Review

Ethylene Biosynthesis and Perception in Fruit

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The gaseous plant hormone ethylene plays an essential role in a number of developmental processes, including seed germination, seedling growth, leaf and petal abscission, fruit ripening, and senescence. 1-Aminocyclopropane-1-carboxylic acid synthase (ACS) is a key enzyme in the ethylene biosynthetic pathway, and its activity increased in response to endogenous and exogenous factors. In fruit, different ACS isogenes are induced and are responsible for differences in ethylene production among cultivars. ACS is phosphorylated in vivo, and phosphorylation confers protein stability. Ethylene is perceived by ethylene receptors, many of which have been identified and are known to be negative regulators of ethylene signaling. This review focuses on transcriptional and post-translational regulation of ACS. Furthermore, the effect of ethylene on the stability of ethylene receptors in apple is described.

Key Words: ACC synthase (ACS), ethylene receptor, protein stability, ripening, stress.

Introduction

Ethylene is a plant hormone with numerous important roles in plant development from germination to senescence. Ethylene is also involved in responses to many environmental stimuli, such as touch, wounding, pathogen attack, and flooding (reviewed by Abeles et al., 1992). Ethylene is biosynthesized by two successive reactions from S-adenosyl-L-methionine (SAM) via 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams and Yang, 1979). The first reaction is catalyzed by ACC synthase (ACS) and the second by ACC oxidase (ACO). ACS is generally considered as a rate-limiting enzyme in the biosynthetic pathway. Genes for ACS have been cloned from numerous species and their expression patterns have been studied. Several reports have shown that different ACS isogenes are expressed in response to endogenous and exogenous factors, such as pathogen infection, plant hormones, wounding, and fruit ripening (reviewed by Kende, 1993; reviewed by Zarembinski and Theologis, 1994). Recent work has led to new insights that in addition to transcriptional regulation of ACS isogenes, ACS activity is also regulated at the post-translational level. The common features of amino acids sequences among ACS isozymes include the presence of seven highly conserved regions, but the C-terminal region of ACS is divergent. Early studies reported that the C-terminal region is involved in determining ACS protein activity, but more recently the C-terminal region has been shown to be involved in determining ACS protein stability.

Ethylene action takes place via the ethylene signaling pathway. Molecular genetic studies of ethylene receptors have been performed using Arabidopsis (etr1) and tomato (Nr) mutants, which are insensitive to ethylene, and identified genes encoding a bacterial two-component histidine kinase-like receptor (Chang et al., 1993; Schaller and Bleecker, 1995; Wilkinson et al., 1995). Five and six ethylene receptors have been isolated from Arabidopsis and tomato, respectively, and each has a similar overall modular structure (reviewed by Bleecker, 1999). A large number of studies have revealed how ethylene receptors function in signaling.

1-Methylcyclopropene (1-MCP), an ethylene action inhibitor, binds to ethylene receptors more strongly than ethylene and thereby prevents ethylene-dependent responses in many plants (Sisler and Serek, 1997; reviewed by Blankenship and Dole, 2003; reviewed by Watkins, 2006). The application of 1-MCP extends the freshness of various fruits, vegetables, and flowers in which senescence is accelerated by ethylene (reviewed by Blankenship and Dole, 2003; reviewed by Watkins, 2006). Since 1-MCP binds irreversibly to ethylene receptors (reviewed by Blankenship and Dole, 2003), this chemical has been used to investigate the physiological and biochemical bases of ethylene-
mediated responses in fruits and vegetables (reviewed by Watkins, 2006). Furthermore, 1-MCP is useful for investigating the expression of genes encoding proteins that are involved in ethylene-mediated plant development, such as ripening and senescence.

In this review, the regulation of ACS genes at the transcriptional level during fruit ripening, and in response to touch and wound stresses are described. Furthermore, the post-translational regulation of ACS by phosphorylation and protein binding are described. Also, gene expression and protein accumulation of apple ethylene receptors are described.

1. Transcriptional regulation of ethylene biosynthesis during fruit ripening

Two systems are assumed to regulate ethylene production in higher plants. System 1 functions during normal vegetative growth and is responsible for producing the basal levels of ethylene detectable in all tissues, including non-climacteric fruit where ethylene inhibits its own biosynthesis. System 2 is responsible for the upsurge in ethylene production during ripening of climacteric fruit when ethylene biosynthesis is autocatalytic (Barry et al., 2000; reviewed by Inaba, 2007; reviewed by Lelièvre et al., 1997). In climacteric fruit, such as apple, tomato, and peach, increased levels of system 2 ethylene production occur simultaneously with a burst in respiration, which is required for normal fruit ripening and senescence. During fruit development in many plants, ACS isogenes are induced and responsible for ethylene production to regulate different stages of fruit development and ripening.

