Gene Expression of Pectic Polysaccharide Degrading Enzymes in On-tree Softened ‘Hiratanenashi’ Persimmon Fruit

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The fruit of the ‘Hiratanenashi’ persimmon (*Diospyros kaki* Thunb.) over-ripen on the tree by late November as a result of senescence processes. On-tree over-ripened fruits were harvested at this time, and the degree of fruit softening was measured. The ‘softening degree’ index ranged from 1.5 (firm to moderately soft) to 4 (very soft, or part of the peel ruptured). To clarify the fruit softening processes resulting from on-tree senescence, internal ethylene concentrations, gene expression of ethylene synthesis, and cell-wall-degrading enzymes were investigated in ‘Hiratanenashi’ persimmon. The internal ethylene concentration increased concomitantly with softening progress. The expression of two ACC synthase (ACS) genes, *DkACS1* and *DkACS2*, and two ACC oxidase (ACO) genes, *DkACO1* and *DkACO2* were investigated using quantitative PCR. The ACS genes were rate limiting for ethylene production, but ACO genes were constitutive. Genes encoding three polygalacturonases (PG), two pectin methylesterases (PE), two β-galactosidases (Gal), and one α-arabinofuranosidase (Arf) were isolated from an expressed sequence tag (EST) library of on-tree softened pulp, and their expression was investigated. Among these eight isolated genes, the expression of *DkPG1*, *DkPG2*, *DkGal1*, and *DkArf1* increased concomitantly with softening progress. In contrast, the expression of *DkPG3*, *DkPE1*, *DkPE2*, and *DkGal2* was maintained at low levels throughout the softening progress. These results suggest that *DkPG1*, *DkPG2*, *DkGal1*, and *DkArf1* play a coordinated role in on-tree fruit softening that follows PE action by senescence in ‘Hiratanenashi’. Two of the PG genes are thought to contribute to a jelly- or soup-like texture at softening degree 4.

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**Key words**: cell-wall-degrading enzymes, ethylene, gene expression, softening, persimmon

Fruit softening is a complex process that involves numerous modifications to the cell wall. These modifications may be mediated by polygalacturonase (PG) and pectin methylesterase (PE), which hydrolyze and esterify the main chains of pectic polysaccharides, and β-galactosidase (Gal) and α-arabinofuranosidase (Arf), which hydrolyze side chains of pectic or hemicellulosic polysaccharides. These cell-wall-degrading enzymes have been extensively studied in tomato (*Solanum lycopersicum*), melon (*Cucumis melo*), European pear (*Pyrus communis*), and grape (*Vitis labruscana*).

Fruit softening in persimmons (*Diospyros kaki* Thunb.) is promoted during the postharvest process. This is led by destringency treatment, and the softened fruit begins to lose commercial value. Persimmon fruit may naturally soften on the tree because of occasional physiological disorders or senescence processes during late autumn. In such cases, the over-softened fruit develops a jelly- or soup-like texture, and drops from the tree. Although changes in cell wall components and degrading enzyme activities during fruit softening after harvest or destringency treatments have been...
the focus of much research in persimmons, little is known about senescence-induced on-tree fruit softening.

In persimmons, the softening to a jelly-like texture has been reported in many varieties, including ‘Tonewase’, which is grown by forcing culture, and mature ‘Saijo’, which is rapidly softened after CO2 and dry ice treatment for the removal of astringency. Harvested fruits at the young and late mature stages and immature green stages of ‘Saijo’ persimmon also rapidly softened. This rapid softening after harvest was concomitant with an increase in Gal or Arf activity. In contrast, the cooperative activities of PE, Gal, and Arf increased during the on-tree softening of ‘Saijo’ fruits in late October.

Molecular analysis revealed that genes related to cell-wall-degrading enzymes are involved in ‘HIRATANENASHI’ and ‘Saijo’ in the softening processes of postharvest fruits. Propylene- and ethanol-treated mature ‘HIRATANENASHI’ fruits rapidly softened, and there was a parallel increase in DkCel 3 (cellulase), DkPG1, and DkExp2 (expansin) gene expression. Mature ‘Saijo’ persimmons rapidly softened within six days of dry ice treatment for deastringency, and the expression of two xylanase endotransglycosylase/hydrolase (XTH) genes, DkXTH1 and DkXTH2, coincided with fruit softening. The contribution of the activity or gene expression of cell-wall-degrading enzymes, such as PG, PE, Gal, and Arf, seemed to be different for each softening process induced by deastringent treatment and ripening, although fruit changed to a jelly- or soup-like texture, called ‘Jukushi’ in Japanese, via the degradation of pectic and hemicellulosic polysaccharides by postharvest treatment, ripening, or senescence in persimmons.

