

# An Allele of the Ripening-Specific 1-Aminocyclopropane-1-Carboxylic Acid Synthase Gene (*ACS1*) in Apple Fruit with a Long Storage Life<sup>1</sup>

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An allele of the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene (*Md-ACS1*), the transcript and translated product of which have been identified in ripening apples (*Malus domestica*), was isolated from a genomic library of the apple cultivar, Golden Delicious. The predicted coding region of this allele (*ACS1-2*) showed that seven nucleotide substitutions in the corresponding region of *ACS1-1* resulted in just one amino acid transition. A 162-bp sequence characterized as a short interspersed repetitive element retrotransposon was inserted in the 5'-flanking region of *ACS1-2* corresponding to position –781 in *ACS1-1*. The *XhoI* site located near the 3' end of the predicted coding region of *ACS1-2* was absent from the reverse transcriptase-polymerase chain reaction product, revealing that exclusive transcription from *ACS1-1* occurs during ripening of cv Golden Delicious fruit. DNA gel-blot and polymerase chain reaction analyses of genomic DNAs showed clearly that apple cultivars were either heterozygous for *ACS1-1* and *ACS1-2* or homozygous for each type. RNA gel-blot analysis of the *ACS1-2* homozygous Fuji apple, which produces little ethylene and has a long storage life, demonstrated that the level of transcription from *ACS1-2* during the ripening stage was very low.

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Ethylene is a gaseous plant hormone that regulates many physiological processes in plant growth and development. Its synthesis is induced not only by stress, such as wounding, but also during unique developmental stages, such as seed germination, fruit ripening, and leaf and flower abscission (Yang and Hoffman, 1984; Ky et al., 1992; Rodrigues-Pousasa et al., 1993). Because large amounts of fruit and vegetables are lost due to the effect of ethylene on plant senescence, the significance of controlling the synthesis of ethylene is obvious (Theologis, 1992). The enzyme that limits ethylene production is ACC synthase (EC 4.1.1.14), which catalyzes the formation of ACC, the immediate precursor of ethylene (Gussman et al., 1993; Gorny and Kader, 1997). In tomato ACS is encoded by at least six divergent genes (Lincoln et al., 1993), two of which, *LE-ACS2* and *LE-ACS4*, are expressed during fruit ripening. Expression of antisense RNA derived from *LE-ACS2* results in almost complete inhibition of the mRNA of both these

genes and extends the longevity of the fruit (Oeller et al., 1991).

The apple is also a typical climacteric fruit, and therefore it appears to be possible to extend its storage life by inhibiting ethylene biosynthesis using antisense RNA technology (Hamilton et al., 1990). Furthermore, the molecular mechanisms responsible for the different storage properties of apple (*Malus domestica*) cultivars are of interest. At the commercial harvesting stage, the cv Golden Delicious already produces a considerable amount of ethylene, and the amount increases by about 5-fold or more at the climacteric stage, even during cold storage. On the other hand, some cultivars produce very little ethylene during storage and can be stored for up to 1 year at a low temperature with little or no deterioration in the physical quality of the fruit (Gussman et al., 1993). Although a clear relationship between ripening and ethylene production has been demonstrated, much less is known about the molecular mechanism responsible for good, long-term storage properties. Gussman et al. (1993) studied ethylene production and the fruit-softening rates of several apple variants as they ripened. One variant with a long storage life produced only a small amount of ethylene at the time of harvesting and throughout most of an 86-d storage period at 4°C, whereas fruit of the control, cv Golden Delicious, produced large amounts of ethylene under the same conditions. Because the variant converted ACC, but not Met, to ethylene, it is considered to lack the ability to synthesize ACC. Fuji, the most popular cultivar in Japan, has a long storage life and produces very little ethylene at harvest time and during subsequent storage at low temperature (Kondo et al., 1991). However, the molecular mechanisms responsible for this low level of ethylene production are unclear.

