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Islam El-Sharkawy, Brian Jones, Zhengguo Li, Jean-Marc Lelièvre, Jean-Claude Pech, et al.. Isolation and characterization of four ethylene perception elements and their expression during ripening in pears (*Pyrus communis* L.) with/without cold requirement. *Journal of Experimental Botany*, 2003, 5 (387), pp.1615-1625. 10.1093/jxb/erg158 . hal-03483368

HAL Id: hal-03483368

<https://hal.science/hal-03483368>

Submitted on 16 Dec 2021

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Isolation and characterization of four ethylene perception elements and their expression during ripening in pears (*Pyrus communis* L.) with/without cold requirement

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Abstract

Pear (*Pyrus communis* L.) are climacteric fruit: their ripening is associated with a burst of autocatalytic ethylene production. Some late pear cultivars, such as Passe-Crassane (PC) require a long (80 d) chilling treatment before the fruit will produce autocatalytic ethylene and ripen. As the cold requirement is linked to the capacity to respond to ethylene (or its analogue, propylene), three pear cDNAs homologous to the *Arabidopsis* ethylene receptor genes *At-ETR1*, *At-ERS1*, and *At-ETR2*, designated *Pc-ETR1a* (AF386509), *Pc-ERS1a* (AF386515), and *Pc-ETR5* (AF386511), respectively, have been isolated. A pear homologue of the *Arabidopsis* ethylene signal transduction pathway gene *At-CTR1*, called *Pc-CTR1* (AF386508) has also been isolated. The search of the genomic sequences for *Pc-ETR1a* and *Pc-ERS1a* resulted in the isolation of four related genomic clones *Pc-DETR1a* (AF386525), *Pc-DETR1b* (AF386520), *Pc-DERS1a* (AF386517), and *Pc-DERS1b* (AF386522). Analysis of transcript levels for the four cDNAs in PC and pear fruit genotypes with little or no cold requirement revealed that *Pc-ETR1a* expression increased during chilling treatment, and *Pc-ETR1a*, *Pc-ERS1a*, *Pc-ETR5*, and *Pc-CTR1* expression increased during fruit ripening and after ethylene treatment. Whether the differences in the ethylene response elements studied here are the cause or an effect of the cold requirement in PC fruit is discussed.

Key words: Cold requirement, ethylene perception elements, exogenous ethylene, fruit ripening, pear.

Introduction

Both the biosynthesis and the perception of the phytohormone ethylene are modulated during development of plant tissues. In climacteric fruit, such as the pear, avocado, and melon a majority of ripening processes are driven by ethylene-regulated changes in gene expression (Abeles *et al.*, 1992; Lelièvre *et al.*, 1997a; Giovannoni, 2001). However, in some fruit species such as pears, cold treatment can hasten the rate of ripening on or off the tree (Knee, 1993; Itai *et al.*, 1999). Late ripening pears require several weeks at low temperatures and will then ripen rapidly at higher temperatures (Gerasopoulos and Richardson, 1997b). In a few genotypes such as Passe-Crassane (PC), unchilled fruit will generally not ripen normally (Ulrich, 1961; Leblond, 1975). In all cases, cold treatment stimulates ethylene biosynthesis that leads to ripening (Knee, 1993; Gerasopoulos and Richardson, 1997a). Treatment of cold-treated PC fruit with ethylene or its analogue, propylene, is able to advance ripening. However, the duration of propylene treatment, necessary to induce ripening-related changes in PC fruit is, unusually, quite long, from 20 d to 35 d. Non-chilled PC fruit often remain unable to produce autocatalytic ethylene during propylene treatment even though fruit softening and the induction of ACO1, the final enzyme in the ethylene biosynthetic pathway, can be observed (Lelièvre *et al.*, 1997b). These results suggest that the capacity for autocatalytic ethylene production and ripening is developmentally and/or environmentally controlled in mature PC and related pear fruit. The mechanisms underlying the induction by low temperature of autocatalytic ethylene production are not well understood. In this work, the object

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Abbreviations: PC, Passe-Crassane; OH, Old Home; ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ORF, open reading frame.

was to determine whether the cold requirement is related to changes in ethylene perception and signal transduction.

Ethylene perception and signal transduction (Bleecker and Schaller, 1996) have been extensively studied at the biochemical and molecular genetic levels in *Arabidopsis thaliana* and other species (Chang *et al.*, 1993; Knee, 1993; Lanahan *et al.*, 1994; Giovannoni, 2001). 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 and Meyerowitz, 1998), have been identified in *Arabidopsis*. Functional *ETR1* homologues have been isolated from several plant species (Lanahan *et al.*, 1994; Müller *et al.*, 2000; Sato-Nara *et al.*, 1999). Ethylene receptor genes are differentially regulated throughout plant development in *Arabidopsis* and tomato (Hua and Meyerowitz, 1998; Lashbrook *et al.*, 1998). Importantly, *At-ERS1* (Hua and Meyerowitz, 1998) and *NR* (Wilkinson *et al.*, 1995; Payton *et al.*, 1996) are involved in the autoregulation of ethylene perception as their expression is up-regulated by the phytohormone. Ethylene/receptor binding leads to a plant response through 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 with the ethylene receptor proteins, is a negative regulator of the ethylene response. Mutant forms of *ctr1* confer a constitutive ethylene response in air.

In this paper the isolation and characterization of four putative pear ethylene perception response elements, three ethylene receptors (Hua and Meyerowitz, 1998; Bleecker and Kende, 2000) and a *CTR1*-like protein (Kieber *et al.*, 1993; Clark *et al.*, 1998) are described. Their expression was studied during ripening in PC fruit and in pears that have a reduced or no cold requirement in order to determine whether there are dissimilarities that could account for the diversity in ripening behaviour. The molecular and physiological results have been used to build a preliminary genetic model.

Materials and methods

Plant material and post-harvest treatments

Pear (*Pyrus communis* L. cv. Passe-Crassane) fruits were harvested and treated as described previously (Lelièvre *et al.*, 1997b) except that, for the 1-methylcyclopropene (1-MCP) treatment, the 1-MCP (1 µl l⁻¹) was applied overnight immediately before cold treatment. Fruit of Old Home (OH) and Old Home×PC hybrid trees P7R7A16 (A16) and P7R6A50 (A50) were obtained from INRA Angers (Garonne valley), France. PC, OH, A16, and A50 were grafted onto quince rootstock BA27. Preclimacteric fruit were harvested as late as possible before fruit ethylene production had risen and the fruit had abscised. After measurements of ethylene production, fruit tissue was frozen as previously described (Lelièvre *et al.*, 1997b). Mixed tissues of two fruit displaying a similar ethylene production at the same age were used for mRNA extraction and analysis.

