Effects of chilling on the expression of ethylene biosynthetic genes in Passe-Crassane pear (*Pyrus communis* L.) fruits

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Abstract

Passe-Crassane pears require a 3-month chilling treatment at 0 °C to be able to produce ethylene and ripen autonomously after subsequent rewarming. The chilling treatment strongly stimulated ACC oxidase activity, and to a lesser extent ACC synthase activity. At the same time, the levels of mRNAs hybridizing to ACC synthase and ACC oxidase probes increased dramatically. Fruit stored at 18 °C immediately after harvest did not exhibit any of these changes, while fruit that had been previously chilled exhibited a burst of ethylene production associated with high activity of ACC oxidase and ACC synthase upon rewarming. ACC oxidase mRNA strongly accumulated in rewarmed fruits, while ACC synthase mRNA level decreased. The chilling-induced accumulation of ACC synthase and ACC oxidase transcripts was strongly reduced when ethylene action was blocked during chilling with 1-methylcyclopropene (1-MCP). Upon rewarming ACC synthase and ACC oxidase transcripts rapidly disappeared in 1-MCP-treated fruits. A five-week treatment of non-chilled fruits with the ethylene analog propylene led to increased expression of ACC oxidase and to ripening. However, ethylene synthesis, ACC synthase activity and ACC synthase mRNAs remained at very low level. Our data indicate that ACC synthase gene expression is regulated by ethylene only during, or after chilling treatment, while ACC oxidase gene expression can be induced separately by either chilling or ethylene.

Introduction

Post-harvest exposure to chilling temperatures is required for normal ripening of some late pear varieties, including Passe-Crassane and Beurré d'Anjou [18, 44, 45]. A cold treatment, albeit not absolutely required, is also capable of hastening and synchronizing the onset of the climacteric rise of ethylene production and thus of ripening of Bartlett pears, both on the tree [48] or detached [30]. Similar effects have also been reported in a variety of pome fruits including Conference pears [23], and Granny Smith [19] and Golden Delicious apples [24]. In Passe Crassane and Beurré d'Anjou pears, low-temperature treatments also stimulate autocatalytic ethylene synthesis [7, 13], a common feature of climacteric fruits [1], and can be substituted by exposure to exogenous ethylene [18, 44, 46, and references cited in 3]. The ethylene biosynthesis pathway proceeds from SAM to ethylene via the synthesis of ACC [53]. The autostimulation of ethylene synthesis in climacteric fruit is generally considered to be related

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X87097 and X87112.

to the regulation of ACC synthase gene expression [20, 42, 43], although recent data indicate that ACC oxidase gene expression is regulated by ethylene as well [26, 36, 52]. Cold acts by stimulating both ACC synthase and ACC oxidase activity [3, 19, 23, 25, 34], and can induce the accumulation of ACC oxidase protein [28]. The effect of chilling treatments has been studied more at the molecular level in climacteric fruit species of tropical or subtropical origin, such as tomato and avocado, fruits that are more susceptible to the damage referred to as 'chilling injury' [31]. In these species, chilling can interfere with fruit ripening [2, and references therein] and modify ethylene biosynthetic gene expression [12, 49]. However, depending on species, cultivar, developmental stage, as well as duration of the chilling treatment, chilling may stimulate, inhibit or fail to modify ethylene production in these tissues [2, 47, 49]. In Ailsa Craig tomato fruit at the breaker stage, ACC oxidase mRNA level only increases during the first 9 days of chilling and rapidly declines thereafter or upon rewarming [49]. Interestingly, ethylene production at 24 °C is unaffected by prior chilling treatment [49].

No molecular data are available for the chilling-induced stimulation of ethylene production in fruit of temperate origin that require chilling for ripening, although cold-induced changes in the mRNA population have been reported in Conference pears [50]. In the present paper, we have isolated ACC synthase and ACC oxidase cDNA probes to evaluate the role of chilling on ethylene biosynthetic gene expression in Passe-Crassane pear fruits. By using a specific inhibitor of ethylene action, 1-methylcyclopropene [15, 39], and an ethylene analogue, propylene [32], we demonstrate that chilling and ethylene interact differentially on ACC synthase and ACC oxidase gene expression.