Dong et al. (1991) isolated a partial ACS cDNA with a predicted translation product identical to an ACS extracted from ripening apple. Lay-Yee and Knighton (1995) reported the sequence of the full-length cDNA of this ACS (*Md-ACS1*, *M. domestica* ACC synthase), and we reported its genomic sequence (Harada et al., 1997). Furthermore,

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Abbreviations: ACS, ACC synthase; IEC, internal ethylene concentration; RT, reverse transcriptase; SINE, short interspersed DNA element.

we have isolated an allelic form of *Md-ACS1* from a genomic library of cv Golden Delicious, and in the present communication, we describe some of its characteristics. A SINE was inserted into the promotor region of this allele, and the transcription product of the allelic gene was not observed during ripening of cv Golden Delicious fruit. Furthermore, cv Fuji, which produces little ethylene, was found to be homozygous for the allelic gene and to contain a very small amount of the transcript, suggesting that this *ACS1* allelic gene may contribute to the good, long-term storage properties of cv Fuji.

## MATERIALS AND METHODS

### Plant Material

Young leaves of apple (*Malus domestica* Borkh.) cultivars and their corresponding wild species were collected from the experimental farms of Hirosaki University (Japan) and the Apple Research Center, National Institute of Fruit Tree Science (Morioka, Japan). The cvs Golden Delicious and Fuji were harvested when they were commercially mature on October 21 and November 8, respectively. Then, one-half of the apples were transported to our laboratory and stored at 20°C for 12 d to allow them to reach the climacteric stage. The IEC, flesh firmness, and titratable acidity of the apple fruits were evaluated to obtain preclimacteric and climacteric stage data. Cortical tissue from which the skin and core parts had been removed was frozen by immersion in liquid N<sub>2</sub> and then stored in a freezer (−80°C) until isolation of the total RNA.

### Measurement of IECs

The internal gas was withdrawn from apple cortical tissue submerged in water under a vacuum, and a 1-mL aliquot of the gas sample was used to evaluate the IEC by GC.

### Genomic Library Screening and Sequence Analysis

Genomic DNA was isolated from leaves using the method of Varadarajan and Prakash (1991) and purified using the ethidium bromide-CsCl method of Sambrook et al. (1989). The genomic DNA from cv Golden Delicious was cloned as partial *Sau3AI* fragments into the *XhoI* site of FIX (Stratagene), and the library was screened with a <sup>32</sup>P-radiolabeled *ACS* probe DNA (*ACS1-A*). The positive phage clones (AP1) were used to make a restriction map using *XhoI* and *ApaI*, and the 8.7-kb *ApaI* fragment (pAP1-2) containing the *ACS1* gene was subcloned into the pBluescriptII KS vector (Stratagene). Nucleotide sequencing of the 5.7-kb *HindIII/EcoRI* fragment of pAP1-2 was carried out by subjecting the secondary subcloned fragments to dideoxynucleotide chain termination, and the sequence was determined by analyzing the overlapping clones and both strands. The accession numbers of *ACS1-1* and *ACS1-2* are U89156 and AB010102, respectively.

### Southern-Blot Hybridization

Genomic DNAs extracted from young leaves were digested with a restriction enzyme, separated by agarose gel electrophoresis, and blotted onto a membrane (Hybond N<sup>+</sup>, Amersham). A fragment of each probe DNA was labeled with <sup>32</sup>P-dCTP using the Prime-It II random primer labeling kit (Stratagene), and the blots were hybridized with the probe at 65°C for 15 h in a hybridization buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 6× SSC, 5× Denhardt's solution, 0.2 mg/mL salmon sperm DNA, 20 mM sodium phosphate buffer, and 1% SDS). The membrane was washed with 2× SSC/0.1% (w/v) SDS at room temperature for 30 min and 0.2× SSC/0.1% (w/v) SDS at 65°C for 15 min twice and then autoradiographed using Kodak x-ray film at −80°C and an intensifying screen.