The relationship between post harvest fruit softening, treatments to remove astringency, and the activities of cell-wall-degrading enzymes have been well reported, but the softening processes caused by senescence on the tree are unclear at the molecular level. To better understand such on-tree fruit softening, it is necessary to investigate the multiple genes of the cell-wall-degrading enzymes, because PE, Gal, and Arf gene expression has not been investigated in on-tree softened persimmon fruits.

Recently, we obtained 4701 expressed sequence tags (ESTs), including clones encoding many cell-wall-degrading enzymes, from on-tree softened pulp of ‘Saijo’ fruits harvested in early October. This study focused on the on-tree softening processes that occur during senescence, and characterizes gene expression, especially encoding enzymes which degrade the main and/or side chains of pectic polysaccharide, during over-ripened fruit softening.

**Materials and methods**

1. **Plant materials**

To simulate the late autumn on-tree senescent fruit-softening process, mature persimmon (Diospyros kaki Thunb. cv. HIRATANENASHI) fruits grown at an experimental field of Shimane University were harvested on November 20, 2008. The harvested fruits were classified into the four different ‘softening degree’ classes (Fig. 1) previously

![Fig. 1 ‘HIRATANENASHI’ fruit at four different softening degree stages](image)

(A) softening degree 1.5, (B) degree 2, (C) degree 3 and (D) degree 4.
proposed by Iwata et al.\textsuperscript{10} : ① firm ; ② moderately soft ; ③ soft enough to be easily crushed with fingers, or part of flesh prone to become jelly- or soup-like; ④ very soft, or part of peel ruptured. As fruits had slightly softened by November 20 on a tree, the fruits were designated as having a softening degree of 1.5 at harvest. Internal ethylene levels of whole fruits were measured in triplicate. The peel was removed from the remaining fruits, and the fruits were frozen at −80°C until required for gene expression analysis.

2. Measurement of internal ethylene

Internal ethylene was measured using whole fruits (replications/fruit; n = 3) at four different stages (softening degree 1.5, 2, 3, and 4)\textsuperscript{10}. The fruits were dipped in a vessel containing NaCl-saturated solution, and decompressed using a vacuum pump (ULVAC, GCD-050XA, Osaka, Japan) to extract the internal gas from the fruits. Two milliliters of gas headspace was withdrawn from the vial by using a glass syringe, and injected into a gas chromatograph (Shimadzu GC-14, Kyoto, Japan) fitted with an activated alumina column (3 mm × 2 m) and a flame ionization detector. The temperatures of the column, injection, and detector, were set at 130, 150, and 150°C, respectively.

3. Blast search

Candidate genes were selected from ESTs (Accession no. FY 982194 – FY 986894 ; DDBJ database) derived from on-tree softened ‘Saijo’ pulp\textsuperscript{5}. The BLAST program was used to search the sequences against the NCBI non-redundant protein database\textsuperscript{20}.

4. Gene expression of ethylene biosynthetic and cell-wall-degrading enzymes

Total RNA for real-time qPCR (RT-qPCR), was extracted from approximately 10 g flesh weight pulps (replications/fruit; n = 3) of ‘Hiratanenashi’ using the hot borate method\textsuperscript{20}. The cDNA was synthesized from 1 μg DNase-treated total RNA using ReverTra Ace reverse transcriptase (Toyobo Co, Osaka, Japan) and oligo (dT) primers (Toyobo Co, Osaka, Japan). The accumulation levels of the transcripts were analyzed using ten-fold diluted cDNA and gene-specific primers, using the Thermal Cycler Dice Real Time System and SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan) according to the manufacturer’s instructions. Gene specific primers used for RT-qPCR were generated based on the Perfect Real Time Support System of the TaKaRa web site, and are given in Table 1. Two ACC synthase (ACS) genes (\textit{DKACS}) and two ACC oxidases (ACO) genes (\textit{DKACO}) were detected using specific primers described by Ortiz et al.\textsuperscript{20}. The quantity of target gene mRNA was determined and normalized using \textit{DKACT} (Accession no. AB746346) mRNA as the reference sample.