Materials and methods

Plant materials and post harvest treatments

Pear (*Pyrus communis* L. cv. Passe-Crassane) fruits were harvested at commercial maturity in an orchard in the Garonne valley (Montauban, France) in 1992 and 1994. Ethylene emission of fruits was measured individually a week later as described elsewhere [28]. The fruits were kept in closed jars under a dry air flow of 3 l/h at either 0 °C or 18 °C. After 70–100 days of cold treatment fruits could ripen upon rewarming at 18 °C in air. After 27 days storage at 0 °C, some fruits were treated overnight in closed jars in the presence of KOH at 2 °C with 4 μl/l of 1-methylcyclopropene [15] freshly synthesized according to Dr Sisler's recommendations. After ventilation, the fruits were returned to 0 °C. Treatment with propylene, a gas used to mimic ethylene action [32], was carried out with air containing 1000 μl/l propylene at 18 °C (Alphagaz, France). After ethylene emission measurements, fruits were peeled and pieces of cortex immediately frozen in liquid nitrogen and stored at –80 °C for RNA analysis and assays for enzyme activities.

Extraction and assay of ACC oxidase and ACC synthase and immunoblot analysis

Extraction of ACC oxidase was performed according to Dupille *et al.* [14] and measured as described by Dong *et al.* [9]. Extraction and assay of ACC synthase was carried out according to Knee [23]. All enzyme activities were expressed in pmol/s per kg of fresh weight. Western blot analysis for ACC oxidase was performed as described elsewhere [14]. Where indicated, antigen-antibody complexes were resolved with the ECL System (Amersham) according to the manufacturer's recommendations.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA extraction was performed essentially as described by Manning [33] except that LiCl precipitation was replaced by sodium acetate (3M) precipitation. The cDNA was synthesized with First Strand cDNA Synthesis Kit (Pharmacia) using 1 to 15 μg of total RNA, followed by PCR with appropriate primers. The oligonucleotide primers were designed from conserved regions of ACC synthase and ACC oxidase available in gene data banks or literature [54]: 5′-AATGATGCGCTTGTGAGAAYTGG-3′ (Olacco-A), 5′-CTTCATGTAGTCATCYAARAG-3′ (Olacco-H), 5′-GATCCATGGGTCTNGCWGARAATCAGCT-3′ (S1A) and 5′-CTGCAGTTANGTKGTRCCYAAKGG RTTTGATGG-3′ (S1B). Amplified fragments were purified by gel electrophoresis and re-amplified before being sequenced using the *fmol* DNA Sequencing System (Promega). For direct cloning of PCR products, pGEM-T Vector Systems (Promega) were used.

