Differential regulation of ACC synthase genes in cold-dependent and -independent ripening in pear fruit

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ABSTRACT

Late pear cultivars such as Passe-Crassane (PC) require a long chilling treatment before they are capable of ripening. Early cultivars such as Old-Home (OH) have no cold prerequisite. The regulation of 1-aminocyclopropane-1-carboxylic acid synthase (*ACS*) genes was studied in OH, PC and in OH × PC hybrids in order to determine the role of this gene family in the cold requirement. Of the seven *Pc-ACS* cDNAs isolated, four (*Pc-ACS1a/b* and *Pc-ACS2a/b*) showed differential expression associated with the cold requirement. *Pc-ACS1a* transcripts accumulated throughout the cold treatment and, with *Pc-ACS2a*, during ripening of cold-dependent cultivars. *Pc-ACS1b* and *Pc-ACS2b* were detected only during ripening of cold-independent genotypes. Furthermore, *Pc-ACS2a* transcript accumulation was negatively regulated by ethylene, whereas *Pc-ACS2b* was positively regulated by the hormone. *Pc-ACS3*, *4* and *5* transcript accumulation was similar in all genotypes. Genetic analyses of OH, PC, and 22 OH × PC progenies demonstrated that late, cold-dependent cultivars were homozygous for *Pc-ACS1a* and *2a* whereas early, cold-independent cultivars were heterozygous for *Pc-ACS1*(*a/b*) and homozygous for *Pc-ACS2b*. A model is presented in which differences in *Pc-ACS* alleles and gene expression between cold- and non-cold-requiring pears are critical in determining the ripening behaviour of the cultivars.

Key-words: 1-aminocyclopropane-1-carboxylic acid (ACC); 1-aminocyclopropane-1-carboxylic acid synthase (ACS) alleles; ACS isoforms; cold requirement; pear fruit ripening; system 1 and system 2 ethylene production.

INTRODUCTION

A wide range of developmental processes and environmental responses are regulated by the plant hormone ethylene (Abeles, Morgan & Saltveit 1992). One of the most studied examples of ethylene regulation is the ripening of climacteric fruit. Climacteric fruit development typically includes a transition phase during which ethylene responsiveness and production levels are dramatically altered. Although the outcome of these changes, the ripening process, has been extensively characterized (Abeles *et al*. 1992; Lelièvre *et al*. 1997a), the developmental processes that lead up to and accompany the changes are less well understood (Giovannoni 2001).

Although tomato has generally been the model of choice in the study of climacteric fruit ripening (Alexander & Grierson 2002), species such as pear have distinct features that are economically important and provide opportunities to dissect specific mechanisms. European pear (*Pyrus communis*) like Asian pear (*Pyrus pyrifolia*) and apple (*Malus domestica*) are climacteric fruit characterized by a large diversity for the date and rate of ripening (Knee 1993; Itai *et al*. 1999). In most climacteric fruit, a short period (several hours to a few days) of ethylene treatment in mature fruit is sufficient to trigger autocatalytic ethylene production and ripening. Late ripening pears, such as Passe-Crassane (PC) and D'Anjou require a period of low temperature storage (0 °C) before they will ripen at higher temperatures (Ulrich 1961; Leblond 1975; Gerasopoulos & Richardson 1997). In PC, this cold requirement can vary from 0 to 110 d, depending on growth conditions (Ulrich 1961; Leblond 1975). Non-chilled fruit are unable to display the respiratory and ethylene climacteric and hence to ripen (Leblond 1975).

Cold treatment stimulates changes in both ethylene responsiveness and biosynthesis (Knee 1993; Gerasopoulos & Richardson 1997). Four putative ethylene perception elements were recently identified in pear, and the expression patterns conferred by these elements were altered in response to cold and ethylene and throughout fruit ripening (El-Sharkawy *et al*. 2003). The results suggested that the primary role of the isolated elements was to temper the ripening process rather than to control it.

Ethylene is synthesized from *S*-adenosyl-L-methionine via 1-aminocyclopropane-1-carboxylic acid (ACC) (Yang & Hoffman 1984). In PC fruit, cold-induced ethylene biosynthesis correlates with an increase in ACC content and in ACC synthase (ACS), and ACC oxidase (ACO) activities (Morin, Rigault & Hartmann 1985; Lelièvre *et al*. 1997b). ACS and ACO are the two key enzymes in the ethylene biosynthesis pathway. In *Arabidopsis*, tomato, and other

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species, ACO and ACS proteins are encoded by small gene families. It has been proposed that specific members of these two families control two systems of ethylene production in plants (Lelièvre *et al*. 1997a). System 1 is the basal low rate of ethylene production that is detected in all tissues including those of non-climacteric fruit. System 2, or autocatalytic ethylene, operates during climacteric fruit ripening. In tomato fruit, ethylene biosynthesis is primarily regulated by three different *Le-ACS* mRNAs, *Le-ACS2*, *Le-ACS4* and *Le-ACS6* (Nakatsuka *et al*. 1998). *Le-ACS2* and *Le-ACS4* are responsible for system 2 autocatalytic ethylene production and *Le-ACS6* is responsible for the auto-inhibitory, low levels of system 1 ethylene (Barry, Llop-Tous & Grierson 2000).

The objective of this study was to understand the role of *ACS* gene family members in cold-dependent pear fruit ripening. *Pc-ACS* transcript accumulation was studied during ripening and under various conditions in cultivars requiring cold treatment and in cultivars with an intermediate or no cold requirement. The aim was to uncover differences that would enable us to identify *Pc-ACS* genes critical to the cold requirement in PC pear fruit.

MATERIALS AND METHODS

Plant material and post-harvest treatments

Pear (*Pyrus communis* L. cv. Passe-Crassane) fruit were harvested and treated as described previously (El-Sharkawy *et al*. 2003). Other genotypes, Old-Home (OH) and OH × PC hybrid trees were obtained from INRA (Loire valley) Angers, France (Table 1). All genotypes were grafted onto quince rootstock BA29. Pre-climacteric fruit of PC, OH, R7A16 (A16), and R6A50 (A50) were harvested as late as possible before ethylene fruit production had risen and fruit had abscised. After treatments, measurements of ethylene production were taken and the fruit were frozen in liquid nitrogen and stored at –80 °C. Mixed tissues of two fruit displaying similar ethylene production at the same age were used for mRNA analysis.

Isolation and *in silico* analysis of pear cDNA sequences

The RNA extraction procedure used has been described previously (Lelièvre *et al*. 1997b). For semi-quantitative polymerase chain reaction (PCR), RNA extracts were treated with DNAse (Promega, Madison, WI, USA). First strand cDNA synthesis was carried out using 10 μg of total RNA in a 50-μL aliquot followed by PCR with appropriate primers using 1 μL of cDNA. Several sets of primers (Jones & Woodson 1999) were used to isolate pear *Pc-ACS* sequences that shared the structural characteristics associated with functional *ACS* genes (Zarembinski & Theologis 1994; Capitani *et al*. 1999). A high fidelity PCR system was used as described previously (El-Sharkawy *et al*. 2003). The isolated fragments were cloned into the pGEM-T vector

Table 1. Cold requirement, maturation time, and ethylene production in Old-Home, Passe-Crassane, and OH × PC hybrid pear fruit

Cultivars	Cold requirement[a]	Maturation time[b]	Maximum ethylene level[c]
Old-Home	–	Early	High
R7A4	–	Early	High
R7A16	–	Early	High
R6A46	+	Intermediate	Moderate
R6A28	+	Intermediate	Moderate
R6A33	+	Intermediate	Moderate
R6A40	+	Intermediate	Moderate
R7A13	+	Intermediate	Moderate
R6A21	+	Intermediate	Moderate
R7A2	+	Intermediate	Moderate
R6A18	+	Intermediate	Moderate
R6A41	+	Intermediate	Moderate
R6A50	+	Intermediate	Moderate
R6A57	+	Intermediate	Moderate
R6A9	+	Intermediate	Moderate
R6A15	+	Intermediate	Moderate
R6A22	+	Intermediate	Moderate
R6A55	+ +	Late	Low
R7A27	+ +	Late	Low
R6A35	+ +	Late	Low
R6A3	+ +	Late	Low
R6A47	+ + +	No	Low
R6A11	+ + +	No	Low
Passe-Crassane	+ + +	No	Low

[a]Cold requirement of pear fruit cultivars: (–) not requiring cold; (+) not requiring cold but cold accelerates ripening; (+ +) absolute requirement (short cold treatment); (+ + +) absolute requirement (long cold treatment). [b]Maturation time for fruit storage at 20 °C. [c]Maximum ethylene production during fruit ripening: high >220 nmol g^{-1} h^{-1}; moderate, 80–130 nmol g^{-1} h^{-1}; low < 10 nmol g^{-1} h^{-1}.

(Promega), sequenced and compared with database sequences using the BLAST program (Altschul *et al*. 1997). Extension of the partial cDNA clones was carried out using the 3′- and 5′-RACE kit (Invitrogen, Paisley, UK). Alignments of the predicted protein sequences were performed using CLUSTALX (Jeanmougin *et al*. 1998) and GENEDOC (Nicholas & Nicholas 1997). The neighbour-joining tree was constructed with PAUP*4.0b3 (Sinauer Associates, Inc., Sunderland, MA, USA). The tree excluded regions of the alignment where poor matching occurred. Bootstrap values from 1000 replicates were obtained. A neighbour-joining tree is shown, with bootstrap confidence values (1000 replications) shown for neighbour-joining algorithms. Bootstrap values of less than 35% are not shown. The tree was visualized with the TREEVIEW program (Page 1996). Finally, a cDNA clone was isolated with homology to an actin sequence (AF386514). It was checked by northern analysis (data not shown) that the actin mRNA level was similar in all treatments. The gene was, thereafter, used as an internal control in gene expression studies.

