Review

β-Galactosidase and α-L-Arabinofuranosidase in Cell Wall Modification Related with Fruit Development and Softening

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Fruit softening and textural changes are two important factors of fruit quality. The loss of galactosyl and arabinosyl residues from cell wall polysaccharides is observed in many fruit species during ripening. The release of neutral sugar residues could change wall polysaccharides properties of accessibility or reactivity for other cell wall hydrolases. β-Galactosidase and α-L-arabinofuranosidase contribute to the loss of neutral sugar residues. Thus, the enzymes alter polysaccharide properties and might contribute to fruit softening or textural changes during ripening. β-Galactosidase is composed of multiple isozymes and they are clearly distinguishable by their substrate specificity and expression pattern of the related gene. A transgenic experiment revealed that a β-galactosidase isozyme plays an important role in tomato fruit softening. In addition to fruit softening, the contribution of β-galactosidase to plant development is also indicated. α-L-Arabinofuranosidase genes constitute a gene family and the isozymes are expressed in various organs and stages. Moreover, several α-L-arabinofuranosidases possess β-xylosidase activity in addition to α-L-arabinofuranosidase activity, therefore, substrate specificity against native polysaccharides is very complex. Arabinose containing polysaccharides seem to contribute to cell to cell adhesion but the crucial roles of α-L-arabinofuranosidase in fruit development or softening remain unclear. Further biochemical and physiological studies of the enzyme are required.

Key Words: arabinose, galactose, ripening, texture, β-xylosidase.

Introduction

Fruit become edible during ripening when accompanied by several phenomena such as sugar accumulation, starch degradation, production of aroma, color change, and softening and textural changes. Fruit softening and textural changes are two important factors that influence fruit quality. Excessive softening limits fruit shelf life and postharvest handling. The structure of cell wall polysaccharides is very complex and their interactions/connections in situ have not been entirely defined. Numerous enzymes, which may contribute to modifications in their architecture, have been found in the cell walls. Moreover, many wall-modifying enzymes constitute multiple isozymes with different functions or roles and it is still difficult to understand the softening mechanism in detail. To elucidate the basic process of softening during fruit ripening, physiological studies are important and show common features of softening. On the other hand, fruit properties such as textural changes vary in fruit species, cultivars, and storage conditions; therefore, it is important to elucidate the unique contribution of each enzyme (isozyme) to the individual character of fruit formed with softening. This would be helpful to develop high quality fruit and horticultural knowledge at the field, handling, and storage levels. Moreover, the modification of cell wall architecture is involved in not only fruit softening but also plant growth, development, and shape formation. Cooperative biosynthesis and degradation of several cell wall components are necessary. Many enzymes are found in the cell wall. In this review, two glycosidases, β-galactosidase, and α-L-arabinofuranosidase are examined. The enzymatic characters of the enzymes and expression pattern of the genes are described and discussed in relation to fruit softening and other cell wall modification.

1. Pectin degradation and the contribution of polygalacturonase

Fruit softening results from modifications of cell wall architecture caused by several cell-wall-metabolizing enzymes (Fischer and Bennett, 1991). Plant cell-wall polysaccharides are generally divided into three classes:
pectin, hemicellulose and cellulose, based on their solubility. During fruit ripening, the modification of pectin is the major phenomenon among the three. The pectic macromolecule, which consists of a polygalacturonic main chain, is degraded to a smaller size during ripening (Brummell and Labavitch, 1997; Huber and O’Donoghue, 1993). It is apparent that the decrease in mechanical strength of pectic polymers caused by the change to small molecules is responsible for fruit softening. Endo-type polygalacturonase is an enzyme which is able to hydrolyze polygalacturan to a small size. Indeed, polygalacturonase activity is not detected in immature fruit while its activity increases with fruit ripening. Corresponding transcripts are also detected in ripening fruit. Until the 1990’s, it had been considered that polygalacturonase was a key enzyme for fruit softening; however, transgenic tomato (Solanum lycopersicum) fruit, in which polygalacturonase activity was suppressed to 1% in the wild type, showed almost same softening pattern as wild-type fruit (Smith et al., 1988). Polygalacturonase activity was recovered up to 60% in transgenic rin (ripening inhibitor) fruit, but did not affect fruit firmness (Giovannoni et al., 1989). These results suggest that polygalacturonase is not the sole determinant of fruit softening. Suppression of polygalacturonase, which prevents the depolymerization of polyuronide, contributed to the improvement of fruit shelf life, decreased cracking and helped processing by increasing the viscosity of paste or juice (Kramer et al., 1992; Langley et al., 1994; Schuch et al., 1991). Therefore, depolymerization of polyuronide mediated by polygalacturonase contributes to fruit texture rather than fruit softening. It is noted that the results of transgenic experiments described above are limited to tomato fruit. Besides tomato fruit, it has also been shown that polygalacturonase expression is regulated by ethylene. Both the expression of polygalacturonase and softening of fruit are severely suppressed in ripening controlled peach (Prunus persica) (Hayama et al., 2006) and melon (Cucumis melo) (Nishiyama et al., 2007) treated with 1-methylecyclopene. Tatsuki and Endo (2006) showed the relationship between ethylene sensitivity and apple (Malus domestica) fruit shelf life. Inaba (2007) summarized the necessity of ethylene for fruit softening and expression of related enzymes. Ethylene is essential for softening of several fruit species and the expression of polygalacturonase; however, direct evidence of role of polygalacturonase in fruit softening is still unclear.

