Review

The linkage between cell wall metabolism and fruit softening: looking to the future

Ariel R Vicente,[1] Montserrat Saladié,[2] Jocelyn KC Rose[2] and John M Labavitch[1]*

[1]*Plant Sciences Department, Mail Stop 5, University of California, Davis, CA 95616, USA*
[2]*Department of Plant Biology, 228 Plant Science Building, Cornell University, Ithaca, NY 14853, USA*

Abstract: The softening that accompanies ripening of commercially important fruits exacerbates damage incurred during shipping and handling and increases pathogen susceptibility. Thus, postharvest biologists have studied fruit softening to identify ways to manage ripening and optimise fruit quality. Studies, generally based on the premise that cell wall polysaccharide breakdown causes ripening-associated softening, have not provided the insights needed to genetically engineer, or selectively breed for, fruits whose softening can be adequately controlled. Herein it is argued that a more holistic view of fruit softening is required. Polysaccharide metabolism is undoubtedly important, but understanding this requires a full appreciation of wall structure and how wall components interact to provide strength. Consideration must be given to wall assembly as well as to wall disassembly. Furthermore, the apoplast must be considered as a developmentally and biochemically distinct, dynamic 'compartment', not just the location of the cell wall structural matrix. New analytical approaches for enhancing the ability to understand wall structure and metabolism are discussed. Fruit cells regulate their turgor pressure as well as cell wall integrity as they ripen, and it is proposed that future studies of fruit softening should include attempts to understand the bases of cell- and tissue-level turgor regulation if the goal of optimising softening control is to be reached. Finally, recent studies show that cell wall breakdown provides sugar substrates that fuel other important cellular pathways and processes. These connections must be explored so that optimisation of softening does not lead to decreases in other aspects of fruit quality.

Keywords: cell wall; polysaccharide; fruit ripening; hydrolase; turgor; apoplast

INTRODUCTION

Three decades ago, conventional wisdom supported the idea that the ripening-associated softening of fleshy fruits is a direct consequence of enzyme-mediated cell wall degradation. This supposition has been tested by a substantial body of research, including studies of the roles of specific cell wall-modifying proteins (CWMPs) through transgenic analysis, a subject that has been thoughtfully reviewed on a regular basis.[1–4] We do not intend to provide another such review, other than to reiterate that genetic manipulation of the expression of genes encoding these proteins individually has typically resulted in only minor changes in the rate of fruit softening. Thus, even though these manipulations have provided some guidance to programmes aimed at improving the texture of ripe tomato fruit, this work has generated more questions than answers. The goal of this review is to ask why prior work has not led to immediately applicable, biotechnological management of fruit softening and to examine data from a range of research fields, some of which are outside the traditional 'wall disassembly leads to fruit softening' arena. The conventional approach to elucidating fruit softening has typically been based on two strategies: (1) the identification of wall components whose solubility increases and/or polymer size decreases in parallel with decreasing fruit firmness; (2) the characterisation of proteins that are expressed during ripening and whose biochemical activities can be mechanistically related to the observed wall changes. Data developed from these studies have guided the selection of genes whose expression has been enhanced or suppressed in transgenic fruits, in order to test whether they have direct roles in controlling softening, primarily using tomato as an experimental system.

* Correspondence to: John M Labavitch, Plant Sciences Department, Mail Stop 5, University of California, Davis, CA 95616, USA
E-mail: jmlabavitch@ucdavis.edu
Contract/grant sponsor: National Science Foundation's Plant Genome Program; contract/grant number: DBI 0606595
Contract/grant sponsor: National Research Initiative of the USDA Cooperative State Research, Education and Extension Service; contract/grant number: 2006-35304-17323
Contract/grant sponsor: CUAES Hatch; contract/grant number: NYC-184485
Contract/grant sponsor: New York State Office of Science, Technology and Academic Research (NYSTAR)
Contract/grant sponsor: California Agricultural Experiment Station
Contract/grant sponsor: USDA Regional Research; contract/grant number: NE-1018
Contract/grant sponsor: SECYT (Argentina)
Contract/grant sponsor: Fulbright Commission
(*Received 31 August 2006; accepted 25 October 2006*)
Published online 2 April 2007; DOI: 10.1002/jsfa.2837

We focus on tomato in this review because it represents the predominant model for studying fruit ripening,[5] it has been employed in studies emphasising the use of transgenes since the 1980s, and probably more is known about the structure and metabolism of cell walls from tomato fruit than those of any other plant species. However, it is important to emphasise that, while many of the general ideas about how fruit softening is regulated have been drawn from the 'tomato model', some of which may be applicable to other fruits, there are clearly features that vary between species. 'Softening' should therefore be defined by studies of each fruit of interest, and specific analyses of fruits other than tomato will be mentioned.

Early models of fruit softening are now clearly undergoing substantial revision, and this paper provides an overview of some new ideas and approaches that we believe will result in the next generation of models. In particular, we highlight some important unanswered questions that seem central to our understanding of wall architecture and the regulation of its reorganisation, and some new technologies that will accelerate the ability to address those questions. Lastly, we suggest that the 'experimental landscape' must be broadened, taking advantage of insights that can be provided by biophysical and physiological analyses and an improved understanding of the apoplastic microenvironment. In many cases we have gone well beyond the 'fruit-softening' literature to illustrate the research approaches that might be taken to build a more holistic view of ripening-associated fruit softening, which ultimately might be used for more effective biotechnological manipulations. Our discussion of these issues cannot be extensive because of space limitations, but the goal of this 'look to the future' is to expand the ways in which fruit softening is studied and perhaps to stimulate the development of collaborations with scientists outside the traditional cell wall research community; collaborations that we feel will provide a more complete understanding of a developmentally interesting and economically important aspect of plant biology.

CELL WALL MODELS: CAN THEY HELP US BETTER UNDERSTAND FRUIT SOFTENING?

