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Short communication

Exploring the apple genome reveals six ACC synthase genes expressed during fruit ripening

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ABSTRACT

Fruit ripening of apple is regulated by a plant hormone ethylene. Ethylene signaling is based on its biosynthesis in which ACC synthase (ACS) is the rate-limited enzyme and plays the most important role. By exploring the apple whole genome, we identified 19 *ACS* genes, and their expressions in fruit were investigated. Six *ACS* genes were expressed in fruit, including four new sequences. Out of these six genes, three were totally inhibited by 1-MCP (1-methylcyclopropene, an ethylene inhibitor), which were considered to work in System 2 ethylene; the others did not respond to 1-MCP, being considered to work in System 1 ethylene biosynthesis. This study showed that the whole genome can be used as a potential resource to identify new members of a multi-gene family important for a specific trait in apple.

1. Introduction

The release of entire genome sequence of apple (Velasco et al., 2010) provides a new resource to understand a certain research area such as ethylene biosynthesis. The availability of complete genomic sequence makes it easier to identify and characterize each gene of a multi-gene family. And it becomes a very important way to study the gene networks in regulation of fruit ripening.

Ethylene plays an essential role in the ripening of climacteric fruits (Martel et al., 2011). Ethylene biosynthesis has been studied extensively in several plant species including apple (Yang and Hoffman, 1984; Seymour et al., 1993). The formation of ACC (1-aminocyclopropane-1-carboxylic acid), the immediate precursor of ethylene synthesis, and the conversion of ACC to ethylene have

of ACC oxidase (ACO) (Yang and Hoffman, 1984). The formation of ACC is generally considered to be the rate-limiting step in the biosynthesis of ethylene (Kende, 1993). Thus, ACS is the most important enzyme in this pathway.

There are 12 *ACS* genes (*ACS1* to *ACS12*) in *Arabidopsis* and all of them display different expression patterns throughout growth and development, and during various stress conditions (Tsuchisaka and Theologis, 2004; Lin et al., 2009). Tomato (*Lycopersicon esculentum*) has nine *ACS* genes having been reported, *LeACS1A*, *B* and *LeACS2* to *LeACS8* (Lin et al., 2009), in which *LeACS1A*, *LeACS2*, *LeACS4*, and *LeACS6* are expressed in mature and ripening fruit with different expression profiles (Nakatsuka et al., 1998; Barry et al., 2000).

The biosynthesis of ethylene has been divided into two systems in higher plants (McMurchie et al., 1972). System 1 is responsible for the basal level of ethylene production during normal vegetable growth; System 2 operates for the burst of ethylene production during the ripening of climacteric fruit; System-1 ethylene is auto-inhibitory, while the System-2 is auto-stimulatory (Seymour et al., 1993; Lelievre et al., 1997).

In apple, seven *ACS* genes have been reported: *MdACS1* (accession no. U89156), *MdACS3a* (AB243060), *MdACS3b* (AB243061), *MdACS3c* (AB243062), *MdACS4* (Kim et al., 1992), *MdACS5A* (AB034992) and *MdACS5B* (AB034993) (Rosenfield et al., 1996; Sunako et al., 1999, 2000). Among these genes, only *MdACS1* and *MdACS3a* are expressed specifically in fruit tissue and act important roles in regulating fruit ripening (Sunako et al., 1999; Wakasa et al., 2006; Wang et al., 2009b; Varanasi et al., 2011). Based on the investigation of Tan et al. (2012), *MdACS3a* is expressed lower and before fruit ripening, operating for trace amount of ethylene in System 1; while *MdACS1* is expressed abundantly after fruit ripening and responsible for burst of ethylene production in System 2.

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Table 1
Chromosome position for ACS genes in apple.

Chromosome position for ACS genes in apple.