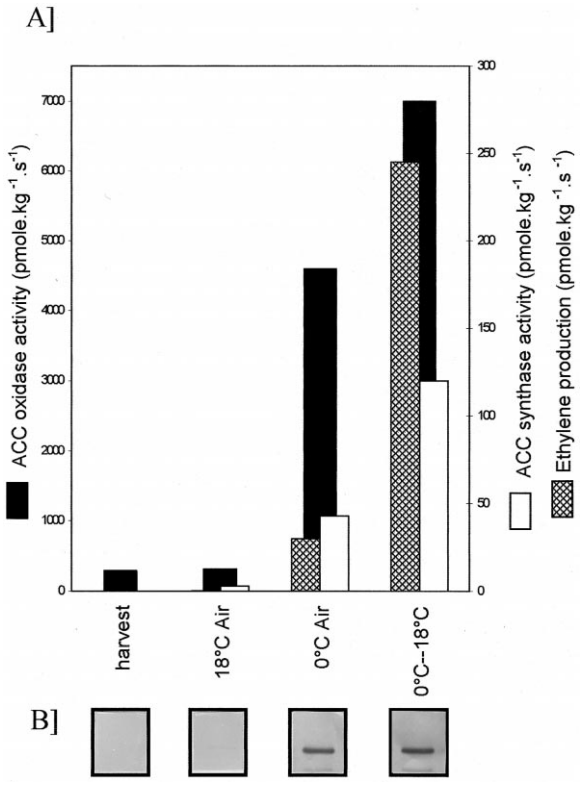

Figure 1. Ethylene production, ACC synthase and ACC oxidase activities (A), and western blot analysis of ACC oxidase protein (B) during post-harvest treatments of Passe-Crassane pear fruits. Total protein (7.5 μg per lane) were separated electrophoretically for immunoblotting with anti-tomato ACC oxidase antibodies.

Construction and screening of the cDNA library

For improving cDNA synthesis, total RNA prepared as described above was re-extracted [8]. Poly(A)$^+$ RNA isolated from fruits stored 100 days at 0 °C was purified with the Poly(A) Tract System (Promega). Oligo-dT primed cDNA was cloned in lambda UniZapII (Stratagene) according to the manufacturer's recommendations. The initial library contained about 10^6 recombinant phage. Amplification of 10 clones randomly selected yielded fragments ranging from 0.5 to 2.3 kb. For screening, phages were transferred onto Nytran membranes (Schleicher and Schuell) and hybridized in 40% formamide, 5× SSC, 0.1% SDS at 42 °C. The filters were finally washed at 65 °C in 1× SSC, 1% SDS. The PCR fragments were labelled according to Feinberg and Vogelstein [16] and used to screen the cDNA library. Rescued phagemids were purified according to standard methods and sequenced with Sequenase (USB-Amersham).

Cloning of ACC synthase and ACC oxidase cDNAs from the chilled Passe-Crassane fruits

Reverse transcription coupled with PCR was carried out using RNA isolated from cold-treated Passe-Crassane fruits. In the presence of S1A and S1B primers, a single fragment of the expected size (ca. 500 bp) was amplified. Partial sequencing showed that this fragment had substantial homology to the apple cDNA encoding ACC synthase [10, 27]. Screening of the pear cDNA library yielded 12 redundant positives. The nucleotide sequence of the longest isolated cDNA insert, pPC-ACS1, encoded a putative polypeptide of 473 amino acid residues. Using a similar RT-PCR approach for ACC oxidase yielded a single fragment of 750 bp whose sequence revealed a strong homology to a known apple cDNA encoding ACC oxidase [11, 37]. In the subsequent screening of the cDNA library, this fragment hybridized with ca. 0.5% of the total clones. When ten of these were selected and sequenced entirely or partially, all of them turned out to correspond to a single species of mRNA. pPC-ACO1, the longest insert isolated (1182 nucleotides), encoded a putative polypeptide of 313 amino acid residues, containing 25 nucleotides in the upstream of the first initiation codon and 239 nucleotides in the 3′-untranslated region.

RNA blot analysis

Total or poly(A)$^+$ RNA was size fractionated on a 1.2% agarose gel containing paraformaldehyde [39]. The RNA was blotted onto Nytran Plus membranes (Scheicher and Schuell) as described in the manufacturer's manual. Final washing was routinely carried out at 2× SSC, 1% SDS at 60–65 °C.

Results

Effects of chilling on ethylene production, activity of ACC synthase and ACC oxidase and level of ACC oxidase

Figure 1A shows that Passe Crassane fruits held for up to 100 days at 18 °C in air after harvest failed to produce significant amounts of ethylene. In addition, ACC synthase and ACC oxidase activities remained at very low levels comparable to that of freshly harvested fruits. In contrast, ethylene production of fruit stored for 100 days at 0 °C and measured at this temperature

was about 30 pmol kg^{-1} s^{-1}. Both ACC oxidase and ACC synthase activities were strongly stimulated by cold treatment, with ACC oxidase increasing from 280 pmol kg^{-1} s^{-1}at harvest to 4600 pmol kg^{-1} s^{-1} and ACC synthase from 5 to 43 pmol kg^{-1} s^{-1}. When fruits that had been chilled for 100 days were rewarmed at 18 °C, an 8-fold increase in ethylene production was observed which was parallel to a 3-fold stimulation of ACC synthase activity. At the same time, ACC oxidase activity was only stimulated by 1.5-fold. Western blot analysis indicated that the chilling-induced increase in ACC oxidase activity was correlated with an accumulation of the corresponding 38 kDa protein (Fig. 1). The ACC oxidase antigen was barely detectable in unchilled fruits.

