ARTICLE

Assessing the allelotypic effect of two aminocyclopropane carboxylic acid synthase-encoding genes *MdACS1* and *MdACS3a* on fruit ethylene production and softening in *Malus*Laura Dougherty¹, Yuandi Zhu^{1,2} and Kenong Xu

Phytohormone ethylene largely determines apple fruit shelf life and storability. Previous studies demonstrated that *MdACS1* and *MdACS3a*, which encode 1-aminocyclopropane-1-carboxylic acid synthases (ACS), are crucial in apple fruit ethylene production. *MdACS1* is well-known to be intimately involved in the climacteric ethylene burst in fruit ripening, while *MdACS3a* has been regarded a main regulator for ethylene production transition from system 1 (during fruit development) to system 2 (during fruit ripening). However, *MdACS3a* was also shown to have limited roles in initiating the ripening process lately. To better assess their roles, fruit ethylene production and softening were evaluated at five time points during a 20-day post-harvest period in 97 *Malus* accessions and in 34 progeny from 2 controlled crosses. Allelotyping was accomplished using an existing marker (ACS1) for *MdACS1* and two markers (CAPS₈₆₆ and CAPS₈₇₀) developed here to specifically detect the two null alleles (ACS3a-G289V and *Mdacs3a*) of *MdACS3a*. In total, 952 *Malus* accessions were allelotyped with the three markers. The major findings included: The effect of *MdACS1* was significant on fruit ethylene production and softening while that of *MdACS3a* was less detectable; allele *MdACS1*-2 was significantly associated with low ethylene and slow softening; under the same background of the *MdACS1* allelotypes, null allele *Mdacs3a* (not ACS3a-G289V) could confer a significant delay of ethylene peak; alleles *MdACS1*-2 and *Mdacs3a* (excluding ACS3a-G289V) were highly enriched in *M. domestica* and *M. hybrid* when compared with those in *M. sieversii*. These findings are of practical implications in developing apples of low and delayed ethylene profiles by utilizing the beneficial alleles *MdACS1*-2 and *Mdacs3a*.

Horticulture Research (2016) 3, 16024; doi:10.1038/hortres.2016.24; Published online 18 May 2016

INTRODUCTION

To make fresh apple fruit available year-round for consumers, the controlled atmosphere (CA) storage technology has been adapted widely in the apple industry. The technology primarily employs low temperature, low O₂ and high CO₂ in combination with an ethylene production inhibitor 1-methylcyclopropene and others. Apple fruit can be stored for >10 months under optimal CA conditions. However, physiological disorders associated with CA storage, such as injuries induced by cold and CO₂ and flesh browning induced by 1-methylcyclopropene, can cause substantial loss for storage operators.^{1–3} Such storage disorders have been reported for major apple varieties such as ‘Empire’ and ‘McIntosh’^{2,4} and for rising cultivars such as ‘Honeycrisp’.³ A strong need for new apples of long-shelf life and improved keeping quality with few or no storage disorders exists.

The gaseous phytohormone ethylene plays an important role in climacteric fruit ripening. The shelf life and storability of apple fruit are closely correlated with their ethylene production levels. Plant ethylene biosynthesis has been well-defined in Yang cycle that involves three enzymes: S-adenosylmethionine synthase, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO).⁵ The enzymes ACS and ACO have been the subject of extensive studies to better understand plant ethylene production. Studies in many plant species including tomato and apple have shown that ACS and ACO are encoded by gene

families of multiple members, that is, the ACS family and the ACO family, respectively.

There are two systems of ethylene production in plants: system 1 occurs during plant/fruit growth and development; and system 2 is defined exclusively for the floral senescence and fruit-ripening stages.⁶ In tomato, system 1 ethylene biosynthesis involves LeACS6, 1A and LeACO1, 3, 4; whereas system 2 uses LeACS2, 4 and ACO1, 4.⁷ In apple, at least five ACS (*MdACS1*–5) and four ACO (*MdACO1*–4) genes have been reported^{8,9} and these genes appear to be operating similarly in the two systems for ethylene production. *MdACS1* is considered a system 2 gene; and its expression is highly correlated with the ethylene production burst in ripening apples. There are two alleles for the *MdACS1* gene, *MdACS1*-1 and *MdACS1*-2, and the former is often associated with high ethylene production while the latter with lower ethylene production during fruit ripening.^{10–14} This observation has led to a marker-assisted selection strategy emphasizing on selection for allelotype (see Discussion for usage of term ‘allelotype’) *MdACS1*-2/2 for long-shelf life apples.¹⁵ Indeed, some evidence suggests that modern apple-breeding practice has unintentionally favored selection for the *MdACS1*-2 allele in commercial apple cultivars,¹⁶ presumably for fruit of low ethylene and long-shelf life.

However, early-ripening cultivars showed faster fruit softening, regardless of their *MdACS1* allelotypes.¹⁰ This is consistent with the observation that the polygalacturonase gene (*MdPG1*) involved in

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Received: 5 March 2016; Revised: 10 April 2016; Accepted: 11 April 2016

softening of fruit flesh is expressed irregularly among apple cultivars of identical *MdACS1* allelotypes.¹² Therefore, there are other factors also affecting fruit shelf life in addition to *MdACS1*. Interestingly, findings in a recent report have suggested that allele variations of another ACS gene (U73816),¹⁷ designated *MdACS3a* (AB243060), are an essential factor regulating apple fruit ripening and shelf life.¹⁸ There are two natural mutant alleles of the wild-type allele *MdACS3a*: One is the functional null allele *MdACS3a-G289V*, arising from a point mutation that leads to an amino-acid substitution from G₂₈₉ to V₂₈₉ at an active region for the *MdACS3a* enzyme activity, resulting in a functionally inactive enzyme. In melon, a similar point mutation in a conserved active region of an ACS gene led to andromonoecy, a common sexual system in angiosperms characterized by carrying both male and bisexual flowers.¹⁹ This is an excellent example demonstrating that point mutations in conserved active regions of an ACS enzyme could confer a major phenotypic variation in plants. The other, a transcriptionally null allele *Mdacs3a*, is characterized by non-detectable mRNA. Moreover, combinations of *Mdacs3a* and *MdACS3a-G289V* alleles, regardless of whether they are homozygous or heterozygous, are highly associated with lower ethylene production and long-shelf life. In the six apple varieties/selections of the two null alleles studied, all showed low ethylene production and long-shelf life, irrespective to their *MdACS1* allelotypes and early, mid or late physiological maturation dates.¹⁸ Furthermore, the expression of *MdACS3a* is fruit tissue specific and detectable only during the transition from system 1 to 2 ethylene biosynthesis.^{8,9,18} These observations suggest that *MdACS3a* acts as a main regulator for the transition, and is thereby crucial in regulating the fruit-ripening process.¹⁸

