Mol Gen Genet (1999) 261: 42–49

ORIGINAL PAPER

A. Itai · T. Kawata · K. Tanabe · F. Tamura
M. Uchiyama · M. Tomomitsu · N. Shiraiwa

Identification of 1-aminocyclopropane-1-carboxylic acid synthase genes controlling the ethylene level of ripening fruit in Japanese pear (*Pyrus pyrifolia* Nakai)

Received: 1 July 1998 / Accepted: 6 October 1998

Abstract The shelf life of Japanese pear fruit is determined by its level of ethylene production. Relatively high levels of ethylene reduce storage potential and fruit quality. We have identified RFLP markers tightly linked to the locus that determines the rate of ethylene evolution in ripening fruit of the Japanese pear. The study was carried out using sequences of two types of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase genes (PPACS1 and pPPACS2) and a ACC oxidase gene (PPAOX1) as probes on 35 Japanese pear cultivars expressing different levels of ethylene (0.0∼300 μl/kg fresh weight/h) in ripening fruit. When total DNA was digested with *Hin*dIII and probed with pPPACS1, we identified a band of 2.8 kb which was specific to cultivars having very high ethylene levels (≧10 μ1/kg f.w./h) during fruit ripening. The probe pPPACS2 identified a band of 0.8 kb specific to cultivars with moderate ethylene levels (0.5 μl/kg f.w./h–10 μl/kg f.w./h) during fruit ripening. The cultivars that produce high levels of ethylene possess at least one additional copy of pPPACS1 and those producing moderate levels of ethylene have at least one additional copy of pPPACS2. These results suggest that RFLP analysis with different ACC synthase genes could be useful for predicting the maximum ethylene level during fruit ripening in Japanese pear.

Key words *Pyrus pyrifolia* · ACC synthase · RFLP marker · Ethylene level · Storage potential

Communicated by H. Saedler

A. Itai (✉) · T. Kawata · K. Tanabe · F. Tamura
M. Uchiyama · M. Tomomitsu · N. Shiraiwa
Laboratory of Horticultural Science,
Faculty of Agriculture,
Tottori University, Koyama Minami 4-101,
Tottori 680-8553, Japan
e-mail: itai@pear.agr.tottori-u.ac.jp
Tel./Fax: +81-857-316749

Introduction

The gaseous plant hormone ethylene is involved in fruit ripening and senescence (Brady 1987; Grierson 1987; Fischer and Bennett 1991; Kende 1993; Zarembinski and Theologis 1994; Lelièvre et al. 1997a). In recent years significant progress has been made in our understanding of the biosynthetic pathway and the molecular regulation of ethylene production (Adams and Yang 1979; Kende 1993). Cloning and characterization of genes encoding the two key enzymes involved, ACC synthase and ACC oxidase, has been carried out in many species (Sato and Theologis 1989; Van Der Straeten et al. 1990; Nakajima et al. 1991; Lay-Yee and Knighton 1995). These reports have shown that ACC synthase and ACC oxidase are encoded by a multigene family whose various members are differentially expressed in response to many factors (Olson et al. 1991; Rottmann et al. 1991; Lincoln et al. 1993; Lasserre et al. 1996). Expression of ACC synthase and ACC oxidase may be developmentally regulated, show tissue specificity, and be controlled by signal transduction pathways that respond to ethylene (Kim et al. 1997).

It is well known that ethylene promotes many ripening responses in climacteric fruit. The molecular aspects of fruit ripening have been most widely studied in tomato. In tomato, ACC synthase is encoded by six genes (Olson et al. 1991, Rottmann et al. 1991; Yip et al. 1992; Lincoln et al. 1993); one of these, LE-ACS2, is expressed during fruit ripening and is induced by treatment with exogenous ethylene. LE-ACS4 appears to be expressed at a lower level during these stages (Rottmann et al. 1991).

In Japanese pear, both climacteric and non-climacteric cultivars exist (Tanabe et al. 1994). Climacteric type fruits exhibit a rapid increase in ethylene production and have a low storage potential. In contrast, non-climacteric type fruits show non-detectable levels of ethylene, and fruit quality is maintained for periods of over a month. These findings therefore show that storage potential is closely related to ethylene production in Jap-

anese pear fruit. To permit selection for varieties having good storage potential, a better understanding of the genetics of ethylene biosynthetic pathways in Japanese pear is needed. To this end, we have initiated studies on the genes involved in the ethylene biosynthetic pathway. Since ACC synthase and ACC oxidase play key roles in this pathway, we were interested in understanding their regulation at the molecular level in relation to fruit ripening in Japanese pear. In this paper we report the identification of two ACC synthase genes which are correlated with high ethylene levels in Japanese pear during ripening. Information gained from this study will be useful in developing breeding strategies to improve storage potential in Japanese pear fruit.

