

# **Fruit Ripening**

## **Physiology, Signalling and Genomics**

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*Edited by*

**Pravendra Nath**

*Council of Scientific and Industrial Research-National Botanical Research Institute,  
Lucknow, India*

**Mondher Bouzayen**

*Institut National Polytechnique – ENSA Toulouse, France*

**Autar K. Mattoo**

*Beltsville Agricultural Research Center, USDA, Beltsville, USA*

**Jean Claude Pech**

*Institut National Polytechnique – ENSA Toulouse, France*



# 4 Cell-wall Metabolism and Softening During Ripening

Mark L. Tucker\*

US Department of Agriculture, Beltsville, MD, USA

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## 4.1 Introduction

The final stage of fruit development is ripening, which typically includes transformation from an inedible hard organ into a palatable softer version. Fruit softening is a combination of changes in firmness and texture. Firmness can be defined as compressibility or the force required to deform the surface of the fruit (Brookfield *et al.*, 2011). Texture is defined as a sensory attribute and is more difficult to measure with instrumentation (Mohamed *et al.*, 1982; Garcia-Ramos *et al.*, 2005; Brookfield *et al.*, 2011). Texture includes crispness, viscosity and juiciness (Brookfield *et al.*, 2011). Texture is often best measured by human tasters (Brookfield *et al.*, 2011). Before moving on to the specific details of what happens during ripening to contribute to softening, let us identify a few basic principles to help visualize what softening really is. The edible parts of fruit are not woody (lignified), not even before they ripen. In other words, the cell walls in the edible parts of fruit are not rigid. They can flex. The flexibility of the cell wall is more obvious in thinner structures like leaves. Leaves are not as 'stiff as a board'. The primary force that maintains structure in a leaf and firmness in fruit is turgor

pressure. Although many factors can affect turgor pressure, understanding the consequences of water loss is not too complicated. A partially dehydrated leaf will be more flexible. Similarly, a dehydrated fruit will be softer than a fully hydrated fruit. It also seems fairly obvious that if you loosen up the cell wall to make it more flexible and extensible, the fruit will be softer. However, the cell wall is a complex structure and how specific changes in the cell wall affect softening is not always obvious.

Let us continue with some additional basic concepts that should help us understand the role of the cell wall in a large multicellular organ like a fruit. Let us begin by building a mental picture of the basic function of the cell wall by comparing it to two types of balloons, a latex balloon and a foil balloon. The latex balloon starts out small and continues to expand as you fill it with gas until at some point it explodes. The foil balloon is flat, flimsy and flexible until it is completely full of gas (turgid) and does not expand much more once completely filled but rather explodes if too high a pressure is reached. The fruit cell walls act more like the foil balloon. Once the cell is turgid, it does not readily expand. The key here is

\* mark.tucker@ars.usda.gov

that the cell wall is flexible, as is the foil balloon, but it does not stretch easily unless modified. If the cell loses some of its water through evaporation, it is softer and more flexible, like the foil balloon would be if it lost some of its air. To take this analogy a bit further, the foil balloon can be made to rupture at lower or higher pressures depending on the thickness and strength of the material used. Similarly, the pressure required to rupture a fruit cell depends on the strength and composition of the cell wall. None the less, a fruit is a bit more complicated than the single balloon because the fruit is not just one cell but many cells that can be made to slide past one another, and slippage can also contribute towards softening.

Let us imagine a bag full of marbles. If the marbles are glued tight to each other, they act as one big incompressible rigid block, but loosen the glue between the marbles, and the marbles readily move about inside the bag. The middle lamella between plant cells acts like a glue that adheres adjacent cells. Completely dissolving the middle lamella causes the fruit to become softer. However, if softening were simply dissolution of the middle lamella to allow cells to slide past each other, eating a fruit might be more like eating a bag of sand. The cells must also rupture to allow the ripened contents to escape.

Thus it can be seen that fruit softening is a combination of changes in turgor pressure, primary cell-wall construction that affects the pressure needed to rupture the cell, and cell-to-cell adhesion that affects how readily the cells slide past one another. However, there are limits to how much these parameters can change whilst still maintain a living organ and palatable fruit. At this point, it is worth mentioning a basic principle necessary to maintain a living cell deep inside a fairly large organ like a fruit. All plant cells, including fruit cells, require efficient gas exchange (Ho *et al.*, 2011). Oxygen is taken up for respiration and carbon dioxide is given off. If the environment surrounding the cell is anaerobic (i.e. oxygen depleted) for very

long, the cell will die. Animals facilitate gas exchange by using a circulatory system, but plants typically rely on passive diffusion of oxygen to and from the cell surface. The primary cell wall is filled with liquid, but some portion of the intercellular space between cells must be gaseous (Ho *et al.*, 2011). There must be a contiguous air channel from the surface of the fruit to its centre to facilitate gas diffusion. If the primary cell-wall structure and middle lamella were to be degraded to the point that the intercellular gas space was lost, either from collapse of the fruit under its own weight or swelling of cells to the point that gas exchange was greatly compromised, the fruit would spoil and become rotten. We all know what a rotten fruit looks like. Rotten fruit can be caused by pathogens, mechanical damage, or over-ripening and collapse of the fruit structure.

