ACC Synthase Genes Related to Cold-dependent Ripening in Pear Fruit

I. El-Sharkawy, J.M. Lelièvre, J.C. Pech and A. Latché
UMR 990 INRA/INPT-ENSAT "Génétique et Biotechnologie des fruits"
31326 Castanet-Tolosan Cédex
France

Keywords: 1-aminocyclopropene-1-carboxylic acid (ACC), ACS isoforms, cold requirement, pear fruit ripening

Abstract

The differential regulation of *ACC* synthase genes has been studied in pear cultivars that either require a long chilling treatment before they are capable of ripening ('Passe-Crassane', PC) or not ('Old-Home', OH) and in OH x PC hybrids having no (A16) or intermediate (A50) cold requirement. Among the seven *Pc-ACS* cDNAs isolated, four of them (*Pc-ACS1a/b* and *Pc-ACS2a/b*) showed differential expression in relation with cold requirement. *Pc-ACS1a* transcripts accumulated specifically during chilling and ripening of cold-dependent cultivars while *Pc-ACS1b* transcripts were detected only during ripening of cold-independent genotypes. *Pc-ACS2a* mRNA was expressed specifically in cold-dependent genotypes and negatively regulated by ethylene while *Pc-ACS2b* transcripts accumulated only in cold-independent genotypes and positively regulated by ethylene. *Pc-ACS3*, *4* and *5* transcripts accumulation was similar in all genotypes, independently of cold-requirements.

INTRODUCTION

A wide range of developmental processes and environmental responses are regulated through modifications in the production and responsiveness to the plant hormone ethylene (Abeles et al., 1992). One of the best examples of this is the ripening of climacteric fruit. Pear is climacteric fruit characterized by a large diversity for the date and rate of ripening. In most climacteric fruit, a short period of ethylene treatment in mature fruit is sufficient to trigger autocatalytic ethylene production and ripening. Late ripening pears, such as 'Passe-Crassane' (PC) require a period of low temperature storage (0°C) before they will ripen at higher temperatures (Lelièvre et al., 1997). In PC, this cold requirement can vary from 0-110 d, depending on growth conditions. Unchilled fruit are unable to display the respiratory and ethylene climacteric and hence to ripen (Lelièvre et al., 1997). Ethylene is synthesized from S-adenosyl-L-methionine via 1-aminocyclopropane-1-carboxylic acid (ACC). In PC fruit, cold-induced ethylene biosynthesis correlates with an increase in ACC content and in ACC synthase (ACS), and ACC oxidase (ACO) activities (Lelièvre et al., 1997). ACS and ACO are the two key enzymes in the ethylene biosynthesis pathway. In tomato and *Arabidopsis* ACO and ACS proteins are encoded by small gene families.

The objective of this study was to uncover the role of *ACS* gene family members in determining the molecular basis of cold-dependent pear fruit ripening. *Pc-ACS* transcript accumulation was studied during ripening of cultivars requiring cold treatment and cultivars with an intermediate or no cold requirement.

MATERIALS AND METHODS

Pear (*Pyrus communis* L. 'Passe-Crassane') fruit were harvested and treated as described previously (El-Sharkawy et al., 2003). Other genotypes, 'Old-Home' and OH x PC hybrid trees, A16 and A50, were obtained from INRA, France. Pre-climacteric fruit of 'Passe-Crassane', 'Old-Home', A16, and A50 were harvested as late as possible before ethylene fruit production had risen. After treatments, measurements of ethylene production were taken and the fruit were frozen in liquid nitrogen and stored at -80°C.

Several sets of primers were used to isolate pear *Pc-ACS* sequences that shared the

structural characteristics associated with functional *ACS* genes. The isolated genes were cloned into the pGEM-T vector (Promega), sequenced and compared with database sequences using the BLAST program. Alignments of the predicted protein sequences were performed using ClustalX and GeneDoc programs. Finally, a cDNA clone with homology to an actin sequence (AF386514) was used as an internal control in gene expression studies. RT-PCR approach was used plus the direct radioactive measurement of the amplified sequences (El-Sharkawy et al., 2003). 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.

RESULTS

Seven 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 1. 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 *b* differed by only 13 amino acids. The percentage similarity between the isolated 5'- and 3'-non-coding regions of these two isoforms was found to be only 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 and 93% similarity, respectively.

