Differential Expression of Cell-wall–Modifying Genes and Novel cDNAs in Apple Fruit During Storage

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ABSTRACT. During postharvest storage, apple [*Malus pumila* P. Mill.] fruit softens and its texture changes noticeably, with adverse effects on fruit quality. These changes are a result of degradation of the cell wall and middle lamella. Enzymes that cause changes in the cell walls have been characterized, but temporal distribution of their activities and their molecular regulation during storage is not well understood. ‘Honeycrisp’ fruit does not soften significantly during storage in contrast to fruit from ‘Macoun’, which softens significantly during storage. Contrasting phenotypes of ‘Honeycrisp’ and ‘Macoun’ were analyzed for changes in transcript levels of four cell-wall–modifying genes in fresh and 3-month-stored fruit from both cultivars. A suppression-subtractive hybridization experiment identified 15 cDNAs differentially expressed in fresh or 3-month-stored ‘Macoun’ fruit. Transcript levels of these 15 cDNAs were further quantified by quantitative real-time polymerase chain reaction (qRT-PCR) in fresh and 3-month-stored fruit from both ‘Macoun’ and ‘Honeycrisp’. The combination of a late increase in *MdEXPA2* and decreased levels of *MdPG* and *MdAFase1* transcript levels in ‘Honeycrisp’ fruit during storage may lead to its nonsoftening phenotype. Three cDNAs, potentially important for postharvest changes in apple fruit were also identified based on their different expression patterns in fresh and 3-month-stored ‘Macoun’ and ‘Honeycrisp’ fruit.

Apple fruit undergoes three stages of postharvest fruit softening: an initial slow softening phase, followed by a rapid softening phase, and a final slow softening phase, thus adversely affecting fruit quality (Johnston et al., 2001; Knee, 1975). At the cell-wall level, fruit softening is a result of changes in interactions among cell-wall polysaccharides and their breakdown (Knee, 1975). These changes in cell walls result from the activities of cell-wall–modifying enzymes. Ripening-specific or postharvest softening-specific genes for various cell-wall–modifying enzymes have been characterized or cloned from apple, but data on how transcript levels of these genes change during storage is lacking.

β -Galactosidase increases wall porosity and enables other cell-wall–modifying enzymes to access cell-wall polysaccharides (Brummell and Harpster, 2001). It is known that the initial slow softening phase of apple fruit softening is marked by the loss of galactose (Knee, 1975; Peña and Carpita, 2004) and an increased transcript accumulation of the fruit ripening-specific β -galactosidase gene, *PABG1* (Ross et al., 1994).

Xyloglucan *endo*-transglycosylase/hydrolase (XET/H) can break down hemicelluloses of the cell wall (Rose and Bennett, 1999). XET/H activity increased in ripe apple fruit and appeared to be positively correlated with the initial slow softening phase of postharvest apple fruit softening (Johnston et al., 2002; Percy et al., 1996). Data on transcript accumulation of the XET/H gene during postharvest storage are lacking.

Arabinose loss precedes the rapid softening phase during storage (Peña and Carpita, 2004; Tong et al., 1999). Arabinose is removed from pectic polysaccharides by arabinofuranosidase. A gene for arabinofuranosidase, *MdAFase1*, has been cloned from overripe ‘Royal Gala’ apple fruit (National Center for Biotechnology Information accession number AY309436).

Expansins are enzymes that allow loosening of the cell wall by breaking hydrogen bonds between cellulose and hemicellulose, making cell-wall polysaccharides accessible to the other cell-wall–degrading enzymes (Brummell et al., 1999; McQueen-Mason and Cosgrove, 1994). mRNA for *MdEXPA2*, an apple expansin gene, is detected primarily after harvest and for up to 12 d of storage in ‘Golden Delicious’ fruit (Wakasa et al., 2003).

Cell-wall polysaccharides exposed by expansin are further acted upon by *endo*-polygalacturonase, an enzyme that breaks down pectic polysaccharides of the cell wall and the middle lamella (Brummell et al., 1999). The loss of polygalacturonic acid, a pectic polysaccharide, is correlated with the rapid softening phase in apple fruit (Knee, 1975). *MdPG* is an apple fruit ripening specific gene for *endo*-polygalacturonase (Atkinson, 1994; Wu et al., 1993).