2. Post polygalacturonase

Following polygalacturonase, many researchers tried to mine new candidates for fruit-softening enzymes. Other cell-wall-metabolizing enzymes or proteins, such as pectin methyltransferase (Tieman et al., 1992), xyloglucan endotransglucosylase/hydrolase (XTH) (Arrowsmith and de Silva, 1995), \( \beta-1,4 \)-glucanase (Brummell et al., 1999a), and expansin (Brummell et al., 1999b), were also examined for their contribution to fruit softening using transgenic experiments in which gene expression was suppressed; however, except for expansin, these enzymes do not contribute to the softening of tomato fruit (Brummell and Harpsper, 2001). Although the changes were small, expansin-suppressed fruit showed rather limited fruit softening and over-expressed fruit showed softening progress compared to the wild type (Brummell et al., 1999b). The precise action of expansin is unclear. Its role in polysaccharides may be partial and transgenic fruit showed complex changes in cell-wall polysaccharides.

Besides studies of cell-wall-modifying enzymes, changes in cell-wall polysaccharides during fruit ripening have been often reported. Polyuronide depolymerization mediated by polygalacturonase is observed during ripening although its extent seems to vary depending on the fruit species (Brummell, 2006). Polyuronide is considerably depolymerized into small molecules in melting or highly softened stages in fruit such as avocado (Persea americana) (Huber and O’Donoghue, 1993; Sakurai and Nevis, 1997; Wakabayashi et al., 2000), tomato (Brummell and Labavitch, 1997; Huber and O’Donoghue, 1993) and kiwifruit (Actinidia delicosa) (Redgwell et al., 1992; Terasaki et al., 2001). Extremely limited depolymerization of polyuronide during ripening was even observed in strawberry (Fragaria × ananassa) (Huber, 1984), apple (Yoshioka et al., 1992), banana (Musa spp.) (Wade et al., 1992), and pepper (Capsicum annuum) (Harpsper et al., 2002). Recently, Goulao and Oliveira (2008) summarized the extent of depolymerization of pectic polysaccharides and other wall components in various fruit species during ripening. Depolymerization of polyuronide occurs at a relatively late stage of ripening; therefore, it may contribute to cell-wall breakdown with over-ripening or may alter fruit textural properties. In addition to polyuronide depolymerization during ripening, the solubilization of pectic polysaccharides is also observed (Brummell and Labavitch, 1997; Huber and O’Donoghue, 1993). ‘Solubilization’ can be defined as polysaccharides, which previously could not dissolve in water or a certain buffer, become soluble the solutions. It is clear that cell-wall materials (mass) decrease with fruit ripening. Increasing the solubility of cell polysaccharides seems to be a general event during fruit softening. Moreover, the solubilization of wall polysaccharides occurs without a degree of polymerization in kiwifruit and nectarine (Prunus persica) (Dowson et al., 1992; Redgwell et al., 1992). Corresponding to the solubilization of pectic polymer, the loss of arabinosyl and galactosyl residues from wall polysaccharides is widely observed in many kinds of fruit (Gross and Sams, 1984). Except for homogalacturonan, almost all pectic or hemicellulosic polysaccharide backbones constituting the cell wall possess branched side chains. The release of neutral sugar residues, which may be located on the
surface of wall polysaccharides, attributes to changes in the structure of side chains and the interaction of neighboring polysaccharides. The changed wall polysaccharide properties are assumed to change the sensitivity of enzymatic degradation or accessibility of other glycan hydrolases to polysaccharide substrates internally. Indeed, the release of galactosyl residues from side chains of pectic polysaccharides is at least involved in fruit softening (Smith et al., 2002, see details below). Therefore, enzymes addressing side chains, such as β-galactosidase and α-L-arabinofuranosidase, might play important roles in fruit softening and textural change during ripening. Pectic polysaccharides and related enzymes are shown schematically in Figure 1.