In a more recent report, however, the allelotypes of *MdACS3a* were demonstrated to affect the ripening initiation of late-maturing cultivars only, but not the early- or mid-maturing cultivars.²⁰ To better assess the roles of *MdACS1* and *MdACS3a*, two approaches were taken in this study. The first approach was to estimate the allelotypic effect of the two genes by evaluating fruit ethylene production levels and softening rates in 97 diverse *Malus* accessions and 34 progeny from 2 controlled crosses. The second approach was to examine how variations in their allelotypic effect were associated with the frequency changes of the *MdACS1* and *MdACS3a* alleles in *M. domestica* and *M. hybrid* as compared with those in *M. sieversii*, the major progenitor species of domestic apples, in 952 *Malus* accessions covering 53 *Malus* species. Allelotyping (see Discussion for usage of term 'allelotyping') of *MdACS1* and *MdACS3a* was conducted using an existing marker for *MdACS1* and two CAPS (cleaved amplified polymorphic sequence) markers specifically developed here to detect alleles *ACS3a-G289V* and *Mdacs3a*.

MATERIALS AND METHODS

Plant materials

Two sets of *Malus* accessions were used in this study, which have been planted and maintained in the *Malus* germplasm repository of the US Department of Agriculture (USDA) in Geneva, New York. The first set included a total of 952 accessions, covering 53 *Malus* species (Supplementary Table S1). Among them, *Malus domestica* of 508 accessions, *M. hybrid* (the breeding selections derived from crosses between *M. domestica* and other *Malus* species) of 146 and *M. sieversii* (the major progenitor species of *M. domestica*) of 78 were most commonly represented (Supplementary Table S1). The second set comprised 34 half-sib progeny selected from 2 interspecific crosses GMAL4592 ('Royal Gala' × PI613978) and GMAL4593 ('Royal Gala' × PI613981). 'Royal Gala', a widely grown apple cultivar (*M. domestica*), has an allelotype *MdACS1-2/2* and *MdACS3a/MdACS3a-G289V* for genes *MdACS1* and *MdACS3a*, respectively. PI613978 and PI613981 are among the elite selections of *M. sieversii* collected from Kazakhstan,²¹ and they have the same allelotypes for the two ACS genes, that is, *MdACS1-1/1* and *MdACS3a/MdACS3a-G289V*. Population GMAL4592 was used in one of our previous studies.²² Both

GMAL4592 and GMAL4593 were planted on their own seedling roots in 2004.

Measurements of fruit ethylene production and firmness

Fruit ethylene production and flesh firmness were measured for 97 of 952 *Malus* accessions in the first set and the 34 half-sib progeny in the second set as described previously.²³ Briefly, for each accession, at least 25 fruits were harvested at a target maturity level as determined by the starch index of 4–6 according to the Cornell Starch Chart.²⁴ The 25 fruits were evenly divided into 5 groups and were stored for 0, 5, 10, 15 and 20 days at room temperature (20–25 °C), respectively. Each fruit was weighed then enclosed in a gas-tight container (1.2 l) and kept for 1 h at room temperature. One milliliter of gas was sampled from the headspace in the container using a BD syringe (No. 309602, BD, Franklin Lakes, NJ, USA). The gas sample's ethylene concentration was measured with a gas chromatograph HP 5890 series II (Hewlett-Packard, Palo Alto, CA, USA) equipped with a flame ionization detector. Before the gas samples were assayed, the gas chromatograph was calibrated with standard ethylene gas (NO. 34489, Restek, Bellefonte, PA, USA) at a series of concentrations—0.01, 0.1, 0.5, 1, 5, 10 and 100 p.p.m.—to obtain the linear relation between ethylene peak area and concentration. The fruit ethylene production was calculated with the following formula:

$$E = [C_2H_4] \times (V_1 - V_2) / W / T$$

Where *E* stands for fruit ethylene production rate in nanoliter per gram of fresh weight per hour (nL g⁻¹ h⁻¹), [C₂H₄] for ethylene concentration in p.p.m., *V*₁ for the volume of container in mL, *V*₂ for the volume of fruit in mL equivalent to fresh weight (*W*) in grams and *T* stands for the time in hours kept in the container.

Fruit flesh firmness was measured using a penetrometer (Fruit Tester, Wagner FTK100, Greenwich, CT, USA) with a probe of 11 mm in diameter. The probe tip was pressed vertically into the fruit pulp (after skin-disc removal) to a depth of 10 mm. For larger fruits, four skin discs were removed from opposite sides of each fruit along the equator, and for smaller fruits, three skin discs were removed at roughly equal distance. The firmness readings were expressed in kg cm⁻², and firmness loss was measured by the percentage (%) of firmness reduced at days 5 to 20 as compared with the firmness at day 0. After the firmness was measured, fruits were sliced in half along the equator, dipped into a iodine-potassium iodide (I₂-KI) solution, and then allowed the staining reaction for > 1 min before reading Cornell Starch Index.²⁴

