Review

Studies on the Internal Feedback Regulation of Ethylene Biosynthesis and Signal Transduction during Fruit Ripening, and the Improvement of Fruit Quality

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A brief account of research carried out in our laboratory over the past decade on the postharvest ripening of several fruits is given. Physiological and molecular biological studies reviewed include those relating to ethylene biosynthesis and flesh softening during fruit ripening. Work relating to the development of postharvest treatments and handling procedures aimed at maintaining the quality and extending the shelf-life of ‘Tonewase’ Japanese persimmon fruit during distribution is also summarized.

Key Words: ethylene biosynthesis, flesh softening, fruit ripening, gene expression, 1-methylcyclopropene.

Introduction

Fruits can be classified as climacteric and non-climacteric depending on the presence or absence of massive ethylene production during ripening and on their response to exogenous ethylene. Postharvest handling techniques of fruits belonging to the two classes are basically different. Since Burg and Burg (1962) demonstrated the production of ethylene from fruit using a gas-chromatograph, our knowledge of the roles of ethylene in fruit ripening has progressed greatly. The discovery of 1-aminocyclopropane-1-carboxylic acid (ACC) as a precursor of ethylene by Adams and Yang (1979) provided the impetus for the subsequent research on this important plant hormone. Just after this discovery, the ACC-formation enzyme, ACC synthase (ACS), was identified from tomato fruit (Boller et al., 1979). The conversion of ACC to ethylene by the enzyme, ACC oxidase (ACO), had been determined in vivo as the ethylene-forming enzyme (EFE) because the activity was undetectable in cell-free systems. Later, it was identified in homogenates of melon fruits in the presence of iron and ascorbate (Ververidis and Jhon, 1991). With the advancement of molecular biology, many members of ACS and ACO gene families have been isolated and characterized in various plant organs. Furthermore, based on the mutant analysis of Arabidopsis, the ethylene signaling pathway has been established as follows: ETR1→CTR1→EIN2→EIN3→ERF (Brummel, 2005; Klee, 2004).

On the other hand, it is well known that ethylene biosynthesis is subjected to both positive and negative feedback regulation (Kende, 1993). Positive feedback regulation is a characteristic feature of ripening fruits and senescing flowers. Negative feedback regulation occurs mostly in instances of auxin- or stress-induced ethylene production in various plant organs. Thus, there are two types of high ethylene production regulated in opposite feedback directions. In the analysis of feedback regulation on ethylene production or ethylene-dependency of gene expression, 1-methylcyclopropene (1-MCP) has been used as a powerful tool. 1-MCP has been identified as a highly potent inhibitor of ethylene action (Sisler and Serek, 1997) that is thought to bind irreversibly to, and thus inactivate, ethylene signaling. In this article, our recent research related to fruit ripening is summarized in a review style focused on ethylene biosynthesis and fruit softening.

1. Transition from system-1 to system-2 ethylene production in tomato fruit

Even in climacteric fruit, ethylene production is generally very low until the commencement of ripening. At the onset of ripening, fruits exhibit a climacteric increase in respiration, with a concomitant burst of ethylene production. Based on the level of ethylene production during fruit development, McMurchie et al. (1972) introduced the concept of system-1 and system-2 ethylene. System-1 is the low basal rate of ethylene production in pre-climacteric fruits or vegetative tissues,
and system-2 is the high rate of ethylene production observed during ripening in climacteric fruits and in certain senescent flowers. It has been thought for a long time that the rate of ethylene production in system-1 gradually increases in developing fruit up to the stage immediately before the onset of ripening. This increased system-1 ethylene could induce system-2 ethylene through a positive feedback mechanism. We identified an ACS gene related to the production of system-1 ethylene and a possible transition mechanism of system-1 to system-2 ethylene production in tomato fruit.

