Distribution of *MdACS3* null alleles in apple (*Malus × domestica* Borkh.) and its relevance to the fruit ripening characters

**Songling Bai**<sup>1,2</sup>, **Aide Wang**<sup>1,2,6</sup>, **Megumi Igarashi**<sup>3</sup>, **Tomoyuki Kon**<sup>4</sup>, **Tomoko Fukasawa-Akada**<sup>4</sup>, **Tianzhong Li**<sup>5</sup>, **Takeo Harada**<sup>1,2</sup> and **Yoshimichi Hatsuyama**<sup>*3</sup>

1) Faculty of Agriculture and Life Science, Hirosaki University, 3-Bunkyo, Hirosaki, Aomori 036-8561, Japan
2) The United Graduate School of Agricultural Sciences, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan
3) Hirosaki Industrial Research Institute, Aomori Prefectural Industrial Technology Research Center, 80 Fukuromachi, Hirosaki, Aomori 036-8363, Japan
4) Apple Research Institute, Aomori Prefectural Industrial Technology Research Center, 82-9 Tanaka, Kuroishi, Aomori 036-0522, Japan
5) College of Agronomy and Biotechnology, China Agricultural University, Beijing 100193, China
6) Present address: Shenyang Agricultural University, China

Expression of *MdACS3a*, one of the ripening-related ACC synthase genes, plays a pivotal role in initiating the burst of ethylene production by *MdACS1* in apple fruit. Although previous studies have demonstrated the presence of *MdACS3a*-null alleles through deficiency of transcription activity or loss of enzyme activity due to amino acid substitution, which may affect the storage properties of certain fruit cultivars, an overall picture of these null alleles in cultivars is still lacking. The present study investigated the distribution of null allelic genes in 103 cultivars and 172 breeding selections by using a simple sequence repeat (SSR) marker linked to them. The results indicated that both allelic genes were widely distributed throughout the examined cultivars and selections, some occurring as the null genotype, either homozygously or heterozygously, with each null allele. The implications of *MdACS3a* distribution results and the influence of its null allelotypes in fruit characters are discussed.

**Key Words:** *MdACS3*, apple, ethylene, ripening, allelotypes, breeding.

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**Introduction**

In climacteric fruits, the process of ripening is accompanied by a marked increase of respiration, which is preceded by ethylene production (Seymour et al. 1993). Since both an ethylene receptor antagonist and RNAi directed against genes linked to ethylene biosynthesis inhibit the ripening process (Dandekar et al. 2004, Guis et al. 1997, Watkins et al. 2000), it is generally accepted that ethylene production is the primary factor leading to the onset of ripening. The first step is the formation of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, from S-adenosyl-L-methionine (SAM) by ACC synthase (ACS). The second step converts ACC to ethylene through the action of ACC oxidase (ACO). In general, the former is the rate-limiting enzyme for ethylene synthesis (Gorny and Kader 1997, Gussman et al. 1993, Yang and Hoffman 1984).

Two systems of ethylene production in higher plants have been proposed (McMurchie et al. 1972). System 1, the ethylene auto-inhibitory system, is considered to function during normal vegetable growth and to be responsible for the basal level of ethylene production. System 2 is instrumental in the upsurge of ethylene production during the ripening of climacteric fruit when ethylene is auto-stimulatory (Barry et al. 2000, Lelievre et al. 1997, Seymour et al. 1993). The transition of ethylene production from system 1 to system 2 is considered to be an important step during fruit ripening, and is developmentally regulated (Pech et al. 2008). In tomato (*Solanum lycopersicum*), which is the primary model for climacteric fruit ripening, system 1 ethylene production is regulated by the expression of *LeACS1A* and *LeACS6*. During the transition period, *LeACS1A* expression is increased, and this is followed by induction of *LeACS4* and *LeACS2* expression in system 2 (Barry et al. 2000, Nakatsuka et al. 1998). In the case of apple (*Malus × domestica*), though four *MdACS* gene families were reported until present (Wang et al. 2009), *MdACS1*, which is predominantly expressed in climacteric fruit, is considered to be involved in system 2 ethylene biosynthesis (Sunako et al. 1999) because its expression is enhanced by ethylene.