Relative quantification of mRNA based on reverse transcriptase-PCR

The reverse transcriptase (RT)-PCR approach was used plus the direct radioactive measurement of the amplified sequences (El-Sharkawy *et al*. 2003) because the mRNA level for several of the genes was low. Moreover, all RT-PCR experiments were repeated at least three times with three different cDNA synthesized from three different RNA extractions for the same sample. The required number of cycles necessary for exponential, but non-saturated PCR amplification was determined for each clone using the cDNA from the highest expressing sample. Ethylene biosynthesis component gene-specific primers were added to the PCR either before or after the actin primers, depending on the relative abundance of the two mRNA species (Table 2). The PCR products were separated on a 2% agarose gel, transferred to a nylon membrane, and hybridized with an [α^{32}P] dCTP labelled gene specific probe in 50% (w/v) deionized formamide, 4× SSPE (40 mM $NaH_2PO_4{\cdot}H_2O$, 0.6 M NaCl, 4 mM EDTA, pH 7.4), 1% sodium dodecyl sulphate (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 gene-specific fragments were labelled by incorporation of [α^{32}P] dCTP using Ready-To-Go DNA Labeling Beads (Amersham Biosciences, Freiburg, Germany). The membranes were finally washed at 68 °C in 4× SSPE for 5 min and radioactivity corresponding to each band was counted directly with the Ambis 100 β-counter (Ambis, San Diego, CA, USA). The expression level for each cDNA is given as the percentage relative to the maximum expression level MAX and the expression ratio for actin : % Relative abundance of transcript Y in situation S = 100 × (number of total counts for gene Y 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 Y in situation MAX)$^{-1}$.

Southern analysis and promoter isolation

Genomic DNA (gDNA) was extracted from immature leaves according to the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The gDNA (15 μg) was digested with *Bam*HI, *Eco*RI, *Eco*RV and *Hin*dIII. Digested gDNA was separated on a 0.8% agarose gel and transferred to a nylon membrane. The membranes were hybridized and *Pc-ACS1* and *Pc-ACS2* fragments labelled as described above. Hybridized membranes were washed at 68 °C in 2× SSC and 0.1% SDS for 15 min and twice for 15 min each in 0.2× SSC and 0.1% SDS. The blots were then exposed to X-ray

Table 2. 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
Pc-Actin-F	5′-ATGACGAAAGAGATTACAGCCTTG-3′	25	326 pb	3′ TR[a]
Pc-Actin-R	5′-AGAGAAATCGACATGAATGAAATTC-3′			3′ UTR[b]
Pc-ACS1a-F	5′-TCCTTTCCACACTTCTGTCTTACAGC-3′	21	485 pb	5′ UTR
Pc-ACS1a-R	5′-GGGTAGGAATAAGAAGAACTTCGCCGG-3′			5′-TR
Pc-ACS1b-F	5′-CACACTTTTGTGTTATAGCTTGTC-3′	20	500 pb	5′ UTR
Pc-ACS1b-R	5′-TCGCCACTTGAGGTCTCTATCAAATCC-3′			5′-TR
Pc-ACS2a-F	5′-TTTCAAAGGTTTTTCATTCAAACAATTC-3′	30	531 pb	5′ UTR
Pc-ACS2a-R	5′-TAGCTCAATTGCTCGAGTTGCACC-3′			5′-TR
Pc-ACS2b-F	5′-CTTTCTTGGAGGCCAAAGTGCTTT-3′	28	472 pb	5′ UTR
Pc-ACS2b-R	5′-GACGAAAGTCAATAGCTCATTTGCTGC-3′			5′-TR
Pc-ACS3-F	5′- GAATTCACGGAAAACTACATAAAG –3′	32	437 pb	3′ TR
Pc-ACS3-R	5′- AAGAGTAAATTGGGTTTTGTCCTC –3′			3′ UTR
Pc-ACS4-F	5′- CTTTTCTGTTGGATGGACTTAAGG –3′	34	491 pb	3′ TR
Pc-ACS4-R	5′- GAAATTAAAAACATATTCAACCTCAG –3′			3′ UTR
Pc-ACS5-F	5′- TGTGGAGAGATTTATAGCACAAAG –3′	28	527 pb	3′ TR
Pc-ACS5-R	5′- CATCATGATCAACATTTGTAGCTG –3′			3′ UTR

[a]TR, translated region. [b]UTR, untranslated region.

film at –80 °C overnight. The probes of *Pc-ACS1* and *Pc-ACS2* used corresponded to the 3′ end of the cDNA (including part of the 3′ non-coding region). The polymorphisms were obtained for *Pc-ACS1* and *Pc-ACS2* genes with the *Eco*RI and *Hin*dIII-digested gDNA, respectively.

Promoters of *Pc-ACS1* (*Pc-DACS1a*/*b*) and *Pc-ACS2* (*Pc-DACS2a*/*b*) were isolated from PC and OH genotypes using the Universal Genome Walker Kit (Clontech, Palo Alto, CA, USA). In order to identify the presence of the different *Pc-ACS1* and *Pc-ACS2* promoters in OH, PC, and 22 OH × PC progeny, four specific PCR primers for *Pc-DACS1a* and *b* promoters, ACS1a-F [-275] (5′-ATGT CATTTTGAGATAATATTCTTATC-3′); ACS1a-R (5′-GCCATTGGAGCTTGTGCAGTGAATGGG-3′) [510]; ACS1b-F [–271] (5′-ATAGAGCATAAATGTTCCCT TGTTTGTC-3′); ACS1b-R (5′-AGACCCATCTGAAT AATCCCGTTTGTGTTG-3′) [134], and four specific primers for *Pc-DACS2a* and *b* promoters, ACS2a-F [–1260] (5′-CTGGTGCATTTCCTCCACTGAAAAC-3′); ACS2a-R (5′-GCCACCTAACATGATAAATAAATG-3′) [–994]; ACS2b-F [–1588] (5′-GGATTAAAAGCTCCAATGA AGGGC-3′); ACS2b-R (5′-AGGATGAATATTCACAA CGATTAAAAAGG-3′) [–1151], were designed from each promoter sequence. The reaction mixture (100 μL) contained about 150 ng of template gDNA was carried out by using Advantage Genomic PCR Kit (Clontech) following the instructions provided by the manufacturer.

RESULTS

ACC synthase gene structure and organization

ACS genes and proteins have been isolated from a wide variety of plant species. Although sequence identity can be as low as 45% in ACS proteins, there are highly conserved signature elements. Only one pear *ACS* mRNA, *Pc-ACS1a*, had been isolated prior to this study (Lelièvre *et al*. 1997b). Six novel *Pc-ACS* sequences were isolated from a variety of pear cultivars using an RT-PCR approach in order to investigate the involvement of ACS proteins in pear fruit ripening (Fig. 1). Eleven out of 12 amino acid residues conserved in aminotransferase and ACS proteins are present in all of the pear sequences. The four important residues (G205, D230, K273, R407) that have been studied by site-directed mutagenesis in the apple *MdACS-1* protein (White *et al*. 1994) are conserved in the predicted pear sequences (Fig. 1).

The relationships between the predicted amino acid sequences, as indicated by percentage identity over the whole sequence, are presented in Table 3. Although there was considerable divergence among the *Pc-ACS* mRNAs, there were also highly homologous sequences putatively coding for closely related isozymes. Sequences related to the isolated pear cDNAs have previously been identified in apple and Asian pear (Harada *et al*. 1997; Itai *et al*. 1999). Strong sequence identity between specific pear, apple and Asian pear sequences (94–97%, Table 3) indicates that these sequences are likely to be orthologous.

Two *Pc-ACS1* isoforms, *a* and *b*, were identified in the pear cultivars, PC and OH, respectively. The predicted amino acid sequences of *Pc-ACS1a* and *1b* differed by only 13 amino acids. These comprised 12 amino acid substitutions and *Pc-ACS1b* has one more amino acid than *Pc-ACS1a*, a Thr at position 456 (Fig. 1). The percentage similarity between the isolated 5′- and 3′- non-coding regions of these two isoforms was found to be 50 and 94%, respectively. Similarly, two closely related *Pc-ACS2* isoforms isolated from PC (*Pc-ACS2a*) and OH (*Pc-ACS2b*) exhibited divergence in the 5′- and 3′- non-coding regions, with 87

Table 3. Amino acid sequence comparison between the predicted full length *Pyrus communis*, *Pyrus pirofolia*, and *Malus domestica* ACC synthase proteins

		Amino acid identity percentage						
	Protein size	Pc-ACS1a	Pc-ACS1b	Pc-ACS2a	Pc-ACS2b	Pc-ACS3	Pc-ACS4	Pc-ACS5
Pyrus communis								
Pc-ACS1a	473	100						
Pc-ACS1b	474	96	100					
Pc-ACS2a	446	51	52	100				
Pc-ACS2b	446	52	53	96	100			
Pc-ACS3	446	52	53	94	95	100		
Pc-ACS4	495	46	46	47	48	47	100	
Pc-ACS5	487	47	47	48	48	48	67	100
Pyrus pirofolia								
pPPACS1	473	97	97	52	53	53	47	47
pPPACS2	446	52	53	94	96	96	48	48
pPPACS3	495	46	46	47	48	47	97	68
Malus domestica								
MdACS-1	473	96	97	52	54	53	48	48
MdACS-3	446	52	53	94	95	96	48	49
MdACS-5 A	487	48	47	49	49	49	68	94
MdACS-5B	487	48	47	48	48	48	69	96