Cell wall models are typically representations of the wall structural components, their orientations, interactions and often their relative abundances, which collectively provide a static view of the overall architecture. One of the most attractive aspects of these models is that they generally collate and incorporate the information of research conducted in many divergent aspects of wall structure and composition. Several models have been proposed in the past three decades.[6–8] However, while fundamental components are common to all these models, there is less agreement in terms of the bonding interactions and their distributions within the wall. The most frequently cited models were presented by Carpita and Gibeaut[8] and describe the cell wall as composed of two polysaccharide networks. One comprises cellulose microfibrils crosslinked by hemicelluloses (most often xyloglucans or xylans); a simple analogy of this network is that of the steel and wire grids in a reinforced concrete slab, while the other network, the pectin polysaccharides, would be the concrete. The linkages that integrate the pectin superstructure in the wall would include Ca^{2+} bridges between uronic acid carboxyl functions, creating the so-called pectin 'egg-box', and borate diesters of two rhamnogalacturonan II (RG-II) monomers.[9] A more recent wall model[10] suggests that the different classes of pectin are covalently crosslinked to form a single, heterogeneous network. Other studies have described covalent associations between xyloglucan and pectin[11,12] and pectin side chains and cellulose microfibrils.[13] Undoubtedly, detailed analysis of walls from additional species will indicate how generally applicable the models and their refinements are[14] for understanding wall structure and metabolism in different developmental contexts. However, they have proven to be useful starting points for studying wall changes associated with cell elongation[15] and fruit ripening.[16]

The improvement of cell wall models should provide useful information for describing wall changes that occur in ripening fruits and developing plants

As indicated above, in the past three decades the view of cell wall structure has been revised several times as new information about wall components, polymer structures and interpolymer associations has become available. This evolution in our understanding of wall architecture is likely to continue, driven by further examination of the plant genome and proteome and by advances in analytical tools and their availability to researchers. We see several approaches that are likely to improve understanding of the cell wall in the next decade.

Pure carbohydrate-degrading enzymes

Polysaccharide-digesting enzymes provide a powerful tool for the analysis of cell wall structures. Enzymes with rigorously defined specificity are used to partially degrade a polymer of interest into smaller oligomers that can be more easily characterised, to deduce patterns of structural motifs in large glycans, or to isolate a desired polymer from a complex mixture. However, relatively few pure cell wall-degrading enzymes are commercially available and so researchers have previously had to identify, purify and characterise their own glycanases. Recently, Bauer *et al.*[17] reported the creation of a new community resource, comprising a substantial collection of fungal genes encoding glycanases with a range of activities that have been engineered for expression in the *Pichia pastoris* heterologous expression system fused to C-terminal peptide tags, to aid subsequent purification. Since these enzymes can be readily produced without

J Sci Food Agric **87**:1435–1448 (2007)
DOI: 10.1002/jsfa

contaminating activities, they will likely be very useful for the detailed analysis of most polysaccharides that are constituents of the plant cell wall.

Rapid characterisation of changes in wall composition and architecture

Many insights into how cell walls change during growth and development have been provided by careful analysis of polysaccharides in cell wall preparations derived with a series of aqueous extractants.[2] These techniques are extremely valuable, but they are time-consuming and require relatively large amounts of materials. New techniques have been used for cell wall analysis in the last few years, including polysaccharide analysis by carbohydrate gel electrophoresis, oligosaccharide fingerprinting, and Fourier transform infrared (FTIR) and FT Raman spectroscopies. These methods could provide information to complement that obtained in biochemical fractionation procedures and are likely to be more appropriate in high-throughput research projects.

Oligosaccharide fingerprinting. In this method, digestion of complex mixtures of wall polysaccharides with a specific glycanase produces oligosaccharide products that can be identified by matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS) analysis.[18] Although MALDI-TOF analysis does not allow absolute quantitation of oligosaccharide abundance, it is possible to reproducibly obtain relative abundance information on the various oligosaccharide 'signatures'. This technique can be used to deduce the structure of the parent polymer and thus provides a means to rapidly screen for and identify cell wall mutants,[19] or to characterise progressive alterations in specific polysaccharides during developmental changes. MALDI-TOFMS has also been used to characterise the pectin-derived oligosaccharides that accumulate in ripening and pathogen-infected tomato fruits.[20]

Polysaccharide analysis by carbohydrate gel electrophoresis (PACE). This technique involves the enzymatic release of oligosaccharides from a polymer followed by their derivatisation with a fluorophore and then separation by electrophoresis in polyacrylamide gels.[21] The migration of different oligosaccharides will depend on their mass and monosaccharide composition, so polysaccharides will generate a specific fingerprint.[21] Both structural and quantitative information can be obtained with this technique. It has been applied to characterise *Arabidopsis* pectic polysaccharides[20] and could also be useful to study the specificity and kinetics of plant polysaccharide hydrolases.[22]

Most traditional chemical analyses of plant cell walls and the two strategies described above are destructive, in that they involve irreversible breakage of chemical bonds: wall structure is disrupted during extraction and so positional information is lost during sample preparation. Therefore the ability to generate images of the chemical composition from plant cell walls *in situ* and non-destructively would be a significant advance. This is certainly true for studies of ripening fruits, since ripening is not uniform and does not occur simultaneously over the fruit surface, or in different fruit tissues. Some alternative analytical methods that can probe the wall in a native state have been developed.

IR spectroscopy and Raman spectroscopy. IR spectroscopy can be used with underivatised cell walls, and analyses are based on the interaction of the radiation with unextracted cell wall material. With IR spectroscopy the ratio of transmitted to incident radiation is measured, while Raman spectroscopy involves detecting sample-induced radiation scattering.[23] The two techniques are based on different properties of the analytes, so the information generated is complementary, but both involve the identification of spectra that are diagnostic for specific functional groups.[24] The acquisition of a spectrum is rapid, allowing high-throughput screening of walls from a population or developmental series,[25,26] and computational tools have been developed to then assign the spectrum to known compounds, based on previously characterised materials, or to assist with the classification of unknown compounds.