In tomato fruit, ethylene production during the climacteric stage has been demonstrated to be due to the accumulation of two ACS transcripts, LE-ACS2 and LE-ACS4 (Lincoln et al., 1993; Rottmann et al., 1991), and one ACO gene, LE-ACO1 (Barry et al., 1996). We reconfirmed these findings with some additional results by the use of 1-MCP (Nakatsuka et al., 1998). Increase in ethylene production during ripening was prevented to a large extent by treatment with 1-MCP at both the turning or pink stages followed by a slow recovery (Fig. 1). In these fruits, characteristic expression patterns were observed in the members of gene families for ACS and ACO (Fig. 1). The abundance of LE-ACS2 and LE-ACS4 mRNA in the fruit increased beginning at the turning stage, and 1-MCP inhibited this increase. In mature green fruit, transcripts of these genes have been shown to be inducible by treatment with ethylene (Lincoln et al., 1993). In our other study using rin and nor mutant tomatoes with ethylene treatment, expression of the LE-ACS4 gene requires both RIN and NOR, but the accumulation of LE-ACS2 mRNA requires only NOR (Yokotani et al., 2004). These findings together with our data strongly suggest that LE-ACS2 and LE-ACS4 are the dominant genes involved in ripening-ethylene production in tomato fruit, and their expression is regulated in a positive feedback manner. Among the ACO gene family members, LE-ACO1 and LE-ACO4 were the genes involved in ripening-ethylene, and their expression during ripening was prevented to a large extent by 1-MCP treatment (Fig. 1). Therefore, the expression of these two ACO genes is also regulated by a positive feedback mechanism. In contrast, the LE-ACS6 gene was expressed in the fruit from immature green to mature green stages, whereas the expression of this gene was inhibited in the ripening fruit (Fig. 1). This inhibition of gene expression was recovered in fruit treated with 1-MCP. These findings strongly suggest that expression of the LE-ACS6 gene is regulated by a negative feedback mechanism. This concept was clearly demonstrated in immature green fruit, in which the previously detected signals for the LE-ACS6 gene were inhibited by treatment with propylene, an ethylene analog. From the above observations, we postulated that, in tomato fruit, preclimacteric system-1 ethylene production is mediated via the constitutive expression of LE-ACS1A and LE-ACS3 and negative feedback-regulated LE-ACS6 genes with preexisting LE-ACO1 and LE-ACO4 mRNAs. At the onset of the climacteric stage, it shifts to system-2 ethylene production with a large accumulation of LE-ACS2, LE-ACS4, LE-ACO1, and LE-ACO4 mRNAs as a result of positive feedback regulation.

To clarify this postulation, we produced a transgenic tomato with a suppressed LeEIL gene by RNA interference engineering. It has been established that the ethylene response is initially carried out through binding to its receptor, ETR or ERS, localized on the endoplasmic reticulum membrane, and EIN3/EIL families of transcription factors in the nucleus may be located at the downstream position of the signaling. In the tomato, three homologs of the EIN3 gene, LeEIL, were identified, and shown to functionally complement the Arabidopsis ein3 mutation (Tieman et al., 2001). In addition, we cloned a fourth tomato EIL gene, LeEIL4, exhibiting ripening-associated expression (Yokotani et al., 2003). The obtained transgenic tomato showed almost the complete suppression of all LeEIL genes, especially in fruit exhibiting an ethylene-insensitive phenotype, such as no fruit ripening, no triple response of seedlings even under exogenous ethylene, no root formation from cut shoots, and a delay in petal fall after flowering (Fig. 2). Interestingly, despite the fact that no fruit ripening was observed in terms of the absence of red color appearance and flesh softening, transgenic fruit exhibited a low but consistent increase in ethylene production in parallel with the commencement of climacteric ethylene in wild-type fruit (unpublished data). This low level of ethylene in transgenic fruit was derived from a significant but lower increase in the accumulation of LE-ACS2 and
LE-ACS4 transcripts. This limited increase in ethylene production and accumulation of the two ACS mRNAs in transgenic fruit was not affected by treatment with 1-MCP. Furthermore, 1-MCP-treated wild-type fruit showed a similar pattern of ethylene production and ACS gene expression to those of transgenic fruit without climacteric ethylene production. The fact that the pattern and level of ethylene production in 1-MCP-treated transgenic fruit are almost identical to those in non-treated transgenic fruit and 1-MCP-treated wild-type fruit suggests that ethylene production detected in the transgenic fruit is independent of the positive feedback regulation of ethylene. Therefore, in tomato fruit, system-1 ethylene is developmentally programmed to increase gradually at the onset of ripening independent of any feedback regulation. This increased level of ethylene could induce massive system-2 ethylene production through a positive feedback mechanism, which in turn commences fruit ripening.