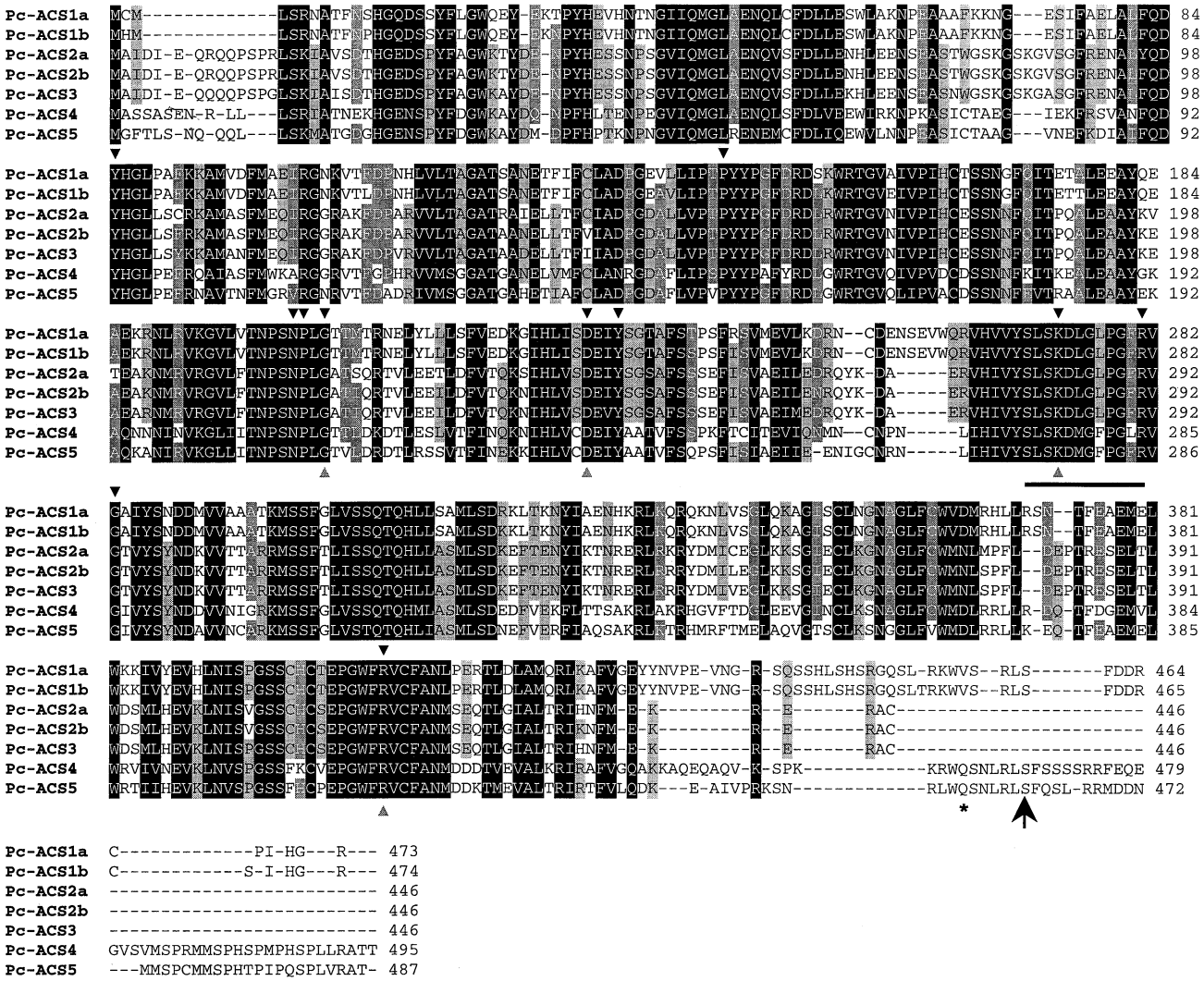

Figure 1. Amino acid sequence alignment of *Pc-ACS1a* (X87112), *Pc-ACS1b* (AY388987), *Pc-ACS2a* (AF386519), *Pc-ACS2b* (AY388989), *Pc-ACS3* (AY388988) *Pc-ACS4* (AF386518), and *Pc-ACS5* (AF386523) using CLUSTALX program. Conserved residues are shaded in black. Dark grey shading indicates similar residues in six out of seven of the sequences and clear grey shading indicates similar residues in five out of seven of the sequences. The 11 black arrows designate the residues that represent the conserved amino acids in aminotransferases. The four grey arrows represent the four residues which have been studied by site-directed mutagenesis in the apple *MdACS-1* sequence (White *et al.* 1994). The underlined amino acids indicate the active site of ACC synthase. The altered residue in *Pc-ACS4* and *Pc-ACS5* is marked with an asterisk. The predicted site of phosphorylation is shown with an arrow (Chae *et al.* 2003).

and 93% similarity, respectively. Both of the predicted *Pc-ACS2* isoforms also shared strong amino acid identity with *Pc-ACS3* (Table 3).

Arabidopsis ACS (At-ACS) proteins have been shown to be post-translationally regulated by the phosphorylation of the valine and serine residues in the C-terminal domain (Chae, Faure & Kieber 2003). Both of the predicted *Pc-ACS1* isoforms have the corresponding valine and serine residues (Fig. 1). In comparison, both of the predicted *Pc-ACS2* isoforms as well as *Pc-ACS3* are C-terminally truncated by 27–49 amino acids and consequently lack both the valine and serine (Fig. 1). The *Pc-ACS4* and *5* predicted proteins have the serine residue but lack the valine. In both these predicted proteins the valine residue is replaced by a glutamine (Fig. 1).

The phylogenetic tree presented in Fig. 2 was obtained by comparing the conserved domains of 35 *ACS* sequences from five species, European pear, *Arabidopsis*, tomato, Asian pear, and apple. It indicates that ACS proteins can be divided into two main subfamilies (I and II). The first subfamily further divided into two branches (I/A and I/B). Genes corresponding to the first branch (I/A), *At-ACS7*, *Pc-ACS2a*, *2b*, *3*, *pPPACS2*, and *MdACS-3*, have a short C-terminal and consequently lack both the valine and serine important for phosphorylation. The second branch (I/B) contained all the At-ACS proteins that have been shown to