Gene	Accession	Published name	Apple predicted protein	Chromosome	Position (Mb)
ACS1	U89156	ACS1	MDP0000370791	LG 0	1.64
ACS3a	AB243060	ACS3a	MDP0000145123	LG 13	32.03
ACS6	-	-	MDP0000133334	LG 1	24.22
ACS7	-	-	MDP0000508068	LG 1	24.29
ACS8	-	-	MDP0000250254	LG 6	10.62
ACS9	-	-	MDP0000166535	LG 3	17.71
ACS3b	AB243061	ACS3b	MDP0000406217	LG 2	5.76
ACS3c	AB243062	ACS3c	MDP0000874578	LG 2	5.74
ACS4	-	ACS4	MDP0000262827	LG 1	9.88
ACS5A	AB034992	ACS5A	MDP0000923426	LG 2	33.73
ACS5B	AB034993	ACS5B	MDP0000435100	LG 7	14.19
ACS10	-	-	MDP0000413933	LG 9	9.97
ACS11	-	-	MDP0000454938	LG 15	1.19
ACS12	-	-	MDP0000321088	LG 2	19.71
ACS13	-	-	MDP0000123248	LG 7	14.20
ACS14	-	-	MDP0000232577	LG 15	1.16
ACS15	-	-	MDP0000408853	LG 1	24.32
ACS16	-	-	MDP0000308887	LG 15	1.18
ACS17	-	-	MDP0000265383	LG 1	22.66

Ethylene biosynthesis is a complicated process in higher plants which involve the cooperation of several *ACS* genes. For example, in tomato, there are four *ACS* genes (*LeACS1A*, *LeACS2*, *LeACS4* and *LeACS6*) working in fruit ethylene biosynthesis (Barry et al., 2000). Based on this knowledge, more *ACS* genes other than *MdACS1* and *MdACS3a* must exist in apple genome for operating the biosynthesis of ethylene.

The release of apple genome provides an easy way to identify new member of a certain gene family. In this study, we identified 19 *ACS* genes by retrieving the apple genome, and their expressions in apple fruit ripening were also investigated. Six of these *ACS* genes including *MdACS1* and *MdACS3a* were expressed during fruit ripening and respond differentially to 1-MCP (1-methylcyclopropene, an ethylene inhibitor). Knowledge on this investigation will be helpful for later research on ethylene biosynthesis in apple fruit.

2. Materials and methods

2.1. Identification of apple ACS genes

The apple genome predicted *ACS* gene sequences (http://www.rosaceae.org) were collected and compared to ACS proteins in other species by a BLASTp retrieve. Only those sequences with high score (>200) were selected. All the selected genes were compared with apple ESTs (expressed sequence tags) to verify their exact sequences.

2.2. Fruit materials

'Golden Delicious' (GD) apple fruits were sampled on commercial harvest day (GD on October 20, 2011). Fruits were kept at room temperature (24 °C) for 20 d and sampled every 5 days for measurements of ethylene production and flesh firmness. GD fruits at 14 days and 60 days before commercial maturity were also collected. The flesh was sliced, frozen quickly in liquid N_2, and stored at −80 °C for RNA extraction. Only the samples of −60, −14, 0, 5, 20 were used for testing ACS expression.

GD fruits, collected on commercial harvest day, were subjected to 1-MCP (1-methylcyclopropene, an ethylene inhibitor) treatment. Fruits were exposed to 1 $\mu l\,l^{-1}$ of 1-MCP (Fresh Doctor, China) for 24 h at 24 °C in an airtight container. After treatment, the fruits were held at 24 °C for 20 days and sampled every 5 days for RNA isolation.

2.3. Quantitative RT-PCR

RNA was isolated using RNA isolation kit (RNAplant Reagent, Cat# DP437, Tiangen Biotech, Beijing, China) according to the manufacturer's instruction. The first strand cDNA was synthesized from 500 ng total RNA, using M-MLV RTase cDNA Synthesis Kit (Cat#D6130, TaKaRa, Tokyo, Japan). The first strand cDNA was diluted ten times with H_2O, and then used as templates for qRT-PCR assays. qRT-PCR was carried out as described in Tan et al. (2012). Specific primers for each *ACS* gene were designed by Primer3 (http://frodo.wi.mit.edu/) and were listed in Table 1. The apple Actin gene (accession number EB136338) was used as internal control. Three replications were conducted.

