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The molecular characterization and expression analyses of ethylene receptor genes from watermelon fruit

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Abstract: Watermelon fruit is very sensitive to ethylene, showing rapid deterioration and water-soaking upon ethylene exposure. The objective of this study was to isolate and determine the expressions of ethylene receptor genes in watermelon fruit. As a result of PCR amplification of cDNAs obtained via RT-PCR of total RNA from watermelon fruit with degenerate primers, 3 partial cDNAs were obtained. After extension of the 3' and 5' ends of the cDNAs via 3' and 5' RACE analysis, 3 full-length genes designated as *CL-ETR1*, *CL-ERS1*, and *CL-ETR2* were obtained. Upon cloning and sequencing, it was determined that the *CL-ETR1*, *CL-ERS1*, and *CL-ETR2* genes were 2343, 2349, and 2550 bp in length, respectively. The structure and sequence analysis showed that the isolated genes had conserved domains found in previously isolated ETR genes. Southern blot analysis showed a single band for *CL-ETR1* and *CL-ETR2* and 2 bands for *CL-ERS1*. Northern blot analyses showed that the expression levels of all 3 receptor genes significantly increased during postharvest storage and in response to ethylene exposure. 1-Methylcyclopropene (1-MCP) treatment significantly reduced the expressions of all receptor genes. The genes also showed different expression patterns during fruit development. The isolated genes could be used in biotechnological studies to develop cultivars with altered ripening behavior that are more tolerant to ethylene-related physiological disorders such as water-soaking.

Key words: Ethylene, gene, receptor, watermelon

1. Introduction

Ethylene is a plant hormone coordinating the plant's responses to physiological events such as senescence, fruit ripening, and fruit abscission, and environmental stresses including water deficit, mechanical wounding, and pathogen attack (Lelievre et al., 1997). Although vegetables are low ethylene-producing species, most of them easily lose their quality and market value upon ethylene exposure. Therefore, vegetables are considered as ethylene-sensitive crops. When exposed to ethylene, the development, ripening, and senescence of vegetables enhance and thus their quality and shelf life decrease (Kasım and Kasım, 2007).

It has also been shown that ethylene causes a physiological disorder known as water-soaking in fruits of Cucurbitaceae (Karakurt and Huber, 2002, 2004; Mao et al., 2004; Lima et al., 2005; Huber, 2008). Studies with immature and ripe watermelon fruits exposed to ethylene reported that fruit cell walls showed extensive solubilization and depolymerization, and the activities and transcript levels of cell wall-degrading enzymes, including polygalacturonase, expansin, and galactosidases, and those of membrane-degrading enzymes including lipoxygenase and phospholipases demonstrated significant increases with ethylene exposure (Karakurt and Huber, 2002, 2004). Exposure of watermelon fruit to ethylene has also caused extensive degradation in phospholipids (Mao et al., 2004). Similar results have also been reported for cucumber fruit (Lima et al., 2005). Moreover, exposure of watermelon fruit to ethylene resulted in differential expression of several gene fragments (cDNAs) participating in ethylene perception and signal transduction (Karakurt and Huber, 2008).

A significant progress in ethylene signal transduction has been achieved with ethylene mutants in *Arabidopsis thaliana* and tomato, and several genes encoding ethylene receptor homologs have been determined (Guzman and Ecker, 1990; Kieber and Ecker, 1993). The characterization of these genes provided the opportunity to determine the expression of ethylene receptors in different organs, species, and developmental stages (Nara et al., 1999). Wilkinson et al. (1997) reported that transgenic plants

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carrying a mutant *etr1-1* gene of *A. thaliana* demonstrated a significant delay in fruit ripening and flower senescence. Thus, genetic manipulation of ethylene receptors could be a good alternative to control ethylene production and delay ripening in fruits and vegetables.

Although important progress has been achieved in ethylene perception and signal transduction in plant tissue (Bleecker and Schaller, 1996), to our knowledge there is no study reporting the characterization of ethylene receptors in watermelon fruit. Thus, the cloning and characterization of ethylene receptor genes in watermelon fruit will give the opportunity to both prevent the physiological disorder of water-soaking and delay ripening, and it will help in understanding the role of ethylene in many physiological processes.

The objectives of this study were to isolate and determine the expression of ethylene receptor genes in watermelon fruit.

2. Materials and methods

2.1. Plant material and RNA isolation

Watermelon fruit samples (*Citrullus lanatus* 'Crimson Sweet') were obtained from a commercial firm (Isparta, Turkey) and used as plant material in the study. Fruits were harvested at the commercial ripening stage (Karakurt and Huber, 2002, 2004) and immediately transported to the laboratory at the Faculty of Agriculture, Süleyman Demirel University, Isparta, Turkey. Fruits were surface-sterilized and treated with 50 µL/L ethylene for 24 hours at 20 °C as previously described (Karakurt and Huber, 2002). Samples were taken from the placental tissue and frozen in liquid nitrogen. Some of the fruits were also treated with 10 µL/L 1-methylcyclopropene (1-MCP) as previously described (Mao et al., 2004). Moreover, fruit tissue samples were also obtained during the developmental period (15–75 days after full flowering) and frozen in liquid nitrogen. Total RNA was isolated from the frozen tissue samples as previously described (Karakurt and Huber, 2004).

The RNA samples from fruit tissue were treated with DNase in order to remove any residual DNA using a commercial kit (Ambion). The DNase-treated RNA samples were converted to cDNA with an Advantage RT-PCR kit following the manufacturer's instructions (Clontech).

2.2. Isolation of ethylene receptor gene fragments

Degenerate primers of 24–37 bp were designed with the primer BLAST program using ethylene receptor gene sequences previously isolated from *Arabidopsis*, tomato, cucumber, and melon (accession numbers: AF13979, AF16250, U21952, U38666, U63291, L24119, U41103) and were synthesized (Metis Biyoteknoloji, Ankara, Turkey). Primers used in PCR reactions to isolate receptor genes were: F1 (5'-GGA TCC AAA ACT CGA GAA TTG ATT CTT AAA AAT AAG GC-3' and R1 (5'-AAG CTT TCA TCA GTC CAC GAG CTG ATC ACG CGC CCG C-3'); F2 (5'-GGA TCC AAA ACT AGA GAG CTC TTT TTG AAG AAC AAG-3') and R2 (5'-AAG CTT TCA TCA CTC CAT TAA GAG ATC TCT AGC CCT C-3'); ETR1-F (5'-GAG ACG GG[ATC] AG[AG] CAT GT[AGCT] AG[AG] ATG-3') and ETR1-R (5'-CAT GGG [AC]GT TCT CAT TTC ATG [AG]TT CAT-3'); and ETR2-F (5'-CAG AAT TGT GCG GTT TGG ATG CCG-3') and ETR2-R (5'-CAC AAC TTT AAC AAT CTC AAT CTC CTG-3').

