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ISOLATION AND CHARACTERIZATION OF THREE ETHYLENE PERCEPTION ELEMENTS AND THEIR EXPRESSIONS DURING LONGAN FRUIT DEVELOPMENT

JIAN-FEI KUANG[1,2] , WANG-JIN LU[2] , YUE-MING JIANG[1] AND JIAN-YE CHEN[2]*

[1]South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, P.R. China
[2]Guangdong Key Laboratory for Postharvest Science, College of Horticultural Science, South China Agricultural University, Guangzhou 510642, P.R. China

Abstract

Ethylene has long been regarded as the major regulator of fruit development. In the present work, three full-length cDNAs homologous to *Arabidopsis* ethylene perception element genes *ethylene response1* (*ETR1*), *ethylene response sensor1* (*ERS1*) and *constitutive triple response1* (*CTR1*), designated as *DlETR1*, *DlERS1* and *DlCTR1*, respectively, were isolated and characterized from fruit of longan, a non-climacteric fruit. Homology analysis showed that DlETR1 and DlERS1 proteins contained three N-terminal membrane-spanning domains and the conserved histidine kinase domain while DlCTR1 protein possessed a conserved serine/threonine kinase domain, an ATP binding site and a serine/threonine kinase catalytic site. Northern blotting demonstrated that mRNA levels of *DlETR1*and *DlERS1* gradually decreased while *DlCTR1* transcript increased steadily during fruit development. Furthermore, treatments with plant growth substances, abscisic acid (ABA) and ethrel, inhibited the accumulation of *DlERS1* transcript. In addition, ABA treatment suppressed the expression of *DlCTR1*. Thus, *DlETR1* and *DlERS1* exhibited a different response to plant growth substances. It was suggested that *DlETR1* and *DlERS1* might play a role in the early stage of longan fruit development, whereas *DlCTR1* was likely to be involved in fruit ripening.

Introduction

The plant hormone ethylene involves in a wide range of developmental and physiological responses including fruit growth and ripening (Guo & Ecker, 2004; Zhu & Guo, 2008; Mahmood *et al*., 2008). The biosynthesis of ethylene in higher plants has been well studied. Ethylene is synthesized from S-adenosyl methionine, which is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase and thence to ethylene by ACC oxidase (Yang & Hoffman, 1984; Shahroona *et al*., 2006). However, the mechanism by which the ethylene signal is perceived and transduced to mediate phenotypic responses is not understood fully (Ezura & Owino, 2008).

Ethylene perception and signal transduction have been extensively studied at the biochemical and molecular genetic levels in *Arabidopsis thaliana* and other species including some climacteric fruits like tomato, pear, banana and kiwifruit (Bleecker & Schaller, 1996; Chang *et al*., 1993; Lanahan *et al*., 1994; Lashbrook *et al*., 1998; Giovannoni, 2001; El-Sharkawy *et al*., 2003; Yin *et al*., 2008). To date, five *ETR1*-like genes, *At-ETR1* (Chang *et al*., 1993), *At-ERS1* (Hua *et al*., 1995), *At-ETR2* (Sakai *et al*., 1998), *At-EIN4* and *At-ERS2* (Hua & Meyerowitz, 1998), have been identified in *Arabidopsis*. Functional *ETR1* homologues have been isolated from several plant species (Lanahan *et al*., 1994; Sato-Nara *et al*., 1999; MÜller *et al*., 2000). Some study indicates that ethylene receptor genes are differentially regulated throughout plant development in *Arabidopsis* and tomato (Hua & Meyerowitz, 1998; Lashbrook *et al*., 1998). Importantly, *At-ERS1* (Hua & Meyerowitz, 1998) and *NR* (Wilkinson *et al*., 1995; Payton *et al*., 1996)

*Corresponding author E-mail: chenjianye@scau.edu.cn
Phone: (+ 86)-020-85280229; Fax: (+86)-020-85282107

are involved in the autoregulation of ethylene perception as their expression is up-regulated by the phytohormone. Ethylene receptor binding leading to a plant response depends on the ethylene signal transduction pathway. The *Arabidopsis* protein, At-CTR1, is one of the early elements of this pathway (Kieber *et al*., 1993). It has been shown to be a Raf-like Ser/Thr protein kinase (MAPKK kinase) and, as the ethylene receptor proteins, is a negative regulator of the ethylene response. Mutant forms of *ctr1* confer a constitutive ethylene response in air (Kieber *et al*., 1993). However, there is little information available on the isolation and characterization of the ethylene perception elements in non-climacteric fruit.

