Studies on the Molecular Mechanism of the Difference of Storage Ability among Apple Cultivars

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Contents

Abbreviations

ACO	1-Aminocyclopropane-1-carboxylate oxidase
ACS	1-Aminocyclopropane-1-carboxylate synthase
CA	Controlled atmosphere
CTR	Constitutive triple response
DAB	Days after bloom
EIN	Ethylene insensitive
ERF	Ethylene response factor
ERS	Ethylene response sensor
ETR	Ethylene receptor
HSP	Heat shock protein
IEC	Internal ethylene concentration
IEF	Isoelectric focusing
MCP	1-Methylcyclopropene
miRNA	microRNA
NFuji	Northern 'Fuji' fruit
ORF	Open reading frame
PCR	Polymerase chain reaction
PG	Polygalacturonase
SFuji	Southern 'Fuji' fruit
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeat

Preface

The domesticated apple (*Malus*×*domestic* Borkh.) is one of the most important fruit crops of the colder and temperate zone of the world (Harris et al. 2002; Newcomb et al. 2006). It was suggested that the wild apple of Kazakstan and its close relatives were the progenitors of the domesticated apple (Harris et al. 2002). The domesticated apple belongs to the family Rosaceae. Together with other commercial fruit and ornamental species, it forms the subfamily Maloideae, which is thought to have evolved by hybridization from the families Spiraeoideae (x = 9) and Prunoideae (x = 8). The resulting allopolyploid has a basic haploid number of x = 17 and an estimated genome size of 743 to 796 Mb (Newcomb et al. 2006).

Apple is recognized by consumers for its flavor, health and nutritional attributes (Harker et al. 2003). The high nutritional value and good taste make apple become the major temperate horticultural fruit crop and a worldwide widespread commodity (Dal Cin et al. 2007). The final crop quality of apple is strongly affected by genotype, agronomic practices and environmental conditions (Adams-Phillips et al. 2004; Dal Cin et al. 2007). The major problem of apple quality is the rate of fruit softening during transportation, storage and on the shelf (Johnston et al. 2002). Although apple fruit have much longer shelf life than other pome fruit, such as quince and pear, there is a marked difference in the storage ability among apple cultivars (Johnston et al. 2002).

In apple industry, two types of storage technology were much used to ensure that consumers receive the best-quality of apple at any time of the year. The regular, cold storage is for short term storage and special, controlled atmosphere (CA) storage is for long term storage. After harvested, the apples are then rushed to cold storage warehouses, consisting of large refrigerated storerooms, where the temperature is kept at 0-3 °C and high humidity (85-95 %) is maintained. This cold temperature slows down but does not stop the ripening process. Hence, most apples put in regular cold storage can not be stored for a longer time. In addition, it is very difficult to maintain the fruit at these optimum temperatures through the entire postharvest handling chain. In 1940, Dr. Robert Smock of Cornell University experimented with reducing oxygen and increasing

carbon dioxide in storage facilities, resulting in the development of a new storage technology called controlled atmosphere (CA) storage. CA storage requires air-tight, refrigerated warehouse rooms that are sealed after the apples are placed inside. The CA process radically reduces the ripening process, keeps the best quality and makes it possible for long term storage of apple fruit, thus is widely applied in fruit-producing areas in order to preserve fruit quality and cover the marketplace year round. CA storage is so far the most effective way to preserve the best quality of apple, but it is more costly for building and maintenance, such that only the very best apples are put into this type of storage (Johnston et al. 2002; Lara et al. 2006).

The ripening of fleshy fruits such as tomato and apple, relates to a series of biochemical, physiological and structural changes that make the fruit edible and attractive to the consumer. Although these processes differ from type to type, fruits can be divided into two broad groups, known as climacteric and non-climacteric (Lelièvre et al 1997). Climacteric fruit, such as tomato, cucurbits, avocado, banana, peaches, plums, and apples, are distinguished from non-climacteric fruits, such as strawberry, grape, and citrus, by their increased respiration and ethylene biosynthesis rates during ripening (Lelièvre et al. 1997; Giovannoni 2001). Although non-climacteric fruits may respond to ethylene, in this group, ethylene is not necessary for fruit ripening. In contrast, ethylene is required for completing ripening in climacteric fruit according to the analysis of inhibitors of ethylene biosynthesis and perception, in transgenic plants blocked in ethylene biosynthesis, and through examination of the *Never-ripe* (*Nr*) ethylene receptor mutant of tomato (Giovannoni 2001).

As a climacteric fruit, apple shows increase of initial ethylene concentration (IEC) when reaching ripening. The role of ethylene in apple softening has been studied via various methods. These include the use of preharvest ethylene releasing sprays, the addition and removal of ethylene from storage atmospheres, preharvest application of an inhibitor of ethylene biosynthesis, postharvest inhibitors of ethylene action, and relating softening with endogenous changes in ethylene concentration through maturation (reviewed by Johnston et al. 2002). The most conclusive evidence that ethylene promotes apple softening derived from experiments using inhibitors of ethylene action.

Apples treated at harvest with 1-methylcyclopropene (MCP) showed slower softening rate and had reduced IEC compared to untreated fruit in storage (Fan et al. 1999; Watkins et al. 2000). It indicated that the decrease of MCP inhibition over time is through a slow release of the inhibitor from the active site, or that the new active sites were generated by fruit (Johnston et al. 2002). Studies attempting to identify relationships between IEC and softening have led to an unclear role for ethylene in the softening process of apple fruit. Ethylene may not be required for initiation of on-tree softening (Lau et al. 1986). A low basal rate of ethylene production may have been sufficient to promote on-tree softening, as has been suggested for the early phases of kiwifruit softening when ethylene production is low (Kim et al. 1999; Johnston et al. 2002). However, on-tree fruit softening may have been caused by cell expansion and increased fruit size that ordinarily occurs during maturation. Once harvested, the firmness of apples generally declines as the elevation of ethylene production (Watkins et al. 2000). Ethylene may induce softening in apples by regulating expression of cell wall modifying enzymes. Increase of PG (polygalacturonase) expression was observed during the early phases of softening for several apple cultivars (Atkinson et al. 1998). Wakasa et al. (2006) has found a clear correlation between expression of *MdPG1* and apple fruit softening rate.

Ethylene response factor (ERF), which functions as a transcription factor in the last step of ethylene signal transduction, has been reported to be involved in modulating the tomato fruit development, maturation, ripening and softening (Li et al. 2007). But in apple, it has not been documented yet.

Besides ethylene, several other factors which are independent of ethylene have been reported to affect fruit ripening such as light, temperature, fruit load and elevation (Giovannoni et al. 2004; Dal Cin et al. 2007). However, it is still unclear that how these ethylene independent factors function and interact with the ethylene pathway in regulation of fruit ripening process. Furthermore, fruit of apple cultivar, 'Fuji', which was bred from a cross of 'Ralls Janet' × 'Delicious' in 1930s, have pretty high storage ability; whereas its sport cultivar, 'Hirosaki Fuji' doesn't show this ability (Harada, unpublished data). Little is known about this difference which is a very important clue

to unravel the mechanism of long shelf life of 'Fuji'.

Here, I studied the regulation of an ACC synthase gene, *MdACS3a*, and two ethylene response factor (ERF) genes on ethylene production and storage ability of apple fruit. The influence of ambient temperature on fruit ripening was also assessed by investigating the difference of apple 'Fuji' and its sport cultivars. This achievement will contribute to understand the molecular mechanism of apple fruit ripening and may lead to obtain a molecular marker of the selection for long shelf life in apple breeding program as well as to develop more effective methods of preventing or controlling the fruit softening rate than conventional technologies.

Chapter I

Allelic genotypes of *MdACS3a* determine the storage ability of apple fruit

I-1 Introduction

Ethylene is a gaseous phytohormone and plays an essential role in many physiological aspects in plant development and growth (Yang and Hoffman 1984; Adams-Phillips et al. 2004). One of the most studied examples of ethylene regulation is the process of fruit ripening. Ethylene production during fruit ripening is altered dramatically and widely considered to be a key factor leading to softening (Abeles and Biles 1991), which subsequently determines the fruit shelf life.

Ethylene biosynthesis has been extensively studied in several plant species (Liang et al. 1992; Lincoln et al. 1993; Capitani et al. 2002). The enzyme that limits ethylene synthesis is ACC synthase (EC 4.1.1.14, ACS), which catalyzes the formation of ACC, the immediate precursor of ethylene synthesis (Gussman et al. 1993; Gorny and Kader 1997). Two systems of ethylene regulation have been speculated to exist in higher plants (McMurchie et al. 1972). System 1, ethylene auto-inhibitory, is considered to function during normal vegetable growth and 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; Lelièvre et al. 1997; Barry et al. 2000). The transition from system-1 to system-2 ethylene production is considered to be an important step during fruit ripening and is developmentally regulated. It is now established that genes responsible for ethylene biosynthesis and signal transduction are stimulated during a climacteric burst of ethylene production and may be tightly associated during the transition from system 1 to system 2 (Nakatsuka et al. 1998).

In tomato, ACS is encoded by at least eight divergent genes (Alexander and

Grierson 2002). System-1 ethylene is regulated by the expression of *LeACS1A* and *LeACS6*. During the transition period, *RIN* gene plays a pivotal role leading to increased expression of *LeACS1A* and induction of *LeACS4*. The system-2 ethylene is subsequently initiated and maintained by the expression of *LeACS2* (Barry et al. 2000).

In apple, at least four genes are found in *ACS* family (*MdACS1*, *MdACS3*, *MdACS4* and *MdACS5*), in which *MdACS4* and *MdACS5* showed no correlation with fruit ripening (Sunako et al. 2000). *MdACS1* is specifically expressed in ripening fruit and one of its alleles (*MdACS1-2*), which possesses an insertion in the promoter region, has low transcription activity compared to its counterpart *MdACS1-1*. Thus *MdACS1-2* homozygous cultivars produce low level of ethylene, whereas cultivars homozygous for *MdACS1-1* produce high level of ethylene during fruit ripening (Sunako et al. 1999; Harada et al. 2000). However, *MdACS1* allelic forms can not explain the different ethylene production and storage ability among apple cultivars (Sunako et al. 1999; Oraguzie et al. 2004).

Another ACS gene family was reported as *MdACS3* (Accession No. U73816). The genomic clone of *MdACS3* (Accession No. AB243060) was isolated through screening an apple genome DNA library of 'Golden Delicious', whose expression was detected prior to the expression of *MdACS1* and *MdACO1* and was proposed to be responsible for system 1 ethylene biosynthesis (Yamakake 2007). Through the analysis of genomic structure, three *MdACS3* genes were cloned, termed *MdACS3a* (Accession No. AB243060), *MdACS3b* (Accession No. AB243061), and *MdACS3c* (Accession No. AB243062), respectively, in *Malus* genome. Two of the subfamily genes, *MdACS3b* and *MdACS3c,* possessed a 333 bp insertion at 5' flanking region (around -570) which is absence in *MdACS3a*. The insertion was thought to be related with no transcribed products from *MdACS3b* and *MdACS3c* (Yamakake 2007). Therefore, only *MdACS3a* functions during ripening in apple. Yamakake (2007) also reported that a single amino acid substitution in an allele of *MdACS3a* and the presence of a null *MdACS3a* allele, *acs3a*. In this study, I reported the enzymatic activity of the mutated ACS3a and effects of the possessing these allelic genes on the ripening ethylene production and fruit softening. The role of ACS3a in the ethylene signaling pathway was discussed.

I-2 Materials and methods

Plant materials

Young leaves of apple (*Malus* × *domestica* Borkh.) cultivars and their corresponding wild species were collected from the experimental farms of Hirosaki University and the farm of Aomori Apple Experiment Station. The cultivars 'Kitaro' and 'Kotaro' were sampled on their respective commercial mature date, October 9 and October 23, respectively. The fruits were transported to our laboratory and stored at 24 °C for 12 d to allow them to reach the climacteric stage. Fruits of 'Gala', 'Koukou' and 'Toki' were also collected at their commercial harvest day, September 29, October 28 and September 29, respectively, and then stored at 24 °C for 10 days and sampled every 5 d. The ethylene production rate and flesh firmness of the apple fruits were evaluated to obtain preclimacteric and climacteric stage data. Cortical tissue from which the skin and core parts had been removed was frozen by immersion in liquid N_2 and then stored in a freezer (−80 °C) until isolation of the total RNA.

Measurements of ethylene production rates

Intact fruit were enclosed in a gas-tight container (0.8 L, 24 °C) equipped with septa, and 1 ml of headspace gas was sampled by means of a syringe. The ethylene concentration was measured with a gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector.

Measurements of flesh firmness

Flesh firmness was measured with a hand-held penetrometer (FT-327; Facchini, Italy) fitted with an 11 mm-diameter probe. Four skin discs (approximately 2.5 cm in diameter) were removed from opposite sides of each fruit. The probe was pressed into the tissue of the cut surface to depth of 8–9 mm in a single smooth motion. Five fruits per sample were measured.

RNA extraction and Northern blot analysis

RNA extraction and gel blot analysis were performed as described by Sunako et al. (1999). The probe for each gene was prepared by PCR from the respective clones according to Wakasa et al. (2003). The primer sequences of *MdACS3*, *MdACS1*, *MdACO1* and *MdPG1* are as described in Table I-1.

RT-PCR analysis of the expression of the ripening related genes

Total RNA extracted from fruit was used for first-strand cDNA synthesis using the Oligo d(T) primer. Then, PCR amplification was carried out using gene-specific primer as described in Table I. The thermal cycling conditions were 3 min at 94 °C; then 24-30 cycles for 30 s at 94 °C, 30 s at respective annealing temperature and 2 min at 72 °C, in which 24 cycles and annealing at 58 °C were for *MdACS3*, 29 cycles and 55 °C for *MdACS1*, 24 cycles and 58 °C for *MdACO1*, 25 cycles and 65 °C for *MdERF2* and 24 cycles and 58 °C for *MdPG1*. To normalize for differences in total RNA, the concentration of actin (Accession No. EB136338) mRNA in each sample was determined using the primers MdACT-F and MdACT-R.

Expression of MdACS1 and MdACS3a in E. coli

cDNAs of *MdACS3a* and *MdACSa3-G289V* were amplified by PCR with *ACS3a* cDNAs as template using the primers ACS3a infu-1 and ACS3a infu-2 (Table I-1). cDNA of *MdACS1* (accession no.U89156) was amplified by the primers: ACS1 infu-1 and ACS1 infu-2 (Table I-1). The PCR products were then ligated to the *Nco* I /*Bam* HI double-digested 5.7 kb pET11d (Fig. I-1, Stratagene) vector by using In-Fusion™ Dry-Down PCR Cloning Kit (Clontech) according to the manufacturer's instructions. Those recombinants containing the correct sequence were identified and retransformed into an *E. coli* host, BL21 (DE3) (Novagen).

BL21 (DE3) cells harboring a pET11d-ACS3 and pET11d-ACS1 recombinant plasmid were grown on a LB plate in the presence of 100 μg/ml ampicillin. LB medium

(5 ml) containing 100 mg/ml of ampicillin was inoculated with a single colony and incubated at 37 °C overnight with constant shaking. The overnight culture (5 ml) was transferred into 500 ml of LB medium. Cells were grown at 30 °C with constant shaking. When the cell density reached OD_{600} of 0.5, IPTG was added to the cell culture to a concentration of 0.1 mm. The culture was then transferred to room temperature (20–25 °C) for 5 more hours with constant shaking. The cells were harvested by centrifugation at 4000 × *g* for 10 min at 4 °C. Cell pellet were stored at -70 °C for later use.

Two-dimensional electrophoresis

Protein concentration was quantified using the BioRad protein assay, using BSA as a standard. Two-dimensional electrophoresis was performed by using the ReadyPrep™ 2-D Starter kit (BioRad) according to the instruction manual. Simply IPG strips (7 cm, pH 3–10, BioRad ReadyStrip, BioRad) were rehydrated overnight with 125 µl of sample buffer containing 87.5 µg of total proteins. Proteins were focused using a PROTEAN IEF Cell (BioRad) at 20 °C for 12 h, applying 250 V (15 min), 4000 V (120 min) and 4000 V for a total of 20 KVh. After IEF (isoelectric focusing), strips were equilibrated in equilibration buffer I and II for 10 min, respectively. Second-dimensional SDS-PAGE was run in acrylamide gels (Ready Gel Precast Gel, 8-16 % Tris-HCl, BioRad) at 200 V for 45 min. Gels were stained by Silver Stain MS kit (Wako, Japan) according to the manufacture's instruction.