To isolate ethylene receptor gene fragments, PCR amplification was performed with the designed primers and watermelon cDNA following the procedure previously described (Basset et al., 2002; El-Sharkawy et al., 2003). PCR amplification products were run on 1.2% agarose gel and 5 amplification products with molecular sizes of 1402, 1190, 740, 528, and 463 bp were obtained. These cDNA fragments were cut from the gel with a sterile razor blade and extracted from the gel using the NucleoTrap gel extraction kit following the manufacturer's instructions (Clontech). The cDNAs were then cloned with the TOPO TA cloning system (Invitrogen). The recombinant colonies were grown overnight at 37 °C and their plasmids were isolated with the PureLink Quick Plasmid Miniprep Kit (Invitrogen) and sequenced (İontek, İstanbul, Turkey). The sequences were compared to the sequences deposited in the NCBI GenBank with the BLAST program, and their homology to the ethylene receptor gene sequences previously isolated from other species was confirmed (Altschul et al., 1997). Gene-specific primers were designed from these sequences and synthesized (Metis Biyoteknoloji). These primers were used in RACE analysis in order to obtain the full-length sequences of the genes. RACE analysis was conducted using the SMART RACE cDNA amplification kit following the manufacturer's instructions (Clontech). Finally, complete full-length cDNAs were amplified using gene-specific primers derived from both ends of the corresponding cDNA sequences determined above.

2.3. Southern blot analysis

Southern blot analysis was performed as described by Sambrook et al. (1989). For this purpose, DNA samples (30 µg) were digested separately with *Eco*RI and *Xba*I restriction enzymes. Digested DNA was separated on 0.8% (w/v) agarose gel and transferred to a Hybond-N+ nylon membrane (Amersham). The blots were hybridized overnight at 45 °C with gene-specific probes containing the 3'-untranslated region (UTR) of the ethylene receptor cDNAs. The DNA probes were labeled with DIG using the DIG DNA labeling kit (Roche). After hybridization and washing, signals were detected using a CDP-Star Kit (Roche) following the manufacturer's instructions.

2.4. The expression analysis of receptor genes

To determine the expressions of receptor genes during storage with northern blot analysis, watermelon fruits (Crimson Sweet) were obtained from a local watermelon production company. The fruits were harvested at commercial ripening stage (Karakurt and Huber, 2002) and stored at 20 °C for 10 days. Fruit samples were removed 4, 8, and 12 h and 1, 2, 4, 6, 8, and 10 days after storage. Total RNA was extracted from each sample (Karakurt and Huber, 2004) and 20 µg of total RNA was used for northern blot analysis (Karakurt and Huber, 2004). Moreover, total RNA was also extracted from fruit treated with ethylene and 1-MCP and from fruit at different developmental stages and used for northern blot analysis. The receptor gene probes were labeled with DIG using the DIG High Prime labeling kit following the manufacturer's instructions (Roche). The results were analyzed with a gel imaging system (Kodak) and converted to numerical values. The expression level of each transcript in the control fruit (day 0, the fruit at the commercial ripening stage) was set to an arbitrary value of 1.0, to which all other time points were normalized to generate an induction value (fold induction) (Karakurt and Huber, 2004).

3. Results

PCR of watermelon cDNA with degenerate primers resulted in the amplification of 5 cDNA fragments with sizes of 1402, 1190, 740, 528, and 463 bp. The amplified fragments were cloned into TOPO-TA cloning vectors and sequenced. The nucleotide sequences of the cDNA fragments showed significant homologies at both the nucleotide and the amino acid levels to the ethylene receptor sequences previously obtained from melon, cucumber, tomato, *Arabidopsis thaliana*, and apple. Therefore, they were used for further analysis. For this purpose, primers were designed from these fragments and used in RACE analysis to obtain their full-length sequences. As a result of 3' and 5' RACE analysis, 3 of the 5 cDNA fragments were successfully amplified and their full-length sequences were obtained. The full-length sequences of these 3 genes were again cloned and sequenced. After the sequence analysis, they were confirmed to be the sequences of ethylene receptors and, based on their similarity to the receptor sequences, they were designated as *CL-ETR1*, *CL-ETR2*, and *CL-ERS1*. The full-length nucleotide and amino acid sequences of these genes were submitted to the NCBI GenBank and given the accession numbers KF670719, KF670720, and KF670721.

The sequence analysis showed that *CL-ETR1*, *CL-ERS1*, and *CL-ETR2* consisted of 2343, 2349, and 2550 bp respectively (see supplementary material: Tables S1–S3 and Figures S1–S3 on the journal's website). *CL-ETR1* contained an open reading frame (ORF) of 2226 nucleotides encoding a protein of 741 amino acids. The 5'-UTR, 3'-UTR, and a poly(A) tail of the gene consisted of 55, 49, and 13, nucleotides, respectively. At the nucleotide level, *CL-ETR1* demonstrated 98%, 97%, and 80% homology to *Cucumis melo* var. *cantalupensis CS-ETR1*, *Cucumis sativus CS-ETR1*, and *Prunus persica PP-ETR1* genes, respectively. The gene also showed 97%, 96%, and 94% homology to *Cucumis sativus* and *Cucumis melo* var. *cantalupensis*, *Prunus persica*, and *Pyrus communis* ETR1 at the amino acid level, respectively. Conserved domain analysis showed that the CL-ETR1 protein contained GAF, histidine kinase, HATPaz C super family (histidine kinase-like ATPases), and signal-receiving domains (Figure 1A). Moreover, the protein had an ATP binding site, a Mg binding site, a phosphorylation site in its histidine kinase domain, and phosphorylation, dimerization, and intermolecular recognition sites in its signal receiving domain. Transmembrane domain analysis of the gene revealed 3 strong transmembrane helices (Figure 1A).

The gene designated as *CL-ERS1* was composed of an ORF of 1914 nucleotides encoding a protein of 637 amino acids (Tables S1–S3 and Figures S1–S3). The 5'-UTR, 3'-UTR, and poly(A) tail of the gene contained 76, 348, and 11 nucleotides. The *CL-ERS1* gene showed 99%, 97%, and 96% nucleotide sequence homology to the corresponding genes of *Cucumis melo* var. *cantalupensis*, *Cucumis melo* var. *reticulatus*, and *Cucumis sativus*, respectively. In terms of amino acid sequence, CL-ERS1 demonstrated 98%, 96%, and 93% similarity to *Cucumis sativus*, *Cucumis melo* var. *reticulatus*, and *Cucumis melo* var. *cantalupensis* ERS protein sequences. CL-ERS1 possessed a GAF domain, a histidine kinase domain, and an HATPase-C domain (Figure 1B). In its histidine kinase domain, it had a phosphorylation site, ATP and Mg binding domains, and a dimerization site. Its HATPase-C domain contained histidine kinase-like ATPases and G-X-G motifs. Moreover, the CL-ERS1 protein had 3 strong transmembrane helices (Figure 1B).