Materials and methods

Plant Materials

Thirty-five cultivars of Japanese pear (*P. pyrifolia* Nakai) grown in the orchard of Tottori University were used in this study (see Table 1). For Southern analysis and studies on ethylene evolution, all 35 cultivars were used. For gene cloning, the cultivars Chojuro, Kikusui and Ninomiya were selected, and for gene expression analysis fruits from eight cultivars (Edoya, Awayuki, Ninomiya, Chojuro, Kikusui, Nijisseiki, Hosui, Shinsetsu) were used.

Ethylene measurement during fruit ripening

Three to five pear fruits each of from 35 cultivars were placed in 1.5-l sealed jars for 2 h at 20° C for ethylene measurement. After 2 h, 2.0 ml headspace gas samples were withdrawn from each jar. Samples were analysed on a gas chromatograph, with a flame ionization detector and 60/80 mesh activated alumina column (Model 163, Hitachi). Ethylene measurements were obtained every 3–4 days for 2 weeks during ripening and were expressed as µl of C_2H_4/kg/f.w./h.

RNA extraction and RT-PCR

Total RNA was extracted by the hot borate method (Wan and Wilkins 1994; Nakatsuka et al. 1997). cDNA was synthesized using the AMV Reverse Transcriptase first-strand cDNA synthesis kit (Life Science) using 5 µg of total RNA from ripe Ninomiya, Chojuro and Kikusui fruit, followed by PCR with appropriate primers. Oligonucleotide primers for ACC synthase were designed

Table 1 Fruit ethylene production and corresponding marker genotypes in 35 Japanese pear cultivars

Cultivars (maturation time)	pPPACS1[a] (2.8-kb)	pPPACS2[b] (0.8-kb)	Genotype[c]	Maximum ethylene level[d]
Ninomiya (Early)	+	+	AB	High
Rokugatsu (Early)	+	–	A-	High
Edoya (Early)	+	–	A-	High
Okuroku (Early)	+	–	A-	High
Sotoorihime (Mid)	+	–	A-	High
Awayuki (Mid)	+	–	A-	High
Shindhu (Early)	–	+	-B	Moderate
Suisei (Mid)	–	+	-B	Moderate
Kimitsukawase (Early)	–	+	-B	Moderate
Ishiiwase (Early)	–	+	-B	Moderate
Shinsui (Early)	–	+	-B	Moderate
Chojuro (Mid)	–	+	-B	Moderate
Kinchaku (Mid)	–	+	-B	Moderate
Yakumo (Early)	–	+	-B	Moderate
Akaho (Early)	–	+	-B	Moderate
Kozo (Mid)	–	+	-B	Moderate
Wasekozo (Mid)	–	+	-B	Moderate
Gion (Mid)	–	+	-B	Moderate
Kosui (Early)	–	+	-B	Moderate
Kikusui (Mid)	–	+	-B	Moderate
Doitsu (Mid)	–	+	-B	Moderate
Ohiromaru (Late)	–	+	-B	Moderate
Inugoroshi (Late)	–	+	-B	Moderate
Waseaka (Late)	–	–	–	Low
Nijisseiki (Mid)	–	–	–	Low
Sekaiichi (Mid)	–	–	–	Low
Hosui (Mid)	–	–	–	Low
Amanokawa (Late)	–	–	–	Low
Imamuraaki (Late)	–	–	–	Low
Okusankichi (Late)	–	–	–	Low
Shinsetsu (Late)	–	–	–	Low
Yokogoshi (Late)	–	–	–	Low
Ruisannashi (Late)	–	–	–	Low
Konpeito (Late)	–	–	–	Low
Niitaka (Late)	–	–	–	Low

[a] +, 2.8-kb band present –, 2.8-kb band absent
[b] 0.8-kb present (+); absent (–)
[c] A, linked to high level of ethylene, B, linked to moderate level of ethylene production
[d] Maximum ethylene production during fruit ripening: High > 10 µl/kg/h; Moderate, 0.5 µl/kg/h–10 µl/kg/h; Low < 0.5 µl/kg/h

as follows. For PPACS1, primers based on the pear sequence (cv. Passe-Crassane; Accession No.: X87097; Lelièvre et al. 1997b) were used. The upstream primer was 5′-ACCTCGAACTCTCTCTCT-AATGC-3′ and the downstream primer was 5′-AATGCATCAT-AATCACAATGGAG-3′. For PPACS2, degenerate primers based on conserved regions of ACC synthases in the Genbank database were used. The upstream primer was 5′-ATICARATGGGIYTI-GCIGARAAYCA-3′ and the downstream primer was 5′-GCRA-ARCAIACICKRAACCAICCIGGYTC-3′. Oligonucleotide primers were also designed for ACC oxidase as follows. For PPAOX1, degenerate primers based on conserved regions in the Genbank database were used. The upstream primer was 5′-SARAAYTGG-GGYTTSTWYGAG-3′ and the downstream primer was 5′-TCR-AABCKYGGYTCYTTNG-3′. PCR conditions for PPACS1 were 40 cycles of 94° C for 1 min, 62° C for 1 min and 72° C for 2 min. Conditions for PPACS2 and PPAOX1 were the same, except that annealing took place at 57° C and 45° C, respectively.