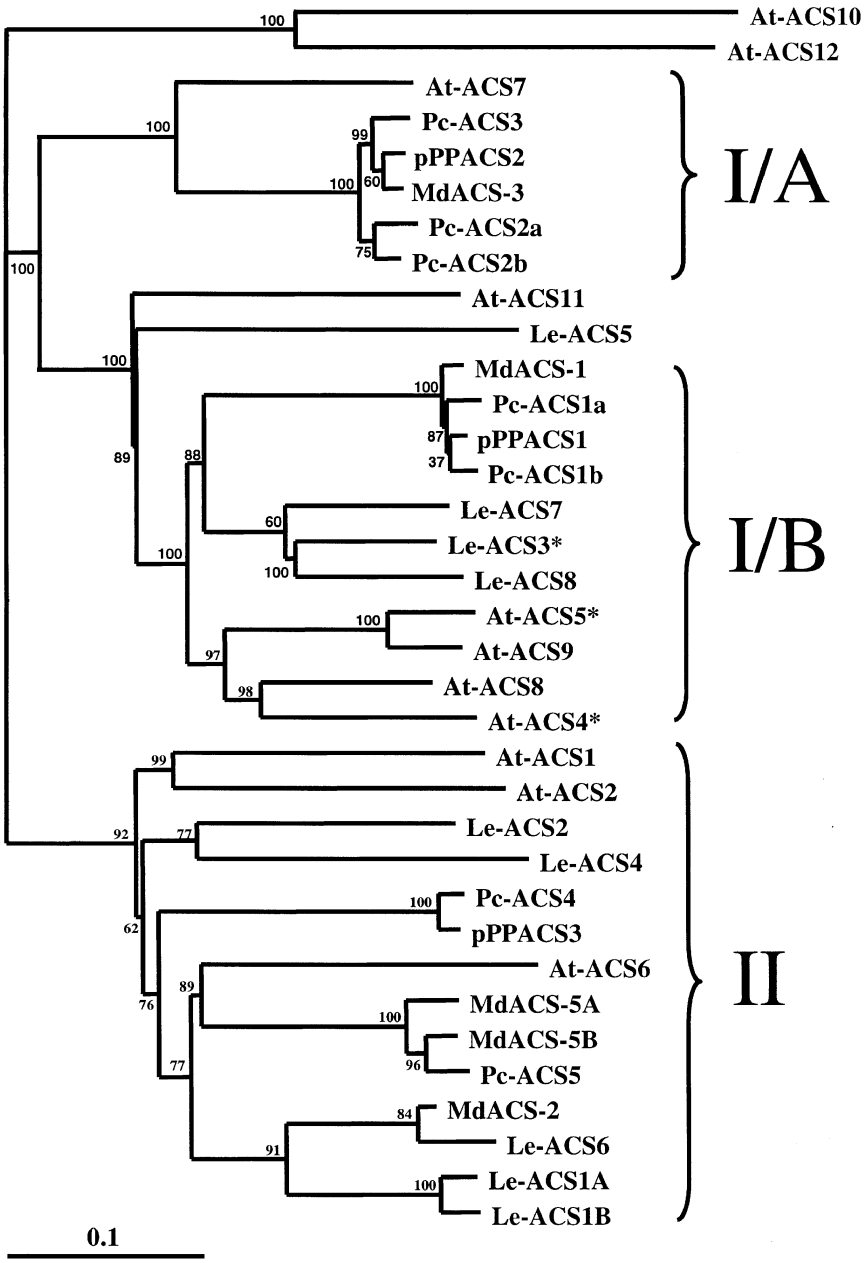

Figure 2. Phylogenetic relationships between *Pyrus communis* [*Pc-ACS1a*, *Pc-ACS1b*, *Pc-ACS2a*, *Pc-ACS2b*, *Pc-ACS3*, *Pc-ACS4*, *Pc-ACS5*], *Arabidopsis thaliana* [*At-ACS1* (AAA96006), *At-ACS2* (Q06402), *At-ACS4* (NP179866), *At-ACS5* (AAG50098), *At-ACS6* (T13019), *At-ACS7* (AAG48754), *At-ACS8* (AAG50090), *At-ACS9* (AAG48755), *At-ACS10* (AAK15546), *At-ACS11* (AF332405), *At-ACS12* (AAG54001)], *Lycopersicon esculentum* [*Le-ACS1A* (AAF97614), *Le-ACS1B* (AAF97615), *Le-ACS2* (CAA41855), *Le-ACS3* (AAB48945), *Le-ACS4* (AAA03164), *Le-ACS5* (AF179246), *Le-ACS6* (BAA34923), *Le-ACS7* (AAC32317), *Le-ACS8* (AAK72431)], *Malus domestica* [*MdACS-1* (AAB68617), *MdACS-2* (AAB67988), *MdACS-3* (AAB67989), *MdACS-5 A* (BAA92350), *MdACS-5B* (BAA92351)], and *Pyrus pirofolia* [*pPPACS1* (BAA76389), *pPPACS2* (BAA76388), *pPPACS3* (BAA78333)] based on amino acid sequence. The asterisk indicates characterized auxin-inducible ACS genes. Bootstrap confidence values from 1000 replicates are indicated. I/A, I/B, and II showed the different ACS proteins subfamilies.

be post-translationally regulated by the phosphorylation of a serine (*At-ACS4*, *5*, *8*, *9*) (Chae *et al*. 2003) and *Pc-ACS1a*, *1b*, *MdACS-1*, *pPPACS1*, *Le-ACS3*, *7* and *8*, indicating the possibility of a common regulatory mechanism for all members of the clade. Genes shown to be induced by auxin, including *At-ACS4*, *5*, and *Le-ACS3*, also fall into this group (Liang *et al*. 1992; Nakatsuka *et al*. 1998). Genes corresponding to the second subfamily (II) have the conserved C-terminal serine residue but lack the valine.

Cold requirement for stimulation of climacteric ethylene biosynthesis in PC pear fruit

Passe-Crassane fruit stored at 20 °C failed to produce significant amounts of ethylene even at 145 d post-harvest (Fig. 3a). The fruit required a long (80 d) cold pretreatment in order to be able to produce autocatalytic ethylene autonomously (Fig. 3b), and to ripen at 20 °C, after a short (approximately 5 d) lag period (Fig. 3c). A post-harvest 1-methylcyclopropene (MCP) treatment immediately before refrigeration eliminated any cold-induced increase in ethylene production (Fig. 3g) and abolished the ethylene burst and ripening in re-warmed fruit (Fig. 3h).

From the *Pc-ACS* mRNAs isolated, only *Pc-ACS1b* and *2b* transcripts were undetectable in PC fruit. In air at 20 °C, where normal ripening could not proceed, transcript levels of all *Pc-ACS* cDNAs remained at a basal level except for those of *Pc-ACS3* that were between 30 and 60% of their maximum levels (Fig. 3d). During cold storage at 0 °C, only *Pc-ACS1a* transcript levels showed a steady increase,

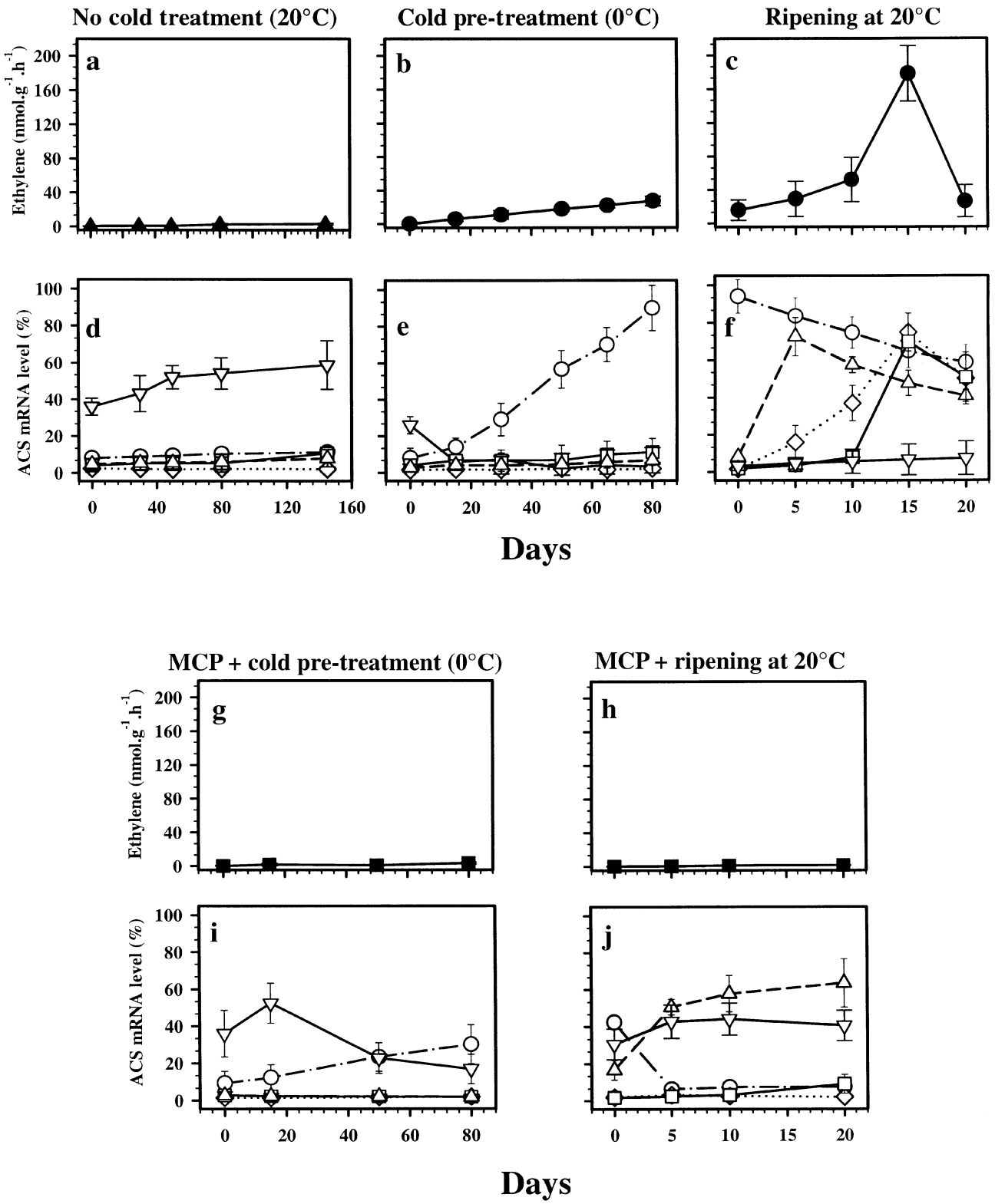

Figure 3. Ethylene production and gene expression in Passe-Crassane pear fruit. Ethylene production (a, b, c, g, k) and steady-state mRNA levels for *Pc-ACS1a* (○), *Pc-ACS2a* (△), *Pc-ACS3* (▽), *Pc-ACS4* (◇), and *Pc-ACS5* (□) genes (d, e, f, i, j): in fruit stored at 20 °C without cold treatment [a (▲), d]; during long-term cold storage [b (●), e]; during ripening at 20 °C after 80 d at 0 °C [c (●), f]; during long-term cold storage after a MCP pretreatment [g (■), i]; and during ripening at 20 °C after 80 d at 0 °C with a MCP pretreatment [h (■), j]. For the MCP treatment, fruit were exposed overnight to 1 μL L^{-1} MCP before the cold treatment. The *x*-axis in each figure represents days of the respective treatment.

whereas those for *Pc-ACS3* decreased in abundance (Fig. 3e). During re-warming at 20 °C, after cold storage for 80 d, transcript levels for *Pc-ACS1a* decreased slightly, whereas those for *Pc-ACS2a* increased to a peak at approximately 5 d. *Pc-ACS3* transcripts were undetectable in ripening PC fruit. *Pc-ACS4* and *5* transcript levels corresponded to the ripening-related peak in ethylene production (Fig. 3f).