ACC synthase activity assay

ACC synthase extract was prepared according to the method of Li et al. (1996). The ACC sythase extract was subsequently concentrated with a Nanosep Centrifugal Devices (PALL, USA) according to the manufacture's instruction. The amount of ACC was determined by the method of Lizada and Yang (1979). ACC synthase activity was assayed by incubating the ACC synthase extract with AdoMet at 30 °C for 1 h in a reaction buffer (Li et al. 1996). One unit of ACC synthase activity is defined as 1 nmol

of ACC formed per hour at 30 °C. Protein concentrations were determined using Bio-Rad protein assay reagent (http://www.bio-rad.com/).

Table I-1 Primer sets used for the PCR amplification in this study

Gene	Primer	Primer sequence
MdACS1	MdACS1F	5'-GATGAAAGGTAGCCTGGTCTGA-3'
	MdACS1R	5'-TACACTAATCACATTGTATGAATC-3'
MdACS3	MdACS3F	5'-GACAAATAGAAAGAGACTGAGGACG-3'
	MdACS3R	5'-CCATCGATTATACAAACTGATTGTG-3'
MdACO1	MdACO1F	5'-TGAAATTCCAAGCCAAGGAG-3'
	MdACO1R	5'-TTCAACTACACAAACAGTGG-3'
MdPG1	MdPG1F	5'-AAAGGTCATGGAATTGATCAGGCC-3'
	MdPG1R	5'-ATGCCCATAATTATGAACCC-3'
MdERF2	MdERF2F	5'-TATGCTGGCAATTGGCGAGC-3'
	MdERF2R	5'-ATGACCAATCCCGCACTCAC-3'
β-Actin	MdACT-F	5'-GGCTGGATTTGCTGGTGATG-3'
	MdACT-R	5'-TGCTCACTATGCCGTGCTCA-3'
	ACS3a infu-1	5'-TATCTGAGTCGCACCCACAG-3'
	ACS3a infu-2	5'-TGGGTTTTGTCCTCCAAAAG-3'
	ACS1 infu-1	5'-CCAAAATGCGCATGTTATCCAG-3'
	ACS1 infu-2	5'-TCAGACCAGGCTACCTTTCATC-3'
	MdACS3a-1F	5'-TGTATGCAGCCCTAGATATC-3'
	MdACS3a-2R	5'-CCATCGATTATACAAACTGATTG TG-3'
	MdACS3a-2F	5'-AGATTTGAGGTGGAGGACTGGTG-3'
	MdACS3a-1R	5'-CTACATGTACCCAACAACTG-3'

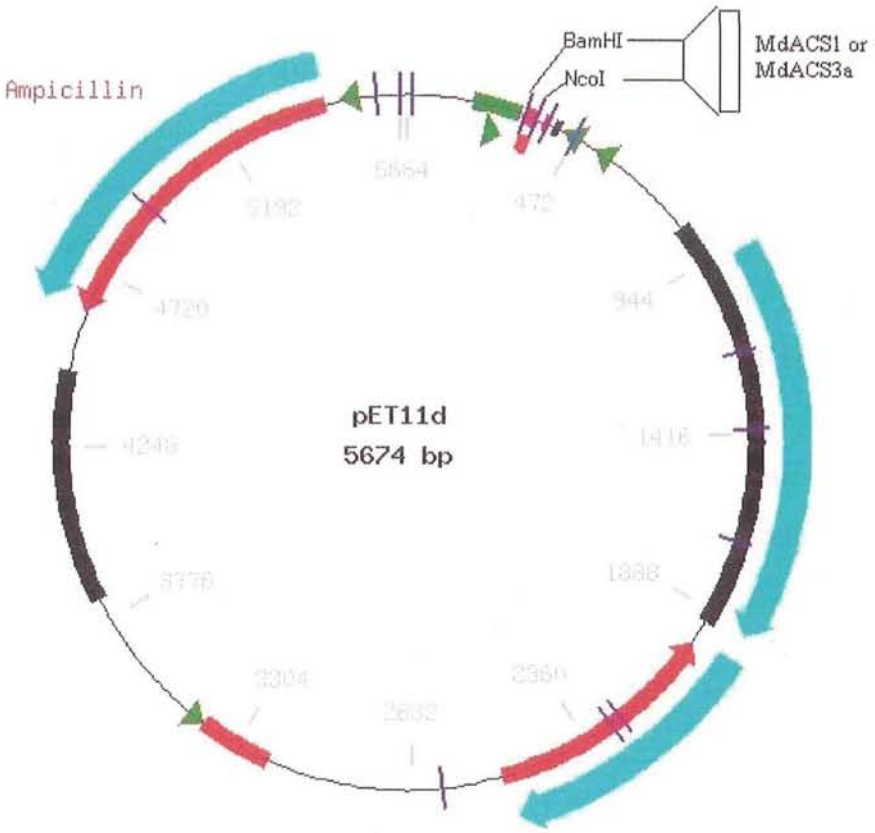

Fig. I-1 Map of pET11d used to express the protein of ACS1 and ACS3a in *E. coli*. The full length cDNAs of *MdACS1* and *MdACS3a* were inserted into the *Nco* I/*Bam* HI double-digested site and the recombinants were introduced into *E. coli*-BL21 (DE3). Figure in http://www.addgene.org/pgvec1?f=v&cmd=showvecinfo&vectorid=4301 was partly modified.

DNA sequence analysis

For sequence analysis of *MdACS3a*, first PCR was performed with primers MdACS3a-1F and MdACS3a-2R (Table I-1) by using the genomic DNA as template. The thermal cycling conditions were 3 min at 94 °C, then 35 cycles of 10 s at 94 °C, 5 s at 58 °C and 3 min at 72 °C, followed by extension at 72 °C for 3 min. For SNPs sequencing, a second PCR were conducted with the primers MdACS3a-2F and MdACS3a-2R by using the first PCR product as the template. For SSR analysis, primers MdACS3a-1F and MdACS3a-1R were used to amplify the SSR region from the first PCR product. The thermal cycling conditions were 3min at 94 °C, then 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C, followed by extension at 72 °C for 3 min. PCR products was run on 6 % polyacrylamide gel and stained by silver according to Bassam et al. (1991). All cycle sequencing reactions for DNA sequence analysis were performed using Big Dye terminator chemistry (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's protocols, and sequences were determined using an ABI 310 automated DNA sequencer.

I-3 Results

SNPs analysis of ACS3a

El-Sharkawy et al. (2003) and Itai et al. (2000) have reported that the ortholog of *MdACS3a* in pear is involved in the fruit ripening. The relatedness between *MdACS3a* and ripening in apple was greatly interesting, and whether the sequence of *MdACS3a* is polymorphic among various apple cultivars was quite attracted. The full-length cDNA of *MdACS3a* (Accession No. AB243060) contains a 1338 bp ORF that encodes a protein of 446 amino acids. PCR was performed to amplify the entire ORF from cDNA synthesized from the total RNA isolated from apple 'Fuji' and 'Golden Delicious' fruit. A single amplification product was obtained from each cultivar and these were sequenced directly. Comparison of the nucleotide sequences of the cDNAs from 'Fuji' and 'Golden Delicious' revealed a single nucleotide polymorphism (SNP) at nucleotide

866 of the ORF. In 'Fuji', only the peak of nucleotide G was found (Fig. I-4A), while in 'Golden Delicious', peaks of both G and T were obtained simultaneously (Fig. I-4C). This result indicated that a nucleotide conversion of G to T occurred at nucleotide 866 of *ACS3a* ORF. Moreover, this nucleotide conversion in the coding region resulted in substitution of Gly289 into Val residue, which is located in the part of active site of ACC synthase (Yip et al. 1990; Liang et al. 1992; Lincoln et al. 1994). Furthermore, this amino acid substitution (G→V) was not found in any *ACS* genes of other plant species reported so far (Yip et al. 1990; Lincoln et al. 1994; Itai et al. 1999; El-Sharkawy et al. 2004, 2008). These results indicated that G289V is a unique mutation which has occurred in *Malus* and caused an allele of *ACS3a*, *ACS3a-G289V*.

Enzyme activity of ACS3a and ACS3a-G289V

To test whether this amino acid substitution affects the enzyme activity of ACS3a, full-length cDNAs of *ACS3a* and *ACS3a-G289V* were constructed into the expression vector pET11d. As a positive control, cDNA of *MdACS1* was also constructed into the vector. DNA sequencing was performed to confirm that no spurious mutations were introduced. Proteins expressed in *E. coli* strain of BL21 (DE3) cells were fractioned and subjected to the enzyme activity assay. Two-dimensional electrophoresis experiment indicated that protein spots of ACS3a and ACS3a-G289V were existed in the position of 50 kD which were identical with the predicted molecular weight of ACS3a (Fig. I-2). The result of enzyme activity assay showed that although the activity of ACS3a was only 1/40 as much as that of ACS1, the activity of ACS3a-G289V was abolished (Fig. I-3). This result suggested that the mutation of G289V inactivated ACS3a.

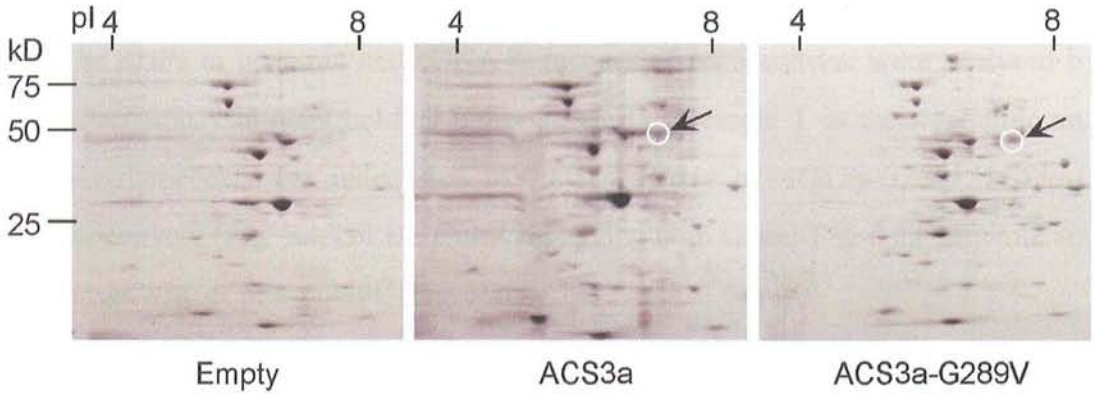

Fig. I-2 Two-dimensional electrophoresis analysis of ACS3a proteins expressed in *E. coli*. Arrows indicate the protein spot of ACS3a and ACS3a-G289V, respectively. Empty means protein made by *E. coli* containing empty vectors (pET11d) and was used as a control. Protein extracts were analyzed in first dimension electrophoresis gel (pH 3–10 linear IPG, 7 cm); second dimension electrophoresis was performed on SDS-PAGE. Protein detection was achieved using colloidal CBB G-250 staining.

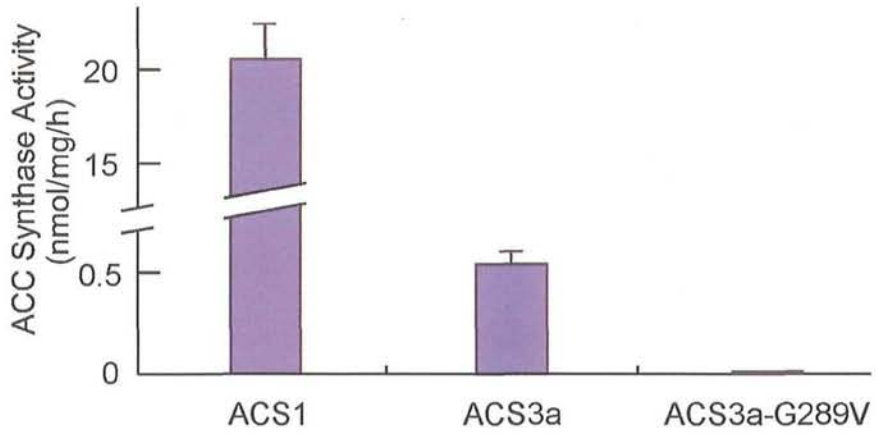

Fig. I-3 Enzyme activity of ACS3a isoenzymes. As a positive control, the activity of ACS1 was measured with the same method.

ACS3a allelic genotypes judged by the genomic and cDNA sequences

Fig. I-4 showed the sequence chart of each *ACS3a* allelic genotype determined by the SNPs. The SNPs in genomic and cDNA sequences of each cultivar were analyzed by direct sequencing. Cultivars holding either single G or single T in their genomic and cDNA simultaneously are indentified as *ACS3a/ACS3a* or *ACS3a-G289V/ACS3a-G 289V*, respectively (Fig. I-4A or B). Cultivars having both G and T in their genomic and cDNA are *ACS3a/ACS3a-G289V* (Fig. I-4C).

During the SNPs analysis, some cultivars were judged as *ACS3a/ACS3a-G289V*, but only *ACS3a-G289V* was found in the cDNA sequences. For example, in the sequence chart of apple 'Kitaro', two peaks (peaks of G and T) appeared in genome DNA but only one peak (peak of T) was found in cDNA (Fig. I-4D). This result suggested that a null gene existed in some apple varieties and it was designated as *acs3a*. Thus the allelic genotype of 'Kitaro' is *acs3a/ACS3a-G289V*. Table I-2 listed the *ACS3a* allelic genotypes of apple cultivars we have investigated. Instead of sequencing, the genotype of apple 'Fuji' was identified by genetic analysis, in which the allelic genotype of 'Kitaro' (*acs3a/ACS3a-G289V*), a progeny derived from the cross between 'Fuji' and 'Hatsuaki' (*ACS3a-G289V/ACS3a-G289V*) suggested that 'Fuji' is *ACS3a/acs3a*.

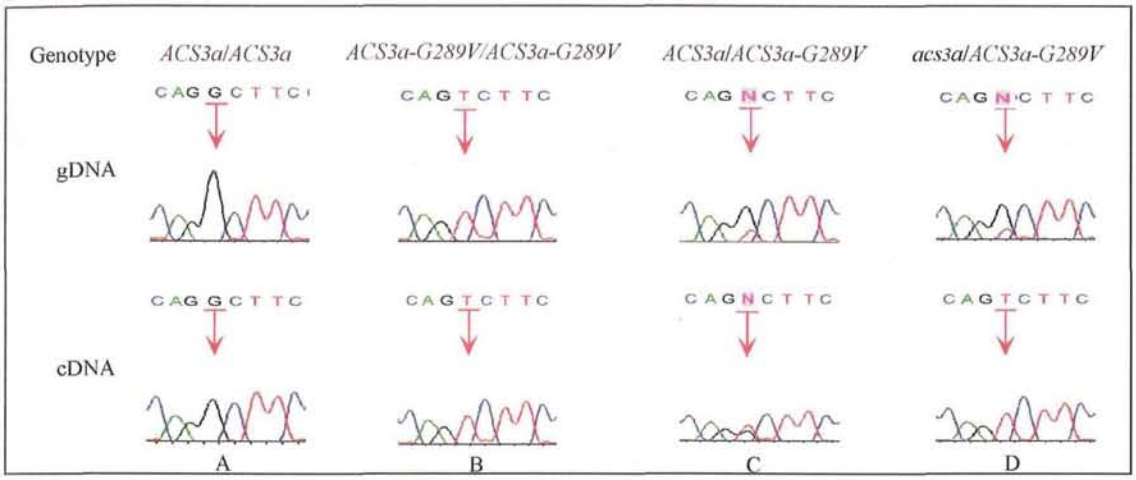

Fig. I-4 Sequence chart of genomic and cDNA of each *ACS3a* allelic genotype. The arrowheads indicate the positions of SNPs which result the alleles of *ACS3a*. The black and red peaks mean the SNPs of G and T, respectively. A and B indicate the sequence chart of alleles homozygous for *ACS3a* and *ACS3a-G289V*, respectively; and C indicates the heterozygous type of *ACS3a* and *ACS3a-G289V*; D showed the heterozygote of a null type, *acs3a* and *ACS3a-G289V*.