Amplified fragments (1.8 kb for PPACS1 from Ninomiya fruit cDNA, 1.1 kb for PPACS2 from Kikusui fruit cDNA and 0.8 kb for PPACS2 from Chojuro fruit cDNA) were purified by gel electrophoresis and ligated into the pGEM-T vector (Promega).

Construction and screening of cDNA libraries

Poly(A)$^+$ RNA was isolated using oligo-dT columns (Pharmacia) from total RNA of Kikusui and Chojuro fruits, extracted as described above. Oligo-dT primed cDNA was cloned in lambda Excell (Pharmacia) and cDNA libraries comprising 120 000 clones from Kikusui and 40 000 clones from Chojuro were constructed. For screening, plaques were transferred to Hybond N$^+$ Nylon membranes (Amersham) and hybridized in 40% formamide, 5 × SSPE, 5 × Denhardt's, and 0.1% SDS at 42° C, with the appropriate probe. Following hybridization, the filters were washed at 65° C in 0.2 × SSC, 0.1% SDS. The cloned 1.1-kb fragment of PPACS2 and 0.8-kb fragment of PPAOX1 were ^{32}P-labelled by the random primer method (Rediprime kit, Amersham) and used for hybridization as described above. The Kikusui cDNA library was screened for the ACC synthase gene and the Chojuro cDNA library was screened for the ACC oxidase gene. Phagemids were released in vivo from positive plaques and DNA extracted by the alkaline-SDS method.

Cloning of two ACC synthase genes and an ACC oxidase gene

Using the PPAOX1 fragment as a probe on the Chojuro cDNA library, ca. 2.5% of the total clones gave positive signals. Ten of these were selected and the longest cDNA insert, pPPAOX1, was sequenced using the Thermo-Sequenase fluoroescent sequencing kit (Amersham) and the ALF Express sequencer (Pharmacia). Using the PPACS2 fragment as a probe on the Kikusui pear cDNA library, six positive clones were detected. The longest cDNA insert, pPPACS2, was sequenced as above. The TA-cloned cDNA insert pPPACS1 was also sequenced. Nucleotide sequences were analysed using DNASIS software (Hitachi).

DNA isolation and Southern analysis

Total DNA was extracted from the immature leaves of the 35 cultivars by a modified SDS method (Teramoto et al. 1994). Ten micrograms of DNA from each cultivar was digested with *Eco*RI, *Hin*dIII, *Eco*RV and *Bam*HI, fractionated by electrophoresis in 0.9% agarose gels, and transferred to Hybond N$^+$ Nylon membranes by Southern blotting. DNA was fixed by UV crosslinking. Membranes were hybridized with ^{32}P-labelled inserts from pPPAOX1, pPPACS1 and pPPACS2 at 42° C overnight in a buffer containing 40% formamide, 5 × SSPE, 5 × Denhardt's, and 0.1% SDS. After hybridization membranes were washed twice for 15 min each at 68° C in 0.2 × SSC and 0.1% SDS, and then exposed to X-ray film.

Northern analysis

Ten micrograms of total RNA was fractionated on a 1.2% agarose gel containing formaldehyde and blotted onto Hybond N$^+$ Nylon membranes. Hybridization and washing conditions were as described above.

Results and discussion

Differences between cultivars in ethylene production

All Japanese cultivars showed no or very low ethylene production until they reached the optimum harvest date. Beyond this point the pattern of ethylene increase was cultivar dependent. Ninomiya, Rokugatsu, Edoya, Okuroku, Awayuki and Sotoorihime pears produced more than 50 µl of C_2H_4/kg f.w./h (Fig. 1), with Edoya