Allelotyping of *MdACS1* and *MdACS3a*

Allelotyping of *MdACS1* was conducted with marker ACS1 using primers ACS1-5F/R (Supplementary Table S2) as reported previously.^{10,15} However, allelotyping of *MdACS3a* was accomplished with two CAPS markers developed in this study using an online tool for identifying appropriate restriction enzymes²⁵ (see Results). These two markers, named CAPS₈₆₆ and CAPS₈₇₀, were capable of detecting the functional null allele *MdACS3a-G289V* and the transcriptional null allele *Mdacs3a*, respectively. In practice, the same primers ACS3a-289F/R (Supplementary Table S2) were used for PCR to amplify the targeted DNA fragment for both CAPS₈₆₆ and CAPS₈₇₀. PCRs were performed with 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, with an initial 94 °C for 5 min and a final extension of 72 °C for 10 min. Each PCR reaction mix was set in 10 µL containing 20 ng genomic DNA, 0.2 mM each dNTP, 0.5 µM of each primer, 2.5 mM MgCl₂, 2 µL 5 × PCR Colorless GoTaq Reaction Buffer and 1 U of GoTaq DNA polymerase (Promega, Madison, WI, USA). To detect alleles *MdACS3a-G289V* and *Mdacs3a*, the PCR products were restricted with enzymes *Bst*NI and *Taq*qi (New England Biolabs, Ipswich, MA, USA) following the manufacturer's instruction, respectively. The restricted PCR products were assayed by electrophoresis on 1.5% agarose gel and then stained with ethidium bromide for visualization and documentation as described previously.²²

Sanger DNA sequencing

The PCR products amplified by primers ACS3a-289F/R (Supplementary Table S2) were directly sequenced using a DNA Sequencer ABI3730XL (Applied Biosystems, Foster City, CA, USA) at the Cornell University Biotechnology Resource Center (Ithaca, NY, USA). The reverse PCR primer ACS3a-289R was used for DNA sequencing. DNA sequence analyses were performed using software Sequencher 5.2 (Gene Codes Corporation, Ann Arbor, MI, USA).

Statistical analysis

Pearson's correlation analysis and one-way analysis of variance (ANOVA) of ethylene production and fruit firmness were conducted with software JMP Pro 10.0 (SAS institute, Cary, NC, USA). Significance levels in comparison of the means were determined by $P < 0.05$ (Student's *t*-test).

RESULTS

Evaluation of fruit ethylene production and softening

Fruit ethylene production and softening were evaluated in 97 of 952 *Malus* accessions (Supplementary Tables S1 and S3). Their mature date was determined by Cornell starch index, which had a mean 5.5 ± 1.4 at harvest. The 97 accessions varied widely not only in maturity date (from 16 August to 8 November 2011; Supplementary Figure S1a) and fruit weight ($25.1\text{--}303.8$ g, Supplementary Figure S1b), but also in ethylene production and firmness at harvest (day 0) and during the 20-day post-harvest period (Figures 1a and b). At day 0, for example, the ethylene levels ranged from 0.7 nL g^{-1} per h of PI588844 ('Fuji', *M. domestica*) to 679.3 nL g^{-1} per h of PI619168 (an accession of *M. sylvestris*), and fruit firmness varied from 3.8 kg cm^{-2} of PI589572 (E14-32, *M. hybrid*) to 12.7 kg cm^{-2} of PI589478 ('Novosibirski Sweet', *M. domestica*). Despite being highly variable,

a trend line of bivariate function could be fit for fruit ethylene production ($r^2 = 0.120$, $P < 0.0001$, Figure 1a) and fruit firmness ($r^2 = 0.147$, $P < 0.0001$, Figure 1b).

The trend line of fruit ethylene showed a peak between days 10 and 15, which was largely a reflection of the mean fruit ethylene levels 75.5 ± 100.5 , 207.3 ± 193.9 , 272.8 ± 249.6 , 247.0 ± 170.8 and 217.3 ± 146.5 ($\text{nL g}^{-1} \text{ h}^{-1}$) at days 0, 5, 10, 15 and 20, respectively (Figure 1a). A majority (59/97, 60.8%) of the 97 *Malus* accessions reached their peak ethylene day at day 10 (25 accessions) or day 15 (34 accessions) while 2, 16 and 20 accessions topped their ethylene production at days 0, 5 and 20 (Supplementary Figure S1c). The peak ethylene reads were spread from 1.7 nL g^{-1} per h of PI589570 (E36-7, *M. hybrid*) at day 20 to 1022.2 nL g^{-1} per h of PI633801 (*M. sieversii*) at day 10 (Supplementary Table S3).

As expected, fruit firmness showed a continuous decreasing trend during the 20-day period (Figure 1b). This was also an approximation of the mean firmness $7.4 \pm 1.7\text{ kg cm}^{-2}$, $6.5 \pm 2.1\text{ kg cm}^{-2}$, $5.8 \pm 2.0\text{ kg cm}^{-2}$, $5.3 \pm 1.99\text{ kg cm}^{-2}$ and $5.3 \pm 1.92\text{ kg cm}^{-2}$ at days 0, 5, 10, 15 and 20, respectively. In other words, the mean fruit firmness was lost by 13.6% at day 5, 22.0% at day 10, 29.2% at day 15 and 29.0% at day 20.

Fruit ethylene production and firmness loss were significantly correlated (Table 1). The strongest correlation ($r = 0.564$, $P = 0$) was

Figure 1. Evaluation of fruit ethylene production (a) and firmness (b) in 97 *Malus* accessions during a 20-day post-harvest period under room temperature. The trend lines (curves in red) and the associated equations and coefficient of determination (R^2) are presented.