The extent of fruit softening varies with genotype (Tong et al., 1999). A comparative study of four different apple cultivars by Tong et al. (1999) established that ‘Honeycrisp’ fruit maintains its texture during storage for 6 months at low temperature, compared with ‘Macoun’ fruit, which is significantly less crisp and less firm after 6 months of storage. Transmission electron micrographs showed little or no cell-wall degradation in ‘Honeycrisp’ after 6 months of storage, whereas the cell walls of ‘Macoun’ fruit had completely deteriorated in that time period. In addition, ‘Honeycrisp’ fruit did not lose uronide or arabinose when compared with ‘Macoun’ fruit after 6 months of storage. An exploration of

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the molecular bases of the above-mentioned phenotypic differences between fruit from 'Honeycrisp' and 'Macoun' could provide us with candidate genes that might regulate or cause postharvest fruit softening in apple.

The pattern of changes in transcript levels of a set of known cell-wall-modifying genes was studied for fresh and 3-month-stored fruit from 'Honeycrisp' and 'Macoun' apples. In an attempt to identify additional genes that might have a role in regulation of postharvest fruit softening in apple, a suppression-subtractive hybridization and cDNA array technique was used. Messenger RNA transcripts with differential accumulation in fresh or 3-month-stored 'Macoun' apple fruit tissue were identified. Differentially accumulated transcripts were further quantified with qRT-PCR from fresh and 3-month-stored fruit from both 'Macoun' and 'Honeycrisp'. Results and discussions of these experiments are presented below.

Materials and Methods

FRUIT HARVEST AND STORAGE. 'Macoun' and 'Honeycrisp' fruit from the Horticultural Research Center (HRC) in Chanhassen, MN, were harvested in Fall 1999 from five or more trees, when the threshold starch index was between 5 and 6 (Blanpied and Silsby, 1992). In Fall 2005, 'Honeycrisp' fruit were also harvested from the HRC in the same way as in 1999, but 'Macoun' fruit were obtained from a commercial orchard and harvested at commercial maturity. Freshly harvested fruit to be used for RNA extractions were peeled, cored, quartered, frozen in liquid nitrogen, and kept at -80°C . Stored fruit were held for 3 months at $0 \pm 0.5^{\circ}\text{C}$ and 95%–99% relative humidity. After the requisite storage period, fruit for RNA extractions were prepared and stored at -80°C as for freshly harvested fruit.

EXPERIMENTAL DESIGN. Fruit from 1999 were used for suppression-subtractive hybridization and qRT-PCR experiments. Fruit from 2005 were used to measure textural changes. For the suppression-subtractive hybridization experiment, total RNA from multiple extractions were pooled for each storage treatment. For qRT-PCR experiments, two independent total RNA samples were prepared for each storage treatment. Each independent RNA sample represented two to four separate RNA extractions. Relative transcript levels were measured in duplicate for each independent RNA sample. Analysis of variance (ANOVA) on qPCR data were performed using SPSS statistical software (SPSS, Chicago). Student's *t* test was used to establish significant differences in transcript accumulation between fresh and stored fruit.

RNA EXTRACTION AND cDNA SYNTHESIS. RNA was extracted from 20-g batches of tissue using a modified Gomez extraction method (Lopez-Gomez and Gomez-Lim, 1992) and then purified using the RNeasy RNA clean-up procedure with DNase digestion (Qiagen, Valencia, CA). The quantity and quality of RNA was measured using a spectrophotometer. For the suppression-subtractive hybridization experiment, mRNA was first converted from total RNA using the Oligotex Direct mRNA Mini Kit (Qiagen) and then reverse-transcribed into cDNA using the Clontech PCR-Select cDNA Subtraction kit (Clontech Laboratories, Palo Alto, CA). For qRT-PCR analysis, total RNA was reverse-transcribed into cDNA using the SuperScript (Invitrogen Corp., Carlsbad, CA) reverse transcriptase kit.