MCP treatment inhibited the cold storage increase in *Pc-ACS1a* transcript levels (Fig. 3i) and the accumulation of *Pc-ACS1a*, *4*, and *5* transcripts during post-cold treatment ripening at 20 °C (Fig. 3j). By contrast, *Pc-ACS2a* and *3* transcripts were detected in re-warmed, MCP-treated fruit (Fig. 3j).

Propylene treatment advances ripening in short-term, cold-treated PC pear fruit

The results presented above suggest that the expression of the *Pc-ACS3* may be under negative feedback regulation in pear fruit. To test this hypothesis, unchilled and chilled PC fruit were treated with 1000 μL L^{-1} propylene at 20 °C for 32 d. Unchilled, propylene-treated fruit displayed no ethylene burst even after 32 d with propylene (Fig. 4a). Fruit that had been cold-treated for short periods (15, 30 or 50 d) produced an ethylene burst and ripened at 20 °C only when treated with propylene during post-chilling storage (Fig. 4b). Fruit that had been stored at 0 °C for 15, 30 and 50 d of chilling, exhibited a climacteric peak after 25, 17 and 10 d of propylene treatment, respectively, with a corresponding ethylene production at the peak of 153, 172, and 215 nmol g^{-1} h^{-1}, respectively (Fig. 4b). In the absence of propylene, PC fruit that had been chilled for 15, 30 or 50 d were unable to ripen autonomously after removal from the cold and their endogenous ethylene production remained low for the following 30 d at 20 °C. In unchilled fruit, *Pc-ACS3* transcript accumulation decreased slightly throughout propylene treatment (Fig. 4c). Transcript levels for the other *Pc-ACS* genes did not respond (Fig. 4c). In contrast, after a short cold storage (30 d) propylene treatment

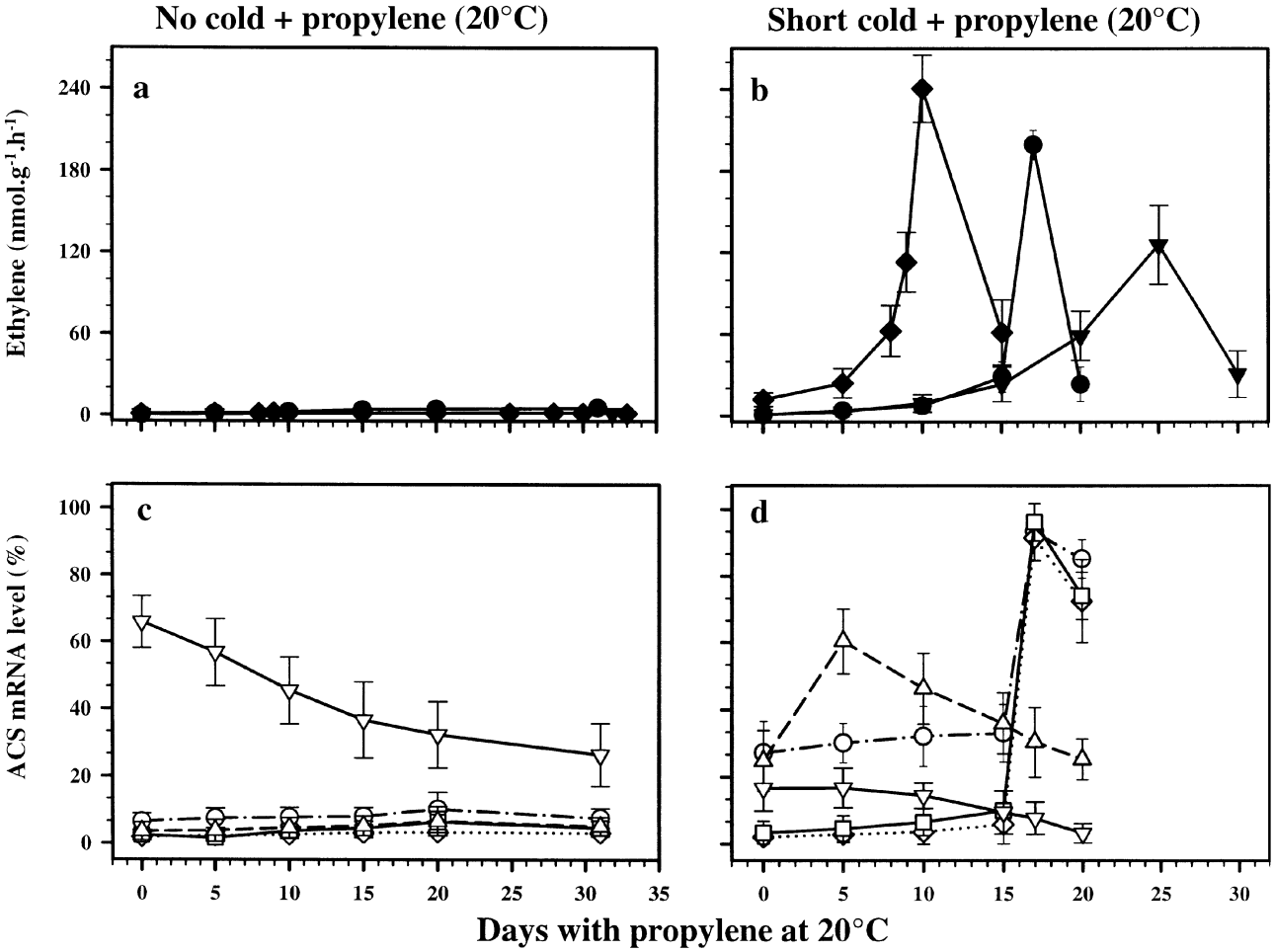

Figure 4. Ethylene production in Passe-Crassane pear fruit treated with propylene (1000 μL L^{-1}) at 20 °C (a) without cold pretreatment (20 °C), or (b) after prechilled at 0 °C for (◆) 15 (●) 30, and (▼) 50 d. The steady state mRNA levels for *Pc-ACS1a* (○), *Pc-ACS2a* (△), *Pc-ACS3* (▽), *Pc-ACS4* (◇), and *Pc-ACS5* (□) genes in the presence of propylene after (c) 30 d at 20 °C, and (d) during ripening after 30 d at 0 °C. The *x*-axis in each figure represents days of propylene treatment.

resulted in a peak in the transcript levels of *Pc-ACS1a*, *4*, and *5* coincident with the peak of endogenous ethylene production. *Pc-ACS2a* transcripts steadily decreased after approximately 5 d of propylene treatment at 20 °C (Fig. 4d).

Expression of *Pc-ACS* genes in different cold requirement pear genotypes

The ripening behaviour of OH and the two OH × PC hybrids, A16 and A50, was studied in order to uncover differences that determine the cold requirement. Old-Home fruit displayed an early, rapid ripening, and a short and rapid (maximal at 6 d) ethylene production profile (Fig. 5a). A16 fruit displayed an early but slower ripening pattern in comparison with OH fruit. Ethylene production in A16 fruit reached a maximum at approximately 15 d post-harvest (Fig. 5b). A50 fruit ripened slower and later than OH and A16, unless if fruit were prechilled. Ethylene production of A50 fruit stored in air at 20 °C reached a maximum after approximately 30 d (Fig. 5c). Ethylene production of A50 fruit chilled for 45 d peaked at approximately 10 d after removal from the cold, while A50 propylene-treated fruit produced an ethylene burst after ~ 30 d of propylene treatment similar to fruit in air at 20 °C (data not shown).

In OH pear fruit, only *Pc-ACS1a* and *2a* transcripts were undetectable. *Pc-ACS* gene expression in fruit of the OH × PC progeny, A16 and A50, were generally similar to OH, with the exception that in A50 fruit, *Pc-ACS4* transcripts remained low throughout the ripening process (Fig. 5f) and *Pc-ACS1a* transcripts accumulated in a cold-treated A50 fruit while, conversely, those of *Pc-ACS1b* were undetectable under these conditions.

Pc-ACS1b, *2b*, and *5* transcript accumulation correlated well with the increase in ethylene production in the different cold requiring genotypes (Fig. 5d, e & f). MCP completely inhibited ripening associated transcription of *Pc-ACS1b*, *4*, and *5* and, in contrast to the situation in PC, also for *Pc-ACS2b* (data not shown). *Pc-ACS3* transcripts were abundant before the onset of ripening in the different cold-requiring cultivars but undetected during ripening. Transcript levels for *Pc-ACS4* increased during ripening in an ethylene-dependent manner in OH and A16 (Fig. 5d & e).

Alleles identified for *Pc-ACS1* and *Pc-ACS2* promoter regions

Because *Pc-ACS1* and *Pc-ACS2* mRNAs showed differential expression between cold-dependent and -independent pear cultivars, further genomic characterization was undertaken in order to determine possible genetic linkages with a cold requirement.

