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Null Mutation of the MdACS3 Gene, Coding for a Ripening-Specific 1-Aminocyclopropane-1-Carboxylate Synthase, Leads to Long Shelf Life in Apple Fruit

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ABSTRACT

Expression of *MdACS1*, coding for 1-aminocyclopropane-1-carboxylate synthase (ACS), parallels the level of ethylene production in ripening apple fruit. Here we show that expression of another ripening-specific ACS gene (*MdACS3*) precedes the initiation of *MdACS1* expression by approximately 3 weeks; *MdACS3* expression then gradually decreases as *MdACS1* expression increases. Because *MdACS3* expression continues in ripening fruit treated with 1-MCP, its transcription appears to be regulated by a negative feedback mechanism. Three genes in the *MdACS3* family (*a*, *b* and *c*) were isolated from a genomic library, but two of them (*MdACS3b* and *c*) possess a 333-bp transposon-like insertion in their 5' flanking region which may prevent transcription of these genes during ripening. An SNP in the coding region of *MdACS3a* results in an amino acid substitution (Gly289→Val) in the active site that inactivates the enzyme. Furthermore, another null allele of *MdACS3a*, *Mdacs3a*, showing no ability to be transcribed, was found by DNA sequencing. Apple cultivars homozygous or heterozygous for both null allelotypes showed no or very low expression of ripening-related genes and maintained fruit firmness. These results suggest that *MdACS3a* plays a crucial role in regulation of fruit ripening in apple, and is a possible determinant of ethylene production and shelf life in apple fruit.
INTRODUCTION

The plant hormone ethylene plays an important role in the regulation of fruit ripening (Capitani et al., 1999). Many ripening processes are driven by ethylene-regulated changes in gene expression, especially in climacteric fruit exhibiting a high respiration rate during ripening, such as apple and tomato (Giovannoni, 2001). The level of ethylene in plants is determined by the activity of the key enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS). In ripening apple fruit, ethylene production is strictly related to the level of *MdACS1* expression (Sunako et al., 1999; Harada et al., 2000). Moreover, differential expression of *MdACS1* allelic forms (*MdACS1*-1 and -2) among different cultivars causes differences in the extent of pre-harvest fruit drop, which is a physiological effect of the ethylene level in the fruit (Harada et al., 2000; Sato et al., 2004). However, the polygalacturonase gene (*MdPG1*), which is involved in softening of fruit flesh (shelf life), was transcribed differently in cultivars with the same *MdACS1* allelic forms (Oraguzie et al., 2004), suggesting that other factor(s) are correlated with the trait of shelf life.

On the basis of the level of ethylene production during fruit development, McMurchie et al. (1972) introduced the concept of system-1 and system-2 ethylene. System 1, ethylene auto-inhibitory, is considered to function during normal vegetative growth and to be responsible for the basal level of ethylene production; System 2 has been proposed to operate in the upsurge of ethylene production during the ripening of climacteric fruit when ethylene is auto-stimulatory (Lelièvre et al., 1997; Barry et al., 2000). A large increase in ethylene production is triggered by exposing fruit to exogenous ethylene, a process that
involves ACC synthase (Sitrit and Bennett, 1998). These observations suggested that system 2 is regulated by a positive feedback mechanism. The increase in the abundance of \( MdACS1 \) mRNA in ripening fruit is prevented to a large extent by treatment with 1-MCP, a potent inhibitor of ethylene action, again indicating the involvement of the system-2 ethylene mechanism. Therefore, it seems that activation of system 2 is a critical step for entering ripening. We have observed a very low level of ethylene generation in fruit before the initiation of the burst of ethylene production.

In pear (\textit{Pyrus pyrifolia} and \textit{Pyrus communis}), other ACS genes in addition to the gene homologous to \( MdACS1 \) have been reported to determine the ripening behaviors of various cultivars (Itai et al., 1999; El-Sharkawy et al., 2004). Other apple ACS genes (including \( MdACS3 \)) were reported by Rosenfield et al. (1996), but their correlation with ripening behavior remains unclear. In this study, we report the genomic structure and expression profile of \( MdACS3 \). Expression of \( MdACS3 \) in apple fruit was consistent with the small amount of ethylene production prior to the initiation of \( MdACS1 \) expression and was regulated by a negative feedback mechanism. In addition, we demonstrate that the allelic forms of \( MdACS3a \) are involved in determining the shelf life of apple fruit. The critical function of \( MdACS3a \) in regulating the transition from system-1 to system-2 ethylene synthesis and apple fruit ripening is discussed.