Table I-2 *ACS3a* allelic genotypes of apple cultivars

Cultivars	***ACS3a***
Delicious	*ACS3a/ACS3a*
Hatsuaki	*ACS3a-G289V/ACS3a-G289V*
Gala	*ACS3a/ACS3a-G289V*
Golden Declicious	*ACS3a/ACS3a-G289V*
Kotaro	*ACS3a/ACS3a-G289V*
Ralls Janet	*ACS3a/ACS3a-G289V*
Hirodai 1	*acs3a/ACS3a-G289V*
Kitaro	*acs3a/ACS3a-G289V*
Koukou	*acs3a/ACS3a-G289V*
Slimred	*acs3a/ACS3a-G289V*
Fuji	*ACS3a/acs3a*

SSR marker for ACS3a-G289V

In order to develop a DNA marker to identify each of *ACS3a* alleles, we analyzed the promoter region of each allele from several cultivars (Fig. I-6). The promoter region (-90 bp – -530 bp) of each cultivar was cloned and sequenced. A dinucleotide 'GA' repeat (SSR, simple sequence repeat) was found locating in -420 bp of the promoter. This SSR was repeated for 20 times in *ACS3a-G289V* but only repeated for 9 times in *ACS3a* (Fig. I-5). Sequencing the counterpart region of *ACS3a* amplified from wild species, *Malus coronaria* (*ACS3a-G289V/ACS3a-G289V*), showed the SSR was repeated for 9 times, suggesting that the SSR linked with the SNPs of *ACS3a* and *ACS3a-G289V*.

A set of primers, MdACS3a-1F and MdACS3a-1R (Table I-1), were designed to amplify the SSR region by using the first PCR product as template. According to the band patterns of the PCR product run on 6 % polyacrylamide gel (Fig. I-6), it is possible to identify whether apple cultivar hold *ACS3a-G289V* allele. Table I-3 showed the presence of *ACS3a-G289V* allele in the wild apple species and cultivars we have investigated. *ACS3a-G289V* was found in wild species, *Malus coronaria*, indicating that this mutation occurred before the domestication of wild apple. In addition, *ACS3a-G289V* not only exists in cultivars bred in Japan, but also exists in those cultivars bred in other countries such as New Zealand, America and Australia (Table I-3).

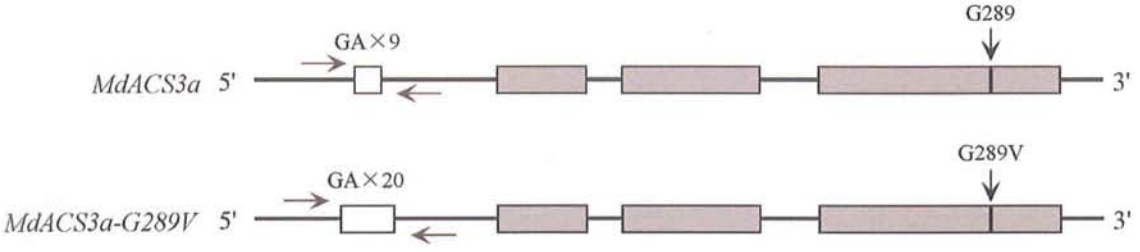

Fig. I-5 Schematic of *ACS3a* and *ACS3a-G289V*. The gray boxes indicate the exons, and white box means the SSRs in the promoter region. The arrows flanking the SSR region indicate the primers used to amplify this region. The vertical lines in the last exon show the position of amino acid substitution.

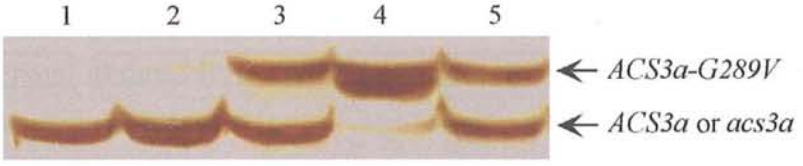

Fig. I-6 Diagnosis of the *ACS3a-G289V* allele in apple cultivars. The SSR region in the promoter was amplified and electrophoresed on a 6 % polyacrylamide gel and stained with silver. The upper band indicates the allele of *ACS3a-G289V* and lower band indicates the alleles of *ACS3a*. The numbers on the top of the lanes mean different cultivars: Lane 1, Delicious; lane 2, Fuji; lane 3, Kitaro; lane 4, Hatsuaki; lane 5, Golden Delicious.

Table I-3 Presence of *ACS3a-G289V* in apple cultivars and wild species

Wild species

Malus arnoldinan, *Malus coronaria*, *Malus floribunda*, *Malus platycarpa*, *Malus robusta*, *Malus transitoria*.

Cultivars

bred in Japan

Aori 4, Fuji, Gold Farm, Golden Melon, Gunma Meigetsu, Hatsuaki, Himekami, Hirodai 1, Hozuri, Indo, Iwakami, Jonagold, Kaori, Kitaro, Kogetsu, Korin, Kotaro, Koukou, Maki 20, Megumi, Mutsu, Orin, Romu 16, Royo 7, Slimred, Toki, Tsugaru.

bred in other countries

Ambishas, Gala, Golden Delicious, Granny Smith, Jonathan, July Red, Melba, Pink Lady, Priam, Prima, Priscilla, Ralls Janet, Red Gold, Rome Beauty.

Open box means the cultivars of *ACS3a/ACS3a-G289V*; blue box means cultivars of *acs3a/ACS3a-G289V*; gray box means cultivars of *ACS3a-G289V/ACS3a-G289V*; the others mean cultivars of *ACS3a/ACS3a* or *ACS3a/acs3a*.

Expression profile of Ripening related genes in 'Kitaro', acs3a/ACS3a –G289V

Fig. I-7 showed the changes in fruit firmness, ethylene production and expression of ripening related genes of apple 'Kitaro' and 'Kotaro'. There was no much change for fruit firmness of 'Kitaro' during the experimental period, but it was obviously decreased in 'Kotaro'. The ethylene production was much higher in apple 'Kotaro' than that in 'Kitaro'. The expression of *ACS1* was not detected in 'Kitaro' by northern, but weak bands were found in 'Kotaro' after storing at 24 °C for 3 -12 d. Weak expression of *ACS3* was detected in 'Kitaro', whereas it was quite strong in 'Kotaro'. Although *ACO1* was expressed in both cultivars, it was much stronger in 'Kotaro'. The expressions of *ERF2*, an ethylene response factor, and *PG1*, which is related to the softening of apple fruit (Wakasa et al. 2006), were not observed in 'Kitaro', but they were expressed strongly in 'Kotaro', although *PG1* was just transiently detected. Furthermore, the almost same result was obtained from the comparison of 'Gala' (*ACS3a/ACS3a-G289V*) and 'Koukou' (*acs3a/ACS3a-G289V*) (Fig. I-8). Fruit of 'Gala' showed higher ethylene production and lower fruit firmness compared to 'Koukou'. The amount of *ACS3a* transcript in 'Gala' was much more than that in 'Koukou'; and although the expression of *ACS1* was not detected in 'Gala' due to its homozygosity of *ACS1-2*, but strong expression of *PG1* was found during the experimental period. These results showed that *acs3a/ACS3a-G289V* cultivars have higher storage ability than *ACS3a/ACS3a-G289V* cultivars, suggesting that the storage ability of apple fruit is under the control of ACS3a allelotypes.

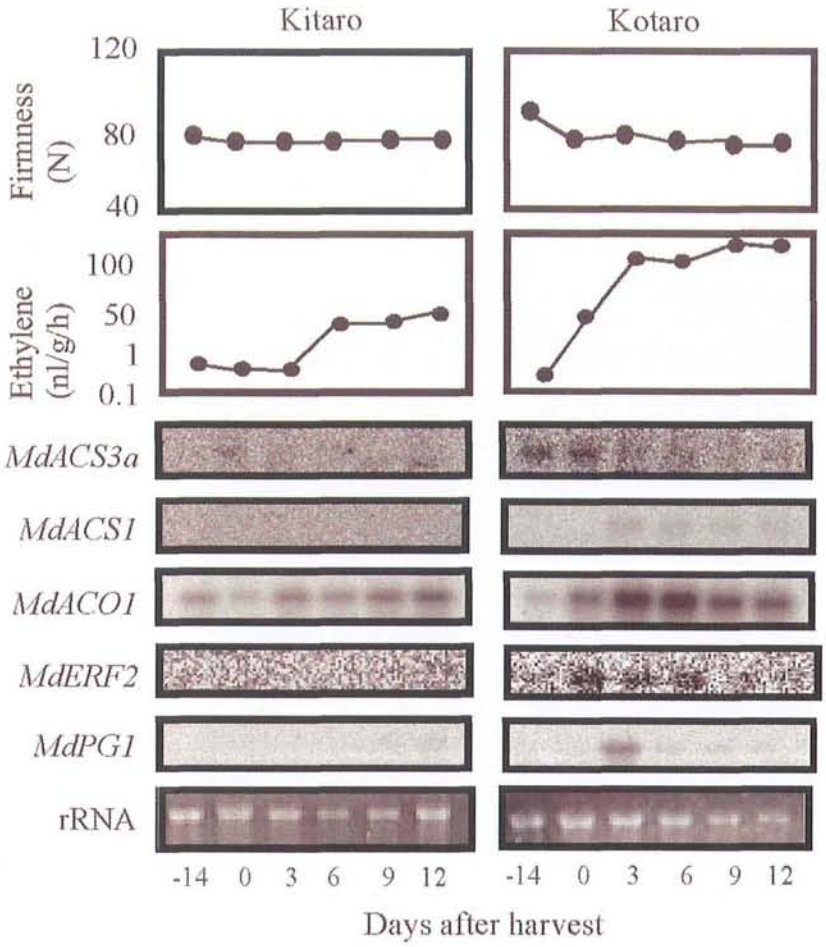

Fig. I-7 Changes in ethylene production, flesh firmness and expression levels of ripening-related genes in 'Kitaro' and 'Kotaro'. Numbers indicate days after commercial harvest (day 0). "–14" means fruit at 14 d before harvest; "3"–"12" mean days of storage at 24 °C. Ribosomal RNA was used as loading controls.

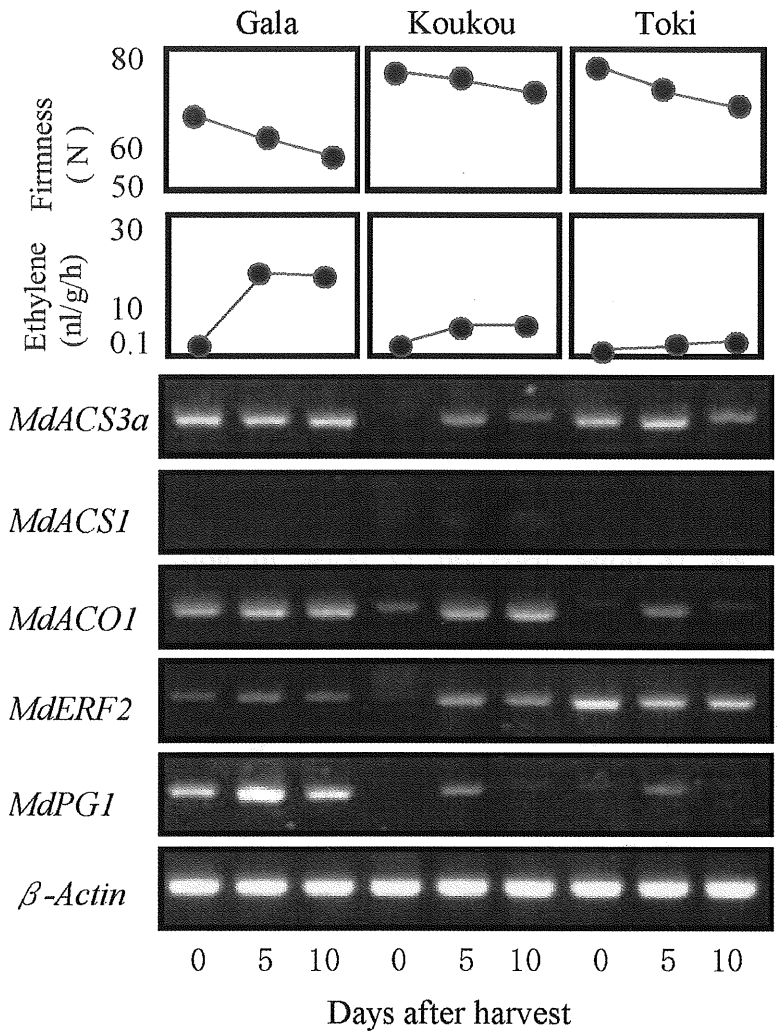

Fig. I-8 Changes in ethylene production, flesh firmness and expression levels of ripening-related genes analyzed by RT-PCR in 'Gala', 'Koukou' and 'Toki'. Numbers at the bottom indicate days after commercial harvest (day 0). "5" and "10" mean days of storage at 24 °C. The Actin gene was used as an internal reference.

I-4 Discussion

Ethylene biosynthesis differs variously among apple cultivars. Although ethylene production can be fundamentally reflected by the alleles of *MdACS1*, it isn't always paralleled with the genotypes (Oraguzie et al. 2004). Through screening a genome DNA library, another ACC synthase gene family of apple, *MdACS3*, was isolated. Two of the subfamily genes, *MdACS3b* and *c*, failed to transcribe to mRNA because of a transposon insertion, thus only *MdACS3a* functioned well. A single amino acid substitution resulted from a single nucleotide acid substitution caused an inactive allelic genotype, *MdACS3a-G289V*. In addition, another allele, *acs3a*, was found as a null allele because of no transcription. The existence of these three allelic genotypes made it possible to elucidate the difference in ethylene production and storage ability among various apple cultivars.

In apple, the expression of *MdACS3* preceded *MdACS1* and *MdACO1*, and decreased after *MdACS1* and *MdACO1* started expressing (Yamakake 2007). My previous study also indicated that the *MdACS3* mRNA was down-regulated in the sport cultivars of 'Fuji' apple (Wang et al. 2008). These results are consistent with the expression patterns of *LeACS1A* and *LeACS6* in tomato (Barry et al. 2000). Taking into account that system 1 ethylene is negatively auto-regulated (Lelièvre et al. 1997; Barry et al. 2000), it is probable that *MdACS3* plays a crucial role in regulating system-1 ethylene biosynthesis and transition to system 2 in apple, and the burst of system-2 ethylene production negatively feeds back to system 1, resulting in the decrease of *ACS3* expression.

Capitani et al. (1999) has reported that Lys283 is one of the 11 conserved amino acid in PLP (pyridoxal-5'-phosphate) dependent enzymes, which is necessary for PLP binding. Moreover, the functional form of ACC synthase is the dimer or theoretically a higher oligomer (Tarun and Theologis 1998; Capitani et al. 2002). In my study, the mutation of G289V inactivated ACS3a (Fig. I-3). Based upon this knowledge, it is reasonable to assume that G289 is important for dimerization which is required for the enzymatic activity due to its close to K283. Apple cultivars were classified into several

genotypes according to the homozygosity for *ACS3a* alleles (Table I-2). For *ACS3a/ACS3a*, the enzymatic activity is normal, but *ACS3a-G289V/ACS3a-G289V* and *acs3a/ACS3a-G289V* genotypes have no activity because of the inactiveness of *ACS3a-G289V* and null type, although the dimer can be formed. For *ACS3a/ACS3a-G289V*, the enzymatic activity may be decreased due to the presence of the mutation which reduces the enzyme activity when forming a dimer (Tarun and Theologis 1998), resulting in that the activity is much lower than that of the *ACS3a/ACS3a* type. However, question arises whether dimers are formed between ACS3a and other members of ACC synthases as ACS1, 4 and 5 are also expressed in apple fruit (Kondo et al. 2008). It is probable that heterodimer is avoided by the presence of specific dimerization site in each enzyme. But most likely heterodimerization is prevented by the asynchronous expression of each gene during fruit ripening (Yamakake 2007; Kondo et al. 2008).

The ethylene production was much lower in 'Kitaro' than that in 'Kotaro', and the expression of ripening related genes was greatly suppressed in 'Kitaro' (Fig. I-7). These differences may be caused by the *ACS3a* allelotypes, because 'Kitaro' is *acs3a/ACS3a-G289V* which is no ACS3a activity, whereas 'Kotaro' is *ACS3a/ACS3a-G289V* type in which ACS3a is still active. The same result was found in the comparison of 'Gala' and 'Koukou' (Fig. I-8). In 'Kitaro' and 'Koukou', system-2 ethylene synthesis may not be initiated because *acs3a/ACS3a-G289V* can not produce sufficient ethylene to initiate the transition to system-2 ethylene synthesis. Based on this consideration, ACS3a probably acts as a switch to initiate the transition from system-1 to system-2 ethylene synthesis and subsequently lead to a burst of ethylene production. Most likely, ACS3a is the main enzyme that controls the ethylene production and storage ability of apple cultivars. To be noted, we can not exclude the possibility that there are SNPs or elements exist in the alleles of *ACS1* which affect the enzyme activity. In addition, the regulation of *ACO1* on ethylene production and storage ability should be considered.