SUPPRESSION-SUBTRACTION HYBRIDIZATION. The two cDNA populations from fresh and stored fruit were reverse- and forward-subtracted according to the Clontech PCR-Select

cDNA Subtraction kit directions. The subtraction procedure amplifies transcripts that are more abundant in one treatment compared with the other and vice-versa and reduces transcripts that are equally abundant in both the treatments via hybridization. In this study, transcripts that were more abundant in stored fruit were designated as the forward-subtracted cDNA population, while transcripts that were more abundant in fresh fruit were designated as the reverse-subtracted cDNA population. Pools of subtracted cDNAs were cloned into the pST Blue1 cloning vector (Novagen, EMD Chemicals, San Diego, CA) for differential screening (Clontech). Reverse- and forward-subtracted cDNA clones were PCR-amplified directly from bacterial cultures using nested primers provided with the kit. PCR product bands were visible for 64 forward-subtracted clones and 92 reverse-subtracted clones, and these were further analyzed using a cDNA array.

One microliter of PCR product for each positive clone was spotted in duplicate on eight nylon membranes, with four blots each for the forward- and reverse-subtracted clones. DNA was fixed to the blots by baking membranes for 1 h at 80°C . Membranes were hybridized with ^{32}P -labeled probes according to the Clontech PCR-Select differential screening procedure. Four ^{32}P -labeled probes corresponding to the forward-subtracted cDNA pool, reverse-subtracted cDNA pool, the unsubtracted tester cDNA (total stored fruit cDNA), and the unsubtracted driver cDNA (total fresh fruit cDNA) were hybridized to the nylon membranes. A Clontech data interpretation key was used to identify cDNAs that were differentially expressed. Differentially expressed cDNAs were sequenced by the Advanced Genomics Analysis Center at the University of Minnesota. BLASTN and BLASTX sequence similarity search algorithms were used to find significant sequence similarities to annotated genes in online genomic databases.

QUANTITATIVE REAL-TIME PCR (qRT-PCR). Using cDNA as template, relative transcript levels of genes for four cell-wall-modifying enzymes, polygalacturonase (*MdPG*, GenBank accession no. L27743), expansin (*MdEXPA2*, AB099927), β -galactosidase (*pABG1*, L29451) and arabinofuranosidase (*MdAFase1*, AY309436), and select cDNAs from the subtractive hybridization experiment were measured using a LightCycler quantitative PCR machine (Roche Applied Science, Indianapolis, IN). Transcripts amplified by sequence-specific primers were detected using Platinum SYBR Green qPCR SuperMix-UDG (Promega Corp., Madison, WI). Transcript levels for apple β -actin were empirically established to be equally abundant in fresh and 3-month-stored fruit of both 'Honeycrisp' and 'Macoun' and were used to normalize the data. Data are presented as transcript concentration relative to that of β -actin for each cultivar. Quantification was based on external standard curves generated with PCR amplification of known amounts of cloned insert DNA or PCR-amplified DNA homologous to each gene or cDNA.

Two micrograms of DNase-free total RNA was reverse-transcribed into cDNA in a 20- μL reaction volume using random hexamers and SuperScript Reverse Transcriptase (Invitrogen). Two microliters of cDNA was used as template for each qRT-PCR reaction in a total volume of 20 μL with 10- μL Platinum SYBR Green qPCR SuperMix-UDG, 0.2 μL of 1 mg·mL⁻¹ bovine serum albumin, 5.8 μL of water, and 400–500 nM each of forward and reverse primers. The PCR program consisted of initial denaturation at 95°C for 2 min followed by 45 cycles of denaturation for 5 s at 94°C , annealing for 15 s at

55 °C, and elongation for 20 s at 68 °C. Fluorescence was measured at 68 °C. Each qRT-PCR reaction was run in duplicate, and each gene was tested twice using cDNA made from independently extracted RNA. Average results from reactions with single melting peaks were reported. Table 1 describes primer sets used for these analyses.

TEXTURE ANALYSIS. A TA.XT2 texture analyzer (Texture Technologies, Scarsdale, NY) was used to measure the work required to break cylindrical apple tissue samples as described in Mann et al. (2005). Work was measured on 10 fruit from each cultivar at week 0 (fresh fruit) and after 12 weeks of storage (3-month-stored fruit).

