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T. Harada · T. Sunako · Y. Wakasa · J. Soejima
T. Satoh · M. Niizeki

An allele of the 1-aminocyclopropane-1-carboxylate synthase gene (*Md-ACS1*) accounts for the low level of ethylene production in climacteric fruits of some apple cultivars

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Abstract An allele of the apple ripening-specific 1-aminocyclopropane-1-carboxylate (ACC) synthase gene (*Md-ACS1–1*) has a 5′-flanking region possessing an inserted retroposon-like sequence. Apple species can be classified into three groups that are heterozygous or homozygous for the *ACS1–1* and *ACS1–2* alleles. We measured the internal ethylene concentration (IEC) in climacteric fruit of 35 apple cultivars with respect to genotype. Eleven *ACS1–2* homozygous cultivars exhibited much lower IECs than cultivars homozygous or heterozygous for *ACS1–1*. Furthermore, F_1 *ACS1–2* homozygous progeny derived from crosses between heterozygous cultivars had fruit with a very low IEC. These results are in accord with previous data indicating the absence of transcription from *ACS1–2* in a heterozygous cultivar. Since the low level of *ACS1* mRNA in climacteric fruit was observed in several *ACS1–2* homozygous cultivars, we conclude that the low level of ethylene production in some cultivars is caused by the mutated allele of *ACS1*, which is the main gene responsible for ethylene production during ripening.

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T. Harada (✉) · T. Sunako · Y. Wakasa · M. Niizeki
Laboratory of Plant Breeding and Genetics,
Faculty of Agriculture and Life Science, Hirosaki University,
Bunkyo-cho 3, Hirosaki 036–8561, Japan
e-mail: tharada@cc.hirosaki-u.ac.jp
Fax: +81-172-39-3750

J. Soejima
Apple Research Center, National Institute of Fruit Tree Science,
Shimokuriyagawa, Morioka 020-0123, Japan

T. Satoh
Aomori Apple Experimental Station, Botandaira,
Kuroishi 036-0332,
Japan

Present address:
T. Sunako, Bioscience Center
and Graduate School of Bioagricultural Sciences,
Nagoya University, Chikusa, Nagoya 464-8601, Japan

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Introduction

Fruits contain a very high percentage of their fresh weight as water. Consequently, they exhibit relatively high metabolic activity when compared with other plant-derived foods such as seeds. This metabolic activity continues after harvest, and thus making most fruits highly perishable commodities (Tucker 1993). Harvesting at the immature or mature green stage and/or using refrigeration and controlled atmosphere storage have been used to solve these post-harvest problems. Though the apple (*Malus*×*domestica* Borkh.) can be stored under controlled conditions for considerably longer periods than most other types of climacteric fruit, its storage capability depends on the cultivar (Chu 1988; Kondo et al. 1991; Gussman et al. 1993; Brackmann and Streif 1994; Suzuki et al. 1997). Some apple cultivars should be on the shelf immediately after harvest because of rapid perishability. In apple breeding programs, genotypes with inherent long-term storage capability have therefore been used to develop new commercially acceptable varieties (Gussman et al. 1993).

Apple is a climacteric fruit, whose ripening is associated with an upsurge in the rate of respiration and ethylene production (Knee 1993). It is generally accepted that ethylene is the key regulator of apple ripening (Theologis 1992), and the suppression of ethylene biosynthesis and its action is one of the primary mechanisms by which controlled atmospheres extend the storage life of apples (Lau et al. 1986; Gorny and Kader 1996, 1997). Some reports have indicated that the rates of ethylene biosynthesis in apple fruits differ considerably among cultivars and have discussed the impact of this variability on their storage capability (Brackmann and Streif 1994; Chu 1988; Kondo et al. 1991; Gussman et al. 1993; Larrigaudiere et al. 1997; Suzuki et al. 1997). However, the molecular mechanism responsible for this difference in ethylene level is unknown. The conversion of *S*-adenosyl-L-methionine to

1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase (EC4.1.1.14) is the first committed step in ethylene biosynthesis and is generally considered to be the rate-limiting step (Lau et al. 1984). This enzyme is encoded as a divergent gene family in all of the plant species analyzed so far. Several ACC synthase genes (*Md-ACS*) have also been reported in apple, but *Md-ACS1* has been found to be expressed predominantly in this climacteric fruit (Dong et al. 1991; Kim et al. 1992; Lay-Yee and Knighton 1995; Rosenfield et al. 1996; Harada et al. 1997).

