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# Characterization and differential expression of ethylene receptor genes during fruit development and dehiscence of durian (*Durio zibethinus*)

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ABSTRACT

Durian (*Durio zibethinus*) is a climacteric fruit. Biochemical changes take place in the durian pulp while the husk is dehiscent during fruit ripening. Fruit development of durian cv. Monthong showed a sigmoid curve and reached a maturity stage week 16 after anthesis, while fruit dehiscence started a week after harvest, was hastened by ethephon treatment, and delayed by 1-methylcyclopropene (1-MCP). 1-MCP treatment delayed dehiscence for 5 days from day 7 to day 12 compared with control fruit at 25 °C. Two ethylene receptors (*DzETR1* and *DzETR2*), a CTR1-like protein (*DzCTR1*) and two ethylene insensitive-like proteins (*DzEIL1* and *DzEIL2*) in the pulp and husk shared high homology to *Theobroma cacao*. The transcript levels of *DzETR2, DzCTR1* and *DzEIL2* slightly decreased during fruit development (week 2 to week 10), while *DzETR2* transcript level showed the highest expression. The gene expression of *DzETR2* in the dehiscence zone, increased at day 12 after harvest, while gene expression of *DzETR1, DzCTR1* and *DzEIL2* slightly increased after harvest. All genes were reduced in expression with 1-MCP treatment. After that, an increase in expression was found at day 9, followed by a gradual decrease until day 12. These results suggest that *DzETR2* plays a role in fruit development and dehiscence of ripening durian.

## 1. Introduction

Ethylene is a gaseous plant hormone and plays important roles in plant growth, development, fruit ripening, organ senescence and stress response (Abeles et al., 1992). Ethylene action is achieved by regulating ethylene receptors and a series of signal transduction events, and by controlling related gene expression (Sisler and Serek, 2003). Ethylene actions are mediated through the ethylene signaling pathway. Ethylene binds to receptors (ETRs, ERSs) after which the signal is transferred to constitutive triple response (CTRs) with both elements acting as negative regulators of ethylene response (Kieber et al., 1993). The signal is then transferred to positive regulators, such as EIN2 (ethylene insensitive 2). In the final step of signal transduction, a nuclear-localized protein, EIN3 acts as a transcription factor and regulates the expression of downstream target genes, such as ethylene response factor 1 (ERF1), and induces the ethylene responses (Chao et al., 1997; Solano et al., 1998).

Durian (*Durio zibethinus* Murr.) is an important economic crop of south-east Asian countries including Thailand. Durian shows self-incompatibility (Subhadrabandhu and Shodal, 1997; Lim and Luders, 1998; Bumrungsri et al., 2009). Durian fruit set after pollination, the ovary has five locules, each locule containing seven to nine ovules, with not all ovules developing to maturity. The spines start to develop from fine soft protuberances two weeks after pollination (Subhadrabandhu and Ketsa, 2001). The pattern of durian fruit growth is expressed in the fruit length, fruit width or fruit volume, follows a simple sigmoid curve. By the second week after pollination, there is little difference between fruit length and width. Durian fruit growth periods depend on cultivar such as 'Khadum' a small fruit cultivar and 'Monthong', a large fruit cultivar that matures at around 90 and 120 days after anthesis, respectively (Siriphanich, 2011). Durian has been categorized as a climacteric fruit and the rise of respiration and ethylene production coincides with ripening (Tongdee et al., 1990; Booncherm and Siriphanich, 1991; Ketsa and Daengkanit, 1998; Amornputti et al.,

*Abbreviations: Dz, Durio zibethinus*; ETR1, ethylene triple response 1; ETR2, ethylene triple response 2; CTR1, constitutive triple response 1; EIL1, ethylene-insensitive 3-like 1; EIL2, ethylene-insensitive 3-like 2

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2016). Ethylene biosynthesis and ethylene perception are modulated by various factors during fruit development (El-Sharkawy et al., 2007).

Durian husk dehiscence occurs in over-ripe stage. Fruit dehiscence takes place along the suture on the back of the locules, which have a dehiscence zone (Sriyook et al., 1994). Ethylene enhances ripening which includes pulp softening and husk dehiscence (Sriyook et al., 1994). Dehiscence of durian occurs in layers of small parenchyma cells at the center of each locule. The dehiscence zone (DZ) runs from the stem to bottom end; when fully ripe fruit dehiscence occurs from inside to outside with splitting starting near the stem to the bottom of fruit (Sriyook et al., 1994; Siriphanich, 2011).

In this study, we isolated and characterized five putative ethylene signaling elements, two ethylene receptors [*DzETR1* (KT026304) and *DzETR2* (KT026305)], a CTR1-like protein [*DzCTR1* (KJ660066)], and two ethylene-insensitive-like genes [*DzEIN1* (KT026306) and before *DzEIN2* (KT026307)] from durian cv. Monthong during fruit growth and ripening. In durian, little molecular information is available about ethylene signal transduction during the physiological changes of durian such as fruit growth and husk dehiscence in over ripe fruit. The molecular and physiological results have been used to build a preliminary genetic model.

