Molecular and enzymatic examination of cell wall-modifying proteins in relation to apple crispness maintenance

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Chapter 1

Literature Review

1.1 Fruit texture and crispness

The texture of apples affects the storage life of the fruit, its susceptibility to bruising and diseases, and its transportability. Additionally, fruit texture, along with taste, is a major factor that contributes to overall sensory perception of apples. Firm, crisp, and juicy apples are generally desired by consumers over soft, mealy, and dry apples (de Smedt et al. 1998).

"Crispness" is perceived by consumers as a fast drop in the force exerted by jaws as the food breaks, accompanied by an acoustic signature that corresponds to the fracture event (Vincent 1998). Tissue can fail at the cell wall, leading to cell rupture, the release of cell contents, and apples that are perceived as crisp and juicy. Apple tissue can also fail at the middle lamella due to dissolution of the middle lamella, leading to cell separation instead of rupture, and apples that are perceived as mealy and dry (Stow 1993; Ben-Arie et al. 1979). Thus, the mealiness, crispness or juiciness of apples will depend on the relative strength of the cell wall compared to the middle lamella, both, in turn, determined by the integrity of their pectic compounds (de Smedt et al. 1998). The acoustic signature that accompanies crisp apple tissue is produced by individual turgid cells with strong cell walls bursting and releasing their contents which were under pressure (Vickers and Bourne 1976).

Though crispness is important to consumers, studies in apple texture (described below) have generally focused on firmness. As the texture of apples changes after harvest, the fruit tend to lose both firmness and crispness. While other fruits tend to soften extensively after harvest, losing up to 100% of firmness, apple fruit do so in a more subtle way, by softening 25-50% (Johnston et al. 2002).

Instrumental techniques are used to eliminate the inherent variation in evaluation of fruit texture by sensory panels. Mechanical measurements can objectively quantify sensory attributes of fruits such as firmness and crispness, but at the same time, they vary in their ability to predict changes in fruit texture that would be perceived by a trained sensory panelist (Harker et al. 2002). In apples, firmness is measured as compression force, while crispness as the fracturability of the apples (Mann et al. 2005). Although measurements of firmness and crispness are correlated, they are not equivalent (Evans et al. 2010; Znudek et al. 2011). A technique to measure crispness in apples is the snapping test described by Tong et al. (1999) and evaluated by Mann et al. (2005). The snapping test used throughout this study measures the Work required to break apart tissue and predicts change in crispness from harvest to storage with r^2=0.76 as well as crispness of stored fruit with r^2=0.82. Sensory firmness is not predicted as accurately with the snapping test, as this trait was found to be correlated to Force with r^2=0.43 (Mann et al. 2005).

1.2 The plant cell wall and fruit softening related changes

The plant cell wall is a complex rigid structure that serves as structural support for cells, bonding them together, and helping them maintain a high turgor pressure. Furthermore, it provides protection against pathogens and regulates materials that enter and exit the cell (Wei et al. 2009). The middle lamella lies between the primary walls of two adjacent cells, and is a layer composed mostly of pectins functioning as an adhesive layer (Cosgrove 2005). Primary cell walls, on the other hand, are composed of cellulose microfibrils tethered together by hemicelluloses and embedded in a matrix of pectins and structural proteins (Reiter 2002; Taiz and Zeiger 2002). The cellulose microfibers are composed of 1→4 linked β-D-glucose chains, arranged in a crystalline structure that adds structural strength to the wall and makes it more inaccessible to enzyme degradation. (Taiz and Zeiger 2002). Hemicellulose fibers, mainly composed of xyloglucans, bind to cellulose with varying strength through hydrogen bonds, forming a load bearing structure (Caffal and Mohnen 2009; Reiter 2002; Fry 2011). The pectins constitute a more soluble gel phase and, in the primary cell wall, they serve as a hydrophilic filler for the cellulose-hemicellulose network, preventing its aggregation and collapse (Zykwinska et al. 2005).

Pectins are a heterogeneous assortment of polysaccharides, including homogalacturonan and rhamnogalacturonan (RGI and RGII), among others (Cosgrove 2005). The rhamnogalacturonan I (RGI) domains are thought to be arranged like beads along a homogalacturonan string, and consist of a backbone with neutral sidechains of β-galactose and α-arabinofuranose (Fry 2011). Homogalacturonan is composed of 1,4 α-D-galacturonic acid (polygalacturonic acid). Charged carboxyl groups (COO-) of neighboring homogalacturonan molecules can be ionically linked by Ca^{2+}, and this epitope is prominent in the middle lamella and cell corners (Taiz and Zeiger 2002; Vincken et al. 2003).

Changes in turgor pressure and degradation of the cell wall and middle lamella leading to loss of cell adhesion are thought to be major proponents of changes in fruit texture, including softening, mealiness, and loss of crispness (Brummel et al. 1999; Goulao et al. 2007). Apple genotypes can vary considerably in their rates of postharvest fruit softening (Johnston et al. 2002; Mann et al. 2005). The 'Honeycrisp' apple (*Malus* x *domestica* Borkh.), developed by the University of Minnesota, is remarkable for its ability to maintain its crisp and firm texture after six months of cold storage, maintaining high texture, flavor and overall liking scores in sensory evaluations. Its fruit showed little cell wall or middle lamella degradation and maintenance of high turgor pressure during storage, in contrast to ‘Macoun’, a genotype with fruit that soften during storage (Tong et al. 1999). Softening tissue was characterized by plasma membranes becoming convoluted and separated from the cell wall, a breakdown of the laminated structure of the cell wall and disorientation or dissolution of the middle lamella (Tong et al. 1999; Ben-Arie et al. 1979).

Cell wall disassembly and depolymerization in fruits is caused by numerous enzymes that act simultaneously on the different types of linkages in the cell walls (Gross and Sams 1984; Peña and Carpita 2004; Johnston et al. 2002). Pectin solubilization is a prominent event during apple ripening and softening, and may be promoted by polygalacturonase (PG), β-galactosidase (BG) and/or α-L-arabinofuranosidase (AF) action (Goulao et al. 2007). Since the middle lamella is rich in homogalacturonan, it can be degraded by loss of Ca^{2+} cross-linking or when it is hydrolyzed by PG (Atkinson et al. 2002; Fry 2011). BG and AF cleave β-galactose and α-arabinofuranose sidechains from pectic rhamnogalacturonan, respectively. Other pectin degrading enzymes include pectate lyase, pectin methylesterase, and β-xylosidase, while hemicellulose modifying enzymes include endo-glucanase and xyloglucan endotransglycosylase/hydrolase (Goulao and Oliveira 2008; Ortiz et al. 2011). On the other hand, expansins are proteins that break noncovalent bonds in the junction between cellulose microfibrils and hemicelluloses in cell walls, promoting cell wall loosening and stress relaxation (Sampedro and Cosgrove 2005).

Each cell wall degrading enzyme can have several isoforms with distinct expression levels and patterns depending on the fruit's developmental stage and sometimes even depending on the cultivar within a species. Thus, studying a single protein or a single genotype may not depict a complete view of the mechanism underlying fruit texture changes. Additionally, since fruit softening is a quantitative trait, suppressing the action of individual enzymes may delay softening or have no effect on fruit softening, but will not likely prevent it completely (Goulao et al. 2007). As a consequence to these limitations, a clear relationship between the expression patterns of cell wall modifying proteins and apple fruit softening rates has not yet been established (Goulao et al. 2007; Wakasa et al. 2006; reviewed by Johnston et al. 2002).

1.3 Polygalacturonase

Apple softening appears to occur by solubilization of pectins, without extensive depolymerization (Goulao and Oliveira 2008). A study by Yoshioka et al. (1992) suggests that polyuronide molecules can be solubilized from the cell wall of softening apples without the depolymerization action of endo-PG and, rather, by de-esterification of higly methoxylated polyuronides by pectin methyl-esterase (PME). The methylester group might prevent the formation of stabilizing Ca^{2+} bridges and the de-esterification of polyuronide molecules would cause them to further repel each other by their negative charges (Yoshioka et al. 1992).

Exo-PG might help to 'loosen' the polyuronide network and promote solubilization by terminal cleavage of polygalacturonic acid which releases D-galacturonic acid residues (Bartley 1978). PME is a prerequisite for exo-PG activity, which only cleaves de-esterified galacturonic acid residues. Alternatively, in the presence of high Ca^{2+} content, PME could increase cell wall integrity by enhancing Ca^{2+} cross-linking, thus restricting access of PG to its substrates (Glenn and Poovaia 1990; Ortiz et al. 2011).

Endo-PG activity has been generally undetected (Bartley 1978; Abeles and Biles 1991; Goulao et al. 2007; with the exception of a study by Wu et al. 1993), while exo-PG activity has been more commonly detected in apple tissue (Bartley 1978; Goulao et al. 2007; Wei et al. 2010). Interestingly, endo-PG (*MdPG1*) expression has been detected in various studies and appears to influence apple texture (Bartley 1978; Abeles and Biles 1991; Goulao et al. 2007; Wu et al. 1993). Transgenic apples that overexpressed *MdPG1* had enhanced cell-to-cell separation in leaf tissue (Atkinson et al. 2002) and enhanced exo-PG activity (suggesting that this gene can have dual endo- and exo- action). Transgenic apples with suppressed endo-PG expression had firmer fruit after cold storage and the apples' rate of softening correlated with *MdPG1* expression (Atkinson et al. 2008). In a survey of 14 apple cultivars maintained at 24° C for up to 12 days (overripe fruit), Wakasa et al. (2006) found that cultivars that lost firmness had higher *MdPG1* expression than cultivars that remained firm. In other studies, 'Honeycrisp' fruit had low *MdPG1* expression compared to the softening cultivars 'McIntosh' (Harb et al. 2012) and 'Macoun' (after cold storage, Mann et al. 2008). *MdPG1* is located on chromosome 10 and co-localized with a quantitative trait locus (QTL) peak for firmness of apples stored at room temperature, explaining 10.7% of phenotypic variance (Costa et al. 2010). Linkage group 10 also contains QTLs for instrumental measurements of fruit firmness at harvest (King et al. 2000; King et al. 2001; Maliepaard et al. 2001; Kenis et al. 2008).

MdPG1 has been identified as an endo-acting polygalacturonase that affects fruit softening. It is, however, not the only PG protein present in apples. A search of the predicted proteins in the published apple genome (Velasco et al. 2010) revealed 138 probable proteins corresponding to polygalacturonase or belonging to the polygalacturonase family, localized on all chromosomes except one (chromosome 14). These proteins may have endo- or exo- activity, or combined activities in a single protein.

Exo-PG activity was first detected in 5-8 month cold stored apples (Bartley 1978). Exo-PG activity levels were lowest in unripe fully expanded ‘Mondial Gala’ fruit (which softens), then increased and maintained similar levels from fruit at harvest to overripe fruit (Goulao et al. 2007). Exo-PG activity was also found to be negatively correlated to firmness in ‘Fuji’ and ‘Golden Delicious’ apples, which remain firm and soften, respectively. PG activity in ‘Fuji’ had a slow and steady increase during room temperature storage, while activity in ‘Golden Delicious’ peaked sooner, then declined. Cold storage delayed softening in ‘Golden Delicious’ and permitted lower PG activity in both cultivars compared to room temperature storage (Wei et al. 2010).

1.4 α-L-arabinofuranosidase and β-galactosidase

The loss of galactose and arabinose neutral sugar sidechains has been observed in ripening and softening apples (described below). The role of arabinans and galactans is possibly to anchor pectins to cellulose, though with less affinity than hemicelluloses, while galactans control pectin pore size (Vincken et al. 2003; Zykwinska et al. 2005). It has been suggested that one of the first steps in cell wall degradation may be an inhibition of aggregation of pectin molecules due to the debranching of rhamnogalacturonan in the primary cell wall (Wei et al. 2010). Loss of the branched pectin structure may also be a pre-requisite for PG action by increasing porosity of the wall and allowing access of this enzyme to its substrates (Gross and Sams 1984).

During ripening of ‘Golden Delicious’ apples, galactose was lost while arabinose content remained fairly steady (Gross and Sams 1984), whereas other studies have detected that both of these neutral sugars are lost during softening. Both galactose and arabinose levels declined during storage of ‘Golden Delicious’ (Glenn and Poovaia 1990). Peña and Carpita (2004), on the other hand, found that galactose was lost during fruit development while a loss of branched arabinan preceded, and was likely a pre-requisite for softening in four overripe cultivars. Tong et al. (1999) observed that arabinose content was maintained in ‘Honeycrisp’ after storage, while arabinose content declined in cultivars that lost crispness after storage. Galactose content was maintained in all softening cultivars and ‘Honeycrisp’ after storage.

The α-L-arabinofuranosidase and β-galactosidase genes from expression studies are located on chromosomes 14 and 15, respectively. Significant QTL with large effects (LOD, logarithm of odds score > 4.5) for crispness were not detected on either of those chromosomes, though QTL with LOD scores > 3.0 describing variation in measurements of sensory firmness were found on linkage groups 14 and 15 (King et al. 2000; King et al. 2001; Maliepaard et al. 2001; Liebhard et al. 2003; Kenis et al. 2008). A more recent study, however, revealed that QTL associated with firmness on chromosomes 14 and 15 were not located near these AF or BG genes (Longhi et al. 2012).

Both BG and AF activity have been detected in ripe and cold stored apples (Ross et al. 1994; Yoshioka et al. 1995) and were found to increase in overripe fruit, compared to levels at harvest (Goulao et al. 2007). Ortiz et al. (2011) found that BG and AF activities were inversely correlated to firmness after 19 weeks of cold storage. BG but not AF activity was lower in ‘Fuji’ fruit, compared to ‘Golden Delicious’, when stored at room temperature (Wei et al. 2010), while ‘Honeycrisp’ fruit had lower AF but not BG expression, compared to ‘McIntosh’ fruit (Harb et al. 2012).

1.5 Expansins

While normally associated with expanding cell walls, expansin expression or activity has also been detected in ripening and softening fruits, and in fully expanded apples at harvest or after storage (peaches: Obenland et al. 2003; pears: Hiwasa et al. 2003; Wakasa et al. 2003; Goulao et al. 2007; Trujillo et al. 2012). Expansins may play a role in fruit softening by facilitating access of other cell wall degrading enzymes to their substrates (Brummel et al. 1999). An expansin that is specifically expressed in ripening fruit was first characterized in tomato (*LeExp1*, Rose et al. 1997). Suppression of *LeExp1* inhibited polyuronide depolymerization and produced firmer fruit in transgenic tomatoes, while overexpression of this gene resulted in softer fruit (Brummel et al. 1999).

For *Malus* x *domestica* Borkh., there are ten expansin accessions in Genbank, corresponding to alpha expansins *MdEXPA1* through *MdEXPA7* (National Institute for Biotechnology Information - GenBank accession Nos. AY083166, AB099928, AB099927, AY083167, AB099926, AF527800, AB099925, AB099929, AB099930, DQ072009). A search for expansin homologues in the apple genome (Velasco et al. 2010) revealed that there are around sixty predicted Expansin A proteins, distributed across the genome’s 17 chromosomes.

Wakasa et al. (2003) determined expression patterns of expansin genes *MdEXPA1* through *MdEXPA6* in apple (expansin names used in this paper follow the nomenclature suggested by the expansin working group; http://homes.bio.psu.edu/expansins/). They found that each one has a unique profile pattern in fruit ontogeny of 'Golden Delicious' apple. *MdEXPA2* was identified as ripening-related because of its increased expression during the early ripening stage and after incubation of harvested fruit at 24° C for 6 days. Furthermore, an ethylene antagonist suppressed the expression of *MdEXPA2*. A study of 14 apple cultivars by Wakasa et al. (2006) found that *MdEXPA2* was expressed after harvest in varying levels among the cultivars, but did not find a clear relationship between *MdEXPA2* transcript levels and loss of firmness of apples kept at room temperature.

Other expansin isoforms have also been considered in apple fruit softening. Expression of *MdEXPA1* and *MdEXPA3* decreased after harvest of 'Golden Delicious' fruit while *MdEXPA4*, *MdEXPA5*, and *MdEXPA6* were not detected at harvest or thereafter (Wakasa et al. 2003). Goulao et al. (2008) isolated only one isoform of expansin, *MdEXPA3*, from 'Mondial Gala' fruit tissue. They found that transcript levels increased until commercial harvest maturity, then decreased but were still detectable in softening and overripe stages in fruit kept at room temperature.

Costa et al. (2008) identified *MdEXPA7*, which they mapped to Linkage Group 1 of the linkage map based on 'Prima' x 'Fiesta' progeny, and found it to be close to a previously identified QTL for apple firmness. An SSR marker was developed based on the lengths of a simple sequence repeat (SSR) in the 5'untranslated region of this gene. Actual expression of the *MdEXPA7* gene has not previously been studied. Its sequence had 99% identity to sequences from the 10 days after full bloom EST library.

Only one study has measured actual expansin activity in apple fruit. Goulao et al. (2007) determined activity of several enzymes in apple fruit protein extractions of ‘Mondial Gala’ from fruit set to overripe fruit. Expansin activity was found to remain stable between fruit at harvest and overripe fruit, though they had different stress relaxation spectra.

1.6 Conclusions

‘Honeycrisp’ fruit have a remarkably crisp texture, even after storage, yet the molecular mechanism by which cell walls in ‘Honeycrisp’ fruit maintain their structural integrity remains unknown. Whether or not a fruit loses crispness or firmness may depend on a delicate balance between the various cell wall modifying proteins, timing of expression, and cell wall conditions. Studies on neutral sugar loss and cell wall modifying proteins can be inconclusive as to the role the proteins play on fruit softening, because they typically study only one protein, or one cultivar. It is difficult to compare results from different laboratories where the methods of fruit texture determination, protein extractions and protein activity quantification differ. Additionally, previous studies in cell wall modifying proteins have focused on their effect on apple firmness, but these findings might not be extrapolated to fruit crispness. The aim throughout this study was to relate expression or activity of several cell wall modifying protein candidates to observed changes in texture of ‘Honeycrisp’ and its progeny.

Chapter 2

Examination of expansin genes as related to apple fruit crispness

2.1 Summary

The possible relationship of seven expansin genes to maintenance of apple (*Malus x domestica* Borkh.) crispness during storage was examined. mRNA accumulation of expansin genes *MdEXPA1, MdEXPA2, MdEXPA3, MdEXPA4, MdEXPA5* and *MdEXPA7* was quantified among apple cultivars that remained crisp or lost crispness between harvest and after 8 weeks of cold storage. *MdEXPA1* transcript levels were present in low quantities in fresh fruit while *MdEXPA4*, *MdEXPA5* and *MdEXPA7* transcripts were not detected among fresh or cold-stored apple cultivars. Among the expansin genes studied, *MdEXPA2* and *MdEXPA3* had the highest mRNA accumulation levels. A new expansin gene, with nucleic acid sequence similarity to *PcEXPA1*, and low mRNA expression levels in fresh and cold-stored apples, was isolated in this study and designated *MdEXPA8*. Two alleles of *MdEXPA2* were identified with different 3'UTR sequences, and 41 apple genotypes were allelotyped for *MdEXPA2* to determine to what extent a relationship existed between allelotype and loss of fruit crispness. Paired t-tests suggested that there was no relationship between allelotype and crispness maintenance. However, genotype sample size was small and may have been inadequate to detect any effect. The gene for 1-aminocyclopropane 1-carboxylate synthase, *MdACS1*, was also allelotyped in the 41 apple genotypes to determine if there might be an additive effect with *MdEXPA2* allelotype, and was found to be unsuitable as a marker for maintenance of apple fruit crispness for the crosses used in this study. Furthermore, fragment analyses using a capillary machine showed that the two

MdEXPA2 alleles did not have different transcript stabilities, and protein blots suggested that *MdEXPA2* allelotype had no effect on translation.