Results

FRUIT TEXTURE. As previously reported (Mann et al., 2005; Tong et al., 1999), ‘Honeycrisp’ fruit was able to maintain its crisp and firm texture during 3 months of storage, whereas ‘Macoun’ fruit was unable to maintain its texture during storage. Figure 1 illustrates the work required to break cylinders of apple fruit mesocarp. We have previously shown that change in work with storage time is able to predict change in crispness with $r^2 = 0.76$ (Mann et al., 2005). Work changed significantly during storage for ‘Macoun’ but not for ‘Honeycrisp’, suggesting that crispness changed significantly in stored ‘Macoun’ but not in stored ‘Honeycrisp’.

EXPRESSION ANALYSES: CELL-WALL-MODIFYING GENES. ANOVA indicated that genotype and storage time, but not

experiment, had major effects on gene expression. Consequently, results from replicate qRT-PCR experiments were combined within genotype and storage time for mean separation analyses. None of the cell-wall genes showed significant differential transcript accumulation between fresh and 3-month-stored ‘Macoun’ fruit (Fig. 2A). However, in ‘Honeycrisp’ fruit, *MdPG* and *MdAFase1* transcripts accumulated at higher abundance in fresh fruit than in 3-month-stored fruit, whereas the transcript for *MdEXPA2* accumulated at a higher abundance in stored ‘Honeycrisp’ fruit when compared with fresh fruit (Fig. 2B).

SUPPRESSION-SUBSTRUCTIVE HYBRIDIZATION. The suppression-subtractive hybridization screen identified 156 subtracted clones, out of which 64 clones were forward-subtracted and 92 clones were reverse-subtracted. All subtracted clones were analyzed using cDNA arrays. Analysis of cDNA array data identified 41 clones that showed differential transcript accumulation in either fresh or 3-month-stored ‘Macoun’ fruit tissue. BLASTN or BLASTX analyses of these 41 cDNA clones revealed that 68% ($n = 28$) of these cDNAs shared a similarity with ribosomal or virus genes and were discarded, and the remaining 32% ($n = 13$) were further tested to confirm differential mRNA transcript accumulation using qRT-PCR. Of the latter 13 cDNAs, five cDNAs remained undetected by the qRT-PCR system. Sequence-similarity data from the remaining eight sequences are presented in Table 2.

Clone DNAs labeled 220 and 124B showed statistically significant differential mRNA transcript accumulation in either fresh or 3-month-stored ‘Macoun’ fruit (Fig. 3A). cDNA 220

Table 1. Primer sets used for quantitative real-time polymerase chain reaction analysis of apple cell-wall-modifying genes and cDNAs from the suppression-subtractive hybridization experiment.

Gene/cDNA	Primer	Sequence	Accession no. (nucleotide range)
<i>MdEXPA2</i>	MdEXPA2F	5' GAGCAACTCTTACCTCAACG 3'	AB099927 (531–823) ^z
	MdEXPA2R	5' GCGGGTGCCAGTTAATTAG 3'	
<i>MdPG</i>	MdPGF	5' GGAATTGATCAGGCCAAGAA 3'	L27743 (442–630) ^z
	MdPGR	5' GCTCCACTGGAAGAACAAGC 3'	
<i>pABG1</i>	pABG1F	5' CATTGCTTTTGAGCGTTTCA 3'	L29451 (141–417) ^z
	pABG1R	5' ATGGCCATTCCAAAACACAT 3'	
<i>MdAFase1</i>	MdAFase1F	5' TCACTGACAGGCTCAGATGG 3'	AY309436 (514–702) ^z
	MdAFase1R	5' GGGAATGGCTGACACTTGAT 3'	
<i>β-Actin</i>	MdACTF	5' ACTCGAGGACGGGGTAGATT 3'	CTG1059321 (1359–1493) ^y
	MdACTR	5' CAAAGATTAGGCAAGGCGAG 3'	
166	166F	5' GCAGTGGAGCTACTTCGACC 3'	46815940 ^x
	166R	5' CAAGTCGTCATCCAAGCAGA 3'	
183	183F	5' GGTACAATGTAGGAGAGCTTCG 3'	46815941 ^x
	183R	5' ATGGCAATCAATAAGGTCGG 3'	
198	1198F	5' TTGGGTCTGAATTTACTACTGC 3'	46815942 ^x
	1198R	5' AAGCTACGAACTCGGTCCA 3'	
220	220F	5' TGTCGCGTAAGTCTGTCGAG 3'	46815943 ^x
	220R	5' ACAACGACCCTTTTCGTTTCA 3'	
250	250F	5' TCTCTTGGGTTGAAAACGTC 3'	46815945 ^x
	250R	5' TGGTCTTGTCTCTCACC 3'	
124B	124BF	5' GGAAGCTGATGATTGGGCTA 3'	46815938 ^x
	124BR	5' TGCATCCCTCAATGTCAAAA 3'	
124T	124TF	5' ACCGGTGGTTTAAATGGTGA 3'	46815939 ^x
	124TR	5' ACCACCTCAGGTTGCTCATC 3'	
210T	210TF	5' ATGCTGAATATTCCCAGCG 3'	46815944 ^x
	210TR	5' ATTATGGACTCCCCAAAGCC 3'	