1) Tomato (Solanum lycopersicum)

In tomato, nine ACS isogenes have been reported and their expression patterns have been well studied (Oetiker et al., 1997; Olson et al., 1991; Rottmann et al., 1991; Shiu et al., 1998; Van der Straeten et al., 1990; Yip et al., 1992). During fruit development and ripening, pre-climacteric system 1 ethylene production is mediated by LeACS1A, LeACS3 and LeACS6 isogenes, and LeACS2 and LeACS4 are responsible for system 2 ethylene production (Barry et al., 2000; reviewed by Inaba, 2007; Nakatsuka et al., 1998). Because LeACS2 is known to be responsible for stress-inducible ethylene production (see section 2. Ethylene biosynthesis in response to external stresses), the induction of LeACS2 during ripening, which includes cell wall degradation concomitant with fruit softening, might result from mechanical damage to tomato fruit.

2) Apple (Malus domestica Borkh.)

Ethylene biosynthesis in apple fruit differs considerably among cultivars (reviewed by Abeles et al., 1992). Five apple ACS isogenes have been isolated and their expression patterns examined. MdACS1 and MdACS3 are expressed in fruit, and MdACS5A and MdACS5B are expressed in leaves (Harada et al., 2000) and fruit abscission zones (Li and Yuan, 2008; Zhu et al., 2008). MdACS1 is expressed predominantly in climacteric fruit (Dong et al., 1991; Harada et al., 2000; Sunako et al., 1999) in which the allelic genotype is responsible for differences in ethylene production among apple cultivars (Sunako et al., 1999). For example, the ‘Orin’ genotype is Md-ACS1-1/1-2, and ‘Fuji’ produces more ethylene than ‘Fuji’, whose genotype is MdACS1-2/1-2. The allelotype of MdACS1 correlates with the rate of fruit drop (Sato et al., 2004), but does not always correlate with the rate of fruit softening (Oraguzie et al., 2004). 1-MCP suppresses apple ethylene production (Fan et al., 1999a, b; Rupasinghe, 2000; Watkins et al., 2000) by inhibiting the expression of MdACS1 (Tatsuki et al., 2007, 2009; Wakasa et al., 2006). The expression of MdACS1 and MdACS3 was examined in 1-MCP-treated apple fruits of two cultivars, ‘Orin’ and ‘Fuji’, that have distinctly different storage characteristics (Tatsuki et al., 2007, 2009). The expression of MdACS1 increased after harvest in non-treated control fruit and was higher in ‘Orin’ than in ‘Fuji’, but decreased in 1-MCP-treated ‘Orin’ fruit and was suppressed for a prolonged time in 1-MCP-treated ‘Fuji’ fruit before any increase in transcription could be measured (Fig. 1). These and previous data indicate that MdACS1 is regulated by positive feedback regulation, and it seems that MdACS1 is involved in system 2 ethylene production. In an earlier study, MdACS3 was reported to be constitutively expressed in fruit (Sunako et al., 1999); however, in our study, MdACS3 was detected only in ‘Fuji’ fruit, where it decreased during storage, and MdACS3 expression was not detected in ‘Orin’ (Fig. 1). Furthermore, the
expression patterns of _MdACS3_ were not affected by 1-MCP treatment. Wiersma et al. (2007) observed that expression of _MdACS3_ increased 100-fold for 4 weeks before harvest and did not change upon ripening. Wang et al. (2009) reported that _MdACS3_ expression preceded _MdACS1_ expression by at least 2 weeks. These results indicated that expression of _MdACS3_ was not affected by ethylene, and that this gene might be responsible for system 1 ethylene production in apple fruit. Because the _MdACS1_ allele type in ‘Fuji’ accounts for the low level of ethylene production, _MdACS3_ might work in place of _MdACS1_ in the early ripening stage before _MdACS1_ expression increases.

