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Rapid identification of 1-aminocyclopropane-1-carboxylate (ACC) synthase genotypes in cultivars of Japanese pear (Pyrus pyrifolia Nakai) using CAPS markers

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Abstract In Japanese pear (*Pyrus pyrifolia* Nakai), fruit storage potential is closely related to the amount of ethylene produced. We have developed a rapid and accurate method for analyzing genes involved in high ethylene production during fruit ripening in Japanese pear. This involves cleaved-amplified polymorphic sequences (CAPS) of two 1-aminocyclopropane-1-carboxylate (ACC) synthase genes (*PPACS1* and *PPACS2*). Two CAPS markers (A for *PPACS1* and B for *PPACS2*), associated with the amount of ethylene produced, were identified. Marker A was associated with high ethylene producers and marker B with moderate ethylene producers. The absence of these two markers enabled the identification of low ethylene producers. Using these markers, we have identified ethylene genotypes for 40 Japanese pear cultivars and two Chinese pear (*P. bretschneideri*) cultivars that are commercially important and used in breeding programs. Furthermore, we performed linkage analysis of these two genes in the F_2 population, which revealed that the recombination frequency between the two markers was 20.8 ± 3.6%. This information is critical to the selection of parents and in breeding strategies to improve storage ability of Japanese pears.

Keywords ACC synthase · Ethylene production · Storage ability · *Pyrus pyrifolia* · CAPS

Introduction

Improving fruit storage potential is of prime interest to the fruit-growing and -distribution industries. Fruits are generally classified as either climacteric or non-climacteric based on differences in ethylene production and respiration rates. Ethylene affects many aspects of ripening, including the rate of ripening of climacteric fruits such as tomato, avocado and banana (Brady and Speirs 1991; Abeles 1992). To impact fruit shelf-life, ethylene production can be manipulated using inhibitors of its synthesis or by genetic engineering. The synthesis of ethylene from S-adenosyl methionine (SAM) is a two-step process. In the first step, 1-aminocyclopropane-1-carboxylate (ACC) synthase converts SAM to ACC; then, ACC oxidase catalyzes the oxidative fragmentation of ACC to form ethylene (Yang and Hoffman 1984). Genes encoding both of these two enzymes have recently been cloned from a number of plant species (Sato and Theologis 1989; Van Der Straeten et al. 1990; Nakajima et al. 1990; Lay-Yee and Knighton 1995). It is clear that both genes are encoded by multigene families and are under developmental regulation (Kende 1993).

In a previous investigation we demonstrated that ethylene production by cultivated Japanese pear fruits varied from 0.1 nl g^{-1} (FW) h^{-1} to 300 nl g^{-1} (FW) h^{-1} during ripening and that there are both climacteric and non-climacteric cultivars of Japanese pear (Kitamura et al. 1981; Itai et al. 1999). Climacteric-type fruits exhibit a rapid increase in ethylene production and have a low storage potential, while non-climacteric fruits show no detectable ethylene production and fruit quality is maintained for over a month in storage. Thus, quality maintenance in Japanese pear fruit is closely associated with ethylene production. To elucidate the reason for the differences in ethylene production among Japanese pear cultivars, we have cloned two ACC synthase genes, *PPACS1, PPACS2*, and an ACC oxidase gene, *PPAOX1* (Itai et al. 1999), and studied their expression during fruit ripening. *PPACS1* is specifically expressed in cultivars exhibiting high ethylene production [>10 nl g^{-1} (FW) h^{-1}], while *PPACS2* is specifically expressed in cultivars with moderate ethylene production [0.5–10 nl g^{-1} (FW) h^{-1}] during fruit ripening (Itai et al. 1999).

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Molecular markers provide a powerful tool in understanding the inheritance of specific traits; they can also increase selection efficiency, especially in tree crops which require long periods of time for breeding. Here, we report on the development of CAPS (cleaved amplified polymorphic sequences) markers for the two ACC synthase genes (*PPACS1* and *PPACS2*) regulating ethylene production during fruit ripening and demonstrate that these markers are useful for the marker-assisted selection (MAS) of Japanese pear cultivars with good storage potential.