**RESULTS**

**Structure and organization of \( MdACS3 \)**
Three positive phage clones were isolated from the genomic library of cv ‘Golden Delicious’ by screening with a probe DNA from the last exon of *MdACS3*. The sequence of one of the three clones corresponded to that of *MdACS3* (accession no. U73816; Rosenfield et al., 1996). The nucleotide sequences of the other two clones were very similar (>90%) to that of *MdACS3* at their predicted exons, but differed markedly at their predicted introns in both sequence (76-43%) and size, as shown in Fig. 1. We termed these three clones *MdACS3a* (accession no. AB243060, corresponding to the cDNA reported by Rosenfield et al. 1996), *MdACS3b* (accession no. AB243061), and *MdACS3c* (accession no. AB243062), respectively. The nucleotide sequences of the 5’ flanking regions showed high homology with *PcACS2a, 2b* and *PcACS3* in *Pyrus communis* (El-Sharkawy et al., 2004). However, all *MdACS3* sequences possessed a GA repeat at positions -424, -426 and -456 of the respective *MdACS3* clones, which is absent from the *Pyrus* genes (El-Sharkawy et al., 2004). Furthermore, *MdACS3b* and *c* possessed a 333 bp insertion, not found in the *MdACS3a* and *Pyrus* genes, at around position -570.

**Three *MdACS3* genes located at distinct loci**

To investigate the relationship among these *MdACS3* genes, we performed CAPS (cleaved amplified polymorphic sequence) analysis using *HindIII* and *EcoRI* sites (Fig. 1) which produce diverse fragments from the respective *MdACS3* genes (Fig. 2). The results indicated that the genomes of the 6 cultivars and 56 strains tested had all three *MdACS3* genes. Then we performed linkage analysis of each *MdACS3* gene using two F1 progenies
MdACS3a was located on the 15th linkage group of cv ‘Ralls Janet’, whereas MdACS3b was located on the 2nd group of cv ‘Delicious’ (data not shown). As both of these two linkage groups appear to possess unique genes, this suggests that MdACS3a and b are unlikely to be different alleles of the same gene. Furthermore, direct sequencing of the coding and flanking regions of these MdACS3s revealed the occurrence of several SNPs (single nucleotide polymorphisms), indicating that MdACS3c must also be an independent gene.

**High amount of inserted sequence in Malus, but not in Pyrus**

As shown in Fig. 1, MdACS3b and c possess extra sequence (333 bp) that is not present in MdACS3a. This sequence is flanked by the 8-bp terminal inverted repeat (TIR) (5’-AATTTTTA-3’) located at -570 of MdACS3a (accession no. AB243060). Querying this insertion in GenBank revealed that it is a MITE (miniature inverted-repeat transposable element; Bureau et al., 1996) in the Malus genome and we designated it as Mahk (*Malus* element identified by one of us; Hisayuki Kudo). Furthermore, the entire sequence is characterized by A-T richness (220/330) (Fig. S1-A, see Supplemental Figure 1) and potential to form a hairpin structure (Fig. S1-B), suggesting that the sequence is an inserted transposon (Wessler et al., 1995). To determine the genomic distribution of the MITE element in Malus, we performed Southern blot hybridization (Fig. 3) using HindIII-digested genomic DNA from eight Malus species, two Pyrus species, two Spiraea species, and one species each of Chaenomeles and Sorbus. Hybridization in all Malus species genomes...
showed a smeared signal, indicating that Mahk elements are present in high copy number. A similar result was obtained with DNA from Chaenomeles lagenaria, whereas the other plants showed no or only very weak (Pyrus pyrifolia, Pyrus communis and Sorbus commixta) smear hybridization signals.

**MdACS3 expression precedes MdACS1 expression**

Northern hybridization using different apple tissues (Fig. 4) revealed no transcription of MdACS3 in non-fruit organs, suggesting that its expression may be fruit-specific. The cDNAs synthesized from total RNA of ‘Golden Delicious’ on-tree fruit were analyzed by CAPS, in which the last exon of MdACS3a was amplified and double digested by HindIII/EcoRI and then electrophoresed on 2% agarose gel. The band pattern of the digested cDNA was the same as that from MdACS3a (Fig. 5), suggesting that only the transcript of MdACS3a is present in apple fruit.

To test the expression pattern of the MdACS3a gene during fruit ontogeny, we carried out northern hybridization of RNA fractions extracted from fruits which were sampled weekly from the full blossom to ripening stages (Fig. S2). Expression of MdACS3a began before the commercial harvest day, but MdACS1 and MdACO1 were expressed only after commercial harvest. These results indicate that MdACS3a functions as a ripening-specific gene, like MdACS1 and MdACO1, at least two weeks before the burst of ethylene production caused by expression of MdACO1 and MdACS1.