2. Feedback regulation system of ethylene biosynthesis in banana fruit

The banana has been widely accepted as a typical climacteric fruit because of its sensitivity to exogenously applied ethylene, which rapidly induces its ripening. The banana also has several unique characteristics compared with other climacteric fruits such as a sharp peak of ethylene production at the onset of ripening (Burg and Burg, 1962, 1965) and high contents of soluble tannins and starch at the pre-climacteric stage. Furthermore, the banana is a monocot and is different from most fruits which are dicots. We clarified the mechanism of the sharp peak of ethylene production at the early climacteric rise first and then showed the differential feedback regulation of ethylene biosynthesis between pulp and peel tissues (Inaba et al., 2007; Liu et al., 1999).

When we started our study, there was no report at that time describing the expression of ACS genes. Therefore, we cloned three genes for ACS and two for ACO from banana fruit. When banana fruit ripened naturally, ethylene production commenced on day-11 and immediately thereafter increased greatly, followed by a rapid decrease. Thus, the sharp peak of ethylene production in banana fruit was detectable only for one-half day (Fig. 3A). A similar sharp peak to that in ethylene production was observed in vivo ACO activity. In northern analysis, MA-ACSI mRNA increased from the onset of the climacteric stage and reached the highest level on day-11, followed by a slight decline (Fig. 3B). The MA-ACO1 gene was already expressed in pre-climacteric fruit and the level of its mRNA increased in ripening fruit. These expression patterns of the genes related to ethylene biosynthesis are not different from those observed in various climacteric fruits. However, changes in ACO activity determined in vivo were not consistent with the abundance of MA-ACO1 mRNA in ripening fruit. The in vivo determination supposedly reflects the in situ condition.
Indeed, ACO activity determined in vitro increased steadily until the fully ripe stage, similar to that observed in other climacteric fruits. As a result, the sudden decrease in the in vivo ACO activity was attributed to the limited availability of its co-factors, especially iron contents, resulting in a sharp peak in ethylene production at the onset of the climacteric stage in banana fruit (Liu et al., 1999).

To further examine ripening characteristics, we developed an extraction method for ACS from banana fruit (Liu et al., 2000) since it had been impossible to determine its activity, probably due to the high concentration of tannins. In this process, Golding et al. (1998) showed interesting features of ethylene production in banana fruit, revealing that 1-MCP eliminated the ripening-induction effect of propylene at the pre-climacteric stage, whereas it had no effect on ethylene production once ripening had commenced. We re-confirmed this finding with further evidence that 1-MCP did not inhibit ethylene production but rather had a stimulatory effect when applied after the onset of ripening. These results suggested that ethylene biosynthesis in banana fruit is regulated in a positive feedback manner at least during the pre-climacteric period, but is either independent from ethylene or is under a negative feedback regulation mechanism at the ripening stage. To confirm this hypothesis, we treated pre-climacteric fruit with different concentrations of propylene and found that higher concentrations had a stronger suppressive effect on ethylene production than lower concentrations despite their earlier ethylene-inducing effect. This induced ethylene may be derived from the pulp because peel tissues did not produce ethylene until day-2 after propylene treatment. Thus, negative feedback regulation may be involved in ripening-ethylene production in banana fruit. A similar proposal based on the suppression of endogenous ethylene production by exogenous ethylene has previously been shown in banana fruit (Domínguez and Vendrell, 1994; McMurchie et al., 1972; Vendrell and MacGlasson, 1971). These observations may introduce a new concept that, in banana fruit, exogenous ethylene does not induce endogenous ethylene production but shifts the physiological condition from the immature stage to ripening stage, and the shift induces endogenous ethylene production which seems to be under a negative feedback regulatory mechanism.