The isolated ETR2-like gene, *CL-ETR2*, consisted of an ORF of 2301 nucleotides encoding a protein of 766 amino acids (Tables S1–S3 and Figures S1–S3). The gene contained 22, 211, and 16 nucleotides in its 5'-UTR, 3'-UTR, and poly(A) tail, respectively. The *CL-ETR2* gene showed 98%, 98%, and 77% homology at the nucleotide level to the ETR2 nucleotide sequences of *Cucumis sativus*, *Cucumis melo* var. *cantalupensis*, and *Pyrus pyrifolia*. Likewise, the amino acid sequence of CL-ETR2 demonstrated 98%, 98%, and 78% similarity to the ETR2-like protein sequences of *Cucumis sativus*, *Cucumis melo* var. *cantalupensis*, and *Prunus persica*. The conserved domain analysis showed that the protein encoded by the gene had a GAF domain, a histidine kinase domain, and a receiver domain. Among those, while the histidine kinase domain possessed a phosphorylation site, the receiver domain

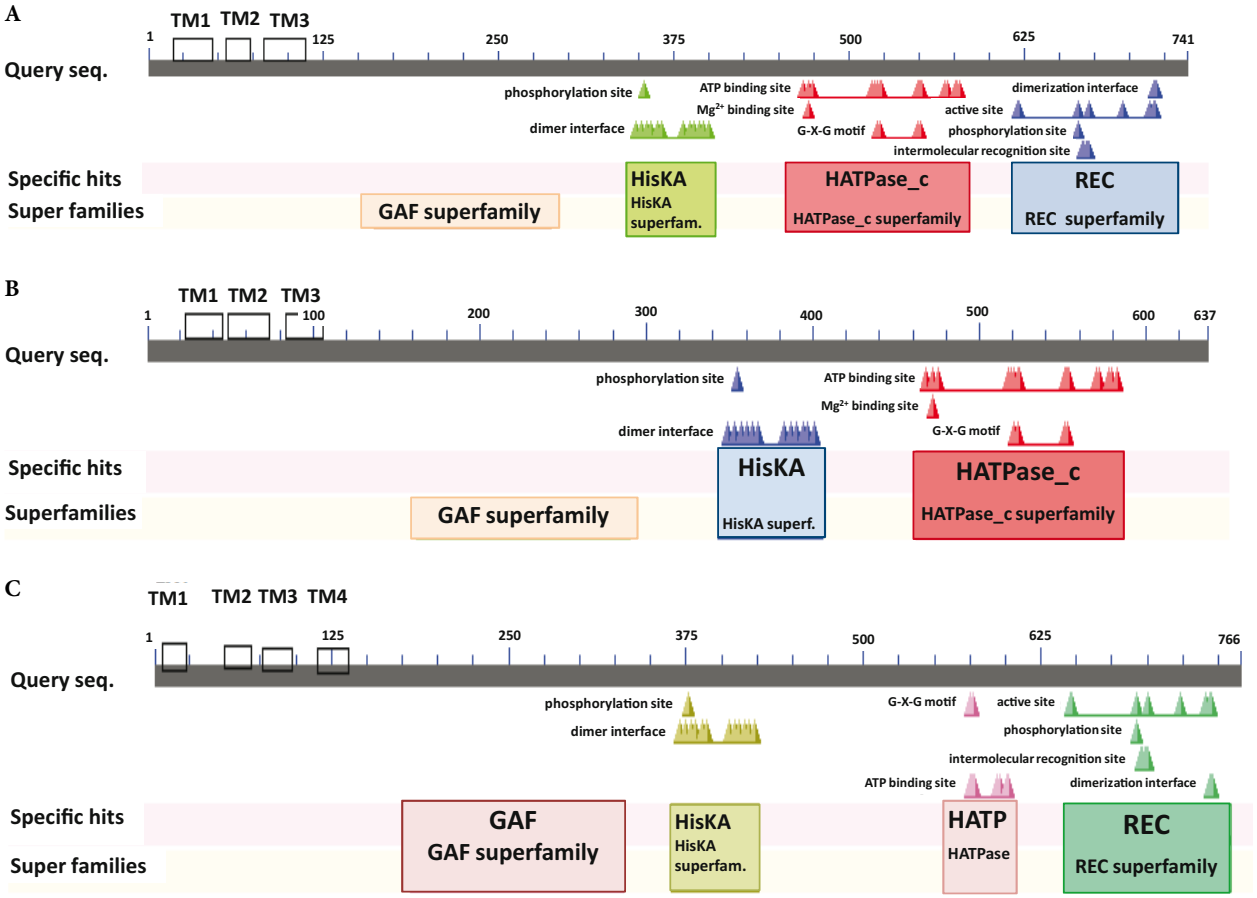

Figure 1. Domain organizations of CL-ETR1 **(A)**, CL-ERS1 **(B)**, and CL-ETR2 **(C)** receptor proteins. Transmembrane domains (TM1, TM2, TM3, and TM4) are shown as open bars.

had phosphorylation and intermolecular recognition sites (Figure 1C). Moreover, transmembrane domain analysis showed that CL-ETR2 contained 4 transmembrane helices (Figure 1C). Mg and ATP binding sites and HTPase-C-like domains found in ETR1-like genes were not available in the *CL-ETR2* gene. Likewise, CL-ERS1 did not possess the receiver domain. Considering the full-length watermelon amino acid sequences, CL-ETR1, CL-ERS1, and CL-ETR2 showed higher homologies to the corresponding proteins isolated from cucumber and melon (Figure 2).

We also compared the sequences of receptor genes to the newly released draft watermelon genome sequence. The results showed that *CL-ETR1*, *CL-ETR2*, and *CL-ERS1* corresponded to the genes designated as *Cla005764*, *Cla015104*, and *Cla021550*, respectively.

The number of genes corresponding to *CL-ETR1*, *CL-ERS1*, and *CL-ETR2* in watermelon fruit was estimated by genomic Southern blot analysis (Figure 3). When the *CL-ETR1* or *CL-ETR2* gene was used as a probe, a single band was observed in each lane of the restriction fragments from watermelon fruit. The use of the *CL-ERS1* gene as a probe revealed a single band in the *Eco*RI restriction fragment and 2 major bands and 1 minor band in the *Xba*I restriction fragments. These results suggest that genes corresponding to *CL-ETR1* and *CL-ETR2* were probably present as a single copy and the gene corresponding to *CL-ERS1* as multiple copies in the watermelon genome (Figure 3). The multiple bands produced by *CL-ERS1* could also result from polymorphisms in the genome that could produce different restriction fragments.

