Europ.J.Hort.Sci., **76** (3). S. 77–83, 2011, ISSN 1611-4426. © Verlag Eugen Ulmer KG, Stuttgart

The Regulatory Role of 1-aminocyclopropane-1-carboxylate Synthase Genes in Apple Fruit Shelf-life

A. Wang[1)], T. Li[2)] and T. Harada[1)]
([1)]Laboratory of Plant Breeding and Genetics, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki, Japan and [2)]Laboratory of Fruit Cell and Molecular Breeding, College of Agronomy and Bio-tech, China Agricultural University, Beijing, P.R. China)

Summary

The postharvest economic quality of mature apple (*Malus* × *domestica* Borkh.) is determined by the fruit shelf-life, which is associated with the fruit softening rate at room temperature. Ethylene is the factor that has been most studied in relation to the regulation of fruit softening. Softening rate and ethylene production differ widely among apple cultivars. 1-aminocyclopropane-1-carboxylic acid synthase (ACS) is one of the key regulatory enzymes involved in the synthesis of ethylene. In apple, at least four members of the *ACS* gene family have been identified, two of which, *MdACS1* and *MdACS3*, have been studied extensively due to their specific expression in fruit tissue. Recent characterization of these genes has provided insight into the molecular basis of *ACS* gene involvement in fruit ripening, and a role of *ACS* genes in the regulation of system-1 and system-2 ethylene biosynthesis has been proposed. This paper reviews advances in our understanding of the relationship between *ACS* gene expression and the fruit shelf-life of apple, and also discusses other factors that influence fruit ripening. In addition, we prospectively overview promising research avenues that could lead to further clarification of the regulatory events involved in ripening.

Key words. apple – fruit – ethylene – acs – ripening – shelf-life

Introduction

Shelf-life determines the economic value of mature apple (*Malus* × *domestica* Borkh.) fruit, which can be stored after harvest, and a good shelf life means specifically a slow breakdown of firmness of the mature fruit at room temperature during ripening (Costa et al. 2005). Cold storage, modified atmosphere (MA) and controlled atmosphere (CA) storage have been widely used to extend the shelf life of fruit after harvest. Most apples placed in regular cold storage cannot be stored for long. The MA and CA technologies radically slow the ripening process and maintain the better fruit quality, making long-term storage of apple fruit possible, but their costs are high in terms of the required building facilities and maintenance (Johnston et al. 2002; Yahia 2009). Therefore it would be highly desirable to develop new commercial cultivars with a longer shelf-life.

Considerable effort has been made to understand the biological mechanisms involved in fruit softening during the ripening process. Ripening is affected by internal and external cues, including developmental gene regulation, hormones, light and temperature (Adams-Phillips et al. 2004; Giovannoni 2004; Dal Cin et al. 2007; Wang et al. 2009a). The most studied factor is ethylene, which is a major plant growth regulator and ripening hormone in climacteric fruits such as apple, pear and peach. The onset of ethylene production during fruit ripening is widely believed to be the key driver of the cellular structural changes that lead to softening (Chaves and de Mello-Farias 2006; Bapat et al. 2010). Ethylene biosynthesis has been extensively studied in several plant species including apple (Liang et al. 1992; Lincoln et al. 1993; Seymour et al. 1993; Capitani et al. 2002). The enzyme that limits ethylene synthesis is ACC (1-aminocyclopropane-1-carboxylic acid) synthase (EC 4.1.1.14, ACS), which catalyzes the formation of ACC, the immediate precursor of ethylene synthesis, and the subsequent oxidation of ACC to ethylene is catalyzed by ACC oxidase (ACO) (Yang and Hoffman 1984; Gussman et al. 1993; Gorny and Kader 1997). ACS is generally the rate-limiting enzyme in the biosynthetic pathway (Kende 1993).

In *Arabidopsis*, there are 12 *ACS* genes (*ACS1* to *ACS12*), all of which display different expression patterns throughout growth and development, and during various stress conditions (Tsuchisaka and Theologis 2004b). For example, the *ACS3* most likely is a pseudogene and *ACS1* encodes a nonfunctional ACS (Liang et al. 1995). *ACS2* expression is high in young tissues and is switched off as the tissue matures, and is also related to lateral root formation (Rodrigues-Pousada et al. 1993). *ACS4* and *ACS5* are responsive to auxin and cytokinin (Abel et al. 1995; Vogel et al. 1998), respectively. And *ACS6* is induced by ozone and other stimuli (Vahala et al. 1998).

Tomato (*Lycopersicon esculentum*), as a model plant for studying fleshy fruits, has nine *ACS* genes, *LeACS1A*, *B*

and from *LeACS2* to *LeACS8* (reviewed by Lin et al. 2009). Out of these nine members, *LeACS1A*, *LeACS2*, *LeACS4*, and *LeACS6* are expressed in mature and ripening fruit, but with distinct expression profiles (Nakatsuka et al. 1998; Barry et al. 2000). Nakatsuka et al. (1998) has reported that the *ACS* family members in tomato respond differently to 1-methylcyclopropene (MCP, an ethylene inhibitor). *LeACS2* and *LeACS4* mRNAs were greatly inhibited by 1-MCP in ripening fruit; *LeACS1A* and *LeACS3* were expressed constitutively throughout fruit development and ripening with no response to 1-MCP treatment; *LeACS6* mRNA was observed in non-ripening fruit and was undetectable in ripening fruit, but was induced by 1-MCP treatment in ripening fruit. In addition, these genes also respond differently to ethylene. *LeACS1A* is expressed constitutively, while *LeACS6* is largely reduced; *LeACS2* mRNA was not detected when treated with ethylene.

A vast amount of work has been done to clarify the regulatory role of *ACS* gene expression in ethylene biosynthesis and the shelf life of apple fruit. This review focuses on recent advances in our knowledge of the relationship of the *ACS* gene family and the shelf-life of apple fruit. Further work is needed to clarify the role of *ACS* gene expression in the individual steps of ethylene biosynthesis and fruit ripening.