Sunako et al. (1999) found that an allele of *Md-ACS1* possesses a retroposon-like insertion in the promoter region and that this allele gene (*ACS1-2*) has very low transcription activity compared with the original allele (*ACS1–1*). Here, we report that homozygosity of *ACS1–2* results in low levels of ethylene production in the fruit of some apple cultivars.

Materials and methods

Plant material

Apples (*Malus×domestica* Borkh.) were harvested at each commercial maturity stage from July to November in both 1997 and 1998 from the experimental farms of Hirosaki University and the Aomori Apple Experimental Station. Fruit of F_1 progenies of a cross between the cultivars Orin and Granny Smith (using Granny Smith as the pollen parent) were harvested on November 5, 1998 from the farm of the Apple Research Center of the National Institute of Fruit Tree Science. In the course of this breeding, 24 of 41 progenies were selected out due to undesirable characteristics such as poor taste or fruit shape, or low disease resistance (*Altenaria* leaf spot). All cultivars were grown on M.26 rootstock, and the breeding lines were on M.27 rootstock.

Measurement of ethylene production rates and internal ethylene concentrations (IEC)

The rate of ethylene production of individual fruit was determined by enclosing a single intact fruit in a gas-tight container (1.23 l) equipped with septa followed by sampling of 1 ml of gas in the container headspace with the aid of a syringe. Determination of IEC in the fruit was carried out as follows. In 1997, harvested samples were stored immediately in a refrigerator (0°C). All were subsequently transferred to an incubator (25°C) on December 12 and placed there for 12 days until they reached the climacteric stage. Samples harvested in 1998 were incubated directly in an incubator (20°C), and the IEC of each sample was measured after 12 days. The internal gas was withdrawn from the cortical tissue submerged in water under vacuum. A 1-ml aliquot of the gas sample was used to evaluate the ethylene concentration using a gas chromatograph equipped with a flame ionization detector. Unless otherwise stated, five fruits per sample were used for measurements.

Determination of *ACS1* allelic forms

Genomic DNA was isolated from young leaves of sample trees using the method of Varadarajan and Prakash (1991). The polymerase chain reaction (PCR) was carried out to identify the *ACS1* allelic forms in the cultivars and breeding selections (Sunako et al. 1999). The reaction mixture (50 µl) contained about 50 ng template genomic DNA, 0.2 µ*M* of each primer, 200 µ*M* of each deoxyribonucleotide triphosphate (dNTP), 1×PCR reaction buffer and 2.5 U Taq DNA polymerase. The amplification program consisted of 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 2.5 min, with a final 10-min extension step at 72°C. The following oligonucleotide primers, the positions being numbered according to accession no. U89156, were used (Sunako et al. 1999): ACS1–5′F 5′AGAGAGATGCCATTTTTGTTCGTAC3′ 861–887; ACS-5′R 5′CCT ACAAACTTGCGTGGGGATTATAAGTGT3′ 1379–1350.

Northern blot hybridization

RNA extraction and gel blot analyses were performed as described by Sunako et al. (1999). The probe used in this experiment was the 3′-untranslated region of *Md-ACS1* (ACS1-B, Sunako et al. 1999).

Results

Ethylene biosynthesis rates in different *ACS1* genotypes

To investigate the difference in ethylene biosynthesis rate between *ACS1* genotypes, we used fruits of Orin (*ACS1–1*/*1–2*) and Fuji (*ACS1–2*/*1–2*) which had been stored at 0°C for about 6 months after harvest. After the start of continuous incubation at 20°C, Orin produced significant amounts of ethylene, with a maximum rate of 128 nl/g per hour. The high rate continued for the first 10 days and then decreased to approximately 30 nl/g per hour. In contrast, Fuji showed only a very slight increase in ethylene production after 3 and 4 days, which then continued at a low level throughout the rest of the incubation (Fig. 1).