Table 1. Correlation coefficients between fruit ethylene production and firmness or firmness loss in 97 *Malus* accessions^a

	$C_2H_4_{d0}$	$C_2H_4_{d5}$	$C_2H_4_{d10}$	$C_2H_4_{d15}$	$C_2H_4_{d20}$	Firmness d0 (kg cm^{-2})	Firmness loss_d5 (%)	Firmness loss_d10 (%)	Firmness loss_d15 (%)	Firmness loss_d20 (%)	Peak C_2H_4 day ^b
$C_2H_4_{d0}$	1.000**										
$C_2H_4_{d5}$	0.434**	1.000**									
$C_2H_4_{d10}$	0.410**	0.695**	1.000**								
$C_2H_4_{d15}$	0.353**	0.734**	0.839**	1.000**							
$C_2H_4_{d20}$	0.265**	0.733**	0.695**	0.871**	1.000**						
Firmness d0 (kg cm^{-2})	-0.208*	-0.261**	-0.164	-0.239*	-0.220*	1.000**					
Firmness loss_d5 (%)	0.324**	0.484**	0.214*	0.334**	0.431**	-0.198	1.000**				
Firmness loss_d10 (%)	0.345**	0.538**	0.493**	0.564**	0.478**	-0.092	0.704**	1.000**			
Firmness loss_d15 (%)	0.284**	0.446**	0.421**	0.481**	0.442**	-0.104	0.699**	0.863**	1.000**		
Firmness loss_d20 (%)	0.306**	0.453**	0.388**	0.481**	0.438**	-0.041	0.596**	0.828**	0.853**	1.000**	
Peak C_2H_4 day ^b	-0.229*	-0.479**	-0.402**	-0.281**	-0.211*	0.219*	-0.112	-0.258*	-0.238*	-0.190	1.000**

^aFruit firmness loss was measured in a 20-day post-harvest period under room temperature. ^bPeak C_2H_4 (ethylene) day: day of peak ethylene production during the 20-day post-harvest storage; signs '*' and '**' stand for significance levels exceeding $P = 0.05$ ($r = 0.1996$, $n = 97$) and $P = 0.01$ ($r = 0.2603$, $n = 97$), respectively.

Figure 2. A chromatogram screenshot of the DNA sequence (partial) of *MdACS3a* encompassing SNPs G₈₆₆/T₈₆₆ and C₈₇₀/T₈₇₀ in six apple cultivars—‘Florina’, ‘Fuji red sport’, ‘Gala’, ‘Golden Delicious’ and ‘Granny Smith’. The oval circles in brown and red indicate the homozygous or heterozygous status at the 866th and 870th nucleotides in the coding sequence of *MdACS3a*, respectively. The recognition sites of restriction enzymes *Bst*NI and *Taq*I are provided to show that the mutation from G₈₆₆ to T₈₆₆ abolishes the restriction site of *Bst*NI while the mutation from C₈₇₀ to T₈₇₀ gives rise to a restriction site for *Taq*I. The right panel shows allelotypes of *MdACS3a* as represented by the SNP alleles, where G₈₆₆ stands for allele *MdACS3a* (wild type), T₈₆₆ for *MdACS3a*-G289V (functional null allele), C₈₇₀ also for allele *MdACS3a* and T₈₇₀ for *Mdacs3a* (transcriptional null allele).

observed between ethylene at day 15 and fruit firmness loss at day 10, while the weakest ($r=0.214$, $P=0.035$) was between ethylene at day 10 and fruit firmness loss at day 5. Peak ethylene day (day of peak ethylene production during the 20-day post-harvest storage) was most significantly correlated with ethylene at day 5 ($r=-0.479$, $P=6.9E-7$), and it also significantly correlated with fruit firmness loss at day 10 ($r=-0.258$, $P=0.011$) and day 15 ($r=-0.238$, $P=0.019$) (Table 1).

Development of allelic specific markers for *MdACS3a*

The null allele *MdACS3a-G289V* is caused by a mutation from G₈₆₆ to T₈₆₆ at the 866th base in the coding sequence of *MdACS3a*.¹⁸ Based on the web-based tool for single nucleotide polymorphism (SNP) analysis,²⁵ the mutation abolishes the recognition site CC₈₆₆WGG of restriction enzyme *Bst*NI (Figure 2). To develop a CAPS marker, two primers (ACS3a-289F/R, Supplementary Table S2) were designed to amplify a DNA fragment (480 bp) covering the SNP (G₈₆₆/T₈₆₆) specifically from *MdACS3a* although the three *MdACS3* member genes *MdACS3a* (AB243060), *MdACS3b* (AB243061) and *MdACS3c* (AB243062) are of high identity in their DNA sequences.¹⁸ The specificity of the primer pair to *MdACS3a* was confirmed by sequencing of the PCR products from 92 of the 97 *Malus* accessions (Figure 2, Supplementary Table S3). Digestion of the PCR products with *Bst*NI yielded restriction bands as expected (Figure 3b), indicating the successful development of a CAPS marker detecting SNP G₈₆₆/T₈₆₆, designated CAPS₈₆₆. Therefore, allele CAPS₈₆₆G represents the wild-type allele *MdACS3a* while CAPS₈₆₆T stands for the functional null allele *MdACS3a-G289V*.

Development of a marker detecting the transcriptional null allele *Mdacs3a* was initially thought to be challenging as the null allele was reported not to show sequence variations from the wild-type allele.¹⁸ However, sequencing analysis of the PCR products amplified by primers ACS3a-289F/R in the 92 accessions (Supplementary Table S3) not only identified the expected SNP

G₈₆₆/T₈₆₆, but also a new SNP C₈₇₀/T₈₇₀ (Figure 2). Importantly, this new SNP can discriminate the two alleles of *MdACS3a* in 'Fuji' (Figure 2), which was known of allelotype *MdACS3a*/*Mdacs3a*.¹⁸ Evidence from this and other studies (see Discussion) indicated that base T₈₇₀ was associated with the *Mdacs3a* allele. Using a similar approach, another CAPS marker, named CAPS₈₇₀, was developed to detect SNP C₈₇₀/T₈₇₀ using restriction enzyme *Taq*^qI along with the same primers ACS3a-289F/R (Figure 3c). Therefore, allele CAPS₈₇₀C corresponds to the wild-type allele *MdACS3a* while CAPS₈₇₀T corresponds to the transcriptional null allele *Mdacs3a*.