3.1. The expression of receptor genes

To determine the possible roles of 3 ethylene receptor genes during fruit development, we analyzed the gene expression during fruit enlargement and ripening. The results showed that the expressions of receptor mRNAs were differentially increased during fruit development (Figure 4). The *CL-ETR1* mRNA increased 15 days after full flowering (DAF) and continued to accumulate from 15 to 30 DAF (Figure 4). A second significant increase in *CL-ETR1* expression was observed during the fruit maturation step at 75 DAF. Similarly, the increase of *CL-ERS1* paralleled that of fruit size, with high levels observed at the middle stage of fruit

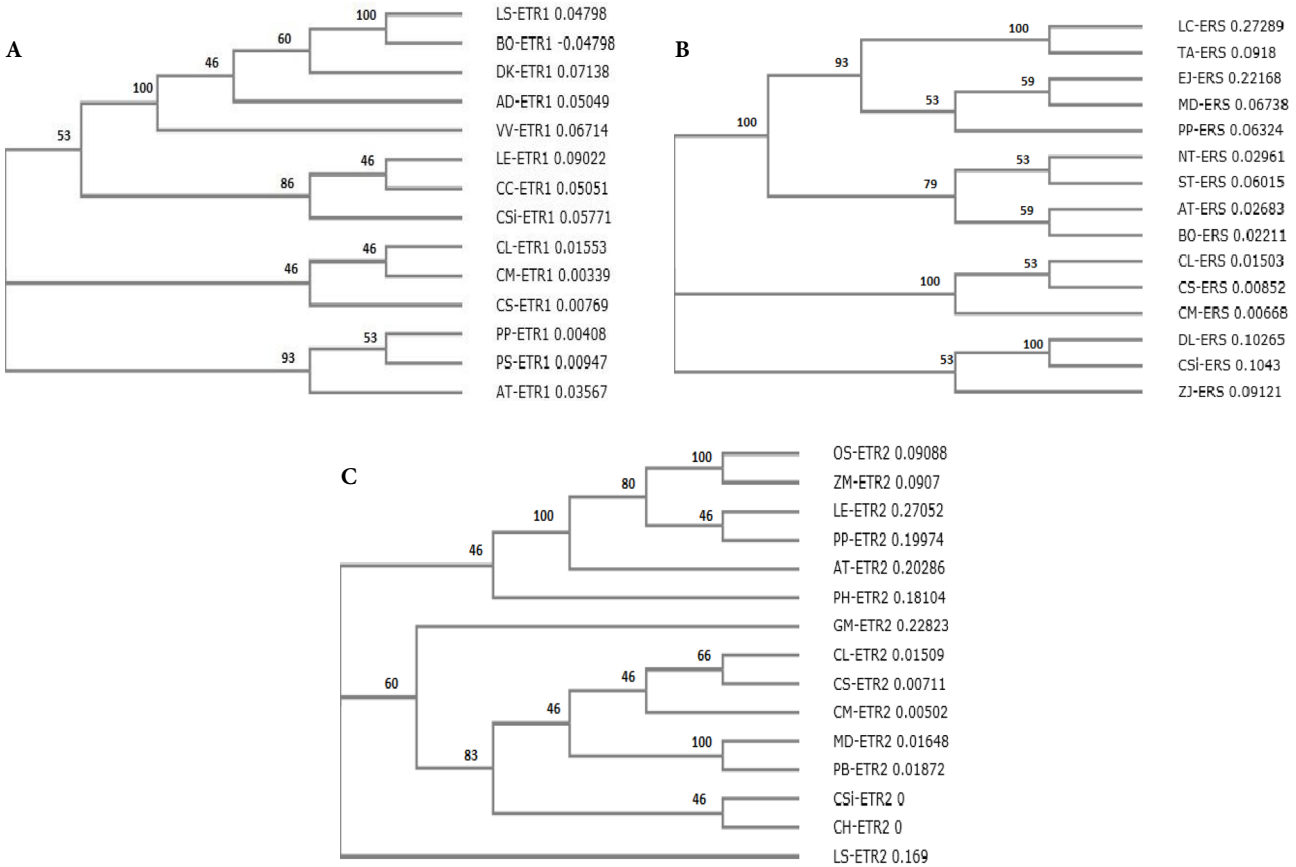

Figure 2. Phylogenetic tree of ETR1 **(A)**, ERS1 **(B)**, and ETR2 **(C)** ethylene receptor proteins isolated from different plant species. The amino acid sequences were compared using the CLUSTALW multiple alignment program, and the phylogenetic tree was generated using the neighbor-joining method. Accession numbers in GenBank sequence databases are as follows: AT-ETR1 (P49333), AT-ERS1(U21952), and AT-ETR2 (AF047975) in *Arabidopsis thaliana*; CL-ETR1 (KF670719), CL-ERS1 (KF670720), and CL-ETR2 (KF670721) in *Citrullus lanatus*; CS-ETR1 (NM_001280633), CS-ERS (AB026499), and CS-ETR2 (AB026500) in *Cucumis sativus*; CM-ETR1 (AF054806), CM-ERS1 (AB049128), and CM-ETR2 (AB294513) in *Cucumis melo*; LE-ETR1 (AF043084) and LE-ETR2 (AF043085) in *Lycopersicon esculentum*; LS-ETR2 (AF350322) in *Lactuca sativa*; MD-ERS (AY083169) and MD-ETR2 (DQ847145) in *Malus domestica*; DK-ETR1 (AB239127) in *Diospyros kaki*; AD-ETR1 (EU170628) in *Actinidia deliciosa*; VV-ETR1 (AF243474) in *Vitis vinifera*; PS-ETR1 (EF585294) in *Prunus salicina*; CC-ETR1 (EF107675) in *Coffea canephora*; BO-ETR1 (AF047476) and BO-ERS (AF047477) in *Brasscia oleracea*; DL-ERS (FJ513323) in *Dimocarpus longan*; LC-ERS (AY311484) in *Litchi chinensis*; EJ-ERS (JX307088) in *Eriobotrya japonica*; ZJ-ERS (DQ229133) in *Ziziphus jujuba*; ST-ERS (AY456694) in *Solanum tuberosum*; CSi-ERS (AF092088) in *Citrus sinensis*; PP-ERS (AF316534) in *Prunus persica*; TA-ERS (HM347272) in *Triticum aestivum*; NT-ERS (AF039921) in *Nicotiana tabacum*; OS-ETR2 (AY136816) in *Oryza sativa*; ZM-ETR2 (NM_001111382) in *Zea mays*; GM-ETR2 (NM_001248060) in *Glycine max*; PH-ETR2 (DQ154119) in *Petunia hybrida*; PH-ETR2 (KF188465) in *Pyrus bretschneideri*; CH-ETR2 (GQ339597) in *Citrus hybrida*; PP-ETR2 (XM_001769438) in *Physcomitrella patens*. Relative support for the relations was assessed with bootstrap analyses (1000 replicates; Felsenstein, 1985). The bootstrap values (%) are shown on the branches.

enlargement (between 15 to 45 DAF) and a significant decrease at the end of fruit enlargement (60 to 75 DAF). The expression of *CL-ETR2* demonstrated similar trends to the expression of *CL-ERS1*, but with higher levels of mRNA accumulation as compared to both *CL-ETR1* and *CL-ERS1* expressions (Figure 4).