2.2 Keywords

Texture, DST element, *Spring* element

2.3 Introduction

Fruit texture, along with taste, is a major factor that contributes to overall sensory perception of apples. It affects the storage life of the fruit, its susceptibility to bruising and diseases, and its transportability. Instrumental techniques are used to objectively measure sensory attributes of fruit texture such as firmness and crispness, and vary in their effectiveness. Firmness is measured as compression force, while crispness as the fracturability of the apples (Mann et al. 2005). Although measurements of the two attributes are correlated, they are not equivalent (Evans et al. 2010; Znudek et al. 2011). As the texture of apples changes after harvest, the fruit tend to lose both firmness and crispness. However, even though crispness is important to consumers, studies in apple texture (described below) have generally focused on firmness.

Changes in turgor pressure and degradation of the cell wall and middle lamella leading to loss of cell adhesion are thought to be major changes that cause fruit softening (Brummel et al. 1999; Goulao et al. 2007). Within apple genotypes there is well-documented variation in rates of postharvest fruit softening (Johnston et al. 2001; Mann et al. 2005). An example is ‘Honeycrisp’, whose fruit showed no cell wall and middle lamella degradation during six months of storage compared to that of ‘Macoun’, a genotype with fruit that soften during storage (Tong et al. 1999).

The plant cell wall is composed of cellulose microfibrils tethered together by hemicelluloses and embedded in a matrix of pectins and structural proteins (Reiter 2002; Taiz and Zeiger 2002). Disassembly and depolymerization of cell wall components occurs during fruit ripening and softening, caused by enzymes that act simultaneously on the different types of linkages in the plant cell walls of fruit. (Brummel et al. 1999; Goulao et al. 2007; Rose et al. 1997). A clear relationship between the expression patterns of these enzymes and apple fruit softening rates has not yet been established (Goulao et al. 2007; Wakasa et al. 2006; reviewed by Johnston et al. 2002).

Expansins are proteins involved in wall loosening that are believed to act by disrupting non-covalent bonds between the cellulose microfibrils and matrix polysaccharides that make up the plant cell wall (McQueen-Mason and Cosgrove 1995). They may play a role in fruit softening by facilitating access of other cell wall degrading enzymes to their substrates (Brummel et al. 1999). An expansin that is specifically expressed in ripening fruit was first characterized in tomato (*LeExp1* transcript, Rose et al. 1997). Suppression of *LeExp1* inhibited polyuronide depolymerization and produced firmer fruit in transgenic tomatoes, while overexpression of this gene resulted in softer fruit (Brummel et al. 1999).

Wakasa et al. (2003) determined the transcript accumulation patterns of expansin genes *MdEXPA1* through *MdEXPA6* in apple (expansin names used in this paper follow the nomenclature suggested by the expansin working group;

http://homes.bio.psu.edu/expansins/). They found that each one has a unique profile pattern in fruit ontogeny of 'Golden Delicious' apple. *MdEXPA2* was identified as ripening-related because of its increased expression during the early ripening stage and after incubation of harvested fruit at 24° C for 6 days. Furthermore, an ethylene antagonist suppressed the expression of *MdEXPA2*. A study of 14 apple cultivars by Wakasa et al. (2006) found that *MdEXPA2* was expressed after harvest in varying levels among the cultivars, but did not find a clear relationship between *MdEXPA2* transcript levels and loss of firmness of apples kept at room temperature.

Other expansin genes have also been considered in apple fruit softening. *MdEXPA1* and *MdEXPA3* transcript levels decreased after harvest of 'Golden Delicious' fruit while *MdEXPA4*, *MdEXPA5*, and *MdEXPA6* transcripts were not detected at harvest or thereafter (Wakasa et al. 2003). Goulao et al. (2008) isolated only one expansin gene, *MdEXPA3*, from 'Mondial Gala' fruit tissue. They found that transcript levels increased until commercial harvest maturity, then decreased but were still detectable in softening and over-ripe stages in fruit kept at room temperature.

Costa et al. (2008) identified *MdEXPA7*, which they mapped to Linkage Group 1 of the linkage map based on 'Prima' x 'Fiesta' progeny, and found it to be close to a previously identified QTL for apple firmness. An SSR marker was developed based on the lengths of a simple sequence repeat (SSR) in the 5'untranslated region of this gene. Actual expression of the *MdEXPA7* gene has not been studied.

Previous studies found no clear relationship of expansin transcript levels to loss of fruit firmness, but these findings might not be extrapolated to fruit crispness.

‘Honeycrisp’ is a genotype with fruit that maintain sensory crispness and cell wall structure, yet can have the same firmness as ‘Delicious’ (Tong et al. 1999). Its fruit showed transcript accumulation of various cell wall modifying genes during storage (Mann et al., 2008), which suggests that allelic variation in regulatory or cell wall modifying genes might be needed to explain the crisp phenotype of ‘Honeycrisp’.

In this study, transcript levels of *MdEXPA1, MdEXPA2, MdEXPA3, MdEXPA4, MdEXPA5* and *MdEXPA7* were compared among genotypes with fruit that remained crisp, or lost crispness, when kept at 0 ± 0.5 °C for up to 8 weeks. The possible influence of two alleles of *MdEXPA2* differing in their 3’UTR region was considered, as well as that of the *MdACS1* gene. Additionally, the publication of the apple genome in 2010 allowed a more extensive search for expansin genes and their locations in the genome.

2.4 Materials and Methods

2.4.1 Plant materials, fruit harvest and storage

Apple fruit were harvested from the Horticultural Research Center in Chanhassen, Minnesota in 2005 and 2006. The eight genotypes used in mRNA accumulation analyses included four parent genotypes – ‘Honeycrisp’, ‘MN1702’, ‘MN1764’ (only harvested in 2006), and ‘MN447’, and their progeny - ‘ED269’ (‘Honeycrisp’ x ‘MN1702’), ‘CD151’ (‘Honeycrisp’ x ‘MN1764’), ‘CC130’ (‘Honeycrisp’ x ‘MN447’), and ‘CC141’ (‘Honeycrisp’ x ‘MN447’). ‘Macoun’ fruit were obtained from a commercial orchard in 2005, harvested at its commercial maturity date. Leaves and fruit from 32 additional genotypes were collected in 2008 for genotyping and texture analysis (listed in Table 2.1). Fruit were harvested for each genotype at a threshold starch index between 5 and 6 (Blanpied and Silsby 1992). Fruit were stored for 8 or 12 weeks at 0 ± 0.5 °C and 95% to 99% relative humidity.

2.4.2 Texture analysis

Fruit texture was measured with a TA.XT2 texture analyzer (Texture Technologies, Scarsdale, NY) after each storage treatment. Change in work required to break cylindrical apple tissue samples was used to analyze changes in fruit crispness during storage (Mann et al. 2005). At least ten fruit per genotype were used for texture analyses.

2.4.3 DNA and RNA extraction and cDNA synthesis

DNA was extracted from young apple leaves using a DNeasy Plant DNA extraction kit according to manufacturer instructions (Qiagen Inc., Valencia, CA). Fruit were peeled, cored and chopped, and pieces pooled from multiple fruit were frozen in liquid nitrogen and kept at –80 ºC for RNA extractions. Total RNA was extracted from 20 g batches of fruit tissue using a modification of a method described by Lopez-Gomez and Gomez-Lim (1992; Mann et al. 2008) and then reverse transcribed into cDNA using SuperScript III^{TM} (Invitrogen Corp., Carlsbad, CA) reverse transcriptase and oligo (dT) primers according to manufacturer instructions. cDNA was made from independently extracted RNA from fruit tissue harvested in two different years, 2005 and 2006.

2.4.4 mRNA accumulation analysis of fruit-related expansin genes

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analyses was performed to analyze mRNA transcript accumulation of five fruit-specific expansin genes in fruit stored for 0 or 8 weeks from eight apple genotypes. Gene specific primers for *MdEXPA3* (AB099926) and PCR conditions were used as described by Wakasa et al. (2003). PCR conditions for quantification of *MdEXPA1*, *MdEXPA2, MdEXPA5* and *MdEXPA7* were initial denaturation for 3 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 45 s at 59 °C, and 30 s at 72 °C. The primer sets were as follows: *MdEXPA1* (F: 5'-CCTTCCAAGTCACCACCAGT-3', R: 5'-GCTCACCTCAGCAAACCTTC-3'); *MdEXPA2* (*MdEXPA2F*: 5'-GAGCAACTCTTACCTCAACGG-3', *MdEXPA2RN*: 5'-TATAGGACTAGCGGGTGCCA-3')*; MdEXPA5* (F: 5'-TTTGCTTCAAAGTGAGTGACC-3', R: 5'-CCTCATGAATTAACAGCGGTG-3')*; MdEXPA7* (F: 5'-GCTCATGCCACCTTCTATGG-3', R: 5'-GAGTTCGAAGCAAGCTCCAC-3').

Transcript abundance was measured using a LightCycler quantitative PCR machine (Roche Applied Science, Indianapolis, IN) and Platinum® SYBR® Green qPCR SuperMix-UDG (Promega Corp., Madison, WI), or using the IQ SYBR Green kit (Bio-Rad, Hercules, CA) in 20 uL total reactions using a Bio-Rad CFX96 Real Time Systems thermocycler, according to manufacturer instructions. Transcript abundance was normalized to β-actin transcript abundance for each genotype and storage time (*MdACTF*: 5'-ACTCGAGGACGGGGTAGATT-3', MdACTR 5'-CAAAGATTAGGCAAGGCGAG-3'; Mann et al. 2008). External standard curves were generated with known quantities of PCR amplified gel-purified templates and gene specific primers. Each qRT-PCR reaction was run in duplicate and each gene was tested using cDNA from at least two biological replicates. Average results from reactions with single melting peaks were reported. Expression of *MdEXPA4* was assessed by semi-quantitative RT-PCR analyses. Gene specific primers for *MdEXPA4* (AB099925) and PCR conditions were used as described by Wakasa et al. (2003).

2.4.5 MdEXPA2 *and* MdEXPA8 *PCR and sequencing*

PCR was performed using forward primer *MdEXPA2F* and reverse primer *MdEXPA2RN,* described above, which were expected to amplify a ~300 bp fragment in the 3'end of the sequence. PCR conditions were the same as those described above for qRT-PCR. Purified PCR-products from all genotypes were cloned into pSTBlue-1 (Novagen, Madison, WI) and sequenced at the BioMedical Genomics Center (BMGC) at the University of Minnesota. The three sequences obtained corresponded to two *MdEXPA2* alleles and *MdEXPA8.* They were compared to published *MdEXPA2* sequences (Trivedi and Solomos, 2002; Wakasa et al., 2003) using the ClustalW (Larkin et al., 2007) sequence alignment tool and GenBank was searched for corresponding expansin genes from other species. To obtain a more complete sequence of *MdEXPA8*, primers were designed based on *PcEXP1* to amplify a 779 bp fragment: *MdEXPA8F*: 5'-GGTGATGCTTCTGGCACAAT-3', *MdEXPA8R*: 5'-TTCCCATCCACGTACAAGGT-3'. PCR was performed (with an annealing temperature of 60 °C) then sequenced as described above.

2.4.6 MdACS1 *allelotyping*

All cultivars were allelotyped for *MdACS1* (1-aminocyclopropane-1-carboxylic acid synthase) gene as described by Zhu and Barritt (2008). Two or more biological replicates were tested per genotype. Homozygosity for allele 2 of *MdACS1* corresponds to a low internal ethylene concentration (Harada et al. 2000).

2.4.7 Fragment analyses

To study mRNA transcript stability, genomic DNA and cDNA samples were analyzed for presence or absence of the *MdEXPA2* alleles and *MdEXPA8* gene in each genotype. PCR using *MdEXPA2F* and *MdEXPA2RN* primers was performed on genomic DNA and cDNA extracted from 0-week fruit or 8-week stored fruit (at least two biological replicates) of all eight genotypes used in expression analyses except 'Macoun'. Serial dilutions of these PCR reactions were analyzed on an ABI 3730xl capillary machine (BMGC) using GeneMapper software and the area of each allele peak was used to estimate the proportion of each fragment in the sample. One to four samples for each biological replicate were used.

2.4.8 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) analysis

Total cell wall-associated protein (150 µg) from apple fruit cortex was separated on a pre-cast 4-15% Tris-HCl polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA) and transferred to a nylon membrane (Brummell et al. 1999; Obenland et al., 2003). The membrane was blocked with 5% Amersham Western blocking agent (Amersham

Biosciences, Piscataway, NJ) in TBS-t buffer containing 100 mM Tris, 0.9 % sodium chloride and 0.1% Tween 20, (pH 7.5) for 1 h at room temperature. After three washes of 10 min each in TBS-t buffer, the membrane was incubated in 0.4 % blocking agent/TBS-t solution containing a 1:1500 dilution of expansin-specific LeExp1 antibody from tomato (Rose et al. 2000) for 14 h with constant shaking at room temperature. Following incubation, the membrane was washed three times for 10 min each in TBS-t buffer and incubated for 1 h at room temperature and constant shaking in goat anti-rabbit alkaline phosphatase. The membrane was washed three times with 10 min per wash and incubated in Amersham detection solution (Amersham Biosciences, Piscataway, NJ) for 1 min, before exposure to the FluorChem 5500 chemiluminesence detection system (Alpha Innotech Corp., San Leandro, CA) for 20 min according to manufacturer protocol. At least two independent protein extracts per genotype were tested.

2.4.9 Survey of expansin genes in the apple genome

A file of non-redundant, best-match predicted protein homologs was searched for putative Expansin A genes in the apple genome (Velasco et al. 2010). The published sequences of expansins 1 through 7 (Wakasa et al. 2003; Goulao et al. 2008; Costa et al. 2008) and *MdEXPA8* were matched to these genes by doing a BLAST (Altschul et al. 1997) search with the Apple Genome V1.0 Contigs (http://www.rosaceae.org/projects/apple_genome).

2.5 Results

2.5.1 MdEXPA2 *and* MdEXPA3 *transcript levels during storage*

The primer combination *MdEXPA2F* and *MdEXPA2RN* used for quantitative RT-PCR amplified two *MdEXPA2* alleles and *MdEXPA8* (discussed below). However, fragment analyses of PCR amplified fragments showed that *MdEXPA8* transcript abundance was very low in comparison to *MdEXPA2* (data not shown), so the data from Figure 2.1 are presented only for *MdEXPA2*. *MdEXPA2* was detected in 0 week-stored fruit of all nine genotypes tested, and in 8 week-stored fruit of all genotypes tested except 'MN1702', 'MN447' and 'ED269' (Fig. 2.1). *MdEXPA2* relative transcript level was noticeably higher in 0 week fruit of 'Macoun' and 'MN1764', which lost crispness, compared to levels in 0 week fruit of other genotypes. 8-week cold-stored fruit of noncrisp 'CD151' had higher transcript levels than 8 week fruit of other genotypes.

Because Goulao et al. (2008) detected *MdEXPA3* transcripts in 'Mondial Gala' fruit at harvest, its expression was examined in this study's genotypes. *MdEXPA3* transcript levels were very variable between the 2005 and 2006 cDNA samples (as shown by error bars) and transcripts were detected in 0 week fruit in 'Honeycrisp', 'MN447', 'ED269', 'CC130', 'CD151' and 'CC141' (Fig. 2.2). On the other hand, compared to 0 week levels, this gene was not as highly expressed in 8 week fruit.

Transcripts of *MdEXPA1, MdEXPA4* and *MdEXPA5* can also potentially be detected during apple fruit ontogeny (Wakasa et al. 2003. Accession Nos. AB099928, AB099925, AB099929). *MdEXPA7* was mapped close to a QTL for apple firmness, but its expression had not been studied (Costa et al. 2008). Therefore, the mRNA accumulation of these expansin genes was examined in fresh and 8 week-stored fruit tissue of nine genotypes. *MdEXPA1, MdEXPA4*, *MdEXPA5* and *MdEXPA7* were not expressed, or had much lower transcript levels than *MdEXPA2* and *MdEXPA3* (data not shown). Therefore, in fresh and stored fruit, *MdEXPA2* and *MdEXPA3* were the predominant expansin transcripts that accumulated.

2.5.2 MdEXPA2 *and* MdEXPA8 *sequence data*

The *MdEXPA2* gene, which has been referred to as 'ripening-related' (Wakasa et al. 2003), was sequenced from several apple cultivars. The *MdEXPA2F* and *MdEXPA2RN* primer combination amplified three sequences. Two of the *MdEXPA2* cDNA sequences obtained corresponded to alleles from 'Golden Delicious' (accession AB099927) and 'Granny Smith' fruit (accession AY083167). 'Granny Smith' is a slow softening genotype, whereas 'Golden Delicious' fruit soften rapidly during storage (Johnston et al. 2001; Wakasa et al., 2003). Both *MdEXPA2* sequences are widely represented in apple EST libraries and are homologous to the *PcEXPA2* gene in European pear (*Pyrus communis*: *PcEXP2*, AB093029) and *EuEXPA3* in loquat (*Eriobotrya japonica*: *EXPA3*, EU123921).

The 'Granny Smith' allele has a region in its 3'UTR with similarity to an mRNA destabilizing element (DST) found in unstable plant transcripts known as small auxin up-RNA (SAUR, Newman et al. 1993). These elements contain three conserved motifs (Fig. 2.3, highlighted in gray, adapted from the soybean *SAUR-15A* gene described by

Feldbrügge et al. 2001), each separated by a 4-11 bp variable region. This separation is necessary for the DST sequences to maintain their function (Newman et al. 1993). The 'Golden Delicious' *MdEXPA2* allele has a 10/11 bp (ATAGTTGGGGG) deletion (shown in bold) from one of the 4-11 bp variable regions and thus lacks the DST-like element. The 3'UTR of the *MdEXPA2* allele from 'Honeycrisp' fruit was more similar to that of the 'Granny Smith' *MdEXPA2* allele with the DST-like element, while the 3'UTR of the 'Macoun' fruit *MdEXPA2* had greater similarity to the 'Golden Delicious' allele lacking the DST-like element (Fig. 2.3).

A transposable element was found in the 3'UTR of the *MdEXPA2* 'Golden Delicious'-like allele of genomic DNA of 'MN447', 'CC130' ('Honeycrisp' x 'MN447') and 'CC141' ('Honeycrisp' x 'MN447'). The sequence was determined to be a miniature inverted-repeat transposable element (MITE) belonging to a family of transposable elements in the apple genome called *Spring* (Han and Korban 2007). It was 148 bp in length, with an 8 bp duplication of the flanking sequence where it was inserted (Fig. 2.3 and 4a). This transposable element was found in fragment analyses of cDNA samples from these cultivars, as well as sequencing data. Thus, instead of 303 bp, fragment analysis showed that the *MdEXPA2F*/*MdEXPA2RN* primer combination amplified a 459 bp fragment in the gDNA of the cultivars listed above (data not shown). However, the transposable element was not always amplified in fragment analysis, and appeared to have been excised out of some of the *MdEXPA2* mRNA or cDNA strands. When excised, the *MdEXPA2F*/*MdEXPA2RN* primer combination produced a 332 bp fragment (303 bp + 8 bp target site duplication + 21 bp palindromic sequence, Fig. 2.4b). Accordingly, both

332 bp and 459 bp fragments were present in the cDNA of the three cultivars, although fragment analyses revealed that the 459 bp fragment was 1.5 to 6 times more abundant than the 332 bp fragment in cDNA (data not shown).

