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**Pectin Cross-Linking Dynamics and Wall Softening
during Fruit Ripening**

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Project Title:

**PECTIN CROSS-LINKING DYNAMICS AND WALL SOFTENING DURING FRUIT
RIPENING**

Investigators

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

**peach, wooliness, cell-wall metabolism, postharvest storage, chilling injury, cellulase, cellulose
apple, highly branched arabinan, cell-cell separation, non methyl esters of polyuronic acid,
phenolic compounds**

Abbreviations commonly used in the report, in alphabetical order:

**ACC, 1-aminocyclopropane-1-carboxylic acid; EGase, endoglucanase; HV, harvest; HVSL,
Shelf-life following harvest; PG, polygalacturonase; PME, pectin methylesterase; RS, regular
stored fruits; RSSL, Shelf life following regular storage.**

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Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

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Refereed (published, in press, accepted)			3	3
Submitted, in review, in preparation	1	2		3
Invited review papers				
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Abstracts		3		3
Not refereed (proceedings, reports, etc.)				

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Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		1		1
Longer Visits (Sabbaticals)				

Description of Cooperation:

-Amnon Lers had spent two weeks learning and applying cell-wall analysis methods in order to analyze the peach wooly tissue. _____

-The peach tissue samples were analyzed in the US laboratory. _____

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted				
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Abstract

Our study was designed to elucidate the chemical determinants of pectin cross-linking in developing fruits of apple and peach and to evaluate the role of breakage cross-linkages in swelling, softening, and cell separation during the ripening. Peaches cell walls soften and swell considerably during the ripening, whereas apples fruit cells maintain wall firmness but cells separate during late stages of ripening. We used a "double-reduction" technique to show that levels of non-methyl esters of polyuronic acid molecules were constant during the development and ripening and decreased only in overripe fruit. In peach, methyl and non-methyl esters increased during the development and decreased markedly during the ripening. Non-methyl ester linkages in both fruit decreased accompanied fruit softening. The identity of the second component of the linkage and its definitive role in the fruit softening remain elusive. In preliminary examination of isolated apples cell walls, we found that phenolic compounds accumulate early in wall development but decrease markedly during ripening. Quantitative texture analysis was used to correlate with changes to wall chemistry from the fresh-picked ripe stage to the stage during storage when the cell separation occurs. Cell wall composition is similar in all cultivars, with arabinose as the principal neutral sugar. Extensive de-branching of these highly branched arabinans pre-stages softening and cell-cell separation during over-ripening of apple. The longer 5-arabinans remain attached to the major pectic polymer rhamnogalacturonan I (RG I) backbone. The degree of RG I branching, as judged from the ratios of 2-Rha:2,4-Rha, also decreases, specially after an extensive arabinan de-branching. Loss of the 4-Rham linkages correlated strongly with the softening of the fruit. Loss of the monomer or polymer linked to the RG I produce directly or indirectly the softening of the fruit. This result will help to understand the fruit softening and to have better control of the textural changes in fruit during the ripening and especially during the storage.

'Woolliness', an undesirable mealy texture that is induced during chilling of some peach cultivars, greatly reduces the fruit storage possibilities. In order to examine the hypothesis that the basis for this disorder is related to abnormality in the cell wall softening process we have carried out a comparative analysis using the resistant cultivar, Sunsnow, and a sensitive one, Hermosa. We investigated the activity of several pectin- and glycan-modifying enzymes and the expression of their genes during ripening, chilling, and subsequent shelf-life. The changes in carbohydrate status and in methyl vs. non-methyl uronate ester levels in the walls of these cultivars were examined as well to provide a basis for comparison of the relevant gene expression that may impact appearance of the wooly character. The activities of the specific polygalacturonase (PGase) and a CMC-cellulase activities are significantly elevated in walls of peaches that have become wooly. Cellulase activities correlated well with increased level of the transcript, but differential expression of PGase did not correspond with the observed pattern of mRNA accumulation. When expression of ethylene biosynthesis related genes was followed no significant differences in ACC synthase gene expression was observed in the wooly fruit while the normal activation of the ACC oxidase was partially repressed in the Hermosa wooly fruits. Normal ripening-related loss of the uronic acid-rich polymers was stalled in the wooly Hermosa inconsistent with the observed elevation in a specific PGase activity but consistent with PG gene expression. In general, analysis of the level of total esterification, degree of methyl esterification and level of non-methyl esters did not reveal any major alterations between the different fruit varieties or between normal and abnormal ripening. Some decrease in the level of uronic acids methyl esterification was observed for both Hermosa and Sunsnow undergoing ripening following storage at low temperature but not in fruits ripening after harvest. Our results support a role for imbalanced cell wall degradation as a basis for the chilling disorder. While these results do not support a role for the imbalance between PG and pectin methyl esterase (PME) activities as the basis for the disorder they suggest a possible role for imbalance between cellulose and other cell wall polymer degradation during the softening process.