3. β-Galactosidase for fruit softening

Generally limited in pectic substances galactose is composed of a side chain of pectic polysaccharides such as arabinogalactan or galactan branched from the rhamnogalacturonan backbone (Fig. 1). Galactose is also found in hemicellulosic polysaccharides. In tomato fruit, the loss of galactosyl residue during fruit ripening is observed in pectic galactan. β-Galactosidases (EC 3.2.1.23) are characterized by their ability to hydrolyze terminal, non-reducing β-D-galactosyl residues from numerous substrates. In higher plants, β-galactosidase is the only enzyme that is able to hydrolyze galactosyl residues from cell wall polysaccharides and no other enzyme capable of cleaving β-1,4-galactan in an endo fashion has been identified (Smith et al., 1998). The activity of β-galactosidase was measured in various fruit species (Table 1). The existence of multiple isoforms (isozymes) was also reported in several kinds of fruit and their different galactosyl-hydrolysing abilities were characterized using native cell-wall polysaccharides and synthetic substrate. Each isozyme discriminated at the protein level by chromatography possesses different substrate specificities and they are able to hydrolyze the galactosyl residue at different positions (linkage). For example, a ripening-related β-galactosidase isozyme is capable of hydrolysing a native substrate, β-1,4-galactan, more effectively than an artificial substrate, 4-nitrophenyl-β-D-galactopyranoside. Among three β-galactosidase isozymes (β-Gal I to III) isolated from tomato fruit, β-Gal II is capable of releasing galactosyl residues from the pectic side chain and activity increased during ripening (Carrington and Pressey, 1996; Pressey, 1983). β-Galactosidases isolated from melon fruit are able to reduce the molecular size of cell-wall polysaccharides, as observed during ripening (Ranwala et al., 1992). DeVeau et al. (1993) also indicated that β-galactosidase purified from avocado fruit is capable of depolymerizing chelator-soluble pectin of tomato. Among three avocado β-galactosidase isoforms, AV-GAL I, AV-GAL II and AV-GAL III, AV-GAL III shows the highest activity of releasing free galactosyl residue from the native cell-wall polysaccharides isolated from fruit and its activity increases with fruit softening whereas AV-GAL I can not hydrolyze native polysaccharides (Tateishi et al., 2001a). Yoshioka et al. (1995) indicated that one β-galactosidase fraction, termed GA-ase I, from apple fruit, whose activity

![Fig. 1. Schematic model of pectic polysaccharides and related enzymes. Polygalacturonase hydrolyzes homogalacturonan, which was de-esterified previously mediated by pectin methylesterase, reducing size of pectin. Prior to depolymerization, pectic substances are solubilized and galactosyl and arabinosyl residues are released. β-galactosidase and α-L-arabinofuranosidase approach the side chains and release neutral sugar residues. Abbreviated hemicellulosic polysaccharides are also likely their substrates.](image-url)
increased during storage, effectively released galactose from pectic polysaccharides but not from larch wood arabinogalactan, whereas GA-ase II, III, and IV were able to hydrolyze the arabinogalactan. Kitagawa et al. (1995) also indicated that five β-galactosidase isoforms (Gal I to V) fractionated from Japanese pear (Pyrus pyrifolia) fruit possessed different activities against native cell-wall polysaccharides. In this case, Gal III has the highest activity of releasing galactose from Na2CO3-soluble pectic polysaccharides, guanidine thiocyanate-soluble pectic polysaccharides, and hemicellulosic polysaccharides isolated from Japanese pear. Lazan et al. (2004) also fractionated β-galactosidase from papaya (Carica papaya) fruit into three isoforms with different substrate specificities and indicated that the enzymes were able to solubilize and depolymerize not only pectic polysaccharides but also hemicellulosic polysaccharides. Thus, β-galactosidase can be clearly separated at the protein level with different substrate specificities. This implies that each isoform plays a different role in the modification of cell-wall architecture during fruit development and ripening. These differences may depend on fruit species with different characters. If we evaluate β-galactosidase in fruit softening, it is necessary to determine softening-related β-galactosidase at the gene level.