IEC of the fruits of 35 cultivars

In order to elucidate the relationship between ethylene production rate and different *ACS1* alleles, we selected 35 apple cultivars (Table 1) after consideration of their harvest season and genotype. All were harvested at their respective commercial maturity from the end of July to the beginning of November 1998, and then placed in an incubator (20°C) for 12 days. These conditions brought

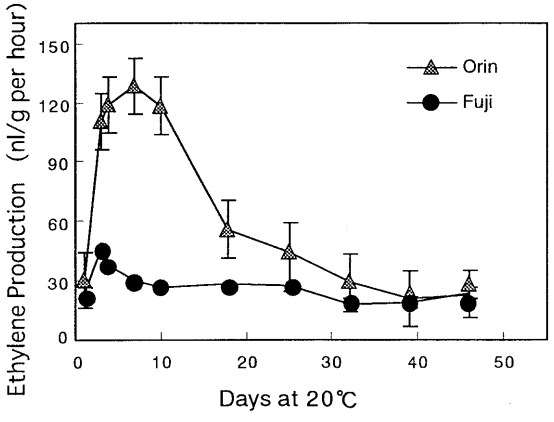

Fig. 1 Changes in ethylene production rates in apple fruits after transfer from cold storage (0°C) to room temperature (20°C). Data points are the means of five fruit samples. *Bars* represent SE

Table 1 Apple cultivars used in this study and their *ACS1* genotype

ACS1 genotype	Number of sample	Name of cultivar
1-1/1-1	1	American Summer Pearmain
	2	Bancroft
	3	Fu Jin
	4	Indo
	5	Jerseymac
	6	Julyred
	7	McIntosh
	8	Puritan
	9	Viking
	10	Vista Bella
1-1/1-2	11	Beacon
	12	Golden Delicious
	13	Golden Melon
	14	Granny Smith
	15	Jonagold
	16	Jonathan
	17	Mikilife
	18	Mutsu
	19	Orin
	20	Raritan
	21	Starking Delicious
	22	Tangier
	23	Tsugaru
	24	Toko
1-2/1-2	25	Akane
	26	Discovery
	27	Fuji
	28	Himekami
	29	Himekomachi
	30	Iwakami
	31	Kaori
	32	Megumi
	33	Narihoko
	34	Ralls Janet
	35	Sansa

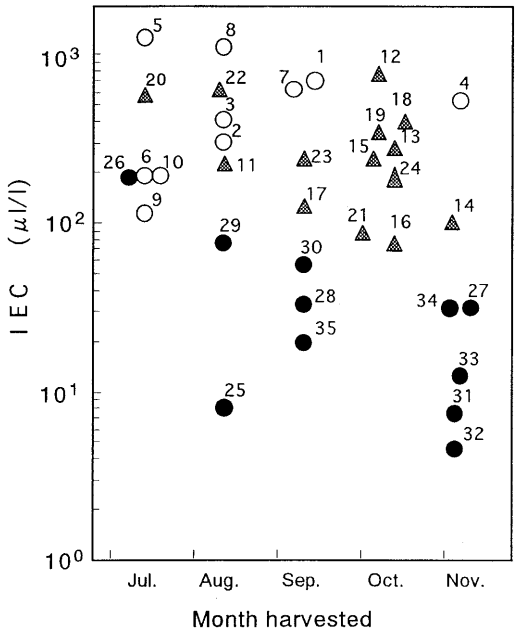

Fig. 2 Internal ethylene concentration (IEC) of the fruits of 35 apple cultivars. IEC values of each cultivar are grouped together by the month harvested. Number of data points represents the number of samples listed in Table 1. Data points are the means of five fruit samples. The genotypes of the apple cultivars are: ○ *ACS1–1/1–1*, ▲ *ACS1–1/1–2*, ● *ACS1–2/1–2*

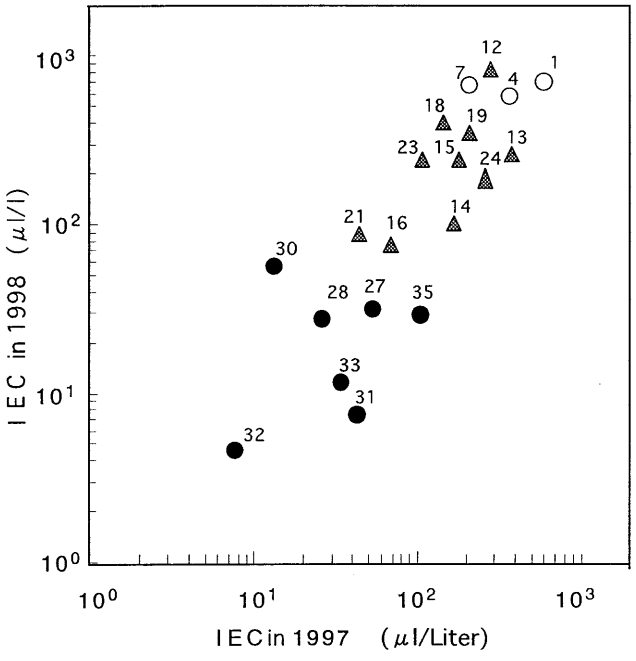

Fig. 3 Relationship between IEC of apples harvested in 1997 and in 1998. Number of data points represents the number of samples listed in Table 1. Data points are the means of five fruit samples. The genotypes of the apple cultivars are: ○ *ACS1–1/1–1*, ▲ *ACS1–1/1–2*, ● *ACS1–2/1–2*