Fig. 6 shows changes in ethylene production and expression of ripening-related genes,
including \textit{MdACS3a}, in ‘Golden Delicious’ fruit. A strong signal of \textit{MdACS3a} was observed at Oct. 9, when \textit{MdACS1}, \textit{MdACO1} and \textit{MdPG1} had not yet begun to be expressed, and persisted to Oct. 27. Expression of \textit{MdACS3a} decreased gradually from Oct. 21 after the massive increase in \textit{MdACO1} and \textit{MdACS1} expression, suggesting that expression of \textit{MdACS3a} is suppressed when fruit enter ripening with a burst of ethylene production. In order to investigate whether the expression of \textit{MdACS3a} is affected by ethylene, we analyzed its transcript in 1-MCP-treated fruit (cv ‘Golden Delicious’). The expression of \textit{MdACS1}, \textit{MdACO1} and \textit{MdPG1} was completely inhibited by 1-MCP treatment, but the signal of \textit{MdACS3a} expression was observed until 12 days (Fig. 7).

\textbf{A null mutation exists in the apple genome}

El-Sharkawy et al. (2004) and Itai et al. (2000) have reported that the allelic pattern of the \textit{MdACS3a} ortholog in pear is involved in fruit ripening, causing us to investigate the relationship between \textit{MdACS3a} and fruit ripening in apple. To analyze the allelic \textit{MdACS3a} genes, we performed direct sequencing of \textit{MdACS3a} genomic DNA of cv ‘Golden Delicious’. The presence of an allele can be identified where a sequenced nucleotide was judged as N due to the appearance of two clear peaks at the same position (Fig. S3-C). Some SNPs (single nucleotide polymorphisms) were found, and one of which was a G→T change in the protein-coding region resulting in replacement of Gly289 by a Val residue. We compared the predicted protein sequence with that of \textit{ACS} genes in other species, revealing that G289 is located in the active site of ACC synthase (Yip et al., 1990; Liang et
Furthermore, this amino acid substitution (G→V) has not been found in any ACS genes of other plant species reported so far (Yip et al., 1990; Lincoln et al., 1993; Itai et al., 1999; El-Sharkawy et al., 2004, 2008). These results indicate that G289V is a unique mutation in Malus causing an allele of ACS3a, ACS3a-G289V.

### Enzyme activity of ACS3a and ACS3a-G289V

To test whether this amino acid substitution affects the enzyme activity of ACS3a, full-length cDNAs of ACS3a and ACS3a-G289V were constructed into the expression vector pET11d. As a positive control, cDNA of MdACS1 (Sunako et al., 1999) was also constructed into the vector. DNA sequencing was performed to confirm that no spurious mutations were introduced. Proteins expressed in E. coli cells were fractioned and assayed for enzyme activity. Two-dimensional electrophoresis was performed to confirm the existence of the ACS3a and ACS3a-G289V proteins. The protein spots corresponded to a molecular mass of 50 kDa, identical with the predicted molecular mass of ACS3a (Fig. S4).

Enzyme activity assay showed that the activity of ACS3a was 1/40 of that of ACS1, whereas ACS3a-G289V was inactive (Fig. 8), suggesting that G289 is essential for the activity of ACS3a.

### MdACS3a allelic genotypes determined by the genomic and cDNA sequences

The ACS3a allelic genotype of each cultivar was determined from the relative abundance of SNPs in genomic and cDNA sequences, which were analyzed by direct sequencing (Fig.
Cultivars with either G alone or T alone in both their genomic and cDNA sequences were identified as $ACS3a/ACS3a$ or $ACS3a\text{-}G289V/ACS3a\text{-}G289V$, respectively (Fig. S3-A or -B). Cultivars having both G and T in their genomic and cDNA sequences were identified as $ACS3a/ACS3a\text{-}G289V$ (Fig. S3-C).

During the SNP analyses, some cultivars were assessed as $ACS3a/ACS3a\text{-}G289V$ in their genomic sequence, but only $ACS3a\text{-}G289V$ was found in the cDNA sequences. For example, in apple ‘Kitaro’, two peaks (G and T) appeared in the sequencing trace for genomic DNA but only one peak (T) appeared in the cDNA sequence (Fig. S3-D). This result suggests that a null gene, with no ability to be transcribed, exists in some apple varieties, and it was designated as $Mdacs3a$. Thus the allelic genotype of ‘Kitaro’ is $acs3a/ACS3a\text{-}G289V$.