To determine the expression levels of receptor genes during postharvest storage, watermelon fruits that had not been treated with ethylene were stored at 20 °C for 10 days and fruit samples were taken at different stages of the storage and used for northern blot analysis. The results showed a significant variation among various storage periods in the accumulation of transcripts (Figure 5). The mRNA levels of all 3 receptor genes started to increase at the beginning of the storage, and these increases continued up to the second day of storage in *CL-ETR1* and *CL-ETR2* and up to day 4 of storage in *CL-ERS1*. In general, the increase in *CL-ETR2* transcripts was higher than those

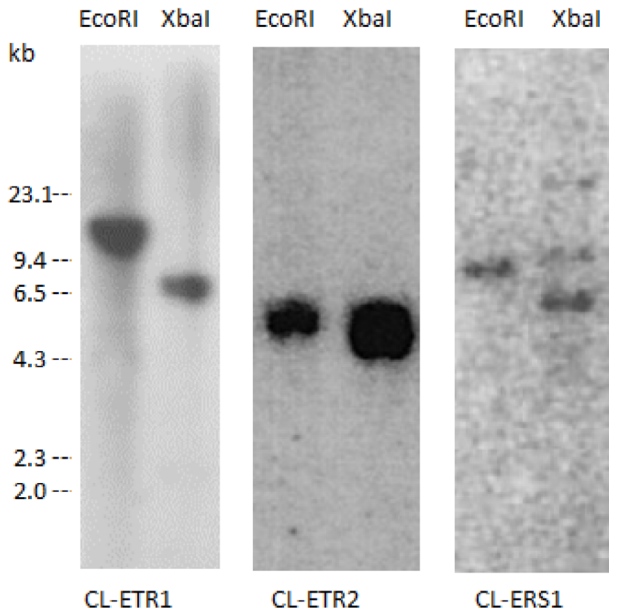

Figure 3. Genomic Southern blot analysis of the *CL-ETR1*, *CL-ETR2*, and *CL-ERS1* genes. Genomic DNA was isolated from watermelon fruit. DNA samples (10 µg) were digested with each restriction enzyme and separated on a 0.8% agarose gel. The gel was blotted onto a Hybond-N+ nylon membrane. The blots were probed with DIG-labeled fragments from the 3'-UTR of the cDNAs. The positions of DNA standards are indicated on the left.

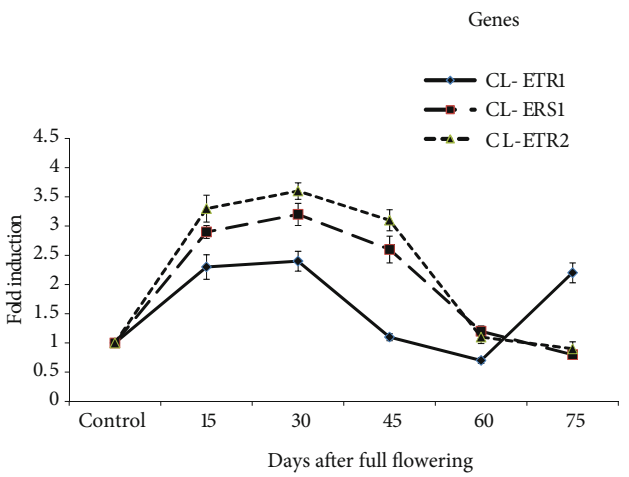

Figure 4. The mRNA transcript levels [fold induction as compared to the expression level in the fruit at the commercial ripening stage (control)] of 3 isolated ethylene receptor genes during fruit development. Data show the averages of 3 replications and standard deviations.

of the other 2 receptor genes. For example, on the second day of storage, the transcript level of *CL-ETR2* increased 3.6-fold as compared to the control (day 0). However, the increases in the mRNA levels of *CL-ETR1* and *CL-ERS1*

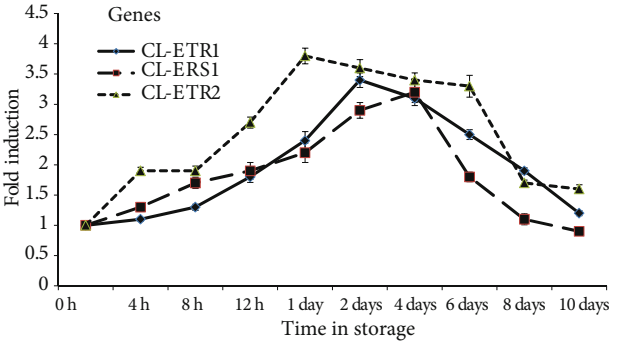

Figure 5. The changes (fold induction as compared to the control) in the mRNA transcript levels of 3 isolated ethylene receptor genes during storage at 20 °C. Data show the averages of 3 replications and standard deviations.

were 3.4- and 3.2-fold, respectively. The mRNA levels of *CL-ETR1* and *CL-ETR2* started to decrease on the second day of storage and that of *CL-ERS1* on the fourth day of storage.

In order to determine whether the expressions of genes are regulated by ethylene, we analyzed the changes in the expressions of all receptor genes in response to ethylene and 1-MCP, an ethylene action inhibitor (Sisler and Serek, 1999). The exposure of watermelon fruit to ethylene for 24 h resulted in significant increases in the mRNA levels of all 3 receptor genes (Figure 6). These increases were significantly reduced by 1-MCP treatment (Figure 6), suggesting their regulation by ethylene. We also stored some of the ethylene-treated fruit at 20 °C in order to determine whether the fruits would develop water-soaking disorder. Visual symptoms of water-soaking were evident only after 4 days of storage and the fruits were totally water-soaked after 7 days of storage (data not shown).

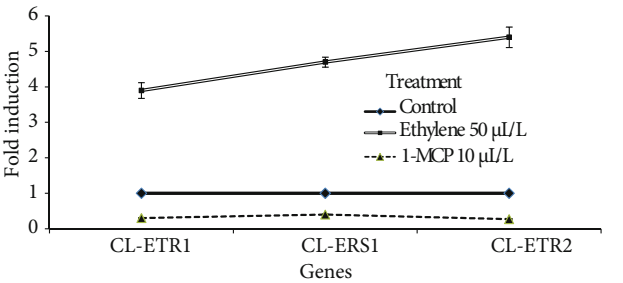

Figure 6. The changes (fold induction as compared to the control) in the mRNA transcript levels of 3 isolated ethylene receptor genes in response to ethylene and 1-MCP treatments. Data show the averages of 3 replications and standard deviations.

