Null Mutation of the \textit{MdACS3} Gene, Coding for a Ripening-Specific 1-Aminocyclopropane-1-Carboxylate Synthase, Leads to Long Shelf Life in Apple Fruit\textsuperscript{1}[W][OA]

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Expression of \textit{MdACS1}, coding for 1-aminocyclopropane-1-carboxylate synthase (ACS), parallels the level of ethylene production in ripening apple (\textit{Malus domestica}) fruit. Here we show that expression of another ripening-specific ACS gene (\textit{MdACS3}) precedes the initiation of \textit{MdACS1} expression by approximately 3 weeks; \textit{MdACS3} expression then gradually decreases as \textit{MdACS1} expression increases. Because \textit{MdACS3} expression continues in ripening fruit treated with 1-methylcyclopropene, its transcription appears to be regulated by a negative feedback mechanism. Three genes in the \textit{MdACS3} family (\textit{a}, \textit{b}, and \textit{c}) were isolated from a genomic library, but two of them (\textit{MdACS3b} and \textit{MdACS3c}) possess a 333-bp transposon-like insertion in their 5' flanking region that may prevent transcription of these genes during ripening. A single nucleotide polymorphism in the coding region of \textit{MdACS3a} results in an amino acid substitution (glycine-289 → valine) in the active site that inactivates the enzyme. Furthermore, another null allele of \textit{MdACS3a}, \textit{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 \textit{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.

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 (\textit{Malus domestica}) and tomato (\textit{Solanum lycopersicum}; 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 \textit{MdACS1} expression (Sunako et al., 1999; Harada et al., 2000). Moreover, differential expression of \textit{MdACS1} allelic forms (\textit{MdACS1-1} and -2) among different cultivars causes differences in the extent of preharvest 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 (\textit{MdPG1}), which is involved in softening of fruit flesh (shelf life), was transcribed differently in cultivars with the same \textit{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 autoinhibitory, 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 autostimulatory (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 \textit{MdACS1} mRNA in ripening fruit is prevented to a large extent by treatment with...
The nucleotide sequences of the 5′ MdACS3c showed high homology with clones size, as shown in Figure 1. We termed these three predicted introns in both sequence (76%–43%) and their predicted exons, but differed markedly at their homologous to MdACS1 et al. (1996), but their correlation with ripening behaviors 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 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 Figure 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, PcACS2b, and PcACS3 in pear (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 MdACS3c 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 cleaved-amplified polymorphic sequence (CAPS) analysis using HindIII and EcoRI sites (Fig. 1) that produce diverse fragments from the respective MdACS3 genes (Fig. 2). The results indicated that the genomes of the six cultivars and 56 strains tested had all three MdACS3 genes. Then we performed linkage analysis of each MdACS3 gene using two F1 progenies (Igarashi et al., 2008). MdACS3a was located on the 15th linkage group of cv Ralls Janet, whereas MdACS3b was located on the second group of cv Delicious (data not shown). As both of these two linkage groups appear to possess unique genes, this suggests that MdACS3a and MdACS3b are unlikely to be different alleles of the same gene. Furthermore, direct sequencing of the coding and flanking regions of these MdACS3 genes revealed the occurrence of several single nucleotide polymorphisms (SNPs), indicating that MdACS3c must also be an independent gene.
ACS3a Alleles Causing Long Shelf Life in Apple Fruit

High Amount of Inserted Sequence in *Malus*, But Not in *Pyrus*

As shown in Figure 1, *MdACS3b* and *MdACS3c* possess extra sequence (333 bp) that is not present in *MdACS3a*. This sequence is flanked by the 8-bp terminal inverted repeat (5′-AATTTTTA-3′) located at −570 of *MdACS3a* (accession no. AB243060). Querying this insertion in GenBank revealed that it is a miniature inverted-repeat transposable element (MITE; 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; Supplemental Fig. S1A) and potential to form a hairpin structure (Supplemental Fig. S1B), 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 quince (*Chaenomeles lagenaria*), whereas the other plants showed no or only very weak (pear, Japanese pear, and rowan [*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 that were sampled weekly from the full blossom to ripening stages (Supplemental 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 2 weeks before the burst of ethylene production caused by expression of *MdACO1* and *MdACS1*.

**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 *MdACS3a* ortholog in pear is involved in fruit ripening, causing us to investigate the relationship between *MdACS3a* and fruit ripening in apple. To analyze the allelic *MdACS3a* genes, we performed direct sequencing of *MdACS3a* genomic DNA of 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 (Supplemental Fig. S3C). Some SNPs were found, one of which was a G → T change in the protein-coding region, resulting in replacement of Gly-289 by a Val residue. We compared the predicted protein sequence with that of ACS genes in other species, revealing that G289 is located in the active site of ACC synthase (Yip et al., 1990; Liang et al., 1992; Lincoln et al., 1993). 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 *Escherichia 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 kD, identical with the predicted molecular mass of *ACS3a* (Supplemental 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 (Supplemental Fig. S3). Cultivars with either G alone or T alone in both their genomic and cDNA sequences were identified as *ACS3a*/*ACS3a* or *ACS3a-G289V*/ACS3a-G 289V, respectively (Supplemental Fig. S3, A or B). Cultivars having both G and T in their genomic and cDNA sequences were identified as *ACS3a*/ACS3a-G289V (Supplemental Fig. S3C).