Regulation of members of the *ACS* gene family in apple fruit

In apple, at least four *ACS* gene families have been reported: *MdACS1* (accession no. U89156), *MdACS3* (accession no. U73816), *MdACS4* (Kim et al. 1992), and *MdACS5* (accession no. AB034992) (Rosenfield et al. 1996; Sunako et al. 1999, 2000). Dong et al. (1991) isolated a partial *ACS* cDNA with a predicted translation product identical to an ACS extracted from ripening apple. Lay-Yee and Knighton (1995) reported the sequence of the full-length cDNA of gene *MdACS1* (accession no. L31347). The cDNA of *MdACS3* (accession no. U73816) in apple had been registered by Rosenfield et al. (1996). A clone of *MdACS4* has been identified by Kim et al. (1992). *MdACS-5A* (accession no. AB034992) and *-5B* (accession no. AB034993) have been reported by Sunako et al. (2000).

Yoshida et al. (2005) have reported three types of *ACS* genes, on the basis of the similarity of their sequences and C-terminal consensus motifs. Type I *ACS* genes possess a Ser residue in the 'RLSF' motif, which is essential for CDPK phosphorylation, and a long C-terminal tail containing three conserved Ser residues that are the targets of MPK6 phosphorylation, but lack a Val residue in the 'WVF' motif. In Type II *ACS* genes, the 'WVF' residues precede the 'RLSF' motif. The Type III isoform lacks the phosphorylation motifs, and the C-terminus is truncated by 24–47 amino acids. ETO1 (ethylene-overproducer 1), which acts as a negative regulator of ethylene production, regulates Type II *ACS* genes negatively by targeting the TOE (target of ETO1) sequence, promoting their degradation via a proteasome pathway (Chae et al. 2003).

In apple, the predicted amino acid sequence of *MdACS1* (AAB68617) has a 'WVS' instead of a 'WVF' motif preceding the 'RLSF' motif, suggesting that it could belong to Type II, but it is unclear whether *MdACS1* interacts with ETO1. *MdACS3* lacks the C-terminal sequence and the 'RLSF' motif, suggesting a Type III property (Wang et al. 2009b). *MdACS5* is thought to belong to Type I, based on the deduced amino acid sequence, which exhibits the 'RLSF' motif but lacks the 'WVF' motif (Sunako et al. 2000). The group to which *MdACS4* belongs remains unclear, due to the unknown nature of its full-length sequence. In apple, ETO1 and its paralogs have not yet been isolated, and it is necessary to study how they interact with *ACS* genes.

Among these four *ACS* genes isolated so far from apple, *MdACS1* and *MdACS3* occur specifically in fruit tissue, and have been extensively studied (Wakasa et al. 2006; Wang et al. 2009b). Wang et al. (2009a, b) analyzed the gene expression of *MdACS3a* in 'Golden Delicious' and 'Fuji' apple fruit ontogeny. It was found to be expressed two weeks before harvest, earlier than *MdACS1* and *MdACO1*, which were detected only after harvest in 'Golden Delicious'. In 'Fuji' fruit which were stored at room temperature, *MdACS3* was expressed constitutively throughout storage for 12 days (Wang et al. 2009a) and the same result was obtained even during storage for 3 months (Tatsuki et al. 2007). However, in early mature sport cultivars of 'Fuji', its expression decreased during storage (Wang et al. 2009a). These results show that the expression of *MdACS3* differs during fruit development and ripening in various cultivars, suggesting its role in regulation of fruit ripening.

The expression profile of *MdACS1* was clearly different from that of *MdACS3*. Expression of *MdACS1* was detected in apple fruit only after harvest, and was initiated at least two weeks later than that of *MdACS3* (Wang et al. 2009b). In 'Fuji' and its early sport cultivars, expression of *MdACS1* was not detected during storage (Wang et al. 2009a), but in 'Orin', 'Granny Smith', 'Tsugaru' and 'Golden Delicious', it was expressed vigorously during storage accompanied by a burst of ethylene production (Tatsuki et al. 2007; Wakasa et al. 2006; Zhu et al. 2008; Kondo et al. 2009).

Many experiments have been performed to test the response of *MdACS1* and *MdACS3* to treatment with 1-MCP (an ethylene antagonist). The expression of *MdACS1* was greatly inhibited in fruit treated with 1-MCP, whereas that of *MdACS3* was not affected (Wakasa et al. 2006; Tatsuki et al. 2007; Zhu et al. 2008; Asif et al. 2009; Wang et al. 2009a). These results suggest that *MdACS1* is up-regulated, whereas *MdACS3* is probably down-regulated, by ethylene since the expression of *MdACS3* was decreased after the explosive expression of *MdACS1* and *MdACO1* and onset of the ethylene burst (Wang et al. 2009b).

MdACS4 and *MdACS5* have received less attention. Expression of *MdACS4* has been found in apple stigma and receptacle, and is induced by auxin. *MdACS5* mRNA has not been detected in any tissue of apple, but has been observed in wounded tissues (Wakasa and Harada 2001). Neither of these two genes may be involved in apple fruit ripening (Wiersma et al. 2007).

MdACS1 and apple fruit ripening

Fruits obtained from plants silenced for *MdACS1* show reduced autocatalytic ethylene production and are significantly firmer than controls, displaying an increased shelf-life (Dandekar et al. 2004). These data indicate a role of *MdACS1* in regulation of the ethylene production