'Passe-Crassane' fruit stored at 20°C failed to produce significant amounts of ethylene even after 145 d post harvest (Fig. 2A). The fruit required a long (80 d) cold pre-treatment in order to be able to produce autocatalytic ethylene autonomously (Fig. 2B), and to ripen at 20°C (Fig. 2C). A post-harvest MCP treatment immediately before refrigeration eliminated any cold-induced increase in ethylene production and abolished the ethylene burst and ripening in re-warmed fruit (data not shown). 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. 2D and G). During cold storage at 0°C, only *Pc-ACS1a* transcript levels showed a steady increase, while those for *Pc-ACS3* decreased in abundance (Fig. 2E and H). During rewarming at 20°C, after cold storage for 80 d, transcript levels for *Pc-ACS1a* decreased slightly, while those for *Pc-ACS2a* increased to a peak at ~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. 2F, and I). MCP treatment inhibited the cold storage increase in *Pc-ACS1a* transcript levels and the accumulation of *Pc-ACS1a*, *4*, and *5* transcripts during post cold treatment, ripening at 20°C. By contrast, *Pc-ACS2a* and *Pc-ACS3* transcripts were detected in re-warmed MCP-treated fruit (data not shown).

The ripening behavior of 'Old-Home' and the two OH x 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. 3A). A16 fruit displayed an early but slower ripening pattern compared to OH fruit. Ethylene production in A16 fruit reached a maximum after ~ 15 d post harvest (Fig. 3B). A50 fruit ripened slower and later than OH and A16. Ethylene production of A50 fruit reached a maximum after ~ 30 d (Fig. 3C). In OH pear fruit, only *Pc-ACS1a* and *2a* transcripts were undetectable. *Pc-ACS* gene expression in

fruit of the OH x 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. 3F). *Pc-ACS1b*, *2b*, and *5* transcript accumulation correlated well with the increase in ethylene production in the OH, A16, and A50 genotypes (Fig. 3D, E, and 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 OH, A16, and A50 cultivars but decreased strongly during ripening. Transcript levels for *Pc-ACS4* increased during ripening in an ethylene-dependent manner in OH and A16 (Fig. 3D and E).

DISCUSSION

In climacteric fruit such as the pear, melon, and tomato most aspects of the ripening process are triggered and maintained by ethylene (Alexander and Grierson, 2002). There are marked differences in ripening behavior and in ethylene production and responses in the pear cultivars used in this study. Mature 'Old-Home' fruit are capable of producing autocatalytic ethylene and ripening without any cold pre-treatment. In contrast, a long cold treatment is required before 'Passe-Crassane' fruit is capable of producing autocatalytic ethylene and ripening. Fruit of the OH x PC progeny, A16, behave similarly to OH fruit while fruit from the other OH x PC cross, A50, have an intermediate ripening phenotype.

The pear ethylene perception elements (*Pc-ETR1*, *Pc-ERS1*, *Pc-ETR5*, *Pc-CTR1*) have been shown to temper the ripening process rather than to control it (El-Sharkawy et al., 2003). The results suggested that neither pear ethylene perception elements nor ACC oxidase enzyme activity determine a cold requirement of PC fruit to produce autocatalytic ethylene and ripening. So that, seven ethylene biosynthetic mRNAs, putatively encoding 1-aminocyclopropane-1-carboxylic acid synthase (ACS) isozymes, were isolated and characterized in order to determine the molecular basis of the cold requirement. The predicted amino acid sequences had the traits generally associated with ACS activity.

Differences in ripening behavior 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. 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 fruit. While *Pc-ACS3* and *5* transcript accumulation was similar during ripening in all four pear cultivars, the different isoforms of *Pc-ACS1* and *2* 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. In tomato and other climacteric fruit, *ACS* genes play a critical role in the onset and maintenance of ripening. In tomato, *Le-ACS4* triggers the synthesis of *Le-ACS2*, which leads to the production of autocatalytic ethylene and ripening (Alexander and Grierson, 2002). 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. Another difference between the cultivars with potential significance for the variation in ripening behavior 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, in OH, A16, and A50 pears, during ripening in an ethylene-dependent manner. *Pc-ACS3* mRNA accumulated in unchilled and MCP-treated PC fruit and before the onset of ripening in OH, A16, and A50 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.

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 behavior of the cultivars. In the tomato, the *rin* and *nor* mutants fail to produce autocatalytic ethylene and to ripen (Giovannoni 2001). The non-ripening phenotypes are caused by mutations in transcription factors that control the expression of ripening related genes. *Le-ACS6* transcripts were present in *rin* fruit at comparable levels with those detected in mature green fruit. However, the ripening-related changes in the expression of *Le-ACS4* and *2* observed in wild-type fruit did not occur in *rin*. In PC fruit, a cold-dependent, ethylene-independent developmental process operates in addition to the *Pc-ACS* genes to block the transition from system-1 to system-2. Consequently, in addition to *Pc-ACS* expression it is likely that other factors are involved in determining cold dependence in the pear.