Longan (*Dimocarpus longan* Lour.), a non-climacteric fruit, is highly attractive with its commercial value in the international trade (Jiang *et al*., 2002). Plant hormone ethylene is considered as the driving ripening process of climacteric fruit (Abbasi *et al*., 2009) while the ethylene regulation involving in the ripening of non-climacteric fruit remains unknown. In non-climacteric citrus, fruit evolves very low amounts of ethylene during ripening but can respond to exogenous ethylene, as indicated by the changes in the ripening-related pigments and respiration (Goldschmidt *et al*., 1993). Recent study has reported that young citrus fruitlets behave as climacteric fruits equipped with a system II-like ethylene biosynthesis activity, but the fruit loses its potential climacteric-like nature and retains system I activity during growth and maturation (Katz *et al*., 2004). In addition, Trainotti *et al*., (2005) has found that the expression of two type I ethylene receptor genes (i.e., *FaETR1* and *FaERS1*) shows a continuous increment during fruit ripening, suggesting that the ripening of strawberry is somewhat similar to climacteric fruits. These results indicate that the relationship between ethylene and ripening of non-climacteric fruit needs to be well documented.

In the present work, the isolation and characterization of three putative longan fruit ethylene perception response elements, including two ethylene receptors and a CTR1-like protein, were described. In addition, their expression profiles during longan fruit development in relation to their responses to plant growth regulators such as abscisic acid (ABA) and ethylene releaser (ethrel) treated at ripening stage were also investigated. This study can help understand and elucidate the mechanism of non-climacteric fruit development based on the point of ethylene perception.

Material and methods

Plant materials: Ten 5-year-old longan trees of 'Shijia' from a commercial orchard near Guangzhou, China were chosen for this experiment. Developing fruit located in different directions of each tree were collected weekly and were then sampled once a week for a period of 9 weeks, beginning at 14 days after anthesis (DAA) and ending at 77 DAA. Whole fruit tissues (including pericarp, aril and seed) before 35 DAA (14, 21, 28 and 35 DAA) while the separated aril tissues at 42 DAA and thereafter were collected, frozen in liquid nitrogen and then stored at -80°C until use.

ABA and ethrel treatment: Six 5-year-old longan trees were used from the same orchard described above. About 400 fruits at 77 DAA located in different directions of each tree were tagged and dipped for 1 min in a solution containing 0 (control), 200 mg/L ABA or 500 mg/L ethrel. After 0, 6, 12, 24, 36 and 48 hours of each treatment, 60 randomly selected fruits were detached and excised, and the aril tissues were then frozen in liquid nitrogen and stored at -80°C prior to analysis.

RNA extraction and isolation of longan full lengh cDNAs encoding ethylene perception elements: Frozen tissues (10 g) were ground to a fine powder in a mortar using a pestle in the presence of liquid nitrogen. Total RNA was extracted using the hot borate method of Wan & Wilkins (1994). The extracted total RNA was used as templates for RT-PCR. The product (the first-strand cDNA) was subjected to PCR amplification. Degenerate primers of *ETRs* and *ERSs* (i.e., sense: 5′-GAGACG GGHAGRCATGTNAGRAT-3′ and antisense: 5′-CATGGGMGTTCTCATTTCATGRTTCAT-3′) and *CTRs* (i.e., sense: 5′-ATGGAGC AAGAYTTY CATGCTGAGCG-3′ and antisense: 5′-ATCTCGMTKAACTTCNGGT GCCATCC-3′) were designed with reference to the conserved amino acids sequences of *ETRs*, *ERSs* and *CTRs*, respectively. Reactions for the RT-PCR were subjected to one cycle of 94°C for 3 min, 35 cycles each at 94°C for 1 min, 45°C for 2 min and 72°C for 2 min, and then one cycle of 72°C for 10 min. PCR products of the predicted size were purified and cloned into pGEM-T easy vector (Promega, USA). The nucleotide sequences of the cDNA inserts were determined using the thermo sequenase dye terminator cycle sequencing kit and a 3730 DNA sequencer (PerkinElmer Applied Biosystems).