and shelf life of apple fruit. The genomic sequence of *MdACS1* (accession no. U89156) has been reported by HARADA et al. (1997). Furthermore, SUNAKO et al. (1999) have isolated an allelic form of *MdACS1* from a genomic library of 'Golden Delicious'. These two alleles were designated as *MdACS1-1* and *MdACS1-2*. A SINE (shorter interspersed nuclear element, BUREAU et al. 1996) was inserted into the promoter region of *MdACS1-2* allele, leading to low transcription activity in comparison to its counterpart *MdACS1-1*. Thus, cultivars homozygous for *MdACS1-2* produce a low level of ethylene, whereas cultivars heterozygous for *MdACS1-1* and *MdACS1-2* or homozygous for *MdACS1-1* produce a higher level of ethylene during fruit ripening (SUNAKO et al. 1999). ORAGUZIE et al. (2004) studied the inheritance of *MdACS1* using five F_1 populations and found that this gene is inherited in a Mendelian manner. HARADA et al. (2000) have measured the fruit IEC (internal ethylene concentration) of 35 commercial cultivars and 13 F_1 progenies derived from a cross between *MdACS1-1/2* cultivars, and found a strong correlation between *MdACS1* genotypes and IEC values; *MdACS1-2* was considered to be correlated with a long shelf-life. However, there is one exception to this correlation: fruits of 'Megumi' have been reported to show a 52 % decrease in firmness 20 days after harvest, even though this variety is harvested late and the *MdACS1-2/2* cultivar has little ethylene production (HARADA et al. 2000; ORAGUZIE et al. 2004). In addition, considerable differences in the level of IEC have been observed among the same *MdACS1* genotype (HARADA et al. 2000; ORAGUZIE et al. 2004). For example, fruits of the apple cultivar 'Kitaro' have less IEC and a longer shelf life than those of the sibling cultivar, 'Kotaro', even though both of them are heterozygous for *MdACS1-1/2* (WAKASA et al. 2006). Thus, the *MdACS1* genotype alone cannot unambiguously account for the IEC and storage potential of apple fruit.

ORAGUZIE et al. (2007) investigated the influence of the *MdACS1* allelotype (*-1/1*, *-1/2* or *-2/2*) and its interaction with harvest season (early or late) on changes in IEC and fruit softening during cold air storage at 0 °C. This study demonstrated that during medium-term cold air storage, cultivars of the *MdACS1-2/2* allelotype had a slower rate of increase in IEC and slower fruit softening than other *MdACS1* allelotypes, and that fruit of early maturing cultivars softened more rapidly than fruit of late cultivars. These effects were independent of each other, and therefore late *MdACS1-2/2* cultivars had the slowest rate of fruit softening, whereas early *MdACS1-1/1* cultivars had the most rapid rate. However, this finding was still not able to explain the exceptional cases of the apples 'Megumi', 'Kitaro' and 'Kotaro'. Overall, *MdACS1* genotypes can only partially explain the IEC and shelf life of various cultivars, even when taking the harvest season into consideration.

SATO et al. (2004) tested the relationship of *MdACS1* genotype to the softening rate of on-tree fruit by investigating two cross strains, 89–95 (*MdACS1-1/2*) and 94–95 *(MdACS1-2/2*). There was no significant effect of *MdACS1* genotype on the softening rate, and the fruits of the two strains showed the same rate of firmness loss. However, by calculating the pre-harvest fruit drop rate of 40 commercial cultivars, a good correlation between *MdACS1* genotype and fruit drop rate was obtained, cultivars homozygous for *MdACS1-2/2* having a lower pre-harvest drop rate than *MdACS1-1/1* and *MdACS1-1/2* cultivars, even though some cultivars did not follow this rule. It seems most likely that the *MdACS1* genotype has more of an influence on the pre-harvest fruit drop rate.

MdACS3 and apple fruit ripening

The genomic DNA of *MdACS3*, in apple, has been isolated by WANG et al. (2009b). Three subfamily genes of *MdACS3* (*a*, *b* and *c*) were isolated from a genomic library, but two of them (*MdACS3b* and *c*) possessed a transposon-like insertion (333 bp), designated as *Makh*, in their 5' flanking region, which may cause failure of *MdACS3b* and *c* transcription during ripening. The *Makh* insertion was not found in the orthologs of pear, *PcACS3* and *PcACS2* (WANG et al. 2009b).

In pear (*Pyrus communis* L.) and plum (*Prunus salicina* L.), the orthologs of *MdACS3a* are reportedly involved in fruit ripening (ITAI et al. 1999; EL-SHARKAWY et al. 2004, 2008). In apple, *MdACS3a* has been studied extensively for its involvement in fruit ripening (WANG et al. 2009b). An inactive isoform of MdACS3a, MdACS3a-G289V, found in the genomic DNA of apple, is caused by an amino acid substitution in the active site. Furthermore, another null allele of *MdACS3a*, *Mdacs3a*, found by sequencing of the cDNA, shows no transcription. Apple cultivars can be classified into several groups according to their homozygosity or heterozygosity for the *ACS3a* alleles. Apple cultivars homozygous or heterozygous for the two null-allelotypes exhibit no or very low expression of ripening-related genes and maintain their fruit firmness. Cultivars of *acs3a/ACS3a-G289V*, such as 'Kitaro' and 'Koukou', produce less ethylene and have higher storage ability due to absence of ACS3a activity, compared to *ACS3a/ACS3a-G289V* cultivars with active ACS3a, such as 'Kotaro' and 'Megumi'. Through the genotypes of *MdACS3a*, it is possible to explain the difference between 'Kitaro' and 'Kotaro', and the discrepancy observed for 'Megumi'. *ACS3a/acs3a* cultivars also produce little ethylene and have good storage ability. These results demonstrate a strong correlation between *MdACS3a* alleles and fruit shelf-life of apple. *MdACS3a* is now considered to be the determinant of apple fruit shelf-life (WANG et al. 2009b).

Role of *ACS* genes in system-1 and system-2 ethylene synthesis

It has been speculated that two systems of ethylene regulation exist in higher plants (MCMURCHIE et al. 1972). System 1, ethylene auto-inhibitory, is considered to function during normal vegetative growth and to be responsible for the basal level of ethylene production; System 2 has been proposed to operate for the upsurge of ethylene production during the ripening of climacteric fruit, when ethylene is auto-stimulatory (SEYMOUR et al. 1993; LELIEVRE et al. 1997; BARRY et al. 2000). The transition from system-1 to system-2 ethylene production is thought to be an important step during fruit ripening, and is developmentally regulated (LELIEVRE et al. 1997). Genes responsible for ethylene biosynthesis and signal transduction are stimulated during the climacteric burst of ethylene production and may be tightly associated in the transition from system 1 to system 2 (NAKATSUKA et al. 1998). In

tomato, system-1 ethylene is regulated by the expression of *LeACS1A* and *LeACS6*. During the transition period, the *RIN* gene plays a pivotal role leading to increased expression of *LeACS1A* and induction of *LeACS4*. System-2 ethylene production is subsequently initiated and maintained by the expression of *LeACS2* (Barry et al. 2000). Yokotani et al. (2009) recently reported that system 2 ethylene could be triggered by another ethylene- independent factor, and this has opened a new avenue of research into system-1 and system-2 ethylene.

