

Fruit Softening: Revisiting the Role of Pectin

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37 **Review**

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39 **Trends**

40 Cell wall remodeling plays an important role in the texture changes in ripening fruits, but the
41 precise underlying mechanisms have remained somewhat elusive.

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43 Recent studies have identified distinct mechanisms of pectin degradation during ripening and
44 new insights into the structure of primary plant cell walls and the role of pectin.

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46 Experiments with ripening fruits from a range of transgenic plants have demonstrated that
47 softening can be delayed by silencing or knocking out genes encoding pectate lyase and
48 polygalacturonase.

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50 New techniques for studying cell wall structure indicate that pectin polymers can be tightly
51 associated with cellulose and fully integrated into the structure of the extracellular matrix.

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66 Fruit softening, which is a major determinant of shelf life and commercial value, is the
67 consequence of multiple cellular processes, including extensive remodeling of cell wall structure.
68 Recently, it has been shown that pectate lyase, an enzyme that degrades de-esterified pectin in
69 the primary wall, is a major contributing factor in tomato fruit softening. Studies of pectin
70 structure, distribution and dynamics have indicated that pectins are much more tightly
71 integrated with cellulose microfibrils than previously thought and have novel structural features,
72 including branches of the main polymer backbone. Moreover, recent studies of the significance
73 of pectinases, such as pectate lyase (PL) and polygalacturonase (PG), are consistent with a causal
74 relationship between pectin degradation and a major effect on fruit softening.

75 76 **Fruit Texture Determines Shelf life and Quality**

77 Softening is a hallmark of ripening in most fleshy fruits. Depending on species and cultivar, a
78 certain degree of softening is desirable, while excessive softening typically leads to postharvest
79 decay or consumer rejection. Decades of research have attempted to address both the
80 underlying mechanisms of fruit softening and its manipulation. Presently, control of softening is
81 achieved through delay or attenuation of the entire ripening process [1], but it is preferable to
82 uncouple fruit softening from other aspects of ripening that are essential for making fruit edible
83 and appealing. Thus, a major goal has been the development of methods for controlling softening
84 without affecting color, flavor, aroma, or nutritional value.

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86 The structural basis of fleshy fruit texture is complex, but depends on the cell wall's ability to
87 maintain turgor pressure and mediate cellular adhesion. Dynamics in the osmotic state of fruit
88 tissue and remodeling of the cell wall are likely the predominant causes of fruit softening, but a
89 more detailed mechanistic understanding has remained elusive. More generally, while many
90 models have been proposed of how structural components of the cell wall interact and
91 contribute to its biomechanical properties, we are far from a 'universal theory' of plant cell wall
92 mechanics, and the macromolecular structure/function relationships of cell wall polymer
93 networks, and the mechanisms for their assembly and subsequent remodeling, remain an active
94 area of research [2].

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96 **Tomato as a Model System**

97 Much of the work on fleshy fruit ripening has focused on tomato, as its economic importance,
98 genetic resources, and extensive ripening-associated softening contribute to its status as the
99 leading model for fleshy fruit biology [3]. As with most plant primary cell walls, those of tomato
100 are principally composed of three classes of polysaccharides: cellulose, hemicelluloses (the most
101 abundant of which in tomato cell walls is **xyloglucan**), and pectins. In tomato, and in many other
102 fleshy fruits, some of the most pronounced ripening associated changes occur in the pectic
103 polysaccharides and these changes have therefore received the most sustained attention. Pectins
104 are the most structurally complex plant cell wall polysaccharides and they play an important role
105 in cell-to-cell adhesion. Three major classes of pectins have been identified: homogalacturonan
106 (HG), which is composed of a backbone of 1,4-linked α -D-galacturonosyluronic acid residues;
107 rhamnogalacturonan 1 (RG-I), comprising interspersed α -D-galacturonosyl residues and
108 rhamnosyl residues, with sidechains of galactosyl and arabinosyl residues; and
109 rhamnogalacturonan II (RG-II), which is less abundant than the other two classes, but has a
110 complex composition. RG-II generally exists as an RG-II borate diester dimer, ostensibly linking
111 HG-connected pectin in the wall and structural data indicate that HG, RG-I, and RG-II are
112 interconnected by covalent linkages via their backbones (see recent review by [4]). Degradation
113 of pectic polymers during ripening occurs as a result of the action of several pectin metabolizing
114 enzymes (Figure 1). In this regard, one of the most abundant ripening-induced enzymes is endo-
115 **polygalacturonase** (referred to here as PG, and which is distinct from an exo-acting acting
116 polygalacturonase), which hydrolyzes HG. However, silencing of the gene encoding the major
117 ripening-associated PG isozyme yielded only minimal improvements in slowing the rate of fruit
118 softening [5, 6, 7]. Subsequent research targeted silencing of other ripening-associated pectin
119 metabolic enzymes, including **pectin methylesterase** (PME) [8, 9] and **galactanase** (β -Gase) [10],
120 but only a minor effect on softening was demonstrated. The lack of success in preventing fruit
121 tissue degradation prompted attempts to modify softening through overexpression or silencing
122 of genes encoding other cell wall modifying proteins, including expansin [11]. However, again
123 only modest changes in firmness were observed, suggesting that fruit softening depends on a
124 more complex orchestration of the remodeling of multiple cell wall components.