Raman microspectroscopy. The combination of microscopy and spectroscopy techniques has recently been used to provide detailed non-destructive, *in situ* chemical information on the distribution of components in secondary cell walls.[27] The method has several advantages, since no stains or contrast agents are required and the spectra can be acquired from aqueous samples that are thicker than those used for conventional microscopy, so spatially resolved information can be derived from intact tissue.[28] For example, the technique has recently been used to determine the distribution of cell wall components and to detect changes in orientation of cellulose microfibrils.[27]

Increasing the specificity of tissue-sampling techniques

The data obtained from most traditional cell wall analyses reflect averaged values from different cells and tissues that are mixed during sample preparation. This inevitably obscures any differences in wall composition and can generate artefacts. In order to reduce this problem, some studies have attempted to analyse cell wall components after isolating the regions of interest with a micromanipulator.[29] While providing spatially important data, this approach can be time-consuming and tedious, and an attractive alternative is laser capture microdissection (LCM), a high-precision tool, with a degree of automation, to isolate specific targeted cell populations from tissue specimens. LCM methods can provide valuable information about wall composition from different cell types[30] and are

now being used to characterise the spatial variation in wall composition in ripening tomato fruit (Rose JKC, unpublished). Such analyses should substantially improve our knowledge of the biochemical diversity of plant walls.

Immunolocalisation of cell wall components

Antibodies coupled to fluorescent markers have already been extremely useful for determining the distributions of particular cell wall constituents in specific tissues, cell types and wall microdomains.[31] The characterisation of epitopes recognised by additional, potentially useful monoclonal antibodies is ongoing and should add to the information content of immunolocalisation studies. Suites of new probes are currently in development, such as the carbohydrate-binding modules (CBMs) of cell wall glycoside hydrolases, which can have unique specificities for different wall epitopes, making them a potentially useful complement to monoclonal antibodies for probing cell wall microstructure.[32,33]

WALL MODELS AND WALL STRENGTH: IMPLICATIONS FOR FRUIT-RIPENING STUDIES

It is generally assumed that the biomechanical properties of cell walls change substantially during ripening, i.e. the walls loosen or become weaker. However, to date, the structural bases of those changes have only been inferred and not proven. For example, postharvest biologists have long recognised that bathing fruits in solutions of $CaCl_2$ increases fruit firmness,[34,35] and, since these treatments also reduce pectin solubility, it is reasonable to conclude that strengthening of the pectin 'egg-box' structures will result in a stronger cell wall. However, no direct studies have addressed this question, so it is important to identify the key structures and crosslinks that confer mechanical strength and influence wall integrity.

A number of recent reports have investigated the relationship between specific wall components and wall tensile strength in other organs. These include comparisons of wild-type *Arabidopsis thaliana* hypocotyls with mutants exhibiting altered xyloglucan and RG-II structure,[36,37] efforts to correlate the overall order of cellulose microfibril networks with wall yield strength,[38] and examinations of the physical strength of artificial wall composites.[39] The general conclusions from such studies are that the pectin and cellulose/hemicellulose wall polysaccharide networks both contribute to wall tensile strength. However, the extent to which changes in wall tensile strength are involved in fruit softening is not clear. While the tensile strength of the wall has a critical effect on cell elongation and is typically quantified by uniaxial measurements, fruit softening is generally assessed by measuring the force required to compress or penetrate the fruit surface. The resistance of a plant tissue to compression is affected by intercellular adhesion, shear forces between polymers, and cellular turgor pressure that forces the membranes of individual cells against the cell walls. Ulvskov and co-workers[40–43] manipulated the overall content and structure of branched RG-I pectin in potato cell walls by expressing fungal pectin-digesting enzymes in developing tubers. When cylinders of tuber tissues with modified RG-I arabinan or galactan side chain content were compressed and the fracture forces measured, it was clear that the elimination of these side chains resulted in decreased tuber strength.[43] A common feature of the ripening-associated changes in fruit cell walls is the hydrolytic cleavage of arabinose- and galactose-rich pectin side chains,[44] and studies of fruits of the *Cnr* tomato mutant suggest that metabolism of homogalacturonan backbones and pectin side chains affects the strength of fruit cell–cell associations.[45,46] The use of antibodies that recognise RG-I arabinan epitopes helped to reveal the impact of the *Cnr* mutation on fruit cell walls. These antibodies might also be used for affinity chromatography to purify and more thoroughly characterise the RG-I pectins from mutant and normal fruits. In a similar vein, the observation that transgenic tomato fruits with suppressed β-galactosidase/β-galactanase expression softened more slowly during ripening[47] further supports the idea that enzymes involved in the cleavage of pectin side chains make important contributions to fruit texture. Although no specific strength-conferring structures can be identified from these observations, it is apparent that pectin side chain metabolism contributes to fruit softening.

BROADENING THE LANDSCAPE OF CELL WALL DISASSEMBLY: IDENTIFYING NEW WALL-MODIFYING ENZYMES

Studies over the past few decades have identified many CWMPs in extracts of ripening fruits. Most of these were first identified by incubating protein extracts with readily available model substrates and then applying a simple assay, such as measuring the products of substrate hydrolysis, to guide the purification and subsequent sequencing of the active protein. This reliance on commercial substrates and focus on specific, defined biochemical activities inevitably restricts the range of potential CWMPs that can be identified, and it is likely that crucial proteins are overlooked simply because suitable complex substrates are not readily available, or the appropriate assays have not been developed. Some examples of unanswered questions that relate to this issue and some new approaches to extend our understanding of CWMPs are given below.