Recently, Wang et al. (2009) proposed that not only _MdACS1_, but also the _MdACS3_ allelic genotype, determines the differences in ethylene production among apple cultivars. Their group isolated three members of the _MdACS3_ gene family (a, b, c) and showed that transcription of _MdACS3b_ and _MdACS3c_ was prevented by insertion of transposon-like sequences in the 5’ flanking region. Some apple cultivars possess a different kind of _MdACS3a_, in which the protein coding region has a single nucleotide change causing substitution of Gly with Val at the enzyme’s active site, resulting in a loss of enzymatic activity. Furthermore, a third allele, _Mdacs3a_, was found as a null allele of _MdACS3a_. The existence of three alleles of _MdACS3a_ made it possible to relate differences in ethylene production and shelf-life among apple cultivars. In our study, however, ‘Kitaro’ fruit, which possesses the _Mdacs3a/MdACS3-G289V_ genotype (Wang et al., 2009), produces a large amount of ethylene (159 nL·g⁻¹·FW·h⁻¹ in fruit at 5 days after harvest), and does not have a long shelf-life (Tatsuki et al., data not shown). Differences in ethylene production in harvested apple cultivars might be affected by the genotype combination of two ACS isogenes, _MdACS1_ and _MdACS3_, and by various environmental factors, such as harvest time.

3) Peach (Prunus persica (L.) Batsch)

Although ethylene production is closely related with the softening of peach fruit, flesh textures are affected by the presence of cell-wall-modifying enzymes. In melting-flesh peaches, rapid softening occurs after harvest, resulting in a short shelf-life. In non-melting-flesh peaches, softening is slow, and a significant reduction in flesh firmness does not occur even when the fruit is overripe. There are no significant differences in ethylene production between both peach types. The differences in softening between melting-flesh and non-melting-flesh cultivars are attributed to the presence of endo-polygalacturonase (PG) activity during ripening (Pressey and Avants, 1973); melting-flesh peaches have both endo- and exo-PG activity, whereas non-melting-flesh peaches have only exo-PG activity (Pressey and Avants, 1978).

Stony hard peaches barely soften on the tree or after harvest, although the fruit changes color normally and contains highly soluble solids (Haji et al., 2001, 2004). Genetic analysis indicated that stony hard (hd) is a recessive locus (Yoshida, 1976) and is different from the melting (M/non-melting (m) locus (Haji et al., 2005). It has been assumed that a low level of ethylene production by stony hard peach is responsible for the inhibition of fruit softening, because exogenous ethylene softens them effectively (Haji et al., 2003; Hayama et al., 2003). Since ethylene production occurs and the fruit softens by application of ACC, a precursor of ethylene, ACC oxidase activity and ethylene sensing are normal in stony hard peaches (Haji et al., 2003). For this reason, ACS is believed to be the key factor in causing stony hard peach fruit.

We examined ethylene production and expression patterns of three ACS isogenes, _PpACS1_, _PpACS2_, and _PpACS3_, in the stony hard peach cultivars ‘Yumyeong’, ‘Odoroki’ and ‘Manami’ and the melting-flesh cultivar ‘Akatsuki’. In ‘Akatsuki’, only _PpACS1_ mRNA was significantly induced after harvest, a large amount of ethylene was produced, and the expressions of _PpACS2_ and _PpACS3_ were not detected in ripening fruit. Unlike tomato and apple, only one isogene is expressed during ripening of melting peaches. In stony hard peaches, _PpACS1_ mRNA was not accumulated during the ripening stage, and ethylene production was inhibited (Fig. 2). Some stresses, such as wounding, induce the expression of ACS and ethylene production (see section 2. Ethylene biosynthesis in response to external stresses), and Mathooko et al. (2001) reported that expression of _PpACS1_ increased by wounding. In both ‘Akatsuki’, a melting flesh cultivar, and ‘Yumyeong’, a stony hard cultivar, _PpACS1_ mRNA was induced in wounded leaves (Fig. 2). Furthermore, _PpACS1_ mRNA was induced normally in senescing flowers and wounded immature fruit of ‘Yumyeong’. These results indicate that _PpACS1_ is suppressed only at the ripening stage, and lack of expression is not caused by a defect in the _PpACS1_ gene. Furthermore, suppression of fruit softening in stony hard peach cultivars is caused by a low level of ethylene production that depends on suppressed expression of _PpACS1_ (Tatsuki et al., 2006).

Although peach is a climacteric fruit, 1-MCP treatment does not maintain fruit freshness for a long period, unlike apple (Dal Cin et al., 2006; Hayama et al., 2005). In 1-MCP treated fruit, ethylene production and the expression level of _PpACS1_ is transiently higher than in non-treated fruit (Mathooko et al., 2001; Ziliotto et al., 2008). The expression of _PpACS1_ is possibly negatively regulated by ethylene. Previous reports and our results indicated that _PpACS1_ plays a role in system 2 ethylene production in peach. Because _LeACS2_ and _MdACS1_ that are responsible for system 2 ethylene production are positively regulated, the regulatory mechanism for ethylene production in peach seems somewhat different from apple and tomato.
2. Ethylene biosynthesis in response to external stresses