Consequently, we were able to infer the genotype of apple ‘Fuji’ from that of ‘Kitaro’, the progeny derived from a cross between ‘Fuji’ and ‘Hatsuaki’ ($ACS3a\text{-}G289V/ACS3a\text{-}G289V$), indicating that ‘Fuji’ is $ACS3a/acs3a$. Table S1 (see Supplemental Table 1) lists the $ACS3a$ allelic genotypes of the apple cultivars we have investigated. Thus, we conclude that ACS3a is inactive in $ACS3a\text{-}G289V/ACS3a\text{-}G289V$ and $acs3a/ACS3a\text{-}G289V$ cultivars.

An SSR linked to $ACS3a\text{-}G289V$

In order to develop a DNA marker to identify $MdACS3a$ alleles, we analyzed the promoter region of each allele from several apple cultivars. The promoter region (-90 – -530) of each cultivar was cloned and sequenced. A dinucleotide ‘GA’ repeat sequence
(SSR, simple sequence repeat) was found at -420 of the promoter, and contained 20 repeats in mutated ACS3a-G289V but only 9 repeats in wild-type ACS3a (Fig. 9A). Sequencing the counterpart region of ACS3a amplified from the wild species M. coronaria (ACS3a-G289V/ACS3a-G289V) showed 20 repeats in the SSR, suggesting that the repeat polymorphism is linked to the respective SNPs of ACS3a and ACS3a-G289V.

The SSR region of each cultivar was amplified and run on 6% polyacrylamide gels (Fig. 9B). It is possible to identify from the band pattern whether apple cultivars contain the ACS3a-G289V allele. Fig. S5 shows the presence of ACS3a-G289V allele in the apple cultivars and wild species we have investigated. ACS3a-G289V was found in the wild species Malus coronaria, indicating that this mutation occurred before apple domestication. In addition, ACS3a-G289V exists not only in cultivars bred in Japan but also in those bred in other countries such as New Zealand, America and Australia (Fig. S5).

**Expression profile of ripening-related genes in ‘Kitaro’, acs3a/ACS3a–G289V**

Fig. 10 shows changes in ethylene production and expression of ripening-related genes in apples ‘Kitaro’ and ‘Kotaro’ which are derived from the same cross parents. Ethylene production was much higher in ‘Kotaro’ than in ‘Kitaro’. Expression of MdACS1 was not detected in ‘Kitaro’ by Northern blotting, but distinct bands were observed in ‘Kotaro’ after storage at 24°C for 3-9 d. Trace expression of MdACS3a was detected in ‘Kotaro’, whereas the expression was quite strong in ‘Kotaro’. Although MdACO1 was expressed in both cultivars, it was much stronger in ‘Kotaro’. Expressions of MdPG1, which is related to the
softening of apple fruit (Wakasa et al., 2006), was not detected in ‘Kitaro’ but expressed
clearly in ‘Kotaro’. A similar pattern was obtained from the comparison of ‘Gala’
(ACS3a/ACS3a-G289V) and ‘Koukou’ (acs3a/ACS3a-G289V) (Fig. S6). These results show
that acs3a/ACS3a-G289V cultivars have a longer shelf life than ACS3a/ACS3a-G289V
cultivars, suggesting that the shelf life of apple fruit is under the control of ACS3a
allelotypes.

DISCUSSION

Levels of ethylene production in ripening fruit are broadly in agreement with the
presence of particular MdACS1 allelic forms (Sunako et al., 1999; Harada et al., 2000).
However, there are differences even within the same allelic forms, suggesting the effect of
other ripening-related MdACS gene(s) (Rosenfield et al., 1996), as shown in pear fruit (Itai
et al., 1999; El-Sharkawy et al., 2004). By screening a genome DNA library, we identified
another ACC synthase gene subfamily in apple, MdACS3. Two of the genes in the
subfamily, MdACS3b and c, are not transcribed to mRNA because of a transposon insertion,
and thus only MdACS3a is functional. Furthermore, MdACS3a in some cultivars is present
as a mutant allele, MdACS3a-G289V, in which a single nucleotide polymorphism leading to
a single amino acid substitution results in an inactive protein. In addition, another allele,
Mdacs3a, is found as a null allele because of failure of transcription. The existence of these
three alleles made it possible to relate differences in ethylene production to shelf life among
apple cultivars.
In apple, expression of *MdACS3* preceded that of *MdACS1* and *MdACO1*, and decreased after the robust expression of *MdACS1* and *MdACO1* and the burst of ethylene production (Fig. 6) (Wang et al., 2009). Moreover, 1-MCP treatment did not affect the expression of *MdACS3a* (Fig. 7), similar result was also reported by Tatsuki et al. (2007). These results clearly demonstrate that the expression of *MdACS3a* is regulated by a negative feedback mechanism in apple fruit. This negative regulation has also been reported in tomato (Barry et al., 2000; Nakatsuka et al., 1998). Taking into account that system-1 ethylene is negatively autoregulated (Lelièvre et al., 1997; Barry et al., 2000), it is probable that in apple *MdACS3* functions in regulating system-1 ethylene biosynthesis and the transition to system 2, and that the burst of system-2 ethylene negatively feeds back to system 1, resulting in the decrease in *MdACS3* expression.

