Cloning and Characterization of a cDNA Encoding 1-aminocyclopropane-1-carboxylate (ACC) Synthase (PPACS3) from Ripening Fruit of Japanese pear (Pyrus pyrifolia Nakai)

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Summary

We have isolated a cDNA (PPACS3) encoding ACC synthase from the ripening fruit of the Japanese pear (Pyrus pyrifolia Nakai cv. Nijisseiki). This cDNA clone consisted of 1891 bp, containing an open reading frame of 1485 bp that coded for a polypeptide with 495 amino acid residues. The deduced amino acids of PPACS3 revealed 58% and 53% identity to those of PPACS1 and PPACS2, respectively. Northern blot and RT-PCR analyses showed that the PPACS3 mRNA was expressed at a low level in ripening fruits in all cultivars in Japanese pear. Moreover, wound treatment increased the expression of PPACS3 mRNA, indicating that the PPACS3 gene is associated with fruit ripening and wounding.

Key Words: ACC synthase, fruit ripening, Japanese pear, wound-induced expression.

Introduction

The plant hormone, ethylene, regulates many physiological processes, such as seed germination, fruit ripening, abscission and senescence. The ethylene biosynthetic pathway in plants has been well characterized (Yang and Hoffman, 1984; Kende, 1993). In this pathway, two enzymes, ACC synthase and ACC oxidase are involved. Recently, cDNAs and genomic clones, encoding these two enzymes, have been isolated from various plant species (Dong et al., 1991; Holdsworth et al., 1987; Lay-Yee and Knighton, 1995; Liang et al., 1992; McGarvey et al., 1991; Nakajima et al., 1990; Sato and Theologis, 1989; Van der Straeten et al., 1990). ACC synthase and ACC oxidase are encoded by a highly divergent multigene family whose members are differentially regulated by many factors (Dong et al., 1991; Nakajima et al., 1990; Van der Straeten et al., 1990). Generally, the rate-limiting step in ethylene production has been considered to be ACC synthase (Theologis, 1992). In most cases, an increase in ethylene production is due to enhanced transcription of ACC synthase genes (Kende, 1993). However, recent reports have shown that regulatory mechanisms at the posttranscriptional and posttranslational level are equally important in ethylene production (Peck and Kende, 1998; Spanu et al., 1995).

Previously, we showed that the rate of ethylene evolution in Japanese pear fruit varies from 0.1 to 300 \( \mu l \) C2H4 · kg\(^{-1}\) FW · hr\(^{-1}\). during ripening and that the rate is governed by two ACC synthase genes. One is PPACS1, specifically expressed in cultivars with a high ethylene production rate (\( \geq 10 \mu l \cdot kg^{-1}FW \cdot hr^{-1} \)) and the other is PPACS2, specifically expressed in cultivars with a moderate rate of ethylene evolution (0.5—10 \( \mu l \cdot kg^{-1}FW \cdot hr^{-1} \)) during fruit ripening (Itai et al., 1999).

Moreover, we have demonstrated that ethylene production during fruit ripening is regulated by these ACC synthase genes at the transcriptional level and that the expression of PPACS1 is 20—100 times higher than that of PPACS2. However, we have not yet identified ACC synthase genes whose expressions are specific to low ethylene producing cultivars (\( \leq 0.5 \mu l \cdot kg^{-1}FW \cdot hr^{-1} \)). In this paper we report the identification and characterization of a new cDNA encoding ACC synthase which is correlated with low levels of ethylene production in Japanese pear during ripening.

