

Stage- and Tissue-Specific Expression of Ethylene Receptor Homolog Genes during Fruit Development in Muskmelon¹

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We isolated two muskmelon (*Cucumis melo*) cDNA homologs of the Arabidopsis ethylene receptor genes *ETR1* and *ERS1* and designated them *Cm-ETR1* (*C. melo ETR1*; accession no. AF054806) and *Cm-ERS1* (*C. melo ERS1*; accession no. AF037368), respectively. Northern analysis revealed that the level of *Cm-ERS1* mRNA in the pericarp increased in parallel with the increase in fruit size and then markedly decreased at the end of enlargement. In fully enlarged fruit the level of *Cm-ERS1* mRNA was low in all tissues, whereas that of *Cm-ETR1* mRNA was very high in the seeds and placenta. During ripening *Cm-ERS1* mRNA increased slightly in the pericarp of fruit before the marked increase of *Cm-ETR1* mRNA paralleled climacteric ethylene production. These results indicate that both *Cm-ETR1* and *Cm-ERS1* play specific roles not only in ripening but also in the early development of melon fruit and that they have distinct roles in particular fruit tissues at particular developmental stages.

Ethylene is a gaseous hormone that regulates various developmental processes and stress responses in plants (Abeles et al., 1992). Its action is based on two types of responses: (a) responses to a change in the concentration of cellular ethylene (such as the increase in ethylene production caused by various environmental stresses and developmental processes, e.g. pollination) and (b) responses to a change in the sensitivity of tissue to ethylene (such as in fruit ripening, organ senescence, and abscission). However, ethylene production is also increased even in the latter processes. Since these responses are closely related to each other and are involved in the intricate mechanism of ethylene action, we examined the biosynthesis of ethylene and its perception to identify its regulatory role.

The pathway of ethylene biosynthesis has been elucidated throughout the last few decades, providing the basis for subsequent biochemical and molecular genetic analyses of this pathway (Zarembinski and Theologis, 1994). Many studies have been performed to clarify the physiological and molecular mechanisms of the various events regulated by changes in ethylene production (Barry et al., 1996; Blume and Grierson, 1997; Bouquin et al., 1997; Clark et al., 1997; Heidstra et al., 1997; ten Have and Woltering, 1997;

Trebitsh et al., 1997; Zarembinski and Theologis, 1997; Bui and O'Neill, 1998). The genetic approach has been used for Arabidopsis to study ethylene reception and signal transduction (Bleecker and Schaller, 1996). The ethylene-insensitive mutant *etr1* has been identified, and the *ETR1* gene, which encodes an ethylene receptor, has been isolated and characterized (Bleecker et al., 1988; Chang et al., 1993). The *ETR1* protein has three N-terminal hydrophobic domains and two domains that are homologous to a His kinase and a receiver of the bacterial two-component system. Mutant alleles of *ETR1*, designated *etr1-1*, *etr1-2*, *etr1-3*, and *etr1-4*, cause ethylene insensitivity in the plant (Bleecker and Schaller, 1996). All of these mutations cause single-amino acid replacements in the three putative membrane-spanning hydrophobic domains of the protein. When one of these genes (*etr1-1*) was transformed into wild-type Arabidopsis, the transgenic plants lost ethylene sensitivity (Chang et al., 1993). Wilkinson et al. (1997) recently reported that transgenic tomato (*Lycopersicon esculentum*) and petunia with *etr1-1* also became insensitive to ethylene, showing that the dominant *etr1* mutation generally eliminates ethylene sensitivity in higher plants. Analysis of *ETR1* expressed in yeast cells showed that the hydrophobic regions of the *ETR1* protein are capable of reversibly binding ethylene (Schaller and Bleecker, 1995), which is strong evidence that *ETR1* encodes an ethylene receptor.

To date, five *ETR1*-like genes, *ETR1* (Chang et al., 1993), *ERS1* (Hua et al., 1995, 1998), *ETR2* (Sakai et al., 1998), and *EIN4* and *ERS2* (Hua et al., 1998), have been identified in Arabidopsis. Based on sequence similarity and structural features of the proteins, Hua et al. (1998) classified the former two into the *ETR1*-like subfamily, in which the predicted proteins have three hydrophobic domains at the N terminus, and the latter three into the *ETR2*-like subfamily, in which the predicted proteins have four hydrophobic domains at the N terminus. However, unlike the other three genes, *ERS1* and *ERS2* lack the RD. *ETR1* homologs have also been isolated from several other plants: the *NR* gene (Wilkinson et al., 1995) and *LeETR1* and *LeETR2* cDNAs (Lashbrook et al., 1998) from tomato and the *RP-ERS1* cDNA from *Rumex palustris* (Vriezen et al., 1997). *LeETR1* and *LeETR2* have an RD, whereas *NR* and *RP-ERS1* do not (Bleecker and Schaller, 1996; Vriezen et al., 1997; Lashbrook et al., 1998). Point mutations in the hydro-

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Abbreviations: DAP, days after pollination; RD, receiver domain; RT, reverse transcriptase.

phobic domains of ERS1 (Hua et al., 1995) and NR (Lanahan et al., 1994; Wilkinson et al., 1995) also cause insensitivity to ethylene in Arabidopsis and tomato, respectively, indicating that these homologs share a common function with ETR1.