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126 Recently, a central role for pectin depolymerization in tomato fruit softening was confirmed by
127 the dramatic effect of silencing a ripening-associated pectin degrading enzyme, pectate lyase (PL)
128 [12, 13]. Microscopy studies of fruit pericarp from tomato transgenic lines in which PL activity
129 had been silenced using **RNAi** [12], showed changes in the molecular weight, solubility and
130 distribution of pectic polysaccharides. In wild type tomato fruits, de-esterified pectins are
131 concentrated in the tricellular junction zones between cells and are also present in the middle
132 lamella region, and these pectins usually undergo depolymerization and solubilization from the
133 wall during ripening. However, in the PL depleted lines, these junction zones remain rich in
134 deesterified pectins (Figure 2). Here we reevaluate the role of pectin in fruit texture and softening
135 in the context of recent advances in our understanding of pectin and cell wall structure and
136 highlight some promising future directions for this field.

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138 **Pectin and New Models of Cell Wall Structure**

139 Several models have been proposed of how the major cell wall components, cellulose,
140 hemicellulose and pectin, interact to endow the cell wall with its biophysical properties. Until
141 recently, the most prevalent model was the ‘tethered network’ [14], which proposed that
142 cellulose microfibrils were coated and interlocked by xyloglucan, or other hemicellulose polymers,
143 forming a load-bearing network. Pectin was viewed as making a relatively independent
144 contribution to wall mechanics, primarily through its ability to form so-called ‘egg box’ structures,
145 in which divalent calcium ions cross-linked chains of deesterified HG, leading to strengthening of
146 the gel matrix independent of any cellulose-pectin interactions. Calcium-mediated crosslinking
147 of HG chains is thought to be particularly important in mediating cellular adhesion, as
148 deesterified HG is abundant in the middle lamella [15].

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150 Recent studies have challenged important aspects of this ‘tethered network’ model and instead
151 collectively suggest that cellulose-pectin contacts may be more prevalent and make more
152 important contributions to wall biomechanical properties than was previously thought.
153 Zykwincka et al. [16] demonstrated that the arabinan and galactan sidechains of RG-I can bind

154 cellulose microfibrils *in vitro* with similar affinities as xyloglucan. This result is also supported by
155 experiments showing that exogenously supplied pectin-derived galactan and arabinan bind
156 bacterial cellulose during its synthesis [17, 18]. The most compelling evidence for extensive
157 cellulose-pectin contacts *in planta* has come from **solid-state nuclear magnetic resonance**
158 **spectroscopy (ssNMR)** of ¹³C labeled Arabidopsis cell walls. Mae Hong and coworkers have used
159 this approach to demonstrate that pectin-cellulose interactions are extensive, while cellulose-
160 xyloglucan contacts are less prevalent than previously envisaged [19, 20, 21]. While some pectin
161 was observed to be highly dynamic, and likely filling the space between microfibrils,
162 approximately 25-50% of the cellulose chains exhibited close contact with pectins. Intriguingly,
163 pectin contacts with interior cellulose glucan chains were also detected, suggesting that HG and
164 pectin galactan chains may intercalate within, or between, nascent cellulose microfibrils during
165 their synthesis. Thus, it is proposed that pectins may directly contribute to the crosslinking of
166 cellulose microfibrils in the cell wall, potentially to a greater extent than the classical crosslinking
167 hemicellulose xyloglucan [22].