Thus, although it has been thought that the banana is a typical climacteric fruit, its ripening characteristics are different from conventional climacteric fruits. To understand this complex regulation system, we separated the fruit into pulp and peel tissues because banana fruit has a thick peel tissue. Interestingly, 1-MCP applied to banana fruit after the onset of ripening enhanced ethylene production greatly in the pulp while suppressed it almost completely in the peel, suggesting the existence of a different feedback regulatory mechanism of ethylene biosynthesis between these fruit tissues. This was supported by the activity of ethylene biosynthesis and related gene expression in both tissues. In pulp tissue, ACS activity was enhanced and the expression of MA-ACS1 and MA-ACO1 genes was not affected by 1-MCP treatment (Fig. 4). In contrast, both ACS and ACO activities and the accumulation of MA-ACS1 and MA-ACO1 mRNAs in the peel were greatly suppressed by 1-MCP (Fig. 5). Furthermore, a different regulation mechanism between pulp and peel tissues was involved in the expression of cell wall-modifying enzyme genes such as pectate lyase and expansin (Inaba et al., 2007).

3. Clarification of mechanism and development of protection technique for rapid fruit softening during distribution in ‘Tonewase’ Japanese persimmon

‘Tonewase’ is a bud sport of ‘Hiratanenashi’ whose

![Graph of ethylene production rates and expression of genes in banana fruit during ripening.](image-url)
fruit matures about 2 weeks earlier than ‘Hiratanenashi’. Its production ranks 3rd among Japanese persimmon cultivated in Japan, and more than 60% of its total production is in Wakayama Prefecture. ‘Tonewase’ has been grown under forcing-culture in heated plastic-houses but the fruit often undergoes rapid softening during distribution, and this has limited the enlargement of its cultivation area. In contrast, field-grown ‘Tonewase’ was cultivated without any problem until 1993. Thereafter, however, rapid fruit softening was observed to occur during postharvest distribution and the percentage of spoilt fruit kept on increasing every year, coinciding with the slowing of the temperature decrease in September, the ripening season for the fruit. This led to a serious economic loss in Wakayama Prefecture. We clarified the mechanism of this fruit softening and developed a completely suppressible technique based on that mechanism (Harima et al., 2001, 2002a, 2002b; Nakano et al., 2001a, 2001b, 2002, 2003).

It is known that the harvested persimmon fruit produces large amounts of ethylene at a younger stage and its rate decreases with growth (Takata, 1983). Thereafter, almost no ethylene is produced even in the commercially harvested fruit until severe softening occurs. Thereby, young fruit were used in various preliminary studies. In young fruit, 1-MCP greatly suppressed ethylene production with a simultaneous strong inhibition of fruit softening (Nakano et al., 2001a). This strongly suggests the involvement of ethylene in the softening during distribution in ‘Tonewase’ fruit. Therefore, we cloned three genes for ACS and two for ACO and analyzed their expression profiles (Fig. 6). Among the three ACS genes, only DK-ACS1 was expressed in the fully ripened fruit with severe softening, while expression of this gene was induced by treatment with propylene in pre-climacteric fruit. On the other hand, expression of the DK-ACS2 gene was induced by

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**Fig. 4.** Effect of 1-MCP treatment after the onset of ripening on the ACS activity (A), ACC content (B), *in vivo* ACO activity (C), *in vitro* ACO activity (D), and expression of MA-ACS1 and MA-ACO1 genes (E) in the pulp of banana fruit. Pre-climacteric fruit were treated with propylene for 18 h, followed by treatment with or without 1-MCP for 6 h on day-1, and ripened at 22°C.

**Fig. 5.** Effect of 1-MCP treatment after the onset of ripening on the ACS activity (A), ACC content (B), *in vivo* ACO activity (C), *in vitro* ACO activity (D), and expression of MA-ACS1 and MA-ACO1 genes (E) in the peel of banana fruit. Pre-climacteric fruit were treated with propylene for 18 h, followed by treatment with or without 1-MCP for 6 h on day-1, and ripened at 22°C.