Effect of the allelotypes of *MdACS1* and *MdACS3a* on ethylene production and firmness loss

To evaluate the effect of the allelotypes of *MdACS1* and *MdACS3a*, the 97 *Malus* accessions were assayed with markers ACS1, CAPS₈₆₆ and CAPS₈₇₀ that can detect different alleles of *MdACS1* and *MdACS3a* (Figures 3a–c). As a result, marker ACS1 identified 53, 36 and 8 accessions of allelotypes of *MdACS1*-1/*MdACS1*-1 (*MdACS1*-1/1), *MdACS1*-1/*MdACS1*-2 (*MdACS1*-1/2) and *MdACS1*-2/*MdACS1*-2 (*MdACS1*-2/2), respectively (Supplementary Table S3). Similarly, marker CAPS₈₆₆ detected 75 accessions of allelotype CAPS₈₆₆G/CAPS₈₆₆G (CAPS₈₆₆G/G), 18 of CAPS₈₆₆G/CAPS₈₆₆T (CAPS₈₆₆G/T) and 4 of CAPS₈₆₆T/CAPS₈₆₆T (CAPS₈₆₆T/T); and marker CAPS₈₇₀ uncovered 47 accessions of allelotype CAPS₈₇₀C/CAPS₈₇₀C (CAPS₈₇₀C/C), 40 of CAPS₈₇₀C/CAPS₈₇₀T (CAPS₈₇₀C/T) and 10 of CAPS₈₇₀T/CAPS₈₇₀T (CAPS₈₇₀T/T) (Supplementary Table S3).

A series of one-way ANOVA of the fruit ethylene production and fruit firmness loss over the 20-day period within each of the three allelotype groups (Figure 4) indicated that the most differences were observed among the *MdACS1* allelotypes. Allelotype *MdACS1-1/1* showed significantly higher ethylene production (days 0–20) and firmness loss (days 5–20) than *MdACS1-1/2* and *MdACS1-2/2* allelotypes, but *MdACS1-1/2* and *MdACS1-2/2* did not differ in terms of ethylene production or firmness retention (Figures 4a and d). In contrast, there were no difference among

Figure 3. Agarose gel analyses of markers ACS1 (a), CAPS₈₆₆ (b) and CAPS₈₇₀ (c). For marker ACS1, the PCR products amplified by primers ACS1-5F/R were directly analyzed. Allelotypes *MdACS1*-1/1, *MdACS1*-2/2 and *MdACS1*-1/2 are denoted with '1/1', '2/2' and '1/2', respectively. For marker CAPS₈₆₆, the PCR products were first amplified by primers ACS3a-289F/R and then digested with enzyme *Bst*NI, which restricts the *MdACS3a* (G₈₆₆) allele into the two lower bands. Allelotypes *MdACS3a*/*MdACS3a* (G₈₆₆/G₈₆₆), *MdACS3a*/*MdACS3a*-G289V (G₈₆₆/T₈₆₆) and *MdACS3a*-G289V/G289V (T₈₆₆/T₈₆₆) are noted with 'G/G', 'G/T' and 'T/T', respectively. For marker CAPS₈₇₀, enzyme *Taq*I restricts the *Mdacs3a* (T₈₇₀) allele into the two lower bands. Allelotypes *MdACS3a*/*MdACS3a* (C₈₇₀/C₈₇₀), *MdACS3a*/*mdacs3a* (C₈₇₀/T₈₇₀) and *mdacs3a*/*mdacs3a* (T₈₇₀/T₈₇₀) are noted with 'C/C', 'C/T' and 'T/T', respectively.

the CAPS₈₆₆ allelotypes in fruit ethylene production and firmness loss (Figures 4b and e). Among the CAPS₈₇₀ allelotypes, significant difference was not detected for ethylene production, but there were differences in fruit firmness loss between allelotypes CAPS₈₇₀C/C and CAPS₈₇₀C/T at day 5 and between CAPS₈₇₀C/C and CAPS₈₇₀T/T at day 10 (Figures 4c and f). This indicated that such differences in fruit firmness loss at day 5 and 10 in the CAPS₈₇₀ allelotypes might be caused by other factors rather than their ethylene production levels.

To seek such factors, peak ethylene day, which measures ethylene peak timing, was examined (Figure 5) as this trait was negatively correlated with fruit firmness loss at day 10 ($r = -0.258$, $P = 0.011$) although the correlation was insignificant at day 5 ($r = -0.112$, $P = 0.275$) (Table 1). Encouragingly, the three CAPS₈₇₀ allelotypes showed significant difference from each other, with CAPS₈₇₀C/T having peaked the earliest, CAPS₈₇₀C/C intermediate and CAPS₈₇₀T/T the latest (Figure 5a). These data appeared to suggest that the earlier peak ethylene day of CAPS₈₇₀C/C might have contributed to its greater fruit firmness loss of CAPS₈₇₀C/C as compared with that of CAPS₈₇₀T/T at day 10 (Figure 4f). However,

the lowest fruit firmness loss of CAPS₈₇₀C/T at day 5 remained to be explained. Peak ethylene day was also analyzed in the other two groups of allelotypes. In the allelotypes of *MdACS1*, *MdACS1*-1/1 had an earlier peak ethylene than *MdACS1*-2/2, but showed no difference from *MdACS1*-1/2 (Figure 5a). In the three allelotypes of CAPS₈₆₆, no significant difference was observed (Figure 5a).

It was clear that the effect of *MdACS1* on ethylene production and fruit firmness loss was much stronger than that of *MdACS3a* (Figure 4). To see if the random presence of the *MdACS1* alleles might have obscured the detection of the effect of *MdACS3a* allelotypes (Figures 4b, c, e and f), another series of ANOVA was conducted for the *MdACS3a* allelotypes of five or more accessions (Figure 6) under the same background of *MdACS1* allelotypes *MdACS1*-1/1 and *MdACS1*-1/2, which occurred in 53 and 36 of the 97 accessions (Supplementary Table S3), respectively. The third allelotype *MdACS1*-2/2 was not included in the analysis (Figure 6) due to limited number of 8 accessions.