Materials and Methods

Plant material and ethylene measurement

Fruit were harvested from a 'Nijisseiki' pear tree at 82 days after full bloom (DAF), 120 DAF, 153 DAF (commercial harvest date), 23 days after commercial harvest (23 DAH) and immediately placed in 1.5 liter jars and sealed for 2 hr at 20°C for ethylene measurement. After 2 hr, 2.0 ml gas samples were drawn from the headspace of each jar and analyzed using gas chromatography equipped with a flame ionization detector and 60/80 mesh, activated alumina column (Model 163, Hitachi, Tokyo).
RNA extraction and PCR amplification of ACC synthase 
cDNA fragments

Total RNA was isolated using the hot borate method described by Wan and Wilkins (1994) from over-ripe 'Nijisseiki' fruit at 23 DAH (ethylene production rate was 0.11 nl kg⁻¹ FW hr⁻¹). Poly(A) RNA was purified from the total RNA, using Oligotex-dT30 Super (Takara Shuzo, Kyoto, Japan). cDNA was synthesized, using the AMV reverse transcriptase first-strand cDNA synthesis kit (Takara Shuzo). ACC synthase cDNA fragments were amplified from synthesized first-strand cDNA, using two degenerative primers: 5'-ATICA-
RATGGGYTIÖCIGARAAAYCA-3', and 5'-GCRACA-
RCAIACICKRACCAICCIAGGYTC-3' (I=inosine, R=A or G, Y=C or T, K=A or T). The primers were designed, according to the conserved amino acid residues of ACC synthases in the Genbank database. Synthesized cDNA was amplified with 10 mM Tris-HCl pH 8.0, 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM dNTPs, 10 μM primers, and 2.5 U Taq polymerase (Nippon Gene, Toyama, Japan). The PCR condition was 40 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 2 min with a final extension of 5 min at 72°C. Amplified 1.1 kb fragments were separated by gel electrophoresis and cloned into the pGEM-T vector (Promega, Madison, WI, USA). The TA-cloned cDNA insert was partially sequenced using the ALF Express automatic sequencer (Pharmacia, Uppsala, Sweden) with the Thermo-Sequenase fluorescent sequencing kit (Amersham, Buckinghamshire, UK). Since the partial sequence of cloned insert had high homology with ACC synthase genes from other plant species, the cloned insert was used as a library screening probe.

Construction and screening of a cDNA library

A lambda Excell (Pharmacia) library was constructed with poly(A) RNA purified as above. About 2 x 10⁵ plaques that were transferred to Hybond-N nylon membranes (Amersham), were hybridized in 50% formamide, 5 x SSPE, 5 x Denhardt's solution, and 0.1% SDS at 42°C, with a ³²P-labelled cDNA insert. Following hybridization, the filters were washed at 65°C in 0.2 x SSC and 0.1% SDS. cDNA library screening yielded two positive clones. Phagemids were released in vitro from positive plaques and DNA was extracted by the alkaline-SDS method (Sambrook et al., 1989). The longer cDNA insert, designated PPACS3, was sequenced as above.

DNA isolation and Southern blot analysis

Total DNA was extracted from the immature leaves of 'Nijisseiki' pear and 'Fuji'apple by the modified SDS method (Teramoto et al., 1994). Ten μg aliquots of the DNA were digested with EcoRI and Hind III, and the digests were separated by electrophoresis in 0.9% agarose gels, and transferred to Hybond N⁺ nylon membranes (Amersham). Hybridization of the DNA blots was performed using the full length cDNA insert. Post-hybridization washes were 2 x 15 minutes at 65°C in 0.2 x SSC and 0.1% SDS, after which the membranes were exposed to an imaging plate (Fuji Film, Tokyo, Japan). Signals were detected with an image analyzer (FLA2000, Fuji Film).