4. Discussion

Ethylene receptors are proteins that constitute the first step of ethylene response and the signal transduction pathway and act as negative regulators of the pathway (Kieber et al., 1993). In this study, the full-length nucleotide sequences of 3 ethylene receptor genes were isolated and characterized from watermelon fruit. The isolated genes corresponded to the *Cla005764*, *Cla015104*, and *Cla021550* genes in the watermelon genome. The isolated genes demonstrated significant homologies to the ethylene receptor genes isolated from both species taxonomically close to watermelon such as cucumber and melon and taxonomically far such as peach, tomato, and *Arabidopsis*. The genes designated as *CL-ETR1*, *CL-ERS1*, and *CL-ETR2* showed 75%–99% homology both at the nucleotide and the amino acid level to the receptor genes isolated from other species including *Cucumis melo*, *Cucumis sativus*, *Pyrus communis, Prunus persica*, *Prunus salicina*, *Prunus domestica*, and *Lycopersicon esculentum* (Wang et al., 2006; El-Sharkawy et al., 2007).

In previous studies in *Arabidopsis thaliana*, ethylene receptors were classified into 2 subfamilies (subfamily I and II) in terms of structure and sequence similarity (Hua et al., 1998; Bleecker, 1999). The structural and sequence analyses of the isolated genes showed that *CL-ETR1* and *CL-ERS1* belonged to subfamily I, and *CL-ETR2* belonged to subfamily II. The 3 isolated gene products possessed ethylene-binding domains that use Cu as the cofactor and are required for high-affinity ethylene binding, a conserved histidine kinase domain, and a GAF domain (Rodriguez et al., 1999; Wang et al., 2006). CL-ETR1 and CL-ERS1 proteins contained all motifs (H, N, G1, F, and G2) of a histidine kinase domain. However, CL-ETR2 had a degenerate histidine kinase domain, similar to the ETR2 genes isolated from other species. Among these receptors, only CL-ETR1 and CL-ETR2 had the receiver domain. A similar finding was reported for ETR1 and ETR2 genes isolated from species including cucumber, melon, pear, peach, and plum (El-Sharkawy et al., 2007). Additionally, 3 membrane-binding sites that have been reported for proteins such as ETR1 and ERS1 isolated from *Arabidopsis* were determined in CL-ETR1 and CL-ERS1 isolated from watermelon. Four potential transmembrane domains as reported for ETR2 of *Arabidopsis* were found in the amino end of watermelon CL-ETR2. The conserved aspartate amino acid found in autophosphorylated receiver domain of bacterial 2-component receptors were also available in CL-ETR1 and CL-ETR2 receptors. Other characteristics such as the GAF domain, a component of these types of receptors, was also found in watermelon CL-ETR1, CL-ERS1, and CL-ETR2. The comparison of the polypeptides obtained from these receptor sequences with the ETR1- and ETR2-like sequences from other species demonstrated a high level of conservation among amino acids in the amino termini functioning as ethylene sensing and binding (Wang et al., 2006).

The sequence analysis of the proteins encoded by the isolated receptor genes (*CL-ETR1*, *CL-ERS1* and *CL-ETR2*) showed that they possessed all amino acids required for receptor function, suggesting their participation in ethylene response and signal transduction (Wang et al., 2006). Among the isolated receptors, CL-ERS1 lacked the C-terminal receiver domain, a component of ETR1 proteins, similar to its homolog proteins in *Arabidopsis thaliana* (AT-ERS1), melon (CM-ERS), and cucumber (CS-ERS). It is thought that the aspartate amino acid in the receiver domain is the target for autophosphorylation in ETR1 receiver sequences (Wang et al., 2006).

Genomic Southern analysis with the probes of 3 receptor genes revealed single bands for *CL-ETR1* and *CL-ETR2* and multiple bands for *CL-ERS1*. A single copy number was reported for ETR1-like genes in melon (Sato-Nara et al., 1999) and ETR1- and ETR2-like genes in cucumber (Yamasaki et al., 2000). Cucumber and melon ERS1-like genes were also available in multiple copies (Sato-Nara et al., 1999; Yamasaki et al., 2000). However, 5 ETR1-like genes have been reported in Arabidopsis (Hua et al., 1998; Sakai et al., 1998) and 5 ETR1 homologs have been reported in tomato (Yen et al., 1995).

The isolated genes also shared similar expression trends during postharvest storage. All 3 genes showed significant increases in mRNA transcript levels during storage. This possibly resulted from an increase in ethylene production during storage in watermelon fruit since ethylene exposure of watermelon fruit showed enhanced expression of all 3 receptor genes. Moreover, the application of 1-MCP to watermelon fruits significantly reduced the expression levels of all 3 receptors. In agreement with our findings, it has been reported that there was a correlation between the increase in ethylene production and the increase in transcript levels of the *ETR1* gene in watermelon (Wechter et al., 2008), *ETR2* and *ERS* genes in cucumber (Yamasaki et al., 2000), and ETR1 and ERS1 transcripts in ripening persimmon, peach, pear, and apples (Rasori et al., 2002; El-Sharkawy et al., 2003; Cin et al., 2006; Pang et al., 2007). Similarly, the mRNA accumulation of *Cm-ETR1* correlated with the increase in ethylene production during ripening in melon (Sato-Nara et al., 1999). The increase in mRNA levels of ethylene receptors in ripening fruit was reported to be a natural response against increased ethylene biosynthesis to protect the homeostatic balance and a mechanism to regulate ethylene responses (Klee et al., 2003). The watermelon fruit exposed to ethylene for 24 h developed water-soaking after 7 days of storage at 20 °C. Similar results were reported by Karakurt and Huber

(2002), who observed the development of water-soaking disorder in watermelon fruit (cultivars Cabb and Abott 5244) in response to ethylene exposure after 6 days of storage at 20 °C. The induction of all 3 receptor genes by ethylene suggests that they possibly contribute significantly to the development of ethylene-related physiological disorders, including water-soaking, in watermelon fruit. All 3 receptor genes also showed significant increases in mRNA levels during early fruit enlargement but reductions during full fruit enlargement (Wechter et al., 2008). These results indicate that *CL-ETR1*, *CL-ERS1*, and *CL-ETR2* play specific roles not only in ripening but also in the early development of watermelon fruit (Sato-Nara et al., 1999; Wechter et al., 2008).

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Table S1. The nucleotide and amino acid compositions of the receptor genes isolated from watermelon fruit.

Gene	Nucleotides (bp)	Amino acids	5'-UTR (bp)	Coding region (bp)	3'-UTR (bp)	Poly(A) tail (bp)
CL-ETR1	2343	741	55	2226	49	13
CL-ERS1	2349	637	76	1914	348	11
CL-ETR2	2550	766	22	2301	211	16

Table S2. Nucleotide-based homology search results of watermelon ethylene receptor genes.