#### 4.1.1 Turgor: waxing and packaging

Water relationships are not the focus of this chapter, and factors that affect turgor pressure and water loss are complex. None the less, because water relationships are so important to softening and maintaining fruit quality, we will briefly discuss what has been done to understand and regulate this important parameter. You might be surprised how many fruits and vegetables are waxed: apples, pears, tomatoes, cucumbers and many more (Hardenburg, 1967). The commercial practice of waxing fruits and vegetables to reduce water loss and extend shelf-life has been used for many years (Hardenburg, 1967). In addition to wax, the coating often incorporates fungicides to reduce postharvest infections (Hall, 1989). In some cases, the wax may reduce not only water loss but also gas exchange enough that it may limit oxidative respiration without the fruit becoming anaerobic (Jeong *et al.*, 2003). Limiting oxidative respiration goes hand in hand with reducing metabolic rate and slowing the ripening process, which includes softening (Tucker and Laties, 1985; Asif *et al.*, 2006; Zabalza *et al.*,

2009). In recent years, considerable research has gone into developing modified atmosphere packaging that reduces mechanical damage and water loss, and creates a gaseous environment that limits oxygen exchange but not so much as to create anaerobic conditions within the fruit (Rojas-Graü *et al.*, 2009; Sandhya, 2010; Singh, 2011).

Of particular interest in regard to water relationships is recent research on a tomato variety that has a long shelf-life (remains firm and palatable for an extended period of time) (Saladie *et al.*, 2007). The authors of this study discovered that the long shelf-life was due to a mutation that altered the cuticle that surrounds and protects the fruit. This is of interest because it suggests an avenue for engineering or breeding that can have profound effects on water loss and maintenance of firm fruit.

None the less, the cell wall must ripen along with the rest of the fruit. What is cell-wall ripening? It is changes in the cell wall that make the fruit desirable – the perfect firmness and texture. But desirability depends on which fruit you are eating. An apple or pear is not the same as a grape, blackberry, banana or tomato. However, there are some general concepts about cell structure and cell-wall metabolism that can be applied to many fruits.

#### 4.2 Cell-wall Structure

Before discussing how the cell wall changes during ripening, we must know something about how it formed. When cells divide to increase cell numbers, a cell plate forms between the dividing cells during a process called cytokinesis (Allen, 1901; Carpita and McCann, 2000). The cell plate will become the middle lamella – the glue that binds cells together. The middle lamella consists primarily of pectins. Very little or no cellulose is found in the middle lamella (Allen, 1901; Carpita and McCann, 2000). Soon after the formation of the cell plate, the primary cell wall is synthesized on either side of the plate. The primary cell wall provides structure and tensile strength

to the cell (Carpita and Gibeaut, 1993). However, as mentioned above, there must be a contiguous gas space between cells to sustain adequate exchange of gases. The gas space can, however, vary widely depending on the fruit; for example, in pear it is ~5% (Verboven *et al.*, 2008), in apple ~23% (Drazeta *et al.*, 2004; Verboven *et al.*, 2008) and in tomato ~6% (Calbo and Nery, 1995). It is at this early step in cell division and primary cell-wall synthesis that the air spaces form. The air space forms at the corners where three or four cells make contact with each other. When the middle lamella joins with the middle lamella from an existing cell, this region (corner) is modified. Carbohydrate-specific antibodies have been used to demonstrate that this region of the middle lamella contains more unesterified homogalacturonans (pectin polymers) than the middle lamella that binds adjacent cells together (Ordaz-Ortiz *et al.*, 2009). Although the airspace at these corners may change in volume during fruit development, a contiguous gas space must be maintained during cell expansion and ripening (Ho *et al.*, 2011).