Isolation and in silico analysis of pear cDNA sequences

RNA extraction and the PC cDNA library were as previously described (Lelièvre *et al.*, 1997b). For the isolation of pear homologues of ethylene perception and signal transduction components, the RNA was treated with DNase (Promega) and then first strand cDNA synthesis was carried out using 10 µg of total RNA in a 50 µl. One µl of cDNA was used in a PCR with the appropriate degenerate primers (see below). In order to isolate ethylene perception components from pear, six PCR primers *ETR1-F* and *ETR1-R*, *ETR2-F* and *ETR2-R*, and *CTR1-F* and *CTR1-R* were designed from the conserved regions of *At-ETR1*, *At-ERS1* of *Arabidopsis* (Chang *et al.*, 1993; Hua *et al.*, 1995, 1998), *NR*, *Le-ETR1* of tomato (Wilkinson *et al.*, 1995; Zhou *et al.*, 1996), *At-ETR2*, *At-ERS2*, *At-Ein4* of *Arabidopsis* (Sakai *et al.*, 1998; Hua *et al.*, 1998), and *CTR1* of *Arabidopsis* and tomato (Kieber *et al.*, 1993; Clark *et al.*, 1998; Zegzouti *et al.*, 1999). The oligonucleotides used were *ETR1-F* (5'-GAGACGGG[ATC]AG[AG]CATGT[AG-CT]AG[AG]ATG-3'); *ETR1-R* (5'-CATGGG[AC]GTTCTCATTT-CATG[AG]TTCAT-3'); *ETR2-F* (5'-CAGAATTGTGCGGTTTGCATGCCG-3'); *ETR2-R* (5'-CACAACTTTAACAATCTCAATC-TCCTG-3'); *CTR1-F* (5'-ATGGAGCAAGA[CT]TT[CT]CATGCT-GAGCG-3'); *CTR1-R* (5'-ATCTCG[AC]T[GT]AACTTC[AGCT]-GGTGCCATCC-3'). A high fidelity PCR system (BMB Indianapolis, IN) was used with the following PCR parameters: 1 min 30 s template denaturation at 95 °C, 2 min primer annealing at 58 °C, and 1 min primer extension at 72 °C for five cycles, followed by 15 cycles at 95 °C (30 s), 58 °C (1 min), and 72 °C (1 min 30 s), then 20 cycles at 95 °C (30 s), 58 °C (1 min), and 72 °C (2 min) with a final 7 min extension step at 72 °C. The isolated sequences were compared with database sequence using the BLAST program (Altschul *et al.*, 1997). Alignments and comparison of the predicted protein sequences were performed with ClustalX (Jeanmougin *et al.*, 1998), and GeneDoc (Nicholas and Nicholas, 1997), and the tree was visualized with the Treeview program (<http://taxonomy.zoology.gla.ac.uk/rod/rod.html>).

Two full-length and one partial cDNA clone putatively coding for ethylene receptors, and a partial cDNA homologue of *Arabidopsis At-CTR1* were isolated. Genomic sequences for four putative ethylene receptors were also isolated by PCR. Extension of the partial cDNA clones was carried out by screening a pear fruit cDNA library and by using the 3' and 5' RACE kit from Gibco BRL.

Finally, a cDNA was isolated with homology to actin genes from a wide range of species.

Relative quantification of mRNA based on RT-PCR

A semi-quantitative RT-PCR method was used for the relative quantification of mRNA levels in fruit tissues (Wang *et al.*, 1989; Zegzouti *et al.*, 1999). Complementary DNA was synthesized with a first strand cDNA synthesis kit (Promega) using 1 µl (50 U) M-MuLV Reverse Transcriptase and 10 µg of total RNA. The number (*n*) of cycles necessary for exponential, but non-saturated, PCR amplification (in the presence of 200 µM dNTPs, 2.5 mM Mg²⁺, and 2.5 U of *Taq* DNA polymerase) was determined for each clone using the cDNA from the highest expressing sample. For actin, the required number of PCR cycles was 25. Ethylene signal component gene-specific primers (Table 1) were added to the PCR either before or with the actin primers, depending on the relative abundance of the two mRNA species. The following PCR parameters were used: 1 min 30 s template denaturation at 95 °C, 1 min primer annealing at 58 °C, and 1 min primer extension at 72 °C for five cycles, followed by 15 cycles at 95 °C (30 s), 58 °C (1 min), and 72 °C (1 min 30 s), then the required number of cycles at 95 °C (30 s), 58 °C (1 min), and 72 °C (2 min) with a final 5 min extension step at 72 °C.

The PCR products were separated on a 2% agarose gel, transferred to a nylon membrane, and hybridized with an α³²P dCTP-labelled

Table 1. List of oligonucleotide PCR primers, number of PCR cycles; size of PCR product; and region of gene corresponding to primer sequence

Name	Oligonucleotide sequence.	No. PCR cycles	Size of PCR product	Region of gene corresponding to primer sequence
<i>Pc-Actin</i>	5'-CTATGAACAAGAGCTAGAGACATC-3' 5'-AGAGAAATCGACATGAATGAAATTC-3'	25	597 bp	3' TR ^a 3'nTR
<i>Pc-ETR1a</i>	5'-ATACATGAAAGGTTACAAAACGC-3' 5'-TGCTCTTCACAGGCCAGCGCCATC-3'	25	353 bp	3'TR ^b 3'nTR
<i>Pc-ERS1a</i>	5'-ATCGTTGCATTCATCGTCAAACCTG-3' 5'-GATTAACAGTTATAAACGGCATCG-3'	27	362 bp	3'TR 3'nTR
<i>Pc-ETR5</i>	5'-ATGATTCTCAAAGTAAAAGTGAGAG-3' 5'-CCAACACATCTGGGTCATTAATTG-3'	27	321 bp	5'TR ^c 5'TR
<i>Pc-CTR1</i>	5'-GAAGTCAGATGTTTACAGTTTTGGTG-3' 5'-AAGAATACATATTGAAGGTAATGG-3'	27	405 bp	3'TR 3'nTR

^a 3' TR=3' translated sequence.

^b 3'nTR=3' non-translated sequence.

^c 5' TR = 5' translated sequence.

gene-specific probe in 50% (w/v) deionized formamide, 4× SSPE, 1% SDS, 5× Denhardt's solution, 10% Dextran sulphate-Na salt (MW 500 000), and 100 µg ml⁻¹ denaturated DNA (salmon sperm) at 55 °C. The PCR fragments were labelled by incorporation of [α^{32} P] dCTP using Ready-To-Go DNA Labelling Beads (Amersham Pharmacia Biotech). The membranes were finally washed at 68 °C in 4× SSPE for 5 min and radioactivity corresponding to each band was directly counted with the Ambis 100 β -counter. The expression level for each cDNA was given as the percentage relative to the maximum expression level (MAX) and the expression ratio for actin: % Relative abundance of transcript X in situation S=100×(number of total counts for gene X in situation S) (number of total counts for actin in situation S)⁻¹ (number of total counts for actin in situation MAX) (number of counts for gene X in situation MAX)⁻¹.

Results

Ethylene perception genes structure and organization

PCR amplifications using degenerate oligonucleotide primers resulted in the isolation of partial cDNAs of the expected sizes for fragments of *Pc-ETR1* (625 bp), *Pc-ETR2* (350 bp), and *Pc-CTR1* (320 bp). The amplified fragments were cloned into the pGEM-T vector, sequenced and found to have strong nucleotide sequence similarity to the *Arabidopsis At-ETR1*, *At-ERS1*, *At-ETR2*, and *At-CTR1* mRNAs.