ACKNOWLEDGEMENTS

I. El-Sharkawy has received a postdoctoral fellowship from INRA France.

Literature Cited

Abeles, F.B., Morgan, P.W., and Saltveit, M.E. 1992. Ethylene in Plant Biology. Academic Press, San Diego, CA, USA.

Alexander, L. and Grierson, D. 2002. Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. Journal of Experimental Botany 53: 2039-2055.

El-Sharkawy, I., Jones, B., Li, Z.G., Lelièvre, J.M., Pech, J.C. and Latché, A. 2003. Isolation and characterization of four ethylene perception elements and their expression during ripening in pears (*Pyrus communis* L.) with/without cold requirement. J. Exp. Bot. 54: 1615-1625.

Giovannoni, J. 2001. Molecular biology of fruit maturation and ripening. Annual Rev. Plant Physiol. Plant Molec. Biol. 52: 725-749.

Lelièvre, J.M., Tichit, L., Dao, P., Fillion, L., Nam, Y.,. Pech, J.C. and Latché, A. 1997. Effects of chilling on the expression of ethylene biosynthetic genes in Passe-Crassane pear (*Pyrus communis* L.) fruits. Plant Molec. Biol. 33: 847-855.

White, M.F., Vasquez, J., Yang, S.F. and Kirsch, J.F. 1994. Expression of apple 1-aminocyclopropane-1-carboxylate synthase in *Escherichia coli*: kinetic characterization of wild-type and active-site mutant forms. Proc. National Academy of Sciences USA 26: 12428-12432.

Tables

Table 1. Amino acid sequence comparison between the predicted full length *Pyrus communis* ACC synthase genes.

Protein		Amino acid identity (%)						
		Pc-ACS1a	Pc-ACS1b	Pc-ACS2a	Pc-ACS2b	Pc-ACS3	Pc-ACS4	Pc-ACS5
Name	**Size**							
Pc-ACS1a	473	-						
Pc-ACS1b	474	96	-					
Pc-ACS2a	446	51	52	-				
Pc-ACS2b	446	52	53	96	-			
Pc-ACS3	446	52	53	94	95	-		
Pc-ACS4	495	46	46	47	48	47	-	
Pc-ACS5	487	47	47	48	48	48	67	-