Southern blot hybridizations were carried out in order to identify polymorphisms in the flanking regions of the *Pc-ACS1* and *Pc-ACS2* genes. A labelled *Pc-ACS1* probe

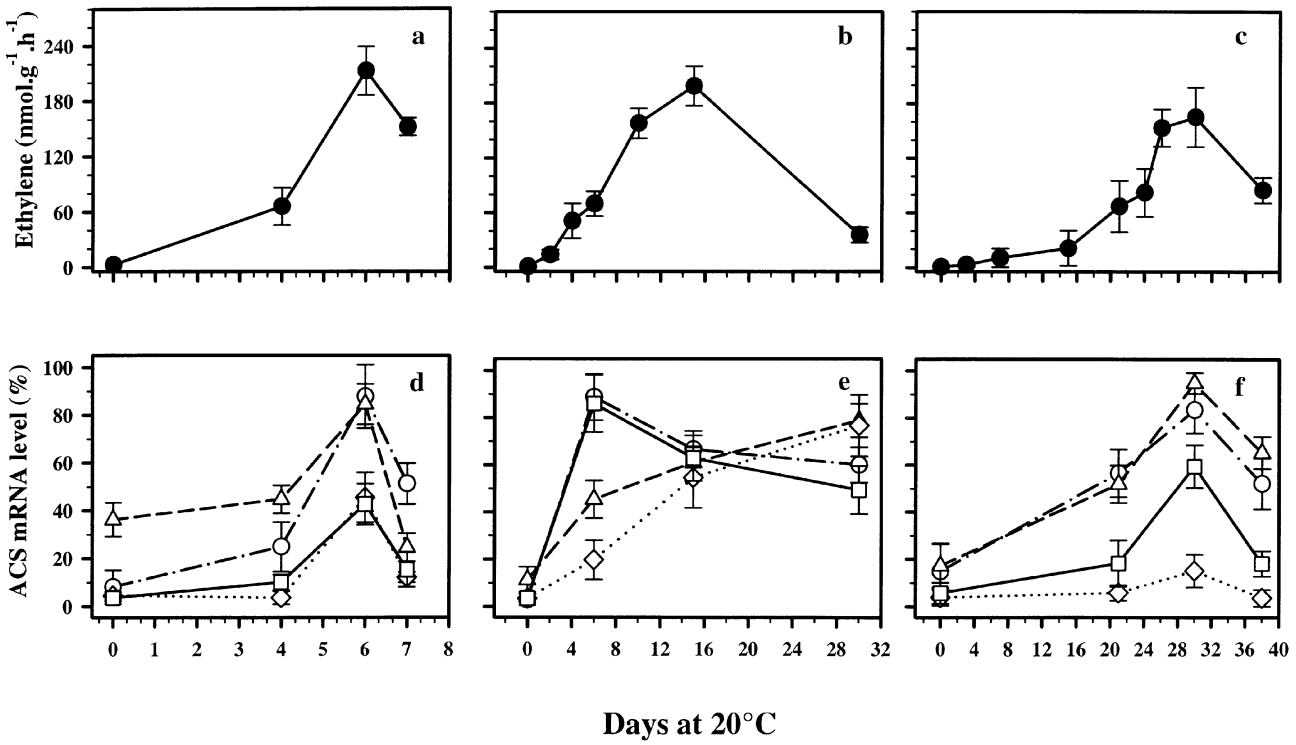

Figure 5. Ethylene production (●) and steady state mRNA levels for *Pc-ACS1b* (○), *Pc-ACS2b* (△), *Pc-ACS4* (◇), and *Pc-ACS5* (□) genes in pears with different cold requirement (OH, A16, A50). Ethylene production and steady state mRNA levels for *Pc-ACS* genes during ripening at 20 °C in Old-Home (a; d), A16 (b; e), and A50 (c; f) pear fruit. The *x*-axis in each figure represents days at 20 °C.

hybridized to one band in PC and two bands in OH in an *Eco*RI-digest of the gDNA. One band at approximately 8.8 kb was common to both genotypes. Another band at approximately 9.4 kb was found only in OH (Fig. 6a). A labelled *Pc-ACS2* probe hybridized to one band in both cultivars in a *Hin*dIII-digest. One band at approximately 3.6 kb was found only in OH and in PC there was a cultivar-specific band at approximately 2.8 kb (Fig. 6c). This indicates a genetic model of one gene per haploid genome with two alleles.

Two alleles of the 5′-non-coding regions of *Pc-ACS1* [*Pc-DACS1a* (–871 bp) and *Pc-DACS1b* (–273 bp)] and *Pc-ACS2* [*Pc-DACS2a* (–1260 bp) and *Pc-DACS2b* (–1588 bp)] genes were identified from the PC and OH cultivars. The percentage similarity between the isolated sections of the 5′- non-coding regions was 50% between *Pc-DACS1a* and *b* and 65% between *Pc-DACS2a* and *b*. Interestingly, the percentage similarity between the two *Pc-DACS2* allelic forms of the 5′- non-coding region up to –900 bp was 97%. Further upstream from this point the sequence conservation was considerably lower. A survey was performed of the *Pc-DACS1a*/*b* and *Pc-DACS2a*/*b* alleles in OH, PC, and 22 OH × PC hybrids. Early, intermediate and late pear phenotypes are associated with specific *Pc-DACS1* and *Pc-DACS2* genotypes (Fig. 6b & d, Table 4). The late genotypes were homozygous for *Pc-DACS1a* and *Pc-DACS2a* genes (Figs 6b & d). The early genotypes were *Pc-DACS1a*/*b* heterozygous and *Pc-DACS2b* homozygous, and the intermediate genotypes were *Pc-DACS1a*/*b* and *Pc-DACS2a*/*b* heterozygous (Fig. 6b & d, Table 4).

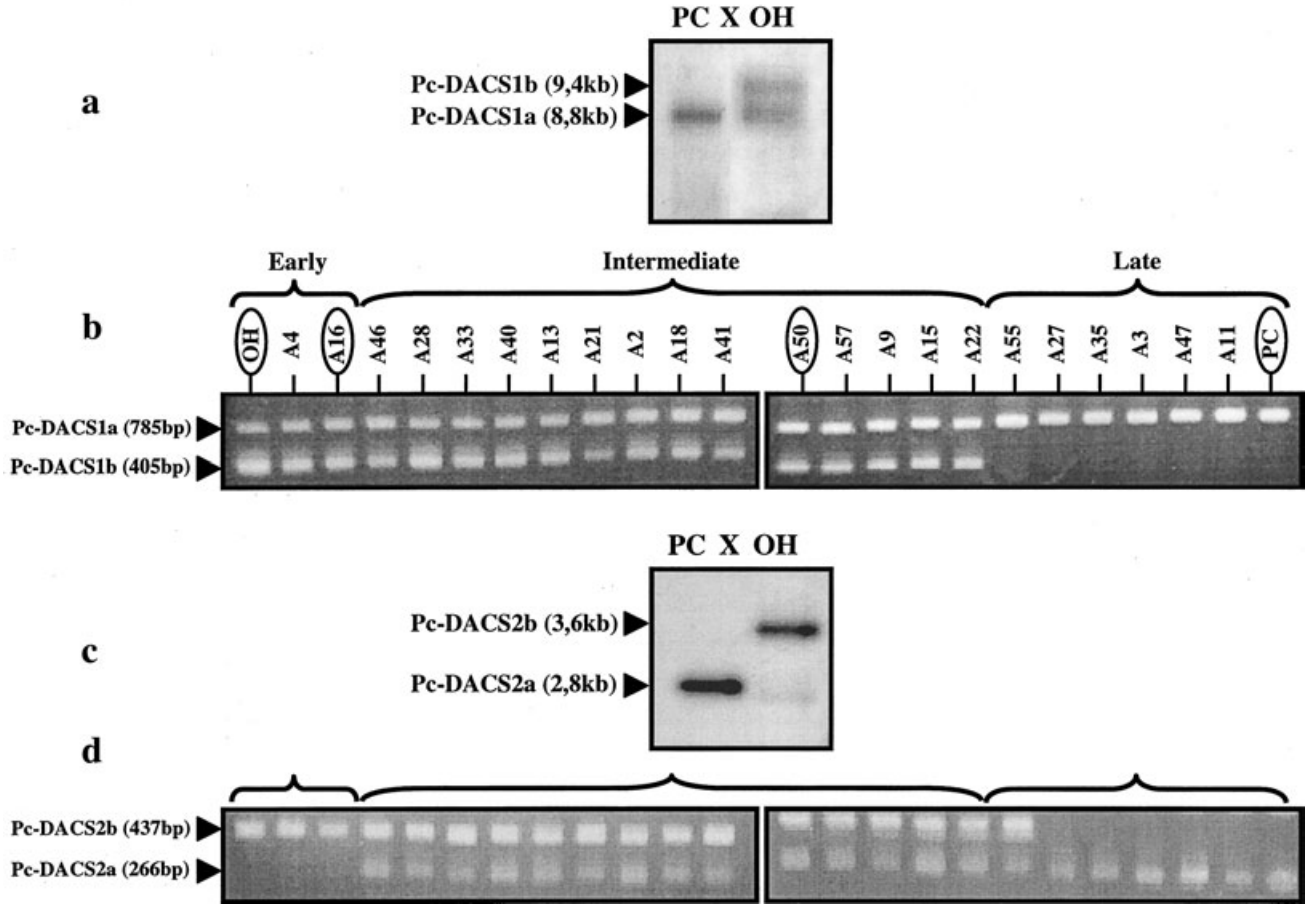

Figure 6. Southern analysis using *Pc-ACS1* (a) and *Pc-ACS2* (c)-specific probes. DNA from Old-Home and Passe-Crassane pear was extracted from leaves and digested by *Bam*HI, *Eco*RI, *Eco*RV and *Hin*dIII. DNA blots were hybridized and washed at high stringency. The polymorphisms were obtained for *Pc-ACS1* and *Pc-ACS2* with the *Eco*RI and *Hin*dIII-digested gDNA, respectively. Estimated sizes of the hybridizing bands are given on the left side. Bands of 8.8 and 9.4 kb in (a) correspond to *Pc-DACS1a* and *b*, respectively. Bands of 2.8 and 3.6 kb in (c) correspond to *Pc-DACS2a* and *b*, respectively. Diagnosis of the two *Pc-DACS1a*/*b* (b) and the two *Pc-DACS2a*/*b* (d) alleles in 24 pear genotypes (Table 4). Specific primers were used to amplify each 5′-flanking region and the PCR products were viewed on a 2% agarose gel. The sizes of the fragments are given on the left side. In (b) bands of 785pb and 405pb correspond to *Pc-DACS1a* (AY514040) and *Pc-DACS1b* (AY514041), respectively. In (d) bands of 266 and 437 bp correspond to *Pc-DACS2a* (AY388991) and *Pc-DACS2b* (AY388992), respectively. The circled cultivars indicate the selected genotypes for mRNA analysis. Early, intermediate and late showed the maturation time, in 24 pear genotypes, for fruit storage at 20 °C.