A cDNA library from fruit stored for 100 d at 0 °C (Lelièvre *et al.*, 1997b) was screened with the *Pc-ETR1* probe in order to isolate the full length cDNA sequences. Two full-length cDNA clones were isolated. The first cDNA was 2852 bp in length with a predicted open reading frame (ORF) encoded a protein of 741 amino acids. The 5'-non-coding, 3'-non-coding, and poly (A⁺) sequences were 324, 282, and 22 bp, respectively. The predicted ORF was more than 82% identical in amino acid sequence to other ethylene receptor genes (Table 2). The predicted amino acid sequence contained putative hydrophobic and histidine (His) kinase regions and a receiver domain, indicating

Table 2. Amino acid sequence comparison between the predicted full length pear, *Arabidopsis* and tomato ethylene receptor sequences

	Protein size (No. of aa)	Amino acid identity (%)			
		<i>Pc-ETR1a</i>	<i>Pc-ETR1b</i>	<i>Pc-ERS1a</i>	<i>Pc-ERS1b</i>
Pear					
<i>Pc-ETR1a</i>	741	100			
<i>Pc-ETR1b</i>	741	94	100		
<i>Pc-ERS1a</i>	638	58	59	100	
<i>Pc-ERS1b</i>	638	58	59	89	100
Arabidopsis					
<i>At-ETR1</i>	738	83	82	56	56
<i>At-ERS1</i>	613	56	57	67	67
Tomato					
<i>Le-ETR1</i>	754	82	81	55	55
<i>Le-ETR2</i>	736	79	78	66	67
<i>NR</i>	635	58	58	69	69

that it is an *At-ETR1*-like ethylene receptor. This clone was therefore designated *Pc-ETR1a*.

The second clone isolated with the *Pc-ETR1* probe had strong sequence similarity to the *Arabidopsis At-ERS1* mRNA. The isolated cDNA was 2510 bp in length with a predicted ORF encoding a protein of 638 amino acids. The 5'-non-coding, 3'-non-coding, and poly (A⁺) sequences were 364, 241, and 18 bp, respectively. The full length predicted protein shared more than 67% amino acid identity with other ethylene receptor genes (Table 2). The predicted amino acid sequence contained the three hydrophobic domains and the His kinase domain, but lacked a receiver domain, indicating that it is a homologue of the *Arabidopsis At-ERS1* protein. It was designated *Pc-ERS1a*. Ethylene receptors can be divided into two subgroups based upon sequence divergence in the conserved domains (Bleecker, 1999) (Fig. 2). *Pc-ETR1a* and *Pc-ERS1a* belong to the *ETR1* subgroup. The *ETR1* subfamily includes pear *Pc-ETR1a/b*, *Pc-ERS1a/b*, the

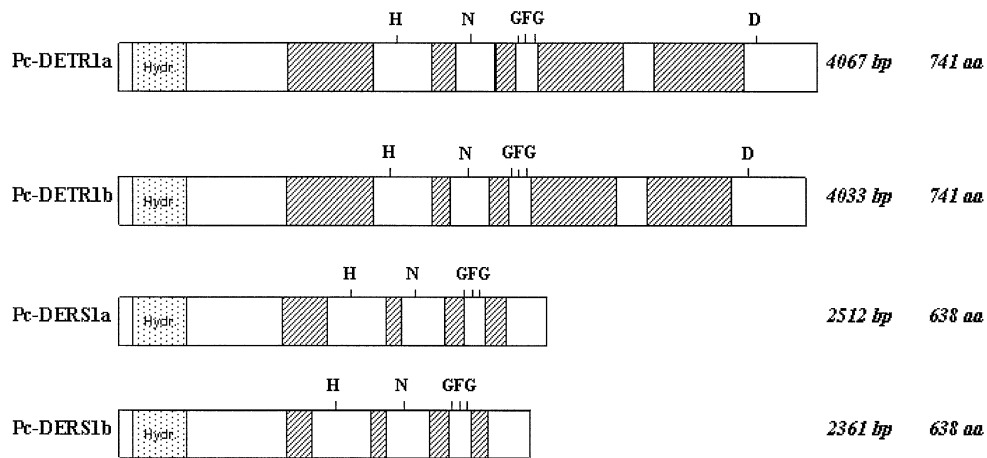


Fig. 1. Structural comparison of exon and intron positions of putative ethylene receptors *Pc-DETR1a*, *Pc-DETR1b*, *Pc-DERS1a*, and *Pc-DERS1b* in PC pear. The unshaded portions represent exons. The point shaded boxes in the N-terminal exon represent the three putative transmembrane hydrophobic domains. Introns are hash shaded. The letters (H, N, G, F, G) indicate the positions of the His kinase domains. The letter D in the C-terminal exons of *Pc-DETR1a* and *Pc-DETR1b* represent the putatively phosphorylated site in the receiver domain. Number of base pairs (bp) = full length genomic sequence from the start codon to the stop codon. Number of amino acid residues (aa) is for the full length predicted protein sequence.

Arabidopsis At-ETR1, *At-ERS1* genes, the apple *Md-ETR1* (AF032448) gene, and the tomato *Le-ETR1*, *Le-ETR2*, and *NR* genes.

Oligonucleotide primers were designed from the 350 bp (*Pc-ETR2*) sequence and were used in 3' and 5' RACE. This resulted in the isolation of a cDNA with strong nucleotide sequence similarity to *Arabidopsis At-ETR2*, *At-EIN4* and *At-ERS2* (Sakai *et al.*, 1998; Hua *et al.*, 1998) and tomato *Le-ETR4* and *Le-ETR5* (Tieman and Klee, 1999) mRNAs. The sequence was labelled *Pc-ETR5* and appears to be incomplete. The predicted *Pc-ETR5* protein (582 amino acid residues) lacks the C-terminal region but has the four hydrophobic domains and the degenerate histidine kinase-encoding domain typical of the second subgroup of ethylene receptors, the *ETR2* subgroup. The *ETR2* subfamily includes *Pc-ETR5*, *Arabidopsis At-ETR2*, *At-EIN4*, *At-ERS2*, and tomato *Le-ETR4*, and *Le-ETR5* genes.

Cloning of the genomic sequences for the putative ethylene receptors, *Pc-ETR1a* and *Pc-ERS1a* resulted in the isolation of four related sequences: *Pc-DETR1a* (4067 bp), *Pc-DETR1b* (4033 bp), *Pc-DERS1a* (2512 bp), and *Pc-DERS1b* (2361 bp). The *Pc-DETR1a* gene corresponds to the *Pc-ETR1a* cDNA and is closely related to a second gene, *Pc-DETR1b* (Fig. 1; Table 3a). There is also a closely related gene to *Pc-DERS1a* (*Pc-ERS1a* cDNA) (Fig. 1; Table 3b). This gene has been labelled *Pc-DERS1b*. It is unknown whether of *Pc-DETR1b* and *Pc-DERS1b* are expressed as all attempts to isolate their cDNAs were unsuccessful.