ETR1 and *ERS1* are expressed ubiquitously in Arabidopsis plants, but each gene has its particular expression pattern in each tissue (Hua et al., 1998). Three genes in tomato are also differentially regulated throughout plant development (Lashbrook et al., 1998). The expression of *ERS1* (Hua et al., 1998), *NR* (Wilkinson et al., 1995; Payton et al., 1996), and *RP-ERS1* (Vriezen et al., 1997) is up-regulated by ethylene, whereas that of *ETR1* (Chang et al., 1993) and *eTAE1* (Zhou et al., 1996; corresponding to *LeETR1*) is not affected by ethylene treatment. *RP-ERS1* is under environmental control, and the mRNA level increases after submergence at low O₂ concentrations and at high CO₂ concentrations (Voesenek et al., 1997; Vriezen et al., 1997). However, because there are only a few reports in which the expression of the two types of receptors in the same plant at the same time have been described, how the two types of ethylene receptor genes share a common function and the specific role of each gene remain unknown. Furthermore, except for reports of the expression of the genes during fruit development and ripening in tomato (Wilkinson et al., 1995; Zhou et al., 1996; Lashbrook et al., 1998), there have been few reports about the other fruits.

Ethylene is essential for ripening of climacteric fruits in plants such as tomato, banana, and melon, because the sharp increase in ethylene production at the onset of ripening promotes the rapid change in sweetness, color, firmness, and aroma from immature solid fruit to ripe fruit (Seymour and McGlasson, 1993; Tucker, 1993). The climacteric increase in ethylene production is coupled with the increase in the activities and gene expression of ACC synthase and ACC oxidase, both of which catalyze the steps in ethylene biosynthesis (Zarembinski and Theologis, 1994).

Melon is a fascinating and important fruit with a sweet and juicy taste, an attractive volatile aroma, and high market value, but it also has a very short shelf-life. Melon has advantages as an experimental material for analyzing fruit development: short generation time, short and fixed fruit development time, three distinctive stages of fruit development (enlargement, maturation, and ripening), and availability of a transformation protocol (Fang and Grumet, 1993; Ezura, 1998). Furthermore, the construction of a genetic map is in progress (Baudracco-Arnas and Pitrat, 1996; Wang et al., 1997). Three ACC synthase genes, *ME-ACS1*, *ME-ACS2*, and *ME-ACS3* (Yamamoto et al., 1995), and three ACC oxidase genes, *CM-ACO1*, *CM-ACO2*, and *CM-ACO3* (Lasserre et al., 1996), have been isolated and recognized in melon for their roles in the pathway of ethylene biosynthesis. These genes are expressed not only in ripening fruit but also in early developing fruit at various levels and in different tissues (Kato et al., 1997). To elucidate the mechanism of the regulation of fruit development by ethylene, it is also important to elucidate the role of the ethylene receptors.

We report the isolation and characterization of cDNAs of two putative ethylene receptor genes in muskmelon (*Cu-*

cumis melo). We also report the differential expression of these cDNAs during early development and ripening of melon fruit and discuss the roles of the two types of ethylene receptors during fruit development.

MATERIALS AND METHODS

Plant Material

Seeds of two varieties of muskmelon (*Cucumis melo* L. *reticulatus*), cvs FuyuA and Natsu4, were soaked in 70% (v/v) ethanol for 15 s and in 1% (v/v) hypochlorite solution for 15 min for surface sterilization and were then rinsed with distilled water three times. For RT-PCR, the surface-sterilized cv FuyuA seeds were germinated on Murashige and Skoog (1962) medium (pH 5.8) with 3% (w/v) Suc and 0.4% (w/v) gellan gum (Gelrite, Wako Pure Chemicals, Osaka, Japan) and grown in darkness at 25°C for 10 d. The seedlings were harvested, frozen in liquid nitrogen, and used for RT-PCR. Other seeds were sown and grown in the greenhouse. Freshly opened female flowers were hand-pollinated and one fruit per plant was allowed to develop. Each fruit was harvested at various times after pollination, and the pericarp tissue was separated from the contents of the seed cavity, sliced, frozen in liquid nitrogen, and stored at -80°C.

Preparation of RNA

Total RNA was isolated from seedlings and fruit tissues by SDS-phenol-chloroform extraction (Ozeki et al., 1990). Poly(A⁺) RNA was isolated using an mRNA purification kit (Pharmacia Biotech).

Cloning of a pKY1.3 cDNA Fragment

For RT-PCR, the first-strand cDNA was synthesized from 0.8 µg of poly(A⁺) RNA isolated from 10-d-old dark-grown seedlings using Moloney murine leukemia virus RT (RNase H⁻, Toyobo, Tokyo, Japan). The cDNA was denatured by heating at 95°C for 2 min and used for PCR amplification. The oligonucleotides 168F (5'-CCGGAATTCGCAGTTTGG[TA]GC[TC]TTTAT-3') and 1205R (5'-CGCGGATCCCATCTTC[AGCT]A[GA][TC]CTAGAAA-3') were derived from the nucleotide sequences of Arabidopsis and tomato (*Lycopersicon esculentum*) ethylene receptor genes. Underlined nucleotides in the primers are built-in restriction sites for *EcoRI* and *BamHI* for later cloning. The amplification was carried out using AmpliTaq and GeneAmp (PCR 9600 system, Perkin-Elmer) for 40 cycles of 30 s at 94°C, 30 s at 50°C, and 2 min at 72°C. The amplified cDNA was purified and amplified again by PCR as described above. The amplified cDNA pKY1.3 was cloned into the pCR2.1 plasmid vector using the TA cloning kit (Invitrogen, Carlsbad, CA).