Materials and methods

Plant material

A total of 40 Japanese pear (*Pyrus pyrifolia*) cultivars and two Chinese pear (*P. bretschneideri*) cultivars (Yali and Cili) were investigated. All cultivars were grown in the orchard of Tottori University in Japan. An F_2 population of 172 trees from the cross Osanijisseiki (marker type: ab) × Cili (marker type: AB) was used for linkage analysis of CAPS markers. Osanijisseiki shows low ethylene production during fruit ripening, whereas Cili is a high ethylene producer.

Ethylene measurement during fruit ripening

Five pear fruits from each of 42 cultivars were analyzed for ethylene production. Fruits of each cultivar were harvested during the fruit ripening season, and immediately placed in 1.5-l sealed jars for 2 h at 20 °C for ethylene measurement. Headspace gas samples 2 ml were then drawn from each jar and analyzed by means of gas chromatography with a flame ionization detector and 60/80-mesh activated alumina column (Model 163, Hitachi, Tokyo, Japan). Ethylene measurements were obtained every 3–4 days for a 2-week period during fruit ripening, and the highest ethylene production level for each fruit was recorded.

DNA extraction

Total DNA was extracted from immature leaves of each cultivar by a modified SDS method (Teramoto et al. 1994). Samples of fresh leaves (2–3 g) were ground in liquid nitrogen to which 40 ml of washing buffer [0.1 *M* Hepes pH 8.0, 0.1% polyvinylpyrolidone (K-30), 2% 2-mercaptoethanol] was added and homogenized. The homogenate was centrifuged at 10,000 *g* for 10 min and the supernatant discarded. The pellet was resuspended in 10 ml extraction buffer (0.5 *M* NaCl, 100 m*M* Tris-HCI pH 8.0, 50 m*M* EDTA-Na, 2% SDS) and incubated at 70 °C for 10 min. After DNA was precipitated with 2-propanol, it was treated with RNase A, adjusted to 100 ng μl^{-1} and used for polymerase chain reaction (PCR) analysis.

Cloning and sequencing of the 5' untranslated region of PPACS1 and development of a CAPS marker in the PPACS1 allele

5' Flanking regions of *PPACS1* (AB015624) were obtained by inverse PCR amplification (Sassa et al. 1997). Genomic DNA (1 μg) of Nijisseiki was digested with *Hin*dIII, self-ligated by T4 ligase and used as template for inverse PCR amplification using *Taq* polymerase (Nippon Gene, Toyama, Japan) and primers ACS1NV1-1 (5'-GGCACCACAATGACCAGAAACG-3') and ACS1NV1-2 (5'-GAGTCTTGGCCGTGAGAGTTGA-3'). Amplified fragments were purified, cloned into pGEMT vector (Promega, Madison, Wis.), and sequenced.