Accumulation of ACC synthase and ACC oxidase transcripts during chilling treatment

RNA blot analysis showed that mRNAs hybridizing to ACC oxidase and ACC synthase probes were present at low and undetectable levels respectively in non-chilled fruit even after 92 days of shelf-life (Fig. 2). In contrast, in chilled fruits ACC oxidase transcripts were present in significant amounts after 40 days of chilling (Fig. 2, lane 5) and strongly increased until 60 days reaching a plateau thereafter. Transcripts hybridizing to the ACC synthase probe started to accumulate later, after 60 days of chilling (Figure 2, lane 6) and steadily increased thereafter. Attempts to detect ACC synthase mRNA by RT-PCR in fruits kept at 18 °C were unsuccessful.

Steady-state level of ethylene biosynthetic transcripts upon rewarming and the effect of 1-methylcyclopropene (1-MCP) treatment

Fruits that had been chilled for 100 days at 0 °C in air exhibited a high level of ACC oxidase mRNA upon rewarming at 18 °C even at very late stages of ripening (Figure 3, lanes 1–6). Senescent fruit after 24 days of shelf-life still had substantial amounts of ACC oxidase mRNA (Figure 3, lane 6) and protein present (Figure 4B). In contrast, the ACC synthase mRNA level decreased drastically during the first few days at 18 °C and then stabilized after 9 days (Figure 3, lane 4). It is noteworthy that ACC synthase transcripts reached a minimum level 9 days after rewarming, at which time ethylene production reached a peak (Figure 3, lane 4). RT-PCR was performed in the presence of S1A and S1B primers and RNA isolated from rewarmed fruits at the climacteric phase (corresponding to lanes 4–5 in Figure 3). Cloning and sequencing showed that amplified fragments corresponded to pPC-ACS1 only.

In order to elucidate the exact role of ethylene, fruits were treated overnight with 1-methylcyclopropene (1-MCP, an ethylene receptor antagonist), after 27 days at 0 °C. The 1-MCP treatment resulted in a complete inhibition of ripening during at least a 26-day rewarming period after 100 days at 0 °C. During chilling, ethylene production, ACC oxidase and ACC synthase activities increased at a substantially reduced rate in 1-MCP-treated fruits (data not shown). ACC oxidase and ACC synthase transcripts were present at the end of the chilling period (Figure 3, lanes 7), but rapidly disappeared upon rewarming (Fig. 3, lanes 8–10). Concomitantly, a collapse in ethylene production was observed (Figure 4A), corresponding to a decrease in ACC oxidase activity from 1400 pmol kg^{-1} s^{-1} at day 0 to 500 pmol kg^{-1} s^{-1} after 20 days at 18 °C. ACC synthase activity remained at a low level of about 25 pmol kg^{-1} s^{-1} throughout the 26-day period. Western blot analysis showed that the ACC oxidase antigen was at a significantly lower level in 1-MCP-treated fruits compared to control fruits (Figure 4B).