### Northern-Blot Hybridization

Total RNA was isolated from apple fruit with guanidinium thiocyanate followed by centrifugation on a CsCl gradient (Lay-Yee et al., 1990). RNA (20 μg per lane) was denatured with 50% (v/v) deionized formamide in 2.2 M formaldehyde at 65°C for 15 min, separated using 2.2 M formaldehyde and 1.2% (w/v) agarose-gel electrophoresis with 40 mM Mops, and then blotted onto membranes (Hybond N<sup>+</sup>). After the blots were fixed under UV light they were prehybridized in a solution comprising 6× SSC, 50% (v/v) formamide, 5× Denhardt's solution, 0.5% (w/v) SDS, and denatured salmon sperm DNA (Sambrook et al., 1989), and then the <sup>32</sup>P-labeled probe DNA was hybridized with the blots at 65°C overnight in the same buffer. The membranes were washed with 2× SSC/0.1% (w/v) SDS at room temperature for 20 min, washed twice with 0.2× SSC/0.1% (w/v) SDS at 65°C for 15 min, and then autoradiographed using Kodak x-ray film at −80°C.

### PCR Amplification

PCR was used to produce probe DNAs for *ACS1-A*, *ACS1-B*, and *ACS3* (Rosenfield et al., 1996). The *ACS1* probes were produced by amplifying the cloned fragments, and the *ACS3* was derived from the genomic DNA of the apple cv McIntosh. PCR was also carried out to identify the *ACS1* allelic forms in apple cultivars and their corresponding wild species. The reaction mixture (50 μL) contained about 50 ng of template genomic DNA, 0.2 μM each primer, 200 μM each deoxyribonucleotide triphosphate, 1× PCR reaction buffer, and 2.5 units of *Taq* DNA polymerase. It was held at 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. RT-PCR was performed to obtain the cDNA from the 3' region of the *ACS1* transcription product as follows. The reaction mixture comprised 1 μg of poly(A<sup>+</sup>) RNA, 40 mM KCl, 1 mM DTT, 20 units of RNase inhibitor, 50 units of RT (Perkin-Elmer) and the primer *ACS1-3'AR*, and reverse transcription was carried out by incubating this mixture at 42°C for 1 h. The tubes were then put on ice, 80 μL of PCR reaction mixture comprising 1× buffer, the forward primer *ACS1-3'AF*, 2.5

units of *Taq* polymerase, and 2.5 mM each dNTP were added, and PCR (30 cycles of 94°C for 2 min, 45°C for 1 min, and 72°C for 3 min) was carried out. The first reaction solution and the ACS1-3'B primers were subjected to a second PCR to obtain the product. The following oligonucleotide primers (the positions being numbered according to accession no. U89156 for *ACS1* and no. U73816 for *ACS3*) were used for the above reactions: ACS1-AF, 5'-TACCATGAGGTCCACAACAC-3' (2302–2321); ACS1-AR, 5'-GGTGAGCACTAAGTGGTTGGG-3' (2813–2793); ACS1-BF, 5'-GATGAAAGGTAGCCTGGTCTGA-3' (4056–4077); ACS1-BR, 5'-TACTACTAATCACATTGTATAGAATC-3' (4513–4489); ACS3-F, 5'-GACAAATAGAAAGAGACCTGAGGACG-3' (1086–1110); ACS3-R, 5'-CCATCGATTATAACAACACTGATTGTG-3' (1573–1549); ACS1-3'AF, 5'-TCCAACAACAACGGGATTATCA-3' (3312–3336); ACS1-3'AR, 5'-AGGCTACCTTTCATCTACCGGGA-3' (4070–4048); ACS1-3'BF, 5'-ATCTTCTCGAGTCATGGCTGGC-3' (2489–2510); ACS1-3'BR, 5'-TTTCATCTACCGGGAATAGGACCGCGG-3' (4062–4036); ACS1-5'F, 5'-AGAGAGATGCCATTTTTGTTCGTAC-3' (861–887); and ACS1-5'R, 5'-CC-TACAAACTTGCCTGGGGATTATAAGTGT-3' (1379–1350).