Endo-1,4-β-glucanases: proteins in search of a substrate

Plant cell walls contain a number of polysaccharides with 1,4-β-D-glucosyl linkages, including cellulose, xyloglucan, glucomannan and mixed linkage glucan. Plant enzymes that can hydrolyse such bonds at

J Sci Food Agric **87**:1435–1448 (2007)
DOI: 10.1002/jsfa

internal positions in polysaccharides are referred to as endo-1,4-β-glucanases (EGases), although they are also termed 'cellulases', by analogy with EGases from microbes that degrade cellulose. However, in most cases the endoglucanase activities that have been reported in ripening fruits should, for the sake of accuracy, be referred to as CMCases, since the artificial substrate carboxymethylcellulose (CMC) has typically been used. CMC is a methylated, and thus soluble, form of cellulose that is commercially available and widely used as an analogue of 1,4-β-linked glucan cell wall polymers. However, it is not possible to directly associate CMCase activity with a corresponding activity against any particular native cell wall polysaccharide *in vivo*. Analyses of the activities of several plant EGases have not revealed a consistent pattern of substrate specificity, and different isozymes exhibit differing affinities for a range of glycan substrates.[3,48–50] However, even with these more detailed studies, native substrates have rarely been used, which further complicates any interpretation. The most promising approach to date has been through examining the composition of the cell walls of fruits in which such genes have been suppressed or up-regulated,[51,52] although even these studies have provided little insight into the substrate of EGases.

The mystery of xyloglucan depolymerisation

Xyloglucan depolymerisation is a characteristic of most ripening fruits, including tomato, and, given its proposed structural importance, cleavage of xyloglucan chains by xyloglucanolytic enzymes provides an attractive wall-loosening mechanism.[2,3,16,53] For many years it was believed that plant EGases were responsible for this activity, since xyloglucan has a 1,4-β-linked glucan backbone and since patterns of EGase gene expression and CMCase activity in softening fruits often correlate and are coincident with xyloglucan depolymerisation. However, this idea is at odds with studies using transgenic plants,[51,52] and a number of groups have now shown that recombinant plant EGases have minimal activity against xyloglucan.[47,48,50] Several distinct xyloglucanase and CMCase activities have been reported in ripening tomato,[54,55] although no corresponding proteins have been purified and so the identity of the enzymes and cognate genes that are responsible for the activities is still unknown. An alternative class of proteins that might catalyse xyloglucan degradation are xyloglucan endotransglucosylase-hydrolases (XTHs), which can act as both xyloglucan hydrolases (XEH activity) and xyloglucan transglucosylases, where xyloglucan chains are cleaved and then re-ligated to other xyloglucan acceptor molecules (XET activity).[56] *XTH* gene expression and associated activities have been reported to increase in several species of ripening fruits,[56] although these studies are correlative and their contributions to xyloglucan depolymerisation *in vivo* have not been confirmed experimentally. A fruit-ripening-related *XTH* gene from tomato was recently reported whose sequence suggested that it would have XEH activity, and, while characterisation of biochemical activity *in vitro* did not support this idea, the activity *in vivo* may be substantially different.[57] The enzymatic basis of xyloglucan degradation in ripening tomato fruit is therefore still entirely unknown and remains an important area of study.

Studies of ripening tomato fruit have indicated that xyloglucan depolymerisation occurs early in the ripening process,[16] although the proteins responsible have not been clearly identified. The oligosaccharide-fingerprinting techniques developed by Pauly *et al*.,[18] specifically for the characterisation of xyloglucan oligosaccharides, could be used in support of studies aimed at determining whether XTH and EGase/CMCase proteins play a role in this early aspect of tomato wall metabolism.

Unravelling pectin breakdown

Pectin metabolism remains the most studied aspect of cell wall biology in tomato fruit, and numerous reports have described the molecular details of pectin structure and modification and the nature of genes and enzymes that are associated with pectin degradation. However, while pectins comprise a highly complex polysaccharide network with a structurally diverse range of glycan chains, glycosidic linkages and other substituents, such as acetyl and methyl groups, most studies of pectin depolymerisation have focused on small group of enzymes: polygalacturonase (PG), pectin methylesterase (PME) and β-galactanase.[2,3] Evidence from various sources now suggests that a number of other classes of enzymes are likely to be involved, although many have not been characterised or the activities detected.

Pectate/pectin lyase

It was originally thought that pectate lyase (PEL) was strictly a microbial enzyme, but PEL activity and *PEL* genes have now been detected in many plant species, often including sequences that are specifically expressed in fruits.[3,58] The expression of PEL genes in ripening tomato fruit has been reported[59] and the unsaturated oligosaccharides characteristic of PEL action have been identified in healthy tissues of *Botrytis cinerea*-infected tomato fruit[20] and in ripening, uninfected tomatoes (An H, Lurie S, Lebrilla C and Labavitch J, unpublished), contradicting a previous report suggesting the absence of PEL in tomato,[60] and demonstrating the value of techniques for isolating and characterizing wall breakdown products in ripening fruit. Suppression of *PEL* gene expression in transgenic strawberry was reported to result in substantially firmer fruits and reduced cell wall swelling,[61] and whether or not this effect can be reproduced in other fruits is an exciting question.

Acetylesterases

Besides the well-studied methyl esterification at C6 of galacturonic acid (GalA) residues, pectins are often

acetyl esterified at C2 and C3, ranging from 0 to 90% depending on the tissue and species.[62,63] Many other plant wall polysaccharides, including xylans and mannans, could also be present in acetylated forms.[64] The significance of wall polymer acetylation is not fully understood, but acetylated pectins are more soluble than non-acetylated polyuronides. Furthermore, as has been shown for C6 methyl-esterified pectins and for acetylation of some bacterial polysaccharides, the increased esterification could significantly reduce the activity of some plant and phytopathogen-derived cell wall-degrading endoglycosidases.[64] Acetyl esterases (AEs) that might catalyse the deacetylation of these polymers have been found in bacteria,[65] fungi[66] and plants.[67–71] The limited studies of plant AEs include a report on the purification of a tomato fruit AE that was a contaminant in a commercially available tomato PME preparation.[71] A number of predicted AE sequences are present in Expressed Sequence Tags (EST) collections derived from ripening tomato and citrus fruits (Rose JKC, unpublished). This suggests a role in wall disassembly, although, to date, their substrates and physiological role have not been described.