According to the phylogenetic relationships of the *Maloideae* reported by Campbell et al. (1995), the position of *Chaemomeles* is closer to that of *Malus* than to that of *Pyrus*. Strong smear hybridization signals for the MITE found in this study were observed in digested genomic DNA of both *Malus* species and *Chaenomeles lagenaria*, but not in that of *Pyrus* (Fig. 3). These data support the result of Campbell et al. (1995). Therefore, it appears that the amplification of *Mahk* occurred in *Malus* at least after the divergence from *Pyrus*.

ACC synthase belongs to the group of pyridoxal-5′-phosphate (PLP)-dependent enzymes, and residue K283 is necessary for PLP binding (Capitani et al., 1999). Moreover, the enzyme functions as a dimer whose active site is formed from the interaction of residues from the monomeric subunits (Tarun and Theologis, 1998). Therefore, if the G289V
mutation in MdACS3a affects the formation of the active site as well as the case of K278A in AtACSs (Tsuchisaka and Theologis, 2004), it would influence heterodimeric interactions with other MdACS, which expresses after MdACS3a in apple fruit. Furthermore, Tsuchisaka and Theologis (2004) reported that AtACS7, a unique ACS missing the hypervariable carboxyl terminus, can form functional heterodimers with other ACS only when it provides the K278 residue. Interestingly, the MdACS3a is the homologue of AtACS7 missing the C terminus. In our study, apple cultivars could be classified into several genotypes according to their homozygosity for ACS3a alleles (Table S2). For ACS3a/ACS3a, the enzymatic activity is normal; however, for the ACS3a-G289V/ACS3a-G289V and acs3a/ACS3a-G289V genotypes, ACS3a must be inactive. For ACS3a/ACS3a-G289V genotype, the ACS3a couple dimer can function, but the ACS3a/ACS3a-G289V dimer would be inactive. The same situation may occur in the case of heterodimerization between MdACS3a-G289V and MdACS1.

Ethylene production was much lower in the ‘Kitaro’ than in ‘Kotaro’, and the expression of ripening-related genes was also greatly suppressed in ‘Kitaro’ (Fig. 10). A similar result was found in the comparison of ‘Gala’ and ‘Koukou’ (Fig. S6). These differences can be explained by the respective MdACS3a allelotypes of these cultivars, because ‘Kitaro’ and ‘Koukou’ are acs3a/ACS3a-G289V with inactive ACS3a, whereas ‘Kotaro’ and ‘Gala’ are ACS3a/ACS3a-G289V with active ACS3a. In ‘Kitaro’ and ‘Koukou’ (Fig. S7), system-2 ethylene synthesis may not occur because acs3a/ACS3a-G289V cannot produce sufficient ethylene to initiate the transition to system-2 ethylene synthesis, and thus downstream genes,
especially *MdPG1*, were not expressed in ‘Kitaro’ and ‘Koukou’. This supports the hypothesis that ACS3a acts as a switch to initiate the transition from system-1 to system-2 ethylene synthesis, subsequently leading to a burst of ethylene production. Thus, ACS3a is likely to be the main enzyme that controls ethylene production and shelf life of apple fruit. This conclusion was also supported from the data of other cultivars (Table S2).

Alignment of *MdACS3a* orthologs of more than 100 plant species revealed that the G289V mutation is found only in *Malus*, including wild species and domesticated cultivars from America, Australia, Europe, New Zealand and Japan (Fig. S5). It was not found in pear, although this species is very closely related to apple. These results indicate that the G289V mutation arose after separation of *Malus* from the Maloideae and was then maintained during domestication and inherited stably among the domesticated cultivars.

The difference between *MdACS3a* and the null gene *Mdacs3a* is still unclear, and understanding the difference will lead to development of a molecular marker for *Mdacs3a*. No transposon or other insertion was found in the promoter region of the null gene, suggesting that the difference might occur much further upstream of the promoter than we have investigated. Matarasso et al. (2005) reported that a Cys protease (LeCp) regulates the expression of tomato *LeACS2* by binding to a *cis* element (TAAAAT motif), and *Cp* was found to be induced by fruit ripening (Alonso and Granell, 1995). Sequence analysis revealed that alleles of *MdACS3a* also possess this motif. Further study is needed to investigate whether mutation of this motif causes the null allele *Mdacs3a*. In addition, other factors that regulate gene expression, such as DNA methylation, should be taken into account.
CONCLUSIONS

This study describes molecular evidence that accounts for the difference of apple fruit shelf life among cultivars. Out of three genes of ripening-specific MdACS3 family, only one (MdACS3a) expresses at the transition from system 1 to system 2 ethylene biosynthesis. We demonstrated here that the existence of three alleles of MdACS3a made it possible to relate differences in ethylene production to shelf life among apple cultivars. These results have allowed us to propose that MdACS3a plays a crucial role in regulation of fruit ripening in apple, and may be the main determinant of ethylene production and shelf life in apple fruit.