## 2. Material and methods

### 2.1. Fruit material

Flowers of durian (*Durio zibethinus* Murr.) cv. Monthong were tagged at an orchard in Chanthaburi province, eastern Thailand. Fruit were harvested every 2 weeks from week 2 to week 16 after anthesis. After harvest, only for mature fruit (16 weeks after anthesis) were submerged in 0.5 mL $L^{-1}$ imazalil solution for 20 s to control fruit rot caused by *Phytophthora palmivora*. Fruit were transported to the laboratory by a temperature-controlled truck (25 °C) and took about 6 h. All samples pulp and husk were kept in liquid nitrogen and storing at −80 °C before extracts total RNA.

### 2.2. Determination of fruit development

Fruit development was determined using fruit weight (fresh), width and length. Fruit weight was measured in individual fruits using a digital balance (Ohaus, NJ, USA) and recorded in kg. Fruit width and length were also measured in cm. For pulp dry weight, the pulp was separated from husk and seed, then incubated in oven at 70 °C for 48–72 h and recorded in gram (g).

### 2.3. Ethephon and 1-MCP treatments

Fruit harvested at week 16 after anthesis, were divided into three groups for post-harvest treatments. One group (100 fruit) served as controls. In another group (100 fruit) an aqueous 480 mL $L^{-1}$ ethephon was brushed at the surface of the cut stalk, applying about 0.2 mL per stalk and dried in air at 25 °C A third group was placed in a 180 sealed container and treated with 500 μL $L^{-1}$ 1-MCP for 12 h at 25 °C. 1-MCP was generated by adding water to 1-MCP (EthylBloc®, Floralife Inc., Walterboro, SC, USA) powder, which was placed a glass vial. This resulted in a final concentration of 500 μL $L^{-1}$ of 1-MCP in the air. Fans were used in the chambers to maintain air circulation. Fruit were stored at 25 °C and 85–90 %RH.

### 2.4. Ethylene production

Measurement of ethylene production followed the method described by Palapol et al. (2015). Individual durian fruit harvested at week 16 after anthesis, were placed into 13.5 L airtight jars for 30 min at 25 °C, after which a 5 mL gas sample was taken from the air space and injected into a gas chromatograph equipped with a flame ionization detector (GC-14, Shimadzu, Tokyo, Japan) for ethylene.

### 2.5. Dehiscence score

At regular intervals nine fruit per replication were visually scored for dehiscence, following Khurnpoon et al. (2008). These scores were: 0 = no dehiscence, 1 = dehiscence up to 1/4 of suture length, 2 = dehiscence up to 1/2 of suture length, 3 = dehiscence up to 3/4 of suture length, and 4 = dehiscence along entire suture (Fig. 3). The dehiscence index of a fruit was calculated as follows:

$$\text{Dehiscence index} = \frac{\text{Dehiscence classification level} \times \text{Number of sutures at the level}}{\text{Number of sutures}}$$

Material from the dehiscence zone of other fruit was collected from the husk (pericarp) by excising a 0.5 cm band of tissue. It was cut into 0.5 × 1.0 × 1.0 $cm^3$ pieces, immediately frozen in liquid nitrogen and stored at -70 °C until further use.

### 2.6. Total RNA extraction and cDNA synthesis

Total RNA extracted from frozen durian pulp according to the CTAB method by Chang et al. (1993) with a modified extraction buffer containing 2% CTAB, 2% PVP 40, 100 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, 2.0 M NaCl, and 500 μL $L^{-1}$ of spermidine. Quality and quantity of the purified total RNA was determined by using the Nano Drop nd-1000 (Thermo Scientific, MA, USA). All samples were treated with DNase using a DNA-free kit following the manufacturer's recommendations protocol (Turbo DNAfreeTM Kit from Ambion, TX, USA). First-strand cDNA synthesis was performed on 1 μg DNase I-treated total RNA as template, using oligo dT as a primer and reverse transcriptase (SuperScript™ III First-Strand Synthesis System for RT-PCR, Invitrogen, USA).

### 2.7. Gene isolation and amino acid sequence analysis

Ethylene receptor and signal transduction genes were isolated from durian pulp and husk tissue (mix RNA), using degenerate forward and reverse primers. Degenerate primers were designed from conserved domain of related genes in other fruit from NCBI sequence database (http://www.ncbi.nlm.nih.gov/) (Supplementary Table 1). The PCR products from each gene were purified by using a gel extraction kit (QIAprep Gel Extraction, QIAGEN, Hilden, Germany) and directly ligated into pGEM-T easy vector (Promega, USA) essentially as recommended by the manufacturer.The recombinant clones were identified by blue/white colony selection then sent to sequencing.Comparisons of nucleotide and deduced amino acid sequences were carried out using basic local alignment search tool (BLAST) programs online (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The 3' untranslated region (3'UTR) was amplified by using protocol of GeneRacerTM kit (Invitrogen, CA, USA) using specific primers based on initial sequence information. The 3'UTR fragment was cloned and compared nucleotide sequence by BLAST program.

For phylogenetic analysis of amino acid sequences from other plants, sequences were retrieved from NCBI public databases (showing their organism and accession number). The amino acids were analyzed using the Geneious program and a phylogenetic tree constructed with MEGA 6.0 using Maximum Likelihood method based on the Poisson correction model and default parameters (Zuckerkandl and Pauling, 1965; Tamura et al., 2013).

### 2.8. qPCR analysis

Primers were designed to produce amplification products within the range of 100–150 nucleotides in 3'untranslated region from each gene (Table 1). Fifty-fold diluted cDNA samples were used for qPCR. qPCR