In apple, *MdACS3* is expressed prior to other ripening-related genes such as *MdACS1* and *MdACO1*, and is suppressed after the burst of ethylene production. Therefore, *MdACS3* most likely functions in the regulation of system 1 and initiates system 2, followed by a burst of ethylene, and system-2 ethylene inhibits the expression of *MdACS3* by negative feedback regulation (Wang et al. 2009b). The results of Tatsuki et al. (2007) and Kondo et al. (2009) also support this possibility. The orthologous genes of pear and plum, *PcACS3* and *PsACS3* respectively, are also known to be important for system-1 ethylene production (El-Sharkawy et al. 2004, 2008).

The expression of *MdACS1* is initiated following the expression of *MdACS3* (Wang et al. 2009b), is abundant in some apple cultivars during the ripening process when a high level of ethylene production is present, and is greatly suppressed by treatment with 1-MCP (Wakasa et al. 2006; Tatsuki et al. 2007; Zhu et al. 2008; Kondo et al. 2009). Moreover, Dandekar et al. (2004) have reported that transgenic apples silenced for *MdACS1* produce less ethylene. These results suggest that *MdACS1* is responsible for the upsurge of ethylene production and functions in system-2 ethylene biosynthesis. In some apple cultivars such as 'Fuji', no expression of *MdACS1* is observed throughout the experimental period (Wakasa et al. 2006; Tatsuki et al. 2007; Wang et al. 2009a). This phenomenon may be caused by differences in the regulation of system 1 by *MdACS3a* alleles, where the produced ethylene is not sufficient to initiate the expression of *MdACS1* and system 2 (Wang et al. 2009b).

Other factors and apple fruit ripening

Ethylene response factor (ERF), which is involved in modulating fruit development, maturation, ripening and softening (Li et al. 2007), has been reported to be regulated by factors independent of ethylene in apple (Wang et al. 2007). Adams-Phillips et al. (2004) and Giovannoni (2004) have also reported that other factors independent of ethylene affect fruit ripening.

Storage temperature affects the post-harvest life of most horticultural produce (Woolf and Ferguson 2000; Johnston et al. 2001). Tacken et al. (2010) studied the cold-regulated control of apple fruit softening and proposed that cold alone might enhance the ethylene response such that background levels of ethylene in the ethylene-suppressed apples is sufficient to induce fruit softening in apples. An increase in the expression of an *sHSP* (small heat shock protein) mRNA and in the production of its protein is associated with high daily flesh temperatures in apple fruit (Ferguson et al. 1998). Wang et al. (2009a) have also found that *MdHSP17.5* (accession No. EU636239) is expressed vigorously in mature red 'Hirosaki Fuji', but not in immature green 'Fuji'. In strawberry and tomato, the orthologous genes of *MdHSP17.5* are expressed in ripening fruits, indicating its involvement in regulation of fruit ripening (Medina-Escobar et al. 1998; Anjanasree et al. 2005). These results indicate that ambient temperature may accelerate the ripening of 'Hirosaki Fuji' fruit via the expression of *MdHSP17.5* (Wang et al. 2009a). Janssen et al. (2008) also found a class II heat shock protein (EG631337) during a middle stage development of apple fruit by using microarray analysis.

The involvement of auxin in fruit ripening has also been reported. Auxin appears to affect fruit softening by acting with ACC synthase genes and inducing ethylene production (Curry and Greene 1993; Gillaspy et al. 1993). Trainotti et al. (2007) have reported crosstalk between ethylene and auxin, and Jones et al. (2002) have shown that Auxin Response Factor (ARF) is expressed differentially in tomato fruits. In apple, auxin has been found to stimulate the expression of *MdACS4*, but the mechanism involved is still unclear (Kondo et al. 2009). Lara and Vendrell (2000) studied the interaction between abscisic acid and ethylene in apple, and demonstrated an increase of the abscisic acid level at the ripening stage, indicating its role in induction of fruit ripening. Light has been documented to affect tomato fruit ripening (Giovannoni 2001, 2004, 2007), but with regard to apple there has been a lack of research in this field.

Model for *ACS* gene regulation during apple fruit ripening

A model of the way in which *ACS* gene expression initiates the transition to autocatalytic ethylene synthesis in apple fruit is shown in Fig. 1. In immature fruit, system-1 ethylene is regulated by developmental pathways with

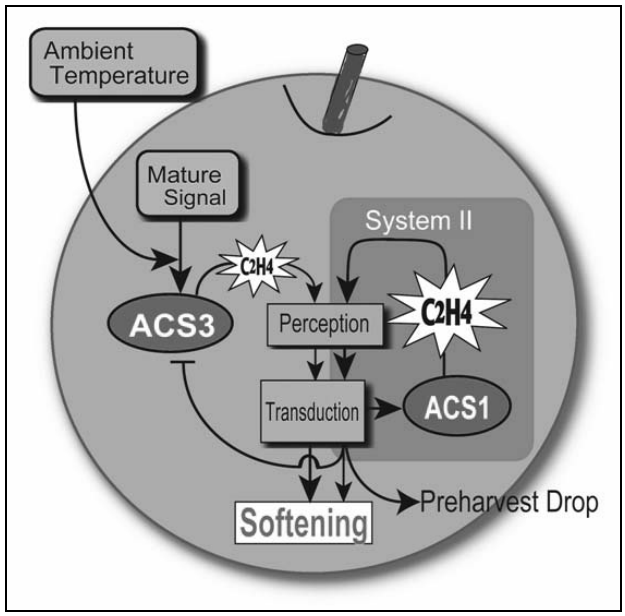


Fig. 1. Model for regulation of *ACS* gene expression during ethylene biosynthesis and fruit ripening in apple. The size of arrows represents amplitude of the signal. The symbols → and ⊤ refer to the action on signaling pathways resulting in stimulation and repression, respectively.

unknown components via *MdACS3* expression. System 1 continues throughout fruit development until a competence to ripen is achieved, at which point a transition occurs. This transition may be initiated by a change in ethylene sensitivity because of the continual ethylene production by system-1. As a result of increased ethylene synthesis, *MdACS1* expression is induced and system-2 autocatalysis is initiated. There is then a huge burst of ethylene production, and this has a negative feedback effect on system-1 ethylene synthesis, leading to reduced expression of *MdACS3*.