By genetic analysis, apple 'Fuji' was judged as *ACS3a/acs3a* type which can not be identified by sequencing the genomic and cDNA only. The previous study revealed that

the *MdACS3a* was expressed constantly in 'Fuji' fruit during storage and the expression of *ACS1* and other ripening related genes could not be detected during the experimental period. Moreover, the ethylene production is quite low for 'Fuji' fruit (Wang et al. 2008). The explanation is that the amount of ACS3a is probably too low to synthesize sufficient ethylene to initiate system-2 ethylene synthesis. This result supported the presumption that ACS3a is the switch to turn on the transition to system-2 ethylene synthesis.

Apple 'Koukou', heterozygous for *MdACS1-1/2*, has been found having better storage ability than 'Fuji' which is homozygous for *MdACS1-2/2* (Prof. Shiozaki, personal communication). My result provided the molecular evidence for this fact that enzymatic activity of ACS3a is higher in *ACS3a/acs3a* ('Fuji') than that in *acs3a/ACS3a-G289V* ('Koukou'). This fact supported the model that ACS3a is the main determinant of storage ability of apple fruit.

Another apple cultivar 'Toki', which is considered to be *ACS3a/acs3a* type, produced less amount of ethylene and expressed ripening related genes stronger than 'Gala' did, although they are all homozygous for *ACS1-2* allele and harvested at same date (Fig. I-8). This phenomenon could also be explained by the model of 'Fuji'.

Alignment of *ACS3a* orthologs of more than 100 of plant species was performed and the mutation of G289V was only found in *Malus* including the wild species and domesticated cultivars from America, Australia, New Zealand Europe and Japan (Table I-3). It was not found in pear although it is very close to apple in genetics. Based upon these results, it appears that *ACS3a-G289V* arose when *Malus* was separated from *Rosales*, maintained during domestication and inherited among the domesticated cultivars.

It is still unknown that why *acs3a* can not be transcribed to mRNA. Understanding this would lead to develop a DNA marker for *acs3a*. We tested the promoter region of the null gene, but no transposon or other insertion was found yet. The difference possibly exists in the much upper stream of the promoter other than we investigated. Matarasso et al. (2005) reported that a Cys protease (LeCp) regulates the expression of tomato *LeACS2* by binding to a *cis* element (TAAAAT motif), and *LeCp* was found to

be induced by fruit ripening (Alonso and Granell 1995). Sequence analysis revealed that alleles of *MdACS3a* also hold this motif. Further study is needed to investigate whether it is the mutation of this motif that resulted in this null allele, *acs3a*. In addition, other factors which regulate the gene expression such as DNA methylation should be taken into consideration.

In some pear cultivars the orthologs of at least two *Pc-ACS1* genes show differential cold requirement and subsequently influence postharvest ethylene production and fruit softening (El-Sharkawy et al. 2004). Due to the close relativeness between pear and apple, it is possible that *ACS1* or *ACS3a* of apple is also correlated with the low temperature which induces the expression of *ACS* gene(s) and then initiates the onset of ethylene production and fruit softening. Further work is needed to investigate the response of *ACS* genes to low temperature.

I-5 Summary

The apple (*Malus×domestic* Borkh.) 1-aminocyclopropane-1-carboxylate (ACC) synthase gene 3 (*MdACS3a*) derived from various cultivars was sequenced at genomic and cDNA level. The SNPs analysis revealed that one of the *ACS3a* allele, *ACS3a-G289V*, was caused by an amino acid substitution (Gly289→Val) which occurred in the active site of ACC synthase gene. Over-expression of *MdACS3a-G289V* in *E. coli* revealed that the mutation of G289V inactivated the ACC synthase. On the other hand, a null gene of *MdACS3a*, *acs3a*, exists in the genome of apple. RNA gel-blot and RT-PCR analysis showed that *acs3a/ACS3a-G289V* cultivars produce little ethylene and have higher storage ability such as 'Kitaro' and 'Koukou', compared to those *ACS3a/ACS3a-G289V* cultivars, such as 'Kotaro'(*MdACS1-1/2*) and 'Gala' (*MdACS1-2/2*), although both 'Kitaro' and 'Koukou' are heterozygous for *MdACS1-1/2*. *ACS3a/acs3a* cultivars also produce little ethylene and have good storage ability. Based upon the ACS3a allelic genotypes, it is obvious that there is the cultivar such as 'Koukou' that has better storage ability than 'Fuji'. An SSR was found to be linked with the SNPs of *ACS3a-G289V*, which can be used as a DNA marker to identify the

cultivars homozygous for *ACS3a-G289V*. Our result suggested that *MdACS3a* plays a crucial role in regulation of ethylene synthesis and determines the storage ability of apple fruit. The role of *MdACS3a* in ethylene signaling was discussed.

Chapter II

MdERFs, two ethylene response factors involved in apple fruit ripening

II-1 Introduction

The involvement of ethylene gas in the ripening of apple fruit is a symbolic phenomenon that has a place in the history of research on plant hormones (Abeles et al. 1992). However, little is still known about the signal transduction mechanism involved. The expression of ripening-related genes (Wakasa et al. 2006), such as cell wall modifying enzymes, is induced through transduction of the ethylene signal from receptors(s) to dedicated transcription factors (Giovannonni 2004). Ethylene response factor (ERF) proteins, which were known formerly as ethylene-responsive element binding proteins (EREBP), function as *trans*-factors at the last step of transduction in the nucleus (Ohme-Takagi and Shinshi 1995). ERF-type transcription factors are specific to plants and belong to the large AP2/ERF superfamily that possesses the DNA-binding domain. Although *Arabidopsis* has 122 predicted ERF genes (Nakano et al. 2006), only a few have been characterized so far (Sakuma et al. 2002). In fact, in tomato, only the role of *SlERF*s in fruit ripening has been reported (Tournier et al. 2003, Pirrello et al. 2006).

We have been studying molecular mechanisms involved in fruit ripening to gain insight into the factors that contribute to differences in flesh softening rate among apple cultivars (Sunako et al. 1999; Harada et al. 2000; Wakasa et al. 2003; Oraguzie et al. 2004; Wakasa et al. 2006). Although the difference in ethylene production rate observed among apple cultivars can be explained by 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene (*MdACS1*) 'allelotype' or 'genotype'(Sunako et al. 1999; Harada et al. 2000), allelic form alone does not explain all the phenotypic variation in fruit softening/storage (Oraguzie et al. 2004). On the other hand, the expression of

ripening-related genes is completely inhibited by the ethylene antagonist 1-methylcyclopropene (1-MCP), indicating that ethylene signaling is indispensable for the expression of these genes. In order to further examine the role of ethylene transduction in apple ripening, two ERF genes (*MdERFs*) isolated from the ripening fruit of 'Golden delicious' apple were characterized, and the features of their expression were compared with those of other ripening-related genes. The results indicated that some factor(s) in addition to ethylene are responsible for regulating the level of *MdERFs* expression.

II-2 Materials and Methods

Plant materials

Samples of the apple (*Malus×domestica* Borkh.) cultivar, 'Golden Delicious', were used in all experimentation, unless otherwise stated. Young leaf, flower, stamen and receptacle were obtained from orchard trees at the Aomori Apple Experimental Station. Tissue samples were collected once a week from May to November and prepared as described by Wakasa et al. (2003). Fruit from other cultivars were also used for comparison of the gene expression patterns with that of 'Golden Delicious'.

Cloning of MdERFs cDNAs

Degenerate oligonucleotides (CCRTGGGGRAAATKKGCGGCK and CATAAGCVAVAKBGCRGCTTCYTC) designed by Tournier et al. (2003) were used in a polymerase chain reaction (PCR) with the cDNA template from ripening fruit. Full-length cDNA clones for *MdERFs* were isolated by screening using a 'Golden Delicious' cDNA library (Sunako et al. 1999).

RNA extraction and northern blot analysis

RNA extraction and gel blot analysis were performed as described by Sunako et al. (1999). The probes for *MdERFs* were prepared by PCR using the respective clones as a

template. The following gene-specific primers were used to amplify their 3'-UTRs: MdERF1F, 5'-ATGACCTGGTGGCATATCAG-3'; MdERF1R, 5'-CACCGTAGCAAA CAACACAC-3'; MdERF2, 5'-TATGCTGGCAATTGGCGAGC-3'; MdERF2R, 5'-AT GACCAATCCCGCACTCAC-3'. The RNA gel blot analysis of *MdACS1, MdACO1* and *MdPG1* was carried out as in Wakasa et al. (2006).

Measurements of ethylene production rates

The method has been described in Chapter I.

1-MCP treatment of fruit

The ethylene antagonist 1-MCP was used to suppress ethylene signal transduction. Fruits were treated with 1 ppm of 1-MCP (EthylBloc, Rohm and Haas, Philadelphia, PA, USA) for 15 h at 24 °C. After treatment, the fruits were held at 24 °C for 12 days for comparison of ethylene production with untreated fruit and expression of ripening-related genes every 3 days (Wakasa et al. 2006).

RT-PCR analysis of MdERF2

Total RNA extracted from fruit collected at 22 or 14 days before harvest and at harvest day was used for first-strand cDNA synthesis using the MdERF2R primer (5'-ATGACCAATCCCGCACTCAC-3'). Then, PCR amplification was carried out using gene-specific primer MdERF2F (5'-TATGCTGGCAATTGGCGAGC-3') and MdERF2R as described by Wakasa et al. (2006). The thermal cycling conditions were 3 min at 94 °C; then 25 cycles of 5 s at 94 °C, 15 s at 65 °C and 20 s at 72 °C. To normalize for differences in total RNA, the concentration of actin (accession number EB136338) mRNA in each sample was determined using the primers 5'-GGCTGGATTTGCTGGTGATG-3' and 5'-TGCTCACTATGCCGTGCTCA-3'.

II-3 Results

Molecular characteristics of MdERF1 and MdERF2

Two partial cDNA clones were isolated from apple cDNAs using degenerate primers targeted to the highly conserved ERF domain, and each of the 3' regions was obtained by 3'RACE. Then, the 3'UTRs were used to screen a cDNA library of ripening 'Golden Delicious' apple fruit. The predicted coding regions of two positive clones possessed a conserved AP/ERF DNA binding domain and a region rich in basic amino acids (P/L-K-K/P-R-R) that could serve as a putative nuclear localization signal (Raikhel, 1992). Therefore, we designated them *MdERF1* (accession number AB288347) and *MdERF2* (accession number AB288348), respectively (Fig. II-1). As shown in Fig. II-2, Southern hybridization to genomic DNA showed that each probe hybridized to one or two of three restriction enzyme fragment(s), revealing that each respective *MdERF* is a single gene in the apple genome.

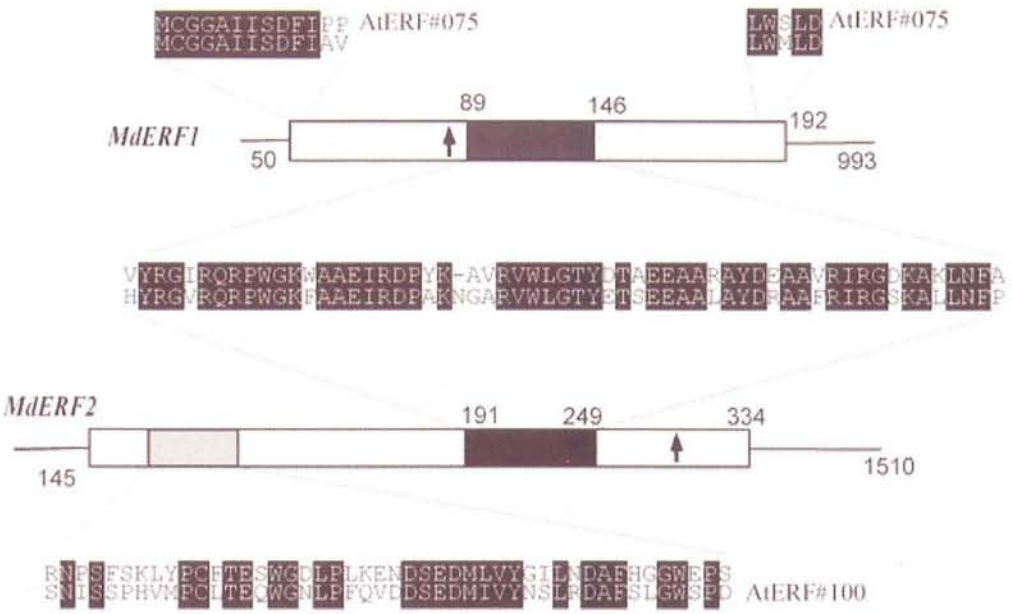

Fig. II-1 A schematic representation of the cDNA structures of *MdERF1* and *MdERF2*. Alignments of the AP2/ERF domains between *MdERFs* and those of the conserved motifs with the corresponding *AtERF* in *Arabidopsis* are shown. Boxes indicate the open reading frames, starting from the first ATG codon, and lines show putative untranslated regions. Block boxes indicate the AP/ERF domain; arrows represent putative nuclear localization signals. Numbers above the line indicate positions of amino acid residues; numbers below the line refer to nucleotide positions. AtERF#75 and AtERF#100 are generic names of the *Arabidopsis ERF* genes (Nakano et al. 2006).

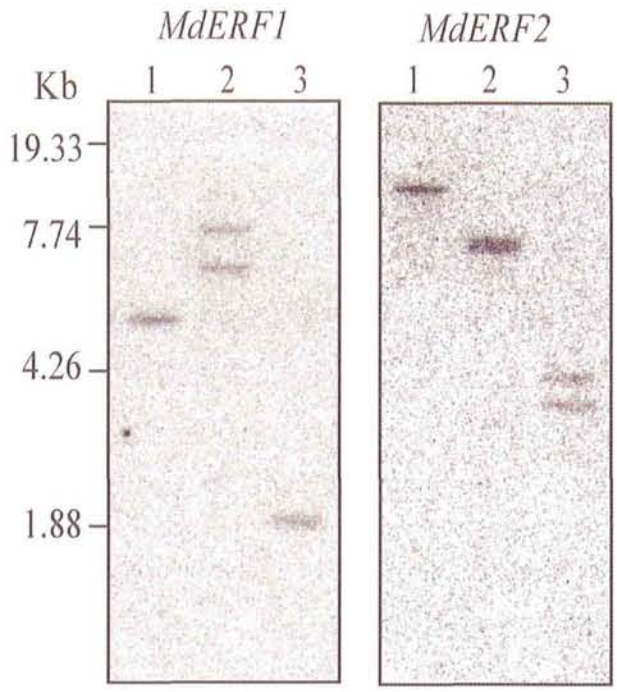

Fig. II-2 DNA gel blot analyses using a specific probe for each *MdERF*. Ten micrograms of genomic DNA was digested with *Hin*dIII (lane 1), *Eco*RI (lane 2) and *Dra*I (lane 3).

MdERF1 and MdERF2 expression in ripening fruit

To clarify the expression pattern of the *MdERFs* at the transcriptional level, northern hybridizations were performed using different apple plant tissues (Fig. II-3). The *MdERF1* signal was clearly detected in ripening fruit and also in root and shoot tissues. On the other hand, a very weak signal of the *MdERF2* transcript was observed only in fruit tissue. The expression level of both *MdERFs* was then analyzed during fruit ontogeny. As shown in Fig. II-4, *MdERF1* was expressed weakly in young fruit in May and continued to be expressed throughout development, and then the level increased at the ripening stage. In the case of *MdERF2*, a detectable transcript was obtained only in the ripening fruit from the end of October. To confirm the exclusive expression of *MdERF2* at the ripening stage, RT-PCR was performed (Fig. II-5). This indicated that the transcript was present at 14 days before commercial maturity (day 0), but was not evident at 22 days before harvest.

Repression of MdERF1 and MdERF2 expression by 1-MCP

To understand the role of ethylene in *MdERFs* expression during ripening, we treated the fruit at commercial maturity with 1-MCP, an ethylene antagonist. The treated fruit showed a marked reduction of the *MdERF1* transcript (Fig. II-6) and complete disappearance of the *MdERF2* signal, indicating that their expression was regulated by ethylene. However, *MdERF1* expression in the treated fruit appeared to increase gradually during ripening, as was the case in the control fruit.

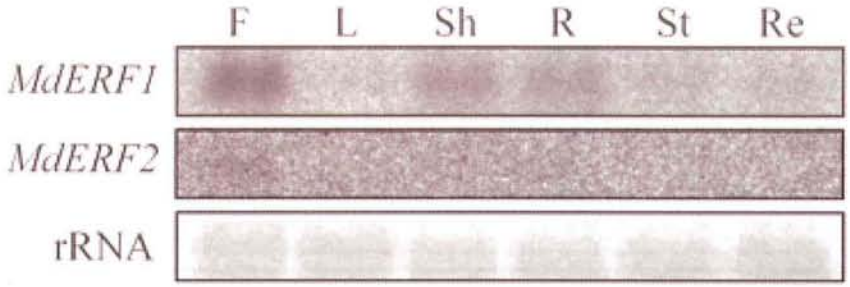

Fig. II-3 RNA gel blot analysis of *MdERF1* and *MdERF2* in ripening fruit and non-fruit tissues. Each lane was loaded with total RNA from fruit (F), young leaves (L), shoots (Sh), root (R), stigmata (St), and receptacles (Re). rRNA is shown as loading control.