Ethylene is involved in regulating plant responses to both biotic and abiotic stresses. Ethylene production is induced in response to many environmental stimuli, such as touching, wounding, pathogen attack, and flooding. Wound-inducible ACS isogenes have been most thoroughly characterized in tomato (Olson et al., 1991; Rottmann et al., 1991; Shiu et al., 1998; Van der Straeten et al., 1990). LeACS2 is expressed in ripening fruit, and transcripts of LeACS2 are accumulated in a large amount in fruit within 2 h after wounding (Lincoln et al., 1993). LeACS2 is thought to be mainly responsible for the production of stress-induced ethylene because its mRNA has been detected abundantly not only in wounded fruit but also in leaves after pathogen attack (Spanu et al., 1993) or exposure to ozone (Tuomainen et al., 1997), and in flooded roots (Olson et al., 1995). Oetiker et al. (1997) showed that not only LeACS2 but also LeACS5 and LeACS6 were markedly expressed in suspension-cultured cells treated with an elicitor. Expressions of LeACS3 and LeACS7 were rapidly induced in flooded roots and in wounded leaves within 1 h after treatment (Shiu et al., 1998). Collectively, these data demonstrate that one or more ACS isogenes are responsible for ethylene production in response to a single stimulus.

We examined the expression patterns of ACS isogenes in tomato plants in response to touch and wound stresses. Tomato seedlings that were touched exhibited a sharp increase in ethylene production within 10 min, the expression of LeACS6 and LeACS1A isogenes increased within 10 min after the stimulus was applied, and mRNAs for both genes disappeared by 2 h (Fig. 3). Thus, the expression of LeACS6 and LeACS1A was transient. Wounding of tomato leaves and fruit also gave rise to transient expression of LeACS6 and LeACS1A. Transcripts of the LeACS2 gene were detected after 2 h in wounded fruit (Fig. 3), but not in touched tissues (data not shown). Ethylene release was more abundant and longer lasting in response to wounding than in response to touch, indicating that wound stress seems to require a larger amount of ethylene to launch a defense reaction. These results indicate that wounding is not essential for eliciting LeACS6 and LeACS1A mRNAs; on the contrary, LeACS2 requires more severe cell damage for expression. Transient expression patterns of LeACS1A and LeACS6 may closely regulate ethylene production, when ethylene is not needed (Tatsuki and Mori, 1999).

We also examined the wound effect on peach ACS isogene expression (Tatsuki et al., 2006). In leaves, the level of PpACS2 mRNA increased rapidly within 0.5 h and then decreased, and the amount of PpACS1 mRNA increased 1 h after wounding (Fig. 2). Expression of PpACS2 was not detected in intact tissues (flowers, leaves, immature fruit, and ripening fruit) (data not shown); thus, this gene may be responsible for stress-

Fig. 2. Ethylene production (A and B, Note different scales on the same graph) of peach fruit during storage, and expression levels of PpACS1 and PpACS2 in ‘Akatsuki’, ‘Yumyeong’, ‘Odoroki’, and ‘Manami’ fruit (C) and ‘Akatsuki’, ‘Yumyeong’ wounded leaves (D). A, B: Fruit were harvested at commercial maturity and stored at 25°C in the air. Vertical bars represent the SE (n = 4). C: Total RNA was extracted from the same sample as in A, D: For wound treatment, leaves were collected in June and were crushed with a hemostat at several sites, and total RNA was extracted from leaves harvested at the indicated times after wounding. Northern analysis was conducted as described in the legend to Figure 1.
inducible ethylene production. Because *PpACS1*, *LeACS1A*, *LeACS2*, and *LeACS6* are induced not only during fruit development and especially at ripening stages, but also by some stresses, expression is required to produce ethylene in response to endogenous and exogenous stimuli.

3. Post-translational regulation of ACC synthase

As described earlier in this review, increases in ACS activity under various conditions correlate with increased levels of ACS mRNA, as shown by Northern blot or RNase protection assays, suggesting that ACS is transcriptionally regulated (reviewed by Kende, 1993). Recent reports, however, suggest that ACS is also regulated post-translationally (reviewed by Argueso et al., 2007; reviewed by Chae and Kieber, 2005). A few early studies implied post-translational regulation of ACS. In cell suspension cultures, ACS activity was induced by stress, but was not sensitive to RNA transcription inhibitors (Chappell et al., 1984; Felix et al., 1991). In other reports, the accumulation of ACS transcripts did not correlate with ACS activity or the rate of ethylene production. This phenomenon was observed in tomato suspension cells treated with an elicitor (Oetiker et al., 1997), in etiolated seedlings of *Arabidopsis* mutants *eto1* and *eto3* that produce elevated levels of ethylene (Woeste et al., 1999), and in cytokinin-treated *Arabidopsis* etiolated seedlings (Vogel et al., 1998).