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wounding treatment, with a large increase by 1-MCP exposure. These results indicate that *DK-ACS1* may be related to ripening-ethylene production and *DK-ACS2* is the gene responsible for ethylene production in response to stress. Surprisingly, in mature ‘Tonewase’ fruit, both *DK-ACS1* and *DK-ACS2* genes were expressed during postharvest distribution. We hypothesized that the harvested ‘Tonewase’ fruit was under some stress that induces stress-ethylene production through *DK-ACS2* expression, and this ethylene induces ripening-ethylene resulting in rapid softening. To elucidate this further, young ‘Hiratanenashi’ fruit was divided into several tissues and ethylene production and related-gene expression were monitored in combination with treatment with 1-MCP (Nakano et al., 2002, 2003). In the pulp, peel, abscission zone, and core tissues, ethylene production was detectable on the 2nd day after harvest, peaked on the 3rd day, and was markedly suppressed by 1-MCP. In contrast, in the calyx, ethylene production started to increase and peaked 1 day earlier than in the other tissues and 1-MCP did not suppress ethylene production, but rather prolonged elevated ethylene levels (Fig. 7). These differences in the ethylene production pattern of the calyx from other fruit tissues were observed in the gene expression (Fig. 7F). On the 3rd day after harvest, accumulation of *DK-ACS2*, *DK-ACO*, and *DK-ACO2* mRNA was detected in each fruit tissue, while the accumulation of *DK-ACS1* mRNA was detected only in the core and pulp. Other than in the calyx, the accumulation of these mRNAs was substantially suppressed by 1-MCP. However, the accumulation of *DK-ACS2* mRNA in the calyx tissue was enhanced by 1-MCP treatment. Furthermore, the mRNA levels of both *DK-ACO* genes in the calyx were not affected by the 1-MCP treatment, different from the other tissues. Thus, expression patterns of the genes related to ethylene biosynthesis in the calyx were completely different from those in the other tissues. Furthermore, ethylene biosynthesis in tissues other than the calyx was found to be regulated by a positive feedback system while that in the calyx was under negative feedback regulation. Thus, interestingly, opposite directions of feedback regulation operated at the same time in different tissues of persimmon fruit, as well as in banana fruit mentioned above.

From the above observations, it is considered that in ‘Tonewase’, some stress functions as a primary signal that triggers stress-ethylene production in the tissue of the calyx, and this ethylene diffuses into the pulp tissue of the fruit where ripening-ethylene production is activated, which, in turn, causes rapid fruit softening. If this is the case, then what stress is operating in the harvested calyx tissue? We hypothesized that harvested fruit experience water loss following detachment from the tree and that this water stress may act as an external stress-related factor that induces ethylene biosynthesis in the calyx. In young ‘Hiratanenashi’, the alleviation of water loss from the fruit by packaging it in perforated polyethylene bags or exposure to humidified airflow markedly delayed the initiation of ethylene production as well as expression of *DK-ACS2* in the calyx together with delaying flesh softening (Nakano et al., 2002, 2003). As expected, this effect of the alleviation of water loss on the delay of ethylene production and fruit softening was observed clearly in commercially harvested ‘Tonewase’ fruit during distribution. This beneficial effect of packaging was observed in a practical transportation test trial (Fig. 8). Softened fruit was not observed until the 26th day post harvest, strongly suggesting the successful application of this technique in commercial distribution. At present, carton boxes laminated with plastic film are being used to alleviate water loss in place of polyethylene bags under practical distribution.

On the other hand, astringent type Japanese persimmon, ‘Saijo’ fruit, are known to soften rapidly after removal of the astringency, and, therefore, its market is restricted to nearby production areas, in spite of its high eating quality. We tried an adaptation of the technique developed for ‘Tonewase’ persimmon to ‘Saijo’ fruit to inhibit its rapid softening (Nakano et al., 2002, 2003).
Without carbon dioxide treatment for the removal of astringency, alleviation of water loss significantly suppressed ethylene production and softening in 'Saijo' as well as in 'Tonewase' fruit. However, this technique could not be adapted commercially to 'Saijo' because considerable amounts of ethylene were induced and softening rapidly occurred in response to carbon dioxide used for the removal of astringency. 1-MCP suppressed fruit softening both in 'Saijo' and 'Tonewase' fruits at commercial levels (Harima et al., 2003).