The third sequence that was obtained (using *MdEXPA2F* and *MdEXPA2RN* primers) was not found among apple EST sequences and was homologous to the 3'UTR of *PcEXPA1* gene (*PcEXP1*, AB093028) in pear and *EuEXPA4* gene (*EXPA4*, EU123922) in loquat (Fig. 2.3). Further sequencing revealed that its coding region differed from that of the two *MdEXPA2* alleles, which were more homologous to the *PcEXPA2* (AB093029) and *EuEXPA3* (EU123921) genes. This third sequence was therefore assumed to be a new expansin gene in apple and was named *MdEXPA8* (accession HQ905438).

2.5.3 MdEXPA2 *and* MdACS1 *allelotyping*

Using a snapping test to measure fruit texture, change in work was found to be highly correlated (r^2=0.76) with change in crispness as determined by a sensory evaluation (Mann et al. 2005). The 41 apple genotypes used in this study were classified based on t-tests ($\alpha = 0.05$) of differences in work values (mJ) between fresh fruit and fruit that were cold-stored for two or three months (Table 2.1), such that genotypes exhibiting no significantly different changes in work were classified as maintaining crispness and those that showed significant decreases in work as ones that lost crispness.

The 'Granny Smith' allele for *MdEXPA2* was present in all genotypes tested except in 'Macoun', while the 'Golden Delicious' allele (GD) was present in 6 crisp cultivars and 12 cultivars that lost crispness (Table 2.1). Since both ‘Honeycrisp’ and ‘MN1702’ were found to be homozygous for the ‘Granny Smith’ allele (GS, Fig. 2.5a), ‘Honeycrisp’ x ‘MN1702’ progeny could not be heterozygous for *MdEXPA2*. Therefore, progeny from this cross were not included in determining the effect of the *MdEXPA2* gene variant on crispness classification (Table 2.2). An insignificant difference was found in work at harvest, work after storage, or change in work between the two *MdEXPA2* allelotypes ($P>0.40$, Table 2.2).

Because the *MdEXPA2* alleles could not by themselves explain the differences in crispness maintenance, and because an ethylene antagonist was found to affect *MdEXPA2* expression (Wakasa et al. 2003), the genotypes were also allelotyped for the 1-aminocyclopropane-1-carboxylate (ACC) synthase gene, *MdACS1.* Allelotypes of this gene are thought to influence internal ethylene concentration (IEC) in apple fruit, with *MdACS1-1/1* and *MdACS1-2/2* coding for high and low ethylene production, respectively (Harada et al. 2000). A correlation between allelotype and fruit firmness was reported (Zhu and Barritt 2008). Given the genotypes of the parents chosen for the crosses (‘Honeycrisp’ x ‘MN447’, ‘MN1702’ or ‘MN1764’), the resulting progeny could only have an *MdACS1* – 1/2 or 2/2 genotype. Nine of the 20 cultivars that maintained crispness were homozygous for allele 2 of *MdACS1* while 11 were heterozygous (Table 2.1). In cultivars that lost crispness, 11 of the 21 cultivars were homozygous for the low ethylene allele, 1 was homozygous for the high ethylene allele, and 9 were heterozygous. A significantly higher work value at harvest was seen in *MdACS1* – 1/2 genotypes than in those homozygous for allele 2 (P = 0.025, Table 2.2). Work at storage and change in work were not significantly different among *MdACS1* – 1/2 or 2/2 genotypes.

Among genotypes that maintained crispness, three exhibited the GD/GS *MdEXPA2* allele with the *MdACS1-2/2* allele, so they would have been expected to lose crispness but produce little ethylene. The low ethylene production could perhaps explain why the GD/GS *MdEXPA2* allelotypes maintained crispness. However, among genotypes that lost crispness, four had the GS/GS *MdEXPA2* allele and the *MdACS1-2/2* allele, suggesting that the combination of allelotypes cannot explain the loss of crispness phenotype.

2.5.4 Effect of MdEXPA2 *allele type on transcription*

To test the hypothesis that the presence of the DST-like element in the 3'UTR of *MdEXPA2* might destabilize transcripts, fragment analyses were done for the eight genotypes: 'Honeycrisp', 'MN1702', 'MN447', 'ED269', 'CC130', 'MN1764', 'CD151' and 'CC141'. The expected peak for the 'Golden Delicious' allele (GD) was ~303 bp, ~310 bp for the 'Granny Smith' allele (GS) and ~293 bp for MdEXPA8. The expected size for the GD allele in'MN447', 'CC130' and 'CC141' was ~459 bp because of the presence of the transposable element.

When both alleles were present in genomic DNA, the proportion of each was around 50% (42 to 58%), suggesting that only one copy of each was present (Fig. 2.5). When both alleles were present in a genotype, both were detected in the cDNA sample, with slight differences in the proportion of each, so it would appear that the DST-like element does not destabilize *MdEXPA2* transcripts.

2.5.5 Effect of the MdEXPA2 *allele on translation*

Total protein from fresh and 8 week-stored fruit from eight genotypes was hybridized against LeExp1 antibody. An expansin protein was detected on Western blots of fresh and 8 week-stored fruit from all eight examined genotypes, except for fresh fruit from ‘CC141’ and 8 week-stored fruit from ‘CD151’ and ‘ED269’, where no expansin was detected (Fig. 2.6). Both ‘CC141’ and ‘CD151’ have the GD/GS *MdEXPA2* allele, but ‘ED269’ has the GS/GS allele. Overall transcript levels of *MdEXPA2* and *MdEXPA3* did not appear to reflect total expansin protein levels detected by tomato expansin LeExp1 antibody. For example, expansin protein was not detected in stored fruit from ‘CD151’ (Fig. 2.6), although quantitative PCR showed that *MdEXPA2* is transcribed at this time point (Fig. 2.1).

2.5.6 Expansin genes in the apple genome

Around sixty predicted proteins were found to be homologous to Expansin A, and found to be distributed across all 17 apple chromosomes except chromosomes 7 and 15. *MdEXPA1* through *MdEXPA7* were located on chromosomes 1, 9, 11, 16 and 17. The newly identified *MdEXPA8* was located on chromosome 4 (Fig. 2.7).

2.6. Discussion

The primary expansin mRNAs that accumulated in stored apples were *MdEXPA2* and *MdEXPA3. MdEXPA2* was present in all cultivars in this study at harvest, but transcript levels of this gene generally dropped to lower or undetectable levels during cold storage. However, it increased in one of the cultivars, 'CD151', which lost crispness during storage. Wakasa et al. (2003) reported that *MdEXPA2* is present in 'Golden Delicious' fruit at harvest and increases up to 12 days postharvest when kept at room temperature. Wakasa et al. (2003) described *MdEXPA2* as ethylene-regulated, but in the present study its transcripts were detected in 'Honeycrisp' fruit, which produces low levels of ethylene (Watkins et al. 2004; DeLong et al. 2004).

Goulao et al. (2007) reported the presence of *MdEXPA3* at harvest and in over-ripe 'Mondial Gala' fruit. However, *MdEXPA3* transcripts were detected in only some of the cultivars in this study at harvest, and transcript levels had a tendency to decline after cold storage. Additionally, mRNA levels of this gene varied greatly from year to year, as seen in Figure 2.3. In all genotypes studied, *MdEXPA1, MdEXPA4,* and *MdEXPA5* mRNA accumulation was very low or undetectable at harvest and after cold storage, which coincides with findings of Wakasa et al. (2003) using 'Golden Delicious'. *MdEXPA7* transcript was not detected in any genotype and at any time point. This suggests that it is not involved in ripening-related processes in apples.

A new apple expansin gene was identified and designated *MdEXPA8* in this study. This gene was not found among apple EST sequences and was originally thought to be an *MdEXPA2* gene, but further sequencing of its coding region revealed that it was more homologous to the pear *PcEXPA1* gene. In pear cv. La France, *PcEXPA1* levels increase during fruit growth until harvest, then remain high during fruit softening (Hiwasa et al. 2003). In pear cv. Rocha, *PcEXPA1* mRNA expression is highest in initial growth stages and after 60 d of cold storage (Fonseca et al. 2005). In this study, the fragment analyses revealed that *MdEXPA8* had very low transcript levels at harvest and after cold storage in apples.

The translated protein sequence of *MdEXPA8* differs from that of *MdEXPA2* by only 9 amino acids, so it could be deduced that these genes are a result of the genome wide duplications that occurred in the apple genome (Velasco et al. 2010). However, *MdEXPA2* is located in chromosome 1 (Fig. 2.7), while *MdEXPA8* is located in chromosome 4, which do not show strong collinearity. Similarly, *MdEXPA1, MdEXPA3, MdEXPA4, MdEXPA5, MdEXPA6* and *MdEXPA7* are located on chromosomes that do not show strong collinearity to each other (Velasco et al. 2010).

In a search of expansin genes in the apple genome, sixty putative genes were found, although it is probable that not all of these represent functional or transcribed expansin genes. They could also be expressed in other tissues besides fruit. *MdEXPA2, MdEXPA6* and *MdEXPA7* were located close to a previously described QTL for apple firmness on linkage group L01, flanking the QTL described by Maliepaard et al. (2001) and referenced by Costa et al. (2008). Another QTL for crispness seems to be close to the QTL for apple firmness on L01, and thus may be linked to *MdEXPA2, MdEXPA6* or *MdEXPA7*. A QTL for apple crispness on L16 did not have any expansin genes close by. There are other QTLs for apple crispness with lower LOD scores located on L05, L10, L12, and L13 (King et al. 2000), although these chromosomes do not harbor expansin genes *MdEXPA1* through *MdEXPA8*.

A *Spring* transposable element in the ‘Golden Delicious’ allele of *MdEXPA2* in‘MN447’, ‘CC130’ and ‘CC141’ was found. Han and Korban (2007) demonstrated the mobility of *Spring* elements by determining that it is present in ACC synthase of apple but not in pear. The present study suggests an even more recent activity for this transposable element, because it is present in only one allele of *MdEXPA2,* and only some of the cultivars with this allele have the transposable element.

The apparent excision of the *Spring* element from *MdEXPA2* mRNA or cDNA (Fig. 2.4b), but not genomic DNA, is also of interest because there is no general consensus on the mechanism of transposition of MITE elements. It is unknown whether transposition occurs through an RNA or DNA intermediate (Han and Korban 2007; Río et al. 1996). The excision of the transposable element from mRNA may be an intermediate step in their transposition, which would suggest a mechanism of transposition that allows their multiplication in the genome. This, however, remains highly speculative.

Two *MdEXPA2* allele types were discovered differing at two locations in the 3’UTRs, and both differences were found to be conserved among apple, pear and loquat (Fig. 2.3). The allelotypes differ mainly in their 3’UTRs and not in their coding regions, so it was hypothesized that any relationship of the ‘Golden Delicious’ allele to fruit crispness may be due to an influence of the 3’UTR on the fate of *MdEXPA2* transcripts within the cell and ultimately in expansin protein abundance.

Part of the sequence in the 'Granny Smith' allele is similar to conserved sequence elements of the SAUR gene mRNA destabilizing element (Gutiérrez et al. 1999, Feldbrügge et al. 2001, Pérez-Amador et al., 2001), which can reduce transcript stability of SAUR genes (Newman et al. 1993). Fragment analyses of the two alleles of *MdEXPA2* revealed that the DST-like element does not appear to play a major role in transcript stability, and the slight variation in the ratios of each allele in the cDNA sample (Fig. 2.5) cannot be linked entirely to maintenance of crispness.

Homozygosity or heterozygosity for the 'Granny Smith' or 'Golden Delicious' *MdEXPA2* alleles did not appear to influence transcript levels of this gene, and a clear association between *MdEXPA2* relative transcript levels and phenotypes that remained crisp, or lost crispness, was not detected. However, it would appear that fruit that maintain crispness have a tendency to overall lower *MdEXPA2* transcript levels, with the exception of 'Honeycrisp' fruit. The sample size of GS/GS genotypes was small ($n < 20$), perhaps obscuring the trend of small work difference correlating with this allelotype.

MdACS1 in combination with *MdEXPA2* allelotypes was studied because *MdEXPA2* allelotype alone could not explain maintenance of crispness, and because of previously reported correlations of allele 2 of *MdACS1* to maintenance of fruit firmness (Zhu and Barritt, 2008). It was anticipated that a higher number of genotypes homozygous for allele 2 of *MdACS1* would be present among progeny with crisp phenotypes. However, in this study, homozygosity for allele 2 of the *MdACS1* gene did not result in significant differences in maintenance of crispness among progeny of 'Honeycrisp' crosses, when compared to heterozygous allelotypes (Table 2.2). Studies by

Orazugie et al. (2004) and Costa et al. (2008) also failed to find a significant correlation between *MdACS1*-2/2 and lower change in firmness at room temperature or cold storage, respectively, while three other studies did (Costa et al. 2005 at room temperature; Orazugie et al. 2007 and Zhu and Barritt 2008 in cold storage). On the other hand, Orazugie et al. (2007) observed that, for late season cultivars, *MdACS1*– 1/1 and 1/2 allelotypes had higher firmness at harvest than *MdACS1*– 2/2 allelotypes, which is consistent with findings in this study but not with findings by Zhu and Barritt (2008). The combination of *MdEXPA2* and *MdACS1* alleles could not explain crispness maintenance.

Expansin protein was detectable in genotypes of both *MdEXPA2* allelotypes (Fig. 2.6). The proteins detected in immunoblots by a tomato expansin LeExp1 antibody would most likely be translated from *MdEXPA2* and *MdEXPA3* transcripts in fresh fruit and from *MdEXPA2* in stored fruit, since all other expansin genes that were studied were expressed at very low levels. mRNA transcript levels and expansin protein detected by SDS PAGE may not reflect actual expansin levels in the cell wall, so a study of expansin activity in different apple genotypes may be necessary to determine whether this protein influences maintenance of apple fruit crispness.

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2.8 Tables

Table 2.1 Work values (mJoules) at harvest and storage and *MdEXPA2* and *MdACS1* gene variants in genomic DNA of 41 apple cultivars. Parental crosses for each genotype are shown in parentheses. Work values are an average for each genotype at each storage treatment, ± standard error values, with the number of sampled fruit shown in parentheses. According to Zhu and Barritt (2008), *MdACS1*-1/1 codes for high ethylene production while *MdACS1*- 2/2 codes for low ethylene production.

Cultivar	Work - harvest	Work - storage	Difference in work	*MdACS1*	*MdEXPA2*
Remained crisp					
Honeycrisp (HC)	1.90± 0.22 (15)	1.66± 0.10 (15)	0.24	1 / 2	GS / GS
MN447	2.23±0.15 (12)	1.96±0.12 (12)	0.27	2 / 2	GD / GS
MN1702	3.05±0.16 (15)	2.86±0.16 (16)	0.19	2 / 2	GS / GS
CC116 (HC x MN447)	2.10± 0.23 (6)	2.64± 0.27 (8)	-0.54	1 / 2	GS / GS
CC130 (HC x MN447)	2.92± 0.19 (13)	3.29± 0.19 (9)	-0.37	1 / 2	GD / GS
BB142* (HC x MN1702)	1.94±0.15 (10)	2.36±0.24 (10)	-0.42	1 / 2	GS / GS
BB143* (HC x MN1702)	1.74±0.16 (10)	2.15±0.25 (10)	-0.41	1 / 2	GS / GS
CP065* (HC x MN1702)	1.98±0.15 (10)	2.03±0.15 (10)	-0.05	1 / 2	GS / GS
CP078* (HC x MN1702)	2.64±0.31 (10)	3.42±0.35 (10)	-0.78	2 / 2	GS / GS
EC310* (HC x MN1702)	1.76±0.13 (10)	1.87±0.16 (10)	-0.11	2 / 2	GS / GS
ED229* (HC x MN1702)	2.27±0.12 (10)	2.63±0.15 (10)	-0.36	2 / 2	GS / GS
ED269 (HC x MN1702)	2.33±0.24 (8)	2.06±0.15 (11)	0.27	1 / 2	GS / GS
ED276* (HC x MN1702)	2.83±0.20 (10)	2.69±0.29 (10)	0.14	1 / 2	GS / GS
ED277* (HC x MN1702)	2.66±0.28 (10)	2.35±0.23 (10)	0.31	1 / 2	GS / GS
ED281* (HC x MN1702)	1.97±0.20 (10)	2.41±0.10 (10)	-0.44	2 / 2	GS / GS
CD138* (HC x MN1764)	2.19±0.18 (10)	3.19±0.59 (5)	-1.00	2 / 2	GD / GS
EF114 (HC x MN1764)	1.58±0.12 (6)	1.51±0.39 (5)	0.09	2 / 2	GS / GS
EF130* (HC x MN1764)	2.20±0.28 (7)	2.73±0.55 (4)	0.13	1 / 2	GD / GS
EG005* (HC x MN1764)	1.48±0.15 (10)	1.52±0.24 (10)	-0.04	2 / 2	GD / GS
EG009* (HC x MN1764)	2.06±0.17 (10)	1.67±0.14 (10)	0.39	1 / 2	GD / GS
Lost crispness					
Macoun	0.88±0.15 (10)	0.25±0.03 (6)	0.63	1 / 1	GD / GD
MN1764	1.31±0.09 (8)	0.72±0.07 (8)	0.59	2 / 2	GD / GS
CC141 (HC x MN447)	3.52±0.25 (15)	1.76±0.18 (15)	1.75	1 / 2	GD / GS
CP087* (HC x MN1702)	1.87±0.21 (10)	1.17±0.16 (7)	0.70	2 / 2	GS / GS
ED225* (HC x MN1702)	2.03±0.21 (10)	1.06±0.12 (10)	0.97	1 / 2	GS / GS
ED267* (HC x MN1702)	1.55±0.26 (10)	0.57±0.05 (10)	0.98	1 / 2	GS / GS
ED272* (HC x MN1702)	1.17±0.19 (10)	0.32±0.05 (10)	0.85	1 / 2	GS / GS
ED280* (HC x MN1702)	2.23±0.19 (10)	0.74±0.06 (10)	1.49	1 / 2	GS / GS
ED286* (HC x MN1702)	1.50±0.09 (10)	0.82±0.07 (10)	0.68	2 / 2	GS / GS
ED316* (HC x MN1702)	2.34±0.19 (10)	1.71±0.14 (10)	0.63	1 / 2	GS / GS
CD150 (HC x MN1764)	2.01±0.25 (4)	0.92±0.23 (6)	1.09	1 / 2	GD / GS
CD151 (HC x MN1764)	1.86± 0.16(13)	1.06± 0.15(14)	0.8	1 / 2	GD / GS
EF096* (HC x MN1764)	2.32±0.23 (10)	0.74±0.05 (9)	1.58	1 / 2	GD / GS
EF120* (HC x MN1764)	1.84±0.09 (10)	1.48± 0.12(10)	0.36	2 / 2	GD / GS

Cultivar	Work - harvest	Work - storage	Difference in work	*MdACS1*	*MdEXPA2*
EF128* (HC x MN1764)	1.27± 0.06(10)	0.86± 0.08(10)	0.41	2 / 2	GD / GS
EF133* (HC x MN1764)	1.93± 0.17(10)	1.17±0.10 (10)	0.76	2 / 2	GD / GS
EG015* (HC x MN1764)	1.77±0.20 (10)	1.12±0.11 (10)	0.65	2 / 2	GD / GS
EG018* (HC x MN1764)	0.93± 0.07(10)	0.62± 0.05(10)	0.31	2 / 2	GS / GS
EG023* (HC x MN1764)	1.42±0.12 (10)	0.98±0.13 (10)	0.48	2 / 2	GD / GS
EG024* (HC x MN1764)	1.69± 0.10(10)	0.76± 0.13(10)	0.93	2 / 2	GS / GS