To reveal post-translational modifications occurring with ACS, the level of ACS protein was analyzed using *LeACS2*, the wound-inducible isozyme in tomato, and an anti-*LeACS2* antibody. Judging from preceding studies, one possible modification of ACS is truncation of the C-terminal region, resulting in increased enzyme activity (Li and Mattoo, 1994); however, immunoblot analysis indicated that the molecular mass of *LeACS2* was 55 kDa (Tatsuki and Mori, 2001), a value consistent with that calculated from the deduced amino acid sequence, indicating that cleavage does not occur in vivo.

Next, it was examined whether ACS is phosphorylated in vivo, because other possible mechanisms for post-translational regulation of ACS proteins include phosphorylation, resulting in a change in activity or stability. For example, in tomato suspension cells, a protein kinase inhibitor blocked elicitor-induced ACS activity whereas a protein phosphatase inhibitor induced a rapid increase in ACS activity (Spanu et al., 1994), and the same phenomenon was observed in ozone-exposed tomato leaves (Tuomainen et al., 1997). To check whether ACS phosphorylation occurs in vivo, crude extracts from wounded tomato fruit fed with [32P]Pi were immunoprecipitated. Phosphorylated *LeACS2* was detected as a 55 kDa protein (Fig. 4), and in vitro...
phosphorylation analysis indicated that phosphorylation occurred at Ser-460 in the C-terminal region. The phosphorylation of recombinant LeACS2 by a partially purified kinase fraction from tomato tissues depended on the available calcium. Furthermore, \textit{in vitro} phosphorylation analysis using other tomato isozymes, LeACS3 and LeACS4, indicated that recombinant LeACS3 was phosphorylated similarly to LeACS2, but LeACS4 was not (Fig. 4). We suggest that LeACS3 is also phosphorylated \textit{in vivo} and that isozymes possessing the amino acid sequence surrounding the LeACS2 phosphorylation site in the C-terminal region can be phosphorylated (Tatsuki and Mori, 2001).

The phosphorylation and dephosphorylation of LeACS2 regulate its stability upstream of the ubiquitin-26S-proteasome degradation pathway (Kamiyoshihara et al., 2010). Pulse-chase experiments coupled with protein kinase and phosphatase inhibitor treatments clearly demonstrated that LeACS2 was stabilized by phosphorylation and immediately degraded after dephosphorylation. The amount of LeACS2 affected by the protein kinase and phosphatase inhibitors significantly influenced cellular ACS activity, ACC content, and ethylene production levels in tomato fruit tissue, suggesting that post-translational regulation by phosphorylation plays an important role in the control of ethylene production (Kamiyoshihara et al., 2010).

Yoshida et al. (2005) reported that ACS isozymes could be classified into three types based on differences in the consensus sequences of C-termini. An \textit{Arabidopsis} ACS isozyme, AtACS6, belongs to type 1 ACS enzymes, as does LeACS2. AtACS6 phosphorylated by mitogen-activating protein kinase (MAPK), MPK6, at three Ser in the C-terminus leads to the accumulation of ACS protein, elevated ACS activity, and ethylene production (Liu and Zhang, 2004). These results suggest a possible model for the regulation of type 1 ACS enzymes. ACS is phosphorylated by CDPK or/and MAPK at the C-terminal region in response to an external stress, thereby stabilizing the protein and preventing degradation via the proteasome pathway (Joo et al., 2008; Kamiyoshihara et al., 2010).

Type 2 ACS enzymes are also regulated post-translationally through a mechanism probably different from that of type 1 ACS enzymes. Study of the \textit{Arabidopsis} mutant, \textit{eto1-3}, supports the idea that ACS protein stability is regulated post-translationally, but via another mechanism. \textit{eto2} and \textit{eto3} are dominant ethylene-overproducing mutants (Vogel et al., 1998), and have mutations in the C-terminal region of two type 2 isozymes, AtACS5 and AtACS9 (Chae et al., 2003). The \textit{eto1} is a recessive ethylene-overproducing mutant (Guzman and Ecker, 1990), and the ETO1 protein is a member of the broad complex/tramtrack/bric-a-brac (BTB) protein subfamily that participates in substrate recognition via the ubiquitin-26S-proteasome system. ETO1 directly binds to the C-terminus of type 2 ACS isozyme in \textit{Arabidopsis}, AtACS5, and interacts with cullin-3, a component of ubiquitin E3 ligase, leading to AtACS5 degradation via the ubiquitin-26S-proteasome pathway (Wang et al., 2004).