## RESULTS

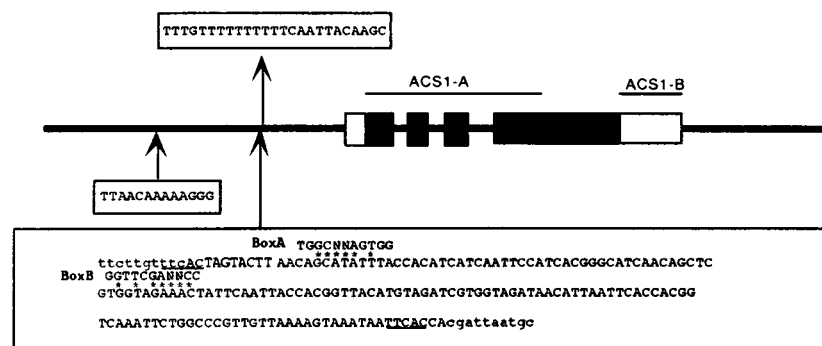
### Allele of ACS1

Plaque hybridization of the apple cv Golden Delicious genomic library with the ACS1-A probe (Fig. 1) yielded 13 positive clones. Restriction mapping of their subcloned fragments suggested that the sequence of one clone was very similar to that of the *Md-ACS1* gene reported previously (Harada et al., 1997). Sequencing of the subcloned fragment revealed an open reading frame that was almost identical to that of the *Md-ACS1* gene and its flanking regions (5676 bp, accession no. AB010102). Therefore, we designated the previously reported sequence *ACS1-1* and this new sequence *ACS1-2*. As shown in Table I, the exons of these sequences are highly homologous and the pre-

**Table I.** Structural characteristics of the *Md-ACS1* genes

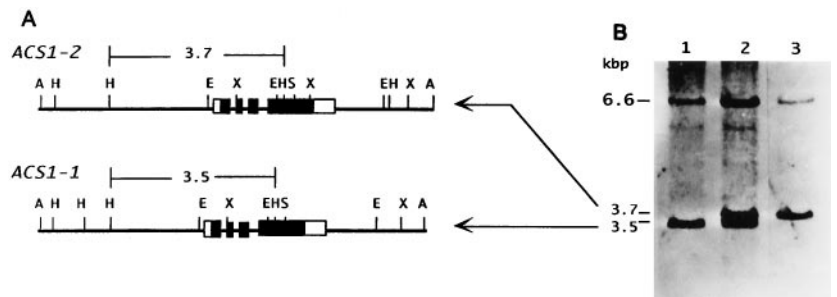
Region	Length		Identity
	<i>ACS1-1</i>	<i>ACS1-2</i>	
	bp		%
5' Flanking	2112	2263	91
5' Untranslated	99	99	100
Exon 1	147	147	100
Intron 1	120	120	99
Exon 2	132	132	100
Intron 2	131	131	100
Exon 3	161	161	99
Intron 3	176	176	100
Exon 4	982	982	99
3' Untranslated	453	454	98
3' Flanking	1013	1011	97

dicted amino acid sequence of *ACS1-2* differs from that of *ACS1-1* by only one amino acid, because six of the seven nucleotide substitutions in the predicted *ACS1-2* exon region did not affect the translation product because of their third positions in the respective codons. A guanine-to-adenine transition at position 3763 of the *ACS1-1* gene changed Gly to Ser in the predicted translation product. By contrast, a significant divergence was found in the 5'-flanking region. A 13-bp sequence from 594 to 607 of *ACS1-1* was tandemly repeated in *ACS1-2*. Furthermore, a 24-bp sequence from 1308 to 1331 of *ACS1-1* was missing from *ACS1-2* and a 162-bp insertion sequence was found at this site in *ACS1-2* (Fig. 1). As shown in Figure 2, Southern blotting with the ACS1-A probe revealed that cv Golden Delicious had three positive fragments in its *Hind*III-digested genomic DNA, and two of them (3.5 and 3.7 kb) were found to correspond to the fragments containing *ACS1-1* and *ACS1-2*, respectively. The other 6.6-kb fragment observed was thought to be derived from the homologous sequence of the gene family. However, some cultivars had either the *ACS1-1* or the *ACS1-2* fragment only (Fig. 2B, lanes 1 and 3). Furthermore, cv Amanishiki, which



**Figure 1.** Structure of *Md-ACS1-2*. Major differences from that of *ACS1-1* are shown schematically. Solid and clear boxes indicate the coding and the untranslated regions, respectively. The lines connecting the solid boxes represent the three introns. The lines connecting the open boxes indicate the 5'- and 3'-flanking regions. The arrow from the 5'-flanking region indicates the *ACS1-1* sequence, which was not observed in *ACS1-2*. Arrows to the 5'-flanking region show the 13-bp tandemly repeated sequence and the 162-bp insertion sequence, which were observed in *ACS1-2*. The flanking sequences of the insertion are also indicated in lowercase letters. Direct repeats at the flanking sequences are underlined, and two promoter motifs for RNA polymerase III are also shown. The extents of the probes used in this study are indicated as lines.