Another ripening fruit-specific *ACS* gene is *MdACS3*, which consists of three subfamily genes (a, b, c) located respective
loci, but two of them (b, c) possess a transposon-like insertion in their 5′-flanking region, which causes failure of their transcriptions (Wang et al. 2009). Therefore, MdACS3a is only functional ACS3 gene in apple. It expresses transiently just before the expression of MdACS1, MdACO1 and other ripening-related genes such as the β-polygalacturonase (Wakasa et al. 2006) and expansin (Wakasa et al. 2003) genes, indicating that the function of MdACS3a is pivotal in regulating the transition from system 1 to system 2 ethylene biosynthesis. Similar involvement of at least two ACS genes has been reported in the ripening process of Pyrus (El-Sharkawy et al. 2004, Itai et al. 1999, 2000).

We have previously reported the presence of two null MdACS3a allelotypes in apple cultivars (Wang et al. 2009). The first is ACS3a-G289V, which has an amino acid substitution altering glycine (GCT) to valine (TCT) in codon 289 at the active site of MdACS3a, resulting in loss of the enzyme activity. The second null allele is mdacs3a, which exhibits an absence of the transcript, indicating that this allelotype is deficient in transcription activity. Rough identification of these allelotypes using a simple sequence repeat (SSR) marker linked to the MdACS3a allelotype has revealed that the differences in fruit storage properties among several apple cultivars may be explained by these allelotypes (Wang et al. 2009). Therefore, the use of DNA markers for the MdACS3a allelotype would be very valuable in apple breeding to select candidates with better storage properties.

Here, we investigated theMdACS3a allelotypes of 103 cultivars and 172 selections using DNA markers. The results indicated that two null allelotypes were widely distributed in the examined cultivars and selections. The relationship of these allelotypes to fruit ripening character is discussed.

Materials and Methods

Plant materials

One hundred three cultivars and 172 selections from the Aomori Apple Research Institute breeding program were randomly selected and used in this study. The parentages of the cultivars are listed in Supplemental Table 1. Young expanding leaves of sampled trees were used as the source of genomic DNA, which was extracted as described by previously (Sunako et al. 1999). Fruits of some cultivars were collected on the day of commercial harvest from the orchard of the Aomori Apple Research Institute, sliced, and then stored at ~80°C until use for RNA extraction.

SSR analysis

Primers MdACS3a-1F2 and -1R for ACS3a were used (Supplemental Table 2). The amplification conditions were as follows: initial denaturation at 94°C for 4 min; 32 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and a final extension for 5 min at 72°C. A 2-μl aliquot of the PCR product was added to 4 μl post-PCR labeling reaction buffer [15 mM Tris-HCl, pH 8.3, 15 mM MgCl2, 0.1 μM TAMRA-ddCTP, 0.006 U μl⁻¹ Klenow fragment and 0.0288 U μl⁻¹ Thermos Sequenase DNA Polymerase] (Kukita and Hayashi 2002). The mixed solution was incubated at 37°C for 5 min, and then at 57°C for 15 min. The reaction was stopped by addition of 6 μl 20 mM EDTA. Appropriate amounts of labeling products were precipitated with ethanol before loading onto an ABI3100 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). The resulting electropherogram was analyzed using GeneScan Analysis Software and Genotyper Software (Applied Biosystem).

Sequencing of MdACS3a allelic genes

The promoter region and the coding region of the MdACS3a allelic genes were amplified with the primers MdACS3a pro-LF/LR and ACS3a-infI-infu-2 (Supplemental Table 2). PCR was conducted with iProof DNA polymerase (Bio-rad) under the following conditions: pre-denaturation at 98°C for 1 min, 35 cycles for denaturation at 98°C for 15 s, annealing at 55°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 5 min. The PCR products were inserted into the pGEM-T easy vector (Promega). At least 8 clones for each apple cultivar were sequenced with the ABI 3500 sequencer, and the sequence data were analyzed with BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The accession numbers (Genbank) are JF833088 and JF833309.

CAPS (cleaved amplified polymorphic sequence)/dCAPS analysis of PCR products

The upstream region of MdACS3a was amplified by PCR using the primers MdACS3a-1F and -1R. The PCR products were treated with ScaI for 2 h at 37°C and then separated on a 1.5% agarose gel. dCAPS (derived cleaved amplified polymorphic sequence) analysis was performed (Neff et al. 1998) as follows. The primers MdACS3a G289V dCAPS-Fw/-Rv were designed using the online software dCAPS finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html) (Neff et al. 2002). PCR was carried out for 30 cycles of denaturation at 94°C for 3 min, annealing at 52°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 3 min. The products were digested with SpeI for 3 h at 37°C and separated on a 2.5% agarose gel. To detect the transcription of the allele, dCAPS primers MdACS3a RT dCAPS -F/-R BamHI, which can yield products with different sizes from the cDNA and genomic DNA because of inclusion of the intron, were designed. The primers and PCR conditions were the same as those described above. The products were digested with BamHI for 3 h at 30°C and separated on a 2.5% agarose gel. All the primers used are listed in Supplemental Table 2.