the fruit to the climacteric stage and the maximum level of ethylene production (Gussman et al. 1993). As shown in Fig. 2, the IEC of these samples ranged from 5 to 1380 µl/l. The highest value was obtained in the *ACS1–1* homozygous cultivar Jerseymac, whereas the lowest was in the *ACS1–2* homozygous cultivar Megumi. From the data in Fig. 2, the values of IEC decreased as the harvest month became later. All *ACS1–2* homozygous cultivars except Discovery exhibited IECs of less than 100 µl/l, and cultivars either homozygous or heterozygous for *ACS1–1* did not show low IECs.

The IECs of 20 cultivars from the list in Table 1 were also measured in 1997 using a similar experimental procedure, and the IEC values of these cultivars (Fig. 2) were compared with those in 1998. As shown in Fig. 3, the rates of ethylene production were consistent between years.

IEC of the fruit of F_1 progenies

Fruit of F_1 progenies between *ACS1–1/1–2* cultivars were used to measure these IECs. Although most F_1 trees produced fewer than five fruits due to their immaturity, being only the seventh year after crossing, IEC values of fruit harvested from the trees showed a strong correlation with their *ACS1* genotypes (Fig. 4). Further-

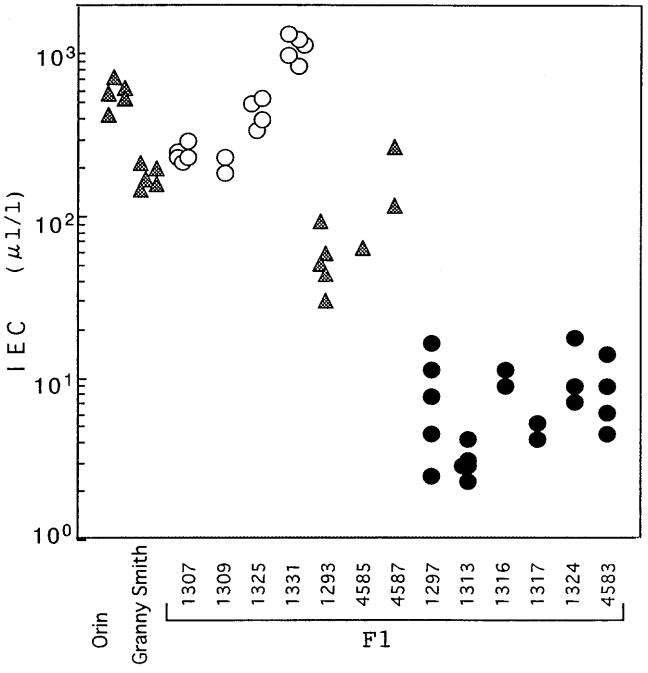

Fig. 4 IEC of apples of Orin, Granny Smith and their advanced breeding selections. Individual F_1 trees are denoted by their *breeding numbers*. Data points are the IEC level of each fruit sample. The genotypes of the apple cultivars are: ○ *ACS1–1/1–1*, ▲ *ACS1–1/1–2*, ● *ACS1–2/1–2*

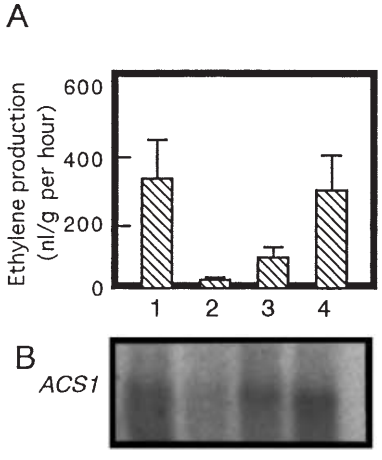

Fig. 5A, B Ethylene production rates (**A**) and the expression levels of *ACS1* (**B**) in the fruit of some early-maturing cultivars. The production values represent the mean of three fruit samples±SE. *1* cv. July Red, *2* cv. Himekami, *3* cv. McIntosh, *4* cv. American Summer Pearmain

more, heterozygous *ACS1* samples had intermediate values between those of each homozygous class.