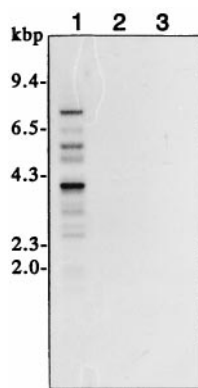
**Figure 2.** A, Restriction maps of the *Apal* fragments of phage clones that contain *ACS1-1* or *ACS1-2*. The exons and the untranslated regions are represented as described in the legend of Figure 1. Restriction sites are as follows: A, *Apal*; H, *HindIII*; E, *EcoRI*; X, *XhoI*; S, *SacI*. B, Genomic DNA gel-blot analyses using probe ACS-A. Lane 1, cv Indo; lane 2, cv Golden Delicious; lane 3, cv Fuji. Genomic DNAs were digested by *HindIII*.



resulted from crossing the respective *ACS1-1* and *ACS1-2* cvs Indo and Ralus Janet, possessed both *ACS1* fragments, as did cv Golden Delicious (data not shown). In light of these results, we concluded that *ACS1-2* is an allele of *ACS1-1* in the apple genome.

### Characteristics of the Insertion SINE Sequence

As shown in Figure 1, we observed that the 162-bp insertion had a flanking 5-bp direct repeat (TTCAC), which led us to assume that this insertion is probably a transposable element. We also detected an A-rich tract at the 3' end and two conserved polymerase III motifs, boxes A and B, which were located 40 bases apart. These are characteristic features of the SINEs that have been identified in some organisms (Umeda et al., 1991; Mochizuki et al., 1992; Deragon et al., 1996). Furthermore, hybridization of the insertion sequence with digested genomic DNAs from apple, rice, and soybean confirmed that this is present only in the apple genome. When the filter was washed under low-stringency conditions after hybridization, the probe was found to hybridize with many fragments and formed no distinct bands but showed a faint, smeared background, whereas under high-stringency conditions, the probe hybridized with several fragments and distinct bands were formed (Fig. 3). These findings indicate that homologous sequences with some degree of sequence heterogeneity are interspersed in the apple genome. Therefore, we designated this element SINE-Md1. Although many families of

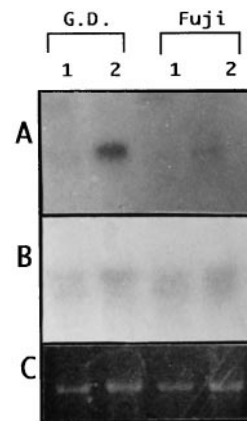


**Figure 3.** DNA gel-blot analysis of the insertion sequence SINE-Md1. Five micrograms of *HindIII*-digested genomic DNA was electrophoresed on a 1.2% agarose gel. Lane 1, Apple (cv Golden Delicious); lane 2, rice (cv Nipponbare); lane 3, soybean (cv Williams).

SINEs are derived from tRNA or tRNA genes (Yoshioka et al., 1993), a search of the GenBank database using the FASTA program revealed no sequence related to SINE-Md1.

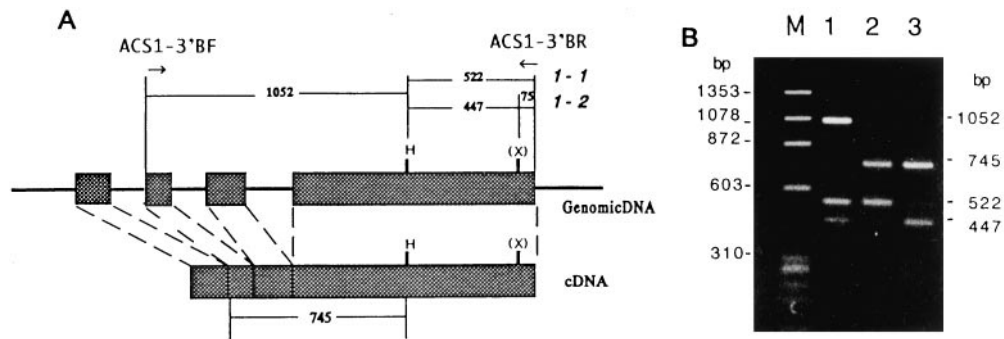
### Absence of or Very Low Level of *ACS1-2* Expression