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169 In addition to these new insights on the interactions between pectin and other cell wall
170 components, **atomic force microscopy (AFM)** analyses of isolated pectin molecules has revealed
171 some unexpected structural features. Specifically, branched structures are often observed that
172 do not correspond to the neutral galactan and arabinan sidechains of RG-I [23, 24]. Rather, these
173 branches are proposed to be HG, based on their recalcitrance to dilute acid hydrolysis [24] and
174 susceptibility to a fungal PG [25]. Careful correlation of the kinetics of hydrolysis of isolated pectin
175 with the loss of structures observed by AFM indicated that conditions that caused hydrolysis of
176 galactan and arabinan sidechains failed to result in the loss of HG branches. These results were
177 interpreted as indicating the HG branches are attached to HG and not an RG-I backbone.
178 Aggregates consisting of HG, RG-I backbone and neutral sugar side-chains were also observed
179 [24]. The occurrence of such aggregates in the cell wall is debatable, especially since the AFM
180 experiments were performed with completely deesterified pectin in the absence of other wall
181 polymers [24]. Nevertheless, the covalently branched structure of HG represents a major revision
182 of existing models of pectin structural organization. Presently, the chemical nature of the bonds

183 involved in initiating such hypothetical HG branches remains unknown (Figure 3). Analysis of
184 these linkages is a compelling, but technically challenging goal since they are relatively scarce in
185 bulk pectin samples. Models to date of the HG domain of pectin present a relatively featureless
186 and uniform structure, in contrast to the complexity of RG-I and RG-II. The new view of a
187 reticulated HG structure that is fully integrated with the cellulose microfibril network of the cell
188 wall suggests that the enzymatic breakdown of pectin during fruit ripening may occur with more
189 surgical precision than has previously been appreciated.

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191 **Pectin metabolism in the fruit-ripening context**

192 Fruit ripening is marked by the secretion of a range of pectin degrading enzymes into the cell wall
193 and changes in pectin structure results from their combined, sequential and synergistic action.
194 There is also evidence that non-enzymatic mechanisms contribute to pectin polymer degradation,
195 but their overall contribution to softening is still unclear [26]. An increase in the solubility of
196 pectic polysaccharides is commonly associated with ripening, along with their depolymerization
197 and loss of neutral sugar sidechains. These soluble pectin molecules are likely derived from a cell
198 wall fraction that becomes more weakly attached to the extracellular matrix as ripening
199 progresses [27]. This pectin solubilization is the result of the collective action of a number of
200 classes of pectin degrading enzymes. Newly synthesized HG is highly methylesterified and, as
201 such, is less susceptible to enzymatic attack. Following secretion of the methylated HG to the
202 apoplast, PME hydrolyze the constituent methyl esters, yielding HG with a low degree of
203 methylation that can then be cleaved by PL or by PG. Accordingly, it was reported that silencing
204 PG in transgenic tomato fruits failed to alter the level of soluble pectin, but the soluble
205 **polyuronide** showed reduced levels of depolymerization [7, 12]. More recently it has been
206 reported that in PL minus fruits, with normal levels of PG, both pectin solubilization and
207 depolymerization were inhibited [12]. These data demonstrate that PL is necessary for pectin
208 solubilization, perhaps through degradation of branched HG, allowing disaggregation of polymers
209 for subsequent depolymerization by PG.