<table>
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<tr>
<th>Target gene</th>
<th>Primer name</th>
<th>Sequence (5’ to 3’ direction)</th>
<th>Amplified size</th>
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<td>DkPG1</td>
<td>DkPG 1-F</td>
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<td>177bp</td>
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<tr>
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<td>DkPG 1-R</td>
<td>TCTTCTCCTCAATCCCTGGT</td>
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<td>DkPG 2-F</td>
<td>AGTTTCATCCCGCTTACAGC</td>
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<tr>
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<td>DkPE2</td>
<td>DkPE 2-R</td>
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<tr>
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<tr>
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<td>DkGal 1-R</td>
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<td>DkGal 2-F</td>
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<td>DkACT</td>
<td>DkACT-F</td>
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<tr>
<td>DkACT</td>
<td>DkACT-R</td>
<td>CAAGGATGTTGGAAGAGA</td>
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Results

1. Isolation of PG, PE, Gal, and Arf genes from the cDNA library

We selected candidate EST clones from a cDNA library of rapidly softened pulp to identify cell-wall-degrading enzyme genes related to on-tree softening senescence (Table 2) [13]. The library was constructed using on-tree softened ‘Saijo’ pulp.

The PG EST clone DkPG1 (KB032_F04; FY984342) from ‘Saijo’ was identical to DkPG1 from ‘Hiratanenashi’ (Pers. comm. Dr. Kubo, Okayama University) and DkPG1 (EU816197) from ‘Fuping Hanshi’; these genes are related to ripening and are ethylene-dependent [10]. Other PG EST clones were named as DkPG2 (KB066_F09; FY986613) and DkPG3 (KB018_A06; FY983373). Both DkPG2 and DkPG3 showed 77% homology with AY280662 from Fragaria × ananassa, which is abundant in soft strawberry cultivars [6], and XM_002509903 from Ricinus communis.

DkPE1 (KB032_C03; FY984336) and DkPE2 (KB062_D03; FY986376) were 75% similar to CapME2 (JN863079), and 78% similar to CapME1 (JN863078) of Coffea arabica, respectively [5].

DkGal1 (KB029_F02; FY984109), DkGal2 (KB002_B09; FY982324), and DkArf1 (KB037_F08; FY984725) showed 83, 94, and 80% similarity to AdgAL1 (HQ108111) of Actinidia delicosa, Dkgy1 (JF440125) of persimmon, and PpARA2 (AB195230) of Pyrus pyrifolia [6], respectively. Of the eight isolated genes retrieved from the persimmon ESTs, seven (the exception being DkPG1 [10]) had unknown expression profiles. Therefore, we investigated their gene expression in ‘Hiratanenashi’ to determine their involvement in on-tree fruit softening.

2. Expression of ethylene biosynthesis genes and pectin-degradation-related enzyme genes in naturally on-tree senescent softened ‘Hiratanenashi’ persimmon fruits

(1) ACS and ACO genes Persimmon fruit at softening degrees 2, 3, and 4 contained 0.04, 0.17, and 0.59 ppm of internal ethylene, respectively (Fig. 2A). DkACS1 and DkACS2 were expressed at softening degree 1.5 (0.03 ppm internal ethylene), and their expression levels were increased at softening degrees 3 and 4; this increase was concomitant with internal ethylene concentrations (Fig. 2B). DkACO1 showed a constant level of expression from softening degree 1.5 to 4 (Fig. 2C). DkACO2 was expressed at softening degree 1.5, maintained the same levels up to softening degree 3, but decreased at softening degree 4.

(2) PG, PE, Gal, and Arf genes DkPG1 and DkPG2 were already expressed at softening degree 1.5. Their signals increased 12- and 10-fold at softening degree 4, respectively (Fig. 3A). The maximum DkPE1 signal was detected at softening degree 1.5, and massively decreased at softening degrees 2, 3, and 4 (Fig. 3B). DkGal1 and DkArf1 were expressed at softening degree 1.5, and their gene expression increased concomitantly with the softening progress (Fig. 3C and D). DkPG3, DkPE2, and DkGal2 expression was low at all softening degree stages.