4. Genes for β-galactosidase

Genes for β-galactosidase have been identified in many kinds of fruit and consist of a small gene family. At least seven β-galactosidase genes are expressed during development and ripening of tomato fruit (Smith and Gross, 2000). A family of β-galactosidase was also reported in strawberry (Trainotti et al., 2001), Japanese pear (Tateishi et al., 2001b, 2005b), avocado (Tateishi et al., 2002, 2007), pear (Pyrus communis) (Mwaniki et al., 2005, 2007; Sekine et al., 2006), and grape (Vitis vinifera) (Nunan et al., 2001). Some gene expressions overlapped. According to an increase in activity during fruit ripening and the ability to hydrolyze native cell-wall polysaccharides, β-galactosidase isoforms isolated from tomato (β-Gal II, Pressey, 1983), apple (Ross et al., 1994), and Japanese pear (Gal III, Kitagawa et al., 1995) are considered as softening-related β-galactosidase. In addition, N-terminal amino acid sequences of the proteins were analyzed and the corresponding cDNA clones were revealed (Ross et al., 1994; Smith et al., 1998; Tateishi et al., 2001b). Accumulation of their mRNAs was mainly specific to ripening fruit. Transgenic experiments using tomato fruit revealed which β-galactosidase isoform is specific to fruit softening. The significant contribution of β-Gal II to tomato fruit softening was reported using antisense suppression of TBG4 which encodes β-Gal II (Smith et al., 2002). Tomato fruit transformed with the antisense TBG4 gene softened with ripening, however, it was firmer than the wild type when fully ripe. Interestingly, total β-galactosidase activity and loss of galactosyl residues were not significantly affected.

Transgenic experiments also identified the roles of other β-galactosidase isoforms in the development and ripening of tomato fruit. Strong accumulation of TBG1 mRNA of another β-galactosidase isoform from tomato was observed at breaker and turning stages; however, down-regulated TBG1 expression caused no effect on fruit softening and activity of β-galactosidase in fruit (Carey et al., 1995, 2001). mRNA of TBG6, which is also a β-galactosidase isoform from tomato, accumulated during the fruit developmental stage, especially 20–30 days after pollination (Smith and Gross, 2000). Fruit with down-regulated TBG6 expression showed cracking on the surface, indicating the contribution of TBG6 to fruit development (Moctezuma et al., 2003). These observations suggest that individual β-galactosidase isoforms play a distinct role in fruit development and ripening, or more broadly, plant development. Senescence-, abscission- and sugar starvation-related β-galactosidase has also been reported in some plant species (de Alcântara et al., 2006; King et al., 1995; Lee et al., 2007; Wu and Burns, 2004).

Table 1. Enzymatic activities of β-galactosidase found in various fruit species.

<table>
<thead>
<tr>
<th>Fruit</th>
<th>References</th>
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<tr>
<td>Apple (Malus domestica)</td>
<td>Bartley, 1974; Dick et al., 1990; Ross et al., 1994; Yoshioka et al., 1995</td>
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<tr>
<td>Avocado (Persea americana)</td>
<td>DeVeau et al., 1993; Tateishi et al., 2001a</td>
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<td>Bell pepper (Capsicum annuum)</td>
<td>Ogasawara et al., 2007</td>
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<tr>
<td>Grape (Vitis vinifera)</td>
<td>Barnavon et al., 2000; Nunan et al., 2001</td>
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<tr>
<td>Japanese pear (Pyrus pyrifolia)</td>
<td>Kitagawa et al., 1995; Mwaniki et al., 2007; Tateishi and Inoue, 2000; Tateishi et al., 2001b, 2005b</td>
</tr>
<tr>
<td>Kiwifruit (Actinidia delicosa)</td>
<td>Bonghi et al., 1996; Wegrzyn and MacRae, 1992</td>
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<tr>
<td>Musk melon (Cucumis melo)</td>
<td>Fils-Lycaon and Buret, 1991; Ranwala et al., 1992</td>
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<tr>
<td>Papaya (Carica papaya)</td>
<td>Ali et al., 1998; Lazan et al., 1995, 2004</td>
</tr>
<tr>
<td>Peach (Prunus persica)</td>
<td>Brummell et al., 2004</td>
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<tr>
<td>Pear (Pyrus communis)</td>
<td>Ahmed and Labavitch, 1980; Mwaniki et al., 2005, 2007</td>
</tr>
<tr>
<td>Tomato (Solanum lycopersicum)</td>
<td>Carey et al., 1995; Smith and Gross, 2000</td>
</tr>
<tr>
<td>Zucchini (Cucurbita pepo)</td>
<td>Balandrán-Quintana et al., 2007</td>
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5. Putative roles of \( \alpha-L \)-arabinofuranosidase and arabinosyl-containing polysaccharides