Conclusions and prospects

This review has focused on regulation of the apple *ACS* gene family during fruit ripening. Studies in this field have shed light on the relationship between *ACS* gene expression and ethylene production, as well as the shelf life of apple fruit. These research achievements have allowed us to propose a model that begins to explain system-1 and -2 ethylene synthesis at the molecular level in apple fruit. This model will be helpful as a basis for clarifying the signaling pathways of the *ACS* gene family regulating the biosynthesis of ethylene during ripening.

Although tremendous progress has been achieved in recent years, much is still unclear about ethylene transduction and its regulatory role in fruit ripening. Janssen et al. (2008) offers an effective genomics approach on apple fruit development and ripening research. Thanks to the release of apple genome (Velasco et al. 2010), which provides more convenience for apple researchers to study on genomic sequences of various genes unique in apple, including *ACS* genes. Further study is needed to clarify the relationship between *MdACS3* and fruit softening in apple, including its inheritance and the development of DNA markers. It is important that work continues to isolate new *ACS* genes involved in ripening, defining the function and characterizing their cooperation with other members of the *ACS* family in regulating fruit ripening. It has been reported that ACC synthase functions as a dimer (Tarun and Theologis 1998). In *Arabidopsis*, *AtACS7* can form functional heterodimers with other ACSs when the K278 residue is available (Tsuchisaka and Theologis 2004a). As a homolog of *AtACS7, MdACS3a* probably forms various dimers with other ACSs to regulate ethylene biosynthesis and fruits shelf life (Wang et al. 2009b). To clarify the details of the regulatory effects of *MdACSs* on ethylene biosynthesis and fruit shelf-life, it will be necessary to study the interactions among these genes.

Characterization of the *cis*- and *trans*- elements associated with *ACS* gene expression, governing the regulation of ethylene biosynthesis and fruit ripening, is expected to lead to great progress in this field. Furthermore, since ETO1 is a negative regulator of ethylene evolution, it would be interesting to study its regulation of fruit ripening. In addition, it would be informative to highlight the interaction between *ACS* genes and environmental factors, especially ambient temperature, during fruit development and maturation.

It is reported that micro RNA (miRNA) is involved in fruit development and ripening (Seymour et al. 2008). Moxon et al. (2008) recently identified a miRNA from tomato targets as belonging to the *CTR* gene family involved in fruit ripening, thus opening a new avenue in the study of fleshy fruit development and ripening. Studies in this field should enable researchers to identify new genetic regulators of ripening in the near future.

Acknowledgement

We thank the Aomori Prefectural Industrial Technology research Center for providing the plant materials. This work was supported by the Program for Promotion of Basic Activities for Innovative Bioscience (PROBRAIN) in Japan.

References

Abel, S., M.D. Nguyen, W. Chow and A. Theologis 1995: *ACS4*, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase in *Arabidopsis thaliana*. J. Biol. Chem. **270**, 19093–19099.

Adams-Phillips, L., C. Barry and J.J. Giovannoni 2004: Signal transduction systems regulating fruit ripening. Trends Plant Sci. **9**, 331–338.

Anjanasree, K.N., A. Srivastava, A. Handa and K.C. Bansal 2005: Identification of differentially expressed ripening-related cDNA clones from tomato (*Lycopersicon esculentum*) using tomato EST array. Curr. Sci. **88**, 792–796.

Asif, M.H., N. Pathak, T. Solomos and P.K. Trivedi 2009: Effect of low oxygen, temperature and 1-methylcyclopropene on the expression of genes regulating ethylene biosynthesis and perception during ripening in apple. S. Afr. J. Bot. **75**, 137–144.

Bapat, V.A., P.K. Trivedi, A. Ghosh, V.A. Sane, T.R. Ganapathi and P. Nath 2010: Ripening of fleshy fruit: molecular insight and the role of ethylene. Biotechnol Adv. **28**, 94–107.

Barry, C.S., M.I. Llop-Tous and D. Grierson 2000: The regulation of 1-aminocyclopropane-1-carboxylic acid synthase gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato. Plant Physiol. **123**, 979–986.

Bureau, T.E., P.C. Ronald and S.R. Wessler 1996: A computer-based systematic survey reveals the predominance of small inverted repeat elements in wild-type rice genome. Proc. Natl. Acad. Sci. U.S.A. **93**, 8524–8529.

Capitani, G., D.L. McCarthy, H. Gut, M.G. Grutte and J.F. Kirsch 2002: Apple 1-aminocyclopropane-1-carboxylate synthase in complex with the inhibitor l-aminoethoxyvinylglycine – evidence for a ketimine intermediate. J. Biol. Chem. **277**, 49735–49742.

Chae, H.S., F. Faure and J.J. Kieber 2003: The *eto1*, *eto2* and *eto3* mutations and cytokinin treatment increase ethylene biosynthesis in *Arabidopsis* by increasing the stability of ACS protein. Plant Cell **15**, 545–559.

Chaves, A.L.S. and P.C. de Mello-Farias 2006: Ethylene and fruit ripening: From illumination gas to the control of gene expression, more than a century of discoveries. Genet. Mol. Biol.**29**, 508–515.

Costa, F., S. Sara, W.E. van de Weg, W. Guerra, M. Cecchinel, J. Dallivina, B. Koller and S. Sansivini 2005: Role of the genes Md-ACO1 and Md-ACS1 in ethylene production and shelf life of apple (*Malus domestica* Borkh.). Euphytica **141**, 181–190.

Curry, E.A. and D.W. Greene 1993: CPPU influences fruit quality, fruit set, return bloom, and preharvest drop of apples. HortScience **28**, 115–119.

Dal Cin, V., M. Danesin, A. Botton, A. Boschetti, A. Dorigoni and A. Ramina 2007: Fruit load and elevation affect ethylene biosynthesis and action in apple fruit (*Malus domestica* L. Borkh.) during development, maturation and ripening. Plant Cell Environ. **30**, 1480–1485.