For CAPS₈₆₆, the ANOVA analyses were conducted for two allelotypes CAPS₈₆₆G/G and CAPS₈₆₆G/T under *MdACS1*-1/1, as well as under *MdACS1*-1/2 (Figures 5b and 6a,c). This allowed us to identify that allelotype CAPS₈₆₆G/T produced significantly higher levels of ethylene than CAPS₈₆₆G/G at day 10 under *MdACS1*-1/1 (Figure 6a). For CAPS₈₇₀, three allelotypes CAPS₈₇₀C/C, CAPS₈₇₀C/T and CAPS₈₇₀T/T under *MdACS1*-1/1 and two allelotypes CAPS₈₇₀C/C and CAPS₈₇₀C/T under *MdACS1*-1/2 were analyzed (Figures 5c and 6b,d). The results showed that allelotype CAPS₈₇₀T/T had significant later peak ethylene day than CAPS₈₇₀C/C and CAPS₈₇₀C/T under *MdACS1*-1/1, and CAPS₈₇₀C/C had significant later peak ethylene than CAPS₈₇₀C/T under *MdACS1*-1/2 (Figure 5c). There were no significant differences detected between the other allelotypes of CAPS₈₆₆ and CAPS₈₇₀ at a given time point (Figures 5b and 6a–d). These observations suggested that the direct effect of *MdACS3a* on ethylene production and firmness loss was limited, but its effect on peak ethylene day was clearly detectable through allele *Mdacs3a* (CAPS₈₇₀T/T).

The analyses also provided information regarding the effect of *MdACS1* under the same background of CAPS₈₆₆ (Figures 5b and 6a,c) or CAPS₈₇₀ (Figures 5c and 6b,d) allelotypes. As expected, allelotype *MdACS1*-1/1 had higher ethylene production (Figures 6a and c) and more firmness loss (Figures 6b and d) than *MdACS1*-2/2, but had similar peak ethylene day as *MdACS1*-1/2 (Figures 5b and c) except under the CAPS₈₇₀C/C background (Figure 5c). These results suggested that the effect of *MdACS1* on peak ethylene day was insignificant under the same background of *MdACS3a*, which was in disagreement with the observation that the effect of *MdACS1* on peak ethylene day was significant when the background of *MdACS3a* was not considered (Figure 5a).

Since the *MdACS3a* allelotype CAPS₈₆₆T/T (*MdACS3a*-G289V/G289V) was present only in 4 of 97 accessions, the 2 controlled crosses GMAL4592 and GMAL4593 segregating for CAPS₈₆₆T/T under the same background of *MdACS1*-1/2 were used for better analysis. In total, 17 progeny of allelotype CAPS₈₆₆G/G (*MdACS3a*/*MdACS3a*) and another 17 of CAPS₈₆₆T/T were similarly evaluated for ethylene production and fruit firmness loss. ANOVA analysis indicated that there were no significant differences between the two allelotypes CAPS₈₆₆G/G and CAPS₈₆₆T/T in ethylene production and fruit firmness loss, nor in peak ethylene day from day 0 to day 20 (Supplementary Figures 2a–c), suggesting that no effect of allelotype CAPS₈₆₆T/T (*MdACS3a*-G289V/G289V) was detectable in this study.

Allelotyping of *MdACS1* and *MdACS3a* in a large set of *Malus* accessions

Additional 855 *Malus* accessions were surveyed with markers ACS1, CAPS₈₆₆ and CAPS₈₇₀, leading to a total of 952 *Malus* accessions allelotyped (Figure 7, Supplementary Table S1). The data showed that the three allelotypes *MdACS1*-1/1, *MdACS1*-1/2

Figure 4. Comparison of the means of fruit ethylene production and firmness or firmness loss among allelotypes of *MdACS1* as defined by marker ACS1 (a and d), and among those of *MdACS3a* as defined by markers CAPS₈₆₆ (b and e) and CAPS₈₇₀ (c and f). The allelotypes are annotated similarly as those in the legend of Figure 3. Colors of column in blue, orange, green, purple and turquoise represent days 0, 5, 10, 15 and 20, respectively. The statistical tests were conducted independently within each of the five storage time points (days 0–20). Significance levels are indicated with letters (shown above the columns in the chart), where different letters indicate $P < 0.05$. The numbers of accessions observed (n) for each allelotype are presented accordingly (shown above the letters for significance). Error bars indicate s.e.

Figure 5. Comparison of the means of peak ethylene day among the allelotypes of *MdACS1* as defined by marker ACS1 (open column) and those of *MdACS3a* as defined by markers CAPS₈₆₆ (dot-filled column) and CAPS₈₇₀ (filled column) (a), and among the allelotypes of *MdACS3a* defined by markers CAPS₈₆₆ (b) and CAPS₈₇₀ (c) under the same background of *MdACS1*-1/1 or *MdACS1*-1/2. The allelotypes, significance levels and observed numbers are represented similarly as those in Figures 3 and 4.

Figure 8. Frequency of the *MdACS1* and *MdACS3a* alleles as defined by markers ACS1, CAPS₈₆₆ and CAPS₈₇₀ in all the 952 *Malus* accessions (a), *M. domestica* (b), *M. hybrid* (c) and *M. sieversii* (d).

and CAPS₈₆₆T of 90.0% and 10.0%, and CAPS₈₇₀C and CAPS₈₇₀T of 57.4% and 42.6%, respectively (Figure 8a).

To investigate whether and how human selection might have favored or repressed these alleles, their frequency in the most represented species *M. domestica* (508 accessions), *M. hybrid* (146) and *M. sieversii* (78), which collectively accounted for 76.9% of the 952 accessions (Supplementary Table S1), were independently estimated (Figures 8b–d). In comparison with *M. sieversii*, *M. domestica* and *M. hybrid* showed the largest allele frequency increases for alleles MdACS1–2 (from 0.6% to 18.8–24.5%) and CAPS₈₇₀T (from 5.1% to 34.3–48.3%), or decreases for allele MdACS1–1 (from 99.4% to 81.2–75.5%) and CAPS₈₇₀C (from 94.9% to 65.7–51.7%), but minimal changes for the CAPS₈₆₆G (from 86.5% to 86.8–94.2%) and CAPS₈₆₆T (from 13.5% to 13.2–5.8%) alleles (Figures 8b–d). These results suggested that apple-breeding practice may have selected for alleles MdACS1–2 and CAPS₈₇₀T (*Mdacs3a*), against alleles MdACS1–1 and CAPS₈₇₀C, and is neutral for alleles CAPS₈₆₆G and CAPS₈₆₆T (*MdACS3a*-G289V). Such human selection for alleles MdACS1–2 and *Mdacs3a* supported their observed significant effect on reduced or delayed ethylene production. Meanwhile, the minimal changes in the frequency of allele *MdACS3a*-G289V reinforced the unfound effect of this allele on ethylene.