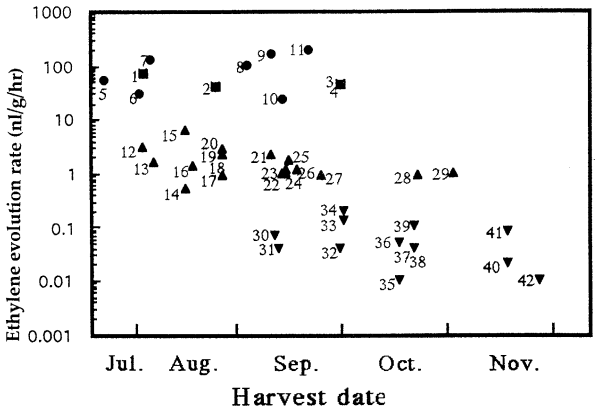

Fig. 1 Ethylene evolution rate of fruits of 40 cultivars of Japanese pear and 2 cultivars of Chinese pear. Each data point represents a cultivar listed below. Data points represent the means of five fruits. The CAPS marker genotype of the cultivars are: ■AB, ●Ab, ▲aB, ▼ab. 1 Ninomiya, 2 Ninomiyahakuri, 3 Yali, 4 Cili, 5 Rokugatsu, 6 Edoya, 7 Okuroku, 8 Awayuki, 9 Rikiya, 10 Gozennashi, 11 Sotoorihime, 12 Kimitsukawase, 13 Shinsui, 14 Yakumo, 15 Ishiiwase, 16 Kosui, 17 Suisei, 18 Akaho, 19 Shinchu, 20 Kinchaku, 21 Chojuro, 22 Taihaku, 23 Wasekozo, 24 Kikusui, 25 Gion, 26 Doitsu, 27 Kozo, 28 Ohiromaru, 29 Inugoroshi, 30 Hosui, 31 Nijisseiki, 32 Niitaka, 33 Waseaka, 34 Sekaiichi, 35 Konpeito, 36 Shinko, 37 Imamuraaki, 38 Amanokawa, 39 Atago, 40 Okusankichi, 41 Yokogoshi, 42 Shinsetsu

Based on sequences of 5' flanking regions of *PPACS1* from Nijisseiki, forward (SYN1PU1: 5'-GATGAAATAAAGTCCACAATCAAG-3') and reverse (SYN1PD1: 5'-CGTTTCTGGATAACATGCG-3') primers (Fig. 2) were designed to amplify cv. Rokugatsu (high-ethylene producer) genomic DNA. This allowed for a comparison of the *PPACS1* sequence from cvs. Nijisseiki and Rokugatsu in order to develop CAPS markers for this gene. The amplified fragments (2.2 kb) from Rokugatsu DNA were purified, cloned into pGEMT vector and sequenced.

Cloning and sequencing of coding region of PPACS2 and development of a CAPS marker in the PPACS2 allele

Based on sequences of *PPACS2* cDNA (AB007631), the SYN2F (5'-GTCACAGAATCAACGATTGA-3') and SYN2R (5'-AGTAGAACG CGAAAACAAAT-3'), primers were designed for amplification of genomic DNA to develop CAPS markers using cvs. Kikusui (moderate-ethylene producer) and Nijisseiki (low-ethylene producer) (Fig. 1). The amplified fragments (2.15 kb) from both cultivars were purified, cloned into pGEMT vector and sequenced.

PCR procedures for CAPS analysis

Primers SYN1PU1 and SYN1PD1 were used for *PPACS1* analysis. The amplification reaction was performed in 50-μl aliquots and contained 100 ng genomic DNA, 1 × *Taq* polymerase buffer, 50 pmol primers, 0.2 m*M* each of the four dNTPs and 1.25 U *Taq* polymerase (Nippon Gene). Amplification was carried out in a TP-2000 thermal cycler (Takara Shuzo, Kyoto, Japan) programmed for 40 cycles of 60 s at 94 °C, 60 s at 60 °C and 90 s at 72 °C, with a final cycle at 72 °C for 10 min. Amplified fragments were digested by *Hin*dIII, separated on 1.2% agarose gels in 1 × TAE buffer and stained with ethidium bromide.

Primers SYN2F and SYN2R were used for *PPACS2* analysis. The PCR reaction was the same as above, except that amplification

was for 40 cycles of 60 s at 94 °C, 60 s at 60 °C and 120 s at 72 °C, with a final cycle at 72 °C for 10 min. Amplified fragments were digested by *Hin*dIII and separated on 1.2% agarose gels in 1 × TAE buffer. After staining with ethidium bromide, gels were observed and photographed under UV light.

Statistical analysis

The fit between observed and expected segregation ratio (3:1) for both markers (A, B) was tested by Chi-square analysis. Recombination frequency was calculated by the maximum likelihood method (Allard 1956).