Preparation of Probes

To prepare the XE0.4 probe a plasmid carrying the *Cm-ERS1* (*C. melo ERS1*; accession no. AF037368) cDNA insert

was treated with the restriction enzymes *EcoRI* and *XhoI* and electrophoresed in an agarose gel. The DNA fragment (XE0.4) of about 450 bp was purified and used as a template to synthesize the probe. PCR was performed to prepare RD and MET0.4 probes. The oligonucleotides E1844F (5'-TGGATGAGAACGGGGTAAGTAGAAT-3') and E2130R (5'-TAGTGATACGGGTTTGAGCAACACA-3') were derived from the nucleotide sequences of *ETR1* and used for the amplification of the RD DNA fragment. The plasmid carrying an *ETR1* cDNA insert (kindly provided by Dr. Hideaki Shinshi, National Institute of Bioscience and Human Technology, Japan) was used as a template. The oligonucleotides MET108F (5'-ATGAGCAGAAGAACCCAGAT-3') and MET463R (5'-TACAAGCATAAGGGCAGTTG-3') were used with the plasmid p587.1 carrying a *Cm-ETR1* (*C. melo ETR1*) cDNA insert for the amplification of the MET0.4 DNA fragment. The amplification was carried out using the PCR systems for 40 cycles of 30 s at 94°C and 1 min at 60°C. After the last cycle the amplification was extended to 15 min at 72°C. The amplified DNAs were separated using agarose gel electrophoresis, purified, and used as templates to synthesize the probe. The radiolabeled DNA probe was synthesized using a DNA-labeling system (Megaprime, Amersham).

DNA-Sequencing Analysis

Nucleotide sequences of the cDNA inserts in the plasmid vectors were determined using a DNA sequencer (model 373A, Applied Biosystems) and a dye primer ready-reaction sequencing kit (PRISM, Applied Biosystems). The sequences were analyzed with Genetix software (version 7.3 for the Mac, Software Development Co., Tokyo, Japan).

Construction and Screening of cDNA Library

Double-stranded cDNA was synthesized from poly(A⁺) RNA from fruit 64 DAP using a cDNA-synthesis module (Amersham); first-strand cDNA synthesis was performed with Moloney murine leukemia virus RT at 37°C for 1 h instead of the avian myeloblastosis virus RT included in the module. cDNA was cloned into λ gt10 (Stratagene) using an *EcoRI/NotI* adaptor (Pharmacia Biotech). About 10⁶ recombinants were packaged in vitro using a gold packaging extract (Gigapack II, Stratagene). Approximately 290,000 plaques were screened with a pKY1.3 probe using a nucleic acid-labeling and detection system (DIG, Boehringer Mannheim) according to the manufacturer's instructions. Twelve positive plaques from the primary screening were purified through a secondary screening, and the clone *Cm-ERS1* was obtained. The phage DNA was isolated and the cDNA insert was subcloned into the *EcoRI* site of the pBluescript II SK⁺ plasmid vector (Stratagene).

Next, 435,000 plaques of the library were rescreened with radiolabeled RD or XE0.4 probes. Sixteen plaques that were positive with both probes on the primary screening were chosen and purified through the secondary screening with an RD probe. Clone *Cm-ETR1* was obtained and subcloned as described above.

Genomic Southern Analysis

Genomic DNA was isolated from young leaves of cv FuyuA by the method of Wagner et al. (1987). Digested DNA (20 μ g each) was electrophoresed in a 0.8% agarose gel and transferred to a Hybond-N⁺ nylon membrane (Amersham). Hybridization and washing of the membrane were performed as recommended by the manufacturer.

Northern Analysis

Total RNA was separated on a 1.0% agarose/formaldehyde gel according to the method of Berk and Sharp (1978) and transferred to Hybond-N⁺ nylon membranes. Northern hybridization was performed overnight at 65°C in a solution containing 10% sodium dextran sulfate, 1% SDS, 1 M NaCl, 100 μ g mL⁻¹ denatured salmon-sperm DNA, and 10⁵ cpm mL⁻¹ ³²P-labeled probe. After hybridization the membranes were rinsed twice for 5 min in a 2 $\times$ SSC solution, washed for 30 min at 65°C in a solution containing 2 $\times$ SSC and 1% SDS, and autoradiographed at -80°C. Signal intensity on the film was quantified using an interpretive densitometer (Master Scan, Scanalytics, Fairfax, VA) and analyzed (RFLP scan version 2.01, CSPI, Minneapolis, MN).

RESULTS

Cloning and Sequencing of *ETR1* and *ERS1* Homologs of Melon

To isolate ethylene receptor homologs from melon, the PCR primers 168F and 1205R were designed on the basis of conserved sequences of *ETR1* and *ERS1* of Arabidopsis (Chang et al., 1993; Hua et al., 1995, 1998) and *NR* and *eTAE1* of tomato (Wilkinson et al., 1995; Zhou et al., 1996). An expected product of about 1 kb in length was amplified by RT-PCR of poly(A⁺) RNA, and the amplified fragment was then cloned into pCR2.1 vector and sequenced. The cDNA fragment, pKY1.3, was 72% and 73% identical in nucleotide sequence to the *ETR1* and *ERS1* genes, respectively, indicating that pKY1.3 is a partial fragment of an *ETR1/ERS1* homolog. The middle-ripe fruit cDNA library was screened with the pKY1.3 probe to isolate a full-length cDNA clone. As a result, a full-length cDNA clone with a region of nucleotide sequences coinciding with that of pKY1.3 was isolated (Fig. 1A).