Table 4. Corresponding marker genotypes of *Pc-DACS1* and *Pc-DACS2* alleles in Old-Home, Passe-Crassane, and OH × PC hybrid pear fruit

Cultivars	Pc-DACS1 genotype	Pc-DACS2 genotype
Early		
Old-Home	ACS1a/b	ACS2b/b
R7A4	ACS1a/b	ACS2b/b
R7A16	ACS1a/b	ACS2b/b
Intermediate		
R6A46	ACS1a/b	ACS2a/b
R6A28	ACS1a/b	ACS2a/b
R6A33	ACS1a/b	ACS2a/b
R6A40	ACS1a/b	ACS2a/b
R7A13	ACS1a/b	ACS2a/b
R6A21	ACS1a/b	ACS2a/b
R7A2	ACS1a/b	ACS2a/b
R6A18	ACS1a/b	ACS2a/b
R6A41	ACS1a/b	ACS2a/b
R6A50	ACS1a/b	ACS2a/b
R6A57	ACS1a/b	ACS2a/b
R6A9	ACS1a/b	ACS2a/b
R6A15	ACS1a/b	ACS2a/b
R6A22	ACS1a/b	ACS2a/b
Late		
R6A55	ACS1a/a	ACS2a/b
R7A27	ACS1a/a	ACS2a/a
R6A35	ACS1a/a	ACS2a/a
R6A3	ACS1a/a	ACS2a/a
R6A47	ACS1a/a	ACS2a/a
R6A11	ACS1a/a	ACS2a/a
Passe-Crassane	ACS1a/a	ACS2a/a

DISCUSSION

In climacteric fruit such as pear, melon, and tomato, most aspects of the ripening process are triggered and maintained by ethylene (Lelièvre *et al*. 1997a). There are marked differences in ripening behaviour and in ethylene production and responses in the pear cultivars used in this study. Mature OH fruit are capable of producing autocatalytic ethylene and ripening without any cold pretreatment. In contrast, a long cold treatment is required before PC fruit is capable of producing autocatalytic ethylene and ripening. Fruit of the OH × PC progeny, A16, behave similarly to OH fruit while fruit from the other OH × PC cross, A50, have an intermediate ripening phenotype. While A50 fruit are capable of ripening without a cold pretreatment, a short cold treatment accelerates the onset of ripening.

The pear ethylene perception elements, *Pc-ETR1*, *Pc-ERS1*, *Pc-ETR5*, and *Pc-CTR1,* have been shown to temper the ripening process rather than to control it (El-Sharkawy *et al*. 2003). Similarly, while some ethylene responses, including *Pc-ACO1* gene expression and enzyme activity, can be induced in unchilled PC fruit, they remain unable to produce autocatalytic ethylene and to ripen during propylene treatment (Lelièvre *et al*. 1997b). This indicates that other factors control the ethylene response and ripening in PC fruit. In tomato, ACS has been shown to control the onset and maintenance of ripening-related autocatalytic ethylene. Seven ethylene biosynthetic mRNAs, putatively encoding 1-aminocyclopropane-1-carboxylic acid synthase (ACS) isozymes, were isolated and characterized in order to determine their role in the cold requirement. The predicted amino acid sequences had the traits generally associated with ACS and related aminotransferase activity.

In the dendrogram, a number of well-defined branches have both *Arabidopsis* and tomato sequences but lack pear sequences, suggesting that there are likely to be as yet unidentified *ACS* genes within the pear genome. In species where multiple *ACS* genes have been characterized, each gene appears to have a unique mode of transcriptional and/or post-translational regulation (Wang, Li & Ecker 2002).

Post-translational regulation has been identified in a number of *Arabidopsis* ACS proteins (Chae *et al*. 2003). The *Pc-ACS1a* and *b* predicted proteins are closely related to *At-ACS5* and *9* and contain the serine and valine amino acid residues essential for *At-ACS4*, *5*, *8* and *9* phosphorylation. The two *Pc-ACS2* isoforms and *Pc-ACS3* have C-terminal regions truncated by 27–49 amino acids compared with the other *Pc-ACS* sequences and consequently they lack the serine and valine residues which are important for phosphorylation-driven degradation. In the predicted *Pc-ACS4* and *5* sequences, replacement of the conserved valine residue with a glutamine suggests that these proteins have different post-translational behaviour to the *Pc-ACS1a* and *b*.

Differences in ripening behaviour between the cultivars reflects an altered capacity to produce and respond to ethylene. In PC fruit, ethylene production increased throughout the chilling treatment. When fruit were exposed to room temperatures after a period in the cold, ethylene production and ripening follow a pattern typical of climacteric fruit. PC fruit that had not been subjected to a cold treatment did not respond to the ethylene analogue propylene, even after 32 d of treatment. Similarly, in A50 fruit, there was no acceleration of ripening when unchilled fruit were treated with propylene at 20 °C. Treating PC fruit with the ethylene perception inhibitor, MCP, prior to the cold treatment resulted in levels of post-chilling ethylene production and *Pc-ACS* gene expression similar to that observed in unchilled, propylene-treated and untreated fruit. In PC, and partially in A50 fruit therefore the capacity to produce autocatalytic ethylene and to ripen was dependent on cold-induced changes in fruit physiology and one of these changes was an altered ethylene sensitivity. Moreover, in PC fruit, the duration of cold storage correlated positively with the precocity of the climacteric phase and the rate of ethylene production at the peak. Taken together, the data suggest that there is a minimum requirement for cold in PC fruit, but once this minimum requirement is met, exogenous ethylene can accelerate the developmental changes required for ripening.

Although *Pc-ACS3* and *5* transcript accumulation was similar during ripening in all four pear cultivars, the different isoforms of *Pc-ACS1* and *Pc-ACS2* were either stage or cultivar-specific. In PC, *Pc-ACS1a* transcript accumulation

was cold- and ethylene-dependent. *Pc-ACS1a* transcripts gradually increased in abundance throughout the cold treatment, prior to the increase in transcripts for the other *Pc-ACS* mRNAs. Early, cold induction of *Pc-ACS1a* suggests that it is important in the capacity of PC fruit to ripen. In OH and A16 fruit, commensurate with the capacity of these fruit to produce autocatalytic ethylene and to ripen, mRNA accumulation of the *Pc-ACS1* isoform expressed in these cultivars, *Pc-ACS1b*, was not cold-dependent. In A50, cold treatment down-regulated *Pc-ACS1b* transcript accumulation and up-regulated *Pc-ACS1a* transcripts. *Pc-ACS1a* was therefore cold inducible whereas its counterpart, *Pc-ACS1b*, was cold inhibited. Different *ACS* isoforms appear to be required to differentially regulate distinct developmental processes and environmental responses. Chilling injury in oranges, for example, causes both the induction (*Cs-ACS1*) and inhibition (*Cs-ACS2*) of *ACS* gene expression (Wong *et al*. 1999). The expression of both the chilling-inducible and the chilling-repressible *Pc-ACS* cDNAs appears to play an important role in determining the level of ethylene production in the post-chilling period.

All pear cultivars examined share a common allele of the *Pc-DACS1* promoter associated with *Pc-DACS1a*, whereas, the second allele, associated with *Pc-DACS1b*, was found only in early and intermediate pear genotypes. Sequences closely related to the two *Pc-ACS1* isoforms are associated with high (autocatalytic) ethylene production in Asian pear (*pPPACS1*) and in apple (*MdACS1-1*) (Itai *et al*. 1999; Sunako *et al*. 1999). One allele of *MdACS-1*, *MdACS1-2*, has an element in its promoter sequence that results in dramatically reduced levels of transcript accumulation. Apple cultivars homozygous for the *MdACS1-2* promoter produce low levels of ripening-associated ethylene and have a long storage life (Sunako *et al*. 1999). It is possible that the differences in promoter sequence between *Pc-DACS1a* and *b* alleles contribute to the cold-dependence disparity.

Another difference between the cultivars with potential significance for the variation in ripening behaviour is the accumulation of *Pc-ACS2* transcripts. In PC fruit, *Pc-ACS2a* transcript levels increased early during the post-cold treatment onset of ripening in an ethylene-independent manner but declined thereafter. By contrast, *Pc-ACS2b* transcripts were induced during ripening in an ethylene-dependent manner in different cold requiring pears. Late and intermediate pear genotypes share a common allele of the *Pc-DACS2* promoter, *Pc-DACS2a*. A second allele, associated with *Pc-DACS2*, *Pc-DACS2b*, was found in intermediate and early genotypes.