RNA extraction from flesh and cDNA synthesis

Total RNA was extracted according to the methods described by (Gasic et al. 2004) with some modification. In brief, the ground apple flesh was first washed in washing buffer (Hu et al. 2002), and then treated twice with phenol-chloroform-isoamyl alcohol (25 : 24 : 1). RNA was
precipitated with isopropanol and washed with 70% ethanol. After resuspension with DEPC-treated water, the RNA solution was then treated with chloroform-isooamyl alcohol (24:1) and precipitated with 2.5 volumes of 99% ethanol. Genomic DNA was eliminated from total RNA with a TURBO DNA-free Kit (Ambion). The cDNAs used for RT-PCR were synthesized from 300 ng of total RNA with a SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer’s instructions.

Results

SSR marker for MdACS3a allelic genotypes

Our previous study revealed that a dinucleotide ‘GA’ repeat SSR locating 420 bp upstream of the start codon shows polymorphism among the alleles of MdACS3a (Wang et al. 2009). Therefore, the lengths of PCR amplicons in cultivars and selections were investigated as possible SSR markers for the allelotypes. A representative electrophoretogram is shown in Supplemental Fig. 1 and the results obtained are listed in Table 1 and Fig. 1. Six kinds of amplified product varying in length from 331 to 361 bp (hereafter expressed as only numbers) were identified among the 275 materials (103 cultivars and 172 selections). The differences in length between respective nucleotide numbers were all multiples of 2 bp, indicating that the length difference was due to dependence on the SSR ‘GA’ repeat number. Five triploid cultivars, including ‘Mutsu’, exhibited three amplified products only numbers) were identified among the 275 materials (103 cultivars and 172 selections). The differences in length between respective nucleotide numbers were all multiples of 2 bp, indicating that the length difference was due to dependence on the SSR ‘GA’ repeat number. Five triploid cultivars, including ‘Mutsu’, exhibited three amplified products.

Presence of MdACS3a-1 and MdACS3a-2 allele groups

To clarify the linkage between the SSR polymorphisms and MdACS3a allele groups, the genomic sequence of MdACS3a in the cultivars ‘Fuji’, ‘Golden Delicious’, ‘Koukou’, ‘Ralls Janet’, ‘Indo’, ‘Jonathan’, ‘McIntosh’ and

Table 1. MdACS3a allelotypes of 103 apple cultivars. Bar (–) indicates none. Single and double underlined cultivars indicate MdACS1-2 heterozygous and homozygous cultivars, respectively. The cultivars with gray background indicate the MdACS3a null cultivars. Triploid cultivars possessing 3 allelotypes are shown in the margin.

| Allelotype | SSR | MdACS3a-1 | MdACS3a-2 |
|------------|-----|-----------|-----------|
| 331        |     |           |           |
| 353        |     | Himekami  |           |
| 359        |     | Early Strawberry, Melba | - |
| 361        |     | Gala, Hanaawai, Kitaro, Kotaro, Sekaiichi, Orei, Raritan, Redgold | Akagi, Fukutami, Hatsuaki, Kagayaki, Megumi |
| 333        |     | Akibi, Akita Gold, Akiyo, Amabure, Amanishiki, Ambitious, Aori 13, Cox’s Orange pippin, Crippy Pink, Esopus Spitzenburg, Freedom, Fuji, Gold Farm, Golden Melon, Hachiyogiku, Hida, Hiroseki Fuji, Hokuto, Hozuri, Iwakami, Jerseymac, Kanki, Kinsel, Kio, Kourin, Kyuto, Mikilife, Oirase, Ori, Ousya, Romu 50, Romu 16, Sansa, Shintindo, Shinano Gold, Shinano Red, Toki, Toko, Vista Bella | Himekachi, Jonathan |
| 335        |     | McIntosh, Priscilla | - |

Triploid Cultivars

| 331 + 361 + 333 | Mutsu, Fukunishiki, HAC-9, Shizuka | 331 + 361 + 333 Santaro |