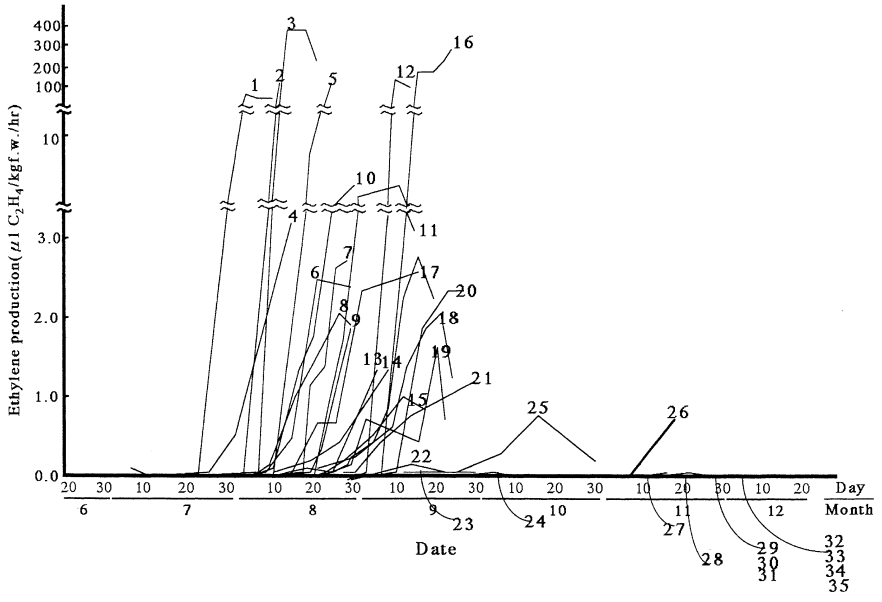

Fig. 1 Ethylene production in 35 Japanese pear cultivars during fruit ripening. The following cultivars were studied: 1, Ninomiya; 2, Rokugatsu; 3, Edoya; 4, Kimitsukawase; 5, Okuroku; 6, Ishiiwase; 7, Suisei; 8, Akaho; 9, Shinsui; 10, Shinchu; 11, Kinchaku; 12, Awayuki; 13, Yakumo; 14, Kosui; 15, Doitsu; 16, Sotoorihime; 17, Wasekozo; 18, Kozo; 19, Gion; 20, Chojuro; 21, Kikusui; 22, Nijisseiki; 23, Sekaiichi; 24, Hosui; 25, Ohiromaru; 26, Inugoroshi; 27, Niitaka; 28, Konpeito; 29, Waseaka; 30, Yokogoshi; 31, Ruisannashi; 32, Okusankichi; 33, Imamuraaki; 34, Amanokawa; 35, Shinsetsu. Cultivars (28–35) show only trace levels of ethylene production

pear peaking at over 300 μl C_2H_4/kg f.w./h. These cultivars are known to have poor storage potential (Kajiura and Sato 1991). Shinsui, Kosui, Chojuro, Yakumo, Kikusui and a further 12 cultivars produced ethylene at moderate levels (0.5–10 μl of C_2H_4/kg f.w./h) and are also known to have a short shelf life. Nijisseki, Hosui, Niitaka, Okusankichi and a further eight cultivars produced very little ethylene (less than 0.5 μl C_2H_4/kg f.w./h) and had long storage potentials. In almost all of these cultivars ethylene production was below the limit (0.1 μl C_2H_4/kg f.w./h) detectable by gas chromatography. Every Japanese pear cultivar showed a characteristic pattern of ethylene evolution, some having a rapid increase in ethylene production and others producing little or no ethylene. These data show that the maximum rate of ethylene production ranges from 0.1 to 300 μl C_2H_4/kg f.w./h during ripening of Japanese pear cultivars. In general, earlier maturing cultivars produce more ethylene than later maturing ones (Abeles et al. 1992) and similar observations have been made on apple cultivars (Hansen 1945). These results are compatible with those obtained in this study.

Isolation of cDNAs for ACC oxidase and ACC synthase

pPPAOX1 is 1288 bp in length, and encodes a putative polypeptide of 314 amino acids (DDBJ Accession No. D67038; data not shown). The coding region of pPPAOX1 is 97% identical to those of Passe-Crassane Pear (pPC-ACO1) and apple ACC oxidase cDNAs (pAP4, pAE12) (Dong et al. 1992; Ross et al. 1992; Lelièvre et al. 1997b). However, the 3′ untranslated region of pPPAOX1 is about 100 bp longer than those of pPC-ACO1, pAP4 and pAE12.

pPPACS1 (1778 bp) was amplified by RT-PCR using primers based on the nucleotide sequence of the pear cv. Passe-Crassane (pPC-ACS1). pPPACS1 encodes a putative polypeptide of 473 amino acids and pPPACS1 shows high homology (98%) to pPC-ACS1. pPPACS2 is 1872 bp in length, and encodes a putative polypeptide of 446 amino acids. The deduced polypeptides encoded by pPPACS1 and pPPACS2 show 58% identity to each other (Fig. 2). Each has the conserved domains (Boxes 1–7) found in other ACC synthases (Kende 1993). In addition the coding region of pPPACS2 is 97% identical to that of MdACS-3 from ripening apple fruit (Rosenfield et al. 1996).