Table 2 Homology search of EST clones by blastn program

<table>
<thead>
<tr>
<th>EST name</th>
<th>Species</th>
<th>Accession No.</th>
<th>Identity (%)</th>
</tr>
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<tr>
<td>DkPG1</td>
<td>Diospyros kaki</td>
<td>EU816197</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>Diospyros kaki</td>
<td>EU816198</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>Diospyros kaki</td>
<td>EU816199</td>
<td>96</td>
</tr>
<tr>
<td>DkPG2</td>
<td>Fragaria × ananassa</td>
<td>AY280662</td>
<td>77</td>
</tr>
<tr>
<td>DkPG3</td>
<td>Ricinus communis</td>
<td>XM_002509903</td>
<td>77</td>
</tr>
<tr>
<td>DkPE1</td>
<td>Coffea arabica</td>
<td>JN863079</td>
<td>75</td>
</tr>
<tr>
<td>DkPE2</td>
<td>Coffea arabica</td>
<td>JN863078</td>
<td>78</td>
</tr>
<tr>
<td>DkGal1</td>
<td>Actinidia delicosa var. delicosa</td>
<td>HQ108111</td>
<td>83</td>
</tr>
<tr>
<td>DkGal2</td>
<td>Diospyros kaki</td>
<td>JF440125</td>
<td>94</td>
</tr>
<tr>
<td>DkArf1</td>
<td>Pyrus pyrifolia</td>
<td>AB195230</td>
<td>80</td>
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</table>
**Fig. 2** Internal ethylene levels (A), and gene expression of DkACS1 and DkACS2 (B), and DkACO1 and DkACO2 (C), in ‘Hiratanenashi’ persimmon fruit at four different softening degree stages. Each bar represents the mean relative expression value of five replicate fruit measurements. The vertical bars represent the SE.

**Fig. 3** Gene expression of PG (A), PE (B), Gal (C), and Arf (D) in ‘Hiratanenashi’ persimmon at four different softening degree stages. Each bar represents the mean relative expression value of five replicate fruit measurements. The vertical bars represent the SE.

**Discussion**

When a persimmon fruit senesces on a tree during late autumn, the flesh firmness decreases and it changes texture; the fruit then naturally drops from the tree. The softening degrees 2 (moderately softened) and 3 (completely softened) classifications describe a slightly melted texture, whereas softening degree 4 describe a jelly- or soup-like texture. Mature ‘Hiratanenashi’ fruit took about
30 days, from mid-October to mid-November, to soften to softening degree 2 (moderately soft) on a tree\(^5\). ‘Hiratanenashi’ fruit with a jelly-like texture (degree 4) appeared on a tree in late November. Generally, ethylene plays an important role in the fruit softening of the persimmons ex ‘Hiratanenashi’\(^20\) and ‘Saijo’\(^20\). In this study, we examined how ethylene production and cell-wall-degrading enzyme genes were induced in on-tree senescent softened fruit.

1. Ethylene and expression of ACS and ACO genes in on-tree softened fruits

In mature ‘Hiratanenashi’ fruit, \(DkACS\) is predominantly expressed after harvest and is thus responsible for the ethylene production associated with fruit ripening\(^26\). In ‘Hiratanenashi’ harvested one month before commercial maturity, \(DkACS1\) and \(DkACS2\) genes were induced by propylene and wounding, respectively\(^6\). \(DkACS2\) expression was induced during postharvest in young intact\(^23\) and mature wounded\(^21\) ‘Hiratanenashi’ persimmons. It has been suggested that \(DkACS1\) may be related to ethylene production during ripening and that \(DkACS2\) is the gene responsible for ethylene production in response to stresses like wounding\(^7\).

In this study, the expression of \(DkACS1\) and \(DkACS2\) increased in on-tree softened ‘Hiratanenashi’ fruits at softening degrees 3 and 4 (Fig. 2). This suggests that \(DkACS1\) and \(DkACS2\) expression is promoted by senescence, consists of ripening and wounding, and is involved in the change to jelly-like flesh observed at softening degree 4. On the other hand, the \(DkACO1\), not \(DkACO2\), gene plays a role in ethylene production in wounded and postharvest softened persimmon fruits\(^20, 23\). Interestingly, \(DkACO2\) expression was predominant in senescent, on-tree softened ‘Hiratanenashi’ (Fig. 2), the same as in ethanol-treated ‘Rendaiji’\(^26\).

