

Identification of Climacteric and Nonclimacteric Phenotypes of Asian Pear Cultivars by CAPS Analysis of 1-Aminocyclopropane-1-Carboxylate Synthase Genes

Akihiro Itai¹ and Naoko Fujita

Laboratory of Horticultural Biotechnology, Faculty of Agriculture, Tottori University, Tottori 680-8553

Additional index words. Asian pears, storage potential, CAPS, ethylene synthesis, marker assisted selection

Abstract. The maximum level of ethylene production is closely related to fruit ripening and storage potential in Asian pears. In a previous study, we identified two markers (A and B) linked to high and moderate ethylene production during fruit ripening, respectively, by restriction fragment-length polymorphism analysis of two 1-aminocyclopropane-1-carboxylate (ACC) synthase genes (*PPACS1* and *PPACS2*). In this study, a total of 152 cultivars were categorized into four marker types (AB, Ab, aB, and ab); types AB and Ab show high levels, aB a moderate level, and ab a low level of ethylene production during fruit ripening. A large number of ab and aB cultivars but few AB and Ab cultivars were observed. It suggests that there has been a decrease in high ethylene producers by artificial selection because of short shelf life. The ab cultivars are a good genetic resource for production of new cultivars with a long shelf life. Such information on marker types is useful for breeding strategies aimed at improving storage ability in Asian pears.

In *Pyrus*, there are three major species, *P. communis* L. (pear or European pear), *P. bretschneideri* Rehd. or *P. usuriensis* Maxim. (Chinese pear), and *P. pyrifolia* Nakai (Japanese pear: Nashi), which are commercially cultivated in temperate zones. The attributes that constitute good quality in one species may differ from that in another, as is the case with European and Asian pears. The attributes of European pears, for example, include its soft buttery texture, whereas those of Asian pears include its juicy and crisp flesh. The most distinctive characteristic of Asian pears is the fact that they mature on the tree in contrast to European pears, which usually require exposure to chilling temperatures for initiation of ripening.

Pear fruits can be classified as climacteric or nonclimacteric according to their ripening characteristics. European pears are climacteric (Jackson, 2003), whereas Asian pears are thought to include climacteric and non-climacteric cultivars because climacteric type fruits showed a rise in respiration and

ethylene production, and nonclimacteric-type fruit did not show a rise in respiration and ethylene production during fruit ripening (Downs et al., 1991; Itai et al., 1999, 2003a; Kitamura et al., 1981). In Asian pears, climacteric-type fruits have a low storage potential, whereas nonclimacteric fruits maintain fruit quality for over 1 month in storage (Itai et al., 1999, 2003a; Kitamura et al., 1981). Therefore, fruit storage potential is closely related to the maximum level of ethylene production in Asian pears.

We previously cloned three 1-aminocyclopropane-1-carboxylate (ACC) synthase genes (*PPACS1*, 2, and 3) and studied their expression during fruit ripening (Itai et al., 1999, 2003b). *PPACS1* was specifically expressed in cultivars showing high ethylene production ($>10 \text{ nL} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$, usually over $50 \text{ nL} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$), and *PPACS2* in cultivars showing moderate ethylene production ($0.5 \text{ nL} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ to $10 \text{ nL} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$). Moreover, we previously identified restriction fragment-length polymorphism (RFLP) markers linked to the ethylene production in ripening fruit using RFLP analysis with two ACC synthase genes (*PPACS1* and *PPACS2*) (Itai et al., 1999). These RFLPs were designated A (2.8 kb of *PPACS1*), which is linked to high levels of ethylene, and B (0.8 kb of *PPACS2*), which is linked to moderate levels. Based on this analysis, we classified 35 Asian pear cultivars into four RFLP types (AB, Ab, aB, and ab), of which types AB and Ab show high levels, aB a moderate level, and ab a low level of

ethylene production during fruit ripening (Itai et al., 1999). We further transformed these two RFLP markers into more convenient easy-to-use polymerase chain reaction (PCR)-based CAPS markers (A: 1.57 kb and 0.63 kb of the *PPACS1* fragment; B, 0.83 kb and 0.35 kb of *PPACS2* fragment) (Itai et al., 2003b).