A partial sequence encoding a predicted polypeptide of 520 amino acid residues was also isolated. The predicted protein displayed strong homology (63% identity, 77%

similarity) (Fig. 3) to the *Arabidopsis At-CTR1* protein (Kieber *et al.*, 1993). The C-terminal region of *Pc-CTR1* has all the features of a Ser/Thr specific protein kinase, including an ATP binding site (VGAGSFGTV) and a IVHWDLKSPN Ser/Thr kinase domain (Fig. 3).

Finally, one actin sequence (AF386514) was isolated. Northern analyses (data not shown) verified that the actin mRNA level remained similar in all treatments. The actin sequence was used as an internal control in RT-PCR gene expression studies.

Separating the competence for climacteric ripening from the cold-induced stimulation of ethylene biosynthesis in Pisse-Crassane

Post-harvest 1-MCP treatment immediately before refrigeration reduced the cold-induced increase in ethylene production (Fig. 4A) and abolished the ethylene burst in rewarmed fruit (Fig. 4B). PC fruit previously chilled for 30 d after harvest produced the ethylene burst, i.e. the climacteric, and fruit ripened after approximately 17 d of propylene treatment (Fig. 4C). In non-chilled fruit, propylene treatment did not result in an ethylene burst after 20 d (Fig. 4C).

Effects of chilling and ethylene on the expression of the four ethylene perception cDNAs in Pisse-Crassane

Semi-quantitative RT-PCR analysis of steady-state mRNA levels were carried out to determine mRNA accumulation patterns during chilling and ripening in pear fruit. *Pc-ETR1a* mRNA accumulate to higher levels and at a greater rate during the cold treatment than *Pc-ERS1a*, *Pc-ETR5*, and *Pc-CTR1* (Fig. 5A). When normal ripening was

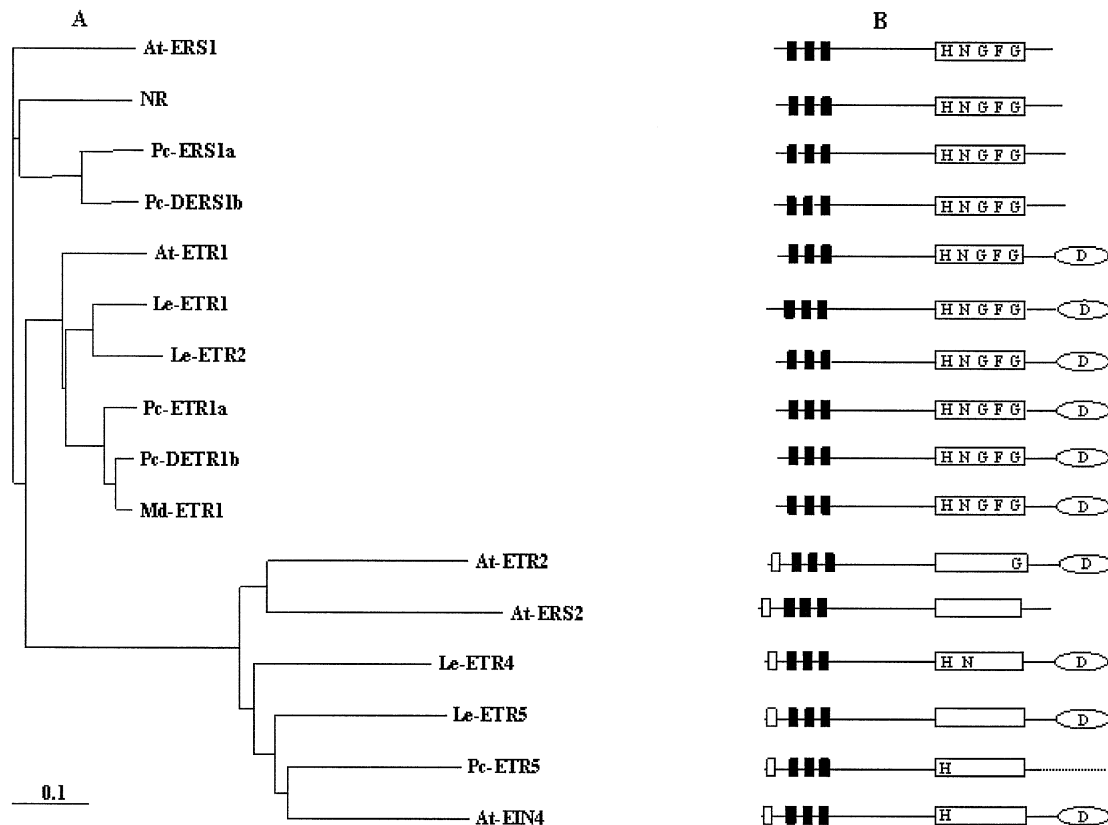


Fig. 2. Relationship between *ETR1*- and *ETR2*-like subfamily members. (A) Phylogenetic relationships between *Arabidopsis thaliana* [*At-ETR1* (P49333), *At-ERS1* (U21952), *At-ETR2* (AF047975), *At-ERS2* (AF047976), and *At-EIN4* (AF048982)], *Pyrus communis* [*Pc-ETR1a* (AF386509), *Pc-DETR1b* (AF386520), *Pc-ERS1a* (AF386515), *Pc-DERS1b* (AF386522), and *Pc-ETR5* (AF386511)], *Lycopersicon esculentum* [*Le-ETR1* (U41103), *Le-ETR2* (AF043085), *NR* (U38666), *Le-ETR4* (AF118843), and *Le-ETR5* (AF118844)], and *Malus domestica* [*Md-ETR1* (AF032448)] based on amino acid sequence divergence in the ethylene-binding and His kinase domains. Sequences were aligned using the ClustalX (Jeanmougin *et al.*, 1998) method and the tree was constructed with the tree view program. (B) Structural domains of proteins corresponding to those adjacent in (A). Domains are represented as small rectangles (white and black); ethylene-binding trans-membrane domains, long open rectangle; histidine-kinase domain (the letters H, N, G, F, G indicate the positions of the non-degenerate His kinase domains), open oval; receiver domain (D in the receiver domain indicates the presence of the putative phosphorylation site). Dotted line at C-terminus of *Pc-ETR5* indicates that the sequence is incomplete.

inhibited (fruit in air at 20 °C) the expression of the four mRNAs remained at basal levels (data not shown). Ethylene is essential for the increase in mRNA levels for *Pc-ETR1a*, *Pc-ERS1a*, *Pc-ETR5*, and *Pc-CTR1* during the cold treatment, as inhibition of ethylene action by 1-MCP resulted in the elimination of any increase (Fig. 5C). *Pc-ETR5* and *Pc-CTR1* mRNA peaked at ~15 d after fruit were placed at 20 °C for fruit previously chilled for 80 d, whereas *Pc-ETR1a* and *Pc-ERS1a* mRNA continued to increase past this peak. The peak of *Pc-ETR5* and *Pc-CTR1* mRNA coincided with the climacteric peak in ethylene production (Figs 4B, 5B). Post-cold-treatment increases were dependent on ethylene action as they were not present when the fruit were pre-treated with 1-MCP (Fig. 5C, D).

