FINAL PROJECT REPORT

Project Title: Genetic controls of apple fruit-specific auxin metabolism

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Budget History:

Item	2010	2011(extended)	Year 3:
Salaries	35,000	36,000	
Benefits	14,000	14,000	
Wages			
Benefits			
Equipment			
Supplies	15,000	15,000	
Travel			
Plot Fees			
Miscellaneous	1,000	1,000	
Total	65,000	66,000	

OBJECTIVES:

1. Elucidate roles for previously identified candidate genes in ethylene, auxin, gibberellin, jasmonate and brassinosteroid metabolism and response during apple fruit ripening.
2. Characterize the relationship between gene expression patterns and specific fruit ripening phenotypes (ripening season, fruit size, fruit texture) in a cross population of 'Honeycrisp' x 'Cripps Pink', as well as other germplasm.
3. Develop a shortlist of candidate genes for hormone metabolism for further validation for use in marker assisted selection.

SIGNIFICANT FINDINGS

1. Most of the selected genes, based on the results from previous microarray or gene chip analysis, showed correlations with apple fruit ripening processes among a wide spectrum of apple germplasm including several commercial cultivars and a segregating population of 'Honeycrisp' x 'Cripps Pink'.

2. The expression patterns of three genes, *MdACS3* (a pre-climacteric ethylene biosynthesis gene), JOM (codes for the enzyme jasmonate O-methyl transferase) and AIP (codes for an auxin induced protein) showed a strong correlation with fruit ripening season.

3. The expression patterns of an AUTR (auxin transporter) encoding gene showed a good correlation with fruit firmness.

4. The expression features of an auxin transporter gene, *MdPIN1-1,* is associated with the timing of climacteric ethylene biosynthesis.

5. Gene activity of *MdGH3.5,* which is related to the availability of active auxin in fruit cortex tissues, showed cultivar-specific regulation patterns during the 10-week on tree maturation and ripening processes.

6. The tissue-specific expression features, i.e. seed-initiated and spreading outward, of the *MdPIN1-1* gene, correspond to starch degradation patterns, and ethylene biosynthesis pathway activation.

7. Overall, variation of auxin metabolism gene activities appear to associate with fruit ripening time or ripening season among apple genotypes studied.

RESULTS AND DISCUSSION

1. Correlations between transcript levels of identified candidate genes and fruit ripening phenotypes within a WSU 'Honeycrisp' x 'Cripps Pink' cross population.

Fruit ripening date, fruit firmness and fruit size were phenotyped based on weekly maturity data for all fruiting trees within 'Honeycrisp' x 'Cripps Pink' cross population. Phenotypic data among individuals were categorized based on the values of ripening date, fruit firmness and fruit size around physiological maturity (average starch pattern index of 3.5, based on 1-6 scale). Fruit from ~12 individual trees (siblings) in each phenotypic group (early or late-ripening; firm or soft fruit; small or large fruit) were selected for the analysis with transcript abundance. Several candidate genes showed correlation with apple fruit ripening processes or quality attributes among individuals in this segregating population. "*" indicated that the transcript abundance of this gene showed statistically significant difference between two groups.

Table 1. Association between gene transcript abundance and sorted phenotypes among individuals in 'Honeycrisp' x 'Cripps Pink' segregating population.

Fruit ripening time

	Early-ripening (Earlier than Sep. 9)	Late-ripening (Later than Oct. 15)	*P* value
*ACS3	20.8 ± 3.5	28.7 ± 3.3	3.20E-05
*JOM	25.6 ± 2.3	31.9 ± 2.3	3.80E-06
*AIP	24.3 ± 1.2	28.3 ± 1.5	1.70E-06
*AUTR	24.5 ± 1.1	22.0 ± 1.2	4.80E-05

Fruit firmness

	Soft fruit (≤ 14.9 Ibs at-harvest)	Firm fruit (≥ 18.2 Ibs at-harvest)	*P* value
JOM	25.6 ± 2.8	28.8 ± 3.5	0.0467
*AUTR	23.0 ± 0.9	24.8 ± 1.2	0.0023
XTH7	24.4 ± 2.4	26.2 ± 2.8	0.1092
BRIP	26.1 ± 3.0	28.4 ± 3.7	0.1495
AQUAPIP	29.3 ± 3.4	29.4 ± 2.1	0.9505