Rhamnogalacturonan (RG-I) hydrolases and lyases

Schols *et al*.[72] reported that rhamnogalacturonase (RGase) was able to cleave within the main chain of RG-I, but, while RGases have been identified in several fungi,[73–75] little attention has been paid to the potential role of RGase in plants. Several genes with high similarity to RGases and RG lyases are present in plant sequence databases, and RG hydrolase has been found in several fruit species,[76] but few studies have analysed the characteristics and potential significance of these enzymes in wall modification. Because RG-I arabinan and galactan side chains are thought to influence cell–cell interactions,[46] as described above, it is logical to assume that disruption of the RG-I backbone would also affect the strength of these associations. Moreover, some of the pectin-derived oligosaccharides that accumulate in ripening tomato fruit contain rhamnose,[77] suggesting that RG-I depolymerisation is an aspect of ripening-associated cell wall metabolism.

Wall-loosening action but no known biochemical activity

Expansins are proteins that were originally described more than 10 years ago as having a wall-loosening activity that contributes to cell expansion,[15,78] but their mechanism of action has still not been determined. Expansin activity can currently be detected only by its effect on the biomechanical properties of plant tissues, rather than by a simple spectrophometric measurement, and only once expansin gene sequences were available was it determined that expansins also likely contribute to fruit softening.[79] Later studies showed that expansin proteins and activity are present in ripening fruit[80] and that expansins appear to influence wall disassembly and aspects of fruit texture,[81,82] but it is important to note that such information would be lacking without the development of the original biomechanical assay. The application of such novel methods to measure wall modification and the use of more complex native substrates will be important areas of future research.

Identification of CWMPs through genomics and proteomics

An alternative strategy to elucidate the complexity of wall metabolism is to take essentially the opposite approach and, rather than focus on one enzyme or activity, characterise the entire proteome, or complete protein population, of the plant wall/apoplast. A complete catalogue of the proteins that are present in the wall during a particular physiological event, such as fruit softening, could then provide a basis to identify new, potentially interesting candidates and, importantly, to determine the suites of CWMPs that are co-regulated. This is a challenging objective, since the isolation of cell wall proteins is notoriously difficult, but several new tools have been developed, including techniques to isolate highly purified wall protein extracts, functional screens for secreted proteins, and bioinformatic analyses, that collectively hold great promise.[83,84] For example, a preliminary analysis of the tomato fruit cell wall proteome, or 'secretome', using a yeast secretion trap screen (described by Yamane *et al*.[85]) revealed that approximately 40% of the identified secreted proteins could be classified as 'new' cell wall proteins. This designation meant that either the protein function was entirely unknown, since there was no revealing sequence homology to a functionally characterised protein, or that the location of the annotated protein was unanticipated, based on its predicted function (Lee H and Rose JKC, unpublished). Bioinformatic prediction has also suggested the existence of far more wall proteins than was previously thought, although, again, in many cases no putative function can be assigned.

While these sets of genome/proteome-scale data do not by themselves advance our understanding of wall modification, they allow the generation of hypotheses that can then be tested experimentally. For example, the Carbohydrate Active Enzymes (CAZY) database (http://afmb.cnrs-mrs.fr/CAZY), a repository of information related to enzymes that interact with carbohydrates, provides a valuable means to link sequence information with catalytic activity. This resource allows large sets of DNA or protein sequences to be screened for putative CWMPs, including those with unusual structural, and hence functional, characteristics. We recently identified and characterised a new subclass of plant EGase proteins with an atypical C-terminal domain that is reminiscent of CBMs from microbial EGases.[50] Such domains are essential for effective EGase-mediated cellulose hydrolysis but have not been reported in plant EGases. Indeed, the reported inability of

J Sci Food Agric **87**:1435–1448 (2007)
DOI: 10.1002/jsfa

plant EGases to degrade crystalline cellulose is generally attributed to the absence of a CBM. We have now shown that the CBM from this new plant EGase subclass binds to cellulose, that it can potentiate cellulose hydrolysis and that the associated catalytic domain can hydrolyse a range of cell wall polysaccharide substrates. Interestingly, a tomato gene encoding this type of EGase is expressed in ripening fruit (Urbanowicz BR, Catalá C and Rose JKC, unpublished), suggesting that it plays a role in wall disassembly, and the nature of the *in vivo* substrate(s) is currently under investigation. These data further reinforce the idea that many new types of enzymes and activities remain to be identified. Moreover, this study illustrates how sequence information and bioinformatics analysis, rather than 'traditional' enzyme purification and analysis, can be exploited and used to catalyse new lines of research. A detailed understanding of the composition of the cell wall proteome of ripening fruits would certainly contribute to a more sophisticated model of the mechanisms of wall disassembly. However, this remains an ambitious and long-term goal, and progress will also rely on the ability to evaluate the function of newly identified wall-localised proteins.

TO WHAT EXTENT IS RIPENING-RELATED WALL DISASSEMBLY A SYNERGISTIC PROCESS?

As discussed above, studies indicate that the cellulose/hemicellulose and pectin polymer networks contribute to the cell wall strength and tissue integrity. Therefore, the most effective way to understand the importance of fruit cell wall metabolism may be to consider the modification of polymer networks, rather than individual polymers. From this perspective, wall restructuring is a synergistic process involving the simultaneous or sequential interaction of numerous families of CWMPs with a particular polysaccharide macromolecular network.