The cDNA was 2363 nucleotides in length, and its open reading frame encoded a protein of 628 amino acids (Fig. 1A). The regions of 5'-noncoding, 3'-noncoding, and poly(A⁺) sequences were 88, 342, and 19 nucleotides in length, respectively. The open reading frame of the cDNA was more than 64% and 68% identical in nucleotide and amino acid sequences to other ethylene receptor genes (Table I). The deduced amino acid sequence had the hydrophobic sequences and His kinase domain but lacked the RD in *ETR1*, indicating that it is a homolog of *ERS1* (Fig. 1B); therefore, the corresponding gene was designated *Cm-ERS1*.

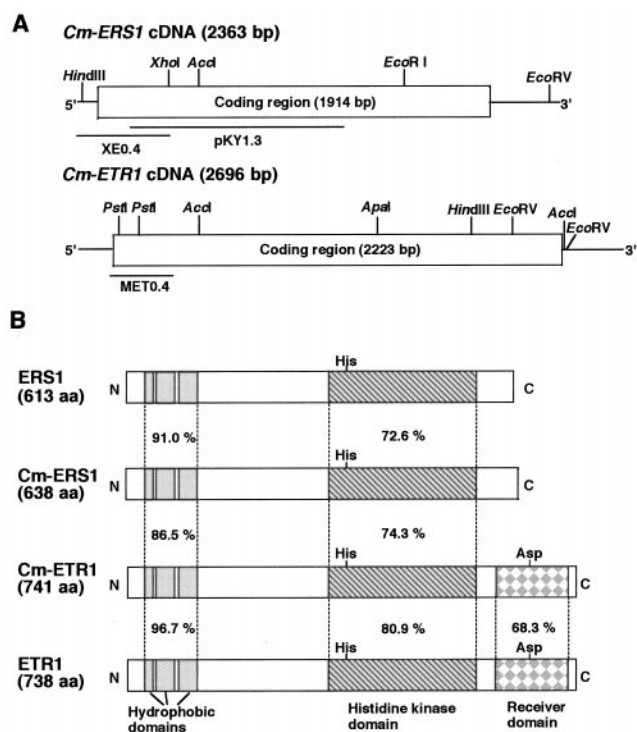


Figure 1. A, Schematic diagram of the *Cm-ERS1* and *Cm-ETR1* cDNAs. The open boxes and the lines represent the open reading frames and flanking regions, respectively. A cDNA fragment (pKY1.3) isolated by RT-PCR and used as a probe to screen the cDNA library is indicated. The regions of XE0.4 and MET0.4 probes and the sites of several restriction enzymes are also shown. B, Structural drawing comparing the deduced amino acid (aa) sequences of melon *Cm-ERS1* and *Cm-ETR1* and Arabidopsis *ERS1* and *ETR1*. The shaded boxes (N termini) represent three hydrophobic domains that are putative transmembrane domains. The shaded boxes (C termini) represent the sequences homologous to the His kinase domain and the RD of bacterial two-component environmental sensor systems. The positions of the conserved His and Asp residues that may be phosphorylated *in vivo* are shown. The amino acid identity between the domains of neighboring proteins is indicated.

An *ETR1* homolog from melon was also isolated. The region coding an RD of *ETR1* was amplified by PCR and used as a probe to screen the fruit cDNA library. XE0.4, which contains the 5'-noncoding sequences and the coding sequences of three hydrophobic domains of *Cm-ERS1*, was also used as a probe (Fig. 1A). The fruit cDNA library was probed with both RD and XE0.4 on the first round of screening and with RD on the second round. A full-length cDNA clone with 74.1% nucleotide homology with *ETR1* was isolated by this procedure (Table I). The deduced amino acid sequence had the RD as well as the other two domains, indicating that it is a homolog of *ETR1* (Fig. 1B); therefore, the corresponding gene was designated *Cm-ETR1*.

The *Cm-ETR1* cDNA was 2696 nucleotides in length, and its open reading frame encoded a protein of 741 amino acids (Fig. 1A). The regions of 5'-noncoding and 3'-noncoding sequences were 164 and 309 nucleotides in length, respectively. The open reading frame of the *Cm-ETR1* cDNA was more than 67% and 69% identical in nucleotide and amino acid sequences to ethylene receptor genes of other plants and 68.6% and 75.3% identical, respectively, to *Cm-ERS1* (Table I). To our knowledge, *Cm-ETR1* and *Cm-ERS1* are the first putative ethylene receptor genes to be isolated from melon.