Results

Ethylene production rate during fruit ripening in 42 cultivars

To investigate differences between cultivars in their ethylene production, we selected 42 cultivars which represented a wide range of harvest seasons. All fruits were harvested at their optimal commercial date, and thereafter ethylene production was recorded every 3–4 days for 2 weeks. Ethylene production rates ranged from undetectable or low [less than 0.2 nl g^{-1} (FW) h^{-1} for Nijisseiki, Hosui, Niitaka, Shinsetsu and nine other cultivars; nos. 30–42 in Fig. 1] to moderate [0.5–10 nl g^{-1} (FW) h^{-1} for Shinsui, Chojuro, Kikusui and 16 other cultivars, nos. 12–29] to high [from 10 nl g^{-1} (FW) h^{-1} to over 100 nl g^{-1} (FW) h^{-1} for Rokugatsu, Ninomiya, Cili, Yali and eight other cultivars, nos. 1–11]. These results confirm a previous report on the existence of a correlation between ethylene production and ripening physiology (Itai et al. 1999).

Generally, high ethylene producers were early- or mid-maturing cultivars – July to September –, while low ethylene producers were mid- or late-maturing cultivars – September to November. These results suggest that harvest season is closely related to maximum ethylene production during fruit ripening.

Development and testing of a CAPS marker for alleles of the *PPACS1* gene

PPACS1 is specifically expressed in cultivars exhibiting high ethylene production during fruit ripening (Itai et al. 1999). Therefore, we tested the possibility that differences between cultivars in the sequence of this gene will be sufficient to enable MAS of cultivars with lower ethylene production. We initially attempted to find a useful CAPS marker for this gene in the coding genomic sequence, however, we failed because the degree of polymorphism between the cultivars was very low (data not shown). Hence, the 5' flanking regions of *PPACS1* of a high and a low ethylene producer were compared as potential sources of a CAPS marker for this gene. Fragments of the 5' flanking region of *PPACS1* were obtained from Rokugatsu (a high ethylene producer) and Nijisseiki (a low ethylene producer) (nos. 5 and No. 31, respectively in Fig. 1) genomic DNA by PCR. The amplified fragments (approx. 2.2 kb) (PPACS1N from Nijisseiki and PPACS1R from Rokugatsu) were then cloned and sequenced (Fig. 2). The identity was 92%, with sequence differences due to small deletions or nucleotide replacements. Comparative searches of DDBJ DNA databases (http://blast.genome.ad.jp) using BLAST showed that both sequences had high identities (88%) with the 5' flanking region of an apple ACC synthase gene (*MdACS1*) (Harada et al. 1997). The sequence from cv. Nijisseiki (PPACS1N) had a mutation at position –1,569 from the ATG initiation codon that disrupted a *Hin*dIII restriction site (Fig. 2). This mutation was absent from the Rokugatsu (PPACS1R) sequence.