3. Results and discussion

3.1. Identification of apple ACS genes

All the predicted *ACS* genes in apple genome were collected and compared with *ACS* genes in other species. Some of these predicted

Table 2
List of primers used in this study.

Gene	Primer	Sequence (5′-3′)
ACS1	MdACS1F	ACAGCCTCTCTAAGGATCTTGGTCT
	MdACS1R	TTTGGTTCTCGGCTATGTAGTTCTT
ACS3a	MdACS3aF	TTTGATAGAGATTTGAGGTGGAGGA
	MdACS3aR	GTGGGTTTGATGGATTTGTGATTAG
ACS6	MD133334F	AGTGGATTAATTCGCTCTTATGGTG
	MD133334R	GGAACATCCTCTGGAGTCAAAGTAG
ACS13	MD123248F	CAAGGAAAGAAAGCTCAAGAACAAG
	MD123248R	AGTGAGACATTGGTGAATGTGGAG
ACS7	MD508068F	GCCGTACTATATCGGGTTGGATAG
	MD508068R	CTACCATCGATTCCATCAAATTCTC
ACS8	MD250254-1F	TTCCAGACACCAAATTTATCCGG
	MD250254-1R	CGTTCCATAACAACCGGAATATCTT
ACS9	MD166535F	CCAGCGCTATCTACTCCTTTAACAA
	MD166535R	TACATTCCACGAAGCCTTTCTCTAC
ACS4	MdACS4F	GCGTGTAATCGTTAATGAAGTGAAG
	MdACS4R	CTTGTTCTTGAGCTTTCTTTCCTTG
ACS5A	MdACS5A-F	GAAACTTTGTGGCTCAATACAAGGA
	MdACS5A-R	GATTGAGGTATCGGTGAATGAGGT
ACS5B	MdACS5B-F	CAGAAGGCAAACATCAGAGTAAAGG
	MdACS5B-R	TTATGAAACTTGGCTGACTGAACAC
ACS10	MD413933F	ATTGAATATCTCTCCCGGTTCTTCT
	MD413933R	CTGGATTTAGACAGTCCTGTTTGCT
ACS11	MD454938F	GCATGCTTTACTCCAACAACCTAAC
	MD454938R	CAGACAAGCATCTCCTTTCTCTCTT
ACS12	MD321088-1F	GCTGCTACAAAAATCACCTTCTCT
	MD321088-1R	CAAAGGCCTTCAGTCTCTGC
ACS13	MD123248F	CAAGGAAAGAAAGCTCAAGAACAAG
	MD123248R	AGTGAGACATTGGTGAATGTGGAG
ACS14	MD232577F	GCATGCTTTACTCCAACAACCTAAC
	MD232577R	CAGACAAGCATCTCCTTTCTCTCTT
ACS15	MD408853F	TTGAAGTACTCTTCCTCCATCCAAG
	MD408853R	GAGGGATTGAGTGAGATTCGAGAG
ACS16	MD308887F	GTTGAATATCTCTCCCGGTTCTTCT
	MD308887R	CTAGATTTGGACAGTCCTGTTTGCT
ACS17	MD265383F	AGGAGATATCCCAAGGCCTCTATTT
	MD265383R	AGGATCAGATATGACTCAACCACCT
Actin	MdAct-F	GGCTGGATTTGCTGGTGATG
	MdAct-R	TGCTCACTATGCCGTGCTCA

genes showed exon–intron difference although they were mapped on the same location in apple genome. In this case, only those sequences matched ESTs (expressed sequenced tags) were selected as our target genes. Based on this information, 19 *ACS* genes were identified in apple genome. Out of these *ACS* genes, seven of them have been reported previously and were labeled with their published names (Table 2). For the other 12 unreported genes, we gave them a name by adding a number to their family name (Table 2).