Propylene induced changes in mRNA levels for the four clones after a lag phase of about 17 d following rewarming. This coincided with the onset of the ethylene climacteric (Fig. 5F). The propylene treatment had no effect in the absence of cold pretreatment (Fig. 5E).

Expression of the four ethylene perception mRNAs in pear fruit without cold requirement

Accumulation of transcripts during fruit ripening were measured on three other pear genotypes. Fruit from OH, A16 and A50 do not have the cold requirement of PC fruit. OH fruit are characterized by an early, rapid ripening, and an early and brief (maximal at 6 d) ethylene production maximum. A16 fruit display an early but slow ripening pattern compared to OH. A16 fruit ethylene production peaks at ~15 d after it begins to rise. Finally, A50 fruit ripen at a slow rate, except if first chilled. A50 fruit chilled for 45 d display a peak of ethylene production 10 d after removal from the cold (Fig. 6A).

In common with ethylene levels, *Pc-ETR1a* and *Pc-ERS1a* mRNA abundance increased during the ripening process (Fig. 6B, C). By contrast, *Pc-ETR5* and *Pc-CTR1* mRNA decreased during ripening of the early pears, OH and A16, but not for the late A50 pear (Fig. 6D, E).

Table 3. Structural characteristics of genomic sequences (A) *Pc-DETR1a* and *Pc-DETR1b*, and (B) *Pc-DERS1a* and *Pc-DERS1b*

Region ^a	Length (bp)		Identity (%)
	<i>Pc-DETR1a</i>	<i>Pc-DETR1b</i>	
A			
Total length	4067	4033	96
Total exon length	2223	2223	94
Exon1	909	909	96
Intron1	526	526	100
Exon2	369	369	95
Intron2	139	103	74
Exon3	264	264	93
Intron3	95	95	100
Exon4	129	129	91
Intron4	529	529	100
Exon5	195	195	96
Intron5	555	527	99
Exon6	357	357	91
B			
Total length	2512	2361	84
Total exon length	1914	1914	92
Exon1	909	909	91
Intron1	271	142	32
Exon2	369	369	92
Intron2	88	88	86
Exon3	267	267	91
Intron3	112	108	71
Exon4	126	126	93
Intron4	127	106	65
Exon5	243	243	92

^a Intron-exon boundaries predicted by using the Blast program (Altschul *et al.*, 1997).

Pc-ETR5 and *Pc-CTR1* basal expression levels were relatively high while ethylene production was low in early pears (OH and A16).

Discussion

Pear fruit are climacteric, producing a burst of autocatalytic ethylene that triggers and maintains many aspects of the ripening process. The fruit from late ripening pear varieties such as PC require a long cold treatment before they can ripen (Gerasopoulos and Richardson, 1997b). Cold treatment enables the fruit to produce autocatalytic ethylene (Lelièvre *et al.*, 1997b), and hence to ripen. The aim of this work was to isolate components of the ethylene response in order to come to an understanding of the developmental control of ethylene perception and production in pears.

Four pear clones were isolated that closely matched known *Arabidopsis* ethylene response element genes. *Pc-ETR1a*, *Pc-ERS1a*, *Pc-ETR5*, and *Pc-CTR1* correspond to the *Arabidopsis* *At-ETR1*, *At-ERS1*, *At-ETR2*, and *At-CTR1* genes, respectively (Chang *et al.*, 1993; Hua *et al.*, 1995, 1998; Kieber *et al.*, 1993). *Pc-ETR1a*, *Pc-ERS1a*, and *Pc-ETR5* are predicted to code for ethylene receptors,

and *Pc-CTR1* for a pear homologue of the *Arabidopsis* Raf-like histidine kinase, *At-CTR1*. Both *At-CTR1* and the ethylene receptors act as negative regulators of the ethylene signal response pathway (Kieber *et al.*, 1993).

The complete *Pc-ETR1a* and *Pc-ERS1a* predicted proteins are 741 and 638 amino acids long, respectively. *Pc-ERS1a*, like its *Arabidopsis* homologue, *At-ERS1*, lacks the C-terminal receiver domain that forms part of *Pc-ETR1a* and the *Arabidopsis*, *At-ETR1* protein. However, *Pc-ETR1a* and *Pc-ERS1a* both contain the conserved motifs (H, N, G1, F, and G2) that characterize two component bacterial His protein kinases (Bleecker, 1999). In addition to *Pc-ETR1a* and *Pc-ERS1a*, a partial cDNA sequence, *Pc-ETR5*, was isolated that has a protein sequence closer to the *Arabidopsis* *At-ETR2*, *At-EIN4*, *At-ERS2* (Sakai *et al.*, 1998; Hua *et al.*, 1998), and tomato *LeETR4* and *LeETR5* (Tieman and Klee, 1999) proteins. Although several of the histidine kinase residues present in *At-ETR1* are degenerate in *At-ETR2* it has been shown to be a fully active ethylene receptor (Sakai *et al.*, 1998). The predicted *Pc-ETR5* protein (582 amino acid residues) lacks its C-terminal region, and has a histidine kinase domain which lacks four of the five histidine kinase residues conserved in the *ETR1*-like subfamily. In theory, these residues are necessary for transmitting the signal to downstream components of the ethylene pathway. However, as *At-ETR2* is a fully functional ethylene receptor, all members of this subgroup, including *Pc-ETR5*, are potentially functional (Bleecker, 1999; Klee, 2002). The three predicted proteins contain all the residues that have been shown to be important for ethylene receptor function (Schaller *et al.*, 1995; Bleecker and Kende, 2000). The predicted *Pc-ETR1a* protein also contains the aspartate residue in the receiver domain that has been predicted to be the target of autophosphorylation in *Arabidopsis* *At-ETR1*, *At-ETR2*, and other receiver sequences.

The N-terminal sequence of *Pc-ETR5* has four hydrophobic domains, as do the *Arabidopsis* *At-ETR2*, *At-ERS2*, *At-EIN4* (Sakai *et al.*, 1998; Hua *et al.*, 1998), and tomato *Le-ETR4* and *Le-ETR5* proteins (Tieman and Klee, 1999). Three of these domains share high sequence similarity with the three hydrophobic domains present in the *Arabidopsis* *At-ETR1/At-ERS1* proteins that are associated with ethylene binding activity (Chang *et al.*, 1993; Hua *et al.*, 1995, 1998). Together, these results strongly indicate that the three isolated sequences code for functional ethylene receptors in pear.