* indicates 3 months of cold storage

Table 2.2 Influence of *Md-ACS1 allelotypes* and *MdEXPA2* variants on apple crispness (mJoules) at harvest and after storage. Different letters indicate a significant difference based on a t-test (α = 0.05)

Allelotype	Work - harvest	Work - storage	Difference in work
Md-ACS1			
1 / 2 (n = 20)	2.18a	1.69	0.49
2 / 2 (n = 20)	1.81b	1.59	0.22
MdEXPA2			
GS / GS (n = 6)	1.88	1.67	0.20
GS / GD (n = 17)	1.99	1.49	0.47

2.9 Figures

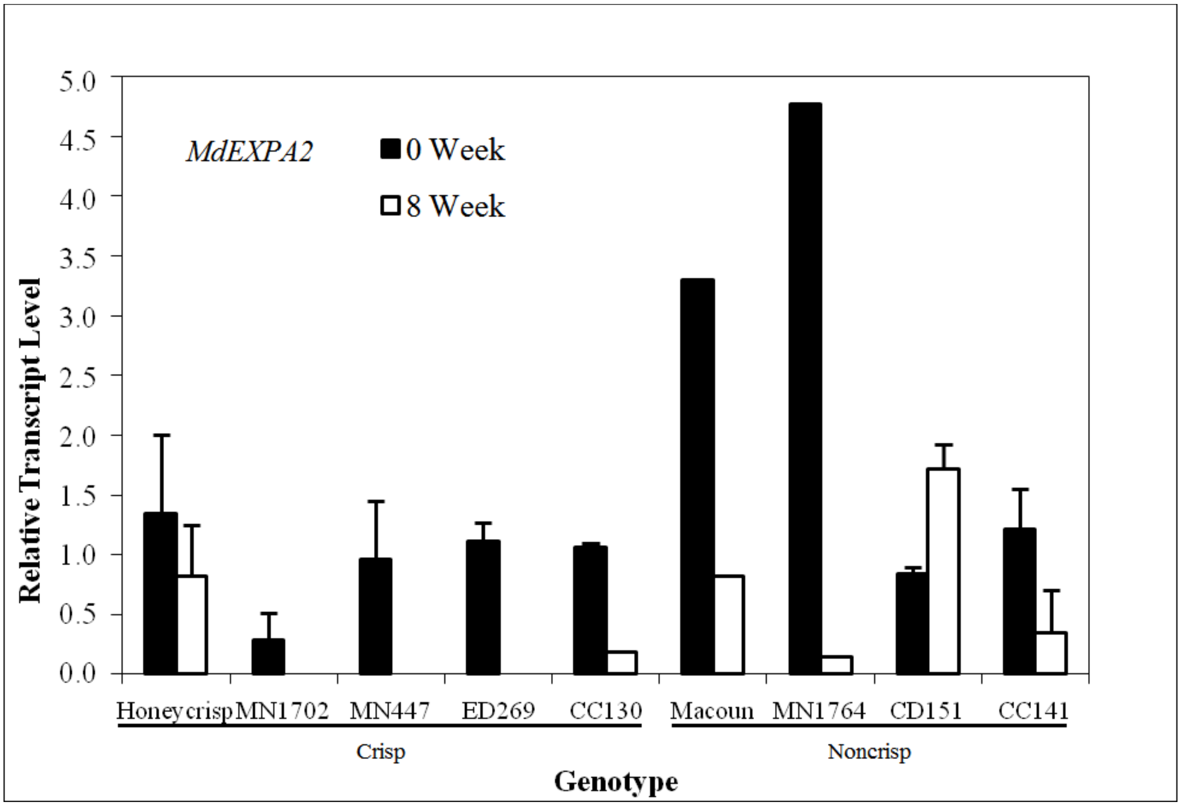

Figure 2.1 Transcript levels of *MdEXPA2* at 0 week and 8 week stored fruit tissue from nine apple genotypes, relative to *β*-actin. Bars represent transcript levels detected by the *MdEXPA2F* and *MdEXPA2RN* primer combination. RNA was extracted from fruit in 2005 and 2006, except for 'Macoun' (only in 2005) and 'MN1764' (only in 2006). Each quantitative Real Time RT-PCR reaction was run in duplicate for at least two biological replicates. Error bars represent standard error for *MdEXPA2* levels over two years (except 'Macoun' and 'MN1764', which do not have error bars).

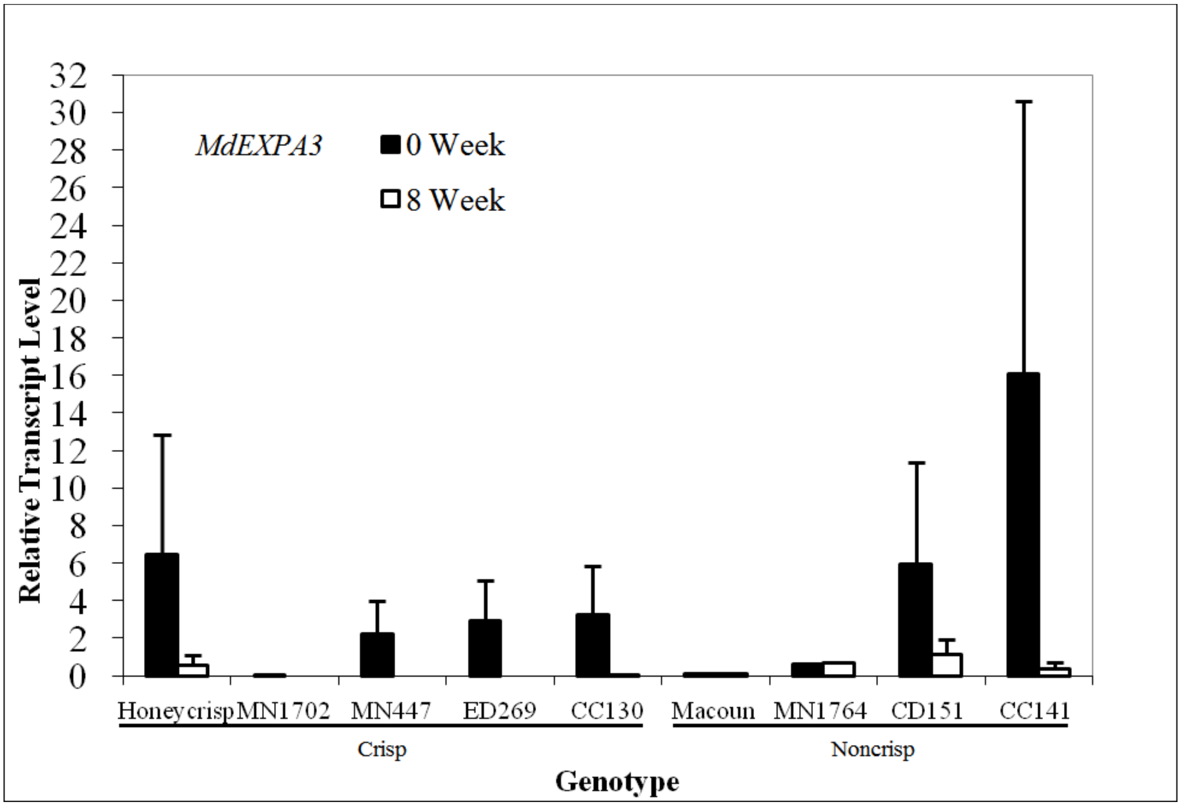

Figure 2.2 Relative transcript levels of *MdEXPA3* at 0 week and 8 week stored fruit tissue from nine apple genotypes, relative to β-actin. Total RNA was extracted on two separate years for all genotypes except for 'Macoun' (only in 2005) and 'MN1764' (only in 2006) and qRT-PCR reactions were run in duplicate. Error bars represent standard error for *MdEXPA3* levels over two years (except 'Macoun' and 'MN1764', which do not have error bars).

```
MdEXPA2.Macoun          CAGTCAATTCTGAGACTCCTCCGGCGGTAAAACAGTTAAAAAGTCTGGTGAATGGTCAAT 180
MdEXPA2.GD.AB099927     CAGTCAATTCTGAGACTCCTCCGGCGGTAAAACAGTTAAAAAGTCTGGTGAATGGTCAAT 180
MdEXPA2.Honeycrisp      CAGTCAATTCTGAGACTCCTCCGGCGGTAAAACAGTTAAAAAGTCTGGTGAATGGTCAAT 180
MdEXPA2.GS.AY083167     CAGTCAATTCTGAGACTCCTCCGGCGGTAAAACAGTTAAAAAGTCTGGTGAATGGTCAAT 180
PcEXPA2.AB093029        CAGTCAATTCTGAGACTCCTCCGGCGGTAAAACAGTTAAAAAGTCTGGTGAATGGTGAAT 180
EUEXPA3.EU123921        CAGTCAATTCTGAGACTCCTCAGGCAGTAAAACTGTAAAAAAGTCTGGTGAAAGGTGGAT 180
PcEXPA1.AB093028        CAGTCAATTCTAAGACTCCTCCGGCAGTAAAACGGTCAAA--GTCTGGTGAACGGTGAAG 178
MdEXPA8.HQ905438        CAGTCAATTCTGAGACTCCTCCGGCGGTAAAACGGTCAAAAAGTCTGGTGAACGGTGAAG 180
EuEXPA4.EU123922        CAGTCAATTCTAAGACTCCTCCGGCAGTAAAACCGTCAAAAAGTCTGGTGAATGCTCAAG 180
                        *********** ********* *** ******* ** ***  ********** * *  *

MdEXPA2.Macoun          GGTGAA-------GGGTGA---ATTGTATATGGTAATTTGAACGTGGATGGGAAGAGGAG 230
MdEXPA2.GD.AB099927     GGTGAA-------GGGTAA---ATTGTATATGGTAATTTGTACGTGGATGGGAAGAGGAG 230
MdEXPA2.Honeycrisp      GGTGAA-------GGGTGA---ATTGTATATGGTAATTTGTACGTGGATGTGAAGAGGAT 230
MdEXPA2.GS.AY083167     GGTGAA-------GGGTGA---ATTGTATATGGTAATTTGTACGTGGATGTGAAGAGGAT 230
PcEXPA2.AB093029        GGTGAATGGTGAAGGGTGA---GTAATTTATGGTAATTTGTACGTGGATGGGAAGAGGAG 237
EUEXPA3.EU123921        GGTGAA-------GGGTGAATTATTGTATATGGTAATTT----GTGGATGGGAAGAGGAG 229
PcEXPA1.AB093028        GGTACA----------------------------ACCTTGTATGCGGATGGGAAGAGGAG 210
MdEXPA8.HQ905438        GGTACA----------------------------ACCTTGTACGTGGATGGGAAGAGGAT 212
EuEXPA4.EU123922        GGTACA---------------------------AGCAATGTGTGTGGATGGGAAGAGGAC 213
                        ***  *                                *    * ***** ********
                                         Consensus SAUR DST Element  GGAGACTGAC---AT

MdEXPA2.Macoun          AG-TTGG------------GGGGTAGTTTTAGGAGAGGCAACTGATTGCTGAGGTGGCTAA 279
MdEXPA2.GD.AB099927     AG-TTGGGGG------------GTAGTTTTAGGAGAGGCAACTGATTGCTGAGGTGGCTAA 278
MdEXPA2.Honeycrisp      AG-TTGGATAGTTGGGGGGGG-GTAGTTTTAGGAGAGGCAACTGATTGCTGAGGTGGCTAA 289
MdEXPA2.GS.AY083167     AG-TTGGATAGTTGGGGGGGG-GTAGTTTTAGGAGAGGCAACTGATTGCTGAGGTGGCTAA 289
PcEXPA2.AB093029        AG-TTTGGGGG-----------GTAGTTTTAGGAGAGGCAACTGATTGCTGAGGTGGCTAA 286
EUEXPA3.EU123921        AG-TTGAGGG------------GTAGTTTTAGGAGAGGCAACTGATTGCTGAGGTGGCTAA 277
PcEXPA1.AB093028        AG-TGGAGGTTTTTTTTTTTTTT---------AGGAGGCAACTGATTGCTGAGGTGGCTAA 261
MdEXPA8.HQ905438        AG-TTGAGGGTTTTTTTTTTTTTTTTTTTTTTAGGAGGCAACTGATTGCTGAGGTGGCTAA 272
EuEXPA4.EU123922        AG-TTGAGGACCCTTTTTTTTTTTTTTT----AGGAGGCTGCTGATTGCTGAGGTGGCTAC 269
                        ** *                              *****  *******************
                        AGATTGGAGGAGACATTTT---GTA

MdEXPA2.Macoun          TTAACTGGCACCCGCTAGTCCTATA 303
MdEXPA2.GD.AB099927     TTAACTGGCACCCGCTAGTCCTATA 303
MdEXPA2.Honeycrisp      ----CTGGCACCCGCTAGTCCTATA 310
MdEXPA2.GS.AY083167     ----CTGGCACCCGCTAGTCCTATA 310
PcEXPA2.AB093029        ----CTGGCACCCGCTAGTCCTATA 307
EUEXPA3.EU123921        ----CTGGCACCCGCTAGTCCTATA 298
PcEXPA1.AB093028        ----TTGGCACCCGCTAGGCCTATC 282
MdEXPA8.HQ905438        ----TTGGCACCCGCTAGTCCTATA 293
EuEXPA4.EU123922        ----TTGGCACCCGCTAGGCCTAT- 289
                             ************* *****
```

Figure 2.3 ClustalW alignment of the 3'UTRs of *MdEXPA2* from 'Macoun', 'Golden Delicious' (GD, AB099927), 'Honeycrisp' and 'Granny Smith' (GS, AY083167), *MdEXPA8* (HQ905438), and expansins from pear (*Pyrus communis*, AB093028 and AB093029), and loquat (*Eriobotrya japonica,* EU123921 and EU123922). Sequence in bold identifies the major differences between AY083167 and AB099927. Dark shaded sequences are similar to the highly conserved portion of consensus SAUR gene 3'UTR DST elements (adapted from Feldbrügge et al. 2001). Light gray shading indicates the area of insertion of a *Spring* transposable element in the 'Golden Delicious' allele of 'MN447', 'CC130' and 'CC141'. Sequences shown here are 5' to 3'relative to mRNA. Underlined sequence in bold indicates the stop codon for each gene.

```
Spring1               -TAATACAAGGGGTGTGATATCCACACACCCCATTTT-CTTCTCACACACCCTTT-TAAT 57
Spring4               TTTTACAAAGGGGTGTGATATCCACACACCCCATTTTACTTCTCACACACTTTTT-TAAT 59
MN447, CC130, CC141   -ATTGTATAGGGGTGTGATACCCACACATCCTATTTTACTTCTCACACACCTTTT-TGAT 58
Spring3               AAATAACATGGGGTGTGCTATCCACACATCTCTTTTTACTTCTCACACACCATCT-TAAT 59
Spring2               TAAATAAAAGGGGTGTGCTATCCACACACCATTTTTTACTTCTCACACACCTCTTGTTAA 60
                       :::  .::********.** ******* *  :**** ************    * * *:

Spring1               TTTCGACCGTCGGATCAGATGAATTGAAGAAGATCAACGGACAGAAATTAACAAGGGGTG 117
Spring4               TTTCAGCCGTCCAATCGGATGAATTTAAGAAAATCAAGGGACAGAAATAATCAAAAGGTA 119
MN447, CC130, CC141   TTTCCGCCGTCGGGTCCCATAAATTTAAGAAGATCAACGGACATAAGTTATCAAGGGGTG 118
Spring3               TTTAGGCCGTCAGATCGAAAAAAATTGAAGAAGACAAATGATATAAATTAATAAGGGGTG 119
Spring2               TTTCTACCATTTGATCTTCTTCAATTCACCCGATCCAAAGGCCAAAACTTAAAAG-GGTG 119
                      ***. .**.*  ..**  .: .*:*  *  ...:*.*  *. . **. ::: **. ***.

Spring1               TGTGAGAAGTAATTTGGGGTGTGTGGATAGCACACCCCTAATACAA- 163
Spring4               TGTGAGAAGTCAAATGGGGTATGTGGATAGCACACCCCTTTTACAAA 166
MN447, CC130, CC141   TGTGAGAAGTAAAAAGGGGTGTGTGGATAGCACACCCCATTGTATA- 164
Spring3               TATGAGAAGTAAAAATGGATATGTGGATACGACACCCGTAAATAAAT 166
Spring2               TGTGAGAAGAAAAAAGGGGTGTGTGGATATCACACCCCTAAATAAAA 166
                      *.*******:.*::: **.*.********  ****** ::: :.:*
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2.4a

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GGTCAATGGTGAAGGGTGAATTGTATAGGGGTGTGGATAGCACACCCCATTGTATATGGTAATTTGTACGTGCATGGGAAGAGGAG
```
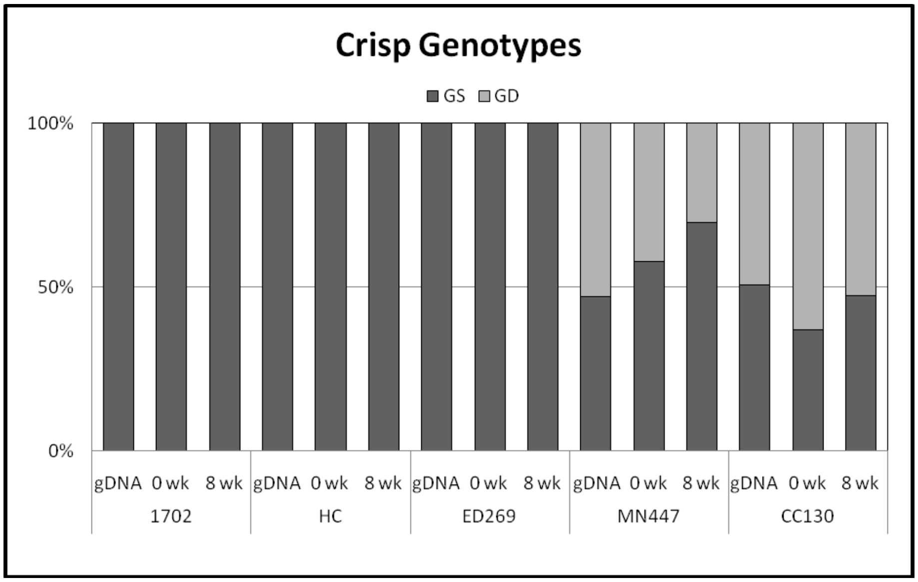
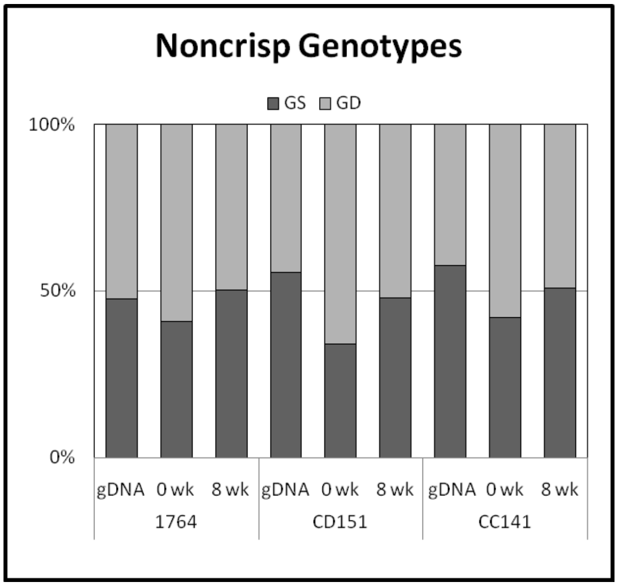

2.4b

Figure 2.4 (a) Comparison of the *Spring* transposable elements described by Han and Korban (2007) to that found in the 'Golden Delicious'-like *MdEXPA2* allele of 'MN447', 'CC130' and 'CC141'. The 8 or 9 bp target site duplication (TSD) is highlighted in gray (b) *MdEXPA2* 3'UTR sequence in which the transposable element is no longer present. The 8 bp TSD flanks a 21 bp palindromic sequence originated from the transposon.