DISCUSSION

The effect of *MdACS1* and *MdACS3a* and beneficial alleles

The allelic effect of *MdACS1* on fruit ethylene production and softening was significant and detectable at nearly all time points tested during the 20-day post-harvest period in the 97 *Malus* accessions. This was consistent with the critical role of *MdACS1* reported in many other studies.^{10–16,26–29} Since the allele

frequency of *MdACS1*–2 was 24.5% in *M. domestica*, 18.8% in *M. hybrid* and only 0.6% in *M. sieversii* (Figure 8), which is the major progenitor species of domestic apples, artificial selection has clearly favored MdACS1–2 over MdACS1–1. In fact, such allele preference of MdACS1–2 over MdACS1–1 was even reported within *M. domestica* when the frequencies of the two alleles in apple cultivars were plotted against their time of introduction.¹⁶ These observations are in accordance with the finding that allele MdACS1–2 is a beneficial allele associated with low ethylene and slow softening (Figures 4 and 6).

MdACS3a was regarded a main regulator for ethylene production transition from system 1 to 2.¹⁸ The gene was also similarly shown to be an accelerator³⁰ or an inducer³¹ of apple fruit ripening based on its gene expression timing and patterns in apple cultivars of varying ethylene levels and softening rates. In this study, such roles of *MdACS3a* were also detected through examining the allelic effect of *Mdacs3a* (CAPS₈₇₀T) on peak ethylene day, which reflects the timing of the climacteric ethylene burst. For example, under the same background of MdACS1–1/1, allelotype *Mdacs3a*/*Mdacs3a* (CAPS₈₇₀T/T) showed a significant delay in peak ethylene day when compared with what was observed for allelotypes *MdACS3a*/*MdACS3a* (CAPS₈₇₀G/G) and *MdACS3a*/*Mdacs3a* (CAPS₈₇₀G/T) (Figure 5c). Moreover, the allele frequency of *Mdacs3a* (CAPS₈₇₀T) was 34.3% in *M. domestica* and 48.3% in *M. hybrid*, a dramatic increase from the corresponding frequency of 5.1% in *M. sieversii*, indicating a strong human selection for allele *Mdacs3a*, presumably for the benefit of delayed ethylene production. Taken together, these data support the regulatory role of *MdACS3a* in ethylene production transition in apple fruit.

However, the allelic effect of *MdACS3a*-G289V on fruit ethylene production, softening and peak ethylene day was shown to be insignificant in the 97 *Malus* accessions, as well as in the 34

progeny from the 2 controlled crosses segregating for allelotype *MdACS3a*-G289V/G289V (*CAPS₈₆₆T/T*) under the same background of *MdACS1* allelotype. Furthermore, the allele frequency of *MdACS3a*-G289V (*CAPS₈₆₆T*) was 13.5% in *M. sieversii*, 13.2% in *M. domestica* and 5.8% in *M. hybrid*, providing no evidence that *MdACS3a*-G289V (*CAPS₈₆₆T*) has been enriched in response to selection. These results were surprising as *MdACS3a*-G289V was shown to be a functional null allele of *MdACS3a*.¹⁸ In a previous study, the two null alleles *MdACS3a*-G289V (*CAPS₈₆₆T*) and *Mdacs3a* (*CAPS₈₇₀T*) were concluded to affect the ripening initiation only in late-season apple cultivars, but not in early- or mid-season ones.²⁰ Such discrepancy in different studies regarding the roles of the two null alleles of *MdACS3a*, particularly *MdACS3a*-G289V, calls for further investigations into the role of *MdACS3a*-G289V. Nevertheless, alleles *MdACS1*-2 and *Mdacs3a* (*CAPS₈₇₀T*) are clearly demonstrated to be beneficial for breeding apples of low or delayed ethylene profiles in this study, a first effort that simultaneously assessed the roles of *MdACS1* and *MdACS3a* in fruit ethylene production and softening in highly diverse *Malus* materials.

Markers ACS1, CAPS₈₆₆ and CAPS₈₇₀

The assessment of the roles of *MdACS1* and *MdACS3a* in apple fruit ethylene production and softening largely relied on the previously developed marker ACS1^{10,11} and the two markers CAPS₈₆₆ and CAPS₈₇₀ developed in this study. Since CAPS₈₆₆ directly detects the mutation SNP G₈₆₆/T₈₆₆, CAPS₈₆₆ is an unequivocal marker for identifying the functionally null allele *MdACS3a*-G289V.¹⁸ Marker CAPS₈₇₀ detects SNP C₈₇₀/T₈₇₀ that does not correspond to a change in the encoding amino acid, that is, CAPS₈₇₀ detects a silent mutation in *MdACS3a*. Regardless of the nature of SNP C₈₇₀/T₈₇₀, T₈₇₀ is a genetic signature for allele *Mdacs3a* as the mutation was identified in 'Fuji', the very source from which the transcriptional null allele *Mdacs3a* was originally defined.¹⁸ Based on the genomic DNA sequences from 'Fuji', alleles *MdACS3a* (JF833309) and *Mdacs3a* (JF833309) differ by 14 nucleotides, and of these, only 4 were within the coding sequence.²⁰ Sequencing of the 92 *Malus* accessions in this study indicated that SNP C₈₇₀/T₈₇₀ is authentic and varying only between 2 nucleotides C₈₇₀ and T₈₇₀ (Figure 2, Supplementary Table S3). These data strongly support that CAPS₈₇₀ is a reliable marker for detecting allele *Mdacs3a*. Since both CAPS₈₆₆ and CAPS₈₇₀ detect the characterized SNPs in the coding sequence of *MdACS3a* and can be simply performed by electrophoresis on agarose gels, the two markers are readily applicable for marker-assisted selection in apple breeding.