In the process of isolating the corresponding genomic sequences, two sequences *Pc-DETR1b* and *Pc-DERS1b*, were isolated that putatively code for close homologues of *Pc-ETR1a* and *Pc-ERS1a*, respectively. *Pc-DETR1b* is closely related in sequence to the genomic clone of *Pc-ETR1a*, *Pc-DETR1a*. Similarly, *Pc-DERS1b* is related to *Pc-ERS1a*. Nucleotide sequence similarity between both

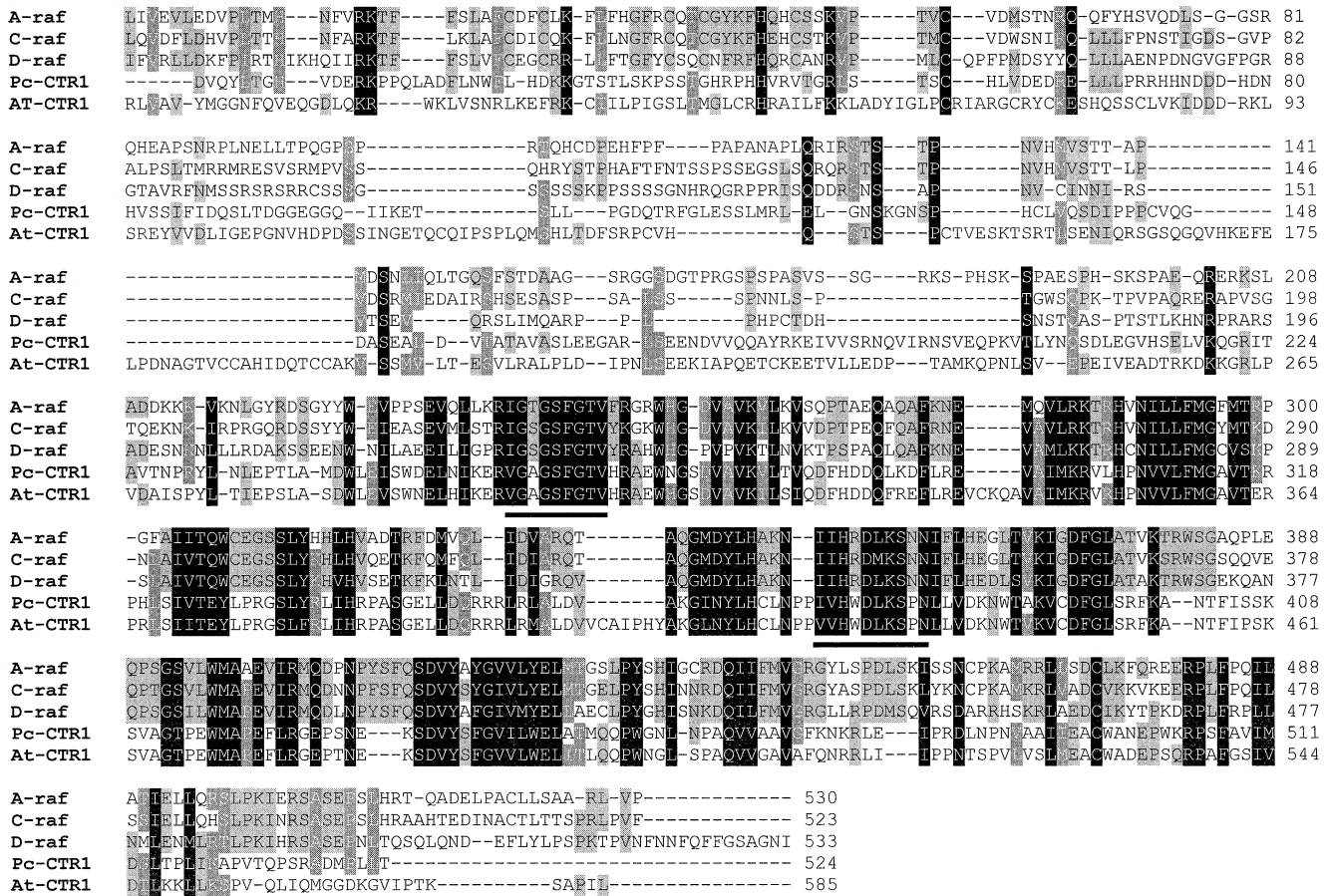


Fig. 3. Alignment of the putative Pc-CTR1 protein (AF386508) with At-CTR1 Ser/Thr protein kinase (T09911) and diverse raf kinase sequences A-raf (X04790), C-raf (X03484) and D-raf (X07181) using the ClustalX. Residues conserved between Pc-CTR1, At-CTR1, A-, C- and D-raf are shaded in black. Dark grey shading indicates similar residues in four out of five of the sequences and clear grey shading indicates similar residues in three out of five of the sequences. The underlined amino acids represent the conserved ATP fixation domain (VGAGSFGTV) and a conserved motif in serine/threonine protein kinases (IVHWDLKSPN).

genes is significant and in the case of *Pc-DETR1a* and *Pc-DETR1b* sequence similarity between the genes is higher for the introns than for the exons, suggesting the possibility that they are duplicated genes and that the introns are important for correct expression of the genes. The sequences of *Pc-DETR1b* and *Pc-DERS1b* also contain all the residues that have been shown to be important for ethylene receptor function (Schaller *et al.*, 1995; Bleecker and Kende, 2000). Attempts to reverse transcribe and amplify sequences corresponding to *Pc-DETR1b* and *Pc-DERS1b* from PC mRNA have been unsuccessful, suggesting that in PC these sequences may be pseudogenes.

The phylogenetic analysis indicates that, similar to *Arabidopsis* sequences (Bleecker, 1999), the pear sequences can be divided into two subfamilies. The pear Pc-ETR1a/b and Pc-ERS1a/b sequences belong to the *ETR1* subfamily, as do the *Arabidopsis* At-ETR1 and At-ERS1 (Chang *et al.*, 1993; Hua *et al.*, 1995), the apple Md-ETR1 (AF032448), and the tomato Le-ETR1, Le-ETR2, and NR

proteins (Wilkinson *et al.*, 1995; Lashbrook *et al.*, 1998; Zhou *et al.*, 1996). Sequence divergence is greater in the second group that includes the predicted Pc-ETR5, *Arabidopsis* At-ETR2, At-EIN4, At-ERS2 (Sakai *et al.*, 1998; Hua *et al.*, 1998) and the tomato Le-ETR4 and Le-ETR5 (Tieman and Klee, 1999) proteins. In tomato, there is a significant increase in *Le-ETR4* and *Le-ETR5* mRNA as fruits mature and ripen. Antisense inhibition of *Le-ETR4* results in a constitutive ethylene response and substantially earlier fruit ripening (Tieman *et al.*, 2000). This indicates that Le-ETR4 is critical for maintaining pre-climacteric ethylene insensitivity, but also that other developmentally-regulated factors are able to override its influence during ripening.