A typical primary cell wall of a dicot cell consists of 50% cellulose/xyloglucan, 30% pectic polysaccharides and 20% structural proteins (Carpita and Gibeaut, 1993). Polymers of cellulose (1,4- $\beta$ -glucans) are twisted together to form a cellulose microfibril (Carpita and Gibeaut, 1993). A single microfibril can include several dozen glucan polymers twisted together to form a very strong thread that is not easily stretched or broken, yet is flexible (Carpita and Gibeaut, 1993). The direction in which the microfibrils are synthesized and laid down in the cell wall largely determines the direction in which the cell can expand. For example, if the microfibrils are laid down in a helical fashion around the cell, the cell expands primarily in the longitudinal direction, much like stretching a spring (Carpita and Gibeaut, 1993). A helical orientation of microfibrils would be the structure found in elongating cells immediately behind the stem or root meristem. If the microfibrils are laid down

in a more random pattern, the cell may expand in all directions, called isodiametric expansion. The cellulose microfibrils give the cell wall its tensile strength (Carpita and Gibeaut, 1993). However, to provide structure to the cell, the microfibrils must be tethered to one another. The polysaccharides that provide this function are often called hemicelluloses and consist primarily of xyloglucans (Brummell, 2006). The cellulose microfibrils and tethering hemicelluloses are both embedded in a pectin matrix. The pectin fraction consists primarily of polygalacturonic acid (homogalacturonans) and rhamnogalacturans (Carpita and Gibeaut, 1993; Brummell, 2006). Pectins add strength to the cell wall and determine pore size in the cell wall, which may limit the access of enzymes to target sites on the xyloglucan matrix and other polymers (Carpita and Gibeaut, 1993). The unesterified charged ends of the individual subunits in polygalacturonic acid are often cross-linked by calcium ions to form a stable egg-box-like structure (Carpita and Gibeaut, 1993). The availability of calcium and esterification of polygalacturonic acid with methyl or acetyl groups limits cross-linking by calcium, which can in turn affect the structural properties of the cell wall. Proteins are another major fraction of cell walls (Carpita and Gibeaut, 1993). There are several types of cell-wall proteins, but the bulk of the protein is made up of extensins – hydroxyproline-rich glycoproteins (Carpita and Gibeaut, 1993). These proteins coalesce into rod-shaped structures that add strength to the cell wall and may play a role in cell-wall assembly, cell shape and formation of the intercellular spaces (Carpita and Gibeaut, 1993; Carpita and McCann, 2000). The rod-shaped protein structures may also anchor the plasma membrane to the cell wall (Knox, 1995).

#### 4.3 Cell-wall Metabolism During Ripening

The tools available to a scientist determine the experiments they can perform. Thus,

research on fruit ripening in the early part of the 20th century focused on respiration. In 1925, Kidd and West reported that apples underwent an increase in respiration during ripening, which they called the climacteric (Laties, 1995). At approximately the same time, it was discovered that a gaseous substance emitted by a ripe apple could stimulate the ripening of an unripe apple, and that exposure to ethylene could induce a similar response (Laties, 1995). In 1934, Gane published a paper demonstrating that some fruit produces ethylene. Assaying for changes in enzyme activity during ripening followed as these tools became available. This included assays for enzymes known to affect the cell wall. In 1979, several articles were published describing an increase in cellulase and polygalacturonase (PG) activity in apples and pears (Ben-Arie and Kislev, 1979), avocado (Awad and Young, 1979) and tomato (Poovaiah and Nukaya, 1979). In apples and pears, it was also noted that, at advanced stages of ripening, the middle lamella was clearly degraded and the orderly arrangement of microfibrils was lost (Ben-Arie and Kislev, 1979). Changes in cell-wall morphology appeared to fit with the rise in cellulase and PG activity. With the advent of tools for cloning mRNA, it was not too long before cellulase and PG were cloned from avocado and tomato and their expression correlated with changes in enzyme activity (Christoffersen *et al.*, 1984; Grierson *et al.*, 1986; Kutsunai *et al.*, 1993; Lashbrook *et al.*, 1994). Clones for PG, cellulases, pectin esterases and xyloglucanases in many different fruit soon followed (Marin-Rodriguez *et al.*, 2002; Rose *et al.*, 2002, 2004; Brummell, 2006; Vicente *et al.*, 2007; Bapat *et al.*, 2010). With improved techniques (Bräutigam *et al.*, 2011; Osorio *et al.*, 2011), we now have the tools to sequence and identify expression patterns for literally hundreds of different cell-wall proteins in practically any fruit we want to examine.

However, expression data alone is simply correlative and does not really tell us how the individual proteins function during fruit softening. The fact that the

gene transcript for a protein increases during ripening suggests it plays a role in ripening, but the true function of the protein is not always obvious. To ascertain a function for the cell-wall proteins, researchers have relied on natural occurring variants (mutants) of a particular fruit or artificially created mutants made by genetic transformation.