Type 3 ACS proteins, such as tomato LeACS4, have a very short C-terminus and no predicted kinase phosphorylation site, and the putative mechanism for

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**Fig. 4.** Detection of \(^{32}\text{P}\)-labeled LeACS2 from wounded fruit (A, B) and \textit{in vitro} phosphorylation of ACS isozymes (C, D). A: radiographic image of \(^{32}\text{P}\)-labeled LeACS2 by \textit{in vivo} phosphorylation. Sliced tomato fruit was incubated for 3 h as described above and then fed with 1 mCi of \([^{32}\text{P}]\text{Pi}\) for 2 h. Radiolabeled proteins were extracted. Total proteins (lane 1), immunoprecipitation with preimmune IgG (lane 2), with the anti-LeACS2 antibody (lane 3), and with the anti-LE-ACS2 antibody and recombinant LeACS2 (10 \(\mu\)g) as a competitor (lane 4). B: Western blot analysis of LeACS2 in total protein (same sample as in A, lane 1). Protein (15 \(\mu\)g/lane) was separated using SDS-PAGE, blotted onto a nitrocellulose membrane, and detected with the anti-LeACS2 antibody. Molecular mass markers are indicated at the left of the lane. Arrows indicate 55 kDa. C: Purified recombinant LeACS2 (lane 1), LeACS3 (lane 2), and LeACS4 (lane 3), expressed in \textit{E. coli}, were incubated with a protein kinase fraction in the presence of \([\gamma-^{32}\text{P}]\text{ATP}\) for 10 min. The reaction was stopped by adding SDS sample buffer, and proteins were separated using SDS-PAGE. C: CBB staining; D: radiographic image. This research was originally published in the Journal of Biological Chemistry by Tatsuki, M. and H. Mori (2001).
post-translational regulation of type 3 ACS enzymes has not been reported yet.

4. Ethylene receptors in apple

Ethylene is perceived by ethylene receptors that are similar to bacterial two-component histidine kinase receptors (reviewed by Bleecker, 1999). Ethylene receptor genes have been classified into two subfamilies (Hua et al., 1998). Subfamily I has three transmembrane domains in the amino-terminal region, and a well-conserved histidine kinase domain in the carboxy-terminal region. Subfamily II has four membrane-spanning domains and a degenerate histidine kinase domain that lacks one or more of the conserved amino acids that are believed to be necessary for catalytic activity, but the importance of histidine kinase activity in ethylene signaling is still unclear. Genetic mutants with a reduced number of ethylene receptors are more sensitive to ethylene (Cancel and Larsen, 2002; Hall and Bleecker, 2003; Hua and Meyerowitz, 1998; Tieman et al., 2000), and vice versa (Ciardi et al., 2000), indicating that ethylene receptors act as negative regulators of the ethylene response, and that ethylene binding inactivates them.

Ethylene receptor genes have been identified in many plant species, and their expression patterns have been examined. Expression is generally regulated depending on tissues, developmental stage, and environmental conditions.

![Graph A](image1)

**Fig. 5.** Levels of *MdERS1* expression (A), *MdERS1* protein (B) and ratio of *MdERS1* protein to mRNA in fruit of apple cultivars ‘Orin’ and ‘Fuji’ (C). A: Total RNA was extracted from fruit that had been treated with (open circle) or without (closed circle) 1-MCP. For 1-MCP treatment, apples at commercial harvest stages were placed in 17-L plastic containers and treated with 1 \( \mu \text{L} \cdot \text{L}^{-1} \) 1-MCP for 12 h at 22°C. The steady-state levels of transcripts were normalized to an *actin* transcript. Data are the mean values ± SD of three individual experiments (n = 3). B: The microsomal membrane fraction was isolated from the samples in A, and 60 \( \mu \text{g} \) protein was separated by SDS-PAGE and blotted. Anti-Bip antibody was used as an ER-localized protein loading control. Numbers beneath columns represent the levels of *MdERS1* relative to the Bip loading control. Protein levels were normalized relative to the highest level in ‘Orin’ and ‘Fuji’ panels set at 1.00. C: ‘Orin’ (open column) and ‘Fuji’ (closed column).
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stimuli (reviewed by Chen et al., 2005). Some ethylene receptor genes are expressed in ethylene-producing tissues, such as ripening fruit, and are upregulated by ethylene. Six genes for apple ethylene receptors (MdETR1, MdETR1b, MdETR2, MdETR5, MdERS1 and MdERS2) have previously been identified (Dal Cin et al., 2005; Tatsuki and Endo, 2006; Wiersma et al., 2007). The amino acid sequence of MdETR1 is 94% identical to that of MdETR1b, which is similar to pear PcETR1a. MdETR1, MdETR1b, MdERS1, and MdERS2 belong to subfamily I; MdETR2 and MdETR5 are subfamily II receptors. Their expression patterns have been examined during fruit abscission, fruit ripening, and storage (Dal Cin et al., 2005, 2006; Tatsuki and Endo, 2006; Tatsuki et al., 2007; Wiersma et al., 2007).