2.5a



2.5b

Figure 2.5 Fragment analyses showing relative abundance of ‘Granny Smith’ (GS) and ‘Golden Delicious’ (GD) *MdEXPA2* transcripts in genotypes that remained crisp (a) or lost crispness (b). Bars show proportions of each transcript variant within genomic DNA (gDNA) or cDNA from fresh (0 wk) or 8 week (8 wk) cold stored fruit. At least two biological replicates were used for each genotype and at each storage treatment.

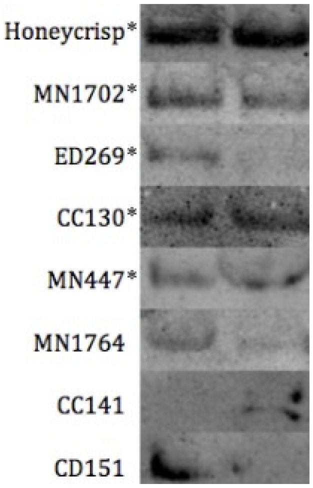


Figure 2.6 Immunoblot analyses of expansin protein of fruit stored for 0 week (left lane) or 8 weeks (right lane) from eight apple genotypes, as determined by hybridization of total apple fruit cell wall-associated protein (150 µg) to LeExp1 antibody. Image is a composite of independent images from at least two replicate extracts per genotype. Asterisks indicate crisp genotypes.

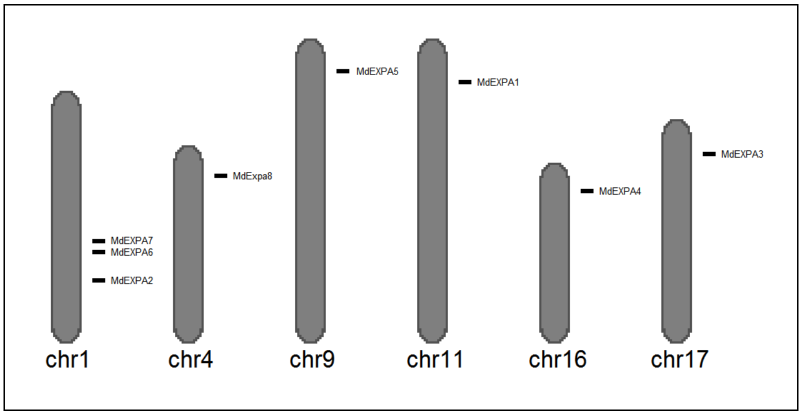


Figure 2.7 Locations of *MdEXPA1 – MdEXPA8* genes on the Malus consensus map (Velasco et al. 2010).

Chapter 3

Relationship of four cell wall modifying proteins to apple crispness maintenance in a 'Honeycrisp' breeding population

3.1 Summary

Cell wall-modifying proteins in fruit are thought to contribute to the loss of desirable fruit texture traits, such as crispness and firmness, after harvest. The activities of four cell wall-modifying proteins were studied for up to four years by quantifying *in vitro* activity from apple genotypes that maintained crispness or lost crispness after cold storage. Arabinofuranosidase (AF), β-galactosidase (BG), and expansin (which was measured in only one year) did not show increasing or decreasing trends in activity between storage treatments across the sampled genotypes, while polygalacturonase (PG) activity generally exhibited an increasing trend after storage. Activity levels of AF and BG were correlated, but neither showed a relationship to expansin or PG activity. There was no significant difference in activity levels among crisp versus noncrisp genotypes for any of the protein activities measured, which puts into question the influence of these proteins on crispness maintenance. Linear regression analysis of individual fruit Work values and the corresponding enzyme activities showed that no one enzyme activity was a good predictor of Work, except possibly PG in apples stored for 8 weeks ($p=0.08$). AF and BG activities showed weak but possibly significant correlations to Force values in fresh ($p=0.09$) and stored ($p=0.002$) apples, respectively, thus suggesting that they may play small roles in loss of fruit firmness.

3.2 Keywords

Arabinofuranosidase, expansin, β-galactosidase, *Malus pumila* Mill., polygalacturonase,

3.3 Introduction

Cell wall disassembly occurs after harvest of apple fruits, and is thought to lead to changes in fruit texture, including softening, mealiness, and loss of crispness (Gross and Sams 1984; Pena and Carpita 2004; Johnston et al. 2002). Apple tissue can fail at the middle lamella due to its dissolution, leading to cell separation and mealiness, or at the cell wall, leading to cell rupture (Stow 1993; Ben-Arie et al. 1979). The primary cell wall is composed of cellulose microfibers imbedded in a matrix that consists of hemicelluloses, pectins, and structural proteins (Taiz and Zeiger 2000). The middle lamella lies between the primary walls of two adjacent cells, and is a layer composed mostly of pectins functioning as an adhesive layer (Cosgrove 2005).

Pectin solubilization is a prominent event during apple ripening and softening, and may be promoted by polygalacturonase (PG), β-galactosidase (BG) and/or ✓-L-arabinofuranosidase (AF) action (Goulao et al. 2007). The middle lamella is rich in homogalacturonan pectin. In its de-esterified form, it can be degraded by loss of Ca^{2+} cross-linking or when hydrolyzed by PG (Atkinson et al. 2002; Fry 2011). In apple tissue, endo-PG activity has been generally undetected (Bartley 1978; Abeles and Biles 1991; Goulao et al. 2007; with the exception of a study by Wu et al. 1993), while exo-PG activity has been more commonly detected (Bartley 1978; Goulao et al. 2007; Wei et al. 2010). Exo-PG activity maintains similar levels from fruit at harvest to over-ripe fruit (Goulao et al. 2007) and was found to be correlated to firmness in ‘Fuji’ and ‘Golden Delicious’ apples, which remain firm and soften, respectively (Wei et al. 2010).

Ben-Arie et al. (1979) observed that tomato endo-PG could dissolve the middle lamella of apple fruit discs. Transgenic apples that overexpressed endo-PG had enhanced cell-to-cell separation in leaf tissue (Atkinson et al. 2002). On the other hand, transgenic apples with suppressed PG expression had firmer fruit after cold storage, and the apples' rate of softening correlated with *MdPG1* transcript levels (Atkinson et al. 2008). Accordingly, in a survey of 14 apple cultivars, Wakasa et al. (2006) found that cultivars that lost firmness had higher *MdPG1* transcript accumulation than cultivars that remained firm. In another study, 'Honeycrisp' fruit had extremely low *MdPG1* mRNA levels compared to the softening cultivar 'McIntosh' (Harb et al. 2012).

β-galactosidase and arabinofuranosidase proteins respectively cleave β-galactose and α-arabinofuranose sidechains from pectic rhamnogalacturonan (Fry 2011). Both BG and AF activities have been detected in ripe and cold stored apples (Ross et al. 1994; Yoshioka et al. 1995) and were found to increase in overripe fruit, compared to fruits at harvest (Goulao et al. 2007). Ortiz et al. (2011) found that after 19 weeks of cold storage, BG and AF activities were inversely correlated to firmness. BG, but not AF, activity was lower in 'Fuji' fruit, compared to that of 'Golden Delicious', when stored at room temperature (Wei et al. 2010), while ripe and overripe 'Honeycrisp' fruit had lower AF but not BG transcript levels, compared to 'McIntosh' fruit (Harb et al. 2012).

During apple ripening, galactose is lost while arabinose content remains fairly steady (Gross and Sams 1984), whereas other studies have determined that both of these neutral sugars are lost during softening. Both galactose and arabinose levels decline during storage of 'Golden Delicious' (Glenn and Poovaia 1990) and Peña and Carpita (2004) found that a loss of branched arabinan preceded softening in four cultivars. Tong et al. (1999) observed that arabinose content was maintained in ‘Honeycrisp’ after storage, while arabinose content declined in cultivars that lost crispness after storage.

Expansins are presumed to act on the junction between cellulose microfibrils and hemicelluloses in cell walls, breaking noncovalent bonds between these two, and promoting stress relaxation of the wall (Sampedro and Cosgrove 2005). While normally associated with expanding cell walls, expansin mRNA accumulation or protein activity has also been detected in fully expanded apples at harvest or after storage (Wakasa et al. 2003; Goulao et al. 2007; Trujillo et al. 2011).

Studies on neutral sugar loss and cell wall modifying proteins can be inconclusive as to the role that cell wall-modifying proteins play on fruit softening, because typically only one protein or one cultivar is studied. It is difficult to compare results from different laboratories where the methods of fruit texture determination, protein extractions and protein activity quantification differ. The aim of this study was to collectively determine activity of four proteins related to cell wall modifications in several apple genotypes (from a breeding population with ‘Honeycrisp’ as a common parent) that remained crisp or lost crispness after storage. The potential relationship of the activities of these cell wall degrading proteins to crispness maintenance was examined.

3.4 Materials and Methods

3.4.1 Plant Materials

Apples were harvested at the Horticultural Research Center in Chanhassen, MN in

2005, 2006, 2010 and 2011. At least eleven fruit were collected for each genotype, which included Gala (a commercial cultivar that loses crispness after storage), the parents Honeycrisp (HC), MN1764, and MN1702, and their progeny CC130, CC141, CD151, ED225, ED269, ED276, ED277, ED280, and EG005. CC130 and CC141 are progeny of a MN447 x HC cross, CD151 and EG005 are progeny of MN1764 x HC, and ED225, ED269, ED276, ED277 and ED280 are progeny of MN1702 x HC. Most genotypes were harvested for three or fewer years, mainly depending on the availability of fruit. Fruit were harvested at a starch index of 5 or 6 out of 8 (Blanpied and Silsby 1992). Half the fruit were processed immediately after harvest ('0 week fruit') , while the other half were processed after storage for 8 weeks at 0 ± 0.5 °C and 95% to 99% relative humidity. At each time point, fruit texture was analyzed (see below), after which fruit were peeled, cored and chopped. Mesocarp tissue was subsequently frozen in liquid nitrogen and kept at –80 ºC until extracted for protein .

3.4.2 Texture analysis

Fruit texture was measured with a TA.XT2 texture analyzer (Texture Technologies, Scarsdale, NY). The Force (g) and Distance (mm) required to break cylindrical apple tissue samples were used to calculate Work (mJoules) and analyze changes in fruit texture during storage (Mann et al. 2005). Work values per se were not used to determine crispness of each individual apple; rather, for each genotype and year, change in Work between 0 (at harvest) and 8 week fruit was analyzed by a two sample two-tailed t-test to determine whether that genotype remained crisp or lost crispness. If there was no significant difference (p= 0.05) between time points, the genotype was deemed crisp, but if Work at 8 weeks was significantly lower than at harvest, then the genotype was deemed as noncrisp. Using this snapping test, change in Work from 0 to 8 stored week fruit was found to be correlated with change in sensory crispness (r^2=0.76, Mann et al. 2005). Most genotypes evidenced at least 1000 mJ of Work at harvest.

3.4.3 Protein extractions

For PG, BG, and AF activity assays, around 25 g of frozen apple fruit cortex tissue was thawed in 200-250 mL cold distilled water for 25-30 min, then ground using a homogenizer (Brinkmann Instruments, Inc., Westbury, NY) for 15 - 20 s (Tong and Gross 1989). Cell wall material was collected using vacuum filtration, then stirred in 25 mL of 50 mM sodium acetate (pH 4.5) containing 1 M sodium chloride for up to 4 h at 4 °C. The solution was filtered through Miracloth (EMD Biosciences Inc. La Jolla, CA) to remove cell wall fibers. The extracted cell wall associated proteins were then precipitated by adding 0.5 g ammonium sulfate per mL of protein extract stirring constantly at 4 °C until the ammonium sulfate was visibly dissolved. The solution was then centrifuged for 1 h at 15000 x g at 4 °C. The protein pellet was dissolved in 1 mL of 50 mM sodium acetate buffer (pH 4.5), then centrifuged for 3 min at 2655 x g at 4 °C to remove suspended particles. The supernatant was desalted at 4 °C with Bio-Gel P-4 (2005 and 2006 assays, Bio-Rad Laboratories, Hercules, CA) in a 1.7 ml bed volume using a capillary tube or with Sephadex G-25 (2020 and 2011 assays, Sigma-Aldrich, Saint Louis, MO) in a 33.3 ml bed volume using a Kontes Flex-column. A 50 mM sodium acetate buffer (pH 4.5) was used to equilibrate columns and as an elution buffer. The eluate was assayed for protein and enzyme activities. Protein content was measured using a bicinchonic acid kit (2005 and 2006, Sigma-Aldrich Co., St Louis, MO) or the Qubit™ protein assay kit (2020 and 2011, Life Technologies Corp., Grand Island, NY) according to manufacturer protocols.

Protein extractions for expansin activity assays were done as described in Goulao et al. (2007), by grinding frozen tissue in liquid nitrogen and preparing acetone powders, then extracting in 25 ml phosphate buffer (200 mM sodium phosphate, pH 8.0, containing 5 mM EDTA and 5 mM DTT) to which 1 mL of 250 mM phenylmethylsulphonylfluoride was added. After one hour of incubation at 4 °C, the resulting homogenates were centrifuged for 1 h at 20000 x g at 4 °C, filtered through miracloth, then desalted with Sephadex G-25 in Kontes Flex-columns (33.3 ml bed volume with water elution), frozen at -40 °C,then lyophilized. Protein powders were resuspended in 50mM sodium acetate buffer immediately prior to expansin activity assays. Protein content was measured with a Qubit™ protein assay kit and fluorometer as per manufacturer instructions.

3.4.4 Enzyme assays

To measure PG activity, 200 µL of the cell wall associated protein extract was incubated with 0.2% (w/v) polygalacturonic acid (Tong and Gross 1989) in 50 mM sodium acetate buffer, pH 4.5, for 14 h at 37 °C. Polygalacturonase activity was determined to be linear in this time range (data not shown). The reaction was stopped by adding 1 mL of 100 mM borate buffer (pH 9.0), after which 200 µL of 1.0 % (w/v) 2-

cyanoacetamide solution (Gross, 1982) was added. The mixture was vortexed and incubated in a boiling water bath for 10 minutes. After allowing the reaction to cool to room temperature, D-galacturonic acid released by polygalacturonase was measured by absorbance at 276 nm. Data were expressed as nM of D-galacturonic acid released·µg protein^{-1}·hr^{-1}.

For BG and AF activity assays, extracted protein was incubated at 37 °C with 0.1% (w/v) o-nitrophenyl-β-D-galactopyranoside in 50 mM sodium acetate buffer, pH 4.5, for 1.5 h or with ρ-nitrophenyl-α-L-arabinopyranoside in 50 mM sodium acetate buffer, pH 4.5, for 5.5 h (Sigma-Aldrich Co., St. Louis, MO) in a 200 µL final reaction volume (Goulao et al., 2007). Activities of both β-galactosidase and α-L-arabinofuranosidase were determined to be linear in this time range (data not shown). Reactions for both enzymes were stopped by adding 1 mL of 200 mM sodium carbonate solution (pH 9.0). Liberation of o-nitrophenol (BG) or p-nitrophenol (AF) during the reaction was measured by its absorbance at 400 nm with a spectrophotometer. Data were expressed as nM of o- or p-nitrophenol released·µg protein^{-1}·hr^{-1}.

Expansin activity was measured for fruit harvested in 2010, using a stress relaxation assay, following the protocol of Goulao et al (2007). A TA.XT2 texture analyzer was fitted with TA-96B grips. A plastic cuvette on the bottom grip was used to hold the protein extract. Cucumber hypocotyls were used as cell wall specimens, and were incubated in protein solution during the assay. To obtain cucumber hypocotyls, ‘Burpee Pickler’ cucumber seedlings were germinated on paper towels moistened with deionized water in darkness until they reached 4-5 cm in height, after which hypocotyls were cut into 3 cm sections and frozen at -80°C. When completely frozen, hypocotyl cuticles were abraded with carborundum. A 1.2 cm-long section was excised from each hypocotyl, flattened between two glass slides and boiled for 15 s to denature native proteins.

For the assay, the hypocotyl was clamped with a distance of 6 mm between the clamps, and incubated in 200 µL of 1.25 µg/µl protein in 50 mM sodium acetate buffer (pH 4.5). The hypocotyl was extended until 20 g of force was reached, and the clamps were then held fixed at the corresponding distance. The subsequent force required to maintain that distance was measured over 5 min. The force at the end of the assay, minus the force of a buffer control, was used to estimate the expansin activity of the sample. Proteins were extracted from oat coleoptiles as described by Cosgrove and Li (1993) to be used as a positive control in stress relaxation assays.

3.4.5 Statistical analysis

All statistical analyses were conducted using R software (R Development Core Team, 2008). Variation of Work by Year, Storage treatment and Genotype was analyzed by Analysis of Variance (ANOVA). Variation of AF, BG, PG and Expansin activities were analyzed in relation to Phenotype, Storage and Year by simplification of linear mixed-effects models using stepwise deletion, with Genotype/Year/Storage as a random effect, using total data or subsets of fresh and stored fruit data. The correlation of protein activities to each other was examined by linear regression. Finally, linear mixed-effects models were used to determine the relationship between raw instrumental measurements

(Work, Force, and Distance) for apple samples and the corresponding protein activities for that sample. Models were constructed using the lme function contained within the nlme package (Pinheiro et al. 2012) for the R statistical software. Models were tested iteratively in a stepwise manner, using restricted maximum likelihood estimates (Pinheiro and Bates, 2000). Conditional *F*-tests were conducted using analyses of variance with the argument type set to 'marginal' to determine the significance of fixed terms in updated models. Two-sample two-tailed t-tests and Tukey's Honestly Significant Difference (HSD) test were used to compare mean values.

3.5 Results

3.5.1 Texture of apple genotypes used in this study

Work, which is correlated to loss of crispness (Mann et al., 2005), was found to vary by Storage and Genotype ($p < 0.001$, Table 5.1). The Storage by Genotype interaction was expected, as the genotypes had varying crisp (Fig. 3.1a) or noncrisp (Fig. 3.1b) phenotypes. However, Work values were generally lower in 2010 and 2011 compared to 2005 and 2006 as determined by Tukey's HSD test, $p = 0.05$. There was a significant ($p < 0.001$, Table 5.1) Year by Storage by Genotype interaction, as some apple genotypes responded differently to storage in some years. Specifically, 'MN1702', 'CC141' and 'CD151', could be classified as crisp or noncrisp phenotypes depending on the year (Fig. 3.1c). The other genotypes in this study exhibited the same phenotype every year they were sampled.

3.5.2 Relationship of protein activities to storage and phenotype

Linear mixed-effects models were used to analyze data from this study with genotypes considered as random samples of phenotypes, allowing analysis of phenotype effects on enzyme activity. Phenotype, aggregated across all genotypes, storage times, and years, did not significantly affect any enzyme activity (Tables 5.2 - 5.5). This suggests that the ability to maintain crispness was not affected by the activity of any one enzyme alone. Storage, aggregated across all phenotypes and years, affected AF, BG, and PG activities ($p \leq 0.10$), but not expansin activity. The effects of Phenotype and Storage on each enzyme activity are described in more detail below. Activity assay results conducted in 2005 and 2006 were significantly different than those done in 2010 and 2011 ($p < 0.001$), so data for 2005 and 2006 were analyzed by linear mixed-effects models separately from those for 2010 and 2011, then compared to assess trends.