#### 4.3.1 Pectin

Let us look at a few examples where the expression of a protein has been altered to see how the loss or overexpression of the protein changed firmness, texture and cell-wall degradation products. PGs are of interest because they are expressed abundantly in many fruits during the softening phase (Crookes and Grierson, 1983). PG can degrade both the middle lamella between cells and the pectin matrix within the primary cell wall (Crookes and Grierson, 1983). Also important is the fact that it is the pectin matrix that determines the porosity of the cell wall and accessibility of enzymes and proteins to the cellulose microfibrils and hemicelluloses embedded in it (Carpita and Gibeaut, 1993). Thus, it seems reasonable to expect that the pectin fraction must first be modified in order to provide protein access to the hemicellulose and cellulose components of the cell wall. Tomato is an important model for genetic manipulations because protocols for genetic transformation of tomato were established fairly early and, unlike tree fruits like apple, pear, avocado, tomato plants bears fruit within a month or two of germination. Two separate research groups used antisense constructs in tomato to suppress accumulation of PG transcripts, one in the USA (Sheehy *et al.*, 1988) and another in the UK (Schuch *et al.*, 1991). These early transformation events are of particular interest because these transgenic plants were the first genetically modified plant products to be commercialized. Calgene in the USA commercialized the transgenic fresh fruit using the trade name

Flavr Savr (Kramer and Redenbaugh, 1994), and Zeneca Seeds in the UK commercialized their product as a tomato purée paste (Khachatourians, 2002). Both groups reported improved shelf-life for the fruit (Sheehy *et al.*, 1988; Schuch *et al.*, 1991). The firmness, as measured by compressibility, of the freshly picked fruit was not much different from the non-transgenic fruit; however, after several days of storage, the transgenic fruit were significantly firmer (Kramer *et al.*, 1992; Brummell and Labavitch, 1997). The fruit also were more resistant to some common fruit pathogens, which improved storage quality. Both groups measured a significant increase in the viscosity of the processed fruit, e.g. purées (Schuch *et al.*, 1991; Kramer *et al.*, 1992). A later study of the Flavr Savr fruit confirmed and extended the earlier work by quantifying the depolymerization of polyuronides in the transgenic and non-transgenic (wild-type) fruit (Brummell and Labavitch, 1997). Depolymerization of polyuronides, as measured by size fractionation of *trans*-1,2-cyclohexanediaminetetraacetic acid (CDTA, a divalent cation chelator)-extractable cell-wall carbohydrate, changed markedly during ripening in both PG-suppressed and wild-type fruit (Brummell and Labavitch, 1997). However, they observed a small difference in depolymerization of polyuronides in PG-suppressed fruit compared with wild-type fruit, which correlated with a small shift in fruit firmness (Brummell and Labavitch, 1997). At least two reasons can be envisaged for only a slight difference in depolymerization of polyuronides at harvest when the polyuronides were clearly fragmented in both lines. It is possible that PG2A, the abundant transcript inhibited in these plants, is not responsible for fragmentation of polyuronides during ripening or that there are additional enzymes that can depolymerize pectins, such as pectate lyases. Giovannoni *et al.* (1989) did an interesting experiment that sheds light on this problem. They transformed a ripening inhibited mutant of tomato, *rin*, with a construct (gene) that could express active PG2A at a develop-

mental stage that corresponded to when the mutant should normally ripen. They demonstrated that PG2A does cause depolymerization of polyuronides, but the fruit did not significantly soften. This indicates that PG2A can fragment pectins but that in the transgenic plants where PG2A is suppressed there are other enzymes that can also fragment pectin.

The results with overexpression of PG2A in *rin* and suppression in Flavr Savr further suggest that PG2A by itself may not be enough to cause significant alterations in tomato fruit firmness, but before moving on to a different topic, let us look at another fruit. In peach, there are naturally occurring varieties that produce fruit called melting flesh (MF) and non-melting flesh (NMF). Examination of NMF fruit found that PGs were not expressed or were not secreted into the cell wall (Callahan *et al.*, 2004; Ghiani *et al.*, 2011). The NMF varieties all had deletions of one form or another in a genetic locus for a family of PGs (Callahan *et al.*, 2004). In this particular case, the correlation between MF texture and the absence of PG was very compelling. Moreover, what is also important was that they found that fruit firmness (compressibility) was not greatly different between the MF and NMF varieties (Ghiani *et al.*, 2011). These authors concluded that cell turgor plays a more important role in changes in firmness but that PG activity is responsible for the marked difference in fruit texture. To restate, texture is defined as a sensory attribute and is more difficult to measure with instrumentation than firmness (Brookfield *et al.*, 2011).