Achievements

Fruit softening is a ripening associated process that affects the fruit quality and post-harvest conservation. Because the pectin substances are enriched in the walls of fruits compared to primary walls of other growing cells and because they constitute a major component of the middle lamellae, these acidic polysaccharides are suspected to play a major role in the wall dynamics during the fruit ripening. Over the years pectin modifications during the fruit ripening were studied extensively but the association with fruit softening was never completely resolved. In experiments with transgenic tomatoes, researchers found that blocking pectin depolymerization by anti-sense inhibition has little impact on wall swelling and softening during ripening. Other studies have shown that the swelling state of several fruit walls may change markedly without significant depolymerization. These data indicate these ripening phenomena may be accompanied by pectin depolymerization events but the events are not requirements for softening and cell separation. One of the surprises that emerged from the completion of the Arabidopsis genome sequence was the size of the repertoire of genes that encode polysaccharide degrading enzymes. For example, Arabidopsis contains over 250 genes that encode enzymes that depolymerize pectins. This significant allotment of the genome toward pectin turnover force to be considered again the role of the pectin in the wall swelling and softening. We proposed the hypothesis that pectins are cross-linked into a rigid form in the fruit walls, and that wall swelling is initiated by breaking these cross-links. Unraveling of the pectin substances may make them more susceptible to depolymerization, but the increase mobility can result in swelling without digestion of the polymers. Our study was designed to elucidate the chemical determinants of pectin cross-linking in developing fruits of apple and peach and to evaluate the role of breakage cross-linkages in swelling, softening, and cell separation during the ripening. Peach and apple present different textures when they are ripe. Peaches soften and swell considerably, whereas ripe apple presents a crispy texture and only softens during the over ripening. Both fruits in the overripe stage present extensive cell-cell separation. This different ripe behavior provided suitable comparative systems to associate wall modification with different styles of fruit softening. We characterized the major polysaccharides, pectic substances and their esters, and phenolic substances during early development, maturation, ripening, and during over-ripening when firm texture is lost.

A major finding from our biochemical and biophysical studies was that fruit growth and maturation is accompanied by a selective loss of pectin-associated (1→4)-linked galactan, whereas over-ripening and loss of firm texture was associated with a selective loss of a highly branched (1→5)-linked arabinan. A second, relative unbranched (1→5)-arabinan did not decrease during over-ripening. Texture analysis was used to determine the breaking strength and resistance to compression of apple during over-ripening of four cultivars that varied markedly in storage life. These experiments allowed us to differentiate between physical behavior and physiological age in our correlations of the biochemical changes in polysaccharide structure. These comparisons revealed that the loss of the highly branched arabinan *pre-staged* the loss of texture. Instead, it was the loss of branching of the RG I that was highly correlated with loss of texture.

In vitro cell-cell separation assays showed that structural proteins, perhaps via their phenolic residues, and homogalacturonans (HGs) potentially contribute to cell contiguity. Our results are consistent with a hypothesis that HGs of the middle lamellae covalently attached to RG I constitute a major cell-cell linkage. The loss of arabinan from RG I before textural changes may give an important clue to a mechanism whereby polysaccharides serve as molecular 'gates' for enzymes that modify wall architecture. Future studies will explore this possibility in systems more amenable to genetic transformation, such as tomato.

The chilling disorder termed "wooliness" ("mealiness") generally develops in susceptible cultivars during a short incubation at ambient temperature ("shelf-life") following 4-5 weeks of cold storage. This disorder is a limiting factor in peach fruit storage. The major symptom of wooliness is the lack of expressible juice and the development of dry, wooly texture. During normal ripening, together with the decrease in firmness due to degradation of cell wall components, there is an increase in expressible juice, which is easily released when the fruit is eaten. In sensitive fruits a long period of low temperature storage, such as 4-5 weeks at 0°C, followed by shelf life period at ambient temperature results with the development of the wooly texture and continuation of the ripening process. We have chosen for our comparative study two cultivars of peach (nectarine) (*Prunus persica* cvs. Hermosa and Sunsnow) that represent a cold-resistant, normal ripening example (Sunsnow) and a cold-sensitive one that develops the wooliness character (Hermosa). It is widely accepted that wooliness results from abnormal ripening process after low temperature storage of the fruit. We propose that when the fruits are returned to ambient temperature there is an imbalance among different enzymatic activities when the ripening processes resume. This imbalance leads to abnormal degradation of the cell wall that results in the mealy texture. In order to learn about the basis for the formation of the wooly tissue, we analyzed the fruits of the two cultivars that ripen either normally or develop the wooliness. During this study we have mainly focused on analysis of cell wall components, hydrolytic enzyme activities involved in cell wall catabolism and the expression of genes encoding for such enzymes.

In order to learn about the involvement of specific hydrolytic enzymes in development of wooliness we used activity gel assays. We followed the activity of pectin methylesterase (PME), polygalacturonase (PGase) and cellulase (endoglucanase, EGase) in tissues of Sunsnow and Hermosa fruits at four different stages including: Harvest (HV), Shelf-life following Harvest (HVSL), Regular-Stored fruits (RS), and Shelf life following Regular Storage (RSSL).