Expression of the *ACS1* gene during fruit ripening was studied by carrying out RNA gel-blot analysis of the total RNAs from apple fruit. The IEC of cv Golden Delicious fruit just after harvesting was  $16 \mu\text{L L}^{-1}$ , and after storage at  $20^\circ\text{C}$  for 12 d it increased to  $296 \mu\text{L L}^{-1}$ , whereas the IECs of cv Fuji were 0.7 and  $57 \mu\text{L L}^{-1}$ , respectively. The total RNAs extracted from these apples were hybridized with the probe ACS1-B (Fig. 1), which DNA gel-blot analysis demonstrated was specific for the *ACS1* gene (data not shown). No positive signals were detected in preclimacteric fruits of either cultivar, whereas clear 2.0-kb signals were detected in climacteric cv Golden Delicious fruit, and those in cv Fuji fruit were very faint (Fig. 4). Even after prolonged autoradiography, no signals were detected in preclimacteric fruit. Then, the same membrane was hybridized with the ACS3 probe, which, in contrast to the ACS1-B probe, yielded very similar signals in both cultivars, irrespective of their ripeness. The poly(A<sup>+</sup>) RNA fractions extracted from climacteric fruit and the primers ACS1-3'A (Fig. 5) were subjected to RT-PCR, and products of the 3' region of the *ACS1* mRNAs were obtained. Distinct PCR



**Figure 4.** Expression of *Md-ACS1* (A) and *Md-ACS3* (B) genes in cv Golden Delicious (G.D.) and cv Fuji fruits. Lanes 1, Preclimacteric stage; lanes 2, climacteric stage. Ethidium bromide-staining results for 18S rRNA were used as an internal control.



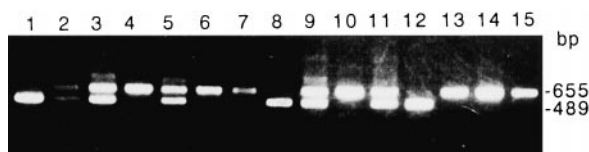


**Figure 5.** A, Theoretical restriction fragment lengths of PCR product from the genomic DNA or cDNA. The structure of *Md*-ACS1 is indicated as described in the legend of Figure 1. The locations of the primers used in the PCR are shown by arrows. H, *Hind*III site; (X), *ACS1-2*-specific *Xho*I site. B, Fragment lengths of *Hind*III/*Xho*I double-digested PCR products. Lane M,  $\phi$ X174/*Ha*III; lane 1, genomic DNA of cv Golden Delicious; lane 2, RT-PCR products from climacteric cv Golden Delicious fruit; lane 3, RT-PCR product from climacteric cv Fuji fruit.

products were amplified by a second PCR using the inside primers (ACS1-3'BF and -3'BR) and first PCR product as the template DNA. Then, we checked whether the product possessed the *Xho*I site, which is located in the 3' region of *ACS1-2* (Fig. 5), because nucleotide transition from adenine to guanine resulted in the yield of the restriction site in *ACS1-2*. As shown in Figure 5B, the digestion patterns show that both alleles were present in cv Golden Delicious genomic DNA, whereas the product from the cDNA fraction of cv Golden Delicious was of the *ACS1-1* type only, indicating that the mRNA had been transcribed exclusively from the *ACS1-1* gene and not from *ACS1-2*. In contrast, the digestion pattern showed that the RT-PCR product from cv Fuji was derived from the *ACS1-2* gene.