Consequently, 3′- or 5′- rapid amplification of cDNA ends (3′- or 5′-RACE-PCR) was performed using cDNA amplification kits (Takara, Shiga, Japan) according to the manufacturer's protocol. In order to amplify 3′-end and 5′-end fragments, the specific primers were designed based on the nucleotide sequences of the cDNA fragments already cloned by RT-PCR and are shown in Table 1. The 3'- and 5'- RACE-PCR products were cloned and sequenced as described above.

DNA sequence analysis, alignment and comparisons: Identification of nucleotide sequences from RT-PCR clones were established using the NCBI Blast program [http://www.ncbi.nlm.nih.gov/BLAST]. Alignment and comparison of sequence were made using the ClustalW program (http://www.ebi.ac.uk/clustalw). Open reading frame and protein prediction were made using NCBI ORF Finder [http://www.ncbi.nlm.nih.gov/gorf/gorf.html]. The mass values for mature peptides were calculated using the PeptideMass program [http://us.expasy.org/tools/peptide-mass.html].

Northern blot analysis: Total RNA (10 μg) was separated on a 1.2% agarose-formadehyde gel and blotted onto positively-charged nylon membrane (Biodyne®B, 0.45 μm, PALL Co. Sarasota, FL). The RNA was fixed to the membrane by baking for 2 h at 80°C and then cross-linked to the membranes using an ultraviolet cross linker (Amersham Biosciences, Piscataway, NJ). The membranes were prehybridized for more than 3 h in SDS buffer [50% deionized formamide (v/v), 5 × SSC, 7% SDS, 2% blocking reagent (Roche Diagnostics, Mannheim, Germany), 50 mM sodium-phosphate (pH 7.0) and 0.1% N-lauroylsarcosine (w/v)], and then hybridization was performed overnight in the same buffer containing the gene-specific digoxin (DIG)-labeled probes at 45°C. Probes were prepared with a DIG probe synthesis kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. All probes were synthesized from the 3′-untranslated regions of these genes. Following hybridization, membranes were washed twice for 10 min with 2 × SSC containing 0.1% SDS at 25°C, followed by washing twice for 30 min in 0.1 × SSC containing 0.1% SDS at 62°C. The signals were detected with chemiluminescence using CDP-Star ™ (Roche Diagnostics) as described by the manufacturer. The specific primers used for synthesis of DIG-labeled probes are listed in Table 2.

Table 1. The sequences of specific primers used for RACE of *DlETR1*, *DlERS1* and *DlCTR1* genes.

Genes	Oligonucleotide sequences
DlETR1- 3RACE1	5′- CTGTGTCTCAATCACTGCCT -3′
DlETR1- 3RACE2	5′- GTCAAAGATTCAGGAACAGG -3′
DlERS1- 3RACE1	5′- GATTCTCCCAACCAACAGTG -3′
DlERS1- 3RACE2	5′- TCTGGAAGAGTCTATGCGGG -3′
DlCTR1- 3RACE1	5′-TCACTCAGCCCCCAAACTTG-3′
DlCTR1- 3RACE2	5′-GTGCGAGAGAGGTGTTGGAT-3′
DlETR1- 5RACE1	5′- ATCCACAAGGCACACTCTTCCAG -3′
DlETR1- 5RACE2	5′- GACACCACAGCAGTCAAAACCT -3′
DlERS1- 5RACE1	5′- CTCCCTGTCAAGTTCTTCGGTCT -3′
DlERS1- 5RACE2	5′- GTGAACAAGCAACAAAGCAGTCG -3′
DlCTR1- 5RACE1	5′- CAAGTTTGGGGGCTGAGTGA -3′
DlCTR1- 5RACE2	5′- ATCCAACACCTCTCTCGCAC -3′

Table 2. The sequences of specific primers used for the syntheses of DIG-labeled *DlETR1, DlERS1* and *DlCTR1* probes.