Genomic Organization of *Cm-ETR1* and *Cm-ERS1*

Genomic Southern analysis was performed to analyze the genomic organization of *Cm-ETR1* and *Cm-ERS1*. With the XE0.4 probe (Fig. 1A) at a low stringency, a strong signal and one or two weak signals were seen (Fig. 2). Hybridization performed at a high stringency revealed a strong signal in each lane and the lower weak signal in the *EcoRI* lane but not the other weak signals (Fig. 2). The disappearance of the weak signals was accompanied by the appearance of strong signals with the MET0.4 probe (Fig. 1A; the sequences coding for the hydrophobic domains of *Cm-ETR1*) at a high stringency. These results

Table I. Homology of the nucleotide sequences and deduced amino acid sequences of ethylene receptor genes from melon and other plants

Gene	Protein Size	Amino Acid Identity		Nucleotide Identity	
		Cm-ERS1	Cm-ETR1	<i>Cm-ERS1</i>	<i>Cm-ETR1</i>
	<i>no. of amino acids</i>			<i>%</i>	
Melon					
<i>Cm-ERS1</i>	638	100	75.3	100	68.6
<i>Cm-ETR1</i>	741	75.3	100	68.6	100
Arabidopsis					
<i>ERS1</i>	613	75.1	71.5	70.2	67.0
<i>ETR1</i>	738	70.7	82.6	64.3	74.1
Tomato					
<i>NR</i>	635	71.2	69.4	70.8	67.6
<i>LeETR1</i>	754	70.1	80.6	68.7	76.2
<i>LeETR2</i>	736	69.3	78.5	67.0	74.8
<i>R. palustris</i>					
<i>RP-ERS1</i>	628	68.8	69.8	69.4	67.8

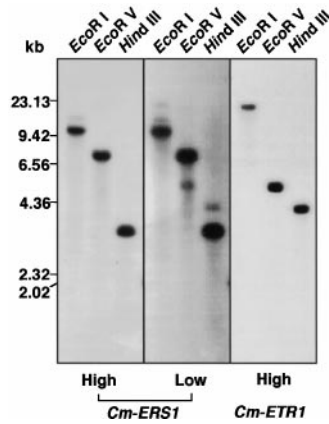


Figure 2. Southern analysis of melon genomic DNA probed with XE0.4 and MET0.4. Genomic DNA digested with each restriction enzyme was electrophoresed on 0.8% agarose gel and transferred to a nylon membrane. First, hybridization was performed with an XE0.4 probe overnight at low stringency (55°C, middle lane). The membrane was washed for 30 min at 55°C and exposed to film for 4 d. Next, the membrane was stripped and reprobed with XE0.4 at high stringency (65°C, left lane). After the routine procedure, the membrane was exposed to film for 2 weeks and stripped and reprobed with MET0.4 at high stringency (65°C, right lane). Molecular size markers are denoted on the left.

indicate that there are at least two ethylene receptor homolog genes in the melon genome corresponding to *Cm-ETR1* and *Cm-ERS1*.

Increased Gene Expression of *Cm-ETR1* and *Cm-ERS1* during Fruit Development

To understand the possible roles of *Cm-ETR1* and *Cm-ERS1* during fruit development, we analyzed the gene expression during fruit enlargement and ripening. First, we compared the stage-specific expression pattern of the *Cm-ETR1* and *Cm-ERS1* mRNAs in two melon varieties, cv FuyuA and cv Natsu4, which have distinct characteristics in fruit development (K. Higashi and H. Ezura, unpublished results). Although these varieties share a closely related genetic background and have very similar characteristics in plant growth, cv FuyuA sets larger fruit than cv Natsu4, because of its longer period of cell proliferation. The enlargement of cv FuyuA fruit stopped between 35 and 49 DAP, whereas that of cv Natsu4 fruit stopped between 21 and 35 DAP (Fig. 3A, arrows).

Northern analysis of *Cm-ETR1* and *Cm-ERS1* demonstrated that the expression of the 2.7- and 2.4-kb mRNAs in the pericarp was differentially increased during fruit de-