In addition to the loss of galactosyl residues, the release of arabinosyl residues during fruit softening was also observed commonly during ripening in many kinds of fruit (Gross, 1984; Gross and Sams, 1984). The extent of arabinose loss during ripening varies between fruit species (Brummell, 2006). For example, extensive loss of arabinosyl residues is observed in pear and blueberry (\textit{Vaccinium} ssp.) but is absent in watermelon (\textit{Citrus} lanatus), apricot (\textit{Prunus armeniaca}) and plum (\textit{Prunus domestica}) (Brummell, 2006; Gross, 1984; Gross and Sams, 1984). Terminal arabinosyl residues are widely distributed in pectic and hemicellulosic polysaccharides such as arabinan, arabinoxylan, arabinogalactan, arabinoxyl glucan, and glucuronoxarabinoylan (Beldman et al., 1997; Saha, 2000; Sozzi et al., 2002b). \( \alpha-L \)-Arabinofuranosidase (\( \alpha-L \)-arabinofuranosidase arabinofuranohydrolase, EC 3.2.1.55) is an enzyme which is able to hydrolyze non-reducing arabinofuranosyl residues.

Increased \( \alpha-L \)-arabinofuranosidase activity during ripening or storage was observed in apple (Yoshioka et al., 1995), Japanese pear fruit (Tateishi et al., 1996), avocado (Tateishi et al., 2001a), tomato (Sozzi et al., 2002a), persimmon (\textit{Diospyros kaki}) (Xu et al., 2003), peach (\textit{Prunus persica}) (Brummell et al., 2004a; Jin et al., 2006), and European and Chinese pear (\textit{Pyrus bretschneideri}) (Mwaniki et al., 2007). Ethylene seems to promote an increase in the activity of climacteric fruit, although an expression pattern of an \( \alpha-L \)-arabinofuranosidase gene did not coincide with the fruit-softening pattern in Chinese pear (Mwaniki et al., 2007). The activity did not increase in several fruit species and some tomato cultivars during ripening (Itai et al., 2003). \( \alpha-L \)-Arabinofuranosidases in fruit have been purified from Japanese pear (Tateishi et al., 1996, 2005a) and partially purified from apple (Yoshioka et al., 1995) and tomato (Sozzi et al., 2002b) and some enzymatic properties have been characterized; however, in \textit{vivo} substrates for \( \alpha-L \)-arabinofuranosidases have not been clarified in detail.

The existence of arabinosyl residues consisting of cell-wall polysaccharides seems to play an important role in the adhesion of each polysaccharide or cell to cell. Iwai et al. (2001, 2002) transformed a T-DNA insertion into \textit{Nicotiana plumbaginifolia} and obtained a non-organogenic callus with loosely attached cells (nolac). Neutral-sugar side chains, composed mainly of linear arabinan, were absent in a nolac mutant, suggesting the role of arabinan in cell adhesion. The presence of arabinan sometimes related to the mealy texture of fruit. A larger amount of tightly bound arabinosyl-containing polysaccharides was observed in softening-suppressed, colorless and non-ripening (\textit{Cur}) mutant tomato fruit, which has a mealy texture (Orfila et al., 2001, 2002; Thompson et al., 1999). In peach fruit, the loss of arabinosyl residues from both loosely and tightly bound matrix glycans was observed in normal ripening fruit, but not in mealy fruit, which showed a decline in the loss of arabinosyl residues containing polysaccharides firmly attached to cellulose (Brummell et al., 2004a, 2004b). In apple fruit, arabinosyl residues decreased during the over-ripening stage (Peña and Carpita, 2004) and a decrease of arabinosyl residues in cell wall polysaccharides was observed in the development of apple fruit mealiness postharvest (Nara et al., 2001). Therefore, arabinosyl residue is usually widely lost during fruit ripening; however, in mealy-textured fruit, the arabinosyl residue becomes tightly bound to matrix glycan, including cellulose, and consequently the loss of arabinosyl residue seems to be suppressed. Although direct evidence was not shown, modification of arabinosyl-containing cell-wall polymer may play an important role in the alteration of fruit texture in relation to cell-to-cell adhesion.

