Fruit Softening: Revisiting the Role of Pectin

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Review

Trends

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

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

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

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

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

Fruit Texture Determines Shelf life and Quality

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

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

Tomato as a Model System

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

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

Pectin and New Models of Cell Wall Structure

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

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

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

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

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

Pectin metabolism in the fruit-ripening context

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

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

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

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

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

Concluding Remarks and Future Perspectives

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

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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.

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

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 (RG-I) shown as multicolored polymers and arabinogalactan and arabinan as red cylinders. These thicker chains reflect branched 'bottlebrush'-like arabinan / arabinogalactan structures. With permission 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.

Outstanding Questions

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

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

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

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

GLOSSARY

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

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

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

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

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

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

Pectin Methylesterase (PME): An enzyme that catalyses the removal of the methyl ester groups from pectin.

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

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

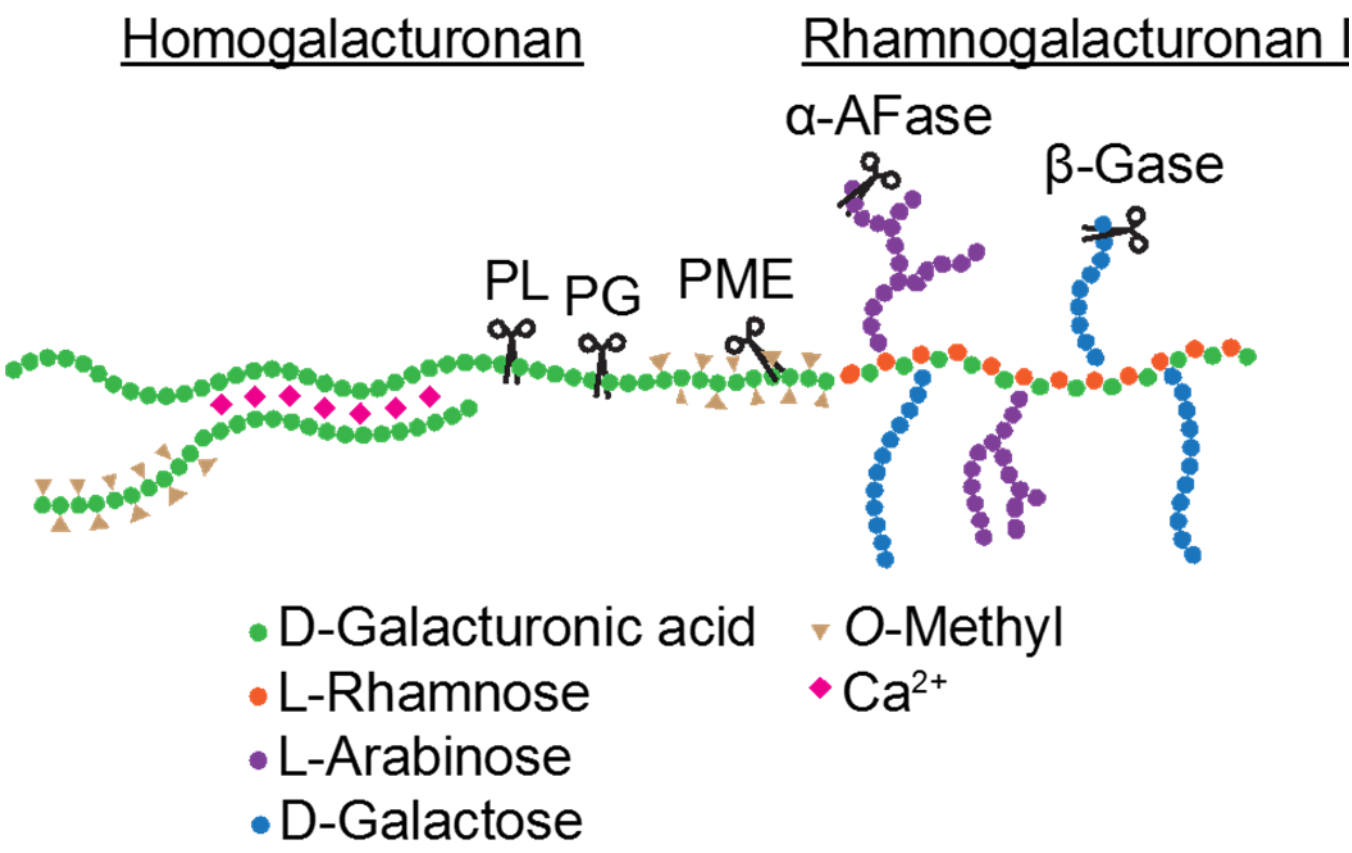
RNAi: RNA interference, a process whereby RNA molecules can be used to target and silence the expression of a specific gene of interest.