Northern blot and RT-PCR analysis

To analyze for the cultivar differences in gene expression, we collected the fruits at comparable ripening stages of 11 cultivars ('Ninomiyahakuri', 'Ninomiya', 'Edoya', 'Rokugatsu', 'Awayuki', 'Shinsui', 'Kikusui', 'Nijisseiki', 'Chojuro', 'Shinsetsu' and 'Housui'); these cultivars have different maximum ethylene production rates during maturation (Itai et al., 1999). For the analysis of gene expression, total RNA was extracted from these samples as above. 'Nijisseiki', 'Housui' and 'Shinsetsu' are low ethylene producers (≤ 0.5 μl kg⁻¹ FW hr⁻¹), 'Kikusui', 'Chojuro' and 'Shinsui' are moderate ethylene producers (0.5 - 10 μl kg⁻¹ FW hr⁻¹), and the remaining 5 cultivars, 'Ninomiyahakuri', 'Ninomiya', 'Edoya', 'Rokugatsu', and 'Awayuki' produce ≥ 10 μl kg⁻¹ FW hr⁻¹. To study the effect of wounding on the expression of ACC synthase genes, four pieces weighing approximately 10 g from overripe 'Nijisseki' fruit (23 DAH) were held in a flask at 20°C; the samples were collected at 0, 6, 12 and 24 hr after wounding for RNA extraction. For Northern blot analysis, 10 μg of total RNA was fractionated on a 1.2% agarose gel, containing formaldehyde and blotted onto Hybond N⁺ nylon membranes (Amersham). Membranes were hybridized with ³²P-labelled PPACS1, PPACS2 and PPACS3 full length cDNA fragments according to Itai et al. (1999). Hybridization and washing conditions were as above.

For RT-PCR analysis, the first strand cDNA was synthesized, using the AMV reverse transcriptase first-strand cDNA synthesis kit from 3 μg of DNaseI-treated RNA (Nippon Gene), and the total RNA was used for Northern blot analysis. Oligonucleotide primers, specific for 3 ACC synthase genes, were designed as follows: for PPACS1, ACS1-1 (5'-ACCCTCGAACCTCTCCTCCTCTATGC-3'), and ACS1-2 (5'-AATGATCATAAATCACAATGGAG-3') for PPACS2, ACS2-1 (5'-GTTCACAGAATCAAAGTGTA-3'), and ACS2-2 (5'-AGTAGAACCGCAAAACAAATT-3'), and for PPACS3, ACS3-1 (5'-CTAGCTGCTTCCAGAATCCAGATA-3') and ACS3-2 (5'-TGATACCAATTGGGGATCCTACAAGC-3'). PCR conditions for PPACS1, PPACS2 and PPACS3 were 40 cycles of of 94°C for 1 min, 62°C for 1 min and 72°C for 2 min, with a final extension of 5 min at 72°C. After the PCR reaction, the products were separated on 0.9% agarose gel, and DNA bands were detected by ethidium bromide staining.
Results

Isolation of PPACS3 cDNA

The cDNA (PPACS3) insert that was 1891 bp in length, contained an open reading frame of 1485 bp, which coded for a protein of 495 amino acids. The predicted molecular mass of the protein encoded by PPACS3 is 55.5 kD. The predicted polypeptide has the same conserved domains (Boxes 1–7) found in other ACC synthases (Kende, 1993) having 72% amino acid identity with an ACC synthase cDNA clone (p-GAC2) from Pelargonium hortorum (Wang and Arteca, 1995), 69% identity with a potato clone (STAC5S) (Schlaghaufer et al., 1997), 68% identity with a tobacco clone (NTACS1) (Bailey et al., 1993), and 68% identity with a carnation clone (CARAS1) (Henskens et al., 1994). The deduced amino acids of PPACS3 revealed 58% and 53% homology to those of PPACS1 and PPACS2, respectively (Fig. 1).