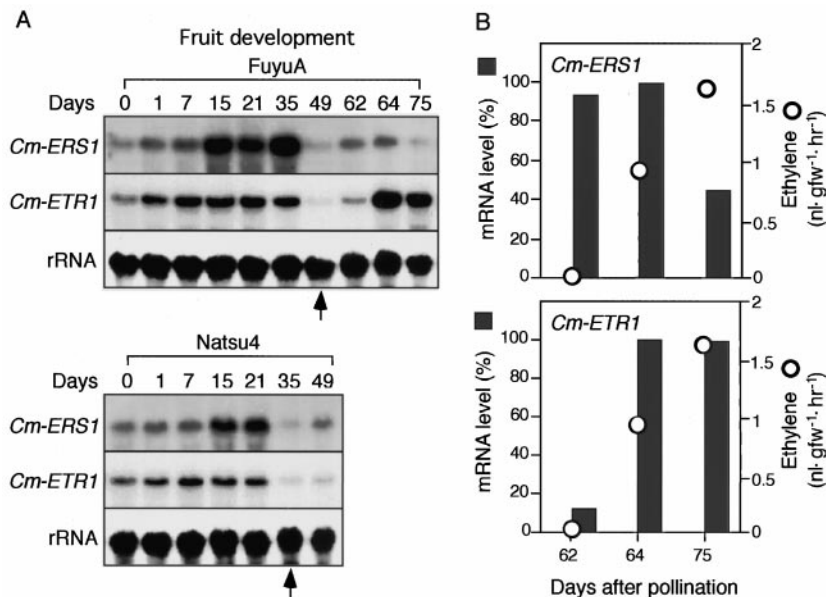


Figure 3. A, Northern analysis of *Cm-ERS1* and *Cm-ETR1*. Total RNA isolated from cv FuyuA fruit at 0, 1, 7, 15, 21, 35, 49, 62, 64, and 75 DAP (top) and cv Natsu4 fruit at 0, 1, 7, 15, 21, 35, and 49 DAP (bottom) was loaded in each lane. Northern analysis was performed using 10 μ g of total RNA per lane and the sample was first probed with XE0.4. After hybridization the membrane was washed and exposed to film. The probe was then removed and the sample was reprobed with a loading control probe rRNA. After the routine procedure the membrane was stripped again and reprobed with MET0.4. B, Increase of ethylene production during ripening and the changes of relative mRNA levels at the early (62 DAP), middle (64 DAP), and late (75 DAP) stages of fruit ripening in cv FuyuA. The amount of ethylene production was measured using a gas chromatograph (GC-14B, Shimadzu, Kyoto, Japan). The mRNA level was quantified and normalized as follows. Signal intensity on the film was quantified using an interpretive densitometer. For normalization the value of *Cm-ERS1* or *Cm-ETR1* was divided by that of rRNA in each sample. Shaded bars in the upper and lower graphs represent the *Cm-ERS1* and *Cm-ETR1* mRNA levels, respectively, relative to each value for fruit 64 DAP. Arrows indicate the stage at which fruit development ended. gfw, Grams fresh weight.

velopment (Fig. 3A). Both mRNAs also accumulated at various levels in the vegetative tissues such as the hypocotyls of the seedlings and the leaves in various developmental stages (data not shown). The *Cm-ETR1* mRNA increased 1 DAP and accumulated at a constant level from 7 to 35 DAP in cv FuyuA and from 7 to 21 DAP in cv Natsu4 (Fig. 3A). Similarly, the increase of the *Cm-ERS1* mRNA paralleled that of the fruit size and the thickness of the pericarp in cvs FuyuA and Natsu4 (Fig. 3A), with high levels observed at the middle stage of fruit enlargement (between 15 and 35 DAP in cv FuyuA and between 15 and 21 DAP in cv Natsu4) and a dramatic decrease at the end of fruit enlargement (49 DAP in cv FuyuA and 35 DAP in cv Natsu4; Fig. 3A).

Next we analyzed the relationship between climacteric ethylene production and the levels of *Cm-ETR1* and *Cm-ERS1* mRNA during fruit ripening. In cv FuyuA fruit at 62 DAP, no ethylene production could be detected (Fig. 3B); the *Cm-ERS1* mRNA level in the pericarp was slightly increased, and the *Cm-ETR1* mRNA level was still low (Fig. 3). Ethylene showed climacteric production between 0.95 and 1.7 nL g⁻¹ fresh weight h⁻¹ in the fruit 64 and 75 DAP, respectively (Fig. 3B). The *Cm-ETR1* mRNA level in the pericarp was also markedly increased in the fruit 64 DAP, with no further increase thereafter. The *Cm-ERS1* mRNA level in the pericarp 64 DAP was the same as that 62 DAP but was lower in the fruit 75 DAP (Fig. 3B).

To investigate the spatial pattern of *Cm-ERS1* and *Cm-ETR1* mRNA expression, we performed northern analysis of various tissues in developing and maturing fruit. In the developing fruit the level of *Cm-ERS1* mRNA was much higher in the pericarp than in immature seeds or placenta, whereas the level of *Cm-ETR1* mRNA was lower in the pericarp than in the other tissues (Fig. 4). In fully enlarged fruit the level of *Cm-ERS1* mRNA was lower in all tissues and that of *Cm-ETR1* mRNA was lower in the pericarp, whereas the level of *Cm-ETR1* mRNA was high in the seeds and placenta (Fig. 4). At the early-ripe stage the level of *Cm-ERS1* mRNA was higher in the epidermis than in the flesh, the seeds, and the placenta (Fig. 4). The level of *Cm-ETR1* mRNA was also high in the epidermis at the early-ripe stage. As the fruit ripened both mRNAs accumulated inside the fruit (Fig. 4). The levels of *Cm-ERS1* mRNA increased in the middle flesh and decreased in the epidermis. At the late-ripe stage the level of *Cm-ETR1* mRNA was high in all tissues except the seeds.

DISCUSSION

Cm-ERS1 and *Cm-ETR1* Are Putative Ethylene Receptors in Melon

We isolated the *Cm-ERS1* and *Cm-ETR1* cDNAs from the fruit of melon. The nucleotide and predicted amino acid sequences of *Cm-ERS1* and *Cm-ETR1* were similar to those of Arabidopsis *ERS1* and *ETR1* genes (Chang et al., 1993; Hua et al., 1995, 1998). Each predicted protein contained three hydrophobic domains and the His kinase domain, and the *Cm-ETR1* protein had the RD in addition to the other domains. Furthermore, *Cm-ERS1* and *Cm-ETR1* pro-