Comparing trends in AF activity across crisp (Fig. 3.2a), noncrisp (Fig. 3.2b) and variable genotypes (Fig. 3.2c) did not reveal any clear relationship between AF activity and crispness maintenance. Crisp genotypes had lower (Honeycrisp in 2011, ED225 in 2011, and ED276 in 2010), higher (CC130 in 2005 and EG005), or no change in activity after storage (Fig 3.2a). Noncrisp genotypes also showed lower (ED280), higher (MN1764) or no change in activity after storage (Fig. 3.2b). Among genotypes with variable crispness (Fig. 3.2c), AF activities in MN1702 were higher in 2010, when it remained crisp, in both 0 week- ($p = 0.03$) and 8 week-stored ($p = 0.005$) fruit than at corresponding storage times in 2011, when it lost crispness. However, AF activity in 'MN1702' fruit did not significantly change between harvest and storage in any year.

There were no significant differences between AF activities of fresh and stored 'CD151' and 'CC141' fruit in 2005 and 2006, while there was a decrease in 'CD151'fruit in 2011.

Similar to Phenotype effects, the effects of Storage on AF activity were variable. In 2005 and 2006, AF activity did not differ significantly for most genotypes between harvest and storage, with the exceptions of CC130 and MN1764, as described above. In 2010, there was a tendency for AF activity to decrease after storage ($p = 0.03$), except for a non significant increase in AF activity for 'ED225'. In 2011, there was no clear trend with storage.

There was no clear relationship between Phenotype and BG activity. As with AF activity, crisp and noncrisp genotypes exhibited lower (ED225 and MN1764 in 2011, ED276 in 2010, and ED280 in both years), higher (ED269 and EG005) or no change in BG activity after storage (Fig. 3.3a and 3.3b). Among variable genotypes, BG activity did not change significantly between harvest and storage except for a decrease in 'CD151' 2011 fruit. Also, BG activity in 8 week-stored 'CD151' fruit was lower in 2006 when it remained crisp, than in 2005 when it lost crispness ($p = 0.08$). No difference was observed in 'CC141' or 'MN1702' BG activities between storage durations or year (Fig. 3.3c).

Storage had no significant affect on BG activity in 2005 and 2006 ($p = 0.13$), but BG activity exhibited a decreasing tendency with storage ($p = 0.06$) in 2010 and 2011, with some genotypic exceptions in 2010 and 2011 as previously described.

Among crisp genotypes, PG activity had a tendency to increase in all genotypes in all years, except for Honeycrisp, in which there was a non-significant decrease in 2010

(Fig. 3.4a). In noncrisp genotypes, a tendency to increase after storage was also observed, except for ‘ED277’ harvested in 2011 and ‘ED280’, in which there appeared to be insignificant decreases in PG activity with storage (Fig. 3.4b). PG activities in fresh ‘CC141’, and fresh and stored ‘CD151’ were higher in 2005, when they lost crispness, than corresponding levels in 2006, when they remained crisp (Fig. 3.4c). PG activities in these genotypes did not change between harvest and storage in any year. PG activity levels at 0 or 8 weeks in ‘MN1702’ did not change significantly between 2010 and 2011. ‘MN1702’ PG levels between harvest and storage did not change in 2005 or 2006, but increased between harvest and storage in 2010 and 2011. Overall, there was an increasing trend in PG activity after cold storage, more significantly in 2010 and 2011 ($p = 0.009$) than in 2005 and 2006 ($p = 0.09$).

Expansin activity was found to differ among genotypes ($p<0.05$), but not among phenotypes or storage times ($p > 0.3$). ‘Honeycrisp’ and ‘ED225’ exhibited decreases in expansin activity with storage (Fig. 3.5, with $p = 0.07$ and $p = 0.04$, respectively, by two-sided t-test). Expansin activity did not change with storage for all other genotypes.

3.5.3 Relationship between BG and AF activities

AF and BG were found to be correlated with $p < 0.001$ (Fig. 5.1a), but with a small correlation coefficient (r^2) of 0.29. A square root transformation of both activity measurements produced a model with better fit ($r^2 = 0.41$, $p<0.001$, Fig. 5.1b), as well as improving the constancy of variance and normality of errors. However, when the linear mixed effects models for AF and BG (above) were refitted with transformed values, Phenotype did not affect transformed AF and BG activities either, reinforcing the conclusion from the models that there is no relationship between these proteins and crispness maintenance in the genotypes used in this study. No relationship was found between either AF or BG activity with either PG or expansin activity.

3.5.4. Relationship between instrumental measurements and cell wall modifying proteins

The relationship of instrumental measurement values and protein activities was analyzed by linear mixed-effects models (Tables 5.6 and 5.7). Work and Force values for each individual apple were not significantly predicted by any of the protein activities in that same apple for the combined 0 and 8 week data. When analyzing the subsets of fresh and stored apples separately, PG was a predictor of Work at 8 weeks of storage ($p = 0.085$, Table 5.6) and AF was a predictor of Force ($p = 0.092$, Table 5.7) at harvest. BG activity was a predictor of Force at 8 weeks of storage ($p = 0.002$, Fig. 5.7).

3.6 Discussion

Of the 13 genotypes used in this study, most maintained their crisp or noncrisp phenotype over all years they were sampled, but three genotypes behaved differently in certain years. This variation may be due to environmental conditions, such as varying rainfall, temperature, or light intensity (Johnston et al. 2002). Rather than a hindrance, this provides an opportunity to compare a genotype’s protein activities from one year to another to determine if a change in activity might have influenced the genotype’s ability to maintain crispness.

MN1702 is a genotype that maintained its crispness in all years except 2011 (Fig. 3.1). For ‘MN1702’, the noticeable differences between crisp and noncrisp years were overall lower AF and BG activities in 2011, when it lost crispness, compared to 2010, when it maintained crispness. PG activities were also lower in 2011, but were not significantly different from 2010 levels. ‘CD151’ and ‘CC141’ were classified as crisp in 2006 but as noncrisp in 2005. In ‘CC141’, the only observable difference was detectable PG activity in fresh 2005 fruit, when it lost crispness, and no PG activity in 2006 fresh fruit. In ‘CD151’ the most prominent difference in 2005 and 2006 fruit were higher BG and PG activities in 2005 when it lost crispness, compared to 2006. It is possible that the expression of cell wall-modifying proteins would be altered in different ways depending on the mechanism by which the environmental condition caused a change in phenotype. For example, the change in phenotype in 'MN1702' may have been spurred by a combination of weather conditions in Minnesota in 2011, which ranged from a very cool and snowy 2010-2011 winter to above average humidity in July and a first frost date that arrived one to two weeks earlier than the median (Minnesota Climatology Working Group 2012).

AF and BG activity were found to be correlated to each other, but not to PG or expansin activity. AF and BG activities may inherently be correlated due to the fact that some isoforms of BG also have arabinofuranosidase activity, as well as β-galactosidase activity (Yoshioka et al. 1995). PG, AF, BG and expansin expression all appear to be ethylene-dependent (Harb et al. 2012; Wakasa et al. 2006; Wei et al. 2010), though it has been observed that PG expression can also be triggered by cold storage (Tacken et al.

2010). In a study by Ortiz et al. (2011), a loading plot of cell wall modifying enzyme activities in 19 week cold stored apples also indicated a correlation between BG and AF activities but not with PG activity. A lack of correlation between AF and BG to expansin activity may be due to expansin activity being measured only in one year (94 expansin activity samples compared to 279 AF, BG and PG activity samples).

A relationship between exo-PG and crispness maintenance was not found in this study. Instead, PG activity was found to increase after storage, regardless of whether the genotype lost crispness or maintained crispness. Tacken et al. (2010) found that PG mRNA accumulation can be induced in ‘Royal Gala’ apples by a cold storage treatment, even if ethylene expression is suppressed, perhaps explaining the increasing activity trend after storage observed in the present study.

Expansin activity in this study was too variable to determine differences in activity between the different genotypes or between storage treatments. However, for two crisp phenotypes, expansin activities decreased with storage.

‘Honeycrisp’ is a cultivar with the ability to maintain crispness, and has subsequently been used in many apple breeding programs since its release in 1991. Tong et al. (1999) found that arabinose content was maintained in ‘Honeycrisp’ fruit after storage, unlike in cultivars with fruit that softened. Also, AF transcript accumulation in ‘Honeycrisp’ is low compared to ‘Macoun’ (Mann et al. 2008) and ‘McIntosh’ (Harb et al. 2012). In this study, ‘Honeycrisp’ fruit had relatively low AF activity, while those of its crisp progeny had AF activity that ranged from very low to very high (Fig. 3.2a). BG activity in ‘Honeycrisp’ fruit was relatively high, despite Tong et al. (1999) finding that galactose content in ‘Honeycrisp’ fruit did not decrease after storage. In another study, BG activity in ‘Honeycrisp’ fruit increased steadily after harvest, reaching higher levels than in fruit of the softening cultivar ‘McIntosh’ (Harb et al. 2012). ’Honeycrisp’ fruit also had higher BG transcript levels than ‘Macoun’ fruit, which soften, at harvest and after storage (Mann et al. 2008). Possibly, BG may be contributing to a loss of firmness and increased porosity in the cell wall (Brummell and Harpster, 2001) but genotypes are able to maintain crispness as long as their middle lamella remains intact. ‘Honeycrisp’ fruit had low *MdPG1* transcript levels compared to fruit of ‘Macoun’ (Mann et al. 2008) and ‘McIntosh’ (Harb et al. 2012). Moderately low PG activity was also found in ‘Honeycrisp’ fruit in this study.

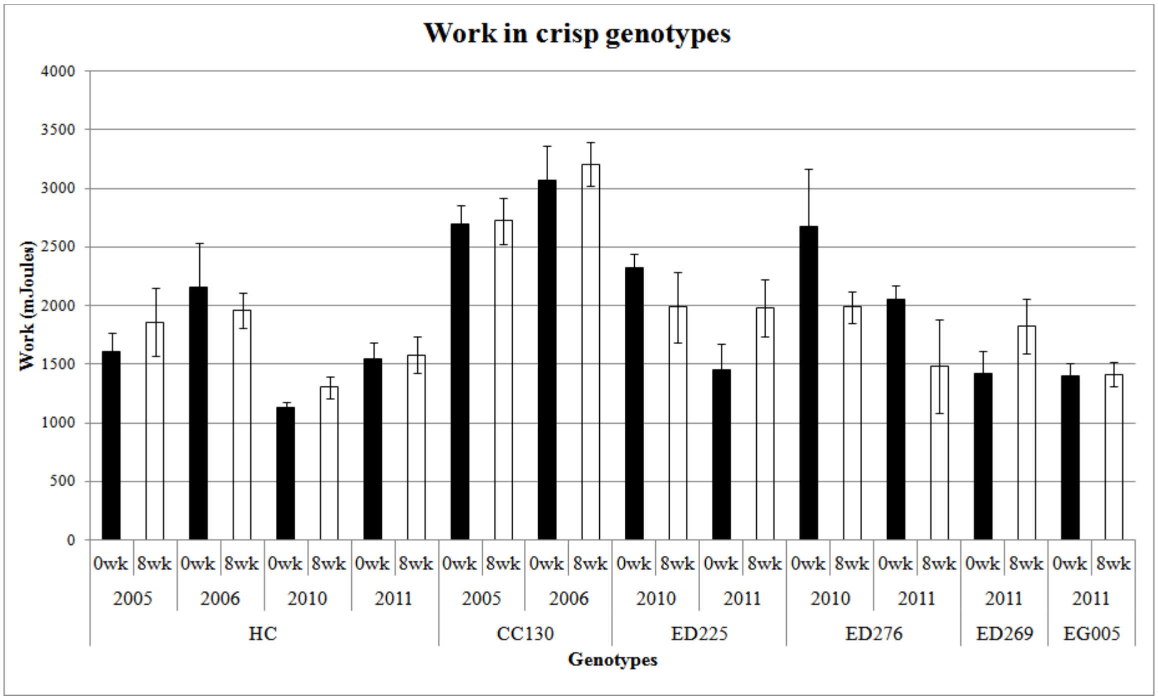
Interestingly, ‘Honeycrisp’ cell wall protein extractions usually yielded much higher protein content than any of the other genotypes in this study (data not shown). ‘Honeycrisp’ fruit might have a unique mechanism of crispness maintenance, in which cell walls with high protein content have highly preserved protein cross-linkages. Depending on the other parental genotype, crisp progeny of ‘Honeycrisp’ may utilize the same or different mechanisms as ‘Honeycrisp’ in maintaining crispness during storage. A future study could investigate whether the integrity of cell wall-associated structural proteins has any relation to crispness maintenance.

Past studies have generally examined cell wall modifying proteins (up to seven) and tried to explain the observed levels in terms of one or two genotypes or cultivars that maintain or lose firmness (Harb et al. 2012; Ortiz et al. 2011; Wei et al. 2010; Goulao et al. 2007). The present study has focused on crispness maintenance rather than firmness, using numerous genotypes and four representative cell wall modifying proteins. This study has shown the importance of incorporating a wider sampling of genotypes for each phenotype of interest, because the conclusions about activity trends that can be drawn from a single genotype generally do not apply to others, even within the same phenotype class. However, even with numerous genotypes, it will be difficult to examine the precise effect of cell wall modifying proteins on crispness maintenance if this is a trait that is determined by numerous small effect proteins or if the genotypes lose crispness by different mechanisms. These data verify that, for the genotypes used in this study, change in AF, BG, PG or expansin activities between harvest and storage was not related to change in Work, which we use to define crispness maintenance.

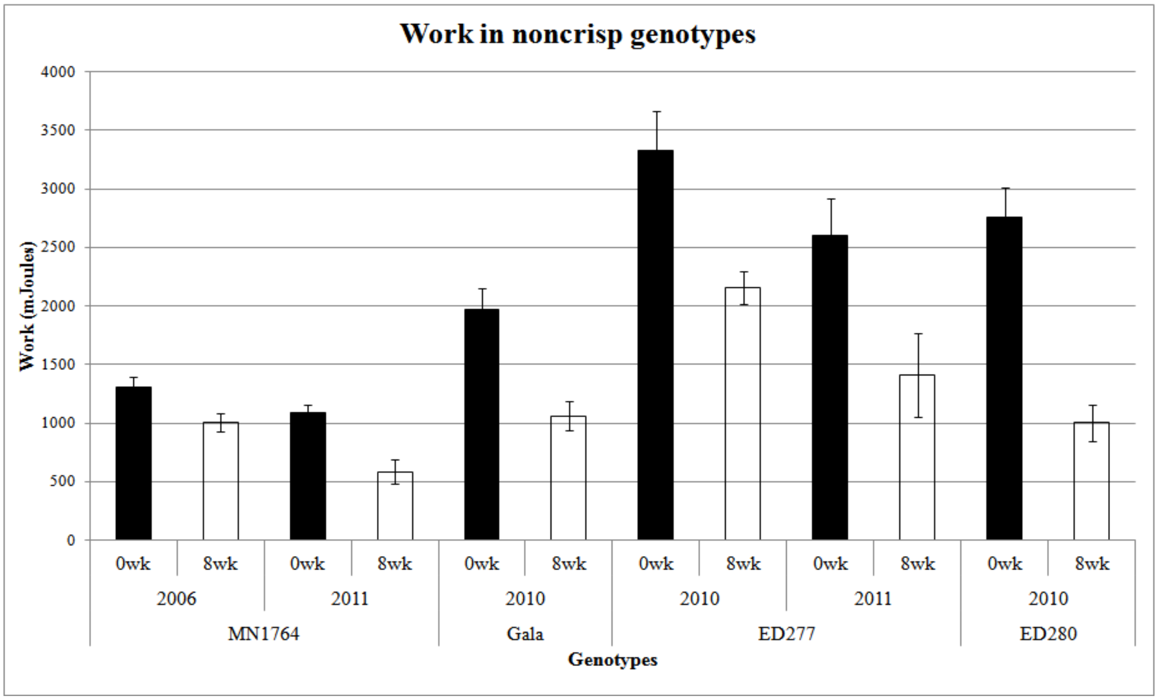
3.7 Acknowledgments

This research was supported by the Minnesota Experiment Station.