Action of propylene on ethylene biosynthetic gene expression in unchilled fruits

Continuous treatment of unchilled Passe-Crassane fruits with 1000 ppm propylene caused a dramatic increase in ACC oxidase activity from 308 to 9600 pmol kg^{-1} s^{-1} after 35 days of treatment. However, there was only a small stimulation of ACC synthase activity from 5 pmol kg^{-1} s^{-1} before treatment to 20 pmol kg^{-1} s^{-1} (Figure 5). Despite the stimulation of ACC oxidase activity, treated fruits never exhibited a climacteric-like rise in ethylene synthesis with the rate of ethylene production remaining between 1 and 10 pmol s^{-1} kg^{-1}. However, this rate was significantly stimulated compared to that of unchilled fruits without propylene treatment, which remained below 0.2 pmol kg^{-1} s^{-1} (data not shown). The amount of ACC oxidase protein continuously increased during the propylene treatment (Figure 5B). ACC oxidase transcripts started to increase after 5 days of propylene treatment while ACC synthase mRNAs were undetectable or only slightly detectable after 35 days of treatment (Figure 6, lanes 6 and 7). In comparison, chilled fruits produced 20 to 200 times more ethylene upon rewarming (Figure 4A) and displayed significant amounts of ACC synthase transcripts (Figure 6, lane 8).

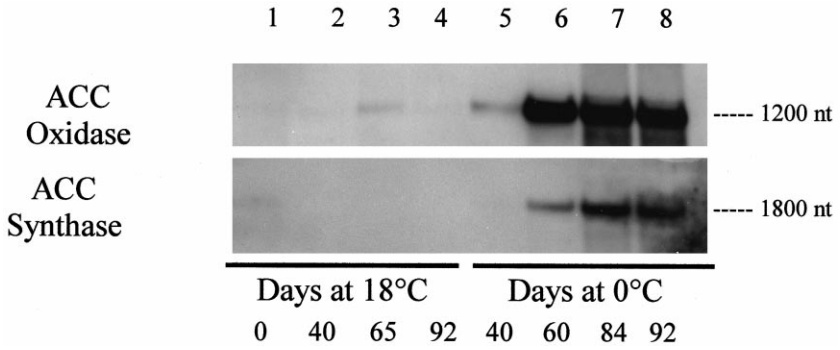

Figure 2. RNA hybridization analysis of postharvest chilled and unchilled Passe-Crassane fruits. The same total RNA (10 μg per lane) blot was probed with purified ^{32}P-labelled probes for PCR fragments of pPC-ACO1 and pPC-ACS1 successively to dose the steady-state level of ACC oxidase and ACC synthase mRNA respectively.

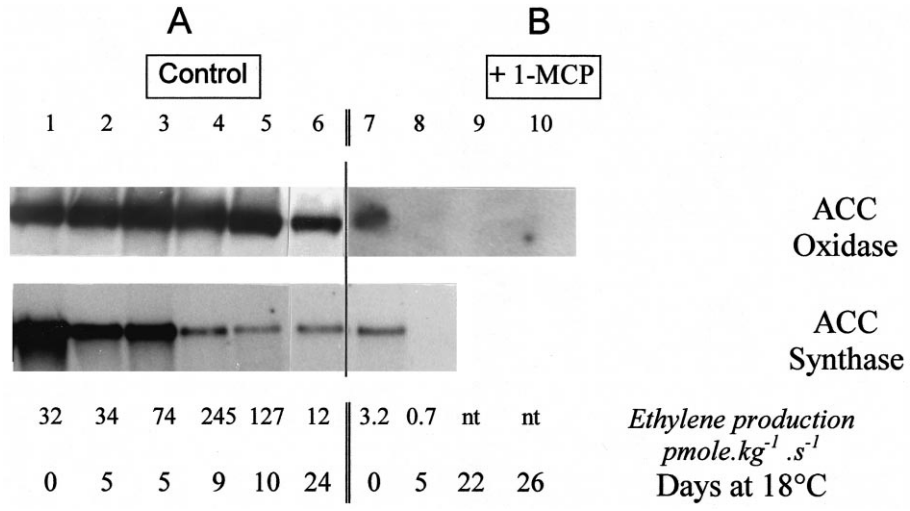

Figure 3. RNA hybridization analysis of pears upon rewarming and effects of 1-MCP treatment. After 100 days of cold treatment, fruits were transfered to 18 °C and their ethylene production measured daily. Its given value corresponds to the production the day the fruit was frozen. In control fruits not treated with 1-MCP (A), the same poly(A)$^+$ RNA blot (1 μg per lane) was probed successively as described in Fig. 2. For 1-MCP treated fruits (B), a total RNA (10 μg per lane) was probed with pPC-ACO1 (ACC oxidase) while a poly(A)$^+$ RNA blot (1 μg per lane) was probed with pPC-ACS1 (ACC synthase).