#### ACS1-2 Homozygosity of Cultivars Producing Little Ethylene

We performed a survey of the *ACS1* allele in apple cultivars and their corresponding wild species. To amplify the 5' region where the *SINE-Md1* was inserted into the *ACS1-2* gene, we designed a set of primers, ACS1-5'F and ACS1-5'R, for the sequences of *ACS1* and carried out PCR using each genomic DNA as the template. According to the pattern of the PCR products (Fig. 6), it was possible to classify the apple species into three groups that were heterozygous or homozygous for the *ACS1* alleles (Table II).



**Figure 6.** Diagnosis of the *ACS1* allelic form in apple cultivars. Primers were used to amplify the *SINE* insert site of the *ACS1* 5'-flanking region and the products were electrophoresed on a 1.5% agarose gel. Lane 1, cv American Summer Peamain; lane 2, cv Golden Delicious; lane 3, cv Granny Smith; lane 4, cv Fuji; lane 5, cv Jonathan; lane 6, cv Kaori; lane 7, cv Megumi; lane 8, cv McIntosh; lane 9, cv Orin; lane 10, cv Narihoko; lane 11, cv Mutsu; lane 12, cv Cox's Orange Pippin; lane 13, cv Ralus Janet; lane 14, cv Sansa; lane 15, cv Himekami.

All of the wild species were either *ACS1-1* homozygous or *ACS1-1/ACS1-2* heterozygous. It is interesting that all of the cultivars that were homozygous for *ACS1-2* produced apples with good, long-term storage properties and/or that produced little ethylene, like cv Fuji.

#### DISCUSSION

An *ACS1* allele characterized by the presence of a *SINE* in its 5'-flanking region was found in apples. *SINEs* are retroposons, copies of which are made by the retroposition of RNA catalyzed by RNA polymerase III (Weiner et al., 1986). Because it has been suggested that random nicking in A+T-rich regions produces staggered breaks that serve as retroposon insertion sites (Rogers, 1985), the deletion of a 24-bp A+T-rich sequence (19/24) from the *SINE* insertion site in the *ACS1-2* gene is considered to be associated with

**Table II.** Types of *ACS1* alleles of apple cultivars and *Malus* species

<i>ACS1-1/ACS1-1</i>	<i>ACS1-1/ACS1-2</i>	<i>ACS1-2/ACS1-2</i>
<i>M. baccata</i>	<i>M. prunifolia</i>	Anbishas
<i>M. florentina</i>	<i>M. toringoides</i>	Fuji
<i>M. floribunda</i>	<i>M. yunnanensis</i>	Himekami
<i>M. hupehensis</i>	Amanishiki	Iwakami
<i>M. sargentii</i>	Antonovka	Kaori
<i>M. sieboldii</i>	Bramley's Seedling	Megumi
<i>M. spectabilis</i>	Delicious	Narihoko
<i>M. pumila</i>	Golden Delicious	Ralus Janet
Amassia	Golden Melon	Sansa
American Summer	Granny Smith	
Pearmain	Jonathan	
Cox's Orange	Mutsu	
Pippin	Orei	
Hatsuaki	Orin	
Indo	Red Gold	
Kitakami	Sturmer Pippin	
Reinette du	Toko	
Canada	Tsugaru	
Rome Beauty	White Winter Pearmain	
Northern Spy	Yellow Newtown	
McIntosh	York Imperial	

the insertion mechanism. The putative A- and B-box sequences, internal *cis* elements for RNA polymerase III, showed low homology with the corresponding elements in the consensus sequence (Galli et al., 1981). Furthermore, our survey for the presence of the SINE in the *ACS1*-flanking region revealed that some wild apple species also possess *ACS1-2*, indicating that the insertion event occurred before the apple cultivars were established. DNA gel-blot analysis showed that the SINE-Md1 homologs were distributed in the genomes of *Pyrus* as well as *Malus* species (data not shown). Although domesticated apples are thought to be complex hybrids of several *Malus* species (Rehder, 1940), very little is known about their phylogenetic relationships. Characterization of the retropositional events at other orthologous loci where SINE-Md1 has integrated will help to resolve such phylogenetic relationships, for which molecular evidence has already been demonstrated in animals (Shimamura et al., 1997).