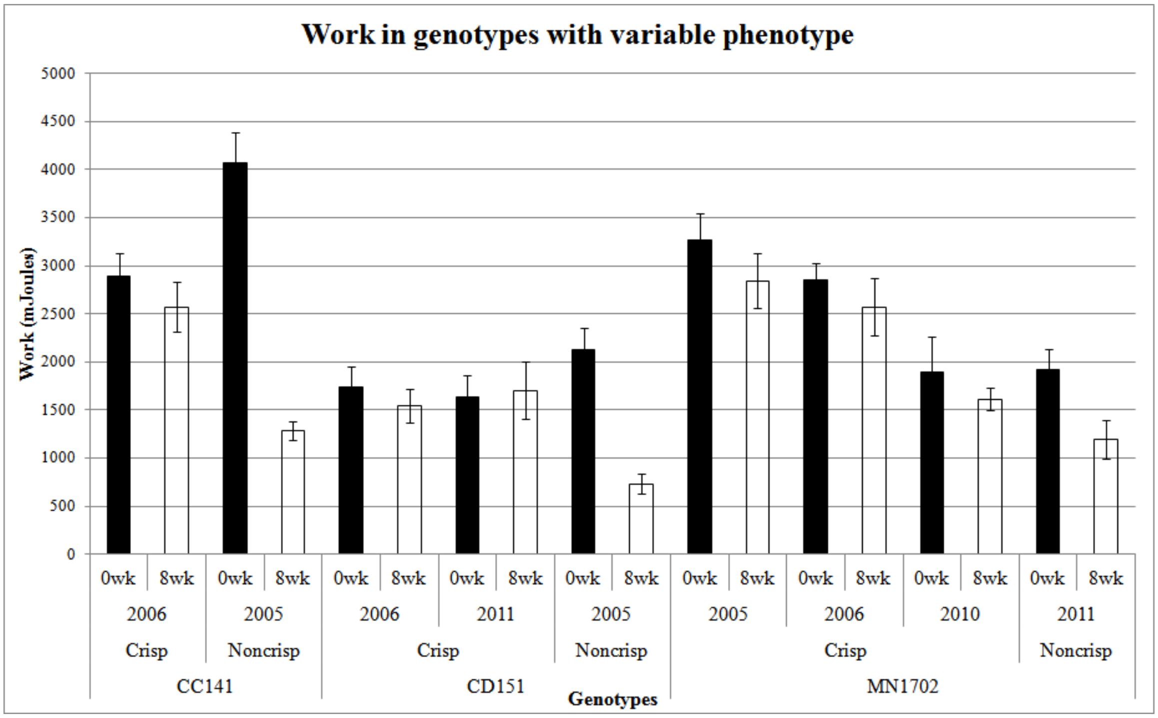
3.8 Figures



3.1a

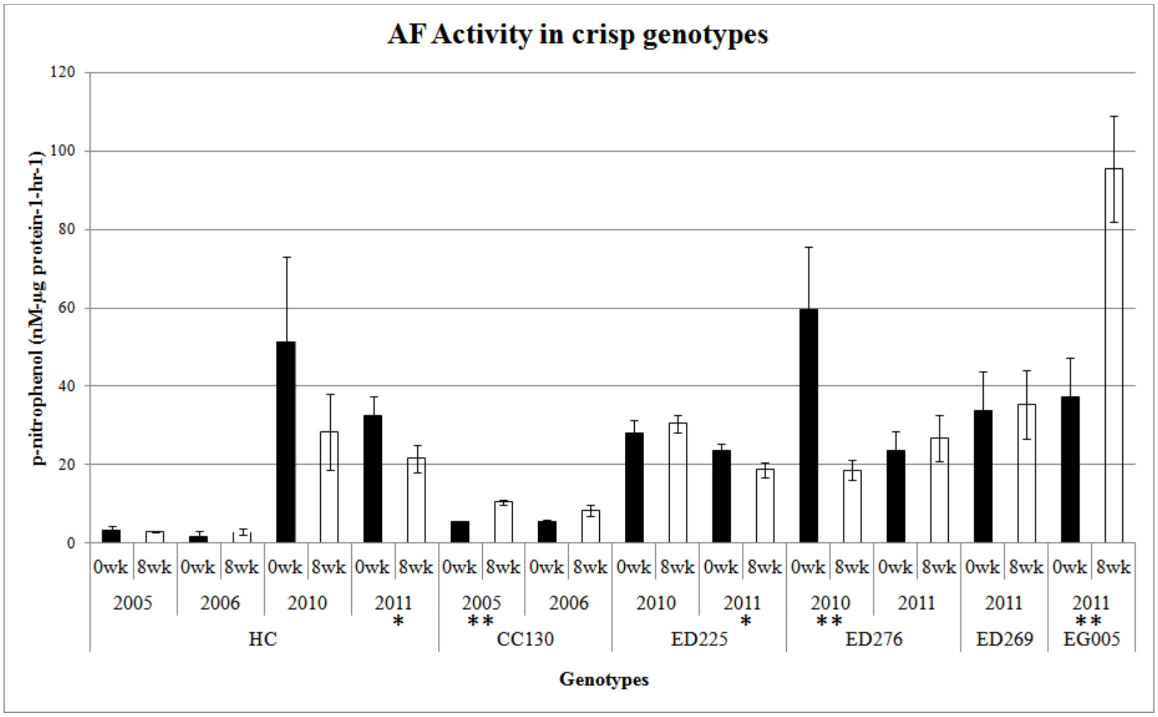


3.1b

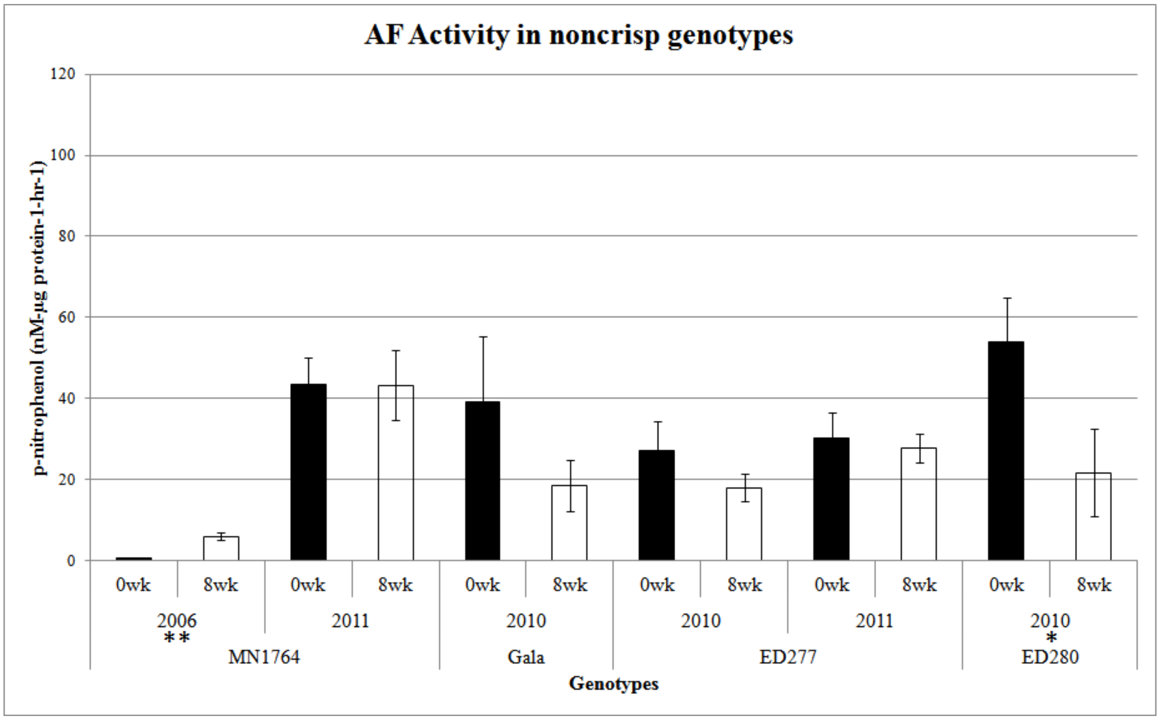


3.1c

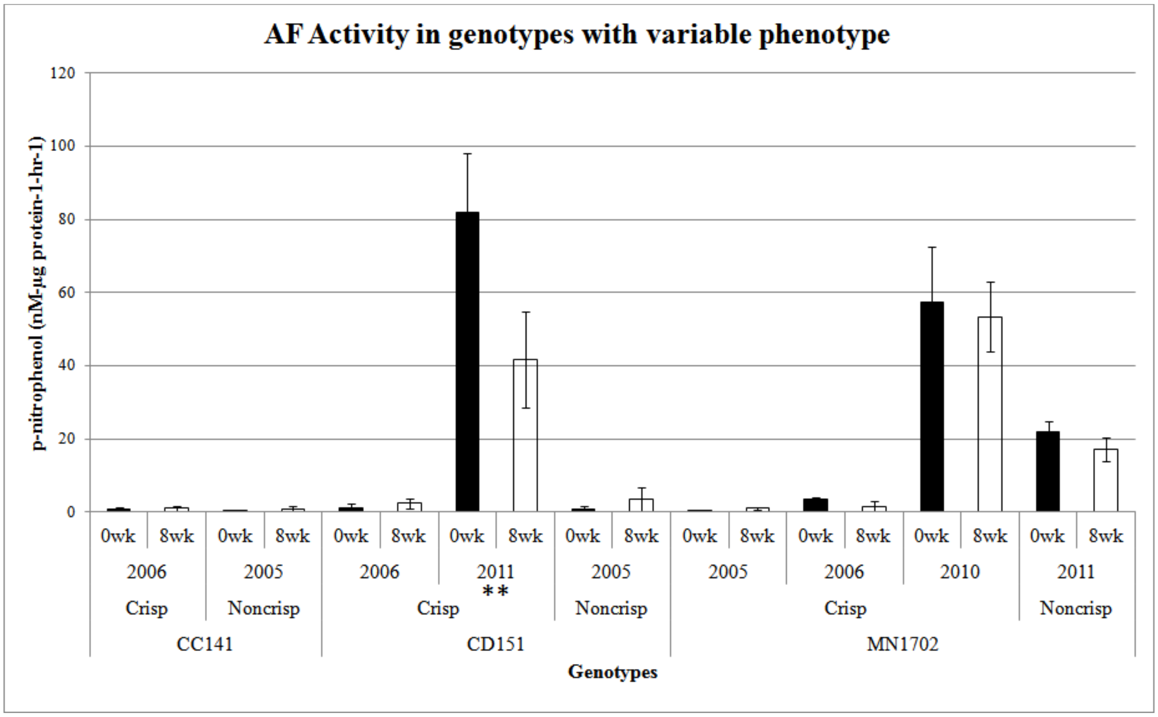
Figure 3.1 Work values for all the genotypes included in this study, as determined by a snapping test. The genotypes were harvested in 2005, 2006, 2010, and 2011 and classified as crisp (a) or noncrisp (b), or had a variable phenotype depending on the year (c). Error bars correspond to Standard Error, determined by a sample of four or more fruit. Apples were processed immediately after harvest (0 wk fruit; black bars) or after a cold storage treatment (8 wk fruit; white bars). 'HC' = Honeycrisp.


AF Activity in crisp genotypes
p-nitrophenol (nM-µg protein-1-hr-1)
120
100
80
60
40
20
0
0wk
8wk
2005
2006
2010
2011
*
**
HC
CC130
ED225
ED276
ED269
EG005
Genotypes

3.2a

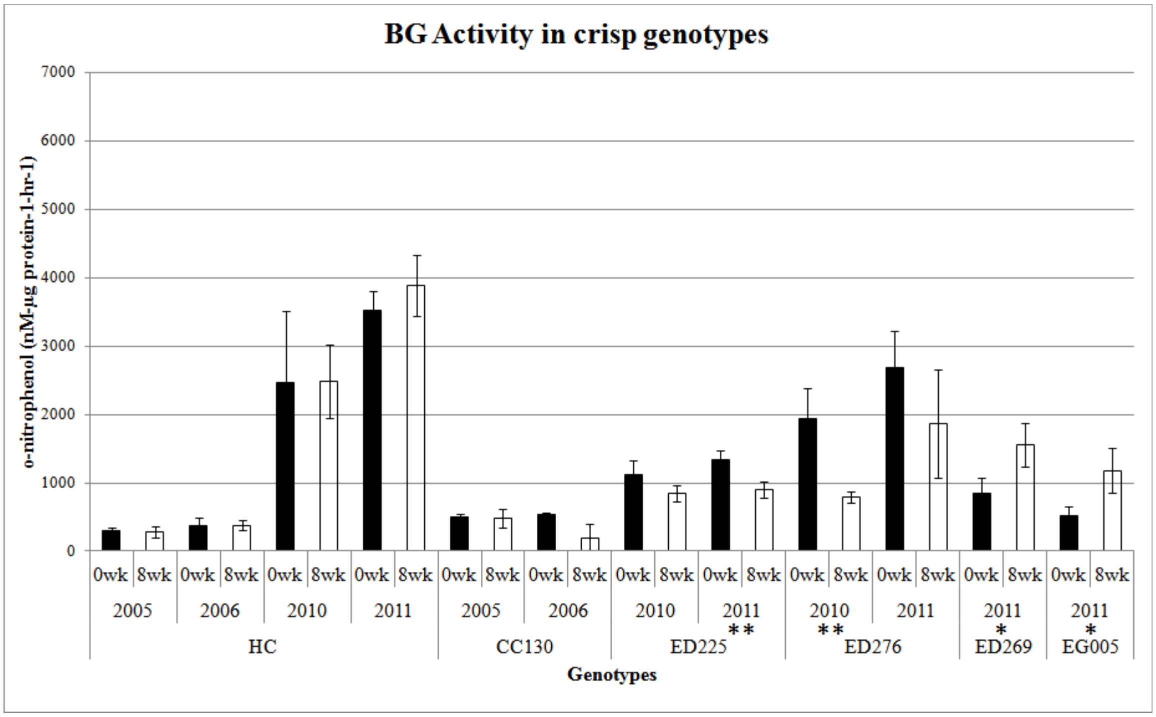

AF Activity in noncrisp genotypes
p-nitrophenol (nM-µg protein-1-hr-1)
120
100
80
60
40
20
0
0wk
8wk
2006
**
2011
2010
*
MN1764
Gala
ED277
ED280
Genotypes

3.2b

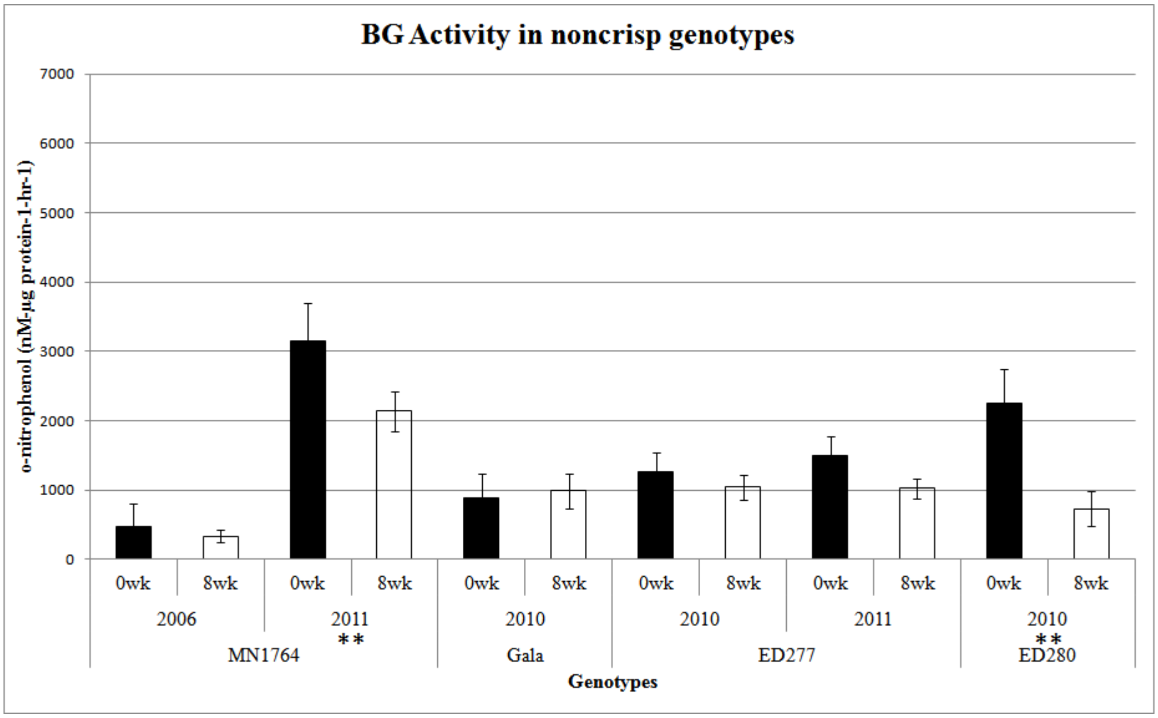


3.2c

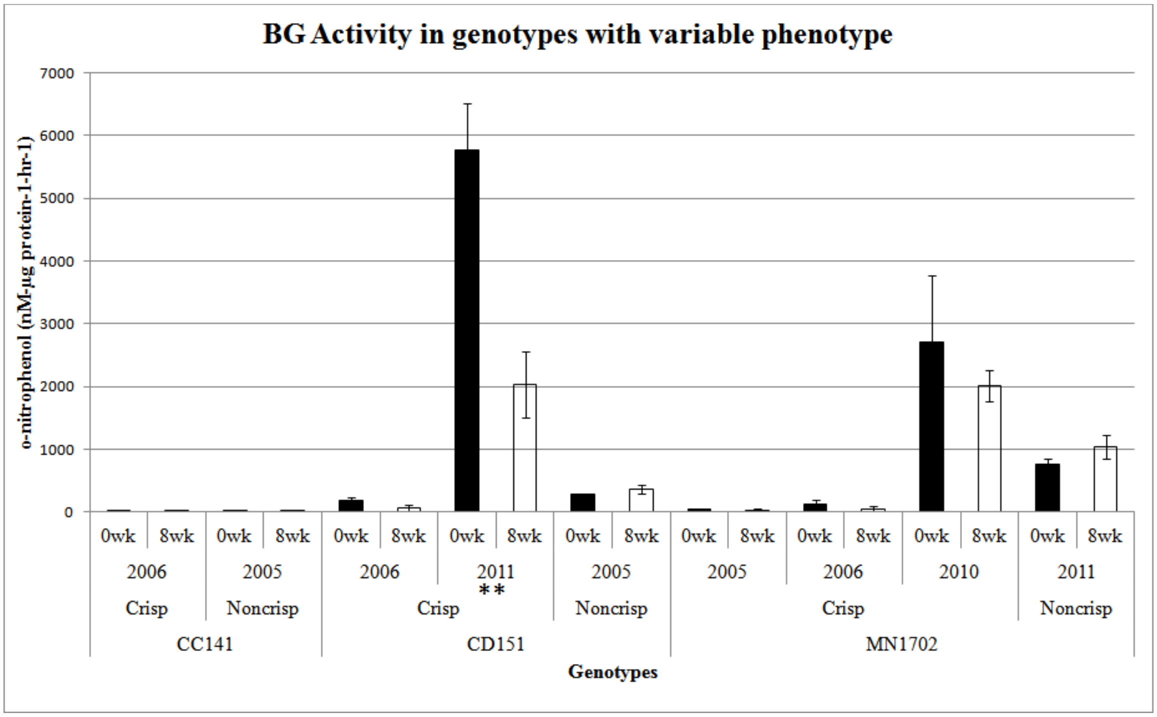
Figure 3.2 Arabinofuranosidase (AF) activity as determined by nM of p-nitrophenol production (nM released - µg protein-1- hour-1) in crisp genotypes (a), noncrisp genotypes (b), and genotypes with varying phenotype (c). Standard Error bars represent two samples for the 2005-2006 data, and five or more samples for 2010-2011. The symbols under each year indicate significant changes in activities after storage, based on two-tailed t-tests, with '*' indicates $p < 0.10$, '**' indicates $p < 0.05$. 'HC' = Honeycrisp.