These markers are useful for predicting the ethylene levels of Asian pear cultivars and they enable identification of low ethylene producers with an enhanced postharvest storage ability. However, available information on ethylene production is limited to certain cultivars. Therefore, in the present study, we conducted CAPS analysis of 152 cultivars using two ACC synthase genes to evaluate the ripening characteristics and to improve breeding and posthandling information on Asian pears.

Materials and Methods

Plant materials. A total of 152 Asian pear cultivars including 35 previously identified cultivars were investigated. All cultivars were grown at the orchard of Tottori University, Japan. Young leaf samples were obtained from one tree per cultivar and were stored at -80°C until DNA extraction.

DNA extraction. The DNA extraction protocol was a modified version of the sodium dodecyl sulfate (SDS) method published in Dellaporta et al. (1983) with some additional washing steps (Itai et al., 2003a). About 100 mg of leaf material was ground in liquid nitrogen using a mortar and pestle and the powder was then transferred to a 1.5-mL tube containing 1 mL of washing buffer [0.1 M Hepes, pH 8.0, 0.1% polyvinylpyrrolidone (K-30), and 2% 2-mercaptoethanol]. The mixed solution was centrifuged at $15,000 \text{ g}_a$ for 10 min and the supernatant was then discarded and the pellet was resuspended in 1 mL of the same washing buffer. This washing step was repeated three times to remove polysaccharides and polyphenols. After washing, the pellet was resuspended in 400 μL of extraction buffer (0.5 M NaCl, 100 mM Tris-HCl, pH 8.0, 50 mM EDTA-Na, and 2% SDS) and was incubated at 70°C for 10 min. After incubation, 120 μL of 5 M potassium acetate was added to the mixture and centrifuged at $15,000 \text{ g}_a$ for 15 min. The supernatant was transferred to a new tube to which an equal volume of 2-propanol was added and the DNA was then precipitated and recentrifuged. The precipitated DNA was dissolved in 20 μL of Tris-EDTA with 1 μL of RNase A (10 mg mL^{-1}), incubated at 37°C for 30 min, and then stored at 4°C .

PCR reaction and CAPS analysis. Forward (SYN1PU1: 5'-GATGAAATAAAGTCCACAATCAAG-3') and reverse (SYN1PD1: 5'-GCGTTTCTGC.ATAACATGCG-3') primers were used for classification of marker A of *PPACS1*, which is linked to high ethylene production in Asian pears (Itai et al., 2003b). The amplification reaction was performed in 50 μL of reaction mixture containing 100 ng of genomic DNA, $1 \times \text{Taq}$

Received for publication 14 June 2007. Accepted for publication 7 Sept. 2007.

This study was partially supported by Grants-in-Aid (Nos. 17380023 and 18780021) from the Ministry of Education, Science, Sports and Culture, Japan, and by a grant from the Venture Business Laboratory (VBL) of Tottori University, Japan.

¹To whom reprint requests should be addressed; e-mail itai@muses.tottori-u.ac.jp

polymerase buffer, 50 pmol of each primer, 0.2 mM each of the four dNTPs, and 1.25 U *Taq* polymerase (Nippon Gene, Toyama, Japan). Amplification was carried out in a TP-2000 thermal cycler (Takara Shuzo, Kyoto, Japan) programmed for 35 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 with *Hind*III for at least 3 h at 37 °C and were then analyzed on a 1.2% agarose gel in 1× TAE buffer and stained with ethidium bromide. Identification of marker A was analyzed by the presence (Fig. 1) or absence of