$\beta$ -Galactans are branch polymers on rhamnogalacturonans, which are a major component, in addition to polygalacturonic acid, in the pectin matrix of the primary cell wall (Carpita and Gibeaut, 1993). Antisense suppression of  $\beta$ -galactosidase 4 reduced the amount of free galactose in ripening fruit and increased fruit firmness (Smith *et al.*, 2002). Another enzyme that can affect pectin structure and fragmentation are pectin esterases (Rose *et al.*, 2004). It is thought that polyuronides are

synthesized as a methyl or acetyl ester that blocks the carboxyl acid group (Carpita and Gibeaut, 1993; Rose *et al.*, 2004). After the polyuronides are laid down in the cell wall; the esterified polyuronides are de-esterified by pectin esterases. The charged carboxyl group can then interact with calcium to form a stable egg-box-like structure (Carpita and Gibeaut, 1993). It is thought that de-esterification of polyuronide is necessary before PGs can act on this substrate (Tieman *et al.*, 1992). Suppression of a pectin methyl esterase (PME) in tomato fruit reduced the de-esterification of polyuronides and its depolymerization during ripening, but, although an increase in soluble solids was observed in the transgenic fruit, no change in firmness or texture were noted (Tieman *et al.*, 1992). A separate group suppressed a different pectin esterase in tomato fruit and also observed a significant increase in esterified pectin but did not identify any significant differences in softening; however, unlike the earlier report on the suppression of a PME, they did not observe any difference in soluble solids (Hall *et al.*, 1993).

#### 4.3.2 Cellulose

Cellulose is another major component of fruit cell walls, and it is the cellulose microfibrils that provide the tensile strength to the cell wall. Intuitively, it makes sense that modification of this fraction of the cell wall would have marked effects on softening. However, in most fruits examined, the cellulose content of the cell wall does not change much during ripening (Maclachlan and Brady, 1994; Newman and Redgwell, 2002; Brummell, 2006). Before discussing the results for mutants with suppressed levels of cellulases, it is worth noting that analysis is complicated by the fact that we do not know the true substrate for the plant enzymes called cellulase. Cellulases are defined by their ability to degrade and reduce the viscosity of carboxymethylcellulose (CMC) in an *in vitro* assay. CMC

is an artificial water-soluble cellulose. For example, purified avocado cellulase readily degrades CMC but when added to microfibrils or crystalline cellulose, the enzyme is incapable of significantly degrading these substrates (O'Donoghue *et al.*, 1994). A similar result has been obtained with other plant cellulases (Urbanowicz *et al.*, 2007; Vicente *et al.*, 2007). It has been suggested that the native substrate for plant cellulases might be xyloglucans (Hatfield and Nevins, 1986); however, subsequent studies with purified avocado cellulase demonstrated that this cellulase could not degrade purified xyloglucans (O'Donoghue *et al.*, 1994). What is the substrate for cellulases? We still do not know for certain; however, incubation of avocado cellulase with cell walls purified from unripe avocado fruit caused a loss in the cohesiveness of the microfibrils (O'Donoghue *et al.*, 1994). These authors concluded that avocado cellulase does not cleave the xyloglucans that tether the cellulose microfibrils but rather cleaves cellulose at accessible sites on the periphery of the microfibrils, which causes a loss of integrity within the fibril structure and alters the binding of associated cell-wall matrix polysaccharides, such as xyloglucans. In avocado, which produces an inordinate amount of cellulase, it was concluded that, although cellulase might not depolymerize crystalline cellulose microfibrils, cellulase might modify the cellulose polymers exposed to the surface of the microfibril, which would then affect the tethering and organization of the microfibrils in the wall (O'Donoghue *et al.*, 1994). Unfortunately, transformation of avocado to suppress cellulase is not currently possible.

Tomato fruit includes two cellulases that are expressed during fruit ripening, Cel1 and Cel2 (Lashbrook *et al.*, 1994). When each gene – Cel1 (Lashbrook *et al.*, 1998) and Cel2 (Brummell *et al.*, 1999a) – was suppressed individually, there was no significant effect on fruit ripening or softening. This result left open the possibility that both genes needed to be suppressed in order to observed and effect

on softening. However, in a subsequent study where both Cel1 and Cel2 were suppressed simultaneously, no obvious change in softening was reported, but there was a clear effect on infection by *Botrytis cinerea* (a common fungal pathogen of fruit), which suggested to the authors that these cellulases altered the cell wall in a manner that made the fruit more susceptible to infection (Flors *et al.*, 2007). Interestingly, expression of Cel2 in the non-ripening *rin* mutant, which does not normally express Cel2, did cause the fruit to partially soften but not ripen (Flors *et al.*, 2007). What this suggests is that Cel2 might affect tethering of the cellulose microfibrils but that, in a normal ripening process, there are other enzymes or processes that have a similar effect on tethering of microfibrils.