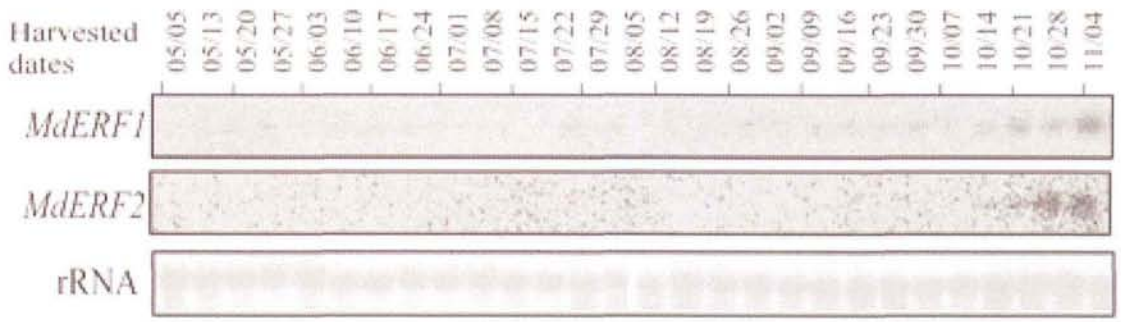

Fig. II-4 Changes in *MdERF1* and *MdERF2* expression during fruit ontogeny. Apple fruit of 'Golden Delicious' were sampled weekly from May to November.

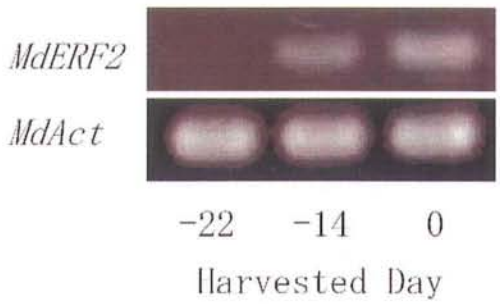

Fig. II-5 RT-PCR analysis of *MdERF2* transcript accumulation during ripening. The *MdAct* gene was used as an internal reference. Times are means day(s) before/after harvest.

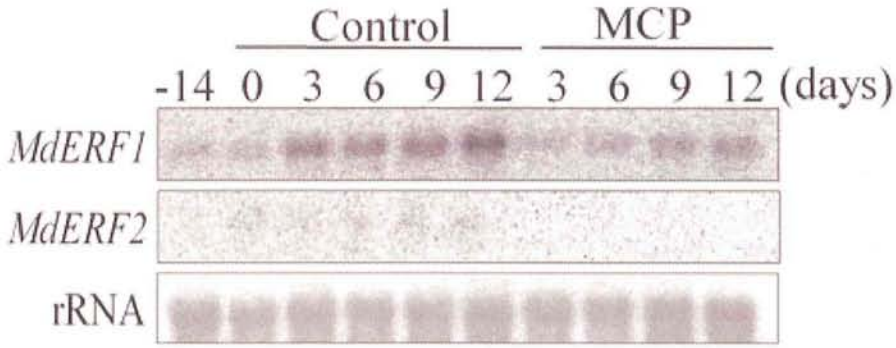

Fig. II-6 Changes in the accumulation of *MdERF1* and *MdERF2* mRNA in 'Golden Delicious' apple fruit with or without 1-MCP treatment. Ribosomal RNA was used to normalize RNA loading. Numbers above each lane indicate the number of storage days after harvest at 24 °C.

MdERFs expression level and ethylene production level

To investigate the relationship between the degree of *MdERFs* expression and the level of ethylene production, fruit of 'Fuji', a representative low-ethylene-producing apple cultivar, was compared with that of 'Golden Delicious'. 'Fuji' fruit produced around 0.5 nl g^{-1}FW h^{-1} ethylene from one month before harvest, whereas in 'Golden Delicious', production increased gradually and reached a plateau at around 70 nl g^{-1}FW h^{-1} (Fig. II-7). Although *MdERF1* and *MdERF2* transcripts were detected in 'Golden Delicious', no signal of either transcript was observed in fruit of 'Fuji'. To clarify whether higher ethylene production is correlated with higher *MdERF1* expression during ripening, we used harvested fruit of various cultivars that had been stored at room temperature (Wakasa et al. 2006). Fig. II-8 shows the changes in the rate of ethylene production and expression of *MdERF1* during ripening in 14 apple cultivars. Because 'Golden Delicious', 'Kotaro', 'Delicious' and 'Kitarou' don't have the transposon of *MdACS1* allele (Harada et al. 2000), their ethylene production rate rose to around 100 nl g^{-1}FW h^{-1}, and a strong expression of *MdERF1* was observed. However, 'Hozuri' and 'Ralls Janet' showed a high expression level that did not correspond with the ethylene production level in each.

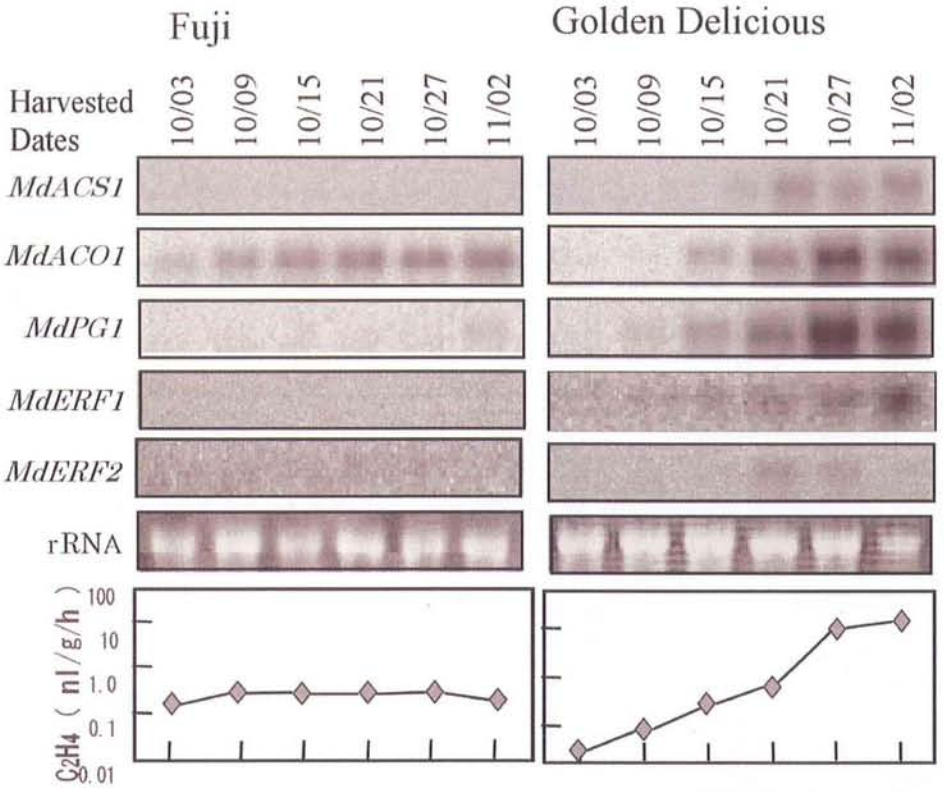

Fig. II-7 Changes in the expression of *MdERF1, MdERF2*, and other ripening-related genes and rate of ethylene production by fruit on a tree. Data obtained from 'Fuji' and 'Golden Delicious' apple fruit harvested from Oct 3 to Nov 2 are shown in order from left to right. Data for different ripening stages (–14, 0, 3, 6, 9, 12 days after harvest) are shown in order from left to right.

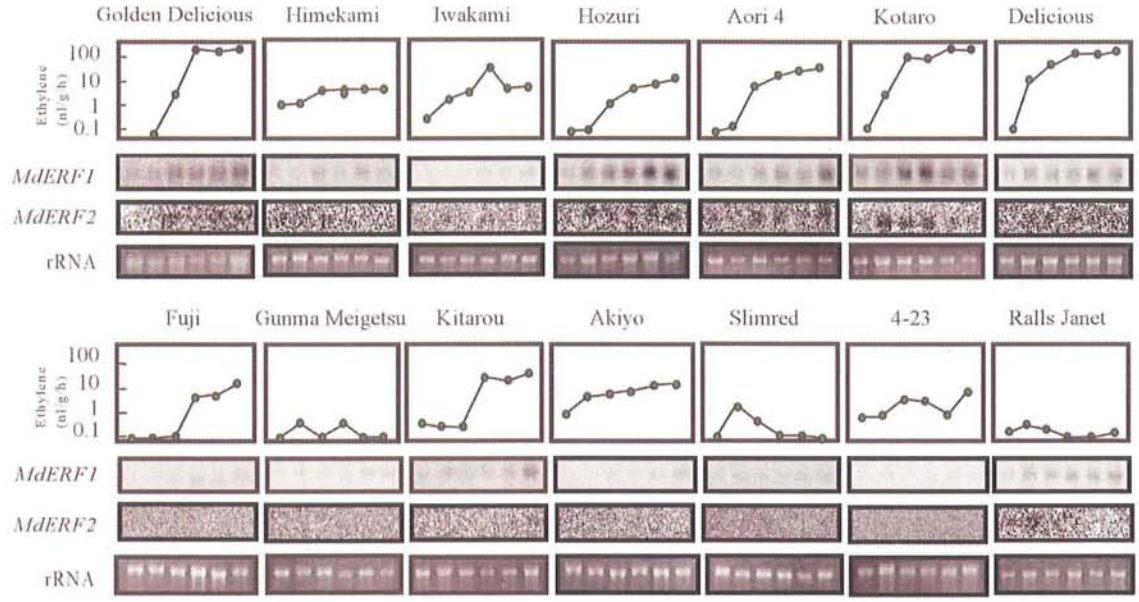

Fig. II-8 Changes in the rate of ethylene production and expression of *MdERF1* during ripening of 14 apple cultivars. Data for different ripening stages (-14, 0, 3, 6, 9 and 12 days after harvest) are shown in order from left to right.

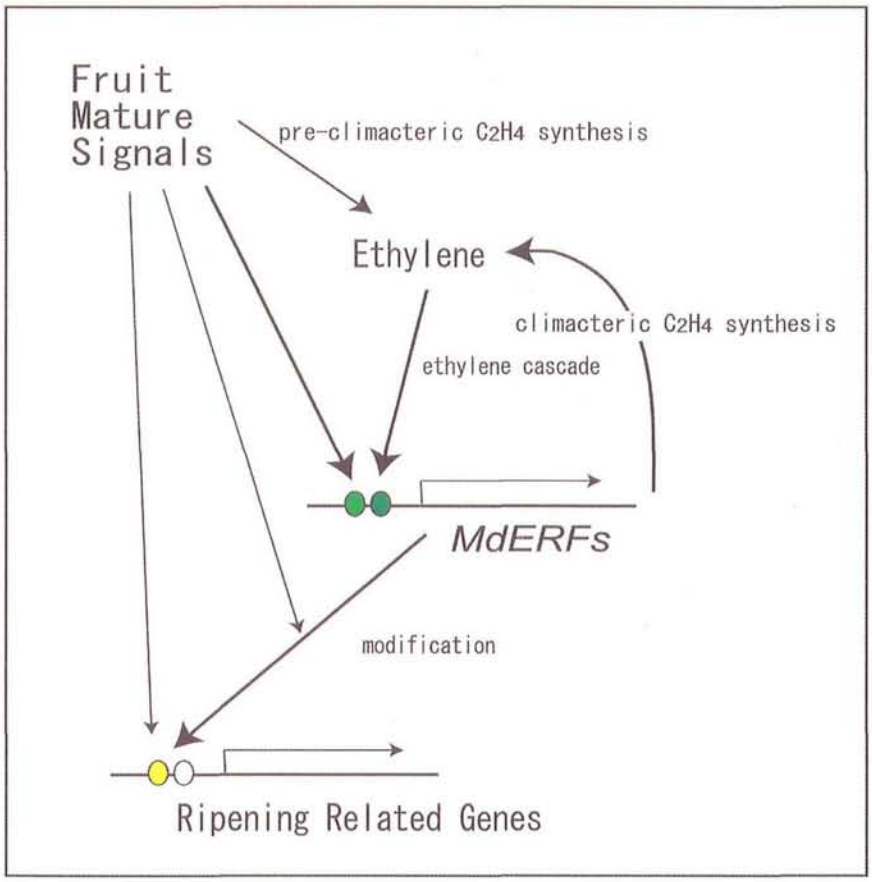

Fig. II-9 A model for signal transduction of ripening-related genes. Expression of *MdERFs* is regulated by at least two factors from the ethylene and maturation cascades. Furthermore, the activity of *MdERFs* as a transcription factor is also modulated by other regulatory factor(s).

II-4 Discussion

Sequence analysis clearly indicated that the two *MdERFs* isolated in this study belong to the ERF family of the AP2/ERF superfamily, which is defined by the ERF/AP2 domain (59 amino acids) that is involved in DNA binding. AP2 family proteins contain two repeated AP2/ERF domains, while ERF family proteins like *MdERFs* contain a single AP2/ERF domain (Riechmann and Meyerowitz, 1998). In *Arabidopsis*, 122 of the 147 genes identified as possibly encoding AP2/ERF domain(s) have been assigned to the ERF family (Nakano et al. 2006). Furthermore, the ERF family is grouped into two major subfamilies, ERF conserving the 14th alanine (A14) and the 19th aspartic acid (D19) and CBF/DREB in which valine (V14) and glutamic acid (E19) are present at the respective positions (Yang et al. 2002; Sakuma et al. 2002; Tournier et al. 2003; Nakano et al. 2006). The predicted amino acid sequences of both *MdERFs* show A14 and E19, indicating that they belong to the ERF subfamily. The N-terminal sequence MCGGAII/L and the C-terminal LWS (I/L/Y) of *MdERF1* are a characteristic signature of group VII among the 12 ERF groups (Nakano et al. 2006). Tomato *SlERF2* possessing these motifs is expressed not only in ripening fruit but also in germinating seed, and a small amount of its transcript accumulates in all plant tissues (Pirrello et al. 2006). The transcript of *MdERF1* was also detected in non-fruit tissue, but showed strongest expression in ripening fruit. On the other hand, *MdERF2* had a conservative group IX motif (CMIX-3), which is considered to function as a transcriptional activation domain (Nakano et al. 2006).

In fruit treated with 1-MCP, a low level of the *MdERF1* transcript was detected and the level increased slightly during ripening. This does not indicate that the increase was not under the control of ethylene, because 1-MCP was applied only transiently before the start of incubation at 24 °C. As ripening progressed, *de novo*-synthesized ethylene receptor(s) could have appeared (El-Sharkawy et al. 2003), and this could have resulted in *de novo* expression of *MdERFs*. Therefore, *MdERF1* expression is considered to be regulated by ethylene.

The most abundant accumulation of the *MdERF1* transcript was observed in

'Golden Delicious', 'Kotaro', and 'Hozuri' fruit incubated at 24 °C. At room temperature, fruit of 'Golden Delicious' and 'Kotaro' produced about 200 nl g^{-1}FW h^{-1} ethylene (Waksasa et al. 2006). Therefore, it is likely that the higher the amount of ethylene production, the greater the expression of *MdERF1* becomes. The apparent lack of *MdERF1* transcript in a low-ethylene-producing cultivar like 'Fuji' may mean that the level of ethylene (around 0.2 nl g^{-1}FW h^{-1}) is below the threshold at which *MdERFs* transcription can be detected by RNA gel blotting. However, clear accumulation of the *MdERF1* transcript was also observed in 'Ralls Janet', which produces a very low level of ethylene, indicating that the ethylene cascade is not an exclusive regulator of *MdERFs* expression. As shown in Fig. II-9, another transcription factor(s) derived from the maturation cascade also appears to function as a regulator of gene expression.

Arabidopsis ethylene-responsive element binding protein (AtEBP), which belongs to group VII, interacts with a bZIP transcription factor to activate the expression of plant defense genes (Büttner and Singh 1997). OsEBP89 of rice, belonging to group VII, also interacts with a Myc transcription factor to regulate expression of the *Wx* gene (Zhu et al. 2003). Furthermore, *Pti4* of tomato, classified into group IX, is phosphorylated by a kinase, thus enhancing its binding to the target DNA (Gu et al. 2000). Therefore, although the expression of *MdERFs* increased almost concomitantly with those of *MdACO1* and *MdPG1* in ripening fruit, *MdERFs* would be necessary to activate the expression of these ripening-related genes through some additional regulatory mechanism involving another regulatory protein.