Dandekar, A.M., G. Teo, B.G. Defilippi, S.L. Uratsu, A.J. Passey, A.A. Kader, J.R. Stow, R.J. Colgan and D.J. James 2004: Effect of down-regulation of ethylene biosynthesis on fruit flavor complex in apple fruit. Transgenic Res. **13**, 373–384.

Dong, J.G., W.T. Kim, W.K. Yip, G.A. Thompson, L. Li, A.B. Bennett and S.F. Yang 1991: Cloning of a cDNA encoding 1-aminocyclopropane-1-carboxylate synthase and expression of its mRNA in ripening apple fruit. Planta **185**, 38–45.

El-Sharkawy, I., B. Jones, L. Gentzbittel, J.M. Lelièvre, J.C. Pech and A. Latché 2004: Differential regulation of ACC synthase genes in cold-dependent and -independent ripening in pear fruit. Plant cell Environ. **27**, 1197–1210.

El-Sharkawy, I., W.S. Kim, S. Jayasankar, A.M. Svircev and D.C.W. Brown 2008: Differential regulation of four members of the ACC synthase gene family in plum. J. Exp. Bot. **59**, 2009–2027.

Ferguson, I.B., W. Snelgarm, Lay-Yee, C.B. Watkins and J.H. Bowen 1998: Expression of heat shock protein genes in apple fruit in the field. Aust. J. Plant Physiol. **25,** 153–163.

Gillaspy, G., H. Ben-David and W. Gruissem 1993: Fruits: a developmental perspective. Plant Cell **5**, 1439–1451.

Giovannoni, J.J. 2001: Molecular biology of fruit maturation and ripening. Annu. Rev. Plant Physiol. Plant Mol. Biol. **52**, 725–49.

Giovannoni, J.J. 2004: Genetic regulation of fruit development and ripening. Plant Cell **16**, 170–180.

Giovannoni, J.J. 2007: Fruit ripening mutants yield insights into ripening control. Curr. Opin. Plant Biol. **10**, 283–289.

Gorny, J.R. and A.A. Kader 1997: Low oxygen and elevated carbon dioxide atmospheres inhibit ethylene biosynthesis in preclimacteric and climacteric apple fruit. J. Am. Soc. Hort. Sci. **122**, 542–546.

Gussman, C.D., J.C. Goffreda and T.J. Gianfagna 1993: Ethylene production and fruit-softening rates in several apple fruit ripening variants. HortSci. **28**, 135–137.

Harada, T., T. Sunako, W. Sakuraba, S. Goto, M. Senda, S. Akada and M. Niizeki 1997: Genomic nucleotide sequence of a ripening-related 1-aminocyclopropane-1-carboxylate synthase gene (MdACS-1) in apple (accession no. U89156) (PGR 97-066). Plant Physiol. **113**, 1465.

Harada, T., T. Sunako, Y. Wakasa, J. Soejima, T. Satoh and M. Niizeki 2000: An allele of 1-aminocyclopropane-1-carboxylate synthase gene (Md-ACS1) accounts for the low ethylene production in climacteric fruits of some apple cultivars. Theor. Appl. Genet. **101**, 742–746.

Itai, A., K. Kawata, K. Tanabe and F. Tamura 1999: Identification of 1-aminocyclopropane-1-carboxylate synthase genes controlling the ethylene level of ripening fruit in Japanese pear (*Pyrus pyrifolia* Nakai). Mol. Gen. Genet. **261**, 42–49.

Janssen, B.J., K. Thodey, R.J. Schaffer, R. Alba, L. Balakrishnan, R. Bishop, J.H. Bowen, R.N. Crowhurst, A.P. Gleave, S. Ledger, S. McArtney, F.B. Pichler, K.C. Snowden and S. Ward 2008: Global gene expression analysis of apple fruit development from the floral bud to ripe fruit. BMC Plant Biol., **8**, 16.

Johnston, J.W., E.W. Hewett, M.L.A.T.M. Hertog and F.R. Harker 2001: Temperature induces differential softening responses in apple cultivars. Postharvest Biol. Technol. **23**, 185–196.

Johnston, J.W., E.W. Hewett and M.L.A.T.M. Hertog 2002: Postharvest softening of apple (*Malus domestica*) fruit. N.Z. J. Crop Hort. Sci. **30**, 145–160.

Jones, B., P. Frasse, E. Olmos, H. Zegzouti, Z.G. Li, A. Latché, J.C. Pech and M. Bouzayen 2002: Down-regulation of DR12, an auxin-response-factor homolog, in the tomato results in a pleiotropic phenotype including dark green and blotchy ripening fruit. Plant J. **32**, 603–613.

Kende, H. 1993: Ethylene biosynthesis. Annu. Rev. Plant Physiol. **44**, 283–307.

Kim, W.T., A. Silverstone, W.K. Yip, J.G. Dong and S.F. Yang 1992: Induction of 1-aminocyclopropane-1-carboxylate synthase mRNA by auxin in mung bean hypocotyls and cultured apple shoots. Plant Physiol. **98**, 465–471.

Kondo, S., S. Meemark, Y. Ban, T. Moriguchi and T. Harada 2009: Effects of auxin and jasmonates on 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase gene expression during ripening of apple fruit. Postharvest Biol. Technol. **51**, 281–284.

Lara, I. and M. Vendrell 2000: Development of ethylene-synthesizing capacity in preclimacteric apples: interaction between abscisic acid and ethylene. J. Am. Soc. Hort. Sci.**125**, 505–512.

Lay-Yee, M. and M.L. Knighton 1995: A full-length cDNA encoding 1-aminocyclopropane-1-carboxylate synthase from apple (accession no. L31347) (PID: g606759). Plant Physiol. **107**, 1017–1018.

Lelievre, J.M., A. Latche, B. Jones, M. Bouzayen and J.C. Pech 1997: Ethylene and fruit ripening. Physiol. Plant. **101**, 727–739.

Li, Y., B. Zhu, W. Xu, H. Zhu, A. Chen, Y. Xie, Y. Shao and Y. Luo 2007: LeERF1 positively modulated ethylene triple response on etiolated seeding, plant development and fruit ripening and softening in tomato. Plant Cell Rep. **26**, 1999–2008.