MATERIALS AND METHODS

Plant materials

Young expanding leaves of apple (Malus × domestica Borkh.) cultivars and wild species sampled from the experimental farms of Aomori Apple Experimental Station (Japan) and Hirosaki University were used as a source of genomic DNA, which was extracted as described by Sunako et al. (1999). Fruits on-tree (cv Golden Delicious) were collected weekly from full-bloom to ripening stage and were treated as described by Wakasa et al. (2003). Fruit materials used for RNA gel blot analysis were obtained as described by Wakasa et al. (2006). Briefly, mature fruits, collected at commercial harvest day from an orchard of the Aomori Apple Experimental Station, were kept at 24°C and sampled every 3...
d. The commercial harvest day of each cultivar is September 29 for ‘Gala’, October 9 for ‘Kitaro’, October 21 for ‘Golden Delicious’, October 23 for ‘Kotaro’ and October 28 for ‘Koukou’, respectively. Young expanding leaves, *in vitro*-cultured shoots, roots emerged from seeds, and receptacles and pistils of full-bloom flowers of ‘Golden Delicious’ were also sampled for analysis of gene expression. Young leaves of the following plants collected at the experimental farms of Hirosaki University and Aomori Apple Experimental Station were also used for Southern blot analysis: cultivated apple (*Malus domestica* cv. Fuji), wild apple species (*M. sieboldii, M. hupehensis, M. transitoria, M. arnoldiana, M. platycarpa, M. robusta, M. floribunda*), Japanese pear (*Pyrus pyrifolia* cv. Kosui), pear (*Pyrus communis* cv. La France), quince (*Chaenomeles lagenaria* Koidz.), rowan (*Sorbus commixta* Hedl.), and spiraea (*Spiraea thunbergii* Sieb.; *Spiraea cantoniensis* Lour.).

**Genomic library screening and sequence analysis**

A genomic DNA library obtained from ‘Golden Delicious’ (Sunako et al., 1999) was screened with a $^{32}$P-radiolabeled ACS3 probe made by PCR using primers ACS3-7 and ACS3-14 (Table S3, see Supplemental Table 1). Genomic DNA from ‘Golden Delicious’ was cloned as described by Sunako et al. (1999). The positive phage clones were used to make a restriction map, and fragments containing the ACS3 gene were subcloned into the pBluescript II KS vector (Stratagene) and sequenced.

**Measurements of ethylene production rates**
Intact fruits were enclosed in a gas-tight container (0.8 L) equipped with septa and kept at 24°C for 1 h, then 1 ml of gas was sampled through the headspace of the container by a syringe. The ethylene concentration in the sample was measured with a gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector. Five fruits per sample were measured.

Measurements of flesh firmness

Flesh firmness was measured with a hand-held penetrometer (FT-327; Facchini, Italy) fitted with an 11mm-diameter probe. Four skin discs (approximately 2.5 cm in diameter) were removed from opposite sides of each fruit. The probe was pressed into the tissue of the cut surface to a depth of 8–9 mm in a single smooth motion. Five fruits per sample were measured.

1-MCP treatment of fruit

‘Golden Delicious’ fruits were treated with 1 μL L⁻¹ of 1-MCP (EthylBloc; Rohm and Haas, Philadelphia, PA, USA) for 15 h at 24°C. After treatment, fruits were held at 24°C and sampled every 3 d.

DNA extraction and Southern blot analysis

Genomic DNA extraction and Southern blot analysis were performed as described by Sunako et al. (1999). Genomic DNA of apple cultivars and wild species and other species
was digested with *HindIII*, separated in a 0.8% agarose gel, transferred onto a nylon membrane, and hybridized with a *Mahk* probe that had been prepared by PCR with the primer ACS3bTE-1 and ACS3bTE-4 (Table S3).

**RNA extraction and Northern blot analysis**

RNA extraction and Northern gel blot analysis were performed as described by Sunako et al. (1999). The probe for ACS3 expression was prepared by PCR using the ACS3a clone and the oligonucleotide primers ACS3-1 and ACS3-2 (Table S3). Probes for other genes were prepared as described in Wakasa et al. (2006).