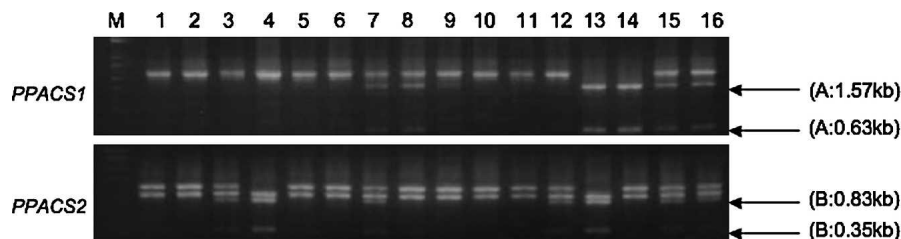


Fig. 1. CAPS analysis of the *PPACS1* and *PPACS2* genes in cultivars of Asian pear. Bands indicated by arrows, A and B, are specific to cultivars that produce high and moderate levels of ethylene during fruit ripening, respectively. M = molecular marker; 1 = Koyuki; 2 = Ruisannashi; 3 = Jianbali; 4 = Asahi; 5 = Aoyagi; 6 = Kousainashi; 7 = Pingli; 8 = Baili; 9 = Hattatsu; 10 = Echigonishiki; 11 = Kuninaga; 12 = Kiyosumi; 13 = Qiubaili; 14 = Huangli; 15 = Shuixiangli; 16 = Kogetsu.

Table 1. Marker types representative of ethylene synthesis during fruit ripening in Asian pear cultivars.