RT-PCR analysis revealed no expression of the *ACS1-2* allele in the ripening fruit of cv Golden Delicious, a result that agrees with the cDNA sequence data reported by two groups (Dong et al., 1991; Lay-Yee and Knighton, 1995); both cDNAs derived from the same cultivar were identical to *ACS1-1*, but not to *ACS1-2*. SINE-Md1 integration into *ACS1-2* occurred at a position corresponding to -751 *ACS1-1* of the wild-type counterpart, and the flanking A+T-rich 24-bp sequence was deleted concomitantly. However, six nucleotide substitutions between the SINE and the transcription start point were detected. Although clear sequence motifs have not been observed in the 5'-flanking regions of the *ACS* genes investigated so far, the possibility that the sequence acting positively as a *cis* element is changed by the substitution cannot be ruled out. We consider that the promoter of the *ACS1-2* allele still functions, since a very low level of transcription was detected in climacteric-stage cv Fuji fruit. Blume and Grierson (1997) carried out experiments on the expression of ACC oxidase promoter-GUS fusion products in transgenic plants and demonstrated that the region containing nucleotides -124 to +97 of the gene was sufficient to increase in the GUS activity markedly during fruit ripening, although the activity levels were very low. However, fusion of 1825 bp of the gene upstream sequence resulted in strong and specific induction of GUS expression. Nicholass et al. (1995) reported that the proximal 150 bp of the tomato polygalacturonase promoter showed no detectable transcriptional activity, although the distal 3.4 kb of the 5' promoter was shown to be necessary for high levels of ripening-specific gene expression. Furthermore, examination of the *Arabidopsis ACS1* promoter-GUS fusion system revealed that 1.4 kb of the 5' region upstream from the ATG start codon was enough to produce high expression levels in a transgenic system (Rodrigues-Pousada et al., 1993). Analysis of the 5'-flanking region of *Md-ACS1* using transgenic or transient assay systems should reveal the causes of low *ACS1-2* expression levels in ripening fruit.

*ACS* gene analysis of higher plants indicated that the *ACS* enzyme is encoded by a highly divergent multigene family, the members of which are subject to differential regulation by more than one inducer (Felix et al., 1991;

Olson et al., 1991; Rottmann et al., 1991; Kim et al., 1992; Liang et al., 1992; Yip et al., 1992; Abel et al., 1995). However, in tomato at least two of the six members of the *ACS* gene family are responsible for the burst of ethylene production during fruit ripening (Lincoln et al., 1993), whereas expression of three of them can be strongly induced by an elicitor (Oetiker et al., 1997). So far, three cDNAs of the *ACS* genes, including *ACS1*, have been isolated from ripening apples (Rosenfield et al., 1996). Our RNA gel-blot experiments showed that the *Md-ACS1* expression was induced strongly during ripening, as described previously (Dong et al., 1991), whereas *Md-ACS3* was expressed constitutively, irrespective of the ripening stage. The expression of *Md-ACS2* was not analyzed because our attempt to obtain the probe was unsuccessful. In any event, the levels of the *ACS1* transcript alone do not seem to account for the different IECs of the cultivars. Analysis of *Md-ACS2* expression is also necessary to clarify the relative contributions of the *ACS* gene family members to ethylene production. On the other hand, the sum of the abundant transcripts of all the *ACS* genes expressed in the tomato did not account for increased ethylene production (Oetiker et al., 1997). Some workers have described mechanisms by which *ACS* activity is regulated posttranslational, such as proteolytic processing (Li et al., 1996) and protein phosphorylation/dephosphorylation (Spanu et al., 1994). Therefore, the *ACS* activity in ripening apples may be regulated by coordinated mechanisms at the transcriptional level and after transcription/translation.

Nevertheless, the allelic gene of *ACS1*, which shows dramatically reduced transcriptional activity in comparison with the parent gene, is considered to contribute to the low level of ethylene production by some cultivars at the ripening stage. In preliminary studies we have found that apple cultivars homozygous for *ACS1-2* have lower IECs than other *ACS1* allelic cultivars at the climacteric stage (T. Harada, T. Sunaka, J. Soejima, T. Sato, and M. Niizeki, unpublished data). To elucidate the relationship between the IECs and *ACS1* allelic forms of apples, studies of many cultivars with different commercial harvesting seasons are now underway in our laboratory.

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