ACS genes are divided into three types according to the similarity of their sequences and C-terminal consensus motifs (Yoshida et al., 2005). Type I *ACS* genes possess 'RLSF' motif and a long C-terminal tail which is essential for CDPK and MPK6 phosphorylation (Liu and Zhang, 2004). Type II *ACS* genes have 'WVF' residues right before the 'RLSF' motif. The Type III isoforms lack the 'WVF' and 'RLSF' concensus sequences. Type II *ACS* genes are regulated negatively by ETO1 (ethylene-overproducer 1), which is a negative regulator of ethylene production and promotes the degradation of *ACS* genes via a proteasome pathway (Chae et al., 2003; Yoshida et al., 2005, 2006). We did an alignment for these six apple *ACS* genes expressed in fruits with tomato *ACS* gene by using their deduced amino acid sequences (Fig. 1). Only *MdACS1* is Type II *ACS* gene and the other five belong to Type III. None of them are Type I *ACS* gene. These results indicated that only *MdACS1* was probably under the regulation of ETO1 in apple. On the other hand, type I *ACS* genes might not be important in fruit ripening.

3.2. Fruit firmness and ethylene production

Only those fruits stored after harvest have been subjected to measurement of fruit firmness and ethylene production. As shown in Fig. 2A, the fruit firmness dropped rapidly after commercial harvest, while fruits treated with 1-MCP maintained very high firmness even when fruits were kept at room temperature for 20 days. Accordingly, the ethylene production was increased to a very high level after fruits were stored at room temperature for 5 and 20 days, however, it was greatly inhibited by 1-MCP treatment, in which the ethylene production was approximately 200 times lower than that in nontreated fruits (Fig. 2A).

3.3. Expression analysis

The expressions of these *ACS* genes were investigated by qPCR (quantitative PCR) before and after fruit harvest, in which −60 and −14 indicated that the fruits were harvested at 60 and 14 days before commercial harvest day, 0 meant commercial harvest day, and 5 and 20 indicated the fruits were stored at room temperature for 5 or 20 days. 1-MCP treated fruits were also used to test whether the expressions of these *ACS* genes were regulated by ethylene.

Out of these nineteen *ACS* genes, six of them were expressed in apple fruits (Fig. 2B). *MdACS1* and *MdACS3a* have been reported in earlier studies (Sunako et al., 1999; Wang et al., 2009b). The transcript of *MdACS1* was only observed in the fruits after harvest and was completely inhibited by 1-MCP treatment; *MdACS3a* was expressed 14 days before commercial harvest, and 1-MCP slightly affected its expression. These results were same as the earlier findings (Sunako et al., 1999; Wiersma et al., 2007; Wang et al., 2009b).

MdACS6, 7, 8 and *9* were newly identified from the apple genome. *MdACS6* was expressed from 60 days before commercial harvest and the expression level increased at 5 days after harvest when climacteric ethylene began evolving. 1-MCP treatment did not affect very much on its expression (Fig. 2B). In fruits at 20 days after 1-MCP treatment, the expression of *MdACS6* was somehow higher than