Southern analysis using pPPAOX1 as a probe

RFLP analysis of Japanese cultivars with pPPAOX1 as a probe against *Eco*RV and *Bam*HI digests showed no length polymorphisms (data not shown). When DNA was digested with *Eco*RI, length polymorphisms were revealed among some cultivars in the 3.5-kb region

Fig. 2 Comparison of amino acid sequences deduced from pPPACS1 and pPPACS2 cDNAs. The *boxes* indicate regions conserved in ACC synthases from various plant species (Kende 1993) Nucleotide sequence data are available in the DDBJ, EMBL and GenBank databases under the accession numbers AB015624 for pPPACS1 and AB007639 for pPPACS2

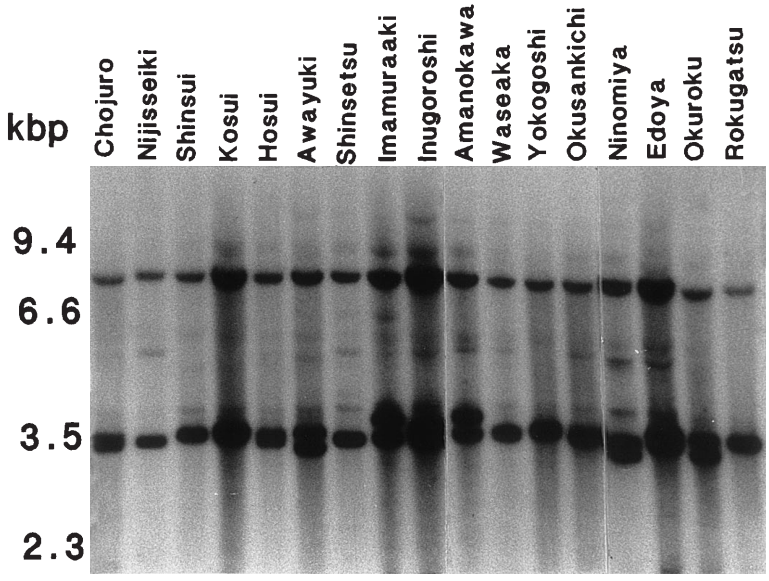

Fig. 3 Southern analysis, using probe pPPAOX1, of *Eco*RI-digested generic DNAs from the indicated Japanese pear cultivars

(Fig. 3). However, no polymorphisms linked to variation in ethylene production during fruit ripening were observed. Similar results were obtained with *Hin*dIII digests (data not shown).

Southern analysis using pPPACS1 as a probe

RFLP analysis with pPPACS1 as a probe against *Eco*RV and *Bam*HI digests showed no length polymorphisms. When DNA was digested with *Hin*dIII, all cultivars showed hybridising bands of 7.7, 3.5, and 1.0 kb (Fig. 4), but cultivars Ninomiya, Rokugatsu, Edoya, Okuroku, Awayuki and Sotoorihime showed an additional band of 2.8 kb, indicating that they have an extra copy of PPACS1. Cultivars showing the additional 2.8 kb band also produced very high levels of ethylene (more than 50 µl C_2H_4/kg f.w./h) during fruit ripening. The extra gene copy therefore distinguishes high ethyl-