Similarly to *Pc-ACS1*, the two *Pc-ACS2* isoforms may play an important role in determining the level of ethylene production and the capacity of the various cultivars to ripen. Southern analysis confirmed that the OH genotype was heterozygous for the *Pc-DACS1* gene (*Pc-DACS1a*/*b*) and that the PC genotype was homozygous for the *Pc-DACS1a* allele. Similar results for *Pc-DACS2* gene supports the hypothesis of one copy of the *Pc-DACS1* and *Pc-DACS2* gene per haploid genome with two alleles. The *Pc-DACS1a*/*b* and *Pc-DACS2a*/*b* promoter alleles may be involved in the differential regulation of the genes in the different cultivars and in the differential ripening behaviour. In this case, it appears that a positive response to ethylene is associated with the promoters found in early and intermediate genotypes (*Pc-DACS1b*/*2b*) but not in late, cold-dependent genotypes (*Pc-DACS1a*/*2a*).

Pc-ACS3 mRNA accumulated in unchilled and MCP-treated PC fruit and before the onset of ripening in different cold-requiring pear fruit. No *Pc-ACS3* transcripts were detected in ripening fruit. Accumulation of *Pc-ACS4* and *5* transcripts accumulated in response to ethylene in all four cultivars, except that for *Pc-ACS4*, transcripts remained low throughout A50 fruit ripening. Pc-ACS4 and 5 proteins may play an important role in stabilizing ACC levels during ripening.

In the tomato, it has been proposed that there are two systems of ethylene production. System 1 ethylene is autoinhibitory and operates in immature fruit and vegetative tissues. System 2, or autocatalytic ethylene, is produced during climacteric fruit ripening (Lelièvre *et al*. 1997a; Barry *et al*. 2000). In tomato and other climacteric fruit, *ACS* genes play a critical role in the transition from system 1 to system 2. In PC, transcript levels for the *Pc-ACS* genes, except that for *Pc-ACS3*, cannot be induced in unchilled, MCP-treated, and unchilled propylene-treated fruit. By analogy with the tomato dual system of ethylene production this indicates that unchilled PC fruit are blocked in system 1. In the tomato, system 1 ethylene is negatively auto-regulated (Barry *et al*. 2000). In unchilled PC fruit, *Pc-ACS3* transcripts are present well above their basal levels. Ethylene and cold resulted in reductions in *Pc-ACS3* transcript abundance. This suggests that *Pc-ACS3* is important for system 1 ethylene production and offers further evidence for the existence of a dual system of ethylene production in the pear.

The model presented in Fig. 7 illustrates our current understanding of the control of ethylene production in pear fruit within the two systems model. In immature pear fruit, system 1 ethylene relies on the expression of *Pc-ACS3*. System 1 ethylene synthesis continues throughout fruit development until the fruit attain a state of physiological maturity at which point a transition occurs. In cold-dependent fruits, this requires that the fruit have been exposed to a sufficient amount of cold. The cold treatment induces and/or represses cold-dependent, ethylene-independent factors causing a transition so that the fruit become responsive to ethylene and gain a competence to ripen at 20 °C. Cold treatment induces *Pc-ACS1a* expression in an ethylene-dependent manner. This results in ethylene production that activates expression of the ethylene-inducible genes *Pc-ACS4*, *5*, and *2a*. Without a cold treatment, cold-dependent pear fruit remain blocked in system 1. The elevated levels of ethylene produced in system 2 results in negative feedback of the system 1 developmental pathway, resulting in reduced *Pc-ACS3* expression. The fact that *Pc-ACS1a* is expressed throughout the cold-induced system 1 to system

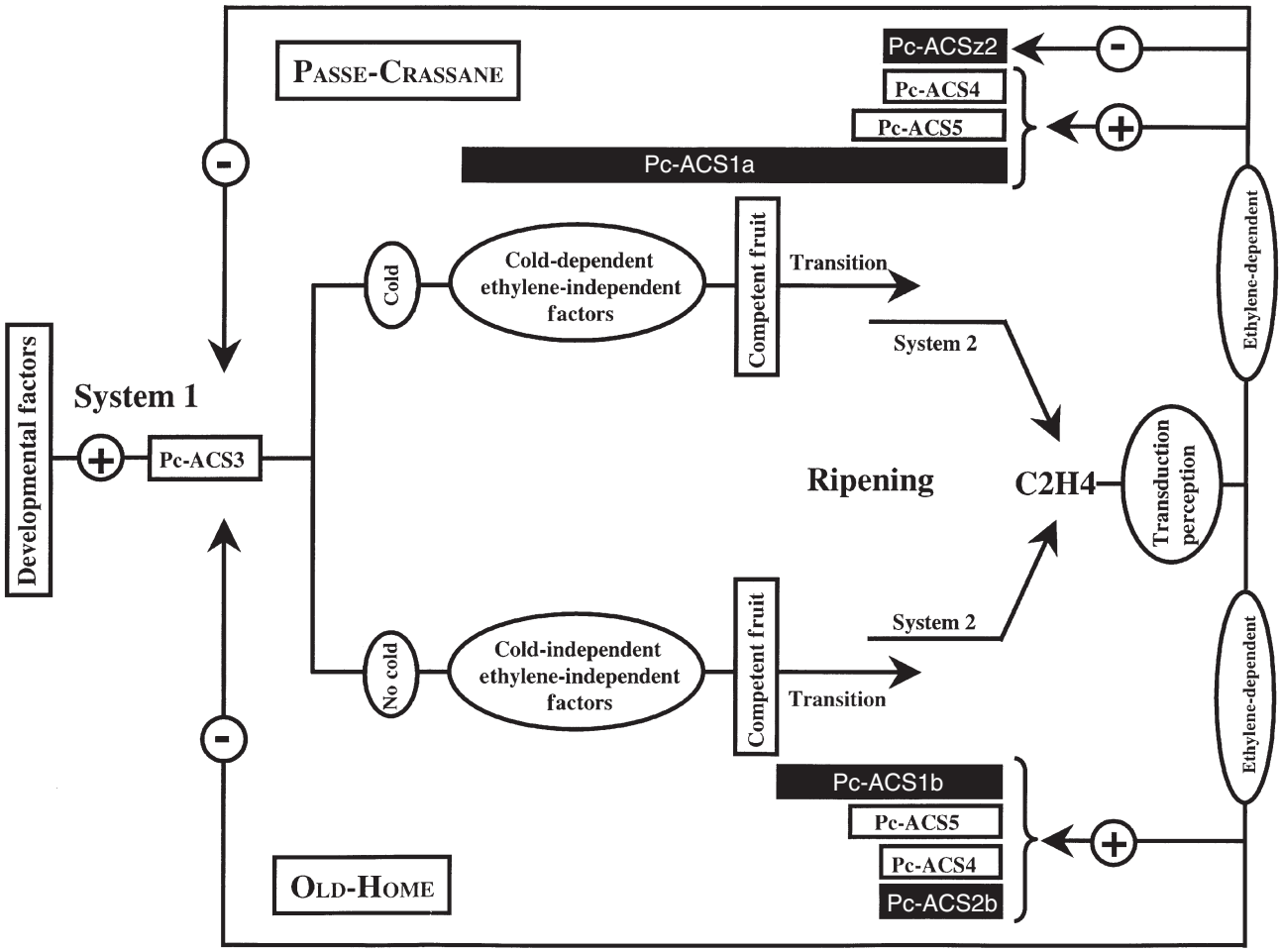

Figure 7. Model for the regulation of *Pc-ACS* gene expression in cold-requiring (Passe-Crassane) and no cold-requiring fruit (Old-Home). The symbols (+) and (–) refer to the positive and negative action of ethylene, respectively, during system 1 and system 2 ethylene synthesis that leads to the activation (+), or repression (–) of *Pc-ACS* gene expression. Filled black rectangles correspond to genes that are differentially regulated in cold-dependent and -independent pear cultivars.

2 transition period in cold-dependent pear fruit suggests that it is a control point in the onset of system 2 ethylene production and ripening. In tomato, transition from system 1 to system 2 is associated with the expression of the *Le-ACS4* gene which triggers the expression of *Le-ACS2* (Alexander & Grierson 2002). *Pc-ACS1b* transcript accumulation is cold-independent in different cold-requiring pears. This suggests that the absolute cold requirement in late pear fruit is at least partially due to the cold requirement of *Pc-ACS1a*. In pear cultivars that do not require cold treatment, the transition from system 1 to system 2 is induced through the increase of *Pc-ACS1b* transcript levels by cold-independent, ethylene-independent developmental factors.

The results presented here suggest that differences in *Pc-ACS* gene expression between cold-dependent and -independent pears is an important determinator in the ripening behaviour of the cultivars. However, in addition to *Pc-ACS* expression it is likely that other factors are involved in determining cold dependence in pear. In tomato, the *rin* mutant fails to produce autocatalytic ethylene and to ripen (Giovannoni 2001). This non-ripening phenotype is caused by mutations in transcription factors that control the expression of ripening-related genes. *Le-ACS4* and *2* transcript accumulation critical for the onset of system 2 ethylene does not occur in *rin* mutant fruit. Similarly, in PC and partially in A50 fruit, a cold-dependent, ethylene-independent developmental process operates to block *Pc-ACS* genes expression, the transition from system 1 to system 2 ethylene production, and ripening.

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