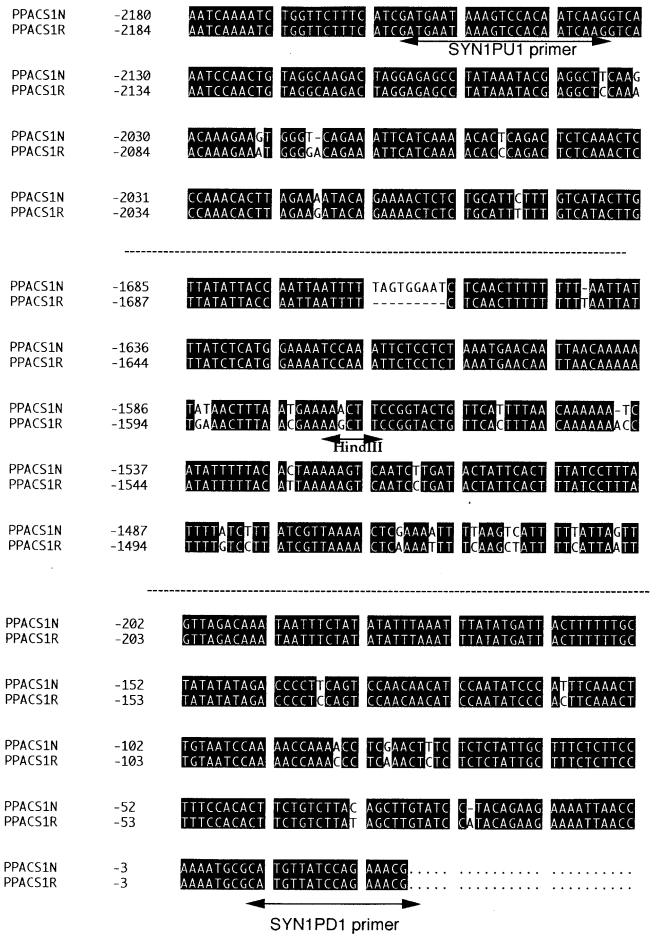

Fig. 2 Nucleotide sequence alignment of the 5' flanking regions of *PPACS1* genes from a high-ethylene producer (cv. Rokugatsu: PPACS1R (Accession No. AB080677)) and a low-ethylene producer (cv. Nijisseiki: PPACS1N (Accession No. AB080678)). Homologous nucleotides are indicated by black shading. The positions of *Hin*dIII site in PPACS1N and primers are underlined

The CAPS maker provided by the restriction enzyme *Hin*dIII was used to screen Japanese and Chinese pear cultivars. The 2.2-kb fragments amplified from 42 pear cultivars (Fig. 1), which included 11 high ethylene producers, by PCR with the same SYN1PU1 and

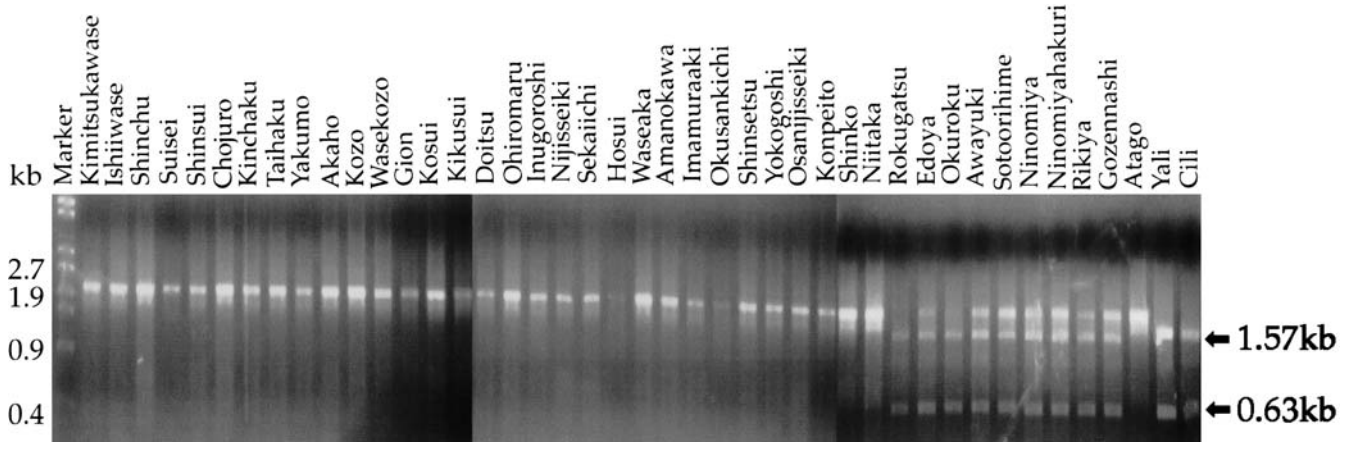

Fig. 3 CAPS analysis for the *PPACS1* gene in the indicated cultivars of Japanese and Chinese pear. PCR products (2.2 kbp) amplified with SYN1PU1 and SYN1PD1 primers were digested with *Hin*dIII. The bands indicated by the arrows are specific to cultivars producing high levels of ethylene during fruit ripening