Fruit size

	Small fruit (≤ 2.12 inches of diameter)	Large fruit (≥ 2.82 inches of diameter)	*P* value
*JOM	31.2 ± 2.3	27.9 ± 2.5	0.003
AUTR	23.9 ± 1.1	24.2 ± 1.3	0.4477

Values in the 2nd and 3rd columns are the average and standard deviation of normalized Ct (stand for cycle threshold of quantitative PCR reaction) values for tested candidate genes, which represent the relative abundance of transcripts or the expression level. Each value is the average based on the gene expression data from the fruits of 10-12 individual trees within the same phenotypic group. Fruit cortex tissues of 3 apples were pooled for RNA isolation and qPCR were carried out in triplicate. Anova analysis was performed between the paired groups. $P < 0.01$ would normally be considered significant and $P < 0.001$ highly significant as indicated by "*" in Table 1. ACS3: a pre-climacteric ethylene biosynthesis gene, JOM: jasmonate O-methyl transferase encoding gene, AIP: auxin induced protein gene, AUTR: auxin transporter gene, BRIP: brassinosteroid induced protein gene, XTH7: xyloglucantransferase/hydrolase gene, and AQUAPIP: aquaporin gene.

2. Physiological characterization of four cultivars with distinct ripening phenotypes

To characterize apple genotype-specific maturation and ripening progression, samples harvested weekly and associated fruit maturity data were collected starting in late July for early ripening cultivar 'SweeTango' and a few weeks later for three later ripening cultivars. The four cultivars studied - 'SweeTango' (ST), 'Golden Delicious' (GD), 'Jazz' (JZ) and 'Cripps Pink' (CP) required 122, 148, 150 and 191 days after full bloom (DAFB) to achieve physiological maturity (Table 2). Physiological maturity was considered to be attained when the average starch rating was approximately 3.5 and this harvest date was designated as week 0. Fruit firmness showed a steady decrease in all cultivars as the fruit matured, although the values and rates of change are cultivar-specific. Fruit internal ethylene concentration (IEC) was variable and fluctuated at a low level and characteristic of pre-climacteric fruit.

3. Expression features of a pre-climacteric ethylene biosynthesis gene *MdACS3* as an indicator of ethylene pathway activation.

The expression profiles of *MdACS3* showed steady up-regulation as maturation progressed for all four cultivars (Fig. 1). The detection of measurable *MdACS3* transcript in GD was observed at week -4 and transcript amount increased through week 1. *MdACS3* transcripts in early-season cultivar ST were detectable even earlier than week -4, while consistent detection in CP was delayed till week -3. The peak expression of *MdACS3* was generally observed at week +1, except CP which did not achieve physiological maturity in 2011 before freezing temperatures. The fold change of *MdACS3* transcript abundance varied greatly along the maturation process depending on genotypes.