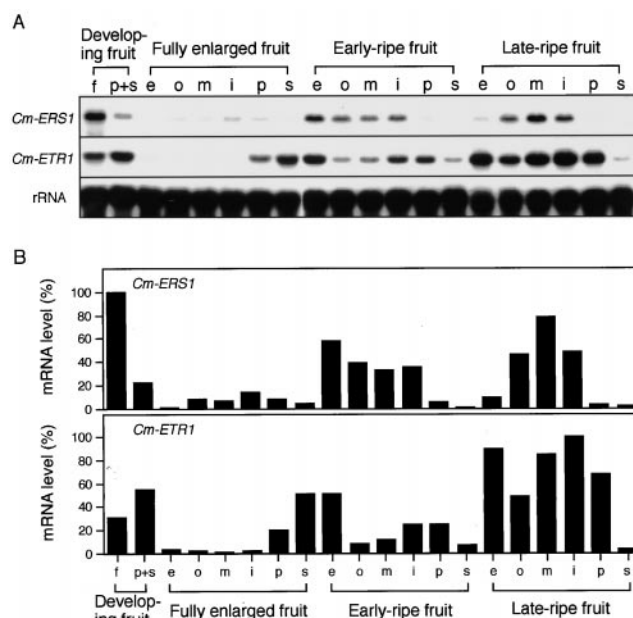


Figure 4. Northern analysis of *Cm-ERS1* and *Cm-ETR1* in melon fruit. A, Northern analysis of *Cm-ERS1* and *Cm-ETR1* in developing, fully enlarged, early-ripe, and late-ripe fruits. Total RNA isolated from flesh (f), placentas containing immature seeds (p+s), epidermis (e), outer flesh (o), middle flesh (m), inner flesh (i), placentas (p), and seeds (s) of melon fruit was loaded in each lane. Northern blotting was performed using 10 μ g of total RNA per lane and the sample was first probed with XE0.4. After hybridization the membrane was washed and exposed to film. The probe was then removed and the membrane was reprobed with MET0.4. After the routine procedure, the membrane was stripped again and reprobed with a loading control probe rRNA. Note that the RNA loaded in lane f is the same RNA as that in lane 21 of the cv FuyuA fruit in Figure 3. B, Relative mRNA level in developing, fully enlarged, early-ripe, and late-ripe fruits. Each shaded bar represents the mRNA level relative to the highest value obtained, flesh in the developing fruit (*Cm-ERS1*) or inner flesh in the late-ripe fruit (*Cm-ETR1*). The mRNA level was quantified and normalized as described in the legend to Figure 3.

teins were conserved at residues thought to be important for the normal function of ETR1 (residues A31, I62, C65, and A102, which correspond to the positions that are mutated in the alleles that cause ethylene insensitivity; Bleecker and Schaller, 1996). Also conserved were the equivalents of ETR1 C4 and C6, which are required for disulfide-linked dimer formation (Schaller et al., 1995), and H354 and D659, which are presumptive sites of autophosphorylation in the His kinase and RDs (Chang et al., 1993). These results indicate that *Cm-ERS1* and *Cm-ETR1* are functional ethylene receptors that have the ability to bind ethylene and kinase activities; however, further experiments are necessary to confirm the hypothesis that these are really ethylene receptors in melon.

Genomic Southern analysis revealed the existence of two ethylene receptor genes in melon that corresponded to *Cm-ERS1* and *Cm-ETR1* and evidence for a third gene (Fig. 2). Five *ETR1*-like genes have been reported in Arabidopsis (Chang et al., 1993; Hua et al., 1995, 1998; Sakai et al., 1998), five *ETR1* homologs have been reported in tomato (Yen et

al., 1995), and a few homologs have been reported in *R. palustris* (Vriezen et al., 1997). The genes in the *ETR2*-like subfamily do not cross-hybridize with the genes in the *ETR1*-like subfamily (A.B. Bleecker, personal communication). It may be that the members of the *ETR2*-like subfamily do not cross-hybridize with *Cm-ERS1* and *Cm-ETR1*, members of the *ETR1*-like subfamily. Thus, three genes belonging to the *ETR1*-like subfamily may exist in melon.

***Cm-ERS1* and *Cm-ETR1* mRNAs Are Expressed in a Stage- and Tissue-Specific Manner during Fruit Development**

The level of *Cm-ERS1* mRNA dynamically increased during early fruit development (Fig. 3A), but ethylene production could not be detected by our method. The undetectable level of ethylene production seems to be in conflict with the high level of *Cm-ERS1* mRNA. Hua and Meyerowitz (1998) showed that the ethylene receptors positively regulate CTR1 (a RAF kinase-like negative regulator; Kieber et al., 1993) in the absence of ethylene and that ethylene binding inhibits this interaction. For complete inhibition of the receptor-CTR1 interaction, many ethylene molecules are required to inhibit the many receptors. Therefore, an increase in receptors may reduce the sensitivity. Ethylene responses in young and developing fruit of melon may be inhibited by an increase in *Cm-ERS1* mRNA, which decreases the sensitivity to ethylene in addition to maintaining a low level of ethylene production. However, how ethylene receptors work to alter ethylene responses is quite complex and remains unknown.

There are two reports indicating that receptors increase while tissue sensitivity to ethylene is also increasing. Vriezen et al. (1997) reported that enhanced levels of ethylene and a low O₂ concentration both stimulate petiole elongation in *R. palustris*. They also reported that treatment with low O₂ concentrations causes both the increase of *RP-ERS1* mRNA level and the enhancement of petiole extension by increasing the sensitivity to ethylene without changing the rate of ethylene production. The level of *NR* mRNA and the sensitivity to ethylene and ethylene production also increase during fruit maturation in tomato (Wilkinson et al., 1995; Lashbrook et al., 1998). Further analysis is required to investigate the relationship between changes in ethylene production and sensitivity and the levels of mRNAs and proteins of the ethylene receptors during fruit development.