Arikawa, Wang, Igarashi, Kon, Fukasawa-Akada, Li, Harada and Hatawama48
‘Worcester Pearmain’ were investigated. The sequences of all 331 alleles from ‘Indo’, ‘Fuji’ and ‘McIntosh’ were completely identical, then the 331 allele was designated as MdACS3a-1 (Accession number JF833308). Furthermore, the same MdACS3a sequence except for the ‘GA’ repeat was obtained from the 353 allele of ‘Jonathan’ and the 359 allele of ‘Worcester Pearmain’ (Table 1), respectively. Therefore, these alleles were also placed in the MdACS3a-1 group. On the other hand, the MdACS3a sequences of the 361 allele, which were found in ‘Ralls Janet’, ‘Worcester Pearmain’, ‘Koukou’ and ‘Golden Delicious’, showed only one nucleotide difference from MdACS3a-1, leading to our previously reported MdACS3a-G289V, in which glycine had been changed to valine at amino acid residue 289 (Wang et al. 2009). Therefore, this allele was hereafter designated MdACS3a-1V, which also belong to the MdACS3a-1 group. The MdACS3a sequence of the 333 allele of ‘Fuji’, ‘Jonathan’, ‘Koukou’ and ‘Ralls Janet’, and the 335 allele of ‘McIntosh’ were identical except for the ‘GA’ repeat (Accession number JF833309). This sequence was 14 nucleotides different from those of MdACS3a-1, which distributed across the whole gene (Fig. 2A). Therefore, we designated them MdACS3a-2.

To distinguish MdACS3a-2 from other alleles, a CAPS was developed. As shown in Fig. 2B, the CAPS DNA marker was able to identify the MdACS3a-2 genotypes easily.

Non-transcriptional null alleleotype, MdACS3a-2

The presence of mdacs3a, a null MdACS3a allele, was identified because it showed no transcript during the ripening stage (Wang et al. 2009). The previous data revealed that the cultivar ‘Koukou’ was Mdacs3a-G289V (MdACS3a-1V in this report)/mdacs3a. Since this cultivar was diagnosed as having a 333 + 361 alleleotype, and 361 was one of MdACS3a-1 (Table 1), the 333 allele identified as MdACS3a-2 was considered to be mdacs3a. To determine whether MdACS3a-2 lacked transcription ability, we carried out RT-dCAPS analysis to amplify the cDNA fragment of MdACS3a with the dCAPS primers (Supplemental Table 2) in the cultivars with the alleles of MdACS3a-1V/MdACS3a-2. As shown in Fig. 3A, the shortened fragment (198 bp) after SpeI digestion of the amplicon indicated the presence of the MdACS3a-1V transcript. The RT-PCR products from ‘Golden Delicious’ and ‘Ralls Janet’, both being 333 + 361, were totally digested, indicating that the cDNA originated from only the MdACS3a-1V transcript. Therefore, transcription from the 333 allele appeared to be absent. We developed a dCAPS marker in the coding region to distinguish MdACS3a-1 and MdACS3a-2 (Fig. 3B). The cDNAs from cultivars possessing both alleleotypes only showed the band corresponding to MdACS3a-1, because the PCR products from the MdACS3a-2 should be cleaved by BamHI. Furthermore, comparative direct sequencing of the PCR products from the genomic DNA and cDNA revealed overlap peaks in gDNA, meaning that MdACS3a-2 allele in the gDNA were missing in the cDNAs (Supplemental Fig. 3). These results indicate that the MdACS3a-2 lost their transcriptional activity during fruit ripening.