We conclude that although ethylene signaling is indispensable for *MdERFs* expression, other factor(s) derived from an ethylene-independent cascade is necessary not only for initiating the expression of *MdERFs* but also as a transcription factor in ripening fruit.

II-5 Summary

Two *MdERFs* (ethylene-response factors) were isolated from ripening apple (*Malus* × *domestica* Borkh. cv. Golden Delicious) fruit. The features of their conserved motifs

indicated that *MdERF1* and *MdERF2* belong to group VII and group IX categorized in *Arabidopsis*, respectively. *MdERF1* was expressed predominantly in ripening fruit, although a small degree of expression was also observed in non-fruit tissues, whereas *MdERF2* was expressed exclusively in ripening fruit. The increased expression in ripening fruit was repressed by treatment with 1-methylcyclopropene (1-MCP: a potent antagonist of ethylene receptor), indicating that transcription is regulated positively by the ethylene signaling system. Indeed, it was a tendency for cultivars with low ethylene production to show lower *MdERFs* expression than those with high ethylene production. On the basis of concomitant analyses of the expression of some genes related to ripening, the functions of *MdERFs* and the role of ethylene in the ripening process were discussed.

Chapter III

Molecular mechanism of distinct ripening profiles in apple fruit of 'Fuji' and its early maturing sports

III-1 Induction

Although apple fruit has a considerably longer shelf life than those of other species such as peach and pear, the fruit quality deteriorates gradually after ripening (knee 1993). However, there is great difference in the rates of deterioration among cultivars. Explaining the difference would allow the identification of molecular markers for apple breeding. Ethylene biosynthesis and ethylene-induced ripening have been studied extensively in tomato fruit at the molecular genetic level (Giovannoni 2004). Using the information from tomato, researchers have gained substantial knowledge about ripening-related genes in apple. The biosynthesis of ethylene in plants is controlled mainly by ACS and ACO enzymes, which are encoded by multigene families (Bleecker and Kende 2000). MdACS1 and MdACO1 are involved in apple fruit ripening (Dong et al. 1991, 1992). A particular allele of *MdACS1* accounts for the low amount of ethylene in some cultivars (Sunako et al. 1999; Harada et al. 2000). Genes encoding ethylene receptors (*MdETR1*, *MdERS1*, *MdERS2*) have been cloned, and dynamic changes in protein levels during ripening were reported (Tatsuki et al. 2007). Two ethylene response factors (*MdERFs*) function as trans-factors in the last step of transduction (Wang et al. 2007). The expression of a polygalacturonase gene (*MdPG1*) is related to the loss of flesh firmness (Wakasa et al. 2006).

Besides ethylene, other factors influence fruit ripening, including ambient temperature and fruit maturity at harvest (Adams-Phillips et al. 2004). Dal Cin et al. (2007) reported that fruit load and elevation also affect apple fruit ripening through the action of ethylene. We revealed that other ethylene-independent factors are necessary for *MdERF* transcription (Wang et al. 2007).

'Fuji', a well-known cultivar which tops world apple production (O'Rourke et al. 2003), shows no loss of firmness for at least one month after harvest, even at room temperature (Gussman et al. 1993; Wakasa et al. 2006). In contrast, 'Hirosaki Fuji', a sport of 'Fuji' that matures about 40 d earlier, softens (Harada, unpublished data). This difference would be due to other effects such as ambient temperature, because 'Hirosaki Fuji' reaches maturity at the end of September, when the average temperature is 17 °C, whereas 'Fuji' matures at the start of November, when the average temperature is 6 °C.

Understanding the reason for the different storage competencies of 'Fuji' and 'Hirosaki Fuji' is an important clue to the mechanism of the long shelf life of 'Fuji'. In this study, we compared the ethylene production rates and expression profiles of ripening-related genes between 'Fuji' and 'Hirosaki Fuji'. We also analysed 'Fuji' fruit grown at different latitudes to assess the influence of ambient temperature on ripening.

III-2 Materials and methods

Fruit materials

In 2005, apple (*Malus* ×*domestica* Borkh.) fruit of 'Fuji' and 'Hirosaki Fuji' were collected at the experimental farms of Hirosaki University and Hirosaki city, respectively. They were harvested 14 d before commercial maturity ('Hirosaki Fuji' on Sep 29 and 'Fuji' on Nov 6) and at commercial maturity (day 0). The fruit collected on day 0 were stored at 24 °C and sampled every 3 d for measurements of ethylene production and flesh firmness; the flesh was sliced, quickly frozen in liquid N_2, and stored for extraction of RNAs and proteins. The other early-maturing 'Fuji' sports ('Benishogun', 'Korin' and 'Ryokanokisetsu') were provided by the Aomori Apple Experimental Station on 2006 Oct 5. In 2007, 'Fuji' fruits picked at the different localities were transported to Hirosaki University in a refrigerator truck within 20 h after harvest. After arrival (day 1), they were stored at 24 °C for 12 d and sampled every 3 d. 'Fuji' and 'Hirosaki Fuji' fruit were sampled weekly from 2007 Aug 25 and stored at −80 °C until RNA extract. Five fruits were used for each experiment.

Measurements of ethylene production rates

The method has been described in Chapter I.

Measurements of flesh firmness

The method has been described in Chapter I.

1-MCP treatment of fruit

The method has been described in Chapter II.

RNA extraction, Northern blot analysis and RT-PCR

RNA extraction and gel blot analysis were performed as described by Sunako et al. (1999). The probe for each gene was prepared by PCR from the respective clones according to Wakasa et al. (2003). The primer sequences of *MdACS1*, *MdACO1* and *MdPG1* and the probe regions are as described in Wakasa et al. (2006), and primer sequences of ethylene receptor genes are presented in Tatsuki and Endo (2006). The primer sequences of *MdACS3* are as follows: MdACS3F 5'-GACAAATAGAAAGA-GACTGAGGACG-3', MdACS3R 5'-CCATCGATTATACAAACTGATTGTG-3'. The probe for *MdHSP17.5* was prepared by PCR using the following gene-specific primers: forward, 5′-GCATAACCTCGATCCGCTAC-3′; reverse, 5′-CGTACATGACAATTGA-CACACC-3′. First-strand cDNA was synthesized by reverse transcriptase from 500 ng total RNA. RT-PCR was carried out with the same primer set. The thermal cycling conditions were 3 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C; and a final extension of 3 min at 72 °C. In order to normalize for differences in total RNA, the relative amount of *actin* (accession No. EB136338) mRNA in each sample was determined with the primers 5′-GGCTGGATTTGCTGGTGATG-3′ and 5′-TGCTCACTATGCCGTGCTCA-3′.

Protein extraction and Western blot analysis

Microsomal membrane fractions were isolated from fruit flesh in extraction buffer containing 100 mM Tris (pH 8.0), 300 mM NaCl, 20 mM EDTA and 20 % v/v glycerol with protease inhibitors (1 mM PMSF, 5 mM DTT). Flesh was homogenized at 4 °C in a homogenizer, and then centrifuged at 9,100×*g* rpm for 15 min. The supernatant was strained through Miracloth, and then centrifuged at 100,000 ×*g* for 1 h. The pellet was resuspended in elution buffer (extraction buffer containing 1 % Triton X-100), kept on ice for 3 h and then centrifuged at 20,400 ×*g* for 15 min. Protein concentrations in the supernatant were determined by Bio-Rad protein assay (http://www.bio-rad.com/). A 50 μg aliquot of total protein was run out for each sample in a 10 % Tris-HCl gel, and proteins were transferred to a PVDF membrane. Membranes were blocked for 1 h in 3 % BSA/TBST at room temperature, washed once for 5 min in TBST, and then incubated overnight with primary anti-ERS1 (1:200) or anti-ERS2 (1:1000) antibody diluted in 3 % BSA/TBST at 4 °C. Membranes were subsequently washed five times for 10 min in TBST, and then incubated for 1 h with peroxidase-conjugated goat anti-rabbit (1:50,000) secondary antibody diluted in 3 % BSA/TBST. Membranes were again washed five times for 10 min in TBST. Signals were visualized using Amersham ECL Plus Western blotting detection reagents according to the manufacturer's instructions before imaging (ChemiDocXRS VP system, Bio-Rad, USA). Values were normalized to an anti-BiP (endoplasmic reticulum immunoglobulin-binding protein) antibody (STA-818, STR, Japan), which was used as an ER-localized loading control.

III-3 Results

Ripening profiles of 'Fuji' and 'Hirosaki Fuji'

Both 'Fuji' and 'Hirosaki Fuji' produced very small amounts of ethylene at harvest. The level remained low until at least 6 d from the start of incubation at 24 °C before gradually increasing (Fig. III-1). The level was a little higher in 'Hirosaki Fuji', although it was <50 $nL \cdot g^{-1} \cdot h^{-1}$ even at 12 d. On the other hand, the firmness loss was

distinct between cultivars (Fig. III-1): 'Fuji' showed no firmness loss at least until 12 d, whereas 'Hirosaki Fuji' showed a rapid firmness loss from about 78 N (almost the same as 'Fuji') to 62 N.

MdACS1 expression was not detected (data not shown). On the other hand, differential expression of *MdACS3*, which is also responsible for ripening (Tatsuki et al. 2007), were pointed out. Although the expression of *MdACS3* in 'Fuji' remained constant during the experiment, it was high at harvest then decreased rapidly in 'Hirosaki Fuji' (Fig. III-1). *MdACO1* expression increased gradually from –14 to 9 d, and then decreased, but the intensity was clearly higher in 'Hirosaki Fuji' than in 'Fuji'. Although the expression of *MdETR1* remained constant in all samples, the expression of both *MdERS1* and *MdERS2* increased (although the pattern in 'Hirosaki Fuji' was unclear). *MdPG1* expression was faint in 'Fuji' but strong in 'Hirosaki Fuji' (Fig. III-1). In 'Fuji', MdERS1 protein was detected at day 0 then gradually decreased. In 'Hirosaki Fuji', in contrast, there was no signal by 6 d and only a slight signal at 9 and 12 d. 'Fuji' produced a large amount of MdERS2 from 0 to 12 d, whereas 'Hirosaki Fuji' produced less protein from 3 to 12 d.

Ripening profiles of other early-maturing 'Fuji' sports

In addition to 'Hirosaki Fuji', we tested three early-maturing 'Fuji' sports found independently in Japan. Their harvest dates are almost the same as that of 'Hirosaki Fuji', around the beginning of September. To determine their ripening profiles, we measured their firmness and performed Northern blot analysis of ripening-related genes (Fig. III-2). All sports decreased their flesh firmness during six days' storage at 24 °C. *MdACS3* transcripts were detected on day 0, but were faint on days 3 and 6. Although the levels of *MdETR1* transcripts were almost the same among samples, those of other ripening-related genes (*MdACO1*, *MdERS1*, *MdERS2*) were high at 3 d and 6 d in every sport. Levels of *MdPG1* transcripts were notably high at 3 d. Overall, the expression profiles of all ripening-related genes were very similar to those in 'Hirosaki Fuji'.

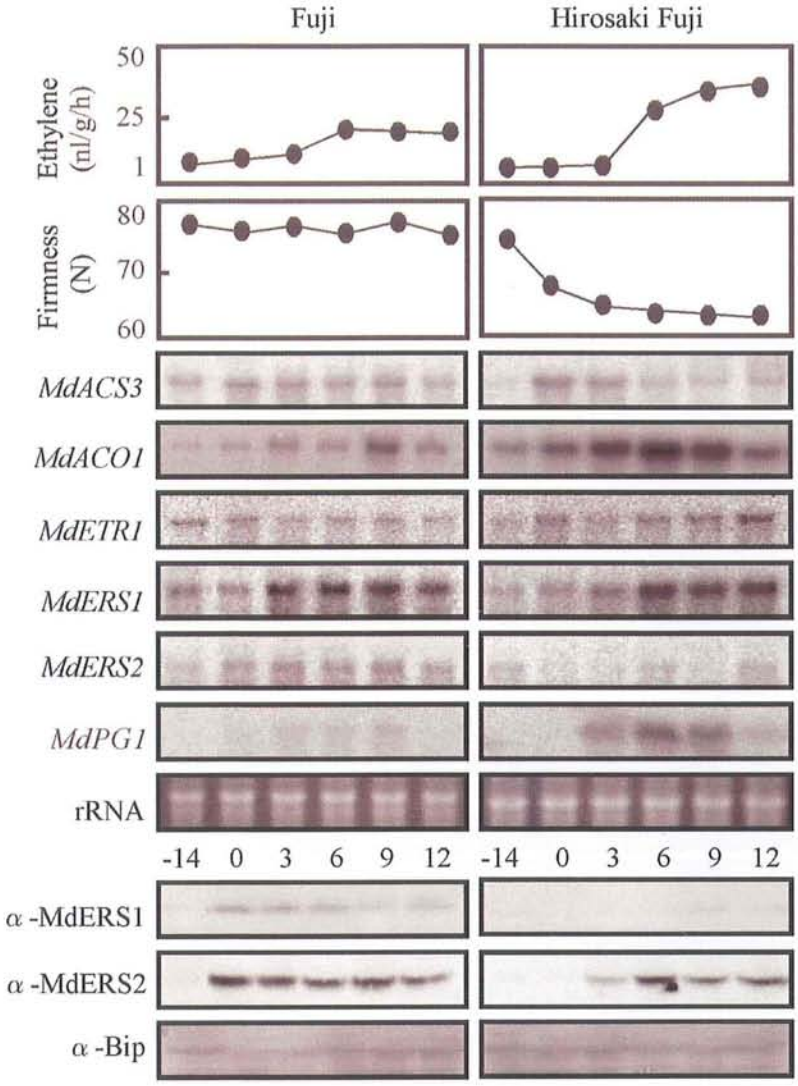

Fig. III-1 Changes in rates of ethylene production, flesh firmness, expression levels of ripening-related and ethylene receptor genes, and amounts of ethylene receptor proteins in 'Fuji' and 'Hirosaki Fuji'. Numbers indicate days after commercial harvest (day 0). "–14" means fruit at 14 d before harvest; "3"–"12" mean days of storage at 24 °C. Ribosomal RNA and BiP antibody were used as loading controls.

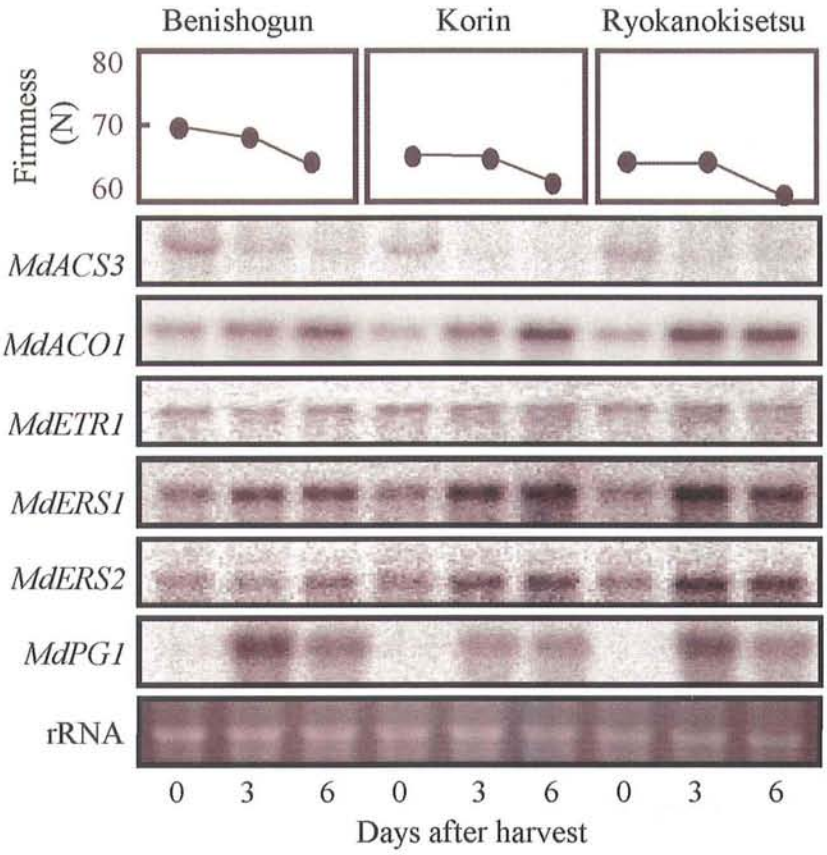

Fig. III-2 Changes in flesh firmness and expression levels of ripening-related genes in three early-maturing 'Fuji' sports. Numbers indicate days after commercial harvest (day 0). "3" and "6" mean days of storage at 24 °C. Ribosomal RNA was used as a loading control.