**CAPS analysis of PCR and RT-PCR products**

*MdACS3* genes in the genome of apple cultivars were amplified by PCR, which was performed with primers ACS3-17 and ACS3-8 for the first PCR, and ACS3-7 and ACS3-14 (Table S3) for the second PCR. The product digested with *EcoRI* and *HindIII* was electrophoresed on a 2% agarose gel. Two μg of total RNA extracted from the fruit was used for first-strand cDNA synthesis (Superscript II RT, Invitrogen) with primer ACS3-8, and the subsequent PCR was carried out with the same primers as for amplification of genomic DNA.

**Over-expression of MdACS1 and MdACS3a in *E. coli***
cDNAs for MdACS3a and MdACSa3-G289V were amplified by PCR with ACS3a cDNA as template and the primers ACS3a infu-1 and ACS3a infu-2 (Table S3). cDNA for MdACS1 (accession no.U89156) was amplified with the primers ACS1 infu-1 and ACS1 infu-2 (Table S3). The PCR products were then ligated to the NcoI/BamHI double-digested 5.7 kb pET11d (Stratagene) vector by using In-Fusion™ Dry-Down PCR Cloning Kit (Clontech) according to the manufacturer’s instructions. Those recombinants containing the correct sequence were identified and retransformed into an E. coli host, BL21 (DE3) (Novagen). BL21 (DE3) cells harboring a pET11d-ACS3 and pET11d-ACS1 recombinant plasmid were grown on an LB plate in the presence of 100 μg/ml ampicillin. LB medium (5 ml) containing 100 mg/ml of ampicillin was inoculated with a single colony and incubated at 37°C overnight with constant shaking. The overnight culture (5 ml) was transferred into 500 ml of LB medium. Cells were grown at 30°C with constant shaking. When the cell density reached OD600 of 0.5, IPTG was added to the cell culture to a concentration of 0.1 mM. The culture was then transferred to room temperature (20-25°C) for 5 more hours with constant shaking. The cells were harvested by centrifugation at 4000×g for 10 min at 4°C. Cell pellets were stored at -70°C for later use.

**Two-dimensional electrophoresis**

Protein concentration was quantified using the BioRad protein assay with BSA as a standard. Two-dimensional electrophoresis was performed with the ReadyPrep™ 2-D Starter kit (BioRad) according to the instruction manual. Briefly, IPG strips (7 cm, pH
3–10; BioRad ReadyStrip, BioRad) were rehydrated overnight with 125 µl of sample buffer containing 87.5 µg of total protein. Proteins were focused in a PROTEAN IEF Cell (BioRad) at 20°C for 12 h, applying 250 V (15 min), 4000 V (120 min) and 4000 V for a total of 20 KVh. After IEF (isoelectric focusing), strips were equilibrated in equilibration buffers I and II for 10 min, respectively. Second-dimensional SDS-PAGE was run in acrylamide gels (Ready Gel Precast Gel, BioRad) at 200 V for 45 min. Gels were stained with colloidal CBB G-250.

**ACC synthase activity assay**

ACC synthase extract was prepared according to the method of Li et al. (1996). The ACC synthase extract was subsequently concentrated with a Nanosep Centrifugal Device (PALL, USA) according to the manufacturer’s instructions. The amount of ACC was determined by the method of Lizada and Yang (1979). ACC synthase activity was assayed by incubating the ACC synthase extract with AdoMet in a reaction buffer for 1 h at 30°C (Li et al., 1996). One unit of ACC synthase activity is defined as 1 nmol of ACC formed per hour at 30°C. Protein concentrations were determined with Bio-Rad protein assay reagent (http://www.bio-rad.com).

**Nucleotide sequence and SSR analysis**

For sequence analysis of genomic DNA of *MdACS3a* to determine the genotype, a first PCR was performed with primers MdACS3a-1F and MdACS3a-2R (Table S3) and genomic
DNA as template. For SNPs sequencing, a second PCR was conducted with the primers MdACS3a-2F and MdACS3a-2R with the first PCR product as the template. All cycle sequencing reactions for DNA sequence analysis were performed using Big Dye terminator chemistry (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocols, and sequences were determined using an ABI 310 automated DNA sequencer.

For SSR analysis, primers MdACS3a-1F and MdACS3a-1R were used to amplify the SSR region from the first PCR product. PCR product was run on 6% polyacrylamide gel and stained with silver according to Bassam et al. (1991).