210

211 Tomato has been the most thoroughly studied fleshy fruit at the molecular and biochemical level,
212 but work in other species ([Table 1](#)) has proved equally important in helping our understanding of
213 pectin degradation and fruit softening; a notable example being research on strawberry (*Fragaria*
214 *× nanassa* Duch.). In contrast to tomato, silencing PG in strawberry seems to have a more
215 pronounced effect on texture than silencing PL [[28](#)]. At present it is not clear why these
216 differences between species are apparent, but they may reflect subtle variations in cell wall
217 structure and, or, activities of other cell wall degrading enzymes. As in tomato, analysis of
218 strawberry pectin in lines engineered to have reduced PL or PG was instructive [[28](#)]. Fruit
219 softening in wild type strawberry, in comparison to the transgenic lines, was characterized by
220 changes in the structure of pectin molecules, as determined by AFM. Softening was associated
221 with reduced abundance of large molecules, as well as, reduced levels of branched and multi-
222 branched polymers and lower branch lengths. These changes were accompanied by a decreased
223 propensity of the pectin molecules to form aggregates, proposed to be formed of HG and RG1
224 [[28](#)]. These may be common events in all fleshy fruits ([Table 1](#)).

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226 Cell wall localized expansins may also play an indirect role in pectin degradation during fruit
227 ripening. These proteins promote non-enzymatic cell wall loosening by binding cellulose and
228 hemicellulose and disrupting non-covalent crosslinking interactions [[29](#)]. Silencing of a ripening-
229 associated expansin in tomato was reported to increase fruit firmness and to attenuate pectin
230 depolymerization [[11](#)]. Thus, it is hypothesized that expansin-promoted wall loosening may
231 enhance accessibility of pectin to enzymatic degradation. However, it is unclear whether
232 expansins may also have a direct effect on softening that is independent of pectin degradation.

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234 The biological and structural significance and consequences of the ripening-related loss of neutral
235 sugar sidechains from RG-I, which has been reported to occur in a variety of fleshy fruits during
236 softening, including tomato [[27](#)], are unclear. However, enzymes that are presumed to be
237 involved in RG-I side chain modification, including ripening-associated β -Gase [[10](#)] and α -
238 arabinofuranosidase [[30](#)] have been characterized. Antisense silencing of the corresponding
239 genes has resulted in only small increases in the retention of firmness during ripening, but their

240 action may enhance access of PME, PL and PG to the pectic polysaccharide backbones. However,
241 in the context of the recent revised models of the cell wall, in which cellulose-pectin interactions
242 are frequent, the action of such enzymes might also, or alternatively, directly contribute to
243 softening by decreasing pectin-mediated tethering of cellulose microfibrils.

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246 **Concluding Remarks and Future Perspectives**

247 In tomato, after nearly 40 years of study, a substantial contribution of HG
248 depolymerization to fruit softening has been demonstrated. Evolving models of primary cell wall
249 structure suggest these results may be interpreted in terms of both breakdown of cell adhesion
250 molecules and the role of pectin in tethering cellulose microfibrils, but further testing of this
251 hypothesis will likely require the application of advanced imaging and analytical techniques, such
252 as AFM and ssNMR, to wild-type and mutant fruits with high spatial and temporal resolution
253 during ripening. We note that to date, null and higher order mutants have not been available for
254 most genes and gene families that encode specific ripening-associated cell wall enzymes, greatly
255 limiting interpretation of reverse genetic experiments. The generation of informative mutants is
256 now relatively straightforward with gene editing technologies, such as CRISPR, which has already
257 been used to confirm experimental results obtained with the PL RNAi lines [12]. Finally, with few
258 exceptions, biochemical and biophysical characterization of fruit softening has relied on bulk
259 samples of pericarp. The relative contribution of different tissue layers to fruit firmness and
260 softening remains unexplored. Towards this end, a more profound understanding of cell- and
261 tissue-specific gene expression in developing and ripening tomato fruit
262 (<http://tea.sgn.cornell.edu/>; [31] will enable the identification of new candidate genes and
263 promoters for the precise manipulation of gene expression to more precisely influence the
264 mechanisms that control cell wall modification and softening.

265

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277 **References**

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512

513

514 **Figure Legends**

515 **Figure 1.** Select structural features of pectin and targets of ripening-related enzymes.
516 Abbreviations used are PL = pectate lyase, PG = endo-polygalacturonase, PME = pectin
517 methylesterase, α -AFase = α -arabinofuranosidase, β -Gase = β -galactanase. Additional structural
518 features of pectin include acetyl, mono-xylosyl, and rhamnogalacturonan II sidechains attached
519 to the homogalacturonan or rhamnogalacturonan backbones. These are not depicted because
520 their relevance to ripening-related cell wall metabolism is unknown. In tomato the major PL and
521 PG enzymes are endo-acting, but the major ripening β -Gase is exo-acting.