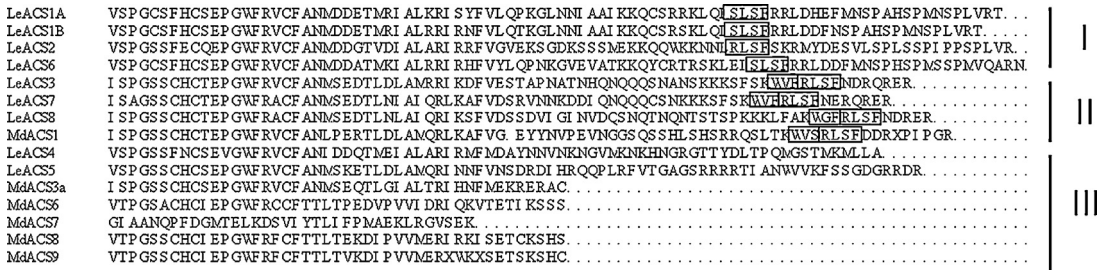

Fig. 1. Alignment of C-ends of *ACS* genes from apple and tomato. Deduced amino acid sequences from apple (MdACS1 U89156, MdACS3a AB243060, MdACS6 MDP0000133334, MdACS7 MDP0000508068, MdACS8 MDP0000250254, MdACS9 MDP0000166535) and tomato (LeACS1A U18056, LeACS1B U18057, LeACS2 X59139, LeACS3 U17972, LeACS4 X59146, LeACS5 AF167425, LeACS6 AF167428, LeACS7 AF043122, LeACS8 AF167427) were used. Three types of ACSs were grouped according to Yoshida et al. (2005). Squares indicate the motifs in *ACS* genes.