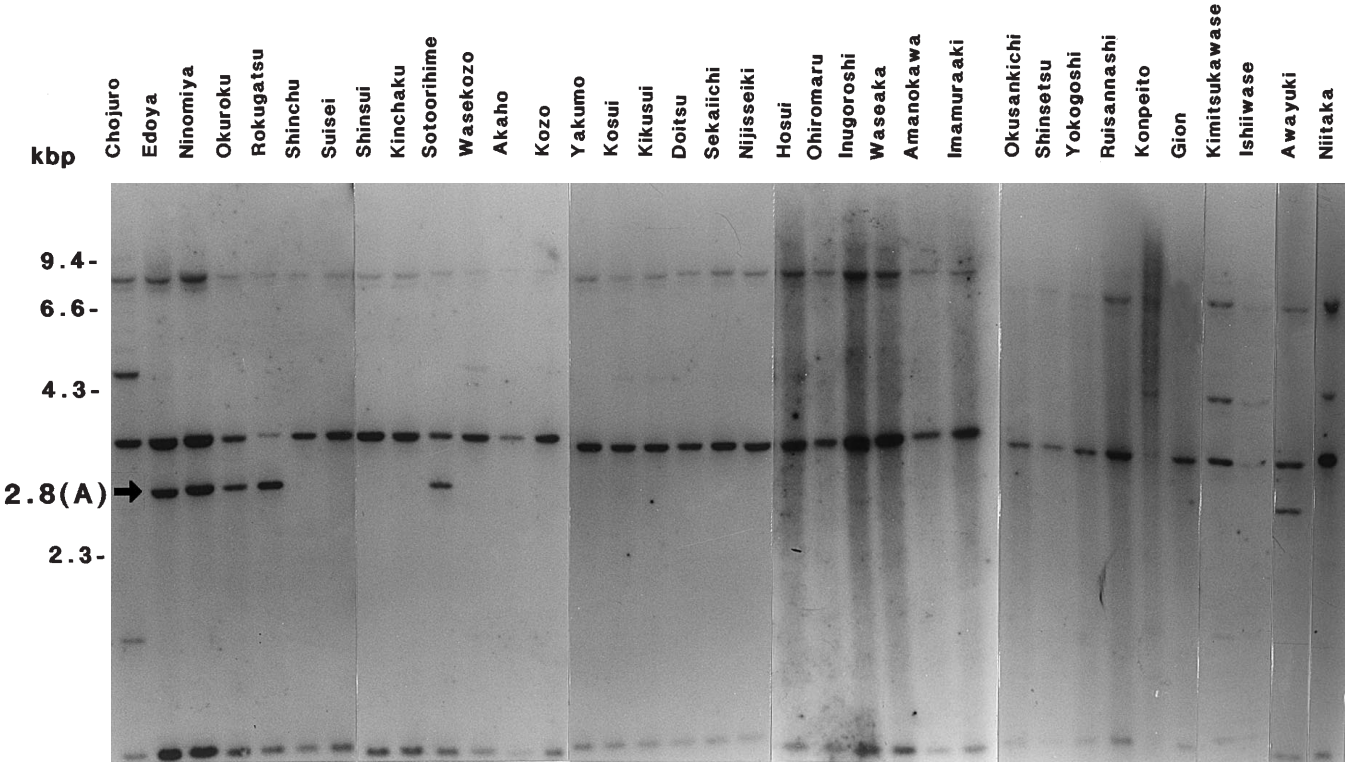

Fig. 4 Southern analysis, using probe pPPACS1, of *Hin*dIII-digested DNAs from 35 Japanese pear cultivars. The band indicated by the *arrow* is specific to cultivars producing high levels of ethylene during fruit ripening

ene producers from moderate and low-level ethylene producers. Southern analysis indicated that, in these cultivars, the additional PPACS1 gene resulted from a gene duplication event similar to those shown for ACC synthase genes in potato (Destéfano-Beltrán et al. 1995) and cucumber (Trebitsh et al. 1997). *Eco*RI digests of genomic DNA from the same high ethylene-producing cultivars revealed an extra band of 9.4 kb in Edoya and Rokugatu and a 6.0 kb band in Ninomiya, Okuroku, Rokugatu, Sotoorihime and Awayuki (Fig. 5). These data also support the gene duplication hypothesis mentioned above, and suggest that the additional ACC synthase gene, homologous to pPPACS1, contributes to the high-level production of ethylene during fruit ripening.

Southern analysis using pPPACS2 as a probe

Southern analysis with pPPACS2 also detected RFLPs in some Japanese pear cultivars (Fig. 6). Cultivars that produce moderate levels of ethylene (1–10 μl C_2H_4/kg f.w./h; Shinsui, Kosui, Chojuro, Kikusui and a further 12 cultivars) contained an extra hybridising *Hin*dIII band of 0.8 kb. This band was absent in all cultivars showing high and low ethylene production, except for Ninomiya. A survey of Japanese pear cultivars revealed a high level of polymorphism in this region, as demonstrated by the bands larger than 6.2 kb in *Hin*dIII digests. Sequence analysis revealed that pPPACS2 has a *Hin*dIII site, and analysis of its restriction digestion pattern demonstrates that pPPACS2 belongs to a divergent multiple gene family. It is possible that the 0.8-kb fragment is specific to cultivars producing moderate amounts of ethylene. These cultivars also have poor storage potential, so the 0.8-kb band may be a useful selective marker for this trait. Cultivars producing high levels of ethylene, such as Rokugatsu, Edoya, Okuroku, Awayuki, and Sotoorihime, lack the 0.8-kb band, and only Ninomiya had both the 0.8-kb fragment of PPACS2 and the 2.8-kb fragment of PPACS1.

Both PPACS1 and PPACS2 appear to be expressed in ripening Ninomiya fruit. Since the PPACS1 gene produces more ethylene than PPACS2, the activity of PPACS1 will mask that of PPACS2. The data presented here indicate that the PPACS2 gene also resulted from a gene duplication event.