The mRNA and protein accumulation of apple ethylene receptors were characterized with 1-MCP-treated and/or ethylene-treated ‘Orin’ and ‘Fuji’ fruit and leaves. MdETR1, MdERS1, and MdERS2 were expressed in ripening fruit, and levels of MdERS1 and MdERS2 transcripts increased after harvest, but their

increase was delayed in 1-MCP-treated fruit (Tatsuki and Endo, 2006; Tatsuki et al., 2007, 2009). Transcription of MdERS1 and MdERS2 was upregulated by ethylene treatment of apple leaves and fruit (Tatsuki and Endo, 2006; Tatsuki et al., 2009). Next, MdERS1 and MdERS2 proteins were examined by immunoblot analysis. MdERS1 decreased gradually in both control and 1-MCP treatments. MdERS2, however, increased gradually in untreated ‘Fuji’ fruit and remained steady in 1-MCP-treated ‘Fuji’ but stayed low in ‘Orin’ (Figs. 5 and 6). The ratio of MdERS1 protein to mRNA in 1-MCP-treated fruit was higher than in the control (Fig. 5). The MdERS2 protein to mRNA ratio was also higher in 1-MCP-treated fruit than in the control within 30 days after harvest (DAH) (Fig. 6). These results, except for the anomalous case of MdERS2 in ‘Fuji’ control fruit at 60 and 90 DAH, suggest that MdERS1 and MdERS2 are more stable in 1-MCP-treated fruit than in untreated fruit, and that stability of MdERS1 and MdERS2 proteins was affected either in the absence or presence of ethylene.

Next, the effect of high concentrations of ethylene on

Fig. 6. Levels of MdERS2 expression (A), MdERS2 protein (B) and the ratio of MdERS2 protein to mRNA in fruit from cultivars ‘Orin’ and ‘Fuji’ (C). Other details are described in Figure 5.
ethylene receptors was examined. Exogenous ethylene treatment of fruit increased \textit{MdERS1} and \textit{MdERS2} expression, but slightly decreased protein levels. The ratios of proteins to mRNAs decreased with ethylene treatment in both cultivars. Similar results were obtained in ethylene-treated leaves. Considering these results, it is likely that \textit{MdERS1} and \textit{MdERS2} are more stable in 1-MCP-treated fruit and leaves, and turnover of these ethylene receptors is probably regulated by ethylene binding, a result consistent with what has been observed in \textit{Arabidopsis} and tomato ethylene receptors (Chen et al., 2007; Kevany et al., 2007). For example, \textit{Arabidopsis} ETR2 is induced at low ethylene concentrations, but is suppressed at high ethylene concentrations (>1 \textmu L·L\(^{-1}\)). Genetic and chemical analyses indicate that ethylene perception inhibits the decrease of ETR2 protein levels. Furthermore, decreases in ETR2 are affected by proteasome inhibitors. Thus, ETR2 is degraded by a 26S-proteasome-dependent pathway in response to ethylene binding (Chen et al., 2007). Tomato ethylene receptors NR, LeETR4, and LeETR6, whose transcripts increase during ripening and are upregulated by ethylene, are degraded after ethylene treatment (Kevany et al., 2007).

The presence of particular alleles of \textit{MdACS1} and \textit{MdACS3} can explain some of the differences in apple fruit ethylene production (see section 1.2 Transcriptional regulation of ethylene biosynthesis during fruit ripening-apple). Differences in ethylene sensitivity or fruit shelf-life among apple cultivars may be correlated with differences in ethylene receptor expression patterns (Wiersma et al., 2007) or protein stability (Tatsuki et al., 2009). For example, cultivar ‘Sunrise’ produces large amounts of ethylene after harvest and has a short shelf-life, and the expression of \textit{MdERS1} is low. On the other hand, \textit{MdERS1} expression is high in ripening ‘Golden Delicious’ fruit that has a longer shelf-life than ‘Sunrise’. Thus, low expression levels of \textit{MdERS1} appear to be correlated with a shorter fruit shelf-life. We quantified both the expression of \textit{MdACS1} and \textit{MdACS2}, and the stability of their products in the presence of ethylene. The ratios of \textit{MdERS1} and \textit{MdERS2} proteins to mRNAs in cultivar ‘Orin’ were lower in harvested fruit and in exogenous ethylene-treated fruit, and the percent reduction was higher in ethylene-treated leaves of ‘Orin’, indicating that ethylene receptor stability in the presence of ethylene differs between the two cultivars, with lower stability in ‘Orin’ than in ‘Fuji’ (Tatsuki et al., 2009).