To date, most of the reverse genetic approaches to understanding the functions of CWMPs have targeted the expression of single genes encoding one enzyme type.[2] With the exception of the antisense suppression of PEL activity in strawberry,[61] where the delay in softening was substantial, the effects on fruit firmness have been limited. One explanation for this could be that the targeted enzymes are not the key factors that influence softening; however, another possibility is that there is functional redundancy involving isozymes within the same protein family, or other divergent proteins whose actions compensate for that of the suppressed enzyme. For instance, PGs and PELs could both contribute to polyuronide backbone degradation as tomatoes ripen; thus, when only PG expression is suppressed, as in the 'FlavrSavr' tomato,[86] at least some pectin degradation continues and the full impact of suppressing pectin breakdown on fruit softening would not be realised. If RGases also act in ripening fruits, then the effect of PG suppression might seem even smaller, because few studies have attempted to discriminate between the breakdown of homogalacturonan and RG pectin backbones.

In addition to this potential for targeting of similar polymer backbones by different enzymes, efficient degradation of polysaccharides can require cooperative or synergistic interactions between the enzymes responsible for cleaving the set of different linkages present in a polymer.[87] The access of PG, PEL or RGases to pectin backbones is likely to be influenced by enzymes that remove backbone substituents, such as PME or AE, or that remove polymer side chains. Thus the reduced tomato fruit softening that resulted from suppressing β-Gal expression[46] may have been the result of prolonged shielding of a pectin backbone from the enzymes that act on that backbone, rather than a specific contribution of galactan side chains to wall integrity. α-Arabinase/arabinosidase (α-Ara) could play a similar role in pectin metabolism.[88]

Rose and Bennett[16] discussed the potential cooperative actions of expansins, EGases, XTHs and glycosidases in disassembly of the tomato cellulose/xyloglucan network; similarly, disassembly of the pectin network certainly involves suites of pectinases acting in concert. However, recent studies in our laboratories suggest a surprising degree of interaction between the major wall polysaccharide networks. For instance, over-expression of PG in fruit of the non-ripening tomato mutant *rin*, which undergoes minimal softening,[5] caused increased depolymerisation of hemicelluloses, in addition to expected effects on pectin integrity. Conversely, *rin* fruit over-expressing expansin, a protein that has been associated with disassembly of the cellulose/hemicellulose network, showed increased pectin degradation. When PG and expansin were co-expressed in *rin* fruit, these effects were magnified and the degree of fruit softening approached that seen in normally ripening fruit (Vicente AR *et al*., manuscript in preparation). Our conclusions about cell wall changes in these transgenic *rin* fruits were based on analyses of serial extracts of wall polysaccharides, a time-consuming process. Interestingly, preliminary studies of intact walls from the *rin* fruits using more rapid FTIR analysis supports several of our conclusions. Clearly, much remains to be learnt about the synergistic nature of wall disassembly. Modification of the expression of combinations of genes/enzymes may be necessary to elucidate such interactions and their influence on wall strength and fruit softening, and approaches for facile characterisation of wall changes will make these studies more manageable.

REGULATION OF WALL MODIFICATION BY THE CHEMICAL ENVIRONMENT OF THE APOPLAST

Identification of the activity of a CWMP in a protein extract has often been the starting point of studies designed to test the linkage between fruit wall metabolism and fruit softening. However, the assessment of enzyme activities is often made by assays

carried out under 'optimum' conditions for a given activity, such as pH 5–6, a value at which most wall hydrolases have the greatest activity, and typically little attempt is made to examine the effects of ions or cofactors. An analysis of the pH of apoplastic fluid from tomato fruits at different ripening stages revealed a change from 6.7 in mature-green (MG) fruits to 4.4 in ripe fruits;[89] similarly, substantial changes in ion concentrations were detected. Both these factors, as well as other aspects of the apoplastic chemical environment, have the potential to affect CWMP activities and wall polymer interactions and to exert a significant influence on wall metabolism.[3] Another important aspect of the apoplastic environment is cell wall porosity, which, based on several measurements, could limit protein movement. The cell walls of some fruits swell during ripening,[90] which presumably would result in an increase in porosity, thus allowing easier access of CWMPs to their substrates. Finally, the probability that fruit firmness is substantially influenced by cellular turgor (see below) suggests that the ripening-regulated movement of solutes across the plasmalemma is another aspect of the regulation of the apoplastic environment that must be better understood.

DOES CELL WALL SYNTHESIS CONTRIBUTE TO FRUIT SOFTENING?

Most studies of wall metabolism in ripening fruits have focused on polysaccharide solubilisation and depolymerisation, based on the presumption that degradation of wall components is likely to weaken the wall. However, Knee[91] measured incorporation of radiolabelled precursors into the pectins of ripening apples and proposed that insertion of new components into the wall might also contribute to wall weakening and fruit softening. More recent work with ripening tomato fruits[92,93] and pericarp explants[94,95] demonstrated that the capacity for synthesis of new cell wall polysaccharides is retained throughout fruit ripening. Whether wall synthesis normally occurs in ripening fruits, and, if so, how it might contribute to fruit softening, is not clear. However, in elongating seedling tissues, continuing wall synthesis is required for maintenance of growth rates above baseline values,[96] and cell extension growth depends on increased wall extensibility and, presumably, wall loosening. Furthermore, many of the wall-modifying proteins that are active in ripening fruits are encoded by genes comprising small families that also have members that are expressed during cell expansion.[2,16] Therefore, if *de novo* wall synthesis plays a role in growth-related wall weakening, it would not be surprising if aspects of softening were also influenced by wall synthesis in ripening fruits.