Genes	DIG-For primers	DIG-Rev primers
DlETR1	5′-ACTGAGTTGACACCTGAGCA-3′	5′-CAAAGGCAGTGATTGAGACA-3′
DlERS1	5′-GAACCACGAAATGAGGACAC-3′	5′-GGGACACCACAGCCTGAATC-3′
DlCTR1	5′-ATCTCCCACACCTCAGCCAA-3′	5′-GCTCAAGTGATGTGCTATGC-3′

Results and Discussion

Isolation and characterization of *DlETR1* and *DLERS1*: In this study**,** two full-length cDNAs encoding ethylene receptors, designated as *DlETR1* and *DlERS1*, were obtained from longan fruit using a combination of RT-PCR and RACE. *DlETR1* cDNA (3230 bp) and *DlERS1* cDNA (2385 bp) consisted of a 5'-untranslated region of 671 bp, an open reading frame (ORF) of 2220 bp, and a 3'-untranslated region of 339 bp, a 5'-untranslated region of 171 bp, an ORF of 1908 bp and a 3'-untranslated region of 306 bp, respectively. They encoded the predicted polypeptides of 740 and 636 amino acids, with the predicted molecular weights of 94.7 and 70.9 kDa, respectively. A BLAST search of GenBank revealed that *DlETR1* shared a high homology with corresponding homologues from other fruit such as mango *MiETR1* (AAF61919, 90% identity) and pear *PcETR1* (AAL66202, 89% identity) while *DlERS1* was homologous to *CsERS1* (AAC99435, 78% identity) and *CpETR1* (AAG41977, 78% identity) isolated from citrus and papaya fruits, respectively, at the protein level.

The optimal multiple sequence alignments of *DlETR1* and *DlERS1* proteins with other homologies are presented in Fig. 1A. It has been well documented that the *ETR1* and *ERS1* genes have three putative membrane-spanning subdomains in the N-terminus, and a histidine (His) kinase domain in C-terminal region (Chang *et al*., 1993; Hua *et al*., 1995). The three predicted transmembrane hydrophobic amino acid sequences at the N-terminus were ^{26}FFIALAYFSIPLELIYFV^{43}, ^{53}WVLIQFGAFIVLCGATHLI^{71} and ^{83}VAIVMTVAKVSTA GVSCATALMLGHIIPDL^{112} according to Hua *et al*., (1998) and Huang *et al*., (2007). As shown in Fig. 1, these three subdomains could be observed in the N-terminus of *DlETR1* and *DlERS1*. In addition, the conserved histidine kinase domain in the C-terminus was also found in both *DlETR1* and *DlERS1* genes. *DlETR1* and *DlERS1* were similar in their coding regions, except that *DlERS1* did not appear to contain a receiver domain (616–733 AA in *DlETR1*). These results indicated that these two ethylene receptor genes of longan fruit shared common features with *ETRs* and *ERSs* obtained from other plants.