Liang, X.W., S. Abel, J.A. Keller, N.F. Shen and A. Theologis 1992: The 1-aminocyclopropane-1-carboxylate synthase gene family of *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. U.S.A. **89**, 11046–11050.

Liang, X., Y. Oono, N.F. Shen, C. Köhler, K. Li, P.A. Scolnik and A. Theologis 1995: Characterization of two members (*ACS1* and *ACS3*) of the 1-aminocyclopropane-1-carboxylate synthase gene family of *Arabidopsis thaliana*. Gene **167**, 17–24.

Lin, Z., S. Zhong and D. Grierson 2009: Recent advances in ethylene research. J. Exp. Bot. **60**, 3311–3336.

Lincoln, J.E., A.D. Campbell, J. Oetiker, W.H. Rottmann, P.W. Oeller, N.F. Shen and A. Theologis 1993: LE-ACS4, a fruit ripening and wound-induced 1-aminocyclopropane-1-carboxylate synthase gene of tomato (*Lycopersicon esculentum*). J. Biol. Chem. **268**, 19422–19430.

McMurchie, E.J., W.B. McGlasson and I.L. Eaks 1972: Treatment of fruit with propylene gives information about the biogenesis of ethylene. Nature **287**, 235–236.

Medina-Escobar, N., J. Cárdenas, J. Muñoz-Blanco and J.L. Caballero 1998: Cloning and molecular characterization of a strawberry fruit ripening-related cDNA corresponding a mRNA for a low-molecular-weight heat-shock protein. Plant Mol. Biol. **36**, 33–42.

Moxon, S., R. Jing, G. Szittya, F. Schwach, R.L.R. Pilcher, V. Moulton and T. Dalmay 2008: Deep sequencing of tomato short RNAs identifies microRNAs targeting genes involved in fruit ripening. Genome Res. **18**, 1602–1609.

Nakatsuka, A., S. Murachi, H. Okunishi, S. Shiomi, R. Nakano, Y. Kubo and A. Inaba 1998: Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, 1-aminocyclopropane-1-carboxylate oxidase, and ethylene receptor genes in tomato fruit during development and ripening. Plant Physiol. **118**, 1295–1305.

Oraguzie, N.C., H. Iwanami, J. Soejima, T. Harada and A. Hall 2004: Inheritance of Md-ACS1 gene and its relationship to fruit softening in apple (*Malus* × *domestica* Borkh.). Theor. Appl. Genet. **108**, 1526–1533.

Oraguzie, N.C., R.K. Volz, C.J. Whitworth, H.C.M. Bassett, A.J. Hall and S.E. Gardiner 2007: Influence of Md-ACS1 allelotype and harvest season within an apple germplasm collection on fruit softening during cold air storage. Postharvest Biol. Technol. **44**, 212–219.

Rodrigues-Pousada, R.A., R. De Rycke, A. Dedonder, W. van Caeneghem, G. Engler, M. van Montagu and D. van Der Straeten 1993: The *Arabidopsis* 1-aminocyclopropane-1-car-boxylate synthase gene 1 is expressed during early development. Plant Cell **5**, 897–911.

Rosenfield, C.L., E. Kiss and G. Hrazdina 1996: MdACS-2 (Accession No. U73815) and Md-ACS3 (Accession No. U73816): two new 1-aminocyclopropane-1-carboxylate synthases in ripening apple fruit. Plant Physiol. **112**, 1735.

Sato, T., Y. Wakasa, T. Kudo, T. Akada, M. Niizeki and T. Harada 2004: Allelotype of a ripening-specific 1-aminocyclopropane-1-carboxylate synthase gene defines the rate of fruit drop in apple. J. Am. Soc. Hort. Sci. **129**, 32–36.

Seymour, G.B., J.E. Taylor and G.A. Tucke 1993: Biochemistry of Fruit Ripening. Chapman & Hall press, London.

Seymour, G., M. Poole, K. Manning and G.J. King 2008: Genetics and epigenetics of fruit development and ripening. Curr. Opin. Plant Biol. **11**, 58–63.

Sunako, T., W. Sakuraba, M. Senda, S. Akada, R. Ishikawa, M. Niizeki and T. Harada 1999: An allele of the ripening-specific 1-aminocyclopropane-1-carboxylate synthase gene (ACS1) in apple fruit with a long storage life. Plant Physiol. **119**, 1297–1303.

Sunako, T., R. Ishikawa, M. Senda, S. Akada, M. Niizeki and T. Harada 2000: MdACS-5A (Accession No. AB034992) and 5B (Accession No. AB034993) – two wound-responsive genes encoding 1-aminocyclopropane-1-carboxylate synthase in apple. Plant Physiol. **122**, 620.

Tacken, E., H. Ireland, K. Gunaseelan, S. Karunairetnam, D. Wang, K. Schultz, J. Bowen, R.G. Atkinson, J.W. Johnston, J. Putterill, R.P. Hellens and R. J. Schaffer 2010: The role of ethylene and cold temperature in the regulation of the apple polygalacturonase1 gene and fruit softening. Plant Physiol. **153**, 294–305.

Tarun, A.S. and A. Theologis 1998: Complementation analysis of mutants of 1-aminocyclopropane-1-carboxylate synthase reveals the enzyme is a dimer with shared active sites. J. Biol. Chem. **273**, 12509–12514.

Tatsuki, M., A. Endo and H. Ohkawa 2007: Influence of time from harvest to 1-MCP treatment on apple fruit quality and expression of genes for ethylene biosynthesis enzymes and ethylene receptors. Postharvest Biol. Technol. **43**, 28–35.

Trainotti, L., A. Tadiello and G. Casadoro 2007: The involvement of auxin in the ripening of climacteric fruits comes of age: the hormone plays a role of its own and has an intense interplay with ethylene in ripening peaches. J. Exp. Bot. **58**, 3299–3308.

Tsuchisaka, A. and A. Theologis 2004a: Heterodimeric interactions among the 1-amino-cyclopropane-1-carboxylate synthase polypeptides encoded by the *Arabidopsis* gene family. Proc. Natl. Acad. Sci. U.S.A. **101**, 2275–2280.