Figures

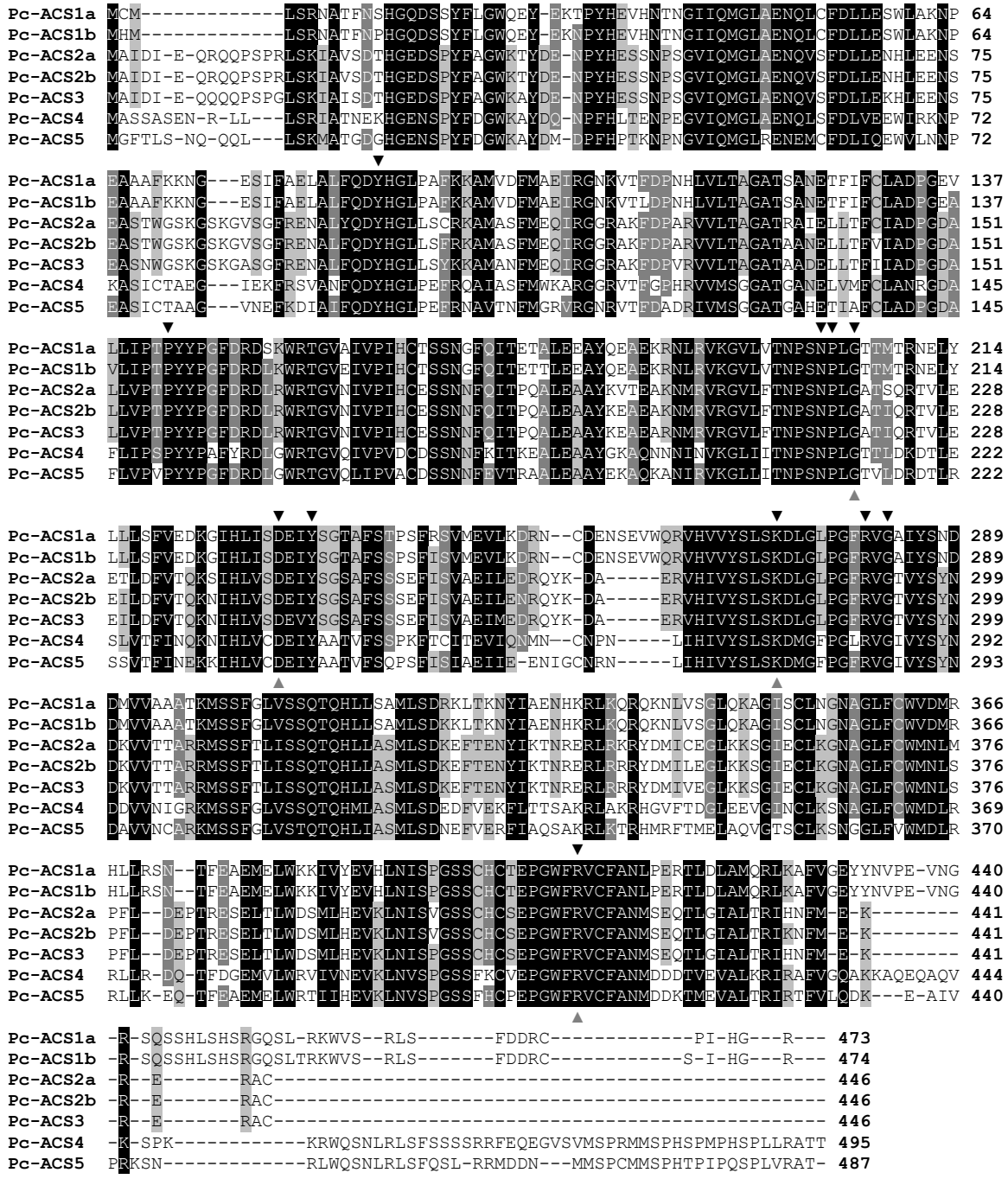

Fig. 1. Amino acid sequence alignment of *Pc-ACS1a* (X87112), *Pc-ACS1b* (AY388987), *Pc-ACS2a* (AF386519), *Pc-ACS2b* (AY388989), *Pc-ACS3* (AY388988) *Pc-ACS4* (AF386518), and *Pc-ACS5* (AF386523) using ClustalX program. Conserved residues are shaded in black. Dark grey shading indicates similar residues 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 4 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.