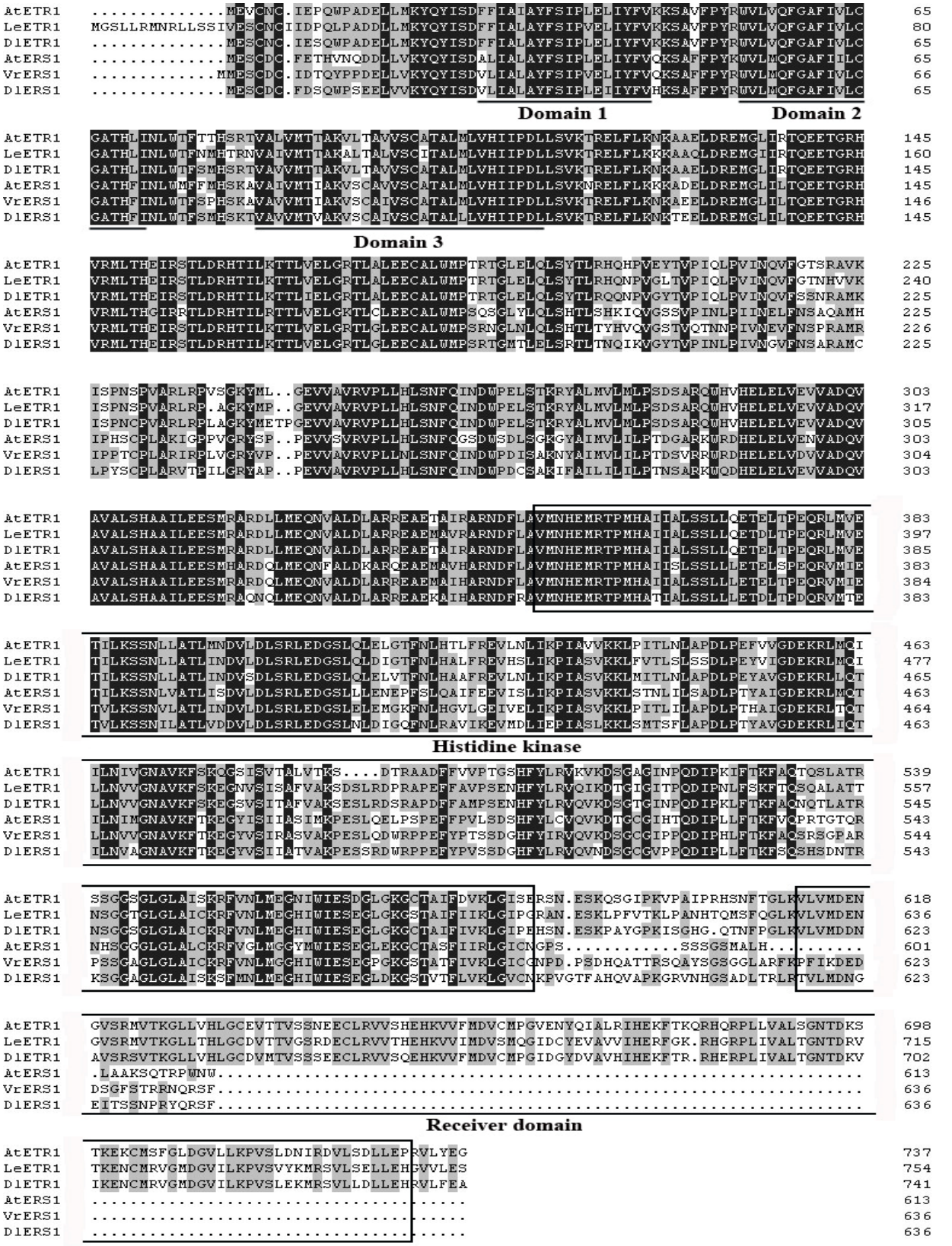

Fig. 1. Alignments of *DlETR1* and *DlERS1* predicted proteins with *Arabidopsis thaliana AtETR1*, *AtERS1*, tomato *LeETR1* and mung bean *VrERS1* proteins. Black shading identified fully conserved residues by the six proteins. Conservative amino acid substitutions were represented by gray shading. Gaps were introduced to optimize alignment. Multiple alignments were made by CLUSTALW, then viewed with BOXSHADE program, and finally manually edited. Three transmembrane hydrophobic amino acid sequences were underlined. The histidine kinase domain and receiver domain were shown in boxes.

Isolation and characterization of *DlCTR1*: *DlETR1* cDNA (3112 bp) consisted of a 5'-untranslated region of 127 bp, an open reading frame (ORF) of 2574 bp, and a 3'-untranslated region of 411 bp. The *DlCTR1* gene encoded a putative protein of 858 amino acids with a predicted molecular weight of 83.04 kDa and an isoelectric point of 7.11.

A BLAST search of GenBank revealed that DlETR1 shared the highest identity of 73% with that of RhCTR1 (AAK40361) from rose and PpCTR1 (ACR2362) from peach. Detailed analysis of the *DlCTR1* proteins with other homologies revealed that the predicted CTR1 protein was a serine/threonine protein kinase that was closely related to the Raf protein kinase family (Schenk & Jagalska, 1999; Kuroda *et al*., 2003). As predicted, the DlCTR1 polypeptide exhibited a complete serine/threonine kinase domain spanning from 584-841 AA containing the ATP-binding site motif (590−611 AA), and the serine/threonine kinase catalytic motif (705−711 AA) (Fig. 2).