Gene	Homology (%)
CL-ETR1	
Cucumis melo var. *cantalupensis* ETR1 gene for ethylene receptor	98
Cucumis sativus mRNA for ethylene receptor *CS-ETR1*	97
Cucumis melo var. *reticulatus Cm-ETR1* mRNA	97
Prunus persica ethylene receptor (ETR1) mRNA	80
Prunus salicina putative ethylene receptor (ETR1) mRNA	80
CL-ERS1	
Cucumis melo var. *cantalupensis* ERS1 gene for ethylene receptor	99
Cucumis melo var. *reticulatus Cm-ERS1* mRNA	97
Cucumis sativus mRNA for ethylene receptor *CS-ERS*	96
Ziziphus jujuba ERS-type ethylene receptor mRNA	78
Dimocarpus longan ethylene receptor (ERS) mRNA	78
CL-ETR2	
Cucumis melo var. *cantalupensis* ETR2 mRNA for ethylene receptor	98
Cucumis sativus mRNA for ethylene receptor *CS-ETR2*	98
Pyrus pyrifolia PPETR2 mRNA for ethylene receptor	77
Vitis vinifera hypothetical protein	76

Table S3. Amino acid-based homology search results of watermelon ethylene receptor proteins.

Protein	Homology (%)
CL-ETR1	
Cucumis sativus	97
Cucumis melo var. *cantalupensis*	96
Prunus persica	94
Pyrus communis	94
Malus domestica	94
CL-ERS1	
Cucumis sativus	98
Cucumis melo var. *reticulatus*	96
Cucumis melo var. *cantalupensis*	93
Vitis vinifera hypothetical protein	80
Pyrus pyrifolia	79
CL-ETR2	
Cucumis sativus	98
Cucumis melo var. *cantalupensis*	98
Prunus persica	78
Malus domestica	77
Fragaria × *ananassa*	77

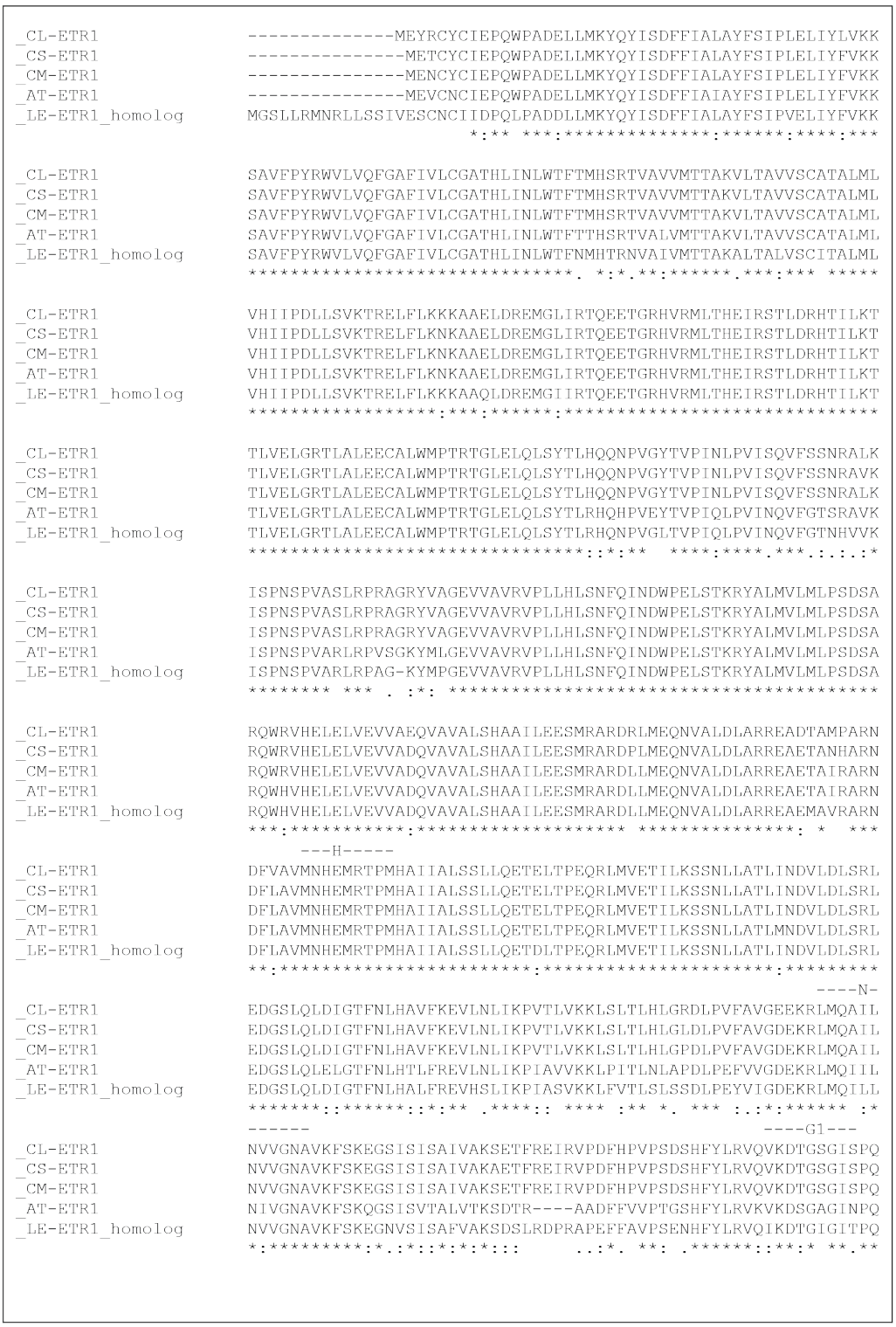

Figure S1. Multiple amino acid sequence alignment of ETR1-like receptor proteins. Histidine kinase domain motifs are shown as H, N, G1, F, and G2. *: Conserved amino acids, .: similar amino acids.

Figure S1. (Continued.)

Figure S2. Multiple amino acid sequence alignment of ERS1-like receptor proteins. Histidine kinase domain motifs are shown as H, N, G1, F, and G2. *: Conserved amino acids, .: similar amino acids.