The pattern of esterase enzyme activities was compared between wooly and healthy tissue. However, at this stage it is not clear which of the few activities observed corresponds to the PME. While in Sunsnow tissue a similar pattern of activities was observed in the chilling sensitive fruits of Hermosa the pattern was somewhat different however, no difference between activities of HVSL (normal ripening) and RSSL (abnormal ripening) was observed. When the activity of PGase was followed it could be detected only in the Hermosa RSSL (wooly) tissue but not in the Hermosa or Sunsnow fruits undergoing normal ripening. A difference was also observed when the cellulase activity was analyzed. The activity was

found to be enhanced in the RSSL Hermosa tissue undergoing abnormal ripening compared to the Hermosa or Sunsnow fruits undergoing normal ripening. The observation that the cellulase activity was found in the RSSL fruits following 2 days of shelf-life, before any wooliness is manifested suggests it has a role in the process which leads to the development of the chilling injury and not just an outcome. Our findings regarding these enzyme activities support the hypothesis that abnormal cell wall catabolism has a role in the development of the wooliness, however, further studies are needed to clarify the enzymes specific role.

The levels of transcripts of genes related to cell wall metabolism and ethylene biosynthesis was measured in order to examine the possibility of altered gene expression related to the development of wooliness. Total RNA was extracted from the Sunsnow and Hermosa cultivars at the different stages mentioned above and used in Northern gel-blot analysis. The ACC oxidase mRNA levels increased during shelf life and ripening in both the Sunsnow and Hermosa cultivars. No differences were detected between fruits that were left to ripen following harvest or following low temperature storage. In contrast, there was a difference in the accumulation pattern of the ACC synthase, which was highly expressed in the Sunsnow cultivar during ripening either following harvest or storage but only slightly increased in its level when wooliness developed in the Hermosa cultivar following RS. Although the general pattern of PGase mRNA was similar between the two varieties its level was somewhat enhanced in the chilling resistant Sunsnow fruits. No major differences between the two cultivars were observed in the accumulation pattern for the pectin esterase mRNA. Major differences were observed between the two cultivars for the pattern of the cellulase mRNA accumulation. In the wooliness-resistant Sunsnow cultivar, a very low level of the transcript was detected while in the sensitive Hermosa cultivar higher level of cellulase mRNA was observed during ripening mainly in the wooly fruits following low temperature storage.

To identify alterations in catabolism of the cell walls of fruits undergoing normal versus wooly ripening we have carried out detailed analysis of some major cell wall components. Analysis of the level of uronic acid-rich polymers revealed that the reduction in their level following abnormal ripening of Hermosa fruits following storage was reduced compared to either normal ripening of Hermosa fruits following harvest or normal ripening of Sunsnow fruits. Similar differences were observed for the level of methyl esters resulting in a similar pattern for the degree of methyl esterification of the uronic acids originating from either the Sunsnow or Hermosa fruits. This level was kept constant with some reduction in fruits of either variety undergoing ripening following storage (RSSL). The most remarkable difference in cell wall components during abnormal ripening leading to wooliness was identified for the cellulose fraction. While normal ripening of either Sunsnow or Hermosa resulted with an elevated content of cellulose in the cell wall fraction in Hermosa fruits undergoing abnormal ripening the cellulose content remained about the same. This observation is consistent with the elevated level of both cellulase enzyme activity and mRNA observed.

Comparative analysis was carried out for determining the relative level of the cell wall basic sugar building blocks. Possible differences between cell wall of fruits undergoing wooliness and either Sunsnow or of Hermosa fruits ripening normally included decreased level of D-xylose in wooly fruits and increased level of galacturonic acid which fits with the determined elevated level of uronic acid-reach polymers in these fruits.

Details of cooperation

Close collaboration between the US and Israeli research groups had focused on the analysis of the cell wall components of Sunsnow and Hermosa fruit tissue undergoing either normal or wooly ripening. The Israeli PI had spent two weeks in the US laboratory for learning the use of cell wall analysis methods taking advantage of both expertise and equipment existing in the US laboratory. This analysis was carried out using the fruit cell wall samples prepared in Israel. Some of this material went through further analysis in parallel in both the US and Israeli laboratories. This cooperation enable the use of up to date dedicated tools and promoted the detailed analysis the alterations in the cell wall components which were complementary to the enzymatic and molecular study carried out mainly in Israel.

List of Publication

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2. Zhou, H., Lurie, S., Lers, A., Khalchitski, A., Sonogo, L. and Ben Arie, R. (2000) Delayed storage and controlled atmosphere storage of nectarines: two strategies to prevent wooliness. *Postharvest Biol. Technol.* 18: 133-141.
3. Zhou, H., Lurie, S., Ben-Arie, R., Dong, L., Burd, S., Weksler, A. and Lers, A. (2001) Intermittent warming of peaches reduces chilling injury by enhancing ethylene production and enzymes mediated by ethylene. *J. Hort. Sci. Biotechnol.* 76: 620-628.
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