Accumulations of *DlETR1*, *DlERS1* and *DlCTR1* mRNAs in aril tissues during longan fruit development: The development of longan fruit can be clearly divided into three stages according to the growth curve (data not shown). Stage I from 0 to 42 DAA showed a slow growth period and mainly maintained pericarp growth, while Stage II was a rapid growth phase from 42 to 70 DAA, in which the fruit primarily exhibited rapid aril growth whereas the pericarp and seed grew slowly, and Stage Ш from 70 to 77 DAA was mature stage and the fruit ceased growing almost (Feng *et al*., 2008). Thus, the maturity of longan fruit can mainly be determined by the aril change (Jiang *et al*., 2002). To understand the possible role of *DlETR1*, *DlERS1*, and *DlCTR1* in fruit development, their expression patterns in aril tissues during fruit development were examined by northern blot analysis. As shown in Fig. 3, *DlETR1*, *DlERS1* and *DlCTR1* exhibited different expression profiles during fruit development. *DlETR1* and *DlERS1* genes were highly expressed at the early stages of fruit development (from 14 to 35 DAA) (Fig. 3). In contrast, a slow increase in the *DlCTR1* transcript levels was observed following fruit development (Fig. 3). A similar expression pattern during fruit development was also reported for other *CTR1* genes, including tomato *LeCTR1* (Adams-Phillips *et al*., 2004), pear *PcCTR1* (El-Sharkawy *et al*., 2003) and kiwifruit *AdCTR1* (Yin *et al*., 2008). Thus, these results suggested that the expression of *DlETR1* and *DlERS1* might play an important role in the early fruit development while *DlCTR1* might be involved in fruit ripening.

Regulation of *DlETR1*, *DlERS1* and *DlCTR1* by ABA and Ethrel treatments: ABA and ethylene are related to fruit ripening. Ethylene plays a critical role in regulating ripening of climacteric fruits (Bondad *et al*., 1970; Brady, 1987). ABA is considered to exhibit the key role in control ripening in some non-climacteric fruits (Coombe & Hale, 1973; Wang *et al.*, 2007). To examine the effect of ABA and ethylene, the accumulation patterns of *DlETR1*, *DlERS1* and *DlCTR1* transcripts in aril tissues of longan fruit at 77 DAA were analyzed. As shown in Fig. 4, ABA and ethrel treatment exhibited little or no effect on the accumulation of *DlETR1* while the accumulation of *DlERS1* was obviously inhibited at 6, 12, 24 and 48 hours after ABA and ethrel treatments. Only ABA treatment suppressed the expression of *DlCTR1* at 2, 6, 12, 24 and 48 hours. In *Arabidopsis*, *AtCTR1* was also repressed by ABA treatment (Arroyo *et al*., 2003). These results suggested that *DlETR1*, *DlERS1*, and *DlCTR1* genes in longan fruit responded differentially to ABA and ethylene treatments.