During the SNP analyses, some cultivars were assessed as *ACS3a*/ACS3a-G289V in their genomic sequence, but only *ACS3a-G289V* was found in the cDNA sequences. For example, in apple cv Kitaro, two peaks (G and T) appeared in the sequencing trace for genomic DNA but only one peak (T) appeared in the cDNA sequence (Supplemental Fig. S3D). 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-G289V. Consequently, we were able to infer the genotype of apple cv Fuji from that of Kitaro, the progeny derived from a cross between cultivars Fuji and Hatsuaki (*ACS3a-G289V*/ACS3a-G289V), indicating that Fuji is *ACS3a*/acs3a. Supplemental Table S1 lists the *ACS3a* allelic genotypes of the apple cultivars we have investigated. Thus, we conclude that *ACS3a* is inactive in *ACS3a-G289V*/ACS3a-G289V and *acs3a*/ACS3a-G289V cultivars.

A Simple Sequence Repeat Linked to *ACS3a-G289V*

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 to −530) of each cultivar was cloned and sequenced. A dinucleotide GA repeat sequence (simple sequence repeat [SSR]) was found at −420 of the promoter, and contained 20 repeats in mutated *ACS3a-G289V* but only nine repeats in wild-type *ACS3a* (Fig. 9A). Sequencing
Expression Profile of Ripening-Related Genes in Kitaro, {\textit{acs3a}}/\textit{ACS3a}-\textit{G289V}

Figure 10 shows changes in ethylene production and expression of ripening-related genes in apple cultivars Kitaro and Kotaro that are derived from the same cross parents. Ethylene production was much higher in Kotaro than in Kitaro. Expression of \textit{MdACS1} was not detected in Kitaro by northern blotting, but distinct bands were observed in Kotaro after storage at 24°C for 3 to 9 d. Trace expression of \textit{MdACS3a} was detected in Kitaro, whereas the expression was quite strong in Kotaro. Although \textit{MdACO1} was expressed in both cultivars, it was much stronger in Kotaro. Expressions of \textit{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 cv Gala (\textit{ACS3a}/\textit{ACS3a}-\textit{G289V}) and cv Koukou (\textit{acs3a}/\textit{ACS3a}-\textit{G289V}) (Supplemental Fig. S6). These results show that \textit{acs3a}/\textit{ACS3a}-\textit{G289V} cultivars have a longer shelf life than \textit{ACS3a}/\textit{ACS3a}-\textit{G289V} cultivars, suggesting that the shelf life of apple fruit is under the control of \textit{ACS3a} allele types.

DISCUSSION

Levels of ethylene production in ripening fruit are broadly in agreement with the presence of particular \textit{MdACS1} allele forms (Sunako et al., 1999; Harada et al., 2000). However, there are differences even within the same allele forms, suggesting the effect of other ripening-related \textit{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, \textit{MdACS3}. Two of the genes in the subfamily, \textit{MdACS3b} and \textit{c}, are not transcribed to mRNA because of a transposon insertion, and thus only \textit{MdACS3a} is functional. Furthermore, \textit{MdACS3a} in some cultivars is present as a mutant allele, \textit{MdACS3a}-\textit{G289V}, in which a single nucleotide polymorphism leading to a single amino acid substitution results in an inactive protein. In addition, another allele, \textit{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 \textit{MdACS3} preceded that of \textit{MdACS1} and \textit{MdACO1}, and decreased after the robust expression of \textit{MdACS1} and \textit{MdACO1} and the burst of ethylene production (Fig. 6; Wang et al., 2009). Moreover, 1-MCP treatment did not affect the expression of \textit{MdACS3a} (Fig. 7), similar result was also reported by Tatsuki et al. (2007). These results clearly demonstrate that the expression of \textit{MdACS3a} is regulated by a negative feedback mechanism in apple fruit. This negative regulation has also been reported in tomato (Nakatsuka et al., 1998; Barry et al., 2000). Taking into account that system-1 ethylene is negatively autoregulated (Leclèvre et al., 1997; Barry et al., 2000), it is probable that in apple \textit{MdACS3} functions in regulating system-1 ethylene biosynthesis and the transition to system 2, and that the burst of system-2 ethylene

**Figure 8.** Enzyme activity of \textit{ACS3a} isoenzymes. As a positive control, the activity of \textit{ACS1} was measured by the same method.
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 quince, 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-dependent enzymes, and residue K283 is necessary for pyridoxal-5'-phosphate 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 homolog of AtACS7 missing the C terminus. In our study, apple cultivars could be classified into several genotypes according to their homozygosity for ACS3a alleles (Supplemental 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 (Supplemental 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 (Supplemental 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 (Supplemental 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 (Supplemental 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 consideration.