Since SNP C₈₇₀/T₈₇₀ is located only four bases downstream of SNP G₈₆₆/T₈₆₆, markers CAPS₈₆₆ and CAPS₈₇₀ were once considered to be used as a single marker in this study. However, such usage would lead to an ambiguous scenario for allelotype G₈₆₆T₈₆₆/C₈₇₀T₈₇₀ as it could be formed by a combination either between gametes G₈₆₆T₈₇₀ and T₈₆₆C₈₇₀ or between gametes G₈₆₆C₈₇₀ and T₈₆₆T₈₇₀. To avoid such possible uncertainty, the two markers were used independently.

Previously, an SSR marker targeting at the promoter region of *MdACS3a* was developed and used to allelotype *MdACS3a* in 103 apple varieties.²⁰ It was shown that three alleles (331, 353, and 359 bp) of the SSR marker corresponded to the wild-type allele *MdACS3a* (that is, *MdACS3a*-1 in ref. 20), two alleles (333 and 335 bp) to *Mdacs3a* (that is, *MdACS3a*-2) and one allele (361 bp) to *MdACS3a*-G289V (that is, *MdACS3a*-1V). This makes the corresponding relationship between the SSR marker alleles and the *MdACS3a* alleles somewhat indirect and inconvenient. Since the size of the SSR marker alleles frequently differ by 2 bp, an automatic DNA sequencer-based detection system is necessary, thereby requiring more sophisticated handling and analysis, compared with the agarose gel-based markers CAPS₈₆₆ and CAPS₈₇₀. However, identical allelotypes were observed for all 19 apple cultivars used

by co-insistence in both studies (Supplementary Table S4), suggesting that the SSR marker and the 2 CAPS markers are useful for allelotyping of *MdACS3a*. As expected, identical allelotypes for *MdACS1* were also obtained for the 19 common apple cultivars between these 2 studies (Supplementary Table S4).

It should be mentioned that two degenerated CAPS (dCAPS) markers were developed to confirm alleles *Mdacs3a* and *MdACS3a*-G289V in cDNA, but the two dCAPS markers were not used for allelotyping the *MdACS3a* alleles.²⁰ Therefore, the applicability of the dCAPS markers is unknown in diverse apples.

Utility of the data

Of the 952 *Malus* accessions, 97 were evaluated for their fruit ethylene production and softening at 5 time points over a 20-day post-harvest period (Supplementary Table S3). Although most accessions seemed to have predictable ethylene-regulated post-harvest behaviors, 'Virginia Gold' (PI588778, *M. domestica*) was unusual as it had minimal firmness loss (comparable to 'Fuji') during the 20-day storage while producing high levels of ethylene (comparable to 'Golden Delicious'). This suggested that the slow softening (long-shelf life) character of 'Virginia Gold' is likely less dependent on ethylene production. More importantly, 'Virginia Gold' has also been shown with an excellent storability.³² To understand the lack of ethylene-related softening in 'Virginia Gold', several preliminary experiments have been initiated by the authors. In melon, it was reported that flesh softening involved both ethylene-dependent and -independent components.³³ In tomato, the ethylene-independent aspects of fruit ripening were evidenced to be regulated by the FRUITFULL homologs.³⁴ It is possible that investigating fruit softening independent of or less dependent on ethylene production would lead to new knowledge for better understanding of the apple fruit-ripening process, promising an interesting research area in apple post-harvest biology.

In addition, the data set of allelotypes for genes *MdACS1* and *MdACS3a* generated in the 952 *Malus* accessions would be useful for other future studies involving *MdACS1* and *MdACS3a*, which are the only 2 apple ACS genes known to be expressed specifically in fruit and associated with apple fruit ethylene production and firmness.^{8,9,13} The data set, together with three markers ACS1, CAPS₈₆₆ and CAPS₈₇₀, would be also useful for planning new crosses for developing improved apples with low ethylene and reduced loss of firmness.

Usage of terms allelotype and allelotyping

Term allelotype is defined as 'the frequency of alleles in a breeding population.' according to 'A Dictionary of Genetics'.³⁵ In this study, allelotype is referred to the allele composition at a specific gene locus, that is, *MdACS1* or *MdACS3a*, in individual accessions, highly similar to term 'genotype' for a given DNA marker. Such usage of allelotype represents a drift from or an expansion for the original definition of allelotype defined in the dictionary. However, the usage offers convenience for describing allele composition at a specific gene locus. Indeed, such usage has been adapted already in literature.^{14,18,20}

The definition for term allelotyping in "Encyclopedia of Genetics, Genomics, Proteomics, and Informatics"³⁶ reads 'Allelotyping is the determination of the spectrum and frequency of allelic variations in a population.' The usage of allelotyping in this study is largely covered by the definition, but an extension to include activities for determining allelotype (allele composition at a specific gene locus) is also practiced.

Conclusions

A substantial effort to simultaneously assess the roles of *MdACS1* and *MdACS3a* in fruit ethylene production and softening in diverse

Malus materials is presented in this study. The most relevant findings include: (1) *MdACS1* had much greater direct influence on fruit ethylene production and softening than *MdACS3a*. (2) Allele *MdACS1-2* was associated with low ethylene and slow softening while *MdACS1-1* with high ethylene and rapid softening. (3) Under the same background of *MdACS1* allelotypes, the transcriptional null allele *Mdacs3a*, rather than the functional null allele *ACS3a-G289V*, significantly delayed the time required to reach the climacteric ethylene peak. (4) Alleles *MdACS1-2* and *Mdacs3a*, but not *ACS3a-G289V*, were highly enriched in *M. domestica* and *M. hybrid* when compared with those in the *M. sieversii*. Overall, this study provides important information as to which alleles of *MdACS1* and *MdACS3a* are beneficial for low and delayed ethylene production and how these beneficial alleles can be selected for apple improvement.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was financially supported in part by the National Plant Germplasm System (NPGS), Apple Crop Germplasm Committee (CGC), Federal Formula Funds, and College of Agriculture and Life Science, Cornell University.

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