However, we should not have to rely on a constructed argument, no matter how logical, to suggest that wall polymer synthesis with polymer incorporation into the wall fabric is a contributor to fruit softening. In the past 10–15 years, studies of natural and laboratory-generated *A. thaliana* lines have resulted in identification of many genes involved in wall polysaccharide synthesis,[14,97,98] and orthologous sequences are present throughout the plant kingdom. Transgenic or naturally occurring mutants in wall biosynthesis-associated genes that are expressed in ripening fruit, examples of which are present in the public tomato sequence and gene expression databases (data not shown), should provide insights into the significance of continued polysaccharide formation and deposition for fruit texture. Tomato would be an excellent system for each of the steps in this analysis.

DOES TURGOR PRESSURE CONTRIBUTE TO FRUIT FIRMNESS?

While the primary focus of research on fruit firmness has been on cell wall metabolism, a few studies have demonstrated that cellular turgor pressure is important in determining firmness, as determined by assessing resistance of intact fruits or tissues to compression. Ripening tomato fruits and explanted pericarp discs show a decline in cellular turgor that roughly parallels the ripening-associated loss of firmness,[99] and, importantly, this reduction in turgor is not a consequence of a loss of membrane integrity. Treatment of ripening pericarp discs with $CaCl_2$ slows tissue softening and, at least in the short term, causes an increase in pericarp firmness even if the calcium treatment is not applied until the discs are approaching the red-ripe stage.[35] Not surprisingly, the treatment increased the amount of cell wall pectin that was extractable by chelator solutions, but it also led to an increase in turgor pressure. This study highlighted the parallel relationship between fruit firmness and turgor and suggested that changes in turgor are under cellular control.

The decrease in fruit cell turgor during ripening is most likely due to both the accumulation of solutes (sugars, organic acids, ions, etc.) in the apoplast, due to export cellular compartments, and accompanying water efflux from the cell and transpirational water loss from the fruit, which would also reduce the water potential of the apoplast and drive water efflux. Levels of apoplastic solutes are known to increase substantially during tomato ripening,[89,99] so identification and characterisation of membrane-localised ion and sugar transporters and channels and aquaporins could provide information leading to genetic or other manipulations of fruits to regulate cell turgor and fruit firmness. Alternatively, the regulation of water transpiration from ripe fruits may also represent an important strategy to prolong fruit firmness. We have recently identified several tomato mutants whose fruits exhibit exceptional shelf life, remaining firm and at an edible texture for many months after reaching a fully ripe stage, and that also appear to be entirely resistant to postharvest pathogen infection.[100] Remarkably, cell wall degradation and

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cell–cell separation occur to the same extent and at the same rate as in normally softening fruits, suggesting that wall disassembly is not the sole and primary cause of fruit softening. We have observed, however, that cellular turgor remains high in these mutants, unlike normal fruits, suggesting that turgor is of critical importance. We have also obtained evidence that the fruit cuticle plays a fundamental role in regulating transpirational water loss and that it undergoes structural modification during ripening. Experiments are now under way to determine the nature of the genetic lesion that regulates the phenotype and to identify key structural components of the cuticle that influence water loss.

THE ULTIMATE STAGE OF WALL DISASSEMBLY AND POSSIBLE ASSOCIATED PLEIOTROPIC EFECTS

As indicated above, in the last few years there has been considerable progress in many aspects of the study of cell wall metabolism, including wall synthesis,[101–104] architecture[12,13] and degradation.[4] One aspect of wall metabolism that has been less explored is the potential contribution of the products of wall disassembly to other cellular processes. There have been several interesting observations in this respect, some of which suggest that the interaction between wall and cell metabolism is broader than previously thought. A key question that appears to be closely linked to this phenomenon is: what happens to wall components that are metabolically removed from the wall – where does the cell wall go?

Cell wall polymer endocytosis

For many years it has been claimed that the initial cell wall deposited during cell division, or cell plate, is produced through delivery of newly synthesised material to the cell surface from the Golgi apparatus by secretory vesicles. However, it has recently been shown that endocytic delivery of cell surface material significantly contributes to cell plate formation during cytokinesis in several plants species.[105] The internalisation of large amounts of pectins and arabinogalactan proteins has been shown to occur in cells undergoing either division or rapid growth,[106] and other studies have shown that pectins that are crosslinked with boron and calcium are internalised into plant cells and apparently recycled via early/recycling endosomes.[107,108] Thus, it seems that cell remodelling can be fuelled by substrates that are supplied by endocytosis.[106] This phenomenon has been observed in expanding and mitotically active cells, where massive amounts of cell wall constituents are required in short periods of time. However, the role of this endocytic wall recycling in other plant developmental processes, such as fruit development and ripening, should be examined.

Simple sugar recycling

A consequence of the *in muro* processes of wall alteration and loosening is the generation of oligomeric and monomeric degradation products. In bacteria it has been established that over half of the lateral wall murein is broken down in each generation and then imported into the cytoplasm, and that both cytoplasmic *N*-acetylglucosamine and anhydro-*N*-acetylmuramic acid derived from cell wall recycling are reused.[109] Recycling of the sugars that are released as a consequence of cell wall turnover indicates that the primary cell wall is not simply a sink for various polysaccharide components, but rather a dynamic structure exhibiting long-term reorganisation and degradation of specific polymers during development.[110] The extent of the recycling of simple sugars generated by plant wall degradation is still unclear, and utilisation of salvaged breakdown products would require their import. Higher plants possess two distinct sugar transporters: those that import sucrose and those that import monosaccharides.[111] Hexose uptake across the plasma membrane is catalysed by monosaccharide/proton symporters, referred to as sugar transport proteins (STPs). STPs have been described at the molecular level in several plant species[111] and can mediate the transport of a range of monosaccharides. For instance, the *Arabidopsis* AtSTP1 protein transports glucose, mannose and galactose, and it has been proposed that it may function in the recovery of monosaccharides liberated by cell wall turnover.[112]