Southern blot analysis

The possibility that PPACS3 probe might cross-hybridize to PPACS1 and 2 genes was examined. PPACS3 cDNA was used as a probe on gel blots of plasmids containing PPACS1 and 2 cDNAs, however, cross-hybridization was not observed (data not shown). Labelled PPACS3 cDNA was hybridized to ‘Nijisseiki’ genomic DNA cut with EcoRI and Hind III (Fig. 2). Digestion of ‘Nijisseiki’ genomic DNA with EcoRI

![Fig. 1. Comparison of deduced amino acid sequences of three ACC synthase genes (PPACS1, PPACS2 and PPACS3). The seven underlined amino acid residues are conserved regions in ACC synthases from various plant species (Kende, 1993). The reverse triangle shows the predicted active site of ACC synthase.](image-url)
Fig. 2. Southern blot analysis using PPACS3 cDNA fragments. (A) Japanese pear (cv. Nijisseiki) and (B) apple (cv. Fuji) genomic DNA (10 μg per lane) were digested with Eco RI (Lane 1) and Hind III (Lane 2), resolved on a 0.9% agarose gel and blotted onto Hybond N’ nylon membrane.

(Fig. 2A, Lane 1) and with Hind III (Lane 2) produced four and six hybridizing genomic fragments, respectively. The cDNA does not contain an EcoRI or Hind III site. The genomic fragment (2.7 kb) was PCR-amplified, using specific primers (ACS3-1 and ACS3-2) for PPACS3 which contains no EcoRI site and one Hind III site (data not shown). The number and the size of hybridizing genomic fragments, therefore, indicate the presence of more than two closely related PPACS3 genes in Japanese pear.

PPACS3 homologues exist in apple genome

To determine whether a PPACS3 homologue exists in apple, genomic DNA blots of ‘Fuji’ apple were probed at high stringency with PPACS3 cDNA. Southern blot analysis revealed four bands with EcoRI digestion and five bands with HindIII digestion, indicating homologous genes to PPACS3 exist in the apple genome.

Ethylene evolution and expression analysis of ACC synthase genes

Ethylene production was not observed before commercial harvest (Fig. 3A), but it became detectable at 153 DAF and 23 DAH. To analyze the expression of three ACC synthase genes, we prepared total RNA from the fruits of ‘Nijisseiki’ at pre-ripening and ripe stages fruits from 11 Japanese pear cultivars and examined them by Northern blot analysis. The expression of the three ACC synthase genes in ‘Nijisseiki’ was not detected by Northern blot analysis at any stage of the fruit development and ripening (Fig. 3A). To re-examine the expression patterns of the three ACC synthase genes, we performed a RT-PCR, using cDNA templates from DnaseI-treated total RNA that was used for Northern blot analysis (Fig. 3B). Neither PPACS1 nor PPACS2 transcript was detected during fruit development and ripening, whereas PPACS3 transcript was detected at the expected size (1.9 kb) in ripe and overripe fruit of ‘Nijisseiki’ pear, which shows that PPACS3 mRNA is expressed at a low level in ripening fruit in ‘Nijisseiki’.

The expression of PPACS1 was detected in ripening fruits of ‘Rinomiyahakuri’, ‘Rinomiya’, ‘Rokugatsu’, ‘Edoya’, and ‘Awayuki’, which produce a very high level of ethylene (≥ 10 μl·kg⁻¹·FW·hr⁻¹) (Fig. 4A). The PPACS2 transcript was detected in ‘Rinomiyahakuri’, ‘Rinomiya’, ‘Kikusui’, ‘Chojuuro’, and ‘Shinsui’, but at a lower level than the PPACS1 transcript. ‘Kikusui’, ‘Chojuuro’, and ‘Shinsui’ produce moderate levels of ethylene during fruit ripening, whereas, ‘Rinomiya’ was the only cultivar to express both PPACS1 and PPACS2 (Itai et al., 1999). In this trial, ‘Rinomiya-
A: Northern blot

