaf tissues (Church and Gilbert, 1984). These restriction enzyme sites were absent in the *EgHOX1* probe sequence.

2.4. Auxin and abiotic stress treatments

For auxin treatments, embryogenic calli was treated as previously described (Baima et al., 1995; Gee et al., 1991). Embryogenic suspensions from auxin supplemented media (MS-basal with 0.1 mg/L NAA and 1 mg/L 2,4-D) were washed by shaking for 4 h in 10 mM potassium phosphate buffer (pH 6), 2% sucrose, to remove endogenous auxins. A representative sample was removed and frozen after this pre-treatment. Half of the calli was then incubated in fresh auxin-supplemented media as above and the other half was incubated in hormone-free MS-basal media. Samples were incubated in the dark for 4 h. Subsequently, the treated samples were collected and frozen in liquid nitrogen and processed for RNA extraction and real time quantitative PCR.

Abiotic stress treatments were similarly conducted as previously described (Kreps et al., 2002). Oil palm young leaves were sliced into strips and used for the different stress treatments. Leaf tissues were obtained from four different palms of the same clone (biological replicates). For dehydration treatment, the leaf slices were placed on a sterile filter paper in a petri dish and incubated in a dehumidifier at 19 °C for 12, 24, 36 and 48 h. Untreated leaf tissues (control) were frozen in liquid nitrogen and stored at −80 °C. For the osmotic stress treatment, leaf slices were placed on MS-basal media supplemented with 0 mM, 200 mM, 400 mM, 500 mM, 600 mM mannitol respectively, and incubated in the dark at 29 °C for four days. For light treatment, leaf slices were incubated on MS-basal media and exposed to continuous light or darkness for 8 h at 29 °C. After the treatments, the tissues were frozen in liquid nitrogen and stored at −80 °C.

2.5. Gene expression analyses

2.5.1. Real time quantitative PCR with FAM-labeled probes

Primers and TaqMan® MGB probe sets were designed and synthesized by Assay-By-Design service (Applied Biosystems) for *EgHOX1* and the endogenous reference gene, *GAPDH* (acc. no. DQ267444). Primers and FAM-labeled locked nucleic acid (LNA) probes were also designed and synthesized by Sigma Proligo for two auxin-related genes, *EgIAA9* (acc. no. EL689638) and a putative *GH3* gene (acc. no. EL689939). The primers and probe sequences for *EgHOX1* are 5′-ACCTCTAGCTTAGATTTCATATATTGATCCCA-3′ (forward primer), 5′-CTCCAGCTTCTCTTTTGACCCTATT-3′ (reverse primer) and 5′-[*FAM*] CTTGGACCACCATTCATC[NFQ]-3′ (probe); for *EgIAA9*, 5′-GCGGTTTC AAATGGACTAAGACTT-3′ (forward primer), 5′-ACCATAGCAGTTACAAA CAGCAAA-3′ (reverse primer) and 5′-[*FAM*]AACATGGCAAACACACAGA CTGAAGGCA[BHQ]-3′ (probe); for EL689939 (putative *GH3* gene), 5′-AAGACCAATGAAGAAGACCTCCTC-3′ (forward primer), 5′-GCTAGTGTA CTCGGTGAGAAGG-3′ (reverse primer) and 5′-[*FAM*]AACACAAGCCAA GCTCCTCCTACAGCC[BHQ]-3′ (probe); and for *GAPDH*, 5′-ACTGCTAC TCAGAAGACTGTTGATG-3′ (forward primer), 5′-TGCTGCTAGGAATGA TGTTAAAGCT-3′ (reverse primer) and 5′-[*FAM*]ACCCCTCCAGTCCTTG [NFQ]-3′ (probe). Contaminating DNA was removed from RNA by RNAse-free DNAse1 (Roche Diagnostics) treatment. Subsequently, integrity and purity of the RNA was analyzed by Agilent 2100 Bioanalyzer. RNA (2 μg) was used for first strand cDNA synthesis using the High Capacity cDNA Archive kit (Applied Biosystems, USA) according to the manufacturer's instructions. Five microliters of cDNA (1:150 dilution with 0.1 mM EDTA) was used to prepare quantitative PCR reactions of 25 μL with Taqman® Universal PCR Master Mix (Applied Biosystems, USA), according to the manufacturer's instructions. Real Time PCR was conducted on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA). Each PCR reaction was performed in triplicates and no-template controls were included.

2.5.2. Real time quantitative PCR with SYBR green dye

For the auxin- and abiotic stress treatment samples, quantitative real time PCR using SYBR® Green dye was conducted. Genes for use as positive controls for the abiotic stress treatments were identified from oil palm EST and transcriptome databases. Primers were designed using Primer3 (version 0.4.0) (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) (Rozen and Skaletsky, 2000) (Table 1). First strand cDNA was synthesized from total RNA with the Quantitect cDNA synthesis kit (Qiagen, Germany). Five microlitres of cDNA (1:50 dilution) were used to prepare PCR reactions of 20 μL with SYBR® Premix Ex Taq™ (Takara Bio Inc., Japan), according to the manufacturer's instructions. Real Time PCR was conducted on an ABI Prism Sequence Detection System 7000 (Applied Biosystems, USA) according to the program of 95 °C for 10 s; and 40 cycles of 95 °C for 5 s and 60 °C for 32 s with a final dissociation cycling stage. Specificity of the primer pairs was evaluated by melt curve analysis. Each PCR reaction was conducted in duplicates and no template controls were included. Selection of endogenous reference genes for normalization of qPCR data was conducted using GeNorm (Ooi et al., 2012a; Vandesompele et al., 2002). Endogenous reference genes identified for auxin-treated tissues were *EA1332*, *PD380* and