6. Expression pattern of \( \alpha-L \)-arabinofuranosidases

There are relatively few reports of \( \alpha-L \)-arabinofuranosidase isolated from fruit with biochemical characteristics compared to \( \beta \)-galactosidase. \( \alpha-L \)-arabinofuranosidases were classified into five glycoside hydrolase (GH) families (family 3, 43, 51, 54, and 62), and biochemically characterized \( \alpha-L \)-arabinofuranosidases from higher plants are found in families 3 and 51 (Fig. 2). This classification is based on the amino acid sequence rather than on substrate specificity (Coutinho and Henrissat, 1999; http://www.cazy.org/, August 10, 2008).

Ferré et al. (2000) purified \( \alpha-L \)-arabinofuranosidase from a monocotyledon, barley (\textit{Hordeum vulgare}) belonging to the GH family 51. The \( \alpha-L \)-arabinofuranosidase is an arabinoxylan arabinofuranohydrolase which is able to release arabinose from both singly and doubly substituted xylose. The enzyme could not release arabinose from linear or branched-chain arabinan, although distinct results of the enzyme activity against arabinan were shown by Lee et al. (2001). \( \alpha-L \)-Arabinofuranosidase isolated by two groups was able to hydrolysis arabinoxylan and produced monomeric arabinose (arabinoxylan arabinofuranohydrolase) (Ferré et al., 2000; Lee et al., 2001). Two \( \alpha-L \)-arabinofuranosidases, ASD1 and ASD2, belonging to family 51, were found in Arabidopsis (\textit{Arabidopsis thaliana}). ASD1 showed higher expression in cell proliferation zones, the vascular system, developing and regressing floral tissues, and floral abscission zones, while ASD2 was expressed in the vasculature of older root tissue and in some floral organs and floral abscission zones (Fulton and Cobbett, 2003). Itai et al. (2003) reported that family 51 \( \alpha-L \)-arabinofuranosidase cloned from tomato fruit were expressed during fruit development and declined during ripening. Sekine et al. (2006) reported that family 51 \( \alpha-L \)-arabinofuranosidase
cloned from pear fruit was expressed constitutively during fruit storage. From only expression analysis of family 51 α-L-arabinofuranosidase, it seems to contribute less to fruit softening, but its detailed role in physiological metabolism remains unclear. α-L-Arabinofuranosidase is also classified into GH family 3 besides family 51, although they are clearly distinguishable based on sequence homology (Fig. 2). Lee et al. (2003) purified a new α-L-arabinofuranosidase from monocotyledonous barley and indicated the enzyme belongs to GH family 3. Following barley, family 3 α-L-arabinofuranosidase was purified from Japanese pear, a dicotyledonous plant (Tateishi et al., 2005a). These were the first reports that α-L-arabinofuranosidases isolated from higher plants belong to GH family 3, although they are clearly distinguishable based on sequence homology (Fig. 2). 

α-L-Arabinofuranosidase is also classified into GH family 3, despite family 51, which seems to contribute less to fruit softening, but its detailed role in physiological metabolism remains unclear. α-L-Arabinofuranosidases and β-xylosidases from Arabidopsis (AY243509 and AY243510), PPARF1 from Japanese pear (AB073311), PpARF2 from pear (AB067643), AXAH-I and AXAH-II from barley (AF320324 and AF320325), XYL1 from strawberry (AY486104), ARAF1 and XYL from barley (AY029259 and AY029260), and LeXYL1 and LeXYL2 from tomato (AB041811 and AB041812) and LeARfXYL to 4 from tomato (unpublished). AtBXL1 to 7 from Arabidopsis, RsArf1 from radish (AB234292) and MsXYL1 from alfalfa (EF569968). 