CONCLUSION

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*) 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 (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* ‘Fuji’), wild apple species (*Malus sieboldii*, *Malus hupehensis*, *Malus transitoria*, *Malus arnoldiana*, *Malus platycarpa*, *Malus robusta*, *Malus floribunda*), Japanese pear (*Pyrus pyrifolia* ‘Kosai’), pear (*Pyrus communis* ‘La France’), quince (Chaenomeles lagenaria), rowan (*Sorbus commixta*), and spiraea (*Spiraea thunbergii*; *Spiraea cantoniensis*).

Genomic Library Screening and Sequence Analysis

A genomic DNA library obtained from Golden Delicious (Sunako et al., 1999) was screened with a 32P-radiolabeled ACS3 probe made by PCR using primers ACS3-7 and ACS3-14 (Supplemental Tables S1 and S3). 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) 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; Facchinetti) fitted with an 11-mm-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 to 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) 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 were separated by agarose gel electrophoresis and transferred to a nylon membrane. Hybridization was performed as described by Sunako et al. (1999). DNA was digested with the enzymes indicated in Supplemental Table S1.
species and other species was digested with HindIII, separated in a 0.8% agarose gel, transferred onto a nylon membrane, and hybridized with a Malaki probe that had been prepared by PCR with the primer ACS3BTE-1 and ACS3BTE-4 (Supplemental 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 (Supplemental Table S3). Probes for other genes were prepared as described in Wakasa et al. (2006).

CAPS Analysis of PCR and Reverse Transcription-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 (Supplemental Table S3) for the second PCR. The product digested with EcoRI and HindIII was electrophoresed on a 2% agarose gel. Two micrograms 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.

Overexpression of MdACS1 and MdACS3a in Escherichia coli

cDNAs for MdACS3a and MdACS3a-G289V were amplified by PCR with ACS3a cDNA as template and the primers ACS3a-inf1 and ACS3a-inf2 (Supplemental Table S3). cDNA for MdACS1 (accession no. U89156) was amplified with the primers ACS1-inf1 and ACS1-inf2 (Supplemental Table S3). The PCR products were then ligated to the NotI/BamHI double-digested 5.7 kb pET1d (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 Escherichia coli host, BL21 (DE3; Novagen), BL21 (DE3); cells harboring a pET11d-ACS3 and pET11d-ACS1 recombinant plasmid were grown on a Luria-Bertani (LB) plate in the presence of 100 μg/mL ampicillin. LB medium (5 mL) containing 100 mg/mL 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, isopropyl-β-d-thiogalactopyranoside was added to the cell culture to a concentration of 0.1 mM. The culture was then transferred to room temperature (20°C–25°C) for 5 more hours with constant shaking. The cells were harvested by centrifugation at 4,000g 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 Bio-Rad protein assay with bovine serum albumin as a standard. Two-dimensional electrophoresis was performed with the ReadyPrep 2-D starter kit (Bio-Rad) according to the instruction manual. Briefly, immobilized pH gradient strips (7 cm, pH 3–10; Bio-Rad ReadyStrip, Bio-Rad) were rehydrated overnight with 125 μL of sample buffer containing 87.5 μg of total protein. Proteins were focused in a PROTEAN IEF cell (Bio-Rad) at 20°C for 12 h, applying 250 V (15 min), 4,000 V (120 min), and 4,000 V for a total of 20 KWh. After isoelectric focusing, strips were equilibrated in equilibration buffers I and II for 10 min at 4°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 (Supplemental Table S3) and genomic DNA as template. For SNP sequencing, a second PCR was conducted with the primers MdACS3a-1F 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) 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).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AB243060, AB243061, and AB243062.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Sequence and secondary structure of Malaki.

Supplemental Figure S2. Changes in MdACS3a expression during fruit ontogeny.

Supplemental Figure S3. Sequencing traces of genomic DNA and cDNA of each MdACS3a allelic genotype.

Supplemental Figure S4. Two-dimensional electrophoresis analysis of ACS3a proteins expressed in E. coli.

Supplemental Figure S5. Presence of ACS3a-G289V in apple cultivars and wild species.

Supplemental Figure S6. Changes in ethylene production, flesh firmness, and ripening-related genes.

Supplemental Figure S7. Fruit of cultivars Kitaro and Koukou.

Supplemental Table S1. ACS3a allelic genotypes of apple cultivars.

Supplemental Table S2. Apple cultivars and these ripening characters.

Supplemental Table S3. Primers used for the PCR amplification in this study.

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