Before moving on to look at other components of the cell wall, it is of interest to examine research with strawberry fruit. Suppression of PGs had a clear effect on fruit firmness when measured by extrusion of the pulp through an orifice during fruit compression (Quesada *et al.*, 2009). The measurement of fruit firmness for strawberry was done differently from that for tomato fruit and might be better compared with the decrease in viscosity of the tomato paste prepared from transgenic tomato with reduced PG activity and also the MF characteristic of peach. Also of comparative interest is that suppression of a cellulase (Cel1) in strawberry, like tomato, had no significant effect on fruit firmness (Woolley *et al.*, 2001).

Whilst on the topic of untethering of microfibrils, it is necessary to discuss work on a protein called expansin. Expansins were first discovered in the stems of elongating seedlings (McQueen-Mason *et al.*, 1992; McQueen-Mason and Cosgrove, 1994) and have since been identified in many plant tissues (Rose *et al.*, 1997; Rose *et al.*, 2000; Cosgrove *et al.*, 2002). Expansins are an interesting class of protein because, although they have no known *in vitro* enzyme activity, they clearly play a role in loosening of the cell wall during cell elongation (Cosgrove,

1999; Cosgrove *et al.*, 2002). However, based on the effect of expansins on plant tissues and cellulose paper, it was concluded that expansins act on hydrogen bonding at the interface of cellulose microfibrils (McQueen-Mason and Cosgrove, 1994). If the tethering of microfibrils to each other is loosened during ripening, a marked effect on both firmness and texture might be expected. Expansins are a family of genes in tomato and several are expressed in fruit but one in particular (EXP1) increases specifically during ripening (Rose *et al.*, 1997, 2000; Brummell *et al.*, 1999c). To examine a possible role for EXP1 in fruit softening its expression was both suppressed and enhanced in tomato fruit (Brummell *et al.*, 1999b). Suppression of EXP1 caused a significant increase in fruit firmness measured at all stages of ripening fruit including over-ripe fruit. Also of interest is that when EXP1 was overexpressed throughout fruit development, the fruit were less firm, even at the mature green stage. Fruit where EXP1 was overexpressed were also smaller and had what was described as a 'rubbery texture'. Thus, results with EXP1 are consistent with expansins affecting the tethering of cellulose microfibrils to each other.

#### 4.3.3 Xyloglucans

Expansins may affect the tethering of cellulose microfibrils, but it is proposed that xyloglucans are the polysaccharide substrate that cross-links and ties the microfibrils together (Carpita and Gibeaut, 1993). Modification or depolymerization of the cross-linking xyloglucan network might also be expected to affect fruit softening. Xyloglucanases, like cellulase and expansins, are another interesting family of enzymes. In addition to hydrolysing xyloglucan polymers, most of these enzymes appear also to be able to join together two xyloglucan polymers. This family of enzymes have been called xyloglucan endotransglucosylase/hydrolases (XTHs) (Rose *et al.*, 2002). Thus, because xylo-

glucan polymers can be broken and rejoined, it is possible for cellulose microfibrils to slide past one another during cell expansion without evidence of xyloglucan depolymerization. Nevertheless, it has been demonstrated that xyloglucans are fragmented (depolymerized) during ripening (Brummell, 2006). In tomato, 25 XTHs have been identified (Ohba *et al.*, 2011) and at least ten are expressed in ripe fruit, but only two appeared to increase with ripening whilst most of the others decreased during ripening (Miedes and Lorences, 2009). Overexpression of XTH1 during fruit development actually caused a slight decrease in depolymerization of xyloglucans during ripening (Miedes *et al.*, 2010). These researchers concluded that the increased endotransglucosylase activity over the hydrolase activity in the XTHs was responsible for the lower xyloglucan depolymerization in fruit and suggested that the role of XTHs during fruit growth and ripening might be to maintain the structural integrity of the cell wall. They suggested that a decrease in activity XTH activity, rather than an increase, during ripening might contribute to fruit softening. A separate group both overexpressed and inhibited expression of XTH1 in tomato and found that the level of expression correlated directly with fruit size (Ohba *et al.*, 2011). These researchers concluded that XTH1 clearly plays a role in cell expansion in fruit development, but they did not identify a clear link to softening; nevertheless, they postulated that a spike in XTH activity at the turning stage early in the ripening process might be linked to xyloglucan depolymerization and a decrease in firmness. Of interest in this regard is recent research using carbohydrate-specific antibodies, which showed that hemicellulose-like polymers are also associated with the cell adhesion layer (middle lamella) and that these polymers tend to disappear in ripe tomato fruit (Ordaz-Ortiz *et al.*, 2009). Currently, it is not clear how important the loss of hemicelluloses in the adhesion layer is to fruit softening.