^zNational Center for Biotechnology Information (NCBI) GenBank.

^yUniversity of California—Davis Genomics Facility.

^xNCBI expressed sequence tag database (dbEST).

Fig. 1. Change in fruit texture during storage represented by work values for fresh and 3-month-stored 'Macoun' and 'Honeycrisp' apple fruit. Bars represent mean work values ($n = 10$); error bars represent SE.

Fig. 2. Transcript levels of cell-wall genes in fresh and 3-month-stored (A) 'Macoun' and (B) 'Honeycrisp' apple fruit as measured by qRT-PCR. Y axis represents transcript level relative to β -actin. Bars represent means; error bars represent SE.

has sequence similarity to a gene for cysteine protease, and its transcript was detected at a greater abundance in 3-month-stored 'Macoun' fruit. cDNA *124B* has sequence similarity to a gene for xyloglucan *endo*-transglycosylase/hydrolase (XET/H), and it was more abundant in fresh 'Macoun' fruit. Transcript levels for the remaining six sequences were not significantly different in fresh or 3-month-stored 'Macoun' fruit.

Transcript levels for six of the eight cDNAs were further quantified in fresh and 3-month-stored 'Honeycrisp' fruit. The six cDNAs include cDNAs with unknown sequence identity (*183*, *198*, and *124T*) and cDNAs potentially important for postharvest changes in apple fruit (*166*, *124B*, and *220*) based on their sequence-similarity results. The remaining two cDNAs (*250* and *210T*) were detected in fresh and 3-month-stored 'Honeycrisp' fruit with nonquantitative RT-PCR (data not shown). They were not quantified because they were deemed to be unimportant in fruit softening based on their sequence similarities to heat shock protein and 6-phosphogluconolactonase, respectively.

Patterns of expression for cDNAs *183* and *198* in fresh and stored 'Honeycrisp' fruit were similar to their patterns in fresh and 3-month-stored 'Macoun' fruit. The transcripts for cDNA *183* were present at extremely low levels in fresh and stored 'Honeycrisp' fruit, and thus appear undetectable in Fig. 3B. Transcripts for cDNA *124T* in 'Honeycrisp' showed an opposite differential accumulation pattern when compared with their pattern of accumulation in fresh and 3-month-stored 'Macoun' fruit. Clone DNA *166* remained undetectable by qRT-PCR in both fresh and 3-month-stored 'Honeycrisp' fruit. cDNA *124B* showed a similar pattern of differential mRNA accumulation in both 'Macoun' and 'Honeycrisp'. Transcripts for cDNA *220* in 'Honeycrisp' fruit showed an opposite differential accumulation pattern when compared with their pattern of accumulation in fresh and 3-month-stored 'Macoun' fruit.