Potential impacts of recycled carbohydrates on non-wall metabolic processes

An issue that may deserve further investigation is the potential introduction of sugars from cell wall turnover into intracellular metabolic pathways. Although little has been done to address this question directly, a few observations using transgenic plants suggest that altered wall metabolism may result in pleiotropic effects. For example, Tieman *et al*.[113] reported that fruits from transgenic plants with down-regulated PME gene expression showed significantly higher levels of soluble solids compared with non-transformed fruits. Whether this resulted from increased solubility of pectins with higher methyl esterification, or by another mechanism was not examined. A second example was provided by studies that linked wall metabolism with a major intracellular biosynthetic pathway. While animals use UDP-D-glucose derived from glycogen as the main substrate for the *de novo* synthesis of ascorbate,[114] the main precursors of ascorbic acid in plants are D-mannose and L-galactose.[115] In addition to this pathway, the existence of an alternative biosynthetic route, via uronic acid intermediates, has been described.[116] Interestingly, in addition to the modification in fruit firmness observed in several independent antisense RNA suppressed PEL strawberry lines,[61] the transformed fruit had a reduced

ascorbic acid content.[117] This led the authors to suggest that D-galacturonic acid (GalA) derived from pectin was reduced to L-galactonic acid, which in turn is readily converted to ascorbate. Several related processes, such as the internalisation of the GalA precursors from the apoplast to the mitochondria, where the final oxidative step of ascorbate synthesis occurs, have not been clearly demonstrated, but in any case these observations suggest a primary involvement of cell wall metabolism-derived products in non-wall traits, such as vitamin synthesis.

NEW SYSTEMS FOR DIRECTLY TESTING GENE FUNCTION ASSOCIATED WITH FRUIT WALL METABOLISM

While modifying the expression of CWMP-encoding genes in stably transformed tomato lines using RNAi, antisense or overexpression technologies is now a relatively straightforward procedure, the process requires substantial effort and time. Moreover, although the simultaneous targeting of multiple genes in the same line can be accomplished and is certainly desirable, given the apparent synergistic nature of wall disassembly, this 'pyramiding' approach is technically more challenging. It would therefore be extremely useful to have techniques to rapidly screen for mutants in specific CWMPs and to assess the potential value of making a particular stable transformant with modified expression of multiple CWMP genes, before investing effort in creating stable transformants. Some examples of such recently developed and emerging technologies include the following.

Transient expression (TE)

Localised, transient gene expression in leaf discs following co-cultivation with *Agrobacterium* is a well-established technique for gene functional studies.[118] This 'agroinfiltration' strategy does not require stable integration of the T-DNA into the host genome, and the desired protein is usually expressed within a few days after infection. A recent report described a similar approach for transiently suppressing or enhancing gene expression in tomato[119] and other fruits.[120] The technique is particularly useful for gene function studies in perennials and species in which fruits are obtained after a long juvenile phase, and a protocol for transient transformation has also been developed in *Citrus* fruits.[121]

Virus-induced gene silencing (VIGS)

This strategy does not require development of stable transformants and offers a rapid alternative for knocking out expression of genes of interest.[122] Using this method, a recombinant virus carrying a partial sequence of a host gene is used to infect the plant.[123] When the virus spreads systemically, the endogenous gene transcripts, which are homologous to the insert in the viral vector (VIGS vector), are degraded by post-transcriptional gene silencing.[124] Improvements to vectors and inoculation techniques have opened the door to large-scale VIGS experiments and theoretically allow the simultaneous suppression of multiple genes. The VIGS technique has been reported to be successful in tomato fruit[125] and represents a powerful tool for studies of fruit ripening-associated genes.

Targeted-induced local lesions in genomes (TILLING)

TILLING provides an alternative non-transgenic approach with great potential for both gene knock-out and structure–function studies and allows relatively easy identification of novel alleles in either mutagenised or natural populations. While this technology was initially used in reverse genetic studies by plant biologists, it is now being applied to crop improvement[126] and holds great potential as a means to both characterise and manipulate enzyme action and function.

Despite the potential advantage of testing the involvement of a number of genes in cell wall metabolism, both TE and VIGS also have several disadvantages. Often these methods do not result in uniform silencing or over-expression of the gene throughout the fruit, and the levels of expression can vary between plants and experiments. In addition, in some cases the identification of a selectable marker that does not interfere with the ripening process or generate spurious phenotypes could be problematic. Finally, several controls are required to assure that the observed modifications result from changes in the level of expression of the gene of interest and are not artefacts. Despite these drawbacks, the development of proper VIGS and TE protocols to be used in fruits could speed up the tests for determining the importance of specific single CWMPs, or combinations of CWMPs, in fruit cell wall breakdown and softening. The combination of such approaches with the creation and screening of large TILLING populations in tomato and other fruits promises to revolutionise the ability to directly and rapidly assess gene function.

CONCLUSIONS

This is an exciting time to study fruit softening and to address the association with cell wall disassembly, for several reasons. A number of dogmatic views in the cell wall/fruit-softening literature have been overturned and now firmly laid to rest, and many new questions are being asked that are bringing together researchers from diverse fields that have not previously had a connection with cell wall biology. There is a general acknowledgment of how little we really know about the nature and regulation of wall disassembly *in vivo*, yet it is apparent that the experimental toolbox has been dramatically expanded. We now have a long list of new and exciting questions and sets of novel

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DOI: 10.1002/jsfa

techniques and resources with which to address them, and there is no doubt that a review of this field a few years from now will describe remarkable developments that are currently inconceivable.

ACKNOWLEDGEMENTS

Research in this area by JKCR is supported by the National Science Foundation's Plant Genome Program (award DBI 0606595), the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service (award 2006-35304-17323), CUAES Hatch project NYC-184485 and a grant from the New York State Office of Science, Technology and Academic Research (NYSTAR). Research at UC Davis (ARV, JML) has been supported by the California Agricultural Experiment Station and USDA Regional Research project NE-1018. Thanks are due to SECYT (Argentina) and the Fulbright Commission for financial support to ARV.

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