#### 4.3.4 Protein

The protein component of cell walls is probably the least well understood part of the cell wall. It seems logical that something that accounts for as much as 20% of the mass of the cell wall (Carpita and Gibeaut, 1993) might be important in softening. Mutants of cell-wall proteins and manipulation of the glycosylation of these proteins clearly affects cell shape and development (Knox, 1995), but how do changes in this component of the cell wall affect fruit softening. Not much is known about this. An early study of tomato demonstrated that total nitrogen content from cell-wall protein changed very little during fruit ripening, but the amount of salt soluble protein that could be extracted from the cell wall increased approximately twofold from mature green to red-ripe fruit (Hobson *et al.*, 1983). However, the increase in extractable protein may have been due to changes in the carbohydrate fraction rather than the proteins themselves. In a recent paper, the change in accumulation of several thousand transcripts and a few hundred proteins was examined during tomato fruit development (Osorio *et al.*, 2011). The results suggested that neither transcription nor the protein content of structural cell-wall proteins changed much during ripening. None the less, it is possible that structural proteins in the cell wall are simply modified, which might affect softening, but this remains to be determined.

#### 4.3.5 Other factors (pH, ionic composition, synthesis and non-uniformity)

The pH of most cell walls is typically between pH 6 and 7 (Almeida and Huber, 1999). When fruit ripen, the pH of the tomato cell-wall fluid (apoplast) drops from 6.5 in mature green fruit to pH 4.5 in ripe fruit (Almeida and Huber, 1999). A drop in the apoplastic pH may be common during fruit ripening. Changes in pH can affect enzyme activity (Chun and Huber, 1998) and possibly ionic interactions with

calcium (Virk and Cleland, 1988). In this regard, an early theory proposed that auxin induced cell growth by acidification of the cell wall, known as the acid growth theory (Rayle and Cleland, 1970; Rayle and Cleland, 1980; Rayle and Cleland, 1992). The acid growth theory was originally explained as an effect on charge-related interactions between polymers or changes in enzyme activity (Rayle and Cleland, 1970). Subsequent to this early work, it was demonstrated that expansins were activated by cell-wall acidification and played a major role in loosening the cell walls during auxin-induced growth (McQueen-Mason and Cosgrove, 1994). As discussed above, expansins probably also play a role in fruit softening, but the role of pH in this process has not been examined (Brummell *et al.*, 1999b). Nevertheless, although the optimum pH for tomato fruit expansins was not reported, the activity of fruit expansin was assayed at pH 4.5 (Rose *et al.*, 2000), presumably because the pH optimum for the tomato fruit expansin is low, as it is for other expansins examined (McQueen-Mason and Cosgrove, 1994). Also of interest in regard to pH is that the activity maximum for the tomato fruit PG is approximately pH 5 and remains relatively high at pH 4.5 (Chun and Huber, 1998), which is the pH of the apoplast in ripe fruit (Almeida and Huber, 1999). At pH 6 and above, the pH of the apoplast in mature green fruit, PG has very low activity (Chun and Huber, 1998). Thus, the pH of the cell wall may be important for the activation of expansin, PG and other enzymes.

A change in the concentration of calcium can also affect cell-wall extensibility (Virk and Cleland, 1988), but the concentration of calcium in the cell wall of tomato does not change much during ripening (Almeida and Huber, 1999). None the less, the importance of calcium in fruit firmness and storage is exemplified by postharvest studies with apple (Mason *et al.*, 1975; Sams and Conway, 1984). More than simply a research interest, fruits and fruit slices are often commercially dipped in a solution containing CaCl<sub>2</sub> to improve

firmness and storage life. Fruits dipped in CaCl<sub>2</sub> include but are not limited to apples (Saftner *et al.*, 1998), pears (Rosen and Kader, 1989), tomatoes (Floros *et al.*, 1992), blueberries (Camire *et al.*, 1994) and strawberries (García *et al.*, 1996).

Up to this point, we have focused on degradative processes in the cell wall, but biosynthesis of cell-wall polysaccharides continues throughout tomato fruit ripening (Mitcham *et al.*, 1989; Greve and Labavitch, 1991). In one study, the researchers injected radioactive sucrose into the pedicel of tomato fruit and then collected pericarp tissue at several stages of fruit development (Mitcham *et al.*, 1989). Radioactivity increased slightly in the pectin and hemicellulose fractions extracted from the fruit pericarp early in tomato fruit ripening and then declined at the red-ripe stage. The greatest increase in label per gram of pericarp was in the pectin fraction. It is not clear, however, whether the polymers synthesized during ripening were different from those synthesized earlier in development and how the newly synthesized polymers affected fruit softening.