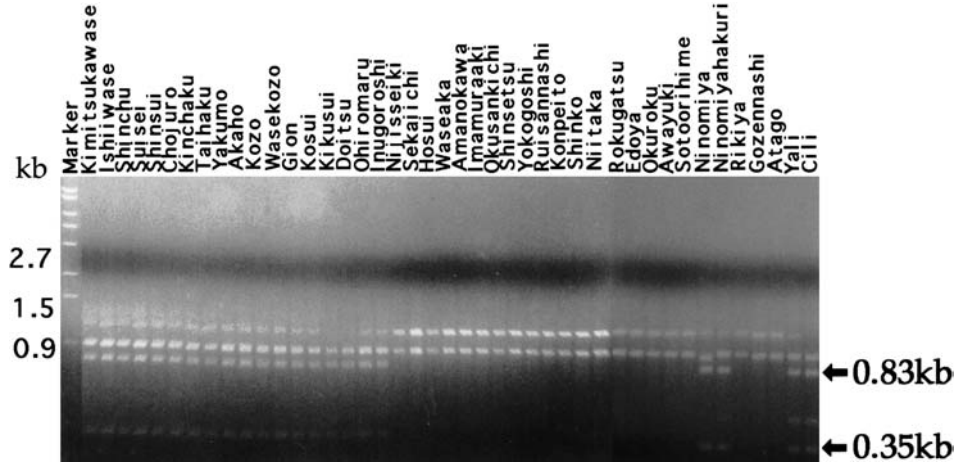

Fig. 4 CAPS analysis for *PPACS2* gene in the indicated cultivars of Japanese and Chinese pear. PCR products (2.15 kbp) amplified with SYN2F and SYN2R primers were digested with *Hin*dIII. The bands indicated by the arrows are specific to cultivars producing moderate levels of ethylene during fruit ripening

SYN1PD1 primers as above were digested with *Hin*dIII. As shown in Fig. 3, the resulting 1.57-kb and 0.63-kb bands were specific to all of the 11 cultivars (Rokugatsu, Okuroku, Ninomiya, Ninomiyahakuri, Awayuki, Sotoorihime, Rikiya, Edoya, Yali and Cili) that produced high levels of ethylene [over 10 nl g^{-1} (FW) h^{-1}] during fruit ripening. Low and moderate ethylene producers showed only a 2.2-kb band. Cultivars having the two fragments resulting from cleavage of the 2.2-kb band were designated as "A", while cultivars having the uncleaved 2.2-kb band were designated as "a". This CAPS marker, A, was 100% accurate in predicting the phenotype of high ethylene producers.

Development and testing of a CAPS marker for alleles of the *PPACS2* gene

PPACS2 is specifically expressed in cultivars showing moderate ethylene production [0.5–10 nl g^{-1} (FW) h^{-1}] during fruit ripening (Itai et al. 1999). We tried to identify a useful CAPS marker for this trait. PCR amplification using primers (SYN2F and SYN2R) produced a fragment of approximately 2.15 kb that was amplified from both cultivars (Kikusui and Nijisseiki). Amplified fragments were cloned, sequenced and compared. The identity of fragments amplified from genomic DNA of a moderate ethylene producer (Kikusui) and that of a low ethylene producer (Nijisseiki) was 98% (data not shown). Potential CAPS markers were identified by computer analysis based on sequence differences between fragments from these two cultivars. Two restriction sites for *Hin*dIII, located at 351 bp and 1182 bp, resulted in 351-, 831-, and 1,063-bp fragments in the moderate ethylene producer Kikusui. Only one cutting site was located at 1,183 bp in the low ethylene producer Nijisseiki, which resulted in 1,183- and 1,062-bp fragments. To test these markers in Japanese and Chinese pear cultivars, the 2.15-kb fragments amplified by PCR from 42 pear cultivars, which included 18 moderate ethylene producers, were cut with *Hin*dIII into fragments of 1.18 kb, 1.06 kb, 0.83 kb and 0.35 kb. As shown in Fig. 4, the 0.83-kb and 0.35-kb

Table 1 Linkage analysis between *PPACS1* (*A*) and *PPACS2* (*B*) in the F_2 population