522

523 **Figure 2.** The effect of PL depletion on deesterified homogalacturonan (HG) pectin in tricellular
524 junction zones in wild type (A) and (B) PL-minus fruits. The middle lamella region between
525 adjacent cells (C) is enriched in deesterified HG (blue). Intracellular turgor forces, indicated by
526 arrows, drive cellular separation. In wild type fruits deesterified HG is solubilized from the middle
527 lamella and tricellular junctions during ripening (A). In PL minus plants (B), deesterified pectin is
528 retained in the middle lamella region and at tricellular junctions to a greater extent than in the
529 wild type control. Figure is adapted from the scheme by Willats et al (2001), incorporating
530 experimental results from Uluisik et al (2016).

531

532 **Figure 3.** Structural representation of types of pectin molecules extracted from tomato pericarp
533 tissue and revealed by atomic force microscopy. The image shows molecular structures of
534 homogalacturonan (HG) alone (brown) and with rhamnosyl residues as rhamnogalacturonan 1
535 (RG-I) shown as multicolored polymers and arabinogalactan and arabinan as red cylinders. These
536 thicker chains reflect branched 'bottlebrush'-like arabinan / arabinogalactan structures. With
537 permission from Round, A.N. et al. (2010) A new view of pectin structure revealed by acid
538 hydrolysis and atomic force microscopy. Carbohydrate Research 345, 487–497 with permission.

539

540

541 Outstanding Questions

542 Transgenic lines in tomato and other fruits where pectin degrading enzymes have been silenced
543 or knocked out still show a considerable degree of softening. Changes in the cell wall and other
544 contributing factors still need to be elucidated.

545 The structure of the primary cell wall is incompletely understood and especially the nature of the
546 covalent links between different polysaccharide classes, including pectin.

547 The relatively recent discovery of covalently branched structure of HG needs further investigation
548 and especially the nature of the bonds involved. The interaction between branched pectin

549 molecules, RG-I and RG-II, and their location within the extracellular matrix is another area of
550 interest.

551

552 GLOSSARY

553 **Atomic Force Microscopy:** A scanning probe microscope where the microscope probe interacts
554 with the sample and allows imaging and measuring of samples at nanoscales. Deflection of the
555 probe by the sample is converted into an electrical signal.

556

557 **Cell Wall:** Highly complex extracellular matrix outside the plasma membrane of plant cells
558 composed of a range of polysaccharides and proteins. The main functions of the cell wall include
559 generating turgor pressure, controlling cell expansion, cell adhesion, support and a protection
560 against mechanical stress.

561

562 **Polygalacturonase (PG):** An enzyme that can hydrolyze the α -1,4 glycosidic bonds in
563 galactosyluronic acid polymers and therefore can hydrolyse HG.

564

565 **Galactanase (β -Gal):** An enzyme that can hydrolyze the β -1,4 glycosidic bonds between galactose
566 residues in pectin β (1-4) galactans that form sidechains on RG-I.

567

568 **Middle Lamella:** A layer between the cell walls of two adjacent plant cells that is rich in pectin.

569

570 **Pectate lyase (PL):** An enzyme that causes eliminative cleavage of polymers of α -1,4
571 galactosyluronic acid molecules to give oligosaccharides with 4-deoxy- α -D-galact-4-enuronosyl
572 groups at their non-reducing ends. PL preferentially acts on HG from which methyl esters have
573 first been removed by PME.

574

575 **Pectin Methyltransferase (PME):** An enzyme that catalyses the removal of the methyl ester groups
576 from pectin.

577 **Pectin:** Also known as pectic polysaccharides are a group of structurally complex molecules with
578 a backbone of α -1,4 galactosyluronic acid residues and which may also have other sugar residues
579 present as sidechains or in the backbone itself. These can include rhamnose, galactose and
580 arabinose. Three main classes of pectins are recognized (1) homogalacturonan (HG), which is
581 composed of a backbone of 1,4-linked α -D-galacturonosyluronic acid residues, (2)
582 rhamnogalacturonan 1 (RG-I), comprising interspersed α -D-galacturonosyl residues and
583 rhamnosyl residues, with sidechains of galactosyl and arabinosyl residues and (3)
584 rhamnogalacturonan II (RG-II), which is less abundant than the other two classes, but has a
585 complex composition.