Expression profiles of ripening-related genes in 'Fuji' grown at different locations

We collected commercially harvested 'Fuji' fruit from two other localities in Japan with different temperature histories. The northern orchard ("NFuji", 43.0 °N) is located on Hokkaido near the northern limit for apple cultivation. The southern orchard ("SFuji", 37.8 °N) is located in the middle of Honshu near the southern limit for cultivation. Our locality, Hirosaki city (40.6 °N), lies approximately halfway between NFuji and SFuji, and the ambient temperatures of the three localities are distinct from each other (Table III-1). We analysed 'Fuji' fruit harvested at each locality to determine whether the preharvest environmental conditions affect ripening rate after harvest. 'Fuji' harvested at Hirosaki exhibited almost the same transcriptional profile as in 2006 (Figs. III-1, 3). Although the firmness decreased very little in NFuji and SFuji, the levels of the firmness were completely different from each other. Overall, it appears that signal strengths of ripening-related genes decreased in the order SFuji > Fuji > NFuji, although NFuji expressed *MdPG1* transiently at 4 d after harvest.

Table III-1. Harvest dates of samples and ambient temperatures.

Sample	Harvest date	Temperature (°C)*		
		Maximum	Minimum	Average
Fuji	Nov 4	15.6	1.8	9.4
Hirosaki Fuji	Sep 29	23.3	10.0	15.8
NFuji	Nov 8	10.0	–1.8	3.7
SFuji	Nov 18	12.9	1.2	6.7

*Temperatures of day before harvest date.

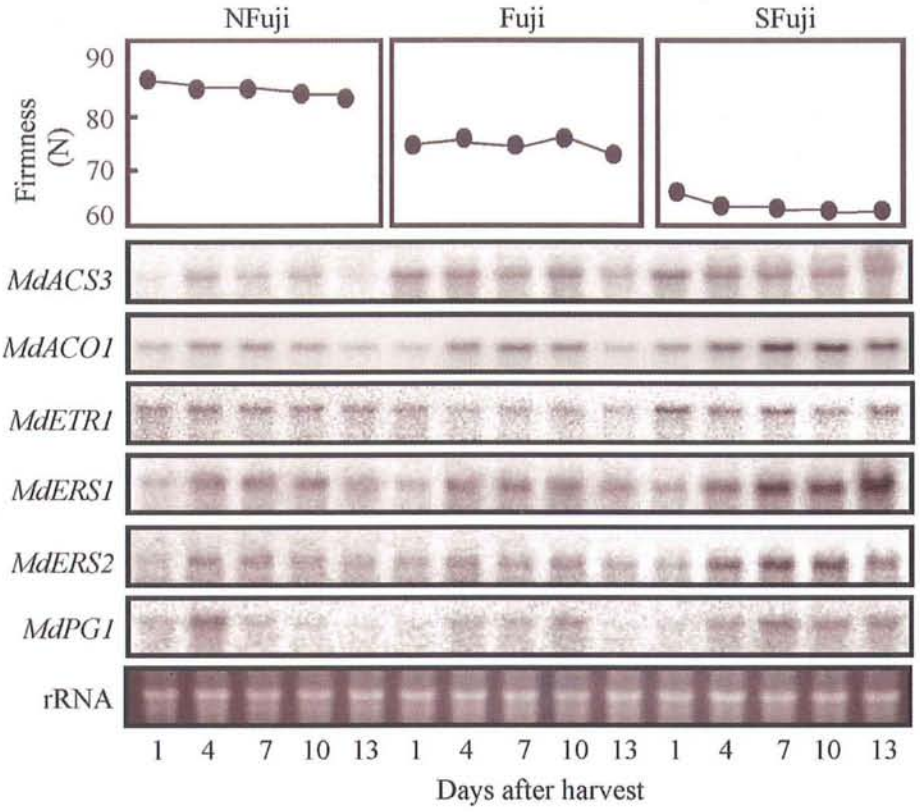

Fig. III-3 Changes in flesh firmness and expression levels of ripening-related genes in NFuji, Fuji and SFuji. Numbers indicate days after harvest. "4" to "13" mean 3 to 12 d of storage at 24 °C (fruit were in transit on day 1).

Expression of a ripening-related sHSP gene

Some small heat shock proteins (sHSPs) are involved in fruit developmental processes, including ripening (Medina-Escobar 1998, Anjanasree et al. 2005, Faurobert et al. 2007). We cloned a cDNA (accession No. EU636239) that is a homologue of a strawberry fruit ripening-related sHSP (accession No. X56138). Based upon the molecular weight of the putative HSP, we designated it *MdHSP17.5*. We analysed the levels of *MdHSP17.5* in 'Fuji' and early-maturing 'Fuji' sports by RT-PCR. 'Fuji' fruit had less *MdHSP17.5* transcript accumulation than the four early-maturing sports (Fig. III-4A). NFuji showed lower expression than SFuji (Fig. III-4B).

To determine the effect of ambient temperature on *MdHSP17.5* expression, we sampled 'Fuji' and 'Hirosaki Fuji' fruit weekly from the middle of August up to the respective harvest dates (Fig. III-5). We also investigated *MdACS3* and *MdACO1* to determine the stage of ripening. Both genes were expressed from 2 to 3 weeks before harvest, indicating that both cultivars had begun ripening. Although *MdHSP17.5* could not be detected in 'Fuji', a strong signal on Sep 11 and positive signals on Aug 21, Sep 18 and Sep 25 were observed in 'Hirosaki Fuji'.

In order to test whether the expression of *MdHSP17.5* is influenced by ethylene, we treated 'Hirosaki Fuji' apple fruit with 1-MCP and analyzed the expression of *MdHSP17.5* and ethylene receptor genes by RT-PCR or northern hybridization (Fig. III-6). The absence of *MdERS1* and *MdERS2* mRNA in 1-MCP treated samples indicated that the treatment inhibited their expression (Fig. III-6A). However, the RT-PCR analysis (Fig. III-6B) showed the treatment didn't affect the expression of *MdHSP17.5*, which means its expression is independent of ethylene.

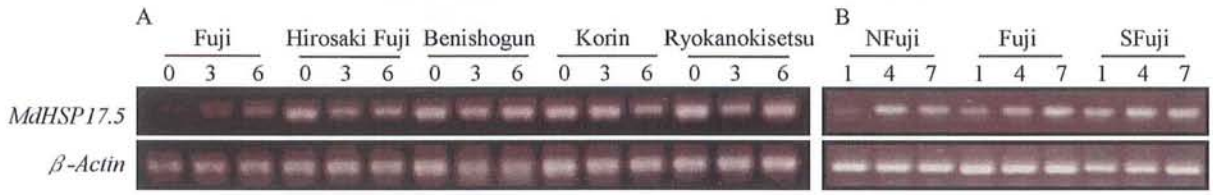

Fig. III-4 RT-PCR analysis of *MdHSP17.5* in ripening fruit of 'Fuji', early-maturing 'Fuji' sports, and 'Fuji' from different localities. Numbers indicate days after commercial harvest (day 0) as described in Figs III-2 and III-3.

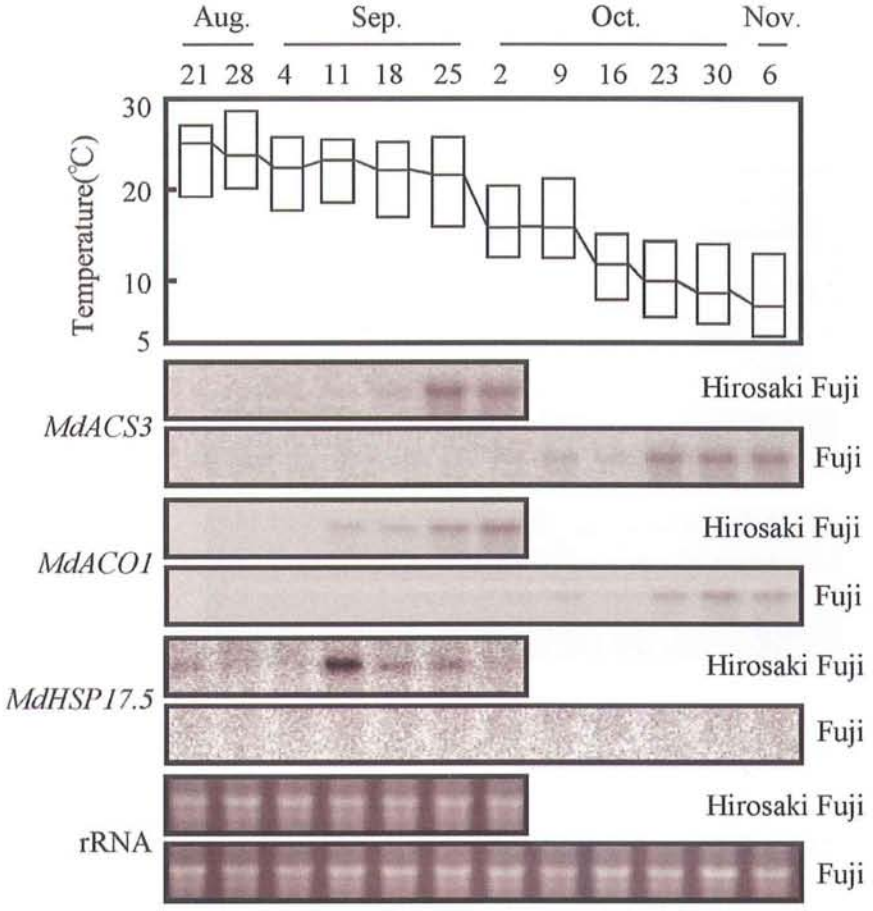

Fig. III-5 Changes in ambient temperature at Hirosaki and RNA gel blot analysis of *MdACS3*, *MdACO1* and *MdHSP17.5*. Fruit were harvested weekly from Aug 21 to Nov 6. Boxes indicate the maximum (upper edge), average (inner line) and minimum (lower edge) temperatures averaged weekly (7 d before harvest day).

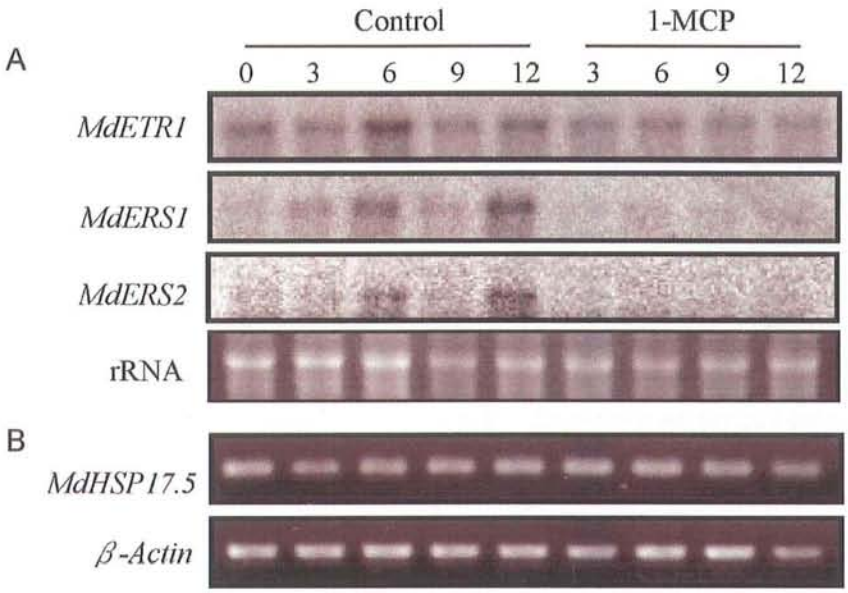

Fig. III-6 Changes in the expression of *MdHSP17.5* and ethylene receptor genes in 'Hirosaki Fuji' apple fruit with or without 1-MCP treatment. Numbers indicate days after commercial harvest (day 0) as described in Figs III-2 and III-3.

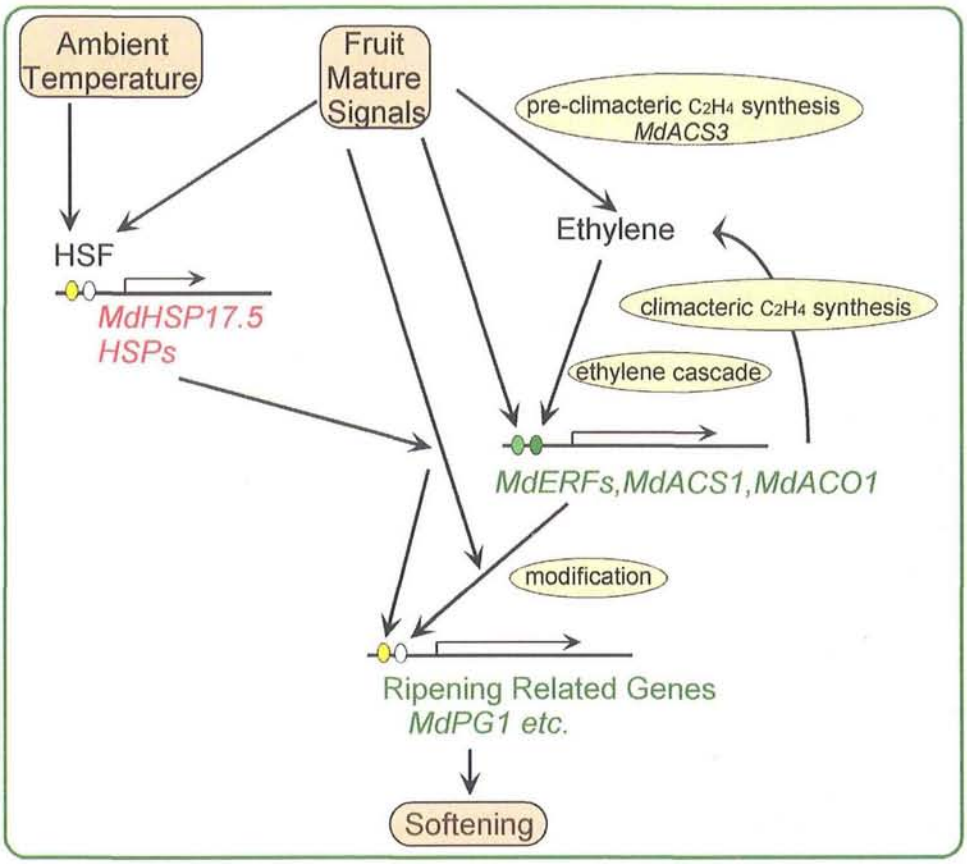

Fig. III-7 Model for signal transduction of ripening related genes in apple. Higher ambient temperature might accelerate the ripening rate via the expression of *MdHSP17.5*.

III-4 Discussion

'Hirosaki Fuji' was discovered as a bud mutation of 'Fuji' in an orchard in Hirosaki city in 1981. It matures at the end of September, about 40 d earlier than 'Fuji'. The fruit diameter of 'Hirosaki Fuji' increases faster than that of 'Fuji' throughout development, although the full-bloom dates are almost the same (Saito, unpublished data). Therefore, the mutation responsible for the early maturation is unrelated to the ripening-related genes in 'Fuji'. Yet despite the absence of a mutation in the ripening-related genes, the cultivars' expression patterns are different. There was little difference in ethylene levels during ripening, but *MdACS1* transcript was undetectable during the experiment in both cultivars. Another *ACS* gene, *MdACS3*, acts in the early stage of ripening in 'Fuji' (Tatsuki et al. 2007). Our recent work with other apple cultivars has revealed that *MdACS3* expression precedes *MdACS1* expression by at least two weeks, and then decreases because of negative control by ethylene (Harada, unpublished data). The decrease in *MdACS3* transcripts in all early-maturing sports may indicate that these fruit produce enough ethylene to regulate *MdACS3* expression negatively.

Although *MdACO1* expression was lower in 'Fuji' than in 'Hirosaki Fuji', ethylene production showed little difference between the two cultivars. This result is the same as in a report in which the expression pattern of *MdACO1* showed no clear relationship with ethylene production (Wakasa et al. 2006). Levels of *MdETR1*, *MdERS1* and *MdERS2* transcripts were similar in 'Fuji' and 'Hirosaki Fuji', yet the protein levels were completely different. The amounts of receptors (MdERS1 and MdERS2) were much lower in 'Hirosaki Fuji' than in 'Fuji'. Since ethylene receptors are negative regulators in ethylene signalling (Hua and Meyerowitz 1998), depletion leads to a progressive increase in ethylene sensitivity. The discrepancy between transcripts and proteins accumulation of receptors may be dependent upon the action of 26S proteasome (Kevany et al. 2007). Lower levels of receptor proteins in 'Hirosaki Fuji' may indicate more active ethylene cascade functions in 'Hirosaki Fuji' than in 'Fuji'. Eventually, the early-maturing sports showed a large amount of *MdPG1* mRNA, the expression of which parallels the degree of fruit flesh softening (Wakasa et al. 2006).