Discussion

A unique and interesting feature of the Passe Crassane pear variety is that a chilling treatment is absolutely required for inducing autocatalytic ethylene production and fruit ripening. We demonstrate in this work that the chilling treatment is responsible for inducing the expression of ACC synthase and ACC oxidase genes. The predicted amino acid sequence of the Passe Crassane ACC synthase cDNA clone that we isolated displayed large conserved regions (96.6%) compared to the corresponding clone from apple fruit [10, 27]. In addition, the non-coding sequences of pear and apple cDNAs share 59% identity to the 3′-untranslated region and 89% to the 5′ region. The ACC oxidase deduced amino acid sequence also showed a striking homology (95.9% identical) to those of apple fruit ACC oxidase cDNAs [11, 37]. Sequence identity with the LE-ACO1 (formerly pTOM13) tomato ACC oxidase cDNA [17] was also high (75.6%). Strong intraspecific [4] and interspecific [55] homology exists among ACC oxidases and it explains why antibodies raised against the recombinant tomato protein derived from LE-ACO1 were capable to recognize apple fruit ACC oxidase [14]. The antigen detected by the same antibodies in

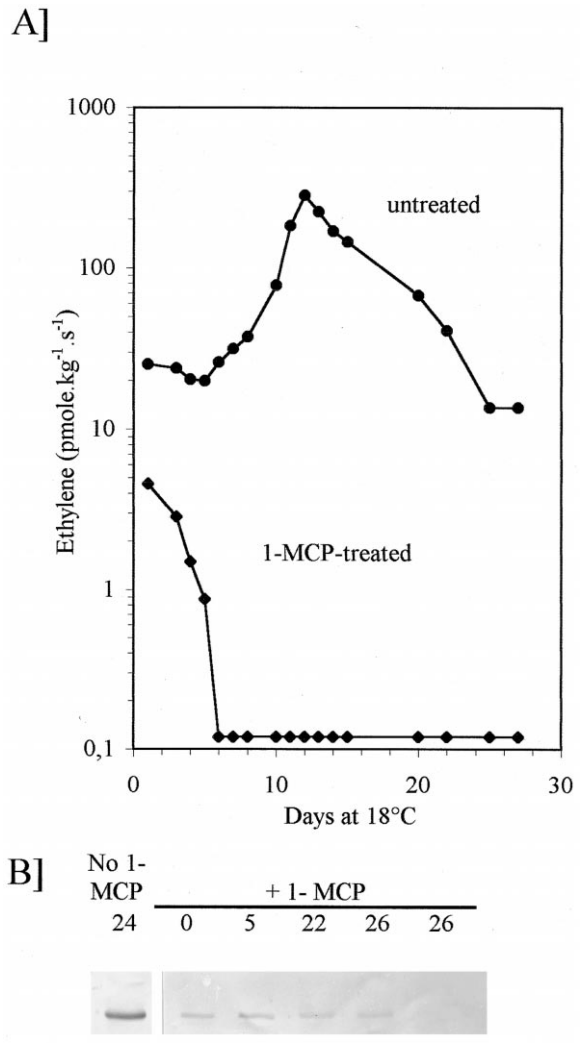

Figure 4. Effect of MCP treatment on ethylene production (A) and ACC oxidase protein level (B) upon rewarming of Passe-Crassane fruits. Because the timing of the onset of the peak of ethylene production varies from fruit to fruit, the rate of ethylene evolution for a single fruit is shown in A. Western blot analysis (B) was performed as described in Fig. 1.