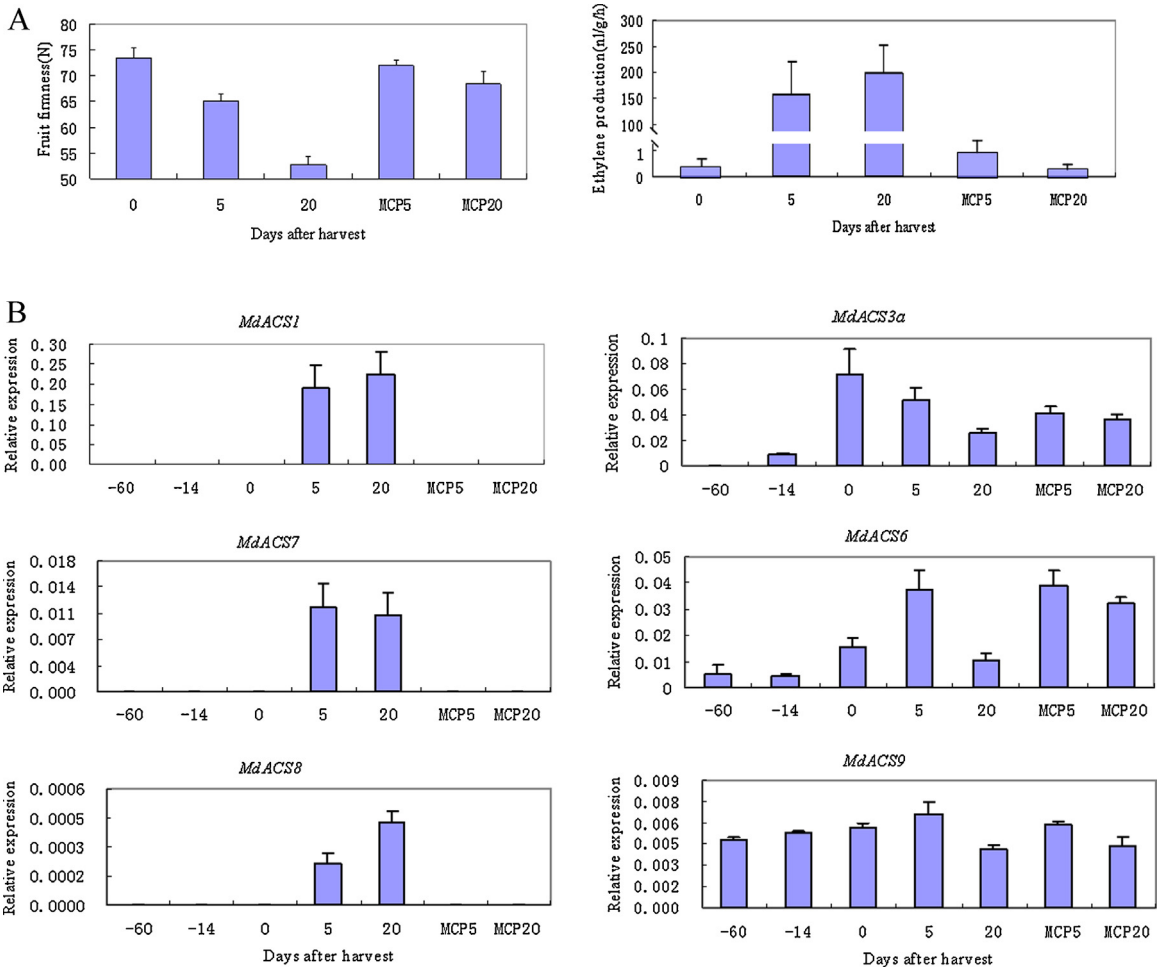

Fig. 2. Fruit firmness, ethylene production and expression of apple *ACS* genes in GD fruits. A, Fruit firmness and ethylene production of GD fruits stored at room temperature after harvest. The numbers under the *X*-axis indicate the fruit samples used in this study. 0; fruits on commercial harvest, −14 and −60, fruits collected at 14 and 60 days before commercial harvest. 5 and 20; fruits stored at room temperature for 5 and 20 days after commercial harvest. 5MCP and 20MCP; fruits treated with 1-MCP and then stored at room temperature for 5 and 20 days. B, Expression of apple ACS genes in GD fruits. Total RNA from GD fruits was used for cDNA synthesis and qRT-PCR as described in Section 2. Expression of each gene is presented as relative fold change. Three replicates were performed for each experiment and the bars represent SE.

in non-treated fruits, probably because the 1-MCP treatment kept the fruits fresher. The early expression of *MdACS6* and its failure to respond to 1-MCP were indications that *MdACS6* might work in System 1 ethylene biosynthesis. However, its expression increased at 5 days after harvest, indicating that it might also work in System 2 ethylene biosynthesis.

Expressions of *MdACS7* and *8* were only observed in fruits after harvest and were totally blocked by 1-MCP treatment. These expression patterns paralleled those *ACS* in System 2 ethylene biosynthesis, indicating that they worked in System 2 ethylene biosynthesis. Therefore, three *ACS* genes including *MdACS1* have been found to be expressed in System 2 ethylene biosynthesis. The expression of *MdACS1* is quite abundant in apple fruit (Wang et al., 2009a), showing its strongest catalyzation in synthesis of ethylene. Some *MdACS1-2* homozygous apple varieties (such as 'Fuji'), in which the expression of the *MaACS1* is attenuated considerably due to a transposon insertion at the promoter (Sunako et al., 1999), produce small amount of ethylene and can finish their ripening process. The part of ethylene production may be from the catalyzation of *MdACS7* and *MdACS8*. *MdACS9* was expressed constitutively throughout the experimental period. Moreover, it did not respond to 1-MCP, indicating that *MdACS9* might be irrelative to fruit ripening.

The action of *ACS* genes is under the regulation of *MADS-box* gene which is a large transcription factor family. Tomato *MADS-box* gene *RIN* plays crucial role in regulation of fruit ripening (Vrebalov et al., 2002). It has been reported that RIN combines with the promoters of *LeACS2* which operates for System 2 ethylene production (Martel et al., 2011). The ortholog of *RIN* in apple has been cloned and the characterization is being conducted by us (unpublished data). It will be quite interesting to study the interaction between *MADS-box* transcription factors and the *ACS* genes in apple and highlight their regulation on apple fruit ripening.

Taken together, a total of 19 *ACS* genes were revealed in apple genome by exploring the genome sequence data. It is the largest *ACS* gene family currently known in plant. Six of these *ACS* genes were expressed in apple fruit, in which four of them were new members of this gene family. In this study, we also found that five of the six apple *ACS* genes were related to fruit ripening, with three of them were regulated by ethylene, suggesting their different roles in ethylene biosynthesis systems. This study also revealed the needs to consider more *ACS* genes in the research on ethylene biosynthesis in apple fruits.

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