**Conclusions**

ACS is the key rate-limiting enzyme for ethylene biosynthesis and is regulated transcriptionally and post-transcriptionally. Expressions of ACS isogenes are differentially regulated in response to endogenous and exogenous factors. In apple, differences in ethylene production among cultivars may be caused by the allelotype of ACS isogenes. Ethylene biosynthesis in Japanese pear and pear fruits also differs considerably among cultivars, and the differences are caused by the induction of different types of ACS isogenes during ripening (Itai et al., 1999; El-sharkawy et al., 2004). Furthermore, the inhibition of fruit softening of stony hard peach cultivars is caused by suppression of \textit{PpACS1} during ripening. Thus, differences in ethylene production among cultivars are determined by the expression levels of different members of the ACS gene family. When plants perceive biotic and abiotic stresses, rapid or/and large amounts of ethylene production are required. Subsequently, the expression of some ACS isogenes is induced rapidly and ethylene is produced, which may play a role in the stress signaling pathway. For example, ethylene production occurs within 10 min of touch stimuli, which is responsible for the rapid induction of ACS isogene expression. Thus, induction of ethylene production is mainly regulated at the transcriptional level for ACS. On the other hand, when ethylene production is not needed, e.g. some stresses have been removed from the plant, ethylene production must be reduced rapidly to prevent additional effects resulting from excessive ethylene. Post-translational regulation of ACS, namely the ACS protein degradation system, seems to be required for fast inactivation of ACS activity and degradation of ACS protein.

Type 3 ACS isozymes have no identified regulatory sequences in the C-terminus. Tomato \textit{LeACS4}, a type 3 ACS isogene, is expressed only in ripening fruit and is assumed to be a ripening-related isogene. Ethylene also affects sex determination in plants. Recently, Boualem et al. (2008, 2009) and Li et al. (2009) reported that mutation of a melon and cucumber ACS isogene (\textit{CmACS-7} and \textit{CsACS2}, respectively) resulted in the transition from monoecy to andromonoecy. Thus, \textit{CmACS-7} (Boualem et al., 2008) and \textit{CsACS2} (Boualem et al., 2009; Li et al., 2009) repress stamen development in female flowers. \textit{CmACS-7} and \textit{CsACS2} are also members of the type 3 ACS isozyme subfamily. These results imply that ACS isozymes related to plant programmed development (such as sex determination and fruit ripening) are included in the type 3 isozyme subfamily and do not need a rapid protein inactivation system for their function.

Details about the mechanisms for ethylene perception and signaling have been revealed mainly using \textit{Arabidopsis} and tomato. In fruit trees such as apple and peach, there are many different cultivars with different levels of ethylene production and shelf-life. Differences in apple ethylene production are mainly due to two ACS isozyme allelotypes. It is assumed that ethylene sensitivity and shelf-life are affected by differences in ethylene receptors; however, to understand the correlation between ethylene sensitivity and ethylene receptor proteins, all members of the apple ethylene receptor gene family have to be analyzed. To date, only subfamily I ethylene receptors in apple have been examined. Because
reduced expression of a single gene, tomato LeETR4 (subfamily II), by the antisense method accelerates the onset of ripening in transgenic tomato fruit (Tiemann et al., 2000), and thus subfamily II is thought to have a critical role in the ethylene signaling pathway, subfamily II ethylene receptors in apple should be examined.

Recent work showed that some ethylene receptors are inactivated and degraded by proteolysis as a result of ethylene binding, but protein levels of other receptors are not regulated by ethylene binding (Chen et al., 2007). There may be uncharacterized regulatory systems for the inactivation of ethylene receptors. Now peach ethylene receptors are being examined at the transcript and protein levels in 1-MCP treated fruit, and it seems that peach ethylene receptor proteins are regulated in a different manner from those found in apple. Further work is needed to elucidate the molecular mechanisms governing the regulation of peach ethylene receptors.

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