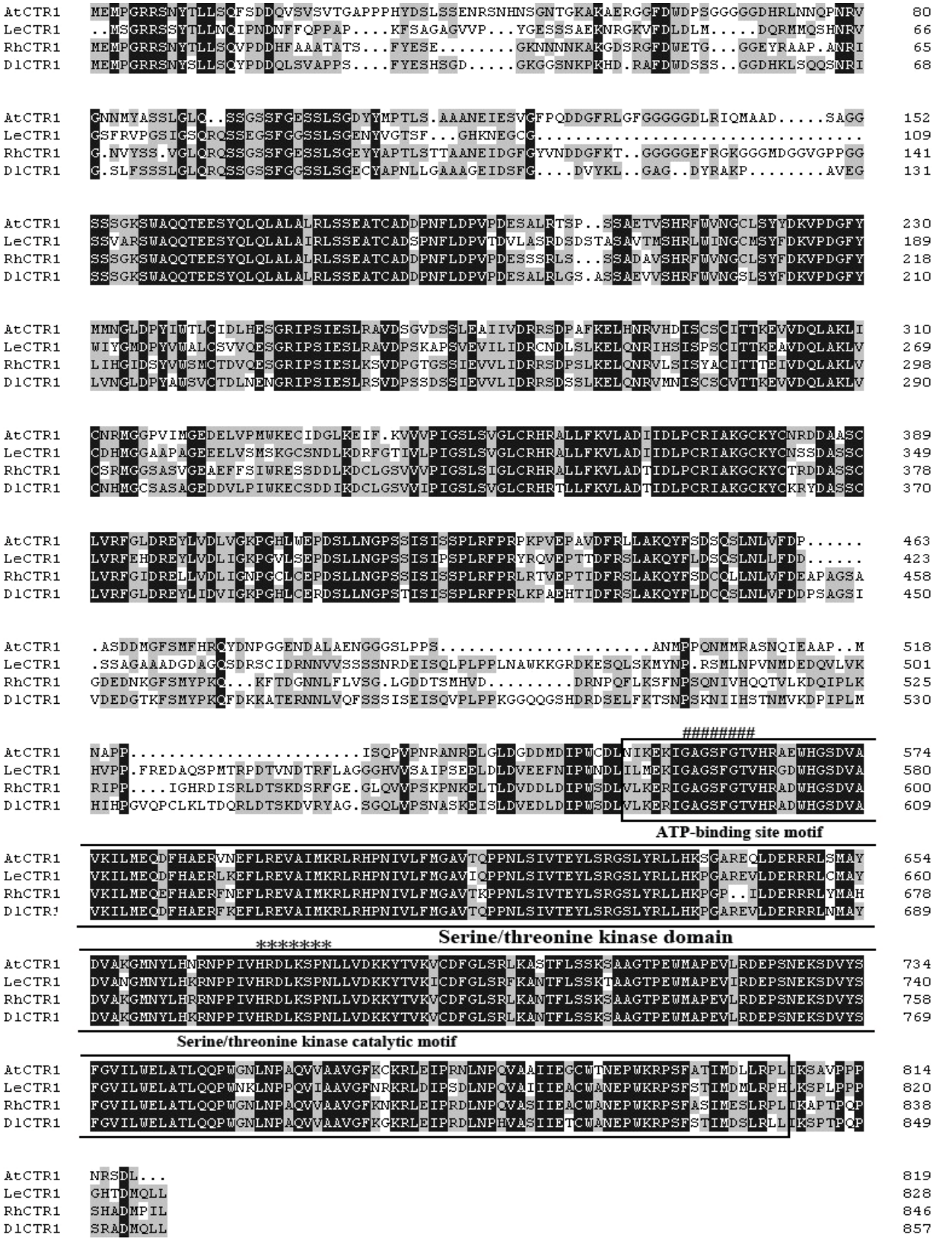

Fig. 2. Alignment of *DlCTR1* predicted protein with *Arabidopsis thaliana AtCTR1*, tomato *LeCTR1*, and rose *RhCTR1* proteins. Black shading identified fully conserved residues by the four proteins. Conservative amino acid substitutions were represented by gray shading. Gaps were introduced to optimize alignment. Multiple alignments were done by CLUSTALW, then viewed with BOXSHADE program, and finally manually edited. The serine/threonine kinase domain in the C-terminal region was boxed. The ATP-binding site motif (GAGSFGTV) and the serine/threonine kinase catalytic motif (HRDLKSPN) were marked with '#' and '*', respectively.