4. Comparison of ethylene production and fruit softening among three different pear cultivars

Among the genus Pyrus, there are several species showing characteristic features in ethylene production and fruit softening. The European pear (Pyrus communis L.) ‘La France’ is a climacteric fruit which undergoes dramatic fruit softening with ethylene production during ripening, resulting in an attractive melting texture. Chinese pear (Pyrus bretschneideri Rehd.) ‘Yali’ fruit shows massive climacteric ethylene production, but does not exhibit fruit softening and its flesh texture remains crisp even at the late ripening stage. Japanese pear (Pyrus pyrifolia Nakai) ‘Nijisseiki’ is classified as a non-climacteric fruit and does not exhibit ethylene production and no striking changes in flesh firmness during ripening. Until now, few comparative studies on ripening and softening using fruit from the same genus have been reported. Therefore, a comparison of differences in softening-related factors among the three pear fruits was examined in relation to their ethylene production patterns (Hiwasa et al., 2003a, 2003b, 2004; Kubo et al., 2003; Mwaniki et al., 2005).

At first, ‘La France’ fruit were treated with propylene or 1-MCP to obtain the basic ripening characteristics of pears, since banana and persimmon fruits had unique feedback regulation systems in ethylene biosynthesis, as mentioned above. Propylene treatment at the pre-climacteric stage stimulated ethylene production and flesh softening, while 1-MCP treatment at the same stage markedly retarded the initiation of the ripening-related events. 1-MCP treatment after the initiation of ripening markedly suppressed ethylene production to trace levels and maintained flesh firmness at steady-state level without further decline. These results indicate that ethylene plays a crucial role in both the initiation and subsequent progression of ripening in pear fruit (Hiwasa et al., 2003a). In ‘La France’, mRNA accumulation of pear polygalacturonase, PC-PG1 and PC-PG2, occurred in parallel with the pattern of fruit softening in both propylene and 1-MCP treatments, suggesting the involvement of ethylene-dependent regulation in these gene expressions. However, expression of PC-EG1 and PC-EG2, pear endo-1,4-β-D-glucanase (EGase) gene, was not regulated by ethylene (Hiwasa et al., 2003a). Furthermore, at least seven α-expansin genes seemed to be operating during fruit development and ripening but their expression overlapped in the ripening fruit and their involvements in flesh softening were unclear (Hiwasa et al., 2003b).

The observations mentioned above suggest that European pear fruit belongs to a typical climacteric group without any exception in terms of ethylene biosynthesis and fruit softening. Softening characteristics were then compared among three types of pear fruits (Hiwasa et al., 2004). ‘La France’ fruit softened dramatically and developed a melting texture during ripening, while ‘Yali’ fruit with and without propylene treatments showed no changes in flesh firmness and texture during ripening.

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**Fig. 8.** Effect of perforated polyethylene bag packaging on fruit softening in ‘Tonewase’ Japanese persimmon fruit during the transportation test. Fruit were transported from Wakayama to Sapporo by airplane and then brought back to Wakayama by truck. The arrow indicates the date when fruit arrived at Wakayama.

**Fig. 9.** Changes in the ethylene production rate (A, C, E) and flesh firmness (B, D, F) in three types of pear fruit during ripening with or without propylene treatment. Fruit were harvested at commercial maturity. Fruit were treated with propylene until day-7 in ‘La France’ (A, B) and ‘Yali’ (C, D) and throughout the experiment in ‘Nijisseiki’ (E, F). Control fruits were maintained in ambient conditions.
Non-treated ‘Nijisseiki’ fruit did not show a detectable decrease in flesh firmness, whereas continuous propylene treatment caused a gradual decrease in firmness resulting in a mealy texture (Fig. 9). In ‘La France’, cell wall polysaccharides revealed distinct solubilization and depolymerization of pectin and hemicellulose during fruit softening. In ‘Nijisseiki’, propylene treatment led to the solubilization and polymerization of pectic polysaccharides to a limited extent, but not of hemicellulose. In ‘Yali’, hemicellulose polysaccharides were depolymerized during ripening, but there were hardly any changes in pectic polysaccharides except in the water-soluble fraction (Hiwasa et al., 2004). Depolymerization patterns of pectic polysaccharides during ripening have been demonstrated to vary in different fruits (Huber and O’Donoghue, 1993) and different tissues (Owino et al., 2003). Depolymerization of hemicellulose polysaccharides during fruit softening has also been shown to occur in various fruits (Rose et al., 1998; Sakurai and Nevins, 1997).