The predicted Pc-CTR1 protein has all the features of a Ser/Thr-specific protein kinase, including the ATP binding site (VGAGSFGTV) and the Ser/Thr kinase domain (IVHWDLKSPN) (Kieber *et al.*, 1993). CTR1, similar to the ethylene receptors, acts as a negative regulator of the

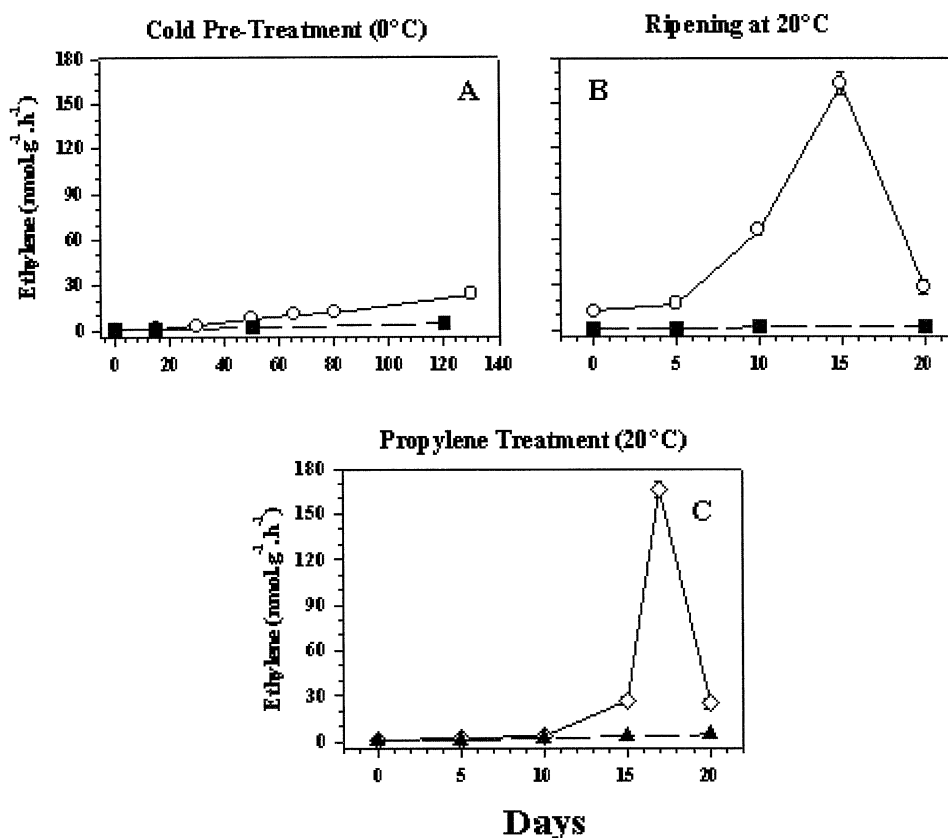


Fig. 4. Ethylene production in Passe-Crassane pear fruit during cold storage and ripening. Ethylene production of (A) untreated (open circles) or MCP-treated (filled squares) PC fruit during long-term cold storage; (B) untreated (open circles) or MCP-treated (filled squares) fruit during ripening at 20 °C after 80 d at 0 °C. MCP-treated fruit were exposed overnight to 1 $\mu\text{l l}^{-1}$ 1-MCP before cold treatment and (C) prechilled (open diamonds) (30 d at 0 °C) or non-chilled (filled triangles) fruit were treated with propylene (1000 $\mu\text{l l}^{-1}$) at 20 °C. The x-axis in each figure represents days of duration of the respective treatment.

ethylene response. Assuming that the sequences isolated in this study encode functional ethylene response regulators, the aim was to determine their expression patterns during pear fruit development in order to determine their involvement in ethylene sensitivity and the capacity to ripen.

Depending on growing season and location, PC fruit can require cold treatment from 0 d to 16 weeks in order to ripen (Ulrich, 1961; Leblond, 1975). This suggests that cold can replace endogenous signal(s) in late pear fruit. Changes in ethylene sensitivity, measured as the ability of PC fruit to generate autocatalytic ethylene in response to exogenous propylene, occurs early (~30 d) in the cold treatment of PC fruit. Ethylene-dependent ethylene production increases during long-term cold storage of PC fruit. It has been proposed for Anjou pears (Gerasopoulos and Richardson, 1997a) that a threshold level of endogenous ethylene may be required to sustain autocatalytic ethylene production. Early removal of the fruit from the cold leads to a fall of ethylene production because the endogenous ethylene level is too low. In PC fruit, cold treatment leads to a gradual increase in ethylene produc-

tion, but there is a commensurate increase in receptor expression. Ethylene/receptor binding changes the conformation of the receptor and effectively ‘switches off’ its inhibition of the signal response pathway (Moller and Chua, 1999). It is probable that a minor increase in ethylene production would be counteracted by the inhibitory effect of an increasing synthesis of ethylene receptors. There is the potential that these changes are involved in the attainment of a capacity to ripen, but given the magnitude of the changes it is unlikely that they are the primary cause of the changes in ethylene sensitivity.

The four putative ethylene perception elements are differentially regulated throughout pear fruit ripening. *Pc-ETR1a* mRNA accumulation is up-regulated by cold and during ripening. *Pc-ERS1a*, *Pc-ETR5*, and *Pc-CTR1* are less affected by cold treatment, but all increase during post-cold-treatment, ethylene-dependent ripening. This is similar to what has been observed for ethylene response elements in tomato and suggests that their primary role during this process is to temper the ethylene response (Klee, 2002).