Discussion

The results from the suppression subtractive hybridization experiment identified novel cDNAs that were differentially expressed in either fresh or 3-month-stored 'Macoun' fruit. Although the suppression subtractive hybridization method could have been used to directly compare 'Macoun' and 'Honeycrisp', some of the differential expression observed in such a comparison could have been due to differences in nontextural traits (e.g., color, flavor, pest resistance, etc.). To simplify data interpretation as much as possible, we limited the use of suppressive subtractive hybridization to a comparison between fresh and stored 'Macoun'. Also, this method preferentially identifies low-abundance transcripts. As all of the cell-wall genes were found at relatively high abundance based on qRT-PCR analysis, this may have kept many cell-wall genes from being identified as differentially expressed in the suppression-subtractive hybridization. We also compared patterns of expression of novel cDNAs and cell-wall-modifying genes in fresh and stored fruit from two genotypes, 'Macoun' and 'Honeycrisp', that have disparate postharvest fruit softening behavior.

Clone DNA *166* showed similarity to a protein phosphatase 2C (PP2C). Plant PP2Cs are known to be components of signaling pathways, including the abscisic acid signaling pathway (Schweighofer et al., 2004). cDNA *166* was expressed at similar levels in both fresh and 3-month-stored 'Macoun' fruit.

Table 2. Putative similarities of the 'Macoun' apple cDNAs identified through suppressive-subtraction hybridization to known genes in National Center for Biotechnology Information (NCBI) databases, as revealed by the BLASTN sequence-similarity tool.

cDNA	NCBI nonredundant (nr) and expressed sequence tag database (dbEST) match
124B	<i>Rosa borboniana</i> Desp. xyloglucan <i>endo</i> -transglucosylase/hydrolase 2 mRNA, complete cds (DQ320658, 8.00E-115) ^y <i>Malus domestica</i> Borkh. cDNA similar to xyloglucan <i>endo</i> -transglycosylase-like protein, mRNA sequence (DR991932, 4.00E-161) ^z
250	<i>Solanum commersonii</i> Dun. Heat shock cognate protein (SCHSP70) mRNA, complete cds (AF002667, 6.00E-121) ^y <i>Solanum lycopersicum</i> L. maturing fruit cDNA clone, mRNA sequence (BP879838, 1.00E-116) ^z
124T	Unknown ^y <i>Prunus persica</i> L. cDNA similar to unknown <i>Arabidopsis thaliana</i> Schur. mRNA sequence (DY646836, 2.00E-130) ^z
210T	<i>A. thaliana</i> putative 6-phosphogluconolactonase mRNA, complete cds (BT001923, 1.00E-23) ^y <i>M. domestica</i> M9 phloem cDNA clone, mRNA sequence (EB153690, 0.00) ^z
183	Unknown ^y <i>Solanum chacoense</i> Bitter. cDNA, mRNA sequence (DN980968, 2.00E-112) ^z
220	<i>Prunus armeniaca</i> Blanco. cysteine protease mRNA, complete cds (U93166, 9.00E-33) ^y <i>M. domestica</i> cDNA similar to <i>P. armeniaca</i> cysteine protease, mRNA (DR994286, 7.00E-166) ^z
198	Unknown ^y <i>M. domestica</i> ('Royal Gala') fruit stored at 0.5 °C for 24 h cDNA clone (CN901999, 8.00E-49) ^z
166	<i>Medicago sativa</i> L. mRNA for protein phosphatase 2C (Y11607, 1.00E-64) ^y <i>Solanum tuberosum</i> L. developing tubers cDNA clone, mRNA sequence (DN907883, 0.00) ^z

^zMatch to NCBI nonredundant nucleotide database.

^yMatch to NCBI dbEST.

Numbers in parentheses represent NCBI accession numbers and E values associated with BLASTN results, respectively. "Unknown" = similarity to an unknown protein or no similarity to any sequence in the NCBI databases.