PC-PG1 and PC-PG2 were expressed in ‘La France’ fruit during ripening, while only PC-PG2 was expressed in ‘Nijisseki’ and neither PC-PG1 nor PC-PG2 was expressed in ‘Yali’ fruit (Fig. 10). The PC-XET1 gene was expressed constitutively during ripening in all three pear types. Therefore, differences in flesh softening during ripening in the three pear fruits may be attributed to the presence or absence of PG gene expression. The softening process has been shown to correlate with the expression of PG genes in melon fruit (Rose et al. 1998).

However, PG-independent softening was also demonstrated in transgenic tomato fruit (Smith et al., 1988). Therefore, we compared PG activity during ripening in the three pears (Fig. 11). When total PG activity was measured by the reducing sugar assay, it increased in all three varieties. However, viscometric measurements showed that the levels of endo-PG activity were high in ‘La France’, low in ‘Nijisseki’, and undetectable in ‘Yali’ fruits. From these observations, it is suggested that, in pears, cell wall degradation is correlated with a decrease in firmness during ripening and the modification of both pectin and hemicellulose are essential for the development of a melting texture. Furthermore, the data suggest that different softening behaviors during ripening among the three pear fruits may be caused by different endo-PG activity and the differential expression of PG genes. To investigate further reasons for the different softening behaviors in the three pear fruits, changes in enzyme activity and gene expression for β-galactosidase and arabinoxylanosidase were analyzed during fruit growth and ripening. The activities of these enzymes and expression of some members among these

Figure 10. Changes in mRNA accumulation for PG and XET genes in three types of pear fruit with or without propylene treatment during ripening. For ‘La France’ and ‘Yali’, only the fruits treated with propylene were used for analysis (A), while for ‘Nijisseki’, both control and propylene-treated fruits were used (B).

Figure 11. A: Changes in total PG activity determined by the reducing sugar assay in three types of pear fruit during ripening. For ‘La France’ and ‘Yali’, only the fruits treated with propylene were used for the assay, while for ‘Nijisseki’, both control and propylene-treated fruits were used. B: Endo-acting PG activity determined by the viscometric assay in three types of pear fruit. Cell-wall-protein solutions were extracted from propylene-treated fruits at day-7 for ‘La France’ and ‘Nijisseki’ and at day-15 for ‘Yali’.
gene families increased during postharvest ripening in all pear fruits. Therefore, these enzymes may be involved in fruit softening to some extent but might be excluded from different softening behaviors in the three pear fruits (Mwaniki et al., 2005).

5. Ethylene production and fruit softening in melon fruits
Similar studies to those in pear fruits were performed using several melon cultivars (Nishiyama et al., 2007). At first, ‘Charentais’ fruits were treated with propylene or 1-MCP to obtain the basic ripening characteristics of melon. This cultivar undergoes remarkably rapid softening, thereby providing an excellent model plant to study fruit ripening and softening. The fruit showed a typical climacteric pattern of ethylene production after propylene exposure and this ethylene was significantly suppressed by 1-MCP treatment after the onset of ripening (Fig. 12A, B). Fruit softening began at the onset of ripening and fruit firmness decreased to half of the initial level within the first 2 days and continued to an over-soft level within the next 2 days. 1-MCP suppressed

![Ethylene Production and Fruit Softening in Melon Fruits](image)

**Fig. 12.** Ethylene production (A), flesh firmness (B), and expression of genes encoding cell wall modifying enzymes (C) in ‘Charentais’ melon fruit treated with or without 1-MCP. All fruit were treated with propylene after harvest for the first 2 days to ripen uniformly. A subset of fruit were treated with 1-MCP overnight after day-2.

this rapid softening with fruits remaining firm until day-10. Among the cell wall-modifying enzyme genes tested, MPG1 showed a well-correlated expression pattern to fruit-softening behaviors, showing an ethylene-dependent expression where its expression was induced by propylene and eliminated for several days by subsequent treatment with 1-MCP (Fig. 12C). CmGal1 also showed an ethylene-dependent expression pattern but its mRNA accumulation was not detected even at later stages when MPG1 mRNA levels had returned to normal. We also observed that MPG1 is not expressed in another melon fruit belonging to the non-climacteric type (unpublished data). Therefore, MPG1 is one of the primary candidates responsible for ethylene-dependent pectin disassembly and fruit softening. An increase of PG activity and mRNA levels has been reported in several fruit species concomitant with the degradation of pectin polysaccharides and softening (Fisher and Bennett, 1991; Huber, 1983). From these observations, it is considered that ethylene biosynthesis in melon fruit may be regulated under a positive feedback mechanism and softening is completely dependent on ethylene.