BG Activity in crisp genotypes
o-nitrophenol (nM-µg protein-1-hr-1)
7000
6000
5000
4000
3000
2000
1000
0
0wk
8wk
2005
2006
2010
2011
HC
CC130
ED225
ED276
ED269
EG005
**
*
Genotypes

3.3a

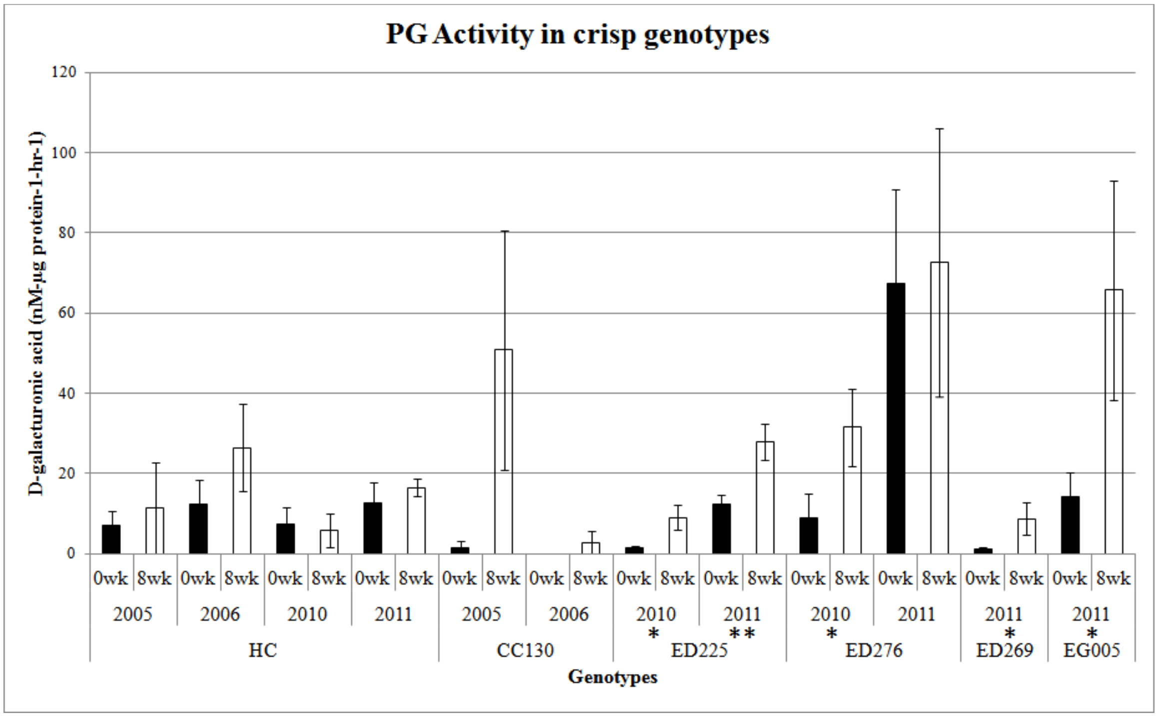

BG Activity in noncrisp genotypes
o-nitrophenol (nM-µg protein-1-hr-1)
7000
6000
5000
4000
3000
2000
1000
0
0wk
8wk
2006
2011
2010
MN1764
Gala
ED277
ED280
**
Genotypes

3.3b

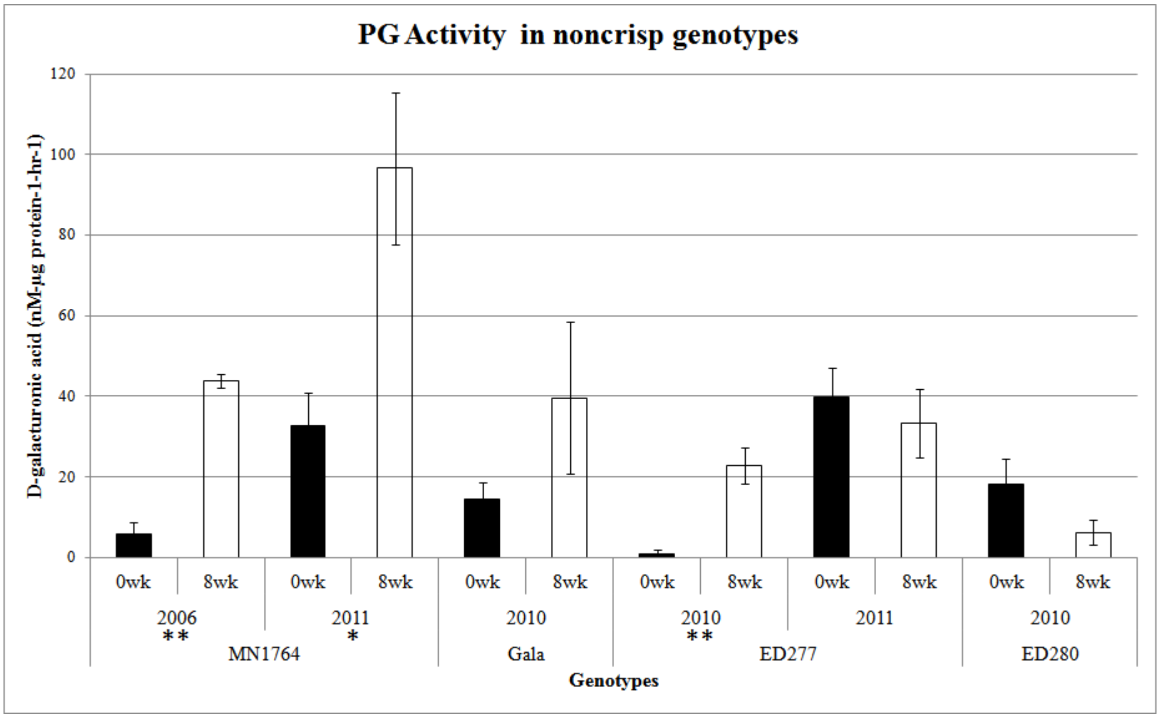


3.3c

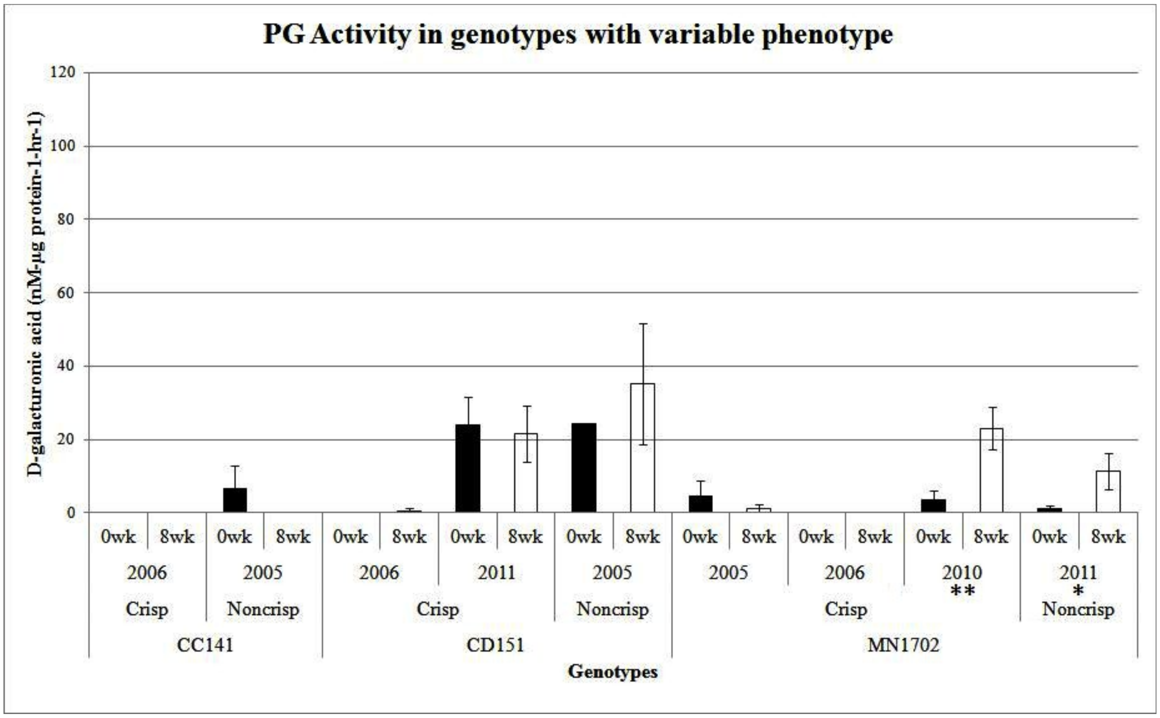
Figure 3.3 β-galactosidase (BG) activity as determined by nM of o-nitrophenol production (nM released - µg protein-1- hour-1) in crisp genotypes (a), noncrisp genotypes (b), and genotypes with varying phenotype (c). Standard Error bars represent two samples for the 2005-2006 data, and five or more samples for 2010-2011. The symbols under each year indicate significant changes in activities after storage based on two-tailed t-tests, with '*' indicates $p < 0.10$, '**' indicates $p < 0.05$. 'HC' = Honeycrisp.


PG Activity in crisp genotypes
D-galacturonic acid (nM-µg protein-1-hr-1)
120
100
80
60
40
20
0
0wk
8wk
2005
2006
2010
2011
HC
CC130
*
**
ED225
ED276
ED269
EG005
Genotypes

3.4a


PG Activity in noncrisp genotypes
D-galacturonic acid (nM-µg protein-1-hr-1)
120
100
80
60
40
20
0
0wk
8wk
2006
2011
2010
**
*
MN1764
Gala
ED277
ED280
Genotypes

3.4b



3.4c

Figure 3.4 Exo-polygalacturonase (PG) activity as determined by nM of D-galacturonic acid production (nM released - µg protein-1- hour-1) in crisp genotypes (a), noncrisp genotypes (b), and genotypes with varying phenotype (c). Standard Error bars represent two samples for the 2005-2006 data, and five or more samples for 2010-2011. The symbols under each year indicate significant changes in activities after storage based on two-tailed t-tests , with '*' indicates $p < 0.10$, '**' indicates $p < 0.05$. 'HC' = Honeycrisp.

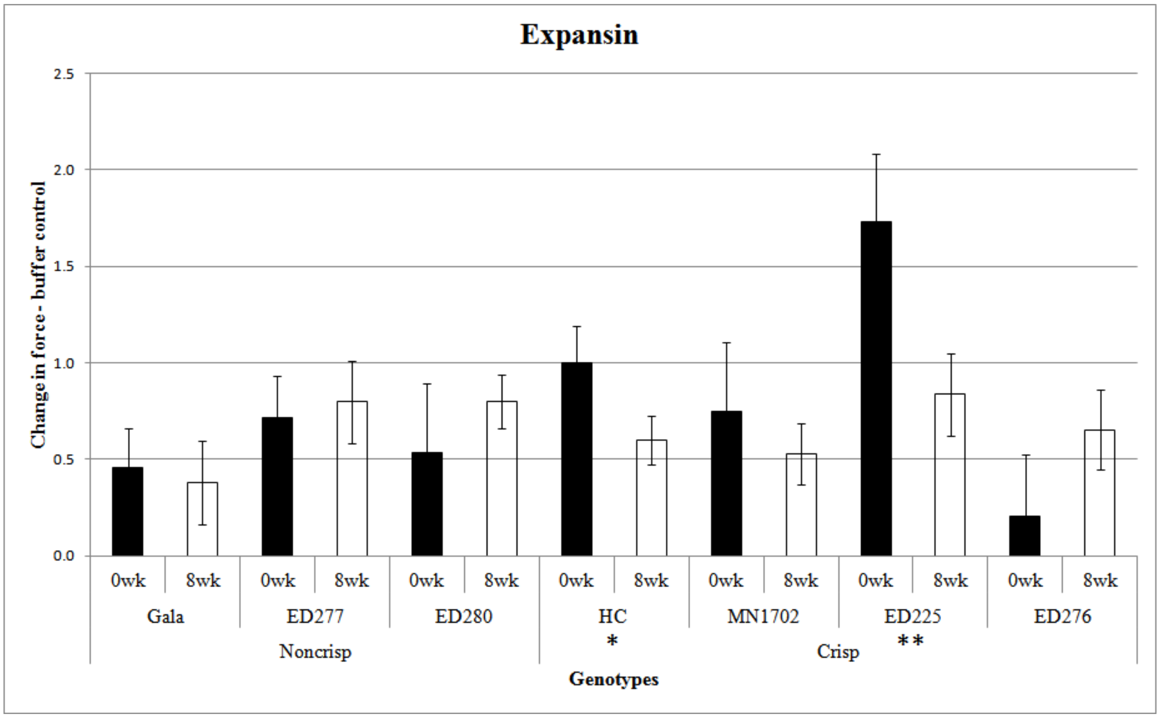


Figure 3.5 Expansin activity measured in 2010, as determined by change in force at the end of the assay, minus the force of a buffer negative control. Standard Error bars represent five or more samples. Symbols under each year indicate significant changes in activities after storage based on two-tailed t-tests, with '*' indicates $p < 0.10$, '**' indicates $p < 0.05$. 'HC' = Honeycrisp.

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5. Appendix

5.1 Tables

5.1.1 Texture of apple genotypes used in this study

Table 5.1 Analysis of variance (ANOVA) table for **Work** measured in fresh or stored fruit over four years in 13 different genotypes. df = degrees of freedom. '***' indicates $p < 0.001$.

Source	df	F value	P value	
Year (Y)	3	23.579	6.95E-14	***
Storage (S)	1	45.519	6.55E-11	***
Genotype (G)	12	22.361	< 2e-16	***
Y x S	3	12.684	7.07E-08	***
Y x G	11	4.09	1.18E-05	***
S x G	12	5.497	1.40E-08	***
Y x S x G	11	3.171	0.000415	***

5.1.2 Relationship of protein activities to storage and phenotype

Table 5.2. Analysis of variance (ANOVA) table associated with the linear mixed effects model for **AF**, with Genotype/Year/Storage as a random effect. df = degrees of freedom; D df = denominator degrees of freedom

Source	df	D df	F value	P value
2005-2006				
Phenotype	1	6	0.275186	0.6187
Storage	1	10	5.413823	0.0423 *
Year	1	2	0.000444	0.9851
2010-2011				
Phenotype	1	10	1.99772	0.1879
Storage	1	14	5.74737	0.031 *
Year	1	3	2.5118	0.2112
Storage x Year	1	14	3.13376	0.0985 .

Table 5.3. Analysis of variance (ANOVA) table associated with the linear mixed effects model for **BG**, with Genotype/Year/Storage as a random effect. df = degrees of freedom; D df = denominator degrees of freedom

Source	df	D df	F value	P value
2005-2006				
Phenotype	1	6	0.173083	0.6919
Storage	1	10	2.652138	0.1345
Year	1	2	0.019637	0.9014
2010-2011				
Phenotype	1	10	0.812086	0.3887
Storage	1	15	3.976056	0.0647 .
Year	1	3	2.428251	0.2171

Table 5.4. Analysis of variance (ANOVA) table associated with the linear mixed effects model for **PG**, with Genotype/Year/Storage as a random effect. df = degrees of freedom; D df = denominator degrees of freedom

Source	df	D df	F value	P value
2005-2006				
Phenotype	1	6	1.636739	0.248
Storage	1	10	3.481975	0.0916 .
Year	1	2	0.293135	0.6425
2010-2011				
Phenotype	1	10	0.619012	0.4497
Storage	1	15	9.164885	0.0085 **
Year	1	3	7.719276	0.0691 .

Table 5.5 Analysis of variance (ANOVA) table associated with the linear mixed effects model for **Expansin**, with Genotype/Storage as a random effect. df = degrees of freedom; D df = denominator degrees of freedom

Source	df	D df	F value	P value
Phenotype (P)	1	5	0.49095	0.5148
Storage (S)	1	5	1.06352	0.3497
P x S	1	5	1.54666	0.2688

5.1.3 Relationship between instrumental measurements and cell wall modifying proteins

Table 5.6 Analysis of variance (ANOVA) table associated with the linear mixed effects model for **Work**, with Genotype/Year/Storage as a random effect. df = degrees of freedom; D df = denominator degrees of freedom; '.' indicates p < 0.10; '*' indicates p < 0.05.

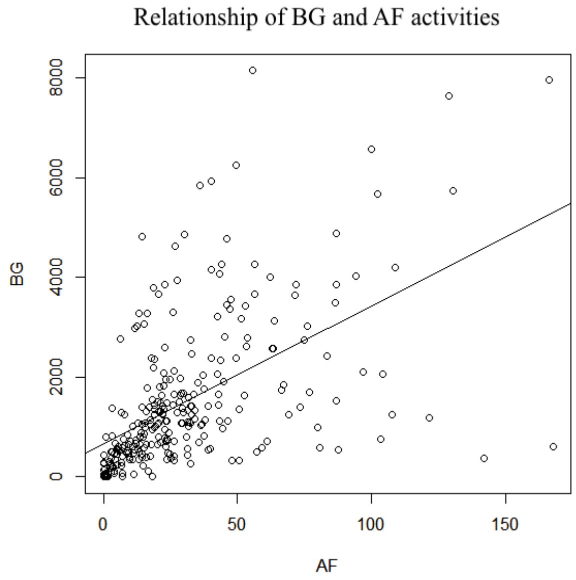
Source	df	D df	F value	P value
All data				
AF	1	196	0.37376	0.5417
BG	1	196	0.0985	0.754
PG	1	196	0.92099	0.3384
Storage	1	15	6.09109	0.0261 *
Year	1	3	2.11507	0.2418
0 Week				
AF	1	90	1.33775	0.2505
BG	1	90	0.20304	0.6534
PG	1	90	0.26308	0.6093
Year	1	3	7.40366	0.0725
8 Week				
AF	1	103	0.00563	0.9403
BG	1	103	0.19449	0.6601
PG	1	103	3.02993	0.0847 .
Year	1	3	0.34129	0.6001

Table 5.7 Analysis of variance (ANOVA) table associated with the linear mixed effects model for **Force**, with Genotype/Year/Storage as a random effect. df = degrees of freedom; D df = denominator degrees of freedom; '**' indicates $p < 0.01$.

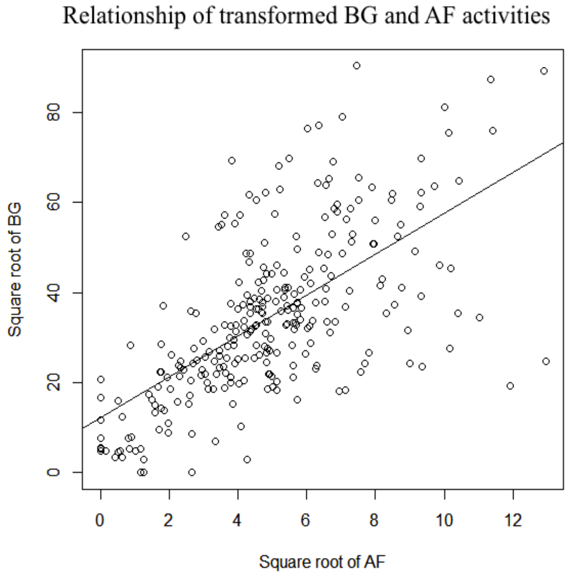
Source	df	D df	F value	P value
All data				
AF	1	196	1.44258	0.2312
BG	1	196	0.19343	0.6606
PG	1	196	0.02047	0.8864
Storage	1	15	12.58337	0.0029 **
Year	1	3	4.57366	0.122
0 Week				
AF	1	90	2.90541	0.0917
BG	1	90	0.58311	0.4471
PG	1	90	0.30263	0.5836
Year	1	3	4.53477	0.1231
8 Week				
BG	1	103	10.21169	0.0019 **
PG	1	103	0.03185	0.8587
AF	1	103	0.11039	0.7404
Year	1	3	1.38978	0.3234

5.2 Figures

5.2.1 Relationship between BG and AF activities



5.1a



5.1b

Figure 5.1 Relationship between BG and AF activity (a) and between their square root transformations (b).

5.2.2 Relationship between instrumental measurements and cell wall modifying proteins

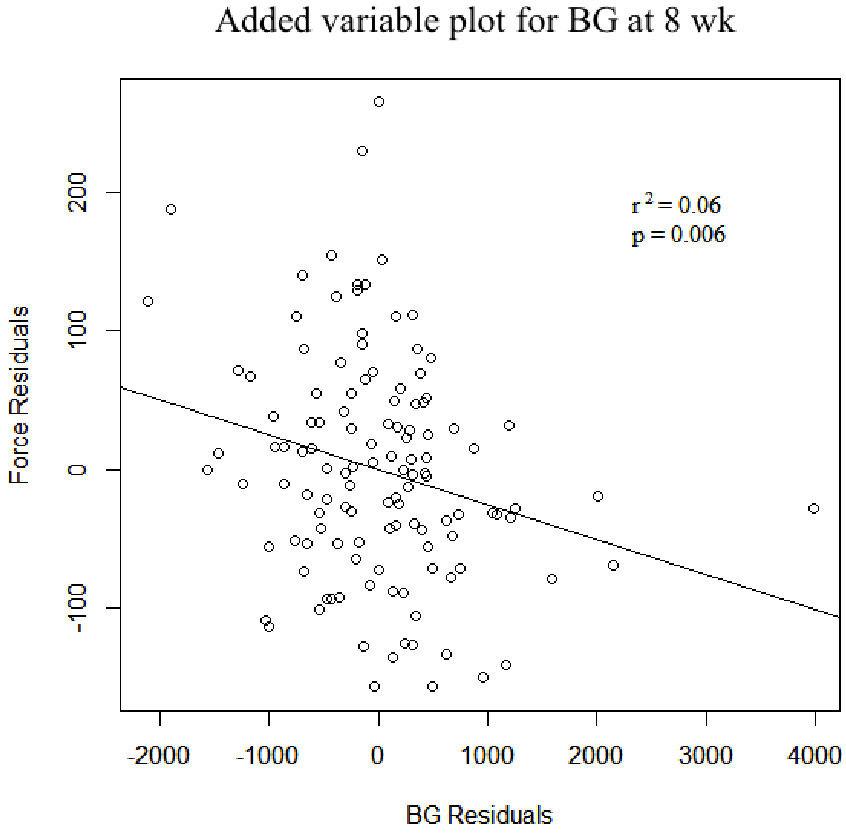


Figure 5.2 Added variable plot showing the unique relationship of BG to Force at 8 weeks, after the effects of AF, PG, Genotype and Year were removed.