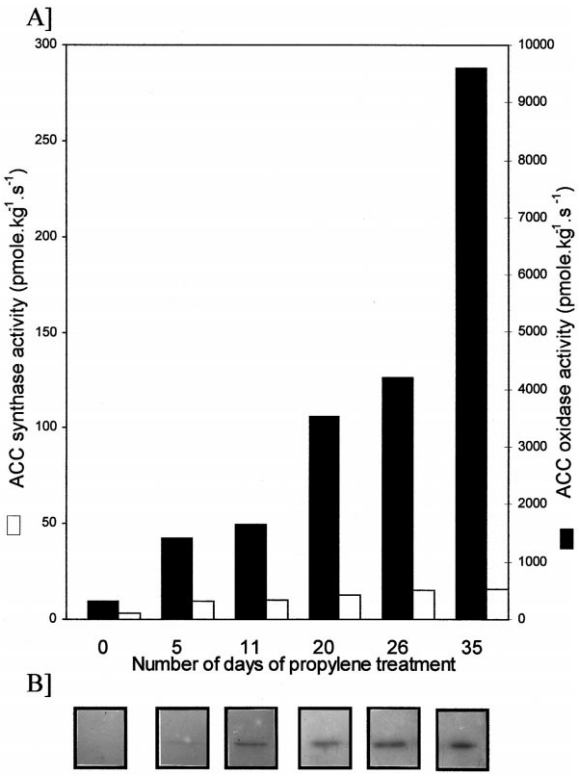

Figure 5. Effect of propylene on ACC oxidase and ACC synthase activities (A) and ACC oxidase protein level (B) of unchilled fruits. After harvest, fruits were exposed at 18 °C to an air flow supplemented with 1000 μl/l propylene. Revelation of ACC oxidase antigen-antibodies complexes were realized with the chemiluminescent ECL system.

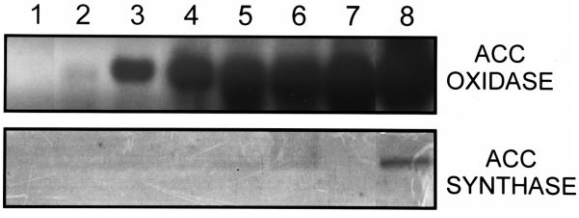

Figure 6. Effect of propylene on ACC oxidase and ACC synthase gene expression in unchilled Passe-Crassane fruits. Propylene treatment was performed as described in Fig. 5. A total RNA blot (10 μg per lane) was probed with pPC-ACO1 (ACC oxidase) and a poly(A)$^+$ RNA blot (0.5 μg per lane) was probed with pPC-ACS1 (ACC synthase). Lanes 1–6 correspond to propylene treated fruits for 0 (lane 1), 5 (lane 2), 11 (lane 3), 20 (lane 4), 26 (lane 5) and 35 (lanes 6 and 7) days. Lane 8 represents the positive control, corresponding to a rewarmed (in air) fruit at the climacteric stage (as lane 4, Fig. 3).

Passe Crassane fruits can therefore be considered as corresponding to the ACC oxidase protein.

Rewarming of the chilled fruits led to a peak of ethylene production associated with fruit ripening which resulted from an increase of ACC synthase activity as shown in this work. The amount of ACC oxidase mRNA remained abundant throughout the rewarming period and was well correlated with both the activity and the amount of the enzyme, as in apple fruit [9]. On the contrary, ACC synthase mRNA level decreased dramatically upon rewarming. This is independent of ethylene action because it is observed also in 1-MCP-treated fruits: upon rewarming, ACC synthase transcripts disappeared in less than five days in fruits