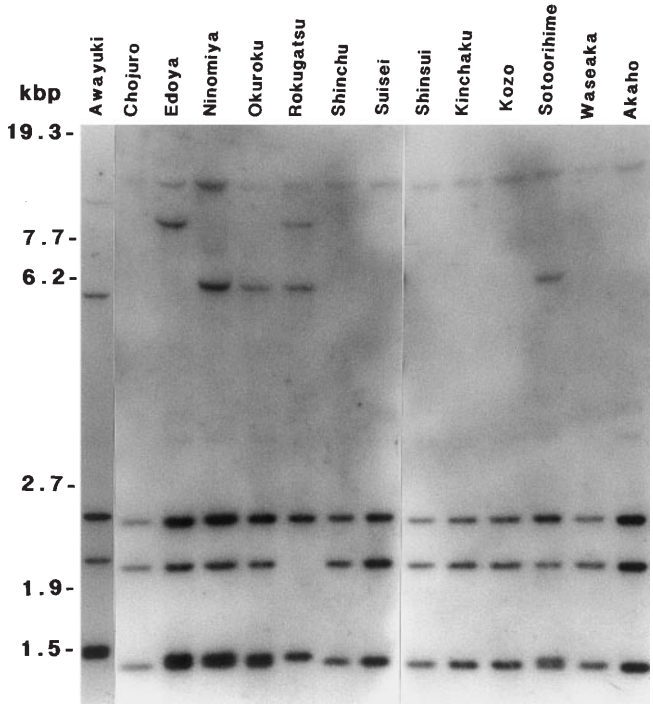

Fig. 5 Southern analysis, using probe pPPACS1, of *Eco*RI-digested DNAs from the indicated Japanese pear cultivars

Expression of PPAOX1, PPACS1 and PPACS2

RNA was isolated from ripening fruits of cultivars producing high levels of ethylene (Ninomiya, Edoya and Awayuki), moderate levels (Kikusui and Chojuro), and low levels (Nijisseiki, Shinsetsu and Hosui). pPPAOX1, pPPACS1 and pPPACS2 fragments were used as probes. High levels of PPAOX1 transcripts were detected in all cultivars, regardless of whether the cultivars produced ethylene or not (Fig. 7). This result shows that the ACC oxidase reaction is not a rate-limiting step in the ethylene biosynthetic pathway of Japanese pear during fruit ripening. The PPACS1 transcripts were highly expressed in the ripening fruit of Ninomiya, Edoya and Awayuki, but not in Kikusui, Chojuro, Nijisseiki, Shinsetsu or Hosui fruits (Fig. 7). The PPACS2 transcript was detected in Ninomiya, Kikusui and Chojuro fruits, but at lower levels than the PPACS1. PPACS2 transcripts was not detected in Edoya, Okuroku, Nijisseiki, Shinsetsu or Hosui fruit. Ninomiya was the only cultivar to express both PPACS1 and PPACS2. These data indicate that the expression of PPACS1 and PPACS2 is differentially regulated in Japanese pear cultivars and correlate with the results of Southern analysis.

Conclusions

Ethylene is an important plant hormone which is synthesized naturally during fruit ripening and reduces storage potential in climacteric fruit. Ethylene levels of cultivated Japanese pear fruit varied from 0.1 to 300 μl C_2H_4/kg f.w./h during ripening and cultivars producing moderate or high levels (0.5 to 300 μl C_2H_4/kg f.w./h) have poor storage potential. Since two enzymes (ACC synthase and ACC oxidase) are involved in ethylene biosynthesis, we were interested in the expression of these genes during ripening of Japanese pear. Two ACC synthase cDNAs and one ACC oxidase cDNA (pPPACS1, pPPACS2 and pPPAOX1) were isolated that are expressed during fruit ripening. Since PPAOX1