Cultivar	Marker type	Cultivar	Marker type	Cultivar	Marker type
Aikansui(P) ^z	aB(M) ^y	Jinjianli(B)	AB(H)	Shinju(P)	aB(M)
Akaho(P)	aB(M)	Kamenashi(P)	Ab(H)	Shinko(P)	ab(L)
Akemizu(P)	aB(M)	Kansaiasahiryu(P)	Ab(H)	Shinseiki(P)	aB(M)
Akiakari(P)	ab(L)	Kansaiichi(P)	Ab(H)	Shinsetsu(P)	ab(L)
Akibae(P)	ab(L)	Kikkou(P)	aB(M)	Shikishima(P)	aB(M)
Akizuki(P)	ab(L)	Kikusui(P)	aB(M)	Shimokaburi(P)	aB(M)
Amanogawa(P)	ab(L)	Kimitsukawase(P)	aB(M)	Shinsui(P)	aB(M)
Aokeiniitaka(P)	ab(L)	Kinchaku(P)	aB(M)	Shirayuki(P)	ab(L)
Aokeishinko(P)	ab(L)	Kiyosumi(P)	aB(M)	Shomyouji(P)	ab(L)
Aoyagi(P)	ab(L)	Kohnowatari(P)	ab(L)	Shugyoku(P)	aB(M)
Asahi(P)	aB(M)	Kogiku(P)	aB(M)	Shuixiangli(B)	AB(H)
Asahiryu(P)	aB(M)	Kogetsu(PB)	AB(H)	Shurei(P)	ab(L)
Awayuki(P)	Ab(H)	Konpeito(P)	ab(L)	Sotoorihime(P)	Ab(H)
Babauchaginashi(P)	aB(M)	Kousainashi(P)	ab(L)	Suisei(P)	aB(M)
Baili(B)	Ab(H)	Koyuki(P)	ab(L)	Taihaku(P)	aB(M)
Balixiang(B)	aB(M)	Kozo(P)	aB(M)	Taihei(P)	ab(L)
Beijingbaili(B)	Ab(H)	Kumoi(P)	aB(M)	Tanponashi(P)	aB(M)
Cheongdangroli(B)	ab(L)	Kuninaga(P)	ab(L)	Tenryu(P)	ab(L)
Cheongsilli(B)	AB(H)	Kunitomi(P)	ab(L)	Tosajo(P)	aB(M)
Choju(P)	aB(M)	Kuroki(P)	aB(M)	Tosanashi(P)	aB(M)
Chojuro(P)	aB(M)	Makezaoli(B)	Ab(H)	Tosanishiki(P)	ab(L)
Chosen(P)	aB(M)	Mili(B)	ab(L)	Tsugaruao(P)	ab(L)
Cili(B)	AB(H)	Mishirazu(P)	ab(L)	Tsukutounashi(P)	ab(L)
Dangshansuili(B)	Ab(H)	Miyadani(P)	aB(M)	Umajiro(P)	ab(L)
Datouhuang(P)	aB(M)	Nakaide(P)	aB(M)	Wakahikari(P)	aB(M)
Doitsu(P)	aB(M)	Nanseichabo(P)	ab(L)	Waseaka(P)	ab(L)
Echigonishiki(P)	ab(L)	Nekogoroshi(P)	AB(H)	Wasekoko(P)	aB(M)
Edoya(P)	Ab(H)	Niitaka(P)	ab(L)	Wasetachio(P)	ab(L)
Enli(B)	AB(H)	Nijisseiki(P)	ab(L)	Whangkeumbae(P)	ab(L)
Fukushima(P)	ab(L)	Nikkori(P)	ab(L)	Wowoli(B)	AB(H)
Gion(P)	aB(M)	Ninomiyahakuri(PB)	AB(H)	Xiangli(B)	Ab(H)
Gold Nijisseiki(P)	ab(L)	Ninomiyahakuri(PB)	AB(H)	Xiangyali(B)	Ab(H)
Hakataao(P)	aB(M)	Oharabeni(PC)	aB(M)	Yahatanishiki(P)	ab(L)
Hakkou(P)	ab(L)	Ohiromaru(P)	aB(M)	Yagoemon(P)	ab(L)
Hamheung(Kou)(B)	Ab(H)	Ohshuu(P)	ab(L)	Yaguali(B)	aB(M)
Hamheung(Otsu)(B)	ab(L)	Ohtani(P)	Ab(H)	Yakumo(P)	aB(M)
Hatsushimo(P)	ab(L)	Okusankichi(P)	ab(L)	Yali(B)	AB(H)
Hattatsu(PB)	Ab(H)	Onba(P)	Ab(H)	Yanaga(P)	ab(L)
Hayatama(P)	aB(M)	Ookoga(P)	ab(L)	Yasato(P)	aB(M)
Hebeili(B)	Ab(H)	Osanijisseiki(P)	ab(L)	Yinbaili(B)	AB(H)
Heishi(P)	ab(L)	Pingli(B)	AB(H)	Yuanbaili(B)	ab(L)
Heiwa(P)	ab(L)	Pingguoli(B)	Ab(H)	Yokogoshi(P)	ab(L)
Hoeryongsaibai(B)	AB(H)	Pingzili(B)	Ab(H)	Zhuzui(B)	Ab(H)
Hokkan(P)	ab(L)	Qiubaili(B)	AB(H)	Zuishu(P)	ab(L)
Hongli(B)	ab(L)	Rokugatsu(P)	Ab(H)		
Hongxiaoli(B)	ab(L)	Ruisannashi(P)	ab(L)		
Hosui(P)	ab(L)	Sagami(P)	aB(M)		
Huangli(B)	Ab(H)	Saizo(P)	ab(L)		
Imamuraaki(P)	ab(L)	Sannashi(P)	Ab(H)		
Imamuranatsu(P)	aB(M)	Seiryu(P)	ab(L)		
Inagi(P)	aB(M)	Sekaiichi(P)	ab(L)		
Inugoroshi(P)	aB(M)	Senryu(P)	aB(M)		
Ishiiwase(P)	aB(M)	Shihyakume(P)	aB(M)		
Jianbali(B)	aB(M)	Shinchu(P)	aB(M)		

^zIn the "Cultivar" column, letters in parentheses represent the species. B = *P. bretschneideri* or *P. ussuriensis*; C = *P. communis*; P = *P. pyrifolia*. Hybrids are indicated by appropriate combinations.

^yIn the "Marker Type" column, letters in parentheses represent the predicted maximum level of ethylene synthesis during fruit ripening. H = high (>10 nL·g⁻¹ fresh weight h⁻¹); M = moderate (0.5–10 nL·g⁻¹ fresh weight h⁻¹); L = low (<0.5 nL·g⁻¹ fresh weight h⁻¹).

the CAPS marker (a 1.57-kb and 0.63-kb band, respectively).