Of possible importance to fruit firmness and more importantly texture, which includes juiciness, is that the cell wall is not the same all around the cell. We have already mentioned that the middle lamella is different where intercellular air spaces form, but the primary cell wall is also non-uniform. Pitfields are one example. Pitfields are regions of the cell wall where plasmodesmata transverse the walls between adjacent cells (Burch-Smith *et al.*, 2011). The plasmodesmata create channels between cells that play a role in cell-to-cell communication (Burch-Smith *et al.*, 2011). Immunoanalysis of the cell wall shows that regions near the plasmodesmata have a different carbohydrate composition from other parts of the wall (Orfila and Knox, 2000). There was an absence of 1,4- $\beta$ -galactan near the plasmodesmata and the pectin material in this region was not easily extracted with calcium chelators (e.g. CDTA). It was concluded that the pectin structure (porosity) around the plasmodesmata might exclude some

enzymes from reaching the plasmodesmata. Ultrastructural studies with apple and pear have indicated that the microdomain around plasmodesmata is indeed resistant to decomposition during ripening (Ben-Arie and Kislev, 1979; Roy *et al.*, 1997).

#### 4.4 Conclusions

Ultrastructural (electron microscopy) studies indicate decomposition and spreading of the middle lamella during ripening of apple and pear (Ben-Arie and Kislev, 1979) and tomato (Crookes and Grierson, 1983). Disassembly of the middle lamella is probably a common theme in fruit in ripening. The same ultrastructural studies also showed a disintegration of the fibrillar arrangement in the primary cell wall late in ripening. The disruption of fibrillar organization is even more pronounced in ripening avocado fruit, which, when ripe, has a MF texture (O'Donoghue *et al.*, 1994). The disruption of fibrillar organization can be accomplished simply by untethering the cellulose microfibrils with expansin (Cosgrove, 1999), depolymerization of the xyloglucan matrix that cross-links the microfibrils (Miedes and Lorences, 2009) or possibly by modification of peripheral cellulose polymers with cellulase (O'Donoghue *et al.*, 1994). However, the porosity of the cell wall is determined largely by the pectin matrix that the cellulose microfibrils and hemicelluloses are embedded in, and partial disassembly of the pectin matrix may be necessary to give enzymes access to the cellulose and hemicellulose polysaccharides (Carpita and Gibeaut, 1993). Extraction of cell-wall fractions at different stages of ripening supports extensive depolymerization of pectin but does not distinguish between the middle lamella and the pectin matrix in the primary cell wall (Brummell, 2006). Depolymerization of the hemicellulose (xyloglucan-rich) fraction does occur during ripening but is not nearly as extensive as the fragmentation of pectins (Brummell, 2006).

The recent immunological observation of hemicelluloses in the middle lamella, which disappear during ripening (Ordaz-Ortiz *et al.*, 2009), may contribute to the slight depolymerization of xyloglucans observed by others (Brummell, 2006).

Much of the research discussed above was performed simply to discover the fundamentals and basic biology of fruit ripening; none the less, the practical goal of using this knowledge to develop shipping and storage practices and to engineer high-quality fruit with a long shelf-life is never forgotten. In considering this practical goal, we must also keep in mind that, although we have selectively bred fruit for human consumption, the fruit has evolved for millions of years for reproduction of the species and not for commercial practice. Not very many fruits have evolved for optimal transcontinental shipping. Seed dispersal by consumption by an animal is one reason for ripening, but sometimes the fruit simply drops to the ground where the seed will then germinate. Over-ripening may be important to create a nursery environment for efficient seed germination. For example, many fruits,

including tomato, will soften to the point that the fruit will collapse under its own weight. However, for human consumption, we typically want firm fruit that can easily be transported and a delay in the over-ripening of the fruit. We currently do this commercially by picking fruit before it is completely ripe, dipping fruit in calcium, waxing fruit or storing the fruit in reduced oxygen atmospheres, but we have also tried to do this through genetic engineering, such as the Flavr Savr tomato. Reducing the expression of a single gene or even two genes may not always produce the desired phenotype. Cell-wall ripening is complex. It may be necessary to suppress multiple genes or identify regulatory genes (proteins) that modulate the expression of several genes that affect the cell wall. None the less, we now have the tools to identify all the changes in gene expression and protein accumulation that occur during ripening and to use this information to engineer fruit with very special characteristics that include delayed softening to improve transport and a delay the over-ripening response to extend shelf-life.

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