Combination	F_1 Genotype	F_2[a]					Chi-square[a]	Recombination frequency (%)
		AB	Ab	aB	ab	Total		
Osanijisseiki (*aabb*) × Cili (*AaBB*)	OT-16 (*AaBb*)	104 (96.75)	21 (32.75)	13 (32.75)	34 (10.75)	172	67.2 (9:3:3:1)	20.8 ± 3.6

[a]Values enclosed in paranthesis are the expected number based on the segregation ratios (9:3:3:1)

bands were specific to 22 cultivars (Kimitsukawase, Shinsui, Yakumo, Ishiiwase, Kosui, Suisei, Akaho, Shinchu, Kinchaku, Chojuro, Taihaku, Wasekozo, Kikusui, Gion, Doitsu, Kozo, Ohiromaru, Inugoroshi, Ninomiya, Ninomiyahakuri, Yali and Cili). Of these, 18 were moderate ethylene producers and four (Ninomiya, Ninomiyahakuri, Yali and Cili) were high ethylene producers. Genomic DNA amplified from cultivars producing low amounts of ethylene during fruit ripening was characterized by the absence of these *Hin*dIII restriction fragments. The restriction fragments in all moderate ethylene producers and some high ethylene producers appear to be generated from a 1.18-kb restriction fragment. Cultivars with fragments resulting from cleavage (0.83 kb and 0.35 kb) were designated as "B", while cultivars lacking these fragments were designated as "b".

Based on the presence or absence of polymorphic fragments, cultivars were classified into four groups AB, Ab, aB, and ab (Fig. 1). For the cultivars tested, there were correlations between these groups and rates of ethylene production. Groups AB and Ab showed high ethylene production during fruit ripening, group aB showed moderate ethylene production, while group ab showed low ethylene production.

Segregation ratios and linkage analysis

A total of 172 individuals were analyzed from the F_2 population. Marker A fit the expected 3:1 segregation (124:48 for A:a, $P > 0.05$). Marker B was close to a 2:1 segregation (118:54 for B:b) but still not significantly different from 3:1 ($P > 0.05$). Consequently, both CAPS markers (A and B) appear to show Mendelian inheritance and to fit a single-gene model. However, when the two markers were analyzed together, they did not fit the expected 9 AB:3 Ab:3 aB:1 ab segregation ratio (Table 1). These data suggest that the A and B markers are linked to each other, and statistical analysis showed that the recombination frequency between the two markers is 20.8 ± 3.6%.

Discussion

In general, climacteric fruit ripening is associated with increased ethylene biosynthesis. Ethylene has a strong influence on the ripening process, and inhibitors of ethylene synthesis and action delay ripening. The manipulation of ethylene production in fruit via transgenic technology has led to an understanding of its role in fruit ripening (Hamilton et al. 1990; Oeller et al. 1991). The European pear is a climacteric fruit, and its ripening is characterized by ethylene production and a high rate of respiration (Knee 1993). In contrast, both climacteric and non-climacteric cultivars exist in Japanese pear (Kitamura et al. 1981; Itai et al. 1999).

Forty-two cultivars, which were selected based on their harvest season, were used for measuring ethylene production and for molecular analysis. The rate of ethylene production differed considerably among these cultivars, confirming the result of previous investigations (Kitamura et al. 1981; Itai et al. 1999). Some cultivars showed undetectable ethylene production during fruit ripening and could be stored for up to 6 months at a low temperature (lower than 10 °C) with no decay (Kajiura and Sato 1990). Other cultivars produced high amounts of ethylene and were best consumed soon after harvest because of rapid deterioration. Harvest season was closely related to the maximum ethylene production during fruit ripening. High and moderate ethylene producers were early- or mid-maturing cultivars, and low ethylene producers were mid- or late-maturing cultivars. Similar observations were also made on apple cultivars (Abeles 1992). In general, late-maturing cultivars of Japanese pear have a good storage potential and are used for longer storage (Kajiura 1981).