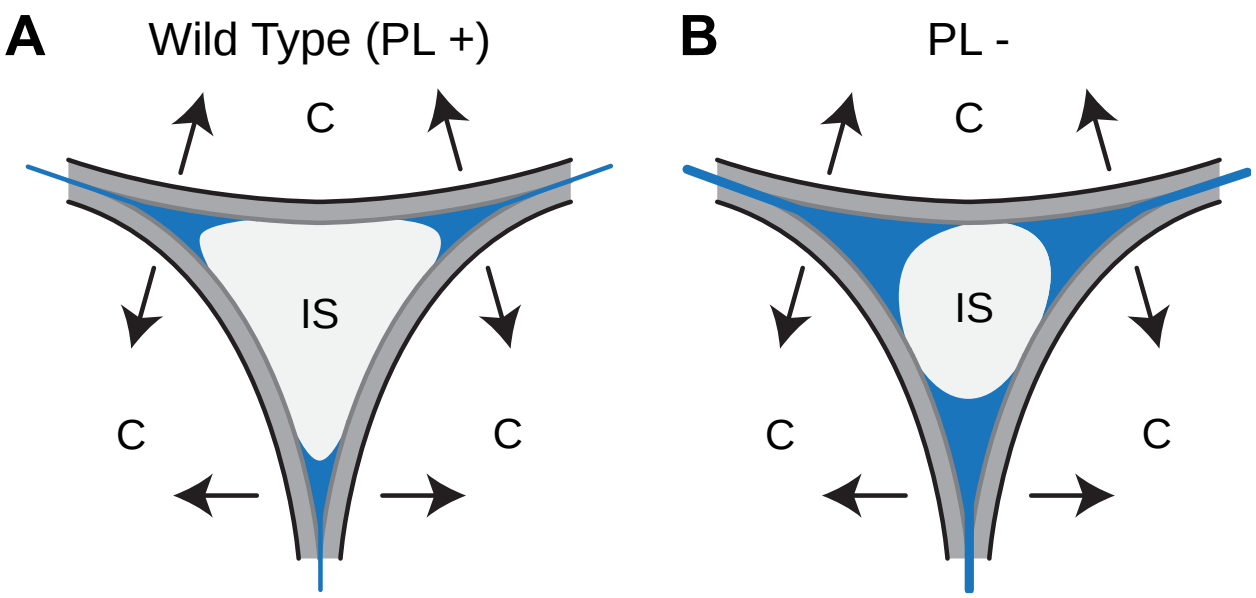
Solid-State Nuclear Magnetic Resonance Spectroscopy (ssNMR): A spectroscopic technique for studying the atomic structure of materials in the solid state by manipulating and correlating spin states of nuclei using strong magnetic fields.

Xyloglucan: A cell wall polysaccharide with a backbone of β1-4-linked glucose residues, most of which are substituted with 1-6 linked xylose sidechains.