When ethylene production increased in mature ‘Saijo’ treated with dry ice\(^28\) and intact young ‘Hiratanenashi’\(^20\) postharvest, flesh firmness decreased to 0 kg within several days. In addition, mature ‘Hiratanenashi’ treated with propylene produced ethylene 1 day after treatment, and then flesh firmness decreased to almost 0 kg within 5 days\(^7\). However, in senescent ‘Hiratanenashi’ on a tree\(^6\), the fruit maintained low ethylene production and took more than 30 days to progress to softening degree 2. These results suggest that ethylene production in postharvest rapidly softened fruit was induced earlier than in on-tree senescent softened fruit, so the softening period in postharvest softened fruit would be shorter.

2. Gene expression of cell-wall-degrading enzymes in on-tree softened fruits

Primary cell wall components may be pectic, hemicellulosic, or cellulotic polysaccharides\(^15, 46\). These polysaccharides are degraded through hydrolysis by numerous cell-wall-degrading enzymes, such as PG, PE, Gal, and Arf. Endo- or exo-PGs can hydrolyze the main chain of pectic polysaccharides (polygalacturonic acid polymers); Gal and Arf act as exo-hydrolytic enzymes by removing galactosyl or arabinosyl residues from the side chains of pectic or hemicellulosic polysaccharides; and PE removes methyl groups from methoxyl residues of the main chain of pectic polysaccharides\(^15, 46, 31\).

In this study, we investigated the expression of the PG, PE, Gal, and Arf genes of on-tree senescent softened ‘Hiratanenashi’ fruits, which were divided into four different softening degree classifications, from slightly softened to a jelly- or soup-like texture (Fig. 3). The \(DkPE1\) gene was expressed at very low levels in the fruits at every softening degree 1. 5 (slightly softened) to 4 (jelly - or soup-like texture) (Fig. 3 B). ROSE et al. (2003)\(^5\) concluded that highly methylated polyuronides represent poor substrates for PG, and that pectin demethylation is an important prelude to PG-mediated mechanisms for regulating the activity of PG, and maybe other pectinases. MATSUMI and KITAGAWA found that the PE activity of ‘Hiratanenashi’ fruits on a tree was highest in September and decreased to a very low level by late October\(^20\). These results suggested that in on-tree over-ripened ‘Hiratanenashi’, PE first acts through pectin de-esterification, and then polyuronide depolymerization by PG occurs. The low expression of PE in the on-tree senescent softened fruit of ‘Hiratanenashi’ (Fig. 3B) is consistent with decreased PE activity in late October\(^20\).

In mature ‘Hiratanenashi’\(^20\), a strong \(DkPG1\) signal was detected 1 day after propylene treatment and then maintained at a high level, and flesh firmness decreased to almost 0 kg 4 days after the treatment. The \(DkPG1\) showed ethylene-dependent gene expression. An early increase in PG activity has also been observed during the postharvest softening of ‘Saijo’ fruits harvested when young\(^20\), immature\(^20\), or mature\(^20\). In the case of senescent on-tree softening, \(DkPG1\) and \(DkPG2\) signals increased.

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3-fold at softening degrees 2 (moderately softened) and 3 (completely softened) compared to those at softening degree 1.5, and showed a maximum level at degree 4, concomitant with internal ethylene (Fig. 3A). This suggests that the softening period differs between postharvest and senescent on-tree fruit because the decrease in flesh firmness and change to a jelly-like texture would be dependent on the role of ethylene-inducible cell-wall-degrading enzyme genes, such as DkPG1.

TATEISHI (2008) concluded that pectinolytic digestion mediated by PG contributes to fruit texture rather than fruit softening. When softening characteristics were investigated in fruits of the European pear ‘La France’, Chinese pear ‘Yali’, and Japanese pear ‘Nijisseiki’, the modification of both pectin and hemicellulose was found to be essential for the development of a melting texture, and this may be caused by different endo-PG activities and the expression of PG genes. A jelly- or soup-like texture is a general feature of over-ripened persimmon fruit, and DkPG1 and DkPG2 expression dramatically increased in softening degree 4 fruits (Fig. 3A). Therefore, these PG genes may contribute to the texture change at softening degree 4 in ‘Hiratanenashi’.