The transcript for 166 remained undetected in 'Honeycrisp' fruit at both time points during storage. Clone DNA 220 showed sequence similarity to a plant cysteine protease, a class of proteins that degrade other proteins during senescence and plant defense responses (Eichmann et al., 2006; van der Hoorn and Jones, 2004; van der Hoorn et al., 2004). Although it is unknown what functions they have in fruit, cysteine proteases have previously been detected in ripe apple and apricot fruit tissue (Grimplet et al., 2005; Park et al., 2006). Clone DNA 124B is a putative gene for XET/H, and a higher abundance of its transcript in fresh 'Honeycrisp' and fresh 'Macoun' fruit compared with 3-month-stored fruit from each cultivar is in line with a published report of its increased activity in fresh apple fruit (Percy et al., 1996). Clone DNA 124T had no known match in National Center for Biotechnology Information nucleotide or EST databases but showed different patterns of transcript accumulation in 'Honeycrisp' and 'Macoun' fruit and may therefore be worth further study.

A decrease in galactose is a feature of postharvest fruit softening. Transcript levels for *PABG1* were not different in fresh and 3-month-stored fruit of 'Macoun' and 'Honeycrisp'. β -Galactosidase activity enables other cell-wall-modifying enzymes to access cell-wall polysaccharides (Brummell and Harpster, 2001). Our results also suggest that β -galactosidase alone might not be able to cause fruit softening.

Uronide levels did not change significantly in 'Honeycrisp' fruit after 6 months of storage, whereas uronide decreased significantly after 6 months of storage in 'Macoun' and 'Honeygold', which softened during storage (Tong et al., 1999), suggesting differences in activity of pectin-degrading enzymes like polygalacturonase in softening and nonsoftening genotypes. 'Honeycrisp' fruit showed a decrease in transcript levels of polygalacturonase gene *MdPG* after 3 months of storage, whereas *MdPG* activity did not change between fresh and 3-month-stored fruit of 'Macoun'. Wakasa et al. (2006)

also reported that decreased *MdPG* expression was correlated with maintenance of texture in 'Fuji' and other apples.

Arabinose loss precedes the rapid softening phase of apple during storage (Peña and Carpita, 2004). Lack of arabinose loss from 6-month-stored 'Honeycrisp' apple fruit was a distinguishing feature and was positively correlated to its nonsoftening behavior (Tong et al., 1999). *MdAFase1* transcript levels did not change among fresh and 3-month-stored 'Macoun' fruit, whereas its levels decreased in 3-month-stored 'Honeycrisp' fruit when compared with fresh 'Honeycrisp' fruit.

Expansins break hydrogen bonding between cellulose and hemicelluloses of the cell wall and may enable polyuronide-degrading enzymes to access their substrates (Brummell et al., 1999). *MdEXPA2* transcripts did not change among fresh and 3-month-stored 'Macoun' fruit, whereas its levels increased in 3-month-stored 'Honeycrisp' fruit when compared with fresh 'Honeycrisp' fruit. Assuming a corresponding activity of expansin enzyme, it appears that expansin may act later during storage in 'Honeycrisp' fruit compared with 'Macoun', and thus polyuronide-degrading enzymes also may have later access to cell-wall polysaccharides in 'Honeycrisp' fruit than in 'Macoun' fruit. In addition, by the time *MdEXPA2* transcript levels might have peaked in 'Honeycrisp' fruit, transcript levels of the polygalacturonase gene *MdPG* may have already declined.

The patterns of expression of genes for various cell-wall-modifying enzymes suggest that a combination of a late increase in *MdEXPA2* and decreased levels of *MdPG* and *MdAFase1* transcript levels in stored 'Honeycrisp' fruit may lead to its nonsoftening phenotype. Fruit softening is a dynamic process, and a snapshot of transcript accumulation at two time points might not fully capture the mechanisms contributing to fruit softening. Data from these experiments suggest that more detailed analyses examining more time points during storage are necessary. Further work including measurements of

Fig. 3. Transcript levels of novel cDNAs in fresh and 3-month-stored (A) 'Macoun' and (B) 'Honeycrisp' apple fruit as measured by qRT-PCR. Y axis represents transcript level relative to β -actin. Note that Y-axis scales are different. Bars represent means; error bars represent SE.

activities of the corresponding enzymes for these genes would also provide more details on the underlying mechanisms of fruit softening in apple. Differences in patterns of transcript accumulation for various genes in Honeycrisp and Macoun also suggest differences in regulatory control of postharvest fruit softening in the two cultivars.

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