Fig. 4. Expressions of three ACC synthase genes during fruit ripening of 11 cultivars showing various levels of ethylene. Lane 1: ‘Ninomiya’, 2: ‘Ninomiyahakuri’, 3: ‘Edoya’, 4: ‘Rokugatsu’, 5: ‘Awayuki’, 6: ‘Shinsui’, 7: ‘Kikusui’, 8: ‘Nijisseiki’, 9: ‘Chojuuro’, 10: ‘Shinsetsu’, 11: ‘Hosui’. ‘Ninomiya’, ‘Ninomiyahakuri’, ‘Edoya’, ‘Rokugatsu’ and ‘Awayuki’ are high ethylene producers (more than 10 μ1 C2H4·kg⁻¹FW·hr⁻¹); ‘Shinsui’, ‘Kikusui’ and ‘Chojuuro’ are moderate ethylene producers (0.5–10 μ1 C2H4·kg⁻¹FW·hr⁻¹); ‘Nijisseiki’, ‘Shinsetsu’ and ‘Hosui’ are low ethylene producers (less than 0.5 μ1 C2H4·kg⁻¹FW·hr⁻¹). (A) Northern blot analysis of total RNA (10 μg). An 18S rDNA probe was used to estimate RNA loading. (B) RT-PCR analysis using specific primers.

B: RT-PCR

Fig. 5. Northern blot analysis of three ACC synthase genes 24 hr after wounding in overripening fruit of ‘Nijisseiki’. 0: control; 6: 6 hr after wounding; 12: 12 hr after wounding; 24: 24 hr after wounding. An 18S rDNA probe was used to estimate RNA loading.

mung bean (Kim et al., 1997) and other species, the ACC synthase is encoded by a multigene family. This is also true for Japanese pear in which three ripening-related ACC synthase cDNA clones were isolated. Two of them, PPACS1 and PPACS2, share 98% and 97% sequence identity with apple ACC synthase cDNA clones, MdACS-1 and MdACS-3, respectively (Lay-Yee et al., 1995; Rosenfield et al., 1996). In apple, to date, five ACC synthase genes (Md-ACS1, 2, 3, 5A and 5B) have been identified. None of the five ACC synthases in apple shares a significant sequence identity with PPACS3. There is a high degree of conservation of most coding regions between pear and apple. For example, the coding regions of a pear ACC oxidase cDNA (pPCACO-1), a pear polygalacturonase inhibitor cDNA and a pear polyphenol oxidase cDNA are 98%, 97% and 93% identical to those of apple homologous counterpart clones (Dong et al., 1992; Haruta et al., 1999; Lelievre et al., 1997; Ross et al., 1992; Stoz et al., 1993). Thus, for any one of the pear genomic or cDNA sequences tested, a homologous counterpart is expected in apple. Based on the evidence that PPACS3 cDNA was hybridized to apple genomic DNA (Fig. 2B), we suppose that MdACS-6 exists as an additional isoform in the apple genome.

To understand the cause of cultivar differences in ethylene production, we investigated the expression of the three ACC synthase genes in ripening fruit of several cultivars at their peak of ethylene production. The expression of PPACS3 in ripening fruit was not detected by Northern blot analysis, but by RT-PCR analysis. Its expression was not observed at the pre-ripening stages which indicates that the expression of PPACS3 was ripening-specific and at a very low level in Japanese pear. The PPACS3 transcript was expressed in all cultivars during fruit ripening, regardless of differences in the amount of ethylene they synthesized. The expression of PPACS1 was specific to cultivars which show a very high level of ethylene production, whereas that of PPACS2 was specific to ‘Ninomiyahakuri’ and ‘Nino-

hakuri’ is also included as a cultivar that express both genes. Although the PPACS3 fragment was used as a probe, no signals were detected in ripe fruits of any cultivars. Cultivar differences in the expression of three ACC synthase genes by RT-PCR analysis are shown in Fig. 4B. The PPACS3 transcript that was detected in ripe fruits of all the cultivars using specific primers ACS3-1 and ACS3-2 (Fig. 4B) indicates that PPACS3 mRNA is expressed at an extremely low level in ripening fruits of all cultivars of Japanese pear. Moreover, the expression of the three ACC synthase genes in response to wounding was monitored by Northern blot analysis. Wounding treatments result in the accumulation of PPACS3 but not of PPACS1 and PPACS2 mRNA (Fig. 5). The PPACS3 mRNA started to accumulate 6 hr after wounding and continued to do so at fast rate for 24 hr. These results indicate that PPACS3 gene is induced by wounding.