Gal and Arf enzymes play important roles in fruit softening and textural changes during ripening, and the loss of arabinosyl and galactosyl residues from wall polysaccharides corresponds to the solubilization of pectic polymers. In the young and mature stages of ‘Saijo’ persimmons, rapid softening was mainly concomitant with the increase in Arf activity following postharvest softening. NAKAMURA et al. reported that postharvest softening in immature ‘Saijo’ fruits is mainly caused by Gal activity. ‘Tonewase’ fruits grown under forcing culture conditions were rapidly soften after the treatment for astringency removal. This postharvest softening is thought to be the result of the decrease in neutral sugars supporting Gal and Arf action for the side groups of pectic polysaccharides and hemicelluloses.

In contrast, both Gal and Arf activities increased during on-tree softening. In this study, RT-qPCR revealed that DkGal 1 and DkArf 1 increased concomitantly with the softening progress in ‘Hiratanenashi’ fruits (Figs. 3C and D). This suggests that Gal and Arf activities are necessary for postharvest and on-tree fruit softening but the contribution of each enzyme to fruit softening would differ depending on the stage, or whether the fruit was detached or attached to the tree.

These changes in enzyme activities, gene expression, and cell wall components support the close relationship between the decrease in flesh firmness or changes in melting texture and Gal and Arf action. On the other hand, biochemically characterized Arf belongs to glycoside hydrolase families 3 and 51 in higher plants, and family 3 enzymes appear to be both single-functional β-xylanase (Xyl), and bi-functional Arf/Xyl for artificial substrates. PPARF2 from the Japanese pear shows bifunctionality and is expressed in ripened fruits. Amino acid sequences of DkArf are similar to PpARF2; therefore, persimmon Arf genes may function through xylose modification in the side chains of pectic polysaccharides and hemicelluloses.

In conclusion, we confirmed the gene expression of four cell-wall-degrading enzymes in on-tree senescence softened ‘Hiratanenashi’ fruits. Our findings suggest that PG, Gal, and Arf may contribute to the overall softening phenomenon after pectin de-esterification by PE, and PG action especially plays a role in the formation of jelly- or soup-like texture of over-ripened ‘Hiratanenashi’ fruit flesh.

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4) GOULAO, L.F. and OLIVEIRA, C.M.: Cell wall modifications during fruit ripening: when a fruit


樹上転化した「平核無」カキ果実におけるベクチン多糖類分解酵素遺伝子の発現解析

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11月下旬に老熟により樹上転化した‘平核無’果実を4段階の軟化度別に収穫した。エチレンおよび細胞壁分解酵素遺伝子間の関連を明らかにするため、軟化度、内部エチレン濃度とエチレンおよび細胞壁分解酵素遺伝子遺伝子発現を調査した。内部エチレン濃度は軟化度の進行とともに増加した。ACC合成酵素遺伝子DkACS1とDkACS2およびACC酸化酵素遺伝子DkACO1とDkACO2の発現を定量的PCRにより調査すると、ACS遺伝子はエチリン生成の律連段階であり、ACO遺伝子は恒常的発現を示した。3つのポリナラクチュロナーゼ（PG）、2つのベクチンメチルエステラーゼ（PE）、2つのβ-ガラクトシダーゼ（Gal）および1つのα-アラビノフランソニダーゼ（Arf）遺伝子は樹上転化果実のESTから単離され、それら8つの遺伝子を発現解析した。DkPG1, DkPG2, DkGal1およびDkArfの発現は軟化段階の進行に伴い増加したが、DkPG2は軟化度1.5で最大を示し、その後減少した。またDkPG3, DkPG2およびDkGal2はほとんど変化がなかった。以上の結果より、樹上で老化的「平核無」ではPEが作用した後にDkPG1, DkPG2, DkGal1およびDkArf遺伝子が果実転化に対して調節的に働いていること、特に2つのPG遺伝子は果実のメルティング性に関与していることが示唆された。

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