586

587 **Polyuronides:** A polymer of uronic acid residues, in plant cell walls these would be composed of
588 a backbone of α -1,4 galactosyluronic acid residues with or without other sugar residues being
589 present. Polyuronides are therefore synonymous with pectin in this context and can refer to any
590 pectic polymers with a high proportion of galactosyluronic acid residues including HG and RG-I.

591

592 **RNAi:** RNA interference, a process whereby RNA molecules can be used to target and silence the
593 expression of a specific gene of interest.

594

595 **Solid-State Nuclear Magnetic Resonance Spectroscopy (ssNMR):** A spectroscopic technique for
596 studying the atomic structure of materials in the solid state by manipulating and correlating spin
597 states of nuclei using strong magnetic fields.

598

599 **Xyloglucan:** A cell wall polysaccharide with a backbone of β 1-4-linked glucose residues, most of
600 which are substituted with 1-6 linked xylose sidechains.

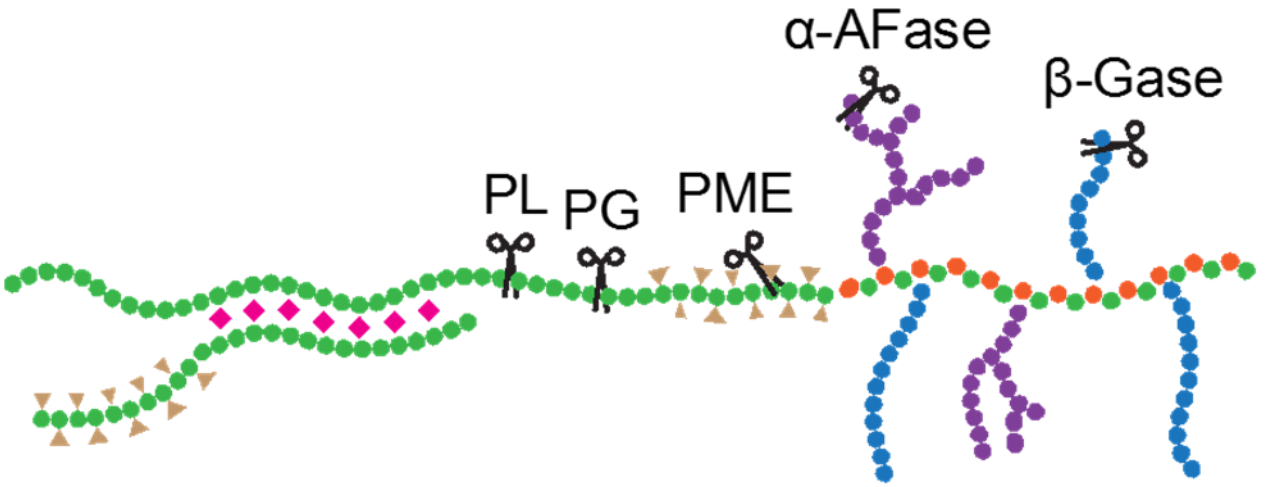
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Table 1. Pectin remodelling activities and, or, gene expression observed in ripening fruits