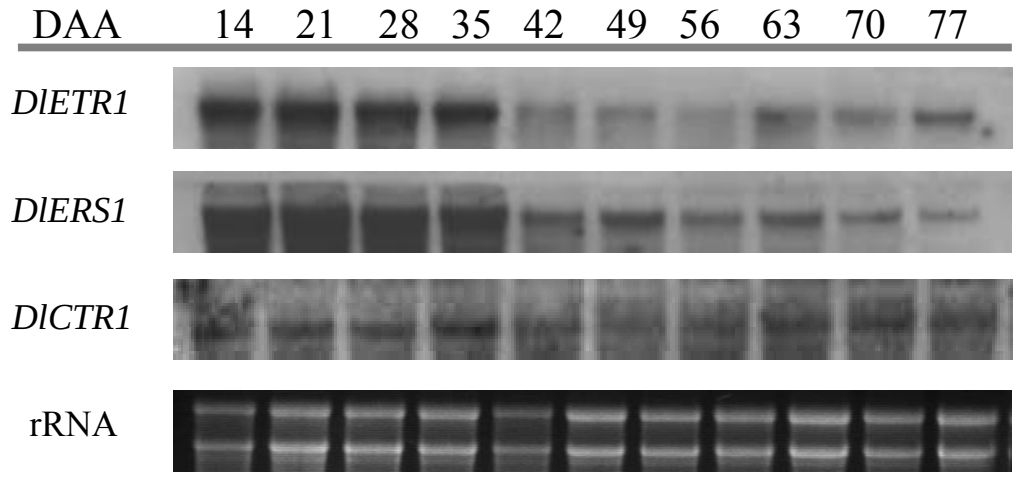

Fig. 3. Differential expression patterns of *DlETR1*, *DlERS* and *DlCTR1* in aril tissues of longan fruit at different developmental stages. Total RNA (10 μg per lane) was used for northern blot analysis and hybridized with gene-specific DIG-labeled probes. Ethidium bromide-stained rRNA was shown as the loading control.

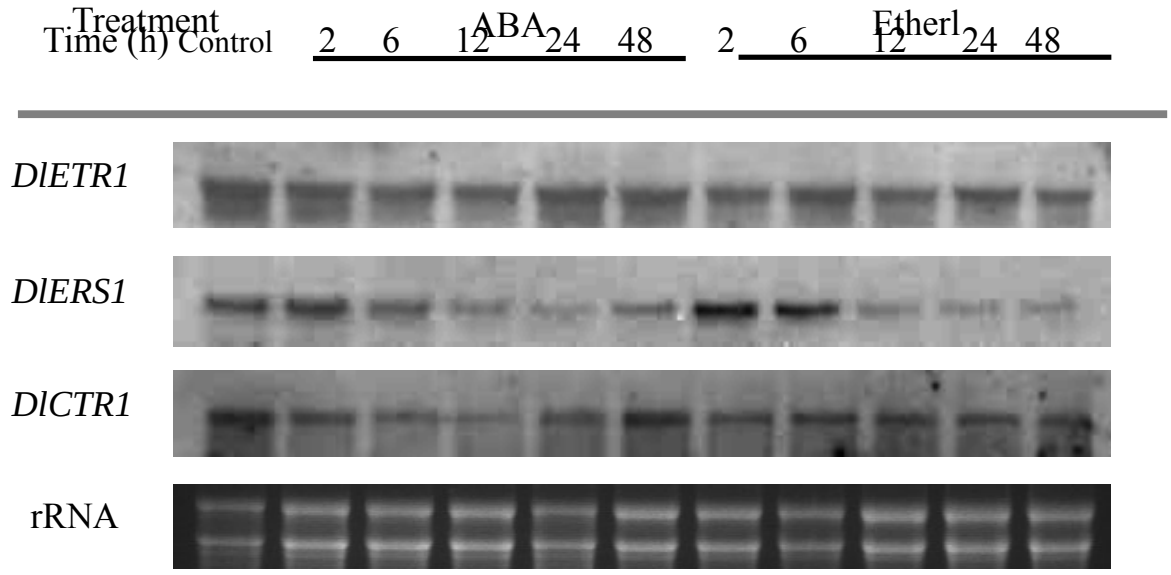

Fig. 4. Differential regulations of *DlETR1*, *DlERS1* and *DlCTR1* in aril tissues of longan fruit after ABA and ethrel treatments at 77 DAA. Fruit were dipped for 1 min in a solution containing 0 (control), 200 mg/L ABA or 500 mg/L ethrel. Total RNA (10 μg per lane) was used for northern blot analysis and hybridized with DIG-labeled probes. Ethidium bromide-stained rRNA was shown as the loading control.

Acknowledgements

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