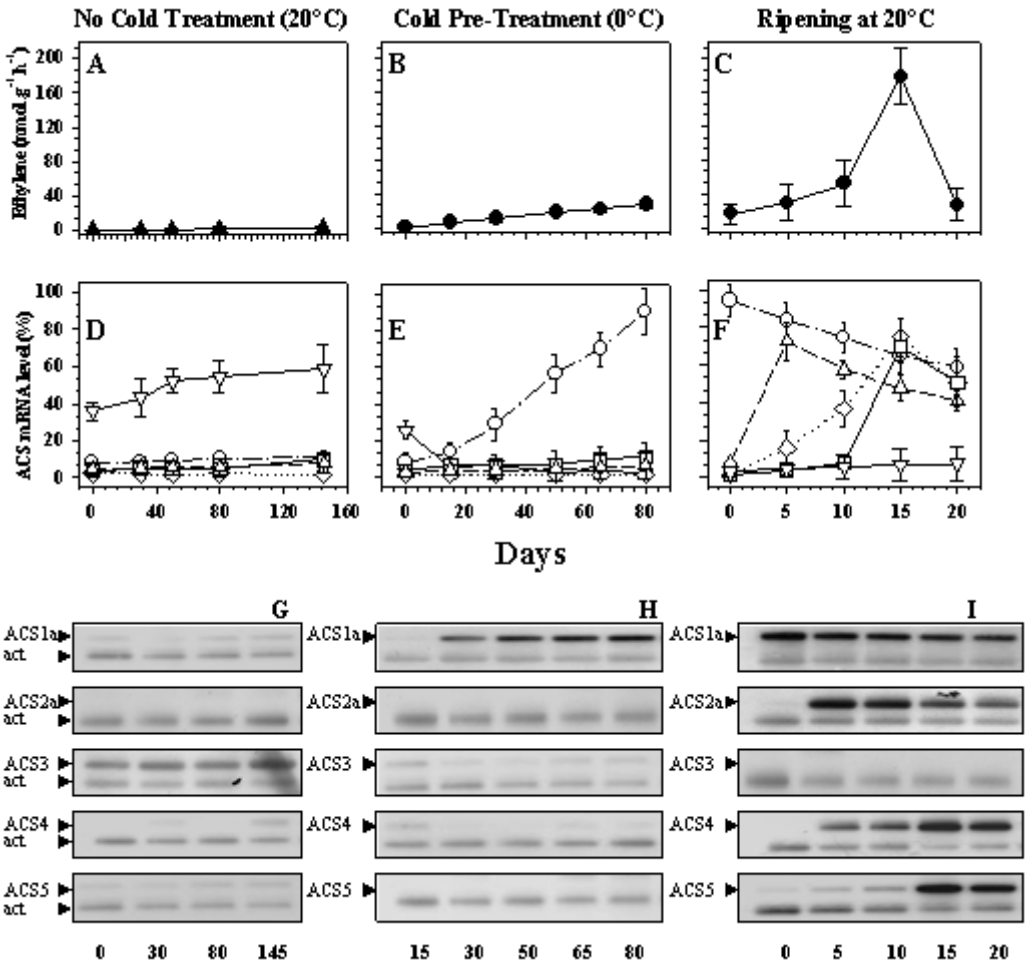

Fig. 2. Ethylene production and gene expression in PC pear fruit. Ethylene production (**A**, **B**, **C**) and steady state mRNA levels for *Pc-ACS1a* (○), *Pc-ACS2a* (△), *Pc-ACS3* (▽), *Pc-ACS4* (◊), and *Pc-ACS5* (□) genes (**D**, **E**, **F**, **G**, **H**, **I**): in fruit stored at 20°C without cold treatment [**A** (▲), **D**, **G**]; during long-term cold storage [**B** (●), **E**, **H**]; and during ripening at 20°C after 80 d at 0°C [**C** (●), **F**, **I**]. The X-axis in each figure represents days of the respective treatment. The upper and lower bands shown in **G**, **H**, **I** correspond to the *Pc-ACS* cDNAs and actin, respectively.

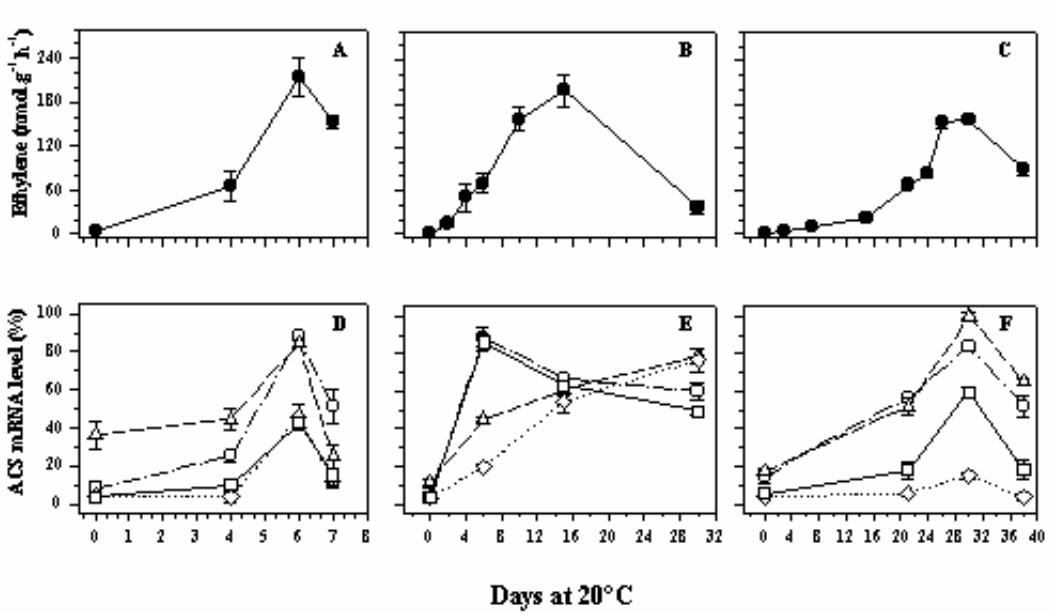

Fig. 3. Ethylene production and steady state mRNA levels for *Pc-ACS1b* (○), *Pc-ACS2b* (△), *Pc-ACS4* (◊), and *Pc-ACS5* (□) genes in pears with different cold requirement (OH, A16, A50). Ethylene production and steady state mRNA levels for *Pc-ACS* genes during ripening at 20°C in OH (A; D), A16 (B; E), and A50 (C; F) pear fruit. The X-axis in each figure represents days at 20°C.