Although there is a clear relationship between ripening and ethylene production, the molecular basis for differences in ethylene production among cultivars is less well understood. We recently isolated two ACC synthase cDNAs (*PPACS1* and *PPACS2*) and demonstrated that the ACC synthase reaction determines maximum ethylene production in Japanese pear cultivars (Itai et al. 1999). Furthermore, *PPACS1* is specifically expressed in high ethylene producers, while *PPACS2* is specifically expressed in moderate ethylene producers.

Here we report the development of a PCR-based method for identifying two markers for high and moderate ethylene production during fruit ripening. This PCR-based method is very rapid, uses a small amount of young leaf tissue and is based on CAPS analysis of the *PPACS1* and *PPACS2* genes. Initially, we cloned the 5' untranslated regions of the *PPACS1* gene from a high ethylene producer (cv Rokugatsu) and from a low ethylene producer (cv. Nijisseiki). These sequences (PPACS1R and PPACS1N) were then compared. PPACS1N has a mutation that disrupts a *Hin*dIII site and is conserved in

all of the cultivars surveyed, except for the high ethylene producers. This makes it possible to distinguish between high, and moderate or low ethylene producers. Furthermore, when we amplified the putative *PPACS2* coding region from the moderate ethylene producer (Kikusui) and from the low ethylene producer (Nijisseiki), based on the sequence of Kikusui cDNA, we found that the sequence from Kikusui has two *Hin*dIII sites, whereas that from Nijisseiki has only a single *Hin*dIII site. Analysis of the whole set of cultivars showed that all low ethylene producers have only one *Hin*dIII site and that all moderate ethylene producers and four high ethylene producers (Ninomiya, Ninomiyahakuri, Yali and Cili) have two *Hin*dIII sites.

It has been reported that the action of *PPACS1* masks the action of *PPACS2* due to its much higher level of expression, leading to 20–100 fold higher ethylene production (Itai et al. 1999). Therefore, after screening of the CAPS marker (A) for *PPACS1*, which is an indicator of high ethylene producers, CAPS marker (B) for *PPACS2* can be used for identifying seedlings or cultivars that are moderate ethylene producers. A combined screening of the CAPS markers (A and B) enables the identification of low ethylene producers.

This selection method has also been used successfully on two Chinese cultivars (*P. bretschneideri*, Yali and Cili). In a preliminary trial, we also succeeded in identifying the marker types in other Chinese cultivars producing different amounts of ethylene (data not shown). Japanese pear is thought to have originated in southeast China (Kajiura 1994) and has a close genetic relationship with Chinese pear (Iketani et al. 1998; Teng et al. 2001). It is likely that mutations in *PPACS1* and *PPACS2* genes are conserved in Chinese pear.

Correlations between restriction fragment length polymorphisms (RFLP) and ethylene production have been reported recently in apple and melon fruits (Sunako et al. 1999; Zheng and Wolff 2000). In Fuji apple, a short interspersed DNA element (SINE) is inserted into the promoter region of *Md-ACS1* (Sunako et al. 1999). This reduces the amount of its transcript, resulting in the production of only a small amount of ethylene. In contrast, in melon, RFLPs using probes for ACC oxidase (MEL1) and ACC synthase (MEACS1) enabled the detection of eight marker types showing variation in ethylene production rate (Zheng and Wolff 2000). These reports indicate that ethylene production during fruit ripening is regulated by variability in the expression of different enzymes in fruits of various species.

Linkage analysis revealed that there is 20.8 ± 3.6% recombination between the two CAPS markers. There are only a few reports on genome mapping in pear species (Iketani et al. 2001; Yamamoto et al. 2002). Iketani et al. (2001) constructed a molecular linkage map of 768 cM using random amplified polymorphic DNA markers, which covered approximately half of the total pear genome. In the future, work is needed to map our identified markers on a molecular linkage map of pear species.

MAS is considered to be a useful tool for increasing selection efficiency by identifying favorable genetic combinations in fruit trees as well as other crops. The major advantage of MAS is the ability to evaluate many traits at the seedling stage in fruit trees that have a long juvenile phase. The markers identified here, which are linked with high and moderate ethylene production, should be useful for the selection of Japanese pear cultivars with enhanced post-harvest keeping ability.

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