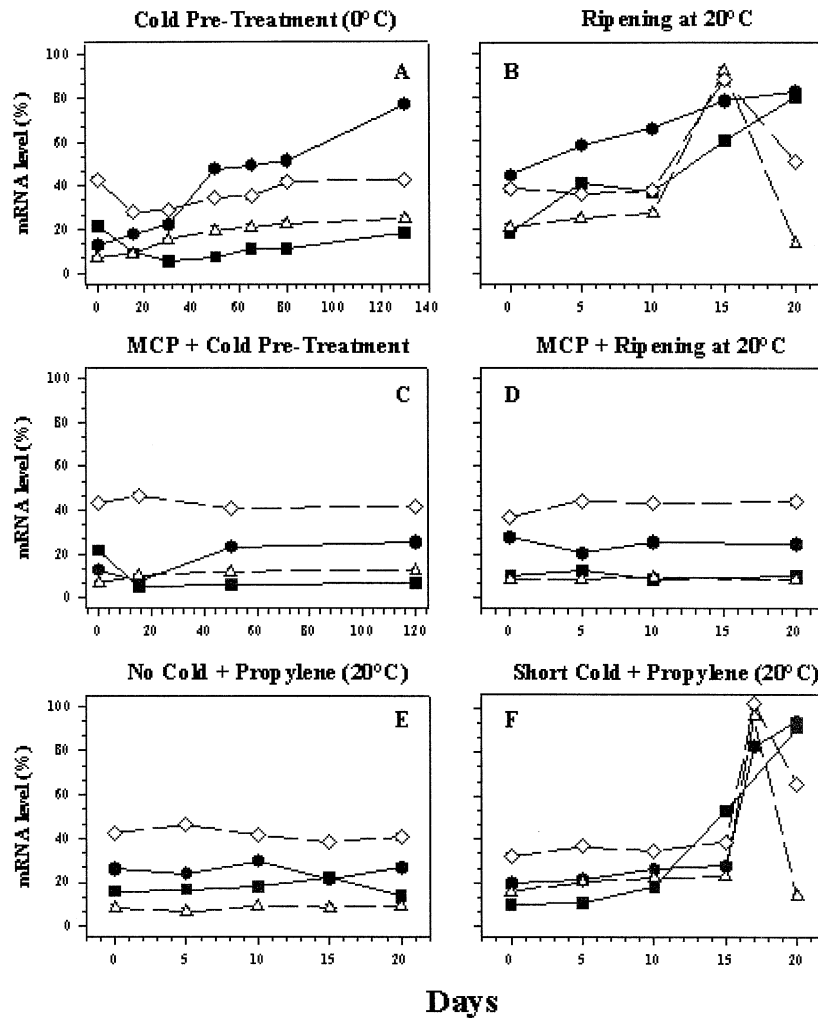


Fig. 5. Steady-state transcript levels for ethylene response elements in PC fruit. Transcript levels for Pc-ETR1a (filled circles), Pc-ERS1a (filled squares), Pc-ETR5 (open triangles), and Pc-CTR1 (open diamonds): (A) during long-term cold storage; (B) during ripening at 20 °C after 80 d at 0 °C; (C) during long-term cold storage after a 1-MCP pre-treatment; (D) during ripening at 20 °C after 80 d at 0 °C with a 1-MCP pre-treatment; (E) in the presence of propylene (1000 $\mu\text{l l}^{-1}$) at 20 °C without cold pre-treatment; and (F) during ripening in the presence of propylene (1000 $\mu\text{l l}^{-1}$) at 20 °C after 30 d at 0 °C. For the 1-MCP treatment fruit were exposed overnight to 1 $\mu\text{l l}^{-1}$ 1-MCP before cold treatment. The y-axis in each figure represents the expression level for each cDNA which was given as the percentage relative to the maximum expression level (MAX) and the expression ratio for actin. The x-axis in each figure represents days of duration of the respective treatment.

The ethylene climacteric and ripening occur without cold treatment in early season pears (A16 and OH) and after a brief cold treatment in winter pears (A50). There is a sharp peak of *Pc-ETR1a* and *Pc-ERS1a* mRNA accumulation during ripening in the early season pears, in contrast to the gradual increase seen in PC fruit. A more pronounced difference between early cultivars and PC fruit can be seen in the behaviour of *Pc-ETR5* and *Pc-CTR1* transcript accumulation. Transcript levels for *Pc-ETR5* and *Pc-CTR1* diminish sharply before and during the ethylene climacteric and ripening of OH and A16 fruit, whereas in PC fruit they increase sharply. A decrease in the expression of a negative regulator could result in an increase in ethylene sensitivity early in the ripening phase of early fruit development. However, given the potential for

redundancy in the ethylene receptor family it remains to be determined whether reduced levels of *Pc-ETR5* affect the overall ethylene sensitivity of early pear fruit.

There are clear differences between early pears and PC fruit in the mRNA accumulation patterns of the ethylene response components isolated in this study. It has been shown that the ethylene receptors and CTR1 act as negative regulators of the ethylene response. It has also been shown that, despite a degree of redundancy in the ethylene receptor family, the expression of ethylene receptors is related to function and therefore to the capacity of plant tissues to respond to ethylene. Differences between ethylene responsiveness between early season and winter pears may determine the ability to ripen, but it is more likely that these differences reflect a

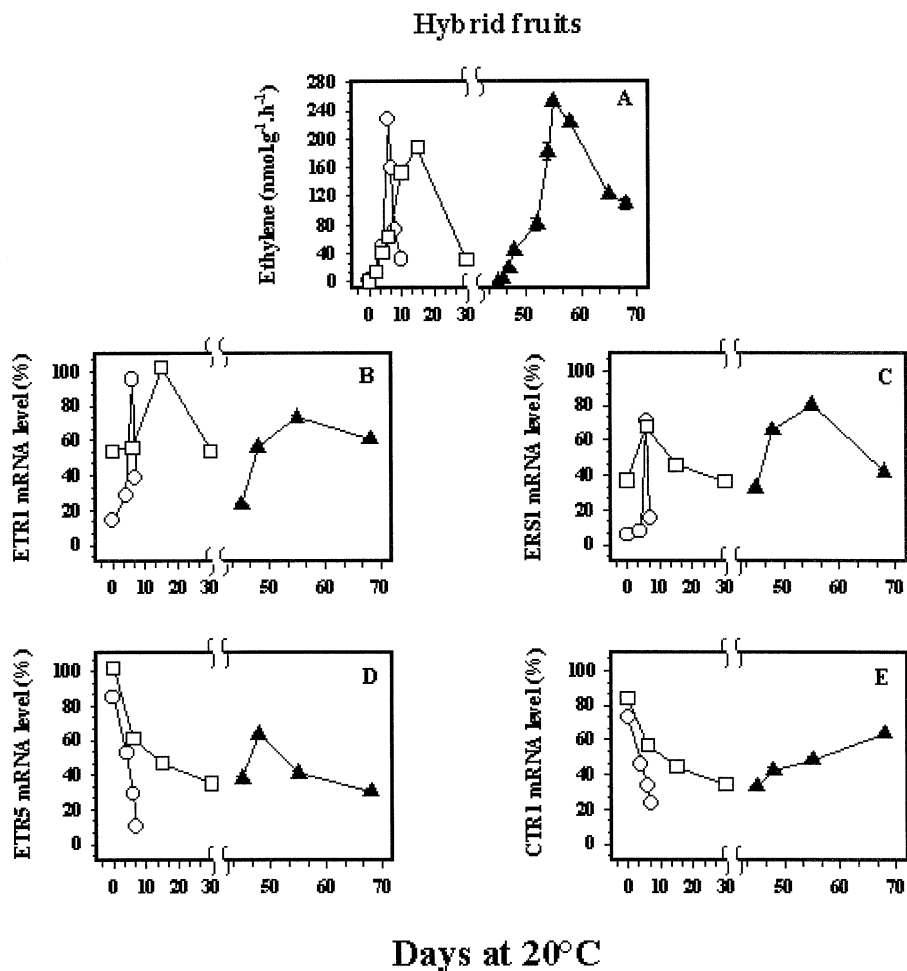


Fig. 6. Ethylene evolution and steady-state transcript levels in pears with reduced (A50, filled triangles) or no cold requirement (OH, open circles; A16, open squares). (A) Ethylene production during ripening; (B) Pc-ETR1a; (C) Pc-ERS1a; (D) Pc-ETR5; and (E) Pc-CTR1 levels during ripening at 20 °C. The relative transcript abundance of the four genes was followed during ripening at 20 °C. Early fruit were studied Old Home, OH (open circles) and A16 (open squares) fruit, and A50 late fruit ripened after 45 d of chilling treatment (filled triangles). A50 fruit ripened after 45 d of chilling treatment. The y-axis in each figure represents the expression level for each cDNA which was given as the percentage relative to the maximum expression level (MAX) and the expression ratio for actin. The x-axis in each figure represents days of duration of the respective treatment.

combination of developmentally or cold-induced changes that occur in the fruit. Further work is needed to determine the precise role of the isolated genes and their products in the capacity to ripen in pear fruit.

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