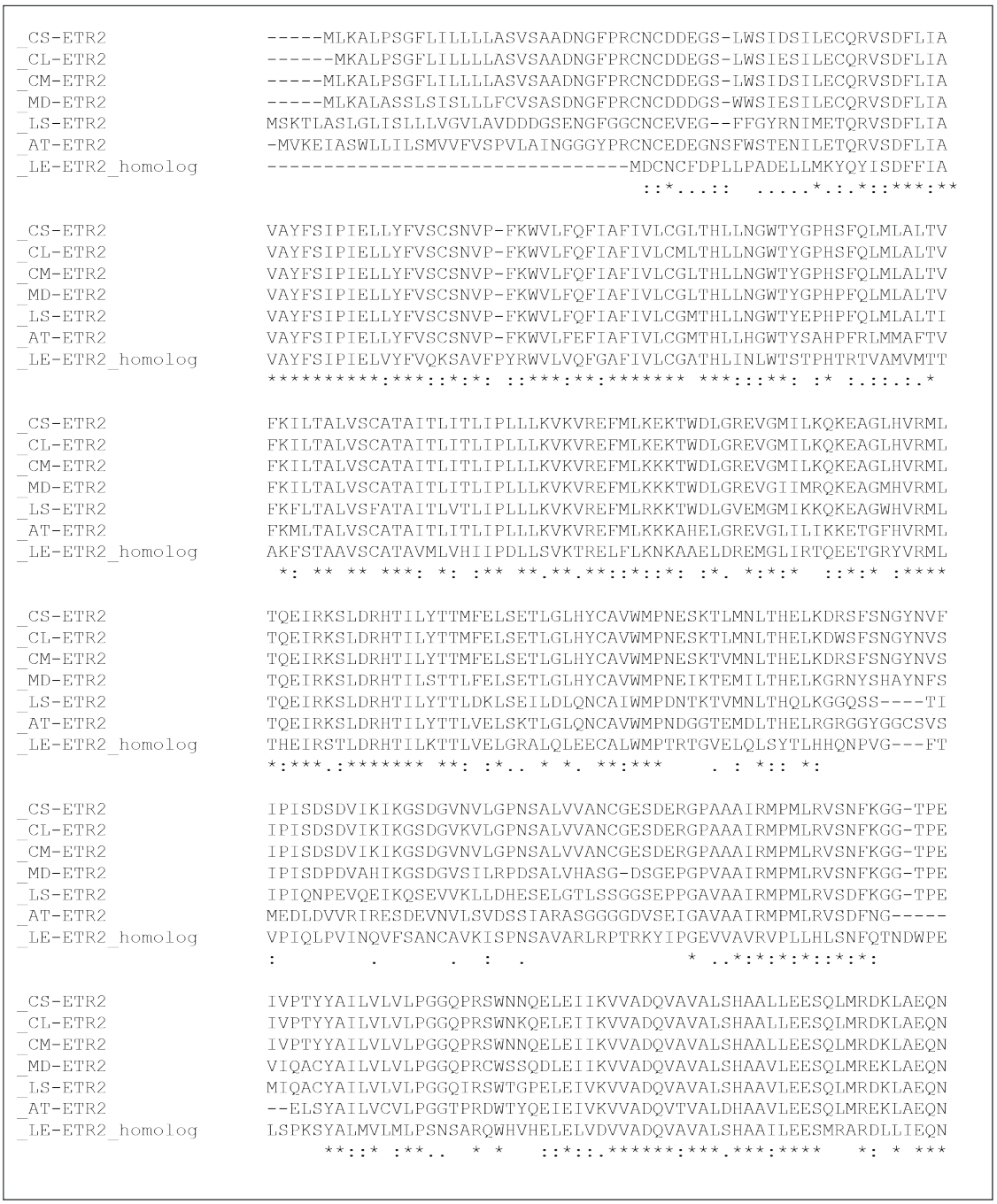

Figure S3. Multiple amino acid sequence alignment of ETR2-like receptor proteins. Histidine kinase domain motifs are shown as H, N, G1, F, and G2. *: Conserved amino acids, .: similar amino acids.

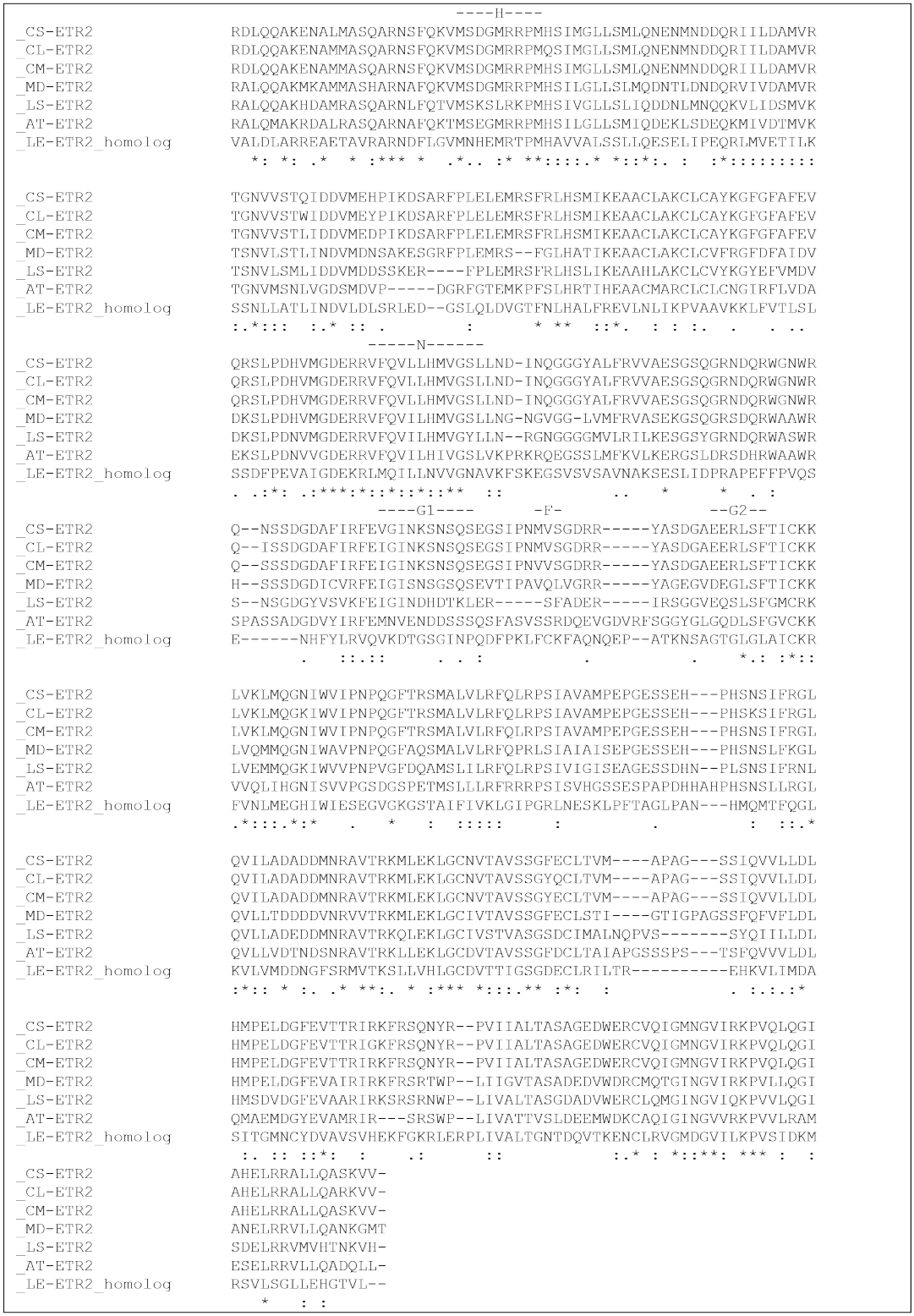

Figure S3. (Continued.)