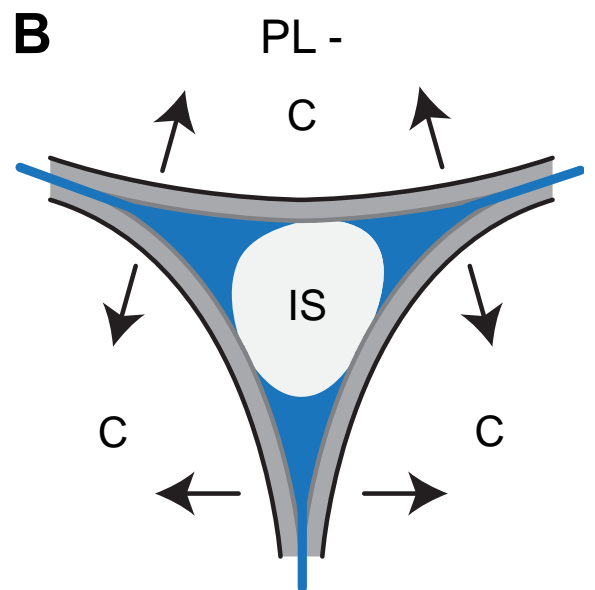
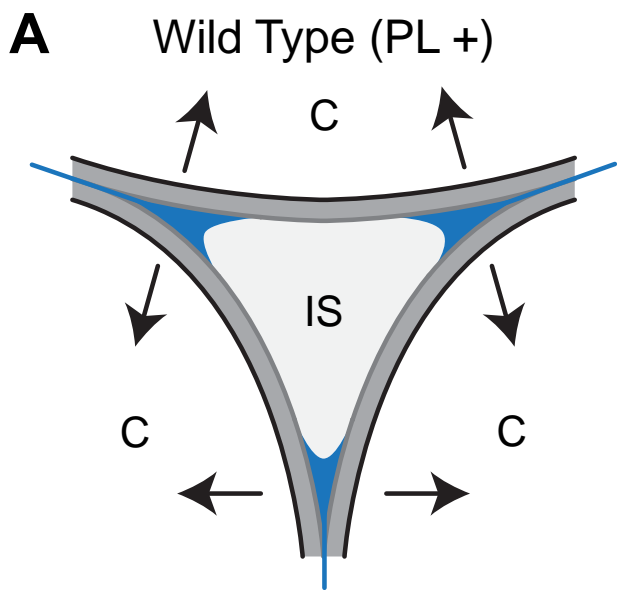
Cell wall enzyme activity or gene expression	Mode of action	Fruit where activity or gene expression during ripening observed
Endo polygalacturonase EC 3.2.1.15 and Exo-polygalacturonase EC 3.2.1.67	Hydrolytic cleavage of α -1,4-galacturonosyl linkages in unesterified pectin Removal of terminal galacturonosyl residues from pectin	Tomato [5], strawberry [32-37], pear [38-40], apple [41-43], papaya [44], raspberry [45], melon [46], peach [47-49], pepper [50].
Pectin methyl esterase EC 3.1.1.11	Removal of methyl groups from esterified pectin	Tomato [51, 52], strawberry [53], avocado [54], apple [43].
Pectate lyase EC 4.2.2.2	The eliminative cleavage of pectate, yielding oligosaccharides with 4-deoxy- α -D-mann-4-enuronosyl groups at their non-reducing ends	Tomato [12, 13], banana [55], mango [56], strawberry [36, 57-59], grape berry [60], raspberry [45].
β-galactosidase EC 3.2.1.23	Removal of galactosyl residues increased from pectin	Tomato [10, 61, 62], persimmon [63, 64], strawberry [65], banana [66], pear [67, 68], papaya [44, 69 – 71], apple [43, 72-74], sweet cherry [75], avocado [76], Carambola fruit [77], pepper [78], grape berry [60].
Arabinofuranosidase (α -l-arafase) EC 3.2.1.55	Release terminal arabinofuranosyl residues from a wide variety of pectic and hemicellulosic polymers	Tomato [79], pear [80, 81], strawberry [82], apple [43, 74, 83-85], persimmon [86].
Rhamnogalacturonan lyase (RG-lyase) EC 4.2.2.23	Catalyses the hydrolysis of a rhamnogalacturonan	Strawberry [87].
Pectin acetyl esterase	Hydrolysis of acetyl esters of pectin, producing pectate, partially esterified pectin	Citrus [88].
Expansin	Wall stress relaxation and irreversible wall extension	Tomato [11, 89-92], strawberry [93, 94], apple [85], pear [95], grape berry [96], Longan fruit [97].