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 a-l,4-galactouronosyl 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 acetylesterase	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
α-AFase
β-Gase
PL
PG
PME
D-Galacturonic acid
L-Rhamnose
L-Arabinose
D-Galactose
O-Methyl
Ca2+


A
Wild Type (PL +)
C
IS
C
C
B
PL -
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IS
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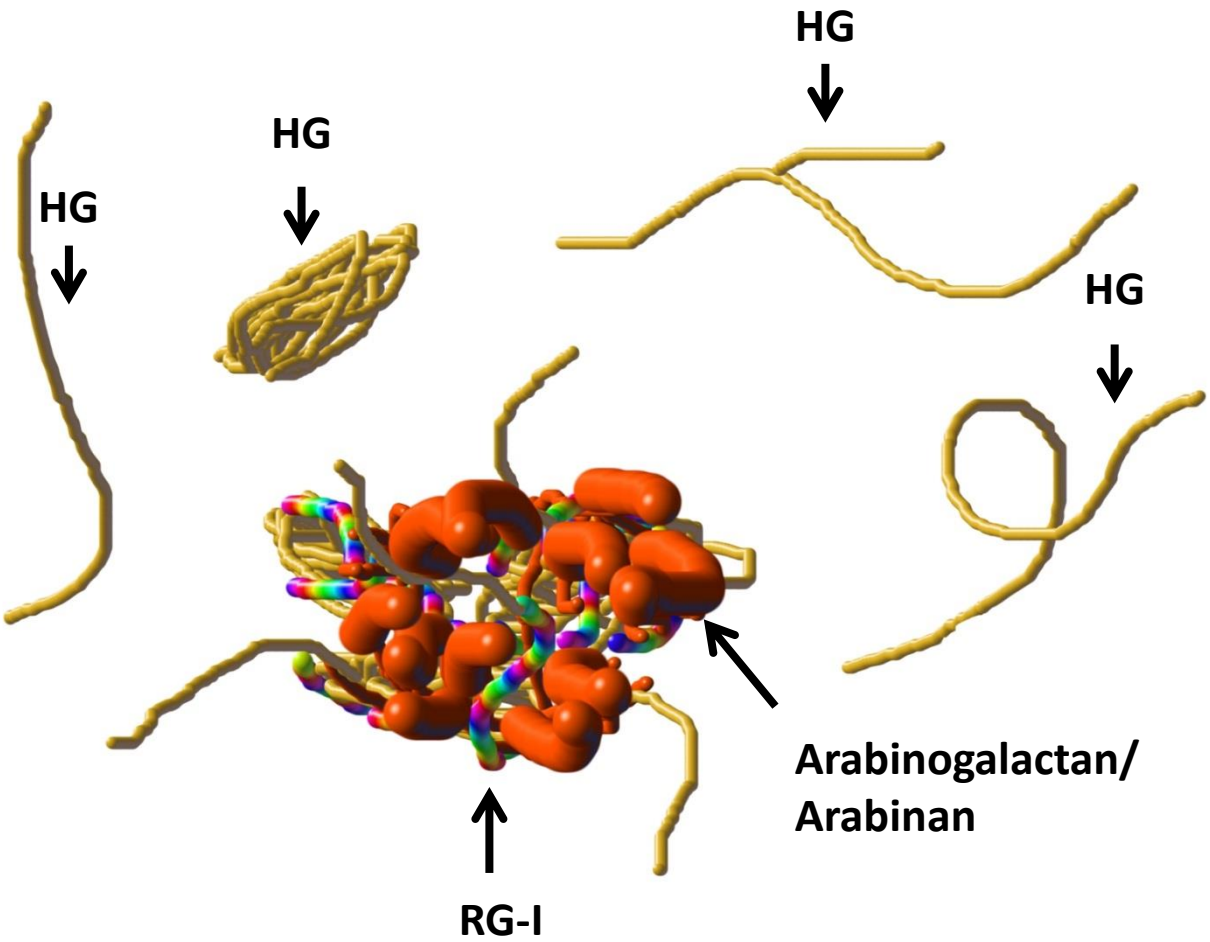

Figure 3. Structural representation of types of pectin molecules extracted from tomato pericarp tissue and revealed by atomic force microscopy. The image shows molecular structures of homogalacturonan (HG) alone (brown) and with rhamnosyl residues as rhamnogalacturonan 1 (RG1) shown as multicolored polymers 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.