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LITERATURE CITED


Rosenfield CL, Kiss E, Hrazdina G (1996) MdACS-2 (Accession No. U73815) and


Figures legends

Fig. 1. Genomic structure of *MdACS3* genes. Solid boxes indicate the coding regions. The lines connecting the solid boxes represent the two introns and the flanking (5’ and 3’) regions. Open boxes indicate the insertion sequence, which was not observed in *ACS3a*. Halftone boxes show GA repeat sequences. The numbers shown under lines and boxes indicate nucleotide numbers. Arrows indicate the position of primers used for CAPS analysis to identify the various genes. H and E indicate the restriction enzyme sites of *HindIII* and *EcoRI* on each exon 3.

Fig. 2. CAPS analysis of the *MdACS3* family. PCR products amplified from part of the last exon were digested by *HindIII*/*EcoRI* and then electrophoresed on 2% agarose gels. Lanes a, b, and c are the products from subcloned fragments containing *MdACS3a*, 3b, and 3c, respectively. Lanes 1 to 6 are the products from the genomic DNA of the following cultivars as the template. Lane 1, cv Golden Delicious; 2, cv Ralls Janet; 3, cv Fuji; 4, cv Delicious; 5, cv Kitaro; 6, cv Kotaro. The signal at 154 bp is a little faint compared with the other bands because the fragment is produced only from *MdACS3b*.

Fig. 3. Southern blot analysis of the genomic distribution of *Mahk* in *Malus* and other species. Genomic DNA was digested with *HindIII*, separated on 0.8% agarose gels,
transferred onto a nylon membrane and probed with Mahk. A, *Malus* species: 1; *M. domestica* cv. Golden Delicious, 2; *M. domestica* cv. Fuji, 3; *M. sieboldii*, 4; *M. hupehensis*, 5; *M. transitoria*, 6; *M. arnoldiana*, 7; *M. platycarpa*, 8; *M. robusta*, 9; *M. floribunda*. B, other species: 10; Japanese pear (*Pyrus pyrifolia* cv. Kosui), 11; pear (*Pyrus communis* cv. La France), 12; quince (*Chaenomeles lagenaria* Koidz), 13; rowan (*Sorbus commixta* Hedl.), 14; spiraea (*Spiraea thunbergii* Sieb), 15; *Spiraea cantoniensis* Lour.

Fig. 4. RNA gel blot analysis of *MdACS3* in ripening fruit and non-fruit tissues. Each lane was loaded with total RNA from fruit (F), young leaves (L), shoots (Sh), roots (R), stigmata (St), and receptacles (Re). Expression of four ripening-related genes in each apple organ is shown. rRNA is shown as a loading control.

Fig. 5. CAPS analysis of RT-PCR product. Lanes a, b, and c are the same as those in Fig. 2. Oct 5 to Nov 2 show the days when harvested ‘Golden Delicious’ fruit was used as material.

Fig. 6. Changes in ethylene production and expression of *MdACS3a* and other ripening-related genes in on-tree fruit (cv. ‘Golden Delicious’). The fruits used here were sampled in different year from those of Fig. S2. Note that ethylene production scale is indicated by logarithmic display.
Fig. 7. Changes in the accumulation of mRNA of ripening-related genes in apple fruit with or without 1-MCP treatment. Ribosomal RNA was used to normalize RNA loading. Numbers above each lane indicate the number of days after harvest.

Fig. 8. Enzyme activity of ACS3a isoenzymes. As a positive control, the activity of ACS1 was measured by the same method.

Fig. 9. Schematic of MdACS3a and MdACS3a-G289V and diagnosis of the MdACS3a-G289V allele in apple cultivars. A, The gray boxes indicate the exons, and white box indicates the SSRs in the promoter region. The arrows flanking the SSR region indicate the primers used to amplify this region. The vertical lines in the last exon show the position of amino acid substitution. B, The SSR region in the promoter was amplified and electrophoresed on a 6% polyacrylamide gel and stained with silver. The upper band indicates the ACS3a-G289V allele and the lower band indicates the ACS3a or acs3a alleles. The numbers on the top of the lanes indicate different cultivars: Lane 1; Delicious, 2; Fuji, 3; Kitaro, 4; Hatsuaki, 5; Golden Delicious.

Fig. 10. Changes in ethylene production and expression levels of ripening-related genes in cultivars ‘Kitaro’ and ‘Kotaro’. Numbers indicate days after commercial harvest (day 0). “–14” means fruit at 14 d before harvest; “3”–“12” mean days of storage at 24°C. Ribosomal RNA was used as loading control.
The graph shows the increase in ethylene (nl g⁻¹ h⁻¹) production over time, with data points at Oct 9, 15, 21, and 27.

The Western blots below correspond to the following genes:
- **MdACS3a**
- **MdACS1**
- **MdACO1**
- **MdPG1**
- **rRNA**