The *MdHSP17.5* expression profile differed between 'Fuji' and the early-maturing sports, even in fruit on the tree before harvest. This difference was also observed between NFuji and SFuji. Ambient temperatures at harvest differed greatly, from 23.3 °C ('Hirosaki Fuji') to –1.8 °C (NFuji). Storage temperature affects the postharvest life of most horticultural produce (Johnston et al. 2001; Woolf and Ferguson 2000), and temperature before storage also greatly affects ethylene biosynthesis and the softening response during ripening (Knee et al. 1983, Larrigaudiere et al. 1997). In response to heat and other stresses, plants synthesize numerous sHSPs that prevent protein aggregation and facilitate recovery from oxidative stress (Sun et al. 2002). Medina-Escobar et al. (1998) reported the expression of an *sHSP* gene in strawberry fruit during ripening. In tomato fruit, the identification of ripening-related cDNA clones through the use of EST arrays revealed that *sHSP* transcripts were greatly upregulated in ripening fruit (Anjanasree et al. 2005). Ramakrishna et al. (2003) reported that an HSP (*VIS*) plays a role in pectin depolymerization in ripening fruit.

An increase in the expression of an *sHSP* mRNA and in the production of its protein was associated with high daily flesh temperatures in apple fruit (Ferguson et al. 1998). The gene for this sHSP is probably the same as *MdHSP17.5* because of sequence homology. High levels of the transcript and products continued to accumulate even though flesh temperatures dropped at night, and fruit flesh temperatures were as much as 15 °C above ambient when fruit were exposed to direct sunlight. Therefore, the temperature history of apple fruit would have a far great influence on the ripening process. Even short-term increases in flesh temperature would be sufficient for metabolic changes to take place in the tissue. A transient strong signal on Sep 11 in 'Hirosaki Fuji' might have come from a sample collected from direct sunlight. The absence of an *MdHSP17.5* signal in 'Fuji' throughout Aug and Sep might have been due to a lower response of sHSPs in immature green 'Fuji' fruit than in mature red 'Hirosaki Fuji' fruit. Taking into account that the expression of *MdHSP17.5* is not related to ethylene, the differences in flesh firmness among fruits from the three localities must also be caused by different daily ambient temperatures.

Our results indicate that a history of high temperature robustly affects fruit quality

through HSPs. We propose that the product of *MdHSP17.5*, along with other HSPs, protects ripening-related proteins against denaturation caused by oxidative stress during ripening. Therefore, sHSPs accumulated in response to the rise in daytime temperature facilitate the ripening of 'Hirosaki Fuji'.

In conclusion, early maturing sports of 'Fuji' lose the intrinsic competence in maintaining of post harvested fruit firmness. Furthermore, distinct ripening profiles of 'Fuji' fruit from three localities are also observed. To clarify the molecular mechanism by which the cause is accounted. We analyzed the expressions of several ripening related genes including a small heat shock protein (*MdHSP17.5*) gene, which is a homologous gene to a strawberry fruit ripening-related HSP. The data obtained in this study indicate the possibility that the expression of the sHSPs in fruit on trees during ambient high temperature affects the ripening profiles of post harvest (Fig. III-7). Further studies are needed to elucidate the molecular mechanism.

III-5 Summary

Fruit of apple (*Malus×domestica* Borkh.) 'Hirosaki Fuji', a sport of 'Fuji' that matures about 40 d earlier, produced almost the same amount of ethylene as 'Fuji' during ripening, but rapidly lost flesh firmness, unlike 'Fuji', which has a long shelf life. Expression profiling of genes encoding ethylene biosynthesis enzymes (*MdACS1* and *MdACO1*), ethylene receptor proteins (*MdETR1*, *MdERS1* and *MdERS2*) and cell wall degradation enzyme (*MdPG1*) in 'Hirosaki Fuji' gave significantly different results from those of 'Fuji'. MdERS1 was more abundant during ripening in 'Fuji' than in 'Hirosaki Fuji'. Profiles of 'Fuji' fruit from two other localities with different ambient temperatures revealed that the more southerly the trees were grown, the more strongly they expressed the ripening-related genes. The gene for a small heat shock protein (*MdHSP17.5*) homologous to a strawberry fruit ripening-related HSP was expressed in 'Hirosaki Fuji' from before harvest on the tree, but was expressed in 'Fuji' only after harvest. The molecular mechanisms explaining these distinct ripening responses are discussed.

General discussion

The main factor that determines the postharvest quality of fruit crops is the rate of softening of the fruit during storage and on shelf (Knee 2000). From the farm to the market, it costs a great deal to prevent fruit softening. Although apples have a much longer shelf life than other pome fruit (Knee 1993), there is a marked difference in the storage ability among cultivars (Johnston et al. 2002). Fruit ripening is influenced by both internal and external cues, such as genetic regulation, hormone, light and temperature, but until recently, most study was limited significantly to the regulation of ethylene biosynthesis (Adams-Phillips et al. 2004). For ethylene regulation, *MdACS1* is the most studied factor so far (Oraguzie et al. 2004). However, *MdACS1* alleles can not completely reflect the ethylene production and firmness of apple fruit (Sunako et al. 1999; Oraguzie et al. 2004; Oraguzie et al. 2007). In this work, it showed a strong correlation between *MdACS3a* genotypes and ethylene production and fruit softening in apple. The wild *ACS3a* together with the loss of activity allele, *ACS3a-G289V*, and null allelic type, *acs3a*, determines the ethylene production and storage ability of apple cultivars. The result in this study has indicated that *MdACS3a* functions in the regulation of system-1 ethylene synthesis in which basal level but sufficient ethylene is synthesized to initiate system-2 ethylene synthesis which is characterized by burst of ethylene production. Therefore, enzymes involved in system-1 ethylene biosynthesis may be the key factors to determine the total ethylene production in ripening apple fruit. The softening profile of apple fruit has been divided into three phases (Johnston et al. 2002). Fruits soften slowly during the first phase (I), more rapidly during the second phase (II), and the again slowly in the final phase (III). Once the second phase softening is initiated, it is difficult to slow down. It has been clear that the rapid phase softening is associated with IEC (Johnston et al. 2001). Thus, the system-1 and -2 ethylene biosynthesis probably refers to the phase I and II fruit softening, respectively. Most likely, controlling the action of enzyme in system 1, MdACS3a, is an effective way to control the ethylene production and fruit softening. Then, it leads to a question that what induces the system-1 ethylene.

The changes of expressions of ethylene transcription factors, *MdERFs*, indicated that factor(s) independent of ethylene influence(s) apple fruit ripening. Furthermore, it is conclusive that the environment factor, ambient temperature, affects the apple fruit ripening process by inducing the expressions of *MdHSP17.5* and other *HSPs*. It is presumed that these HSPs which act as molecular chaperones protect the ripening related proteins from denaturation, although the exactness of this mechanism needs to be confirmed by extra work. Oraguzie et al. (2007) has reported that harvest date also influences the postharvest ethylene production and fruit softening in apple. The earlier the apples are harvested, the easier the fruits suffer from high ambient temperature. In this sense, harvest date (ambient temperature) most likely affects apple ripening via HSPs.

Based on these results, it can be concluded that *MdACS3a* alleles together with ambient temperature (harvest date) determine the postharvest storage ability. All the apple cultivars can be classified into different groups according to the interaction of *MdACS3a* alleles and harvest date (Fig. IV). Cultivars, which are *ACS3a-G289V/ ACS3a-G289V* or *acs3a/ACS3a-G289V* and harvested late included in the red part, have the highest storage ability; and those included in green part with *ACS3a/ACS3a* and harvested early have the worst storage ability; others are in the intermediate. Based upon this system, it is helpful for apple breeders to select the cultivars with long shelf life and subsequently supply the market with apple of high quality, which will be beneficial to decrease the influence of high-cost CA storage on apple price and increase apple growers' income.

Although many experiments have been performed on studying fruit ripening, there is still much necessary work to do in the subsequent research. Ethylene has been thought as typically necessary for fruit ripening (Adams-Phillips et al. 2004), but what initiates the biosynthesis of endogenous ethylene is still unclear. Ambient temperament most likely affects fruit ripening via the action of HSPs, whereas the mechanism by which HSPs regulate fruit development, maturation and ripening needs a lot of work to elucidate. Additionally, those factors affecting the development of apple fruit may have influence on the postharvest fruit ripening, however, little is concentrated on this area so

far. Moxon et al. (2008) recently reported that a micro RNA (miRNA) identified from tomato targets to a CTR family gene involved in fruit ripening, which opens a new avenue in the field of fleshy fruit development and ripening by rising the possibility that miRNA regulates fruit development and ripening. Studies on this field should enable researchers studying fruit ripening to identify new genetic regulators of ripening in the near future.

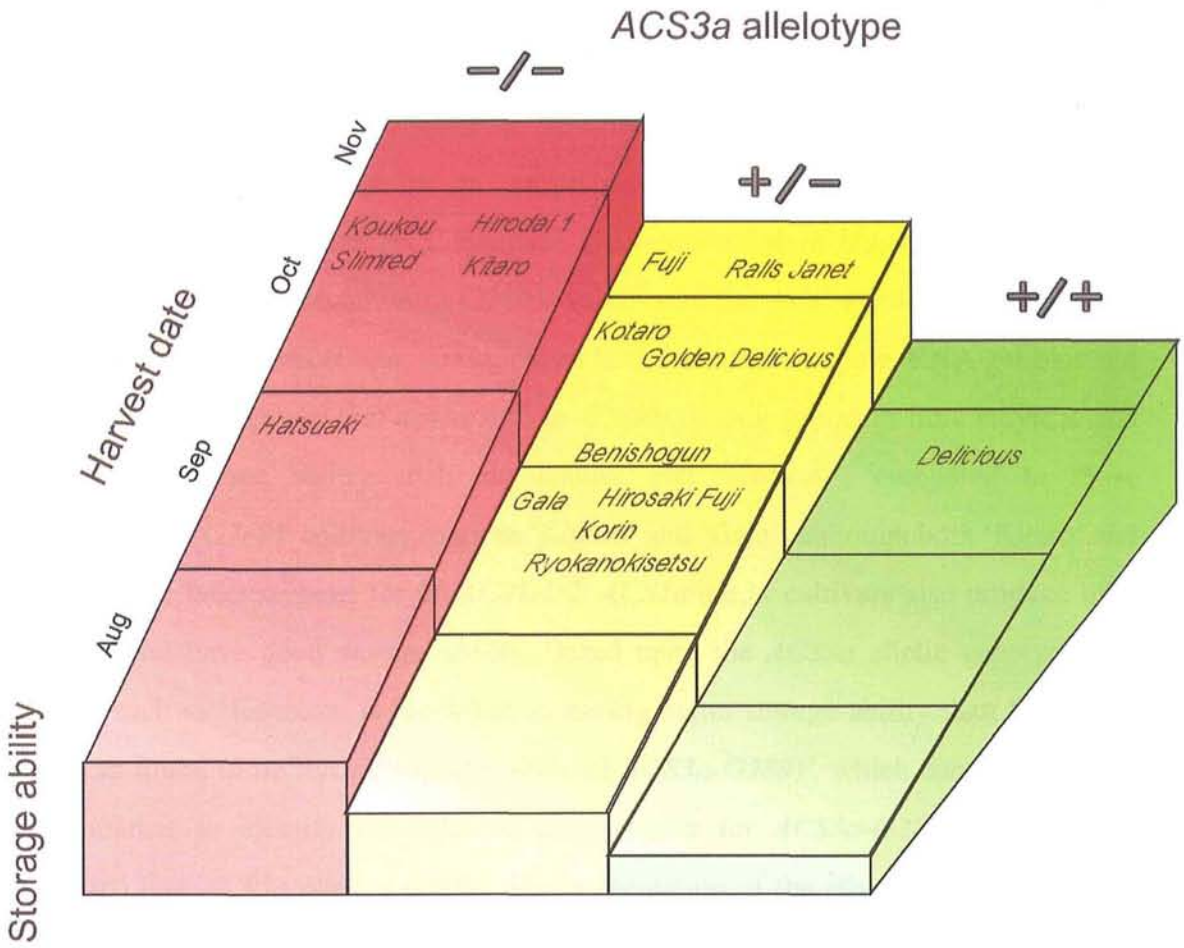

Fig. IV Schematic for apple storage ability determined by interaction of *ACS3a* allelic genotypes and harvest date. The red, green and yellow cuboids indicate the *ACS3a* allelotypes; the deep-colored and light-colored parts in each cuboid represent the late harvested and early harvested cultivars, respectively, as showed in the figure. The symbols "+" and "–" refer to the alleles with *(ACS3a)* or without (*ACS3a-G289V* or *acs3a*) ACS3a activity. The height of cuboid means the storage ability of varies of apple cultivars. A part of the cultivars were labelled in the cuboids which they belong to according to their respective harvest date and *ACS3a* allelotypes.

General summary

The apple (*Malus*×*domestic* Borkh.) 1-aminocyclopropane-1-carboxylate (ACC) synthase gene 3 (*MdACS3a*), which is expressed predominantly at the transition from system 1 to system 2 during apple ripening, derived from various cultivars was sequenced at genomic and cDNA level. The SNPs analysis revealed an *ACS3a* allele, *ACS3a-G289V*, was caused by an amino acid substitution (Gly289→Val) which occurred in the active site of ACC synthase. Over-expression of *MdACS3a-G289V* in *E. coli* indicated that the mutation of G289V inactivated the ACC synthase. On the other hand, a null gene of *MdACS3a*, *acs3a*, exists in the genome of apple. RNA gel-blot and RT-PCR analysis showed that *acs3a/ACS3a-G289V* cultivar produces little ethylene and has higher storage ability such as 'Kitaro' and 'Koukou', compared to those *ACS3a/ACS3a-G289V* cultivars, such as 'Kotaro' and 'Gala', although both 'Kitaro' and 'Koukou' are heterozygous for *MdACS1-1/2*. *ACS3a/acs3a* cultivars also produce little ethylene and have good storage ability. Based upon the *ACS3a* allelic genotype, the cultivar such as 'KouKou' is identified as having better storage ability than 'Fuji'. An SSR was found to be linked with the SNPs of *ACS3a-G289V*, which can be used as a DNA marker to identify the cultivars homozygous for *ACS3a-G289V*. Our results suggested that *ACS3a* plays a crucial role in regulation of the ethylene biosynthesis in apple fruit, and it is the main determinant of ethylene production and fruit storage ability of apple.

Two *MdERFs* (ethylene-response factors) were isolated from ripening apple (cv. Golden Delicious) fruit. The features of their conserved motifs indicated that *MdERF1* and *MdERF2* belong to group VII and group IX categorized in *Arabidopsis*, respectively. *MdERF1* was expressed predominantly in ripening fruit, although a small degree of expression was also observed in non-fruit tissues, whereas *MdERF2* was expressed exclusively in ripening fruit. The increased expression in ripening fruit was repressed by treatment with 1-methylcyclopropene (1-MCP, a potent antagonist of ethylene receptor), indicating that transcription is regulated positively by the ethylene signaling system. Indeed, it was a tendency for cultivars with low ethylene production to show lower

MdERFs expression than those with high ethylene production.

The effect of ambient temperature on the ripening process was also investigated using apple fruit of 'Fuji' and its early sport cultivars. Expression profiling of genes encoding ethylene biosynthesis enzymes (*MdACS1* and *MdACO1*), ethylene receptor proteins (*MdETR1*, *MdERS1* and *MdERS2*) and cell wall degradation enzyme (*MdPG1*) in 'Hirosaki Fuji' gave significantly different results from those of 'Fuji'. The gene for a small heat shock protein (*MdHSP17.5*) homologous to a strawberry fruit ripening-related HSP was expressed in 'Hirosaki Fuji' from before harvest on the tree, but was expressed in 'Fuji' only after harvest. The expression of *MdHSP17.5* was not affected by the treatment of 1-MCP, indicated that *MdHSP17.5* might influence the apple fruit ripening via an ethylene independent pathway. The roles of *MdACS3a*, *MdERFs* and *MdHSP17.5* in apple fruit ripening and the molecular mechanism of how ambient temperature affects apple ripening process were discussed.

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