Tsuchisaka, A. and A. Theologis 2004b: Unique and overlapping expression patterns among the *Arabidopsis* 1-amino-cyclopropane-1-carboxylate synthase gene family members. Plant Physiol. **136**, 2982–3000.

Vahala, J., C.D. Schlagnhaufer and E.J. Pell 1998: Induction of an ACC synthase cDNA by ozone in light-grown *Arabidopsis thaliana* leaves. Physiol. Plant. **103**, 45–50.

Velasco, R., A. Zharkikh, J. Affourtit, A. Dhingra, A. Estaro, A. Kalyanaraman, P. Fontana, S.K. Bhatnagar, M. Troggio, D. Pruss, S. Salvi, M. Pindo, P. Baldi, S. Castelletti, M. Cavaiuolo, G. Coppola, F. Costa, V. Cova, A. Dal Ri, V. Goremykin, M. Komjanc, S. Longhi, P. Magnago, G. Malacarne, M. Malnoy, D. Micheletti, M. Moretto, M. Perazzolli, A. Si-Ammour, S. Vezzulli, E. Zini, G. Eldredge, L.M. Fitzgerald, N. Gutin, J. Lanchbury, T. Macalma, J.T. Mitchell, J. Reid, B. Wardell, C. Kodira, Z. Chen, B. Desany, F. Niazi, M. Palmer, T. Koepke, D. Jiwan, S. Schaeffer, V. Krishnan, C. Wu, V.T. Chu, S. T. King, J. Vick, Q. Tao, A. Mraz, A. Stormo, K. Stormo, R. Bogden, D. Ederle, A. Stella, A. Vecchietti, M.M. Kater, S. Masiero, P. Lasserre, Y. Lespinasse, A.C. Allan, V. Bus, D. Chagné, R.N. Crowhurst, A.P. Gleave, E. Lavezzo, J.A. Fawcett, S. Proost, P. Rouzé, L. Sterck, S. Toppo, B. Lazzari, R.P. Hellens, C.-E. Durel, A. Gutin, R.E. Bumgarner, S.E. Gardiner, M. Skolnick, M. Egholm, Y.V. de Peer, F. Salamini and R. Viola 2010: The genome of the domesticated apple (*Malus* × *domestica* Borkh.) Nat. Genet. **42**, 833–839.

Vogel, J.P., K.W. Woeste, A. Theologis and J.J. Kieber 1998: Recessive and dominant mutations in the ethylene biosynthetic gene *ACS5* of *Arabidopsis* confer cytokinin insensitivity and ethylene overproduction, respectively. Proc. Natl. Acad. Sci. U.S.A. **95**, 4766–4771.

Wakasa, Y. and T. Harada 2001: DNA markers for ethylene production and skin color in apple fruit. Regul. Plant Growth Dev. **36**, 125–130 (in Japanese).

Wakasa, Y., H. Kudo, R. Ishikawa, S. Akada, M. Senda, M. Niizeki and T. Harada 2006: Low expression of an endopolygalacturonase gene in apple fruit with long-term storage potential. Postharvest Biol. Technol. **39**, 193–198.

Wang, A., D. Tan, A. Takahashi, T.Z. Li and T. Harada 2007: MdERFs, two ethylene response factors involved in apple fruit ripening. J. Exp. Bot. **58**, 3743–3748.

Wang, A., D. Tan, M. Tatsuki, A. Kasai, T. Li, H. Saito and T. Harada 2009a: Molecular mechanism of distinct ripening profiles in 'Fuji' apple fruit and its early maturing sports. Postharvest Biol. Technol. **52**, 38–43.

Wang, A., J. Yamakake, H. Kudo, Y. Wakasa, Y. Hatsuyama, M. Igarashi, A. Kasai, T. Li and T. Harada 2009b: Null mutation of the MdACS3 gene, coding for a ripening-specific 1-aminocyclopropane-1-carboxylate synthase, leads to long shelf life in apple fruit. Plant Physiol. **151**: 391–399.

Wiersma, P.A., H. Zhang, C. Lu, A. Quail and P.M.A. Toivonen 2007: Survey of the expression of genes for ethylene synthesis and perception during maturation and ripening of 'Sunrise' and 'Golden Delicious' apple fruit. Postharvest Biol. Technol. **44**, 204–211.

Woolf, A.B. and I.B. Ferguson 2000: Postharvest responses to high fruit temperatures in the field. Postharvest Biol. Technol. **21**, 7–20.

Yahia, E.M. 2009: Modified and Controlled Atmospheres for the Storage, Transportation, and Packaging of Horticultural Commodities. CRC Press. Boca Raton, FL, 1–3.

Yang, S.F. and N.E. Hoffman 1984: Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. **35**, 155–189.

Yokotani, N., R. Nakano, S. Imanishi, M. Nagata, A. Inaba and Y. Kubo 2009: Ripening-associated ethylene biosynthesis in tomato fruit is autocatalytically and developmentally regulated. J. Exp. Bot. **60**, 3433–3442.

Yoshida, H., M. Nagata, K. Saito, K.L.C. Wang and J.R. Ecker 2005: *Arabidopsis* ETO1 specifically interacts with and negatively regulates type 2 1-aminocyclopropane-1-carboxylate synthases. BMC Plant Biol. **5**, 14.

Zhu, Y., D.R. Rudell and J.P. Mattheis 2008: Characterization of cultivar differences in alcohol acyltransferase and 1-aminocyclopropane-1-carboxylate synthase gene expression and volatile ester emission during apple fruit maturation and ripening. Postharvest Biol. Technol. **49**, 330–339.

Received December 29, 2010 / Accepted July 14, 2011

Addresses of authors: Takeo Harada (corresponding author), Laboratory of Plant Breeding and Genetics, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki 036-8561, Japan, Aide Wang, Department of Horticulture, Cornell University, 630 W. North St., Geneva, NY 14456, USA (present address), and Tianzhong Li, Laboratory of Fruit Cell and Molecular Breeding, College of Agronomy and Bio-tech, China Agricultural University, Beijing 100193, P. R. China, e-mail (corresponding author): tharada@cc.hirosaki-u.ac.jp.