The *Cm-ERS1* mRNA level increased during enlargement of both cv FuyuA and cv Natsu4 fruits and decreased at the end of enlargement (Fig. 3A), suggesting that the increase in mRNA is related to developmental events in parallel with fruit enlargement. The increase in fruit volume associated with growth of fruit is usually attributed to a combination of cell division and expansion (Gillaspy et al., 1993). When melon fruits enlarge the pericarp cells mainly divide in the early developmental stage (7 DAP in cv Natsu4 and 14 DAP in cv FuyuA), and mainly expand during the following stage (7–28 DAP in cv Natsu4 and 14–42 DAP in cv FuyuA; K. Higashi and H. Ezura, unpublished results). High accumulation of *Cm-ERS1* mRNA was

observed during the stage of cell expansion in the pericarp, indicating that the change in ethylene sensitivity is related to the regulation of cell expansion. RNA in situ hybridization analysis of various Arabidopsis tissues revealed that the signals of *ERS1* appear higher in younger, smaller cells than in older, more expanded cells (Hua et al., 1998), which is consistent with our data indicating a higher level of *Cm-ERS1* mRNA in the pericarp of the young, expanding fruit in melon. Although the roles of ethylene in the regulation of cell expansion in fruit remain unknown, the direction of cell expansion in the stem of various higher plants has been suggested to be regulated by the combined action of several plant hormones (auxins, GAs, and ethylene; Sibaoka, 1991). The changes in cell expansion and various other events in fruit that occur along with the increase in *Cm-ERS1* mRNA need further investigation.

Lashbrook et al. (1998) reported that *LeETR1* is expressed constitutively in all plant tissues and that *LeETR2* is expressed at low levels throughout the plant except for high levels in imbibing tomato seeds before germination. In melon, however, the level of *Cm-ETR1* mRNA was changed more dynamically in fruit tissues according to the developmental stage, and *Cm-ETR1* had an expression pattern different from that of *Cm-ERS1* (Figs. 3 and 4). The level of *Cm-ETR1* mRNA was high in the seed and placenta of developing and fully enlarged fruit (Fig. 4), whereas the increase of the mRNA level in the pericarp of fruit was concurrent with the beginning of ethylene production during ripening (Fig. 3). Kato et al. (1997) reported that the levels of mRNAs for auxin-responsive ACC synthases (ME-ACS2 and ME-ACS3) were increased in seeds and placenta of immature fruit and that the level for wound ACC synthase (ME-ACS1) was increased in the flesh and placenta during ripening. They also reported that the level of mRNA for ACC oxidase was increased in seeds during seed maturation beginning with the late stage of fruit enlargement. The stages and tissues showing expression of mRNAs for ACC synthase and ACC oxidase are similar to those showing expression of *Cm-ETR1* mRNA (Fig. 4), suggesting that the expression of *Cm-ETR1* and the genes for enzymes of ethylene biosynthesis are closely related to each other and that the mechanism for regulation of fruit development involves the reception and biosynthesis of ethylene.

The levels of *Cm-ERS1* and *Cm-ETR1* mRNAs were low inside the fruit at the early-ripe stage (Fig. 4). Yamamoto et al. (1995) observed an increase in the levels of mRNA and protein of ACC oxidase and *ME-ACS1* mRNA in the placenta of fruit during the preclimacteric stage, whereas it was not observed in the mesocarp until the climacteric stage. The expression pattern of the genes of ACC oxidase and ACC synthase is consistent with the pattern of fruit ripening in melon (the inside of the fruit ripens earlier than the outside). The expression pattern of *Cm-ERS1* and *Cm-ETR1* mRNAs is also consistent with the pattern of fruit ripening. Expression of *Cm-ERS1* and *Cm-ETR1* mRNAs at higher levels on the outside of the early-ripe fruit may lower the sensitivity to ethylene, whereas expression of the mRNA at lower levels inside may increase it. Higher sensitivity to ethylene inside the fruit may promote the ripening from the inside to the outside, in addition to ethylene

production inside. The reason the expression pattern of the ethylene receptor genes is opposite to that of the genes for the ethylene biosynthetic enzymes should be determined to clarify the mechanism of the regulation of fruit ripening by ethylene.

We compared the expression of melon and tomato ethylene receptor genes during fruit development and, like that of *LeETR1* and *LeETR2*, the expression of *Cm-ETR1* differed (Figs. 3 and 4; Lashbrook et al., 1998). The tomato *NR* lacking the RD is expressed at low levels during the early stage of fruit development (Lashbrook et al., 1998), but *Cm-ERS1* is highly expressed at that stage (Fig. 3). During fruit ripening *NR* seems to be expressed in a manner more similar to that of *Cm-ETR1* than that of *Cm-ERS1*, because the increase of both *NR* and *Cm-ETR1* mRNAs occurred simultaneously with the climacteric burst of ethylene production, whereas the increase in *Cm-ERS1* mRNA occurred before the burst (Fig. 3; Wilkinson et al., 1995; Lashbrook et al., 1998). Further studies of the role of each isoform in fruit development in each plant are needed to elucidate the cause of the different pattern of the ethylene receptor genes between melon and tomato.

What roles might *Cm-ERS1* and *Cm-ETR1* play? The increase of *Cm-ERS1* mRNA at a low concentration of ethylene before the increase of *Cm-ETR1* mRNA and ethylene production indicates that *Cm-ERS1* may be sensitive to a low concentration of ethylene, whereas *Cm-ETR1* may be involved in the response at a high concentration of ethylene. To clarify the role of each ethylene receptor on fruit development, the factors regulating the expression of each ethylene receptor gene must be studied.

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