where ethylene action was blocked (see below). Therefore, it seems that most of the cold-induced transcripts were not able to sustain ACC synthase activity during chilling and immediately upon rewarming. In fact, the enzyme activity effectively increased several days after transfer to 18 °C, when the mRNA level has considerably decreased. Selective degradation of cold-induced transcripts upon rewarming has been described in several species [35]. Such a mechanism could also explain the observations made on ACC synthase mRNA level in Passe-Crassane fruit, however, they are intringuing when compared to previous results showing that ethylene production after removal of cold was more sensitive to protein synthesis inhibitors than to transcription inhibitors in *excised* tisssues [23, 46]. It was proposed that ethylene production in rewarmed fruits was therefore due to the translation of ACC synthase transcripts accumulated during chilling. These conclusions can be drawn if one supposes that (1) excised (wounded) tissues behave as intact tissues (2) the transcription synthesis inhibitors are effective, which in both cases needs to be demonstrated (see for example [40]). Another possibility would be that the mRNAs produced during chilling and upon rewarming were different; our probe could detect only the former due to the large sequence divergence among members of ACC synthase multigene family [54]. However, the PC-ACS1 ACC synthase cDNA isolated here is very closely related to the corresponding cDNA found in ripening apple fruit as noted above, and this cDNA sequence is itself substantially divergent from that of the mRNA induced by auxin in apple seedlings [21]. Southern analysis using pPC-ACS1 as probe identified one related gene only in the pear genomic DNA (unpublished results). Nonetheless, we cannot completely exclude that other ACC synthase genes that might be differentially expressed in the fruit during chilling and upon rewarming, were not detected in this study: in tomato, ACC synthase is encoded by at least 9 genes that display significant sequence divergence and whose expression is differentially regulated [54].

Taken together these data suggest that a difference exists in the regulatory mechanism governing the expression of ACC synthase and ACC oxidase genes. Since ethylene is also known to stimulate the expression of genes involved in its own biosynthesis [20, 22, 26, 36, 41, 42], we have studied the role of ethylene vs. that of chilling by manipulating ethylene action with antagonist and analog molecules. Treating fruit during the cold treatment with the potent ethylene antagonist 1-MCP [15, 39], revealed that chilling-induced accumulation of ACC oxidase and ACC synthase transcripts was dramatically lower, but not suppressed, when ethylene action was inhibited. Rewarming of 1-MCP-treated fruits not only abolished the climacteric response, but also the rate of decline of gene expression was similar and resulted in a fast disappearence of ACC synthase and ACC oxidase transcripts. This demonstrates that ethylene-dependent gene expression is required for both enzymes in rewarmed pear fruits. Treatment of unchilled fruits with propylene strongly promoted the accumulation of ACC oxidase transcript and enzyme activity. In contrast, ACC synthase activity remained at a minimal level and its mRNA level was considerably lower than in fruits that were previously chilled. Differential regulation in ACC oxidase and ACC synthase gene expression in ripening fruits has already been suggested. A short ethylene treatment is able to increase ACC oxidase activity in preclimacteric apple [5], melon and tomato [29] fruits, although it fails to induce ethylene production and ACC synthase activity [5, 29]. The increase in ACC oxidase activity was correlated with the increase in enzyme activity and mRNA level [9].

Most studies dealing with the response of plants to low temperature shock at the molecular level have been concerned with cold acclimation [35]. It has been shown that this process involves, in part, the regulation of gene expression mediated by abscisic acid (ABA) [6, 35]. It is noteworthy that exposure to low temperatures induces an increase in the ABA level in pear fruits after harvest [38] or during cold-induced premature ripening on the tree [48]. Cloning of genes and their promoters may help to identify cold-associated signals that mediate ethylene biosynthetic gene expression in fruits.

In summary, we demonstrate in this paper how chilling and ethylene interact to induce ripening in Passe-Crassane fruits. Whereas ACC oxidase gene expression can be induced by either ethylene or chilling, ACC synthase gene expression depends on both chilling and ethylene. First, a cold-related signal is required to induce ACC synthase gene expression. At a higher temperature, ethylene can subsequently induce ACC synthase gene expression associated with the high enzyme activity observed in rewarmed fruits, as it is the case in other climacteric fruits. Therefore, the elucidation of the role of chilling treatment in Passe-Crassane pears will require to understand the mechanisms of ethylene action on ACC synthase gene expression.

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