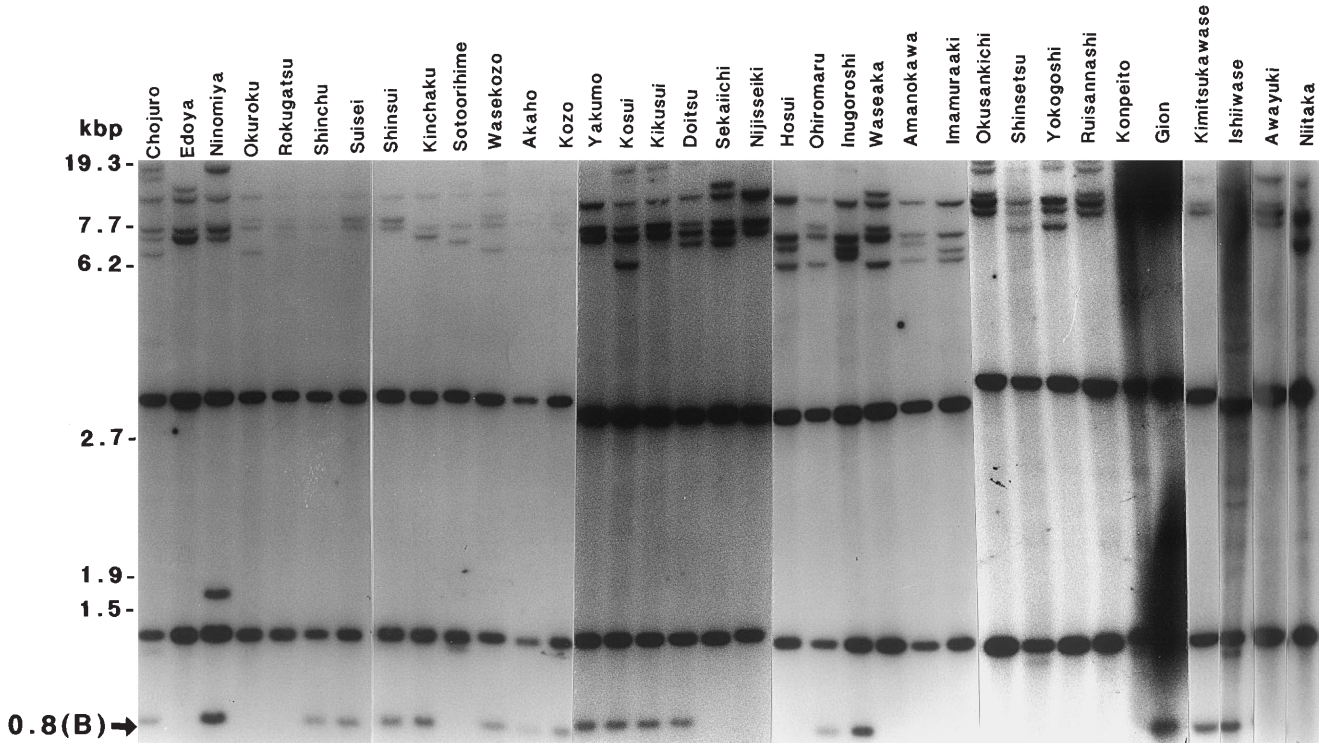

Fig. 6 Southern analysis, using probe pPPACS2, of *Hin*dIII-digested DNAs from 35 Japanese pear cultivars. The band indicated the *arrow* is specific to cultivars producing moderate levels of ethylene during fruit ripening

transcripts were highly expressed in all cultivars during fruit ripening, the ACC oxidase reaction is not a rate-limiting step in determining the maximum ethylene levels in Japanese pear cultivars. Cultivars producing high levels of ethylene possess at least one additional copy of PPACS1, as shown by an additional, *Hin*dIII fragment of 2.8 kb, and those producing moderate levels of ethylene possess at least one additional copy of PPACS2, as shown by an additional *Hin*dIII fragment of 0.8 kb. RFLP analysis of Japanese pear cultivars using the different ACC synthase cDNAs was useful for accurate prediction of maximum ethylene levels during fruit ripening (Table 1). RFLPs were designated as A (2.8 kb pPPACS1), linked to high levels of ethylene, and B (0.8 kb pPPACS2), linked to moderate levels of ethylene. We hypothesize that in Japanese pear the maximum ethylene level during fruit ripening is regulated by the expression of two ACC synthase genes (PPACS1:A and PPACS2:B) and that the action of PPACS1(A) masks that of PPACS2(B) due to its much higher levels of expression, leading to 20–100 times higher ethylene production. Therefore, genotypes AB and A correlate with high levels and genotype B shows moderate levels of ethylene synthesis during fruit ripening.

Although refrigeration systems have been developed, cultivation of cultivars with short storage potential is limited, those having long storage potential being predominant. Progress in Japanese pear breeding is restricted by several factors, such as self-incompatibility, a long juvenile period and the requirement for wide fields. The use of molecular markers should be useful in Japanese pear breeding, increasing selection efficiency by identifying favourable genetic combinations, thereby reducing time and cost as well as field space. To date there is only one molecular marker associated with self-incompatibility in Japanese pear (Sassa et al. 1997; Ishimizu et al. 1996, 1998). This study has identified a second molecular marker which is linked to high ethylene production during fruit ripening and correlates with storage potential in Japanese pear.

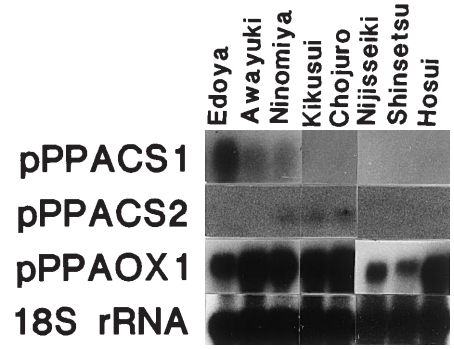

Fig. 7 Northern analysis using the probes pPPACS1, pPPACS2 and pPPAOX1 on total RNAs (10 μg) from ripening fruit from eight cultivars showing various levels of ethylene production during fruit ripening. Edoya, Awayuki and Ninomiya have high levels (more than 10 μl C_2H_4/kg f.w./h); Chojuro and Kikusui have moderate levels (0.5 μl C_2H_4/kg f.w./h–10 μl C_2H_4/kg f.w./h); Nijisseiki, Shinsetsu and Hosui have low levels (less than 0.5 μl C_2H_4/kg f.w./h). An 18S rDNA probe was used to estimate RNA loading

Acknowledgments This study was supported by a Grant-in-Aid (no. 10760019) from the Ministry of Education, Science, Sports and Culture of Japan.

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