Expression of *ACS1* in different *ACS1* genotypes

Expression of the *ACS1* gene during fruit ripening was studied by carrying out RNA gel-blot analysis of the total RNAs from apple fruits. An extremely early-maturing cultivar (July Red) and early-maturing cultivars (American Summer Peamain, McIntosh, Himekami) were harvested on July 30 and September 7, respectively. The sample fruits were then placed in a 20°C incubator for 9–12 days to allow them to reach the climacteric stage. After measuring the level of ethylene production, total RNA was extracted for the RNA gel-blot analysis. As shown in Fig. 5, clear positive signals indicating expression of the *ACS1* were detected in 3 *ACS1–1* homozygous cultivars, whereas a very faint signal was observed in the *ACS1–2* homozygous cultivar (Himekami). A similar result was obtained in several late-maturing cultivars such as Megumi and Fuji (data not shown). Therefore, it was revealed that *ACS1–2* homozygous cultivars have not only lower IECs but also a lower expression of *ACS1* than *ACS1–1* homozygous and *ACS1–1/1–2* heterozygous cultivars at their climacteric stage.

Discussion

Thirty-five cultivars, which had been selected from our experimental farms with respect to the season of ripening and *ACS1* genotype, were used to analyze ethylene production at climacteric stage fruit. Because of the large number of samples (175 fruits), we measured the IEC, which is known to show a very good correlation (r=0.963, P<0.001) with the rate of ethylene production by apple cortical tissue (Gussman et al. 1993), instead of the ethylene production rate. As sample fruits were harvested from the trees of each cultivar when the ground color of the fruits changed or of the fruit turned red, the skin determination of the time to harvest could not be judged precisely. However, storing fruit at room temperature for 12 days is considered to draw out the innate capability for ethylene production in each fruit (Lau et al. 1986). There was, in fact, a good correlation between yearly IEC data in spite of using samples derived from different pretreatments of the fruit sample (see Materials and methods). Therefore, low levels of IEC in the fruit of some apple cultivars means that they have the heritable character of a low ethylene production rate. The relationships between *ACS1* genotypes and levels of IEC in cultivars and F_1 progenies clearly show that *ACS1–2* homozygous apple trees produce fruit in which only a low level of ethylene is synthesized during the climacteric stage.

We determined the *ACS1* genotype of more than 100 *Malus* individuals, including wild species and breeding selections (data not shown). All the wild species were either heterozygous or homozygous for *ACS1–1* (Sunako et al. 1999), while 46% of cultivars and breeding selections were homozygous for *ACS1–1*, 35% were heterozygous *ACS1–1/1–2* and 19% were homozygous for *ACS1–2*. The results suggest that individuals homozygous for *ACS1–2* may have been selected by apple breeding to obtain lines with good storage capability.

The early maturing cultivar Discovery, harvested on 30 July, showed an IEC of 231 μl/l, even though the genotype was homozygous for *ACS1–2*. In general, fruit of early-ripening cultivars are highly susceptible to physiological disorders due to ambient high temperatures

(Westwood 1993). Although there was no visually unusual feature among the samples, the IEC value might be driven by a kind of wounding response due to this character. On the other hand, the lowest IEC was observed in Megumi, which is known to have an exceptionally low rate of preharvest fruit drop (Saito et al. 1994). Thus, it could be interesting to clarify whether there is a correlation between the level of IEC and the dropping phenomenon of preharvest fruit. Nevertheless, considerable differences in the level of IEC were observed among the same *ACS1* genotype. Additional molecular mechanisms would be the presence of other genes involved in ethylene production in apple fruit, such as *Md-ACO* (Dong et al. 1992; Ross et al. 1992) and *Md-ETR1* (Lee et al. 1998).

Interactions between transposable elements and the regulatory sequences of genes can lead to alterations in the level of transcription (Weil and Wessler 1990). The presence of a 162-bp retroposon-like element at position –781 in the *ACS1* allele may reduce its transcript to a very low level. Therefore, there is a possibility that *Md-ACS1–2*, whose promoter is mutated by the insertion, contributes to the long-term storage capability of some apple cultivars by reducing ethylene production during ripening. To elucidate the relationship between the *ACS1* allelic forms and the storage capability of apple, we are currently studying the expression of some ripening-related genes, such as polygalacturonase (Atkinson 1994), in our laboratory.

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