Homogalacturonan

Rhamnogalacturonan I



- D-Galacturonic acid
- L-Rhamnose
- L-Arabinose
- D-Galactose
- ▼ O-Methyl
- ◆ Ca²⁺



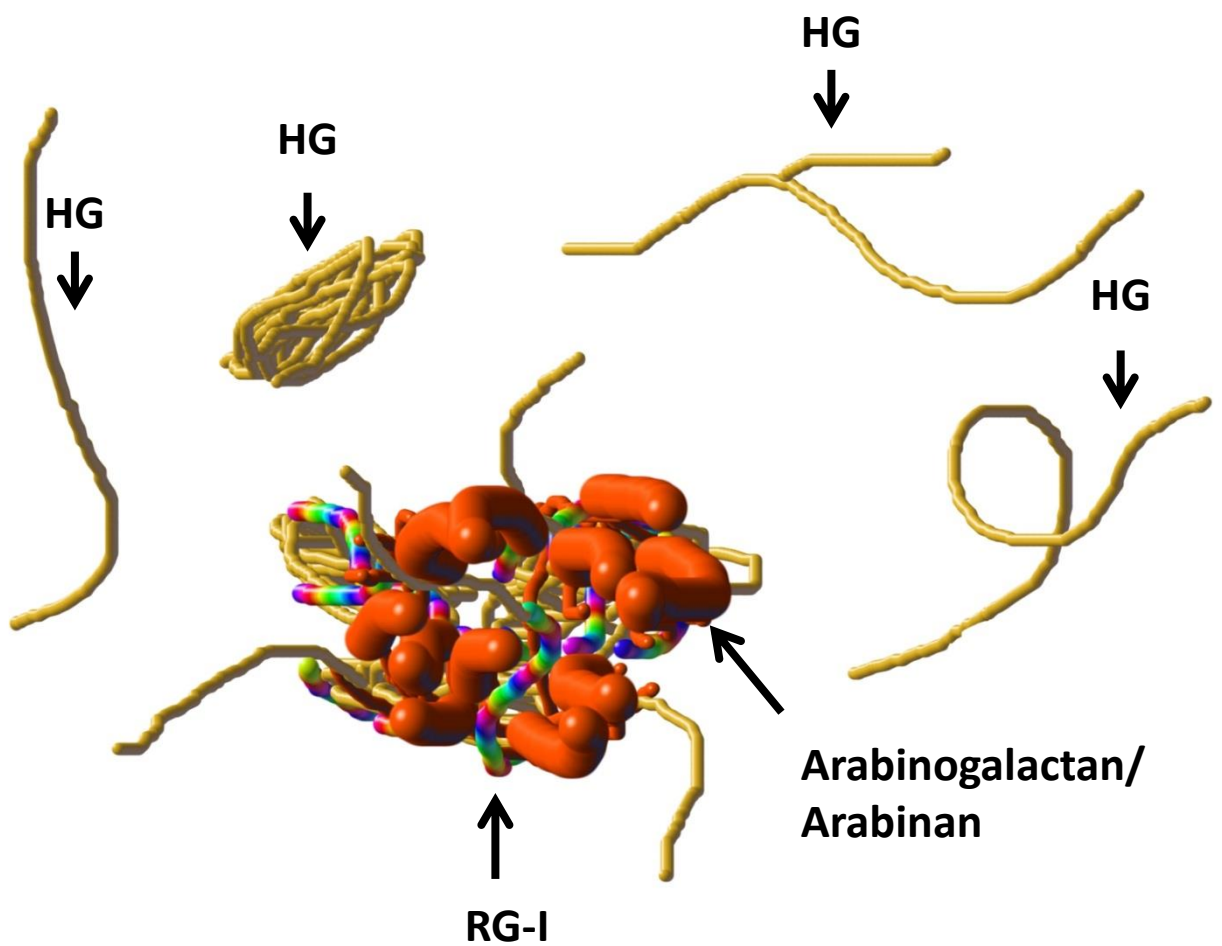


Figure 3. Structural representation of types of pectin molecules extracted from tomato pericarp tissue and revealed by atomic force microscopy. The image shows molecular structures of homogalacturonan (HG) alone (brown) and with rhamnosyl residues as rhamnogalacturonan 1 (RG1) shown as multicolored polymers and arabinogalactan and arabinan as red cylinders. From Round, A.N. et al. (2010) A new view of pectin structure revealed by acid hydrolysis and atomic force microscopy. *Carbohydrate Research* 345, 487–497 with permission.