Both melon and cucumber belong to the Cucurbitaceae family, and the former fruit is mainly climacteric and the latter is non-climacteric. As mentioned above, ethylene production in melon fruit is under positive feedback regulation. Then, we asked ourselves whether cucumber fruit has ACS and ACO gene members related to ripening-ethylene or not. Therefore, we cloned ACS and ACO gene members from cucumber fruit and compared them to those from melons (Shiomi et al., 1998). Among the cloned genes, CS-ACS1 and CS-ACO1 had a high sequence similarity (more than 90%) to ME-ACS1 and CM-ACO1 genes in melon. These melon genes are known to be related to ethylene biosynthesis in ripening fruit (Ayub et al., 1996; Shiomi et al., 1999; Yamamoto et al., 1995). However, these genes in cucumbers were not expressed even in ripening fruit, and were only expressed in response to external stimuli such as wounding and auxin (Shiomi et al., 1998). Therefore, it is considered that even in non-climacteric fruit, ripening-ethylene producible genes are present but fruit lack some factors to express these genes. To examine one of these factors, we cloned promoter regions of ME-ACS1 and CS-ACS1 genes and determined their activities using the GUS-transient assay (Shiomi et al., 2001). GUS activities conferred by the promoters of both genes were detected in melon disks, but only CS-ACS1:GUS was expressed in cucumber disks. This suggests that the difference in ethylene biosynthesis in melon and cucumber during ripening may be due to the difference in their capability of forming trans-acting factors, and not due to their ACS1 promoter activity.

### Conclusion
It has been widely accepted that ethylene production
in climacteric fruits is regulated by a positive feedback mechanism. Thereby, exogenously applied ethylene induces endogenous ethylene and this induced ethylene initiates fruit ripening. This concept is true for tomato, pear, and melon fruits, where ethylene production and the expression of its related genes are under positive feedback regulation. In the tomato, the developmentally programmed elevation of system-1 ethylene production might induce system-2 ethylene production through a positive feedback mechanism. However, a negative feedback regulation system operates in ripening banana pulp despite its sensitive ripening-induction feature in response to applied ethylene. For this reason, at least in banana fruit, a new concept must be introduced in that the applied ethylene induces fruit ripening but does not induce endogenous ethylene directly, and once ripening has commenced, endogenous ethylene production may be initiated. Even in ‘Tonewase’ persimmon fruit belonging to a climacteric group, water loss induces stress-ethylene localized in calyx tissue and this ethylene is regulated under a negative feedback system. We also observed the existence of a similar possibility of negative feedback regulation in peach (Mathooko et al., 2001) and fig (Owino et al., 2002) fruits. Flesh softening in pear and melon fruits occurs in an ethylene-dependent manner in which PG genes have a dominant role. However, such genes are not expressed in ‘Yali’ fruit despite its massive ethylene production. These observations indicate that even in climacteric fruits, different feedback regulation systems of ethylene biosynthesis and different ethylene-dependent manners of ripening-related gene expression operate in different kinds of fruits. Ripening phenomena in non-climacteric fruits are not different from those in climacteric fruits with respect to all events such as sugar accumulation, acid decrease, color development, aroma production, and flesh softening. Even in non-climacteric fruit, cucumbers showed highly similar genes related to ripening ethylene biosynthesis in melons. Through the studies introduced here, we feel that the obtained new results will lead to further new questions. Further studies from various standpoints will be necessary to improve postharvest fruit quality with advanced handling techniques. At this point, 1-MCP may have a potent ability to prolong postharvest quality in several fruits. Recent studies on the ripening mutants of tomatoes have revealed that some novel developmental factors functioning upstream of ethylene could be of essential importance for fruit ripening (Barry and Giovannoni, 2006; Manning et al., 2006; Vrebavlov et al., 2002). These findings may provide a new platform for further research on the ripening mechanisms of fruits including non-climacteric types.

**Literature Cited**


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