Discussion

We isolated a cDNA (PPACS3) from Japanese pear fruit which encodes for ACC synthase and is ripening-related. The identity as ACC synthase was confirmed by the seven highly conserved domains found in other plant species (Kende, 1993). As reported for tomato (Oetiker et al., 1997), potato (Destefano-Beltran et al., 1995),
miya' and others that produced moderate amounts of ethylene. These findings confirm our previous findings (Itai et al., 1999), in which we hypothesized that in Japanese pear the maximum ethylene level during fruit ripening was regulated by the expression of two ACC synthase genes (PPACS1 and PPACS2), and that the action of PPACS1 masked that of PPACS2 due to its much higher levels of expression, leading to 20–100 times higher ethylene production. In this paper, we hypothesize that in Japanese pear, the maximum ethylene production level during fruit ripening is regulated by the expression of three ACC synthase genes (PPACS1, PPACS2 and PPACS3) and that the action of PPACS1 masks the action of PPACS2 and PPACS3 because of its higher activity. We suggest that PPACS3 is responsible in cultivars that produce low levels of ethylene during fruit ripening.

Wounding of ripening fruit resulted in the accumulation of PPACS3, but not of PPACS1 and PPACS2 transcripts. Wounded fruit contained ACC synthase mRNA that was detectable by hybridization with PPACS3. Nakatsuka et al. (1998) reported four genes (LE-AC5A, LE-AC5B, LE-AC5C and LE-AC5D) for ACC synthase that is expressed in tomato during fruit ripening. Two of them, LE-AC5B and LE-AC5D showed a ripening-specific expression; the latter was less active than the former. LE-AC5B responded to wound signals (Olson et al., 1991; Tatsuki and Mori, 1999) which suggests that PPACS3 in Japanese pear has the same functions as LE-AC5B gene in tomato.

In summary, we isolated a cDNA (PPACS3) encoding ACC synthase whose transcript accumulated at a low level during fruit ripening in Japanese pear. Wounding of fruit tissues increased the level of PPACS3 transcript. The PPACS3 transcript was expressed in all cultivars during fruit ripening, independent of the amount of ethylene they synthesized. Cultivars that produces high levels of ethylene (≥ 10 μl·kg⁻¹FW·hr⁻¹) during fruit ripening have either two ACC synthase genes (PPACS1 and PPACS3) or three genes (PPACS1, PPACS2 and PPACS3) that are active. Those showing a moderate levels (0.5–10 μl·kg⁻¹FW·hr⁻¹) expressed both PPACS2 and PPACS3 genes, whereas those that evolve low levels (≤ 0.5 μl·kg⁻¹FW·hr⁻¹) expressed a PPACS3 gene only. These data indicate that PPACS3 is correlated with low levels of ethylene production in Japanese pear during fruit ripening.

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ニホンナシ成熟果実由来 ACC合成酵素 cDNA (PPACS3) のクローニングと発現解析

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摘 要

ニホンナシ品種 '二十世紀' 成熟果実より ACC合成酵素をコードする cDNA クローン (PPACS3) を単離した。推定されるタンパク質の分子量は 55.5 kDa で、495 プロトン酸残基からコードされていた。推定アミノ酸は他の植物由来の ACC 合成酵素でよく保存されている 7つの領域を含み、ニホンナシ果実由来の ACC 合成酵素 (PPACS1, 2) の推定アミノ酸配列と 58%, 55% の相同性を示した。ノーザンプロット及び RT-PCR 解析の結果、PPACS3 遺伝子は成熟果実で非常に低いレベルで発現しており、また供試したすべての品種の成熟果実で発現していた。さらに成熟果実に傷害処理を与えることにより、発現は著しく増大した。これらのことから PPACS3 遺伝子は、成熟及び傷害の両方のエチレン生成に関与するものと思われた。