The following primers were used for classification of marker B of *PPACS2*, which is linked to moderate ethylene production: SYN2F (5'-GTCACAGAATCAA CGATTGA-3') and SYN2R (5'-AGTAGA ACGCGAAAAACAAAT-3'). The PCR reaction was performed as described above, and amplified fragments were also digested with *Hind*III and separated on a 1.2% agarose gel in 1× TAE buffer. After staining with ethidium bromide, gels were observed and photographed under ultraviolet light. Identification of marker B was examined by the presence (Fig. 1) or absence of the CAPS marker (a 0.83-kb and 0.35-kb band, respectively) (Itai et al., 2003b). After analysis of both markers, cultivars were classified into one of the four RFLP types (AB, Ab, aB, or ab).

Results and Discussion

In the present study, a total of 152 cultivars was used to predict maximum ethylene production in Asian pears by CAPS analysis of ACC synthase genes. Based on the presence or absence of polymorphic fragments, cultivars were classified into one of four groups: AB, Ab, aB or ab. The correlations between groups and rates of ethylene production were identified previously (Itai et al., 1999, 2003b); groups AB and Ab showed high ethylene production, group aB showed moderate production, and ab showed low ethylene production. Marker types of a total of 152 cultivars, including 35 cultivars previously identified by RFLP analysis (Itai et al., 1999), are listed in Table 1. Overall, 15, 23, 53, and 61 cultivars were shown to belong to groups AB, Ab, aB, and ab, respectively. A large number of group ab and aB cultivars were listed, but only a small number of AB and Ab cultivars. These data suggest that there has been a decrease in high ethylene producers (marker type of AB or Ab) over the years because of their low storage ability. In line with this, recently bred cultivars such as 'Akemizu', 'Kosui', 'Shinsui', 'Shugyoku', 'Yasato', 'Wakahikari', 'Akia-kari', 'Akibae', 'Akizuki', 'Ohshuu', 'Shurei', and 'Zuishu' have been classified as aB or ab; no newly bred cultivars belong to groups AB or Ab. On the other hand, the high ethylene producers, which belong to groups AB or Ab, include a large number of older cultivars such as 'Awayuki', 'Okuroku', 'Rokugatsu', and 'Sotoorihime' (Kajiura

and Sato, 1990). Despite the development of refrigeration systems, our data indicate that cultivation of cultivars with a short shelf life is still problematic, with cultivars having a long storage potential being preferred.

The ab cultivars lack expression of *PPACS1* and *PPACS2* during fruit ripening and the trait for low ethylene production is recessive (Itai et al., 2002, 2003b). Hybridization between ab cultivars results in seedlings with low ethylene during fruit ripening. If the breeding objective is to increase storage ability, ab cultivars are a good genetic resource for production of new cultivars with a long shelf life. Most Asian pear cultivars are susceptible to scab (*Venturia nasicola*), which, along with black spot, is one of the most prevalent diseases affecting *P. pyrifolia*. Of the examined cultivars, 'Hongli' and 'Mili', low ethylene producers classified as ab, are resistant to scab (Abe and Kotobuki, 1998), and accordingly, have received much attention. Hybridization between 'Osanijiseiki' (a self-compatible mutant of 'Nijisseiki') and 'Hongli' are now in progress with the aim of breeding new self-compatible cultivars resistant to black spot and scab, and with a long shelf life. To date, four molecular markers including ours have been associated with self-incompatibility, black spot (*Alternaria alternata* Japanese pear pathotype), scab, and fruit ethylene production (Banno et al., 1999; Sassa et al., 1997; Takasaki et al., 2004; Terakami et al., 2006). Thus, these markers can be used for marker-assisted selection of these four traits.

In conclusion, measurement of maximum ethylene production is a complex task because determining the accurate time of fruit ripening in Asian pears is often problematic. As a result, underestimation often occurs when using fruits harvested earlier than the optimum date. Our CAPS analysis is a rapid and accurate method for predicting relative levels of ethylene production and gaining posthandling information. Furthermore, information on marker types enables the identification of cultivars for hybridization aimed at enhancing shelf life in Asian pear breeding.

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