**Fig. 2.** Phylogenetic relationships of bi-functional α-L-arabinofuranosidase/β-xylosidases and β-xylosidases classified into GH family 3 and family 51. Amino acid sequences were aligned with the ClustalW program and phylogenetic tree was drawn. ASD1 and ASD2 from Arabidopsis (AY243509 and AY243510), PPARF1 from Japanese pear (AB073311), LeARF from tomato (AB073310), PpARF2 from pear (AB067643), AXAH-I and AXAH-II from barley (AF320324 and AF320325), XYL1 from strawberry (AY486104), ARAF1 and XYL from barley (AY029259 and AY029260), and LeXYL1 and LeXYL2 from tomato (AB041811 and AB041812) and LeARfXYL to 4 from tomato (unpublished). AtBXL1 to 7 from Arabidopsis. RsArf1 from radish (AB234292) and MsXYL1 from alfalfa (EF569968).
### Table 2. Substrate specificities and grouping of α-L-arabinofuranosidase and β-xylosidase.

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<th>Common name</th>
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<td>3</td>
<td>Bifunction (4NPAf &gt; 4NPXp), Xyl from xylanase treated AX</td>
<td>ARA-I</td>
<td>Lee et al., 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>4NPXp, Xyl from xylanase treated AX</td>
<td>XYL</td>
<td>Lee et al., 2003</td>
</tr>
<tr>
<td>Medicago sativa</td>
<td>Alfalfa</td>
<td>3</td>
<td>Bifunction (4NPAf &gt; 4NPXp), Ara from arabinan, Ara and Xyl from CWM and AX, Xyl from xylanase-treated xylan</td>
<td>MsXyl1</td>
<td>Xiong et al., 2007</td>
</tr>
<tr>
<td>Pyrus communis</td>
<td>European pear</td>
<td>51</td>
<td>Unknown</td>
<td>PeARF1</td>
<td>Sekine et al., 2006</td>
</tr>
<tr>
<td>Prunus persica</td>
<td>Peach</td>
<td>3</td>
<td>Unknown</td>
<td>PpARF/XYL</td>
<td>Hayama et al., 2006</td>
</tr>
<tr>
<td>Pyrus pyrifolia</td>
<td>Japanese pear</td>
<td>51</td>
<td>Bifunction (4NPAf &gt; 4NPXp), Ara from arabinan and CWM. No activity against AX</td>
<td>PpARF1</td>
<td>Only database</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>Unknown</td>
<td>PpARF2</td>
<td>Tateishi et al., 2005a</td>
</tr>
<tr>
<td>Raphanus sativus</td>
<td>Radish</td>
<td>3</td>
<td>Bifunction (4NPAf &gt; 4NPXp), Ara from arabinan and AX</td>
<td>RsAraf1</td>
<td>Kotake et al., 2006</td>
</tr>
<tr>
<td>Solanum lycopersicum</td>
<td>Tomato</td>
<td>51</td>
<td>Unknown</td>
<td>LeARF</td>
<td>Itai et al., 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>Unknown</td>
<td>LeXYL1</td>
<td>Itai et al., 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>Unknown</td>
<td>LeXYL2</td>
<td>Itai et al., 2003</td>
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<tr>
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<td>3</td>
<td>Bifunction (4NPAf=4NPXp)</td>
<td>LeARfXYL1</td>
<td>Tateishi et al., (unpublished data)</td>
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<td>Tateishi et al., (unpublished data)</td>
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<td>LeARfXYL3</td>
<td>Tateishi et al., (unpublished data)</td>
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<td>Bifunction (4NPAf&lt;4NPXp)</td>
<td>LeARfXYL4</td>
<td>Tateishi et al., (unpublished data)</td>
</tr>
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</table>

<sup>2</sup> Indicates relatively preferable substrates, although each isozyme showed broad substrate specificities. Activity against oligosaccharides is not shown. Abbreviations: 4NPAf, 4-nitrophenyl α-L-arabinofuranoside; 4NPXp, 4-nitrophenyl β-xylopyranoside; Ara, arabinose; AX, arabinoxylan; CWM, cell-wall materials; Xyl, xylose.
expression of family 3, was also expressed in softened fruit but not
in stony hard peach fruit (Hayama et al., 2006). The expressions of PpARF2 and PpARF/XYL were regulated by ethylene (Mwaniki et al., 2007; Hayama et al., 2006). FaXYL1, which exhibited only β-xylosidase activity isolated from strawberry, was expressed higher and accumulated at an earlier stage in a softer strawberry cultivar (Bustamante et al., 2006). According to expression analysis of family 3 α-L-arabinofuranosidase and β-xylosidase, they seem to play a certain role in fruit softening while they were expressed at a relatively late stage of ripening. A transgenic experiment is necessary to elucidate the role of α-L-arabinofuranosidase in fruit softening or textural changes.