Discussion

By using SSR and dCAPS markers developed in this work, the features of two MdACS3a- null alleleotypes, MdACS3a-1V and MdACS3a-2, which were designated MdACS3a-G289V and mdacs3a in our previous paper (Wang et al. 2009), were investigated in the apple. Although the reason for the lack of transcription from MdACS3a-2 (333 allele) still remained unclear, there is possibility that three
nucleotide substitutions in the promoter region may be responsible, because they locate at -219, -217 and -189 bp from the transcription start site where is normally critical cis-element distribution. Furthermore, with regard to the 335 allele of MdACS3a-2 in ‘McIntosh’ and its progeny ‘Priscilla’, its transcriptional activity could not be examined in this study due to the unavailability of ripening fruit at the time. However, as this allelotype cultivars have been investigated for their internal ethylene concentration in ripening fruit, which is known to parallel the ethylene production rate. Indeed, they reported that the late-harvest cultivars ‘Ralls Janet’, ‘Gunma Meigetsu’, ‘Slimred’ and ‘Narihoko’ exhibit very low ethylene levels as compared with the late-harvest cultivar ‘Fuji’ during storage even at 24°C for 12 days (Harada et al. 2000). Eventually early- and middle-maturing cultivars may easily enter the system 2 stage, regardless of their MdACS3a allelotype (Wang et al. 2007). Therefore, the null genotype is considered to influence the initiation of ripening of only later-maturing cultivars. In our previous studies, some of 22 MdACS3a-null allelotype cultivars have been investigated for their internal ethylene concentration in ripening fruit, which is known to parallel the ethylene production rate. Indeed, they reported that the late-harvest cultivars ‘Ralls Janet’, ‘Gunma Meigetsu’, ‘Slimred’ and ‘Narihoko’ exhibit very low ethylene levels as compared with the late-harvest cultivar ‘Fuji’ during storage even at 24°C for 12 days (Harada et al. 2000, Wakasa et al. 2006). Taken together, we concluded that the null MdACS3a consisting of two types, alleles of no transcription and of no enzymatic activity, affects the ripening initiation of only late harvest cultivars but not early or middle cultivars.

However, Zhu and Barriff (2008) have reported that

hand, each cultivar had been selected by experienced breeders from the respective breeding mass. Since two MdACS3 subfamily genes, MdACS3b and MdACS3c, are of the null type because of a transposon inserted in the promoter region (Wang et al. 2009), the cultivars and selections homozygous for the MdACS3a-null type gene would lose the function of the MdACS3 gene (Fig. 4). Therefore, the MdACS3a-null allelotype likely contributes to the better storability of apple fruit (Wang et al. 2009).

Is there any evidence that cultivars homozygous for the MdACS3a-null genotypes exhibit a long shelf life? Twelve and ten cultivars were found to have the null allelotype of MdACS3a-1V/MdACS3a-2 and MdACS3a-2 homozygosity, respectively (Table 1). Given the fact that MdACS3a functional initiates a burst of ethylene production by MdACS1, the ethylene burst in these cultivars with MdACS3a-null genotypes may be influenced. On the other hand, fruit maturation signal(s) and ambient temperature at the onset of ripening are also known to greatly influence ethylene production (Dal Cin et al. 2007, Kubo et al. 2009). Eventually early- and middle-maturing cultivars may easily enter the system 2 stage, regardless of their MdACS3a allelotype (Wang et al. 2007). Therefore, the null genotype is considered to influence the initiation of ripening of only later-maturing cultivars. In our previous studies, some of 22 MdACS3a-null allelotype cultivars have been investigated for their internal ethylene concentration in ripening fruit, which is known to parallel the ethylene production rate. Indeed, they reported that the late-harvest cultivars ‘Ralls Janet’, ‘Gunma Meigetsu’, ‘Slimred’ and ‘Narihoko’ exhibit very low ethylene levels as compared with the late-harvest cultivar ‘Fuji’ during storage even at 24°C for 12 days (Harada et al. 2000, Wakasa et al. 2006). Taken together, we concluded that the null MdACS3a consisting of two types, alleles of no transcription and of no enzymatic activity, affects the ripening initiation of only late harvest cultivars but not early or middle cultivars.
although the influence of *MdACO1* (ACC oxidase) genotypes plays a minor role in comparison with *MdACS1*, the association between the *MdACS1* and *MdACO1* allelotypes is also considered to influence the storability of harvested fruit. Therefore, the *MdACS3a* genotype alone cannot explain the full spectrum of ethylene production by which ripening progression is controlled. Moreover, the ethylene that is synthesized mediates the ripening process via the receptors (Tatsuki 2010), eventually triggering enzyme activity like that involved in the modification of cell walls (Tacken et al. 2010, Wakasa, et al. 2006). The genes encoding these ripening-related enzymes are probably composed of multiple allelotypes, because of the existence of hemizygous DNA in the heterozygous *Malus* genome (Velasco et al. 2010). Although more studies will be needed for a complete understanding of the differences in fruit storage properties among apple cultivars, knowing the distribution of the *MdACS3a* allelotypes in cultivars and selections would be contributory to elucidate the complex ripening molecular mechanism in apple.

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