7. Substrate specificity of α-L-arabinofuranosidase

Several enzymes belonging to both families 3 and 51 showed bi-functional activity against artificial substrates (4-nitrophenyl α-L-arabinofuranoside or 4-nitrophenyl β-D-xylopyranoside); therefore, the enzymes are considered as bifunctional α-L-arabinofuranosidase/β-xylosidase. It is more difficult and complex to understand substrate specificity against native substrates of the enzymes. The ability of enzymes or isozymes to release both arabinosyl and xylosyl residues from various native substrates in vitro is distinct (summarized in Table 2). There is no correlation between substrate specificities and the primary structure of the enzyme, suggesting the necessity of biochemical properties when using enzyme annotations in a database. Minic et al. (2004, 2006) showed that several β-xylosidase and α-L-arabinofuranosidase from Arabidopsis possessed similar substrate specificities against both arabinan and arabinxyloylan and suggested that these broad substrate specificities are rather convenient for modification of the complex cell wall structure. On the other hand, α-L-arabinofuranosidase isolated from Japanese pear showed limited activity of releasing arabinose from pectic arabinan (Tateishi et al., 2005a). Radish α-L-arabinofuranosidase seems to hydrolyze only arabinosyl residues constituting arabinogalactan protein (Kotake et al., 2006). Although they showed broad substrate specificity in vitro, it suggests that substrates of the enzymes seem to be limited in vivo.

Conclusion

Cell-wall polysaccharides are composed of limited sugar residues; however, linkage diversity complicates its architecture. Moreover, many cell-wall-modifying enzymes consist of multiple isozymes found in the wall with different activities and expression patterns. Several cell-wall-modifying enzymes, such as polygalacturonase, pectin methylesterase, XTH, and β-1,4-glucanase were evaluated by a transgenic technique and showed less contribution of the enzymes to fruit softening. Among them, transgenic fruit, which suppressed the accumulation of an expansin or the activity of β-galactosidase, kept relatively higher mechanical strength than the wild type during ripening; however, fruit softening could not be prevented entirely. Consequently, limited contribution of each enzyme to fruit softening was shown. At present, it is suggested that the degradation of several cell-wall polysaccharides is necessary, and that numerous cell wall-metabolism enzymes are implicated in fruit softening. Therefore, the softening process requires the cooperative action of several enzymes (isozymes) to degrade cell-wall polysaccharides or the sequential degradation of wall polysaccharides.

On the other hand, several enzymes also contribute to the modification of cell-wall structures during plant development in addition to the softening observed during ripening. In the case of β-galactosidase, according to the expression pattern and substrate specificity, each isozyme is active against various galactosyl-containing polysaccharides and plays a role in cell-wall modification in several aspects of plant development besides softening. The roles of α-L-arabinofuranosidase in fruit ripening/softening are still unclear. It may contribute to a change in fruit texture involving softening according to the role of arabinosyl residues in wall polysaccharides, such as cell-to-cell adhesion. In vitro experiments revealed that α-L-arabinofuranosidases possess broad substrate specificities. The expression of α-L-arabinofuranosidase in non-fruit tissues indicates that the enzyme also plays a role in not only fruit softening but also plant development.

Although transgenic experiments are a good tool to assess whether cell-wall-modification enzymes contribute to fruit softening, this technique is not sufficient to elucidate their biochemical characteristics, such as substrate specificity. Moreover, the transgenic technique is useful for limited fruit species such as tomato. Numerous fruit species and cultivars are found with their own unique characters related to their softening pattern and textual changes; therefore, it is necessary to make genetically modified various plant species, in which the introduced individual enzyme (isoyme) gene is regulated, and to evaluate them. It is also necessary to investigate the cooperative effects or interaction of multiple enzymes on fruit softening. Transgenic plants with introduced multiple cell-wall-modifying genes or the progeny of plants crossed by a previously transformed parent may reveal the more precise mechanism of fruit softening. These experiments will also be helpful for producing high quality fruit.

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