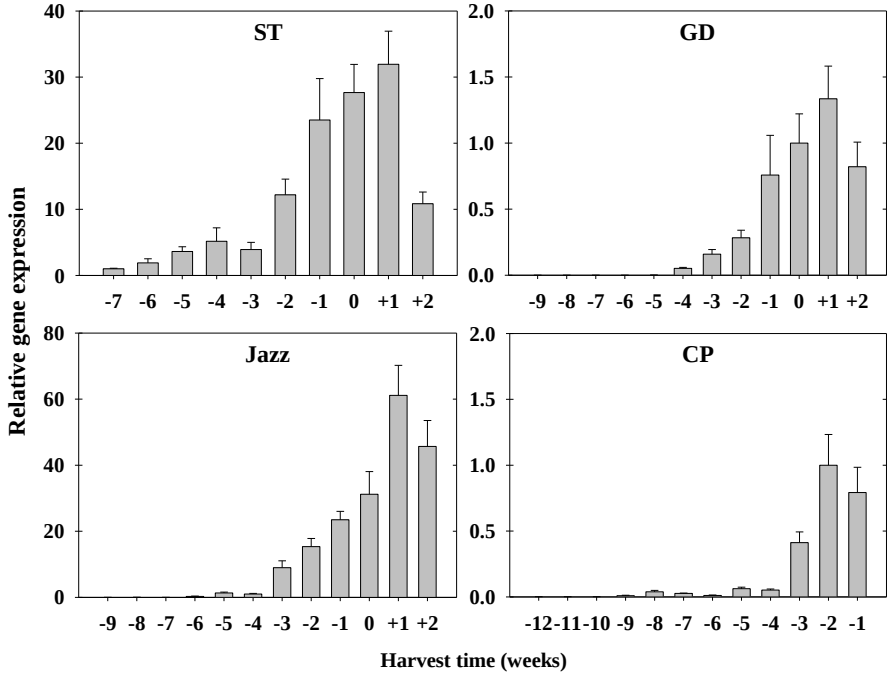

Fig. 1. Expression profile of *MdACS3* during apple fruit maturation and ripening. ST: 'SweeTango', GD: 'Golden Delicious', Jazz: 'Jazz', CP: 'Cripps Pink'. Y axis indicates the fold change in relative gene expression level; X axis indicate week prior to or after physiological maturity (week=0) when fruit were harvested. Each data point represents the means of four replicates with standard error. For better comparison of gene expression levels among cultivars, one comparable Ct value from each cultivar was designated as calibrator for relative gene expression analysis within the sample series for a cultivar. For *MdACS3* gene, week -7 for ST, week 0 for GD, week -4 for Jazz and week -2 for CP were used as calibrator for relative gene expression analysis.

Table 2. Fruit maturity during on-tree development

ST										
Weeks	-7	-6	-5	-4	-3	-2	-1	0	1	2
DAFB	73	80	87	94	101	108	115	122	129	136
IEC (ppm)	0.1±0.05	0	0	0.0±0.03	0.1±0.08	0.1±0.03	0.2±0.04	0.3±0.06	0.7±0.23	0.1±0.02
Firmness (lbs)	17.2±0.4	17.0±0.3	15.9±0.4	15.6±0.4	14.1±0.4	14.0±0.3	13.5±0.3	12.6±0.5	13.2±0.2	13.3±0.4
Starch Index	1.1±0.1	1.4±0.2	1.5±0.1	2.1±0.1	2.1±0.2	2.6±0.2	2.5±0.1	3.4±0.3	4.6±0.1	5.0±0.2
Jazz										
Weeks	-7	-6	-5	-4	-3	-2	-1	0	1	2
DAFB	101	108	115	122	129	136	143	150	157	164
IEC (ppm)	0	0	0.0±0.01	0	0	0.2±0.08	0.1±0.03	1.4±0.74	0.3±0.17	0.1±0.07
Firmness (lbs)	33.1±0.8	30.9±0.8	28.9±0.7	26.7±0.5	25.8±0.6	21.9±0.3	20.6±0.3	19.7±0.4	20.6±0.4	19.7±0.4
Starch Index	1.0±0.0	1.1±0.1	1.0±0.0	1.1±0.1	1.5±0.1	1.9±0.1	2.6±0.2	3.5±0.3	3.7±0.2	5.0±0.1
GD										
Weeks	-7	-6	-5	-4	-3	-2	-1	0	1	2
DAFB	99	106	113	120	127	134	141	148	155	162
IEC (ppm)	0	0.0±0.03	0.1±0.10	0	0.0±0.04	0.0±0.03	0.0±0.01	0.0±0.05	0.1±0.01	0.0±0.01
Firmness (lbs)	17.6±0.4	18.3±0.4	17.7±0.3	17.0±0.4	17.1±0.4	15.9±0.2	16.8±1.0	16.1±0.3	15.3±0.3	15.6±0.4
Starch Index	1.2±0.1	1.1±0.0	1.2±0.0	1.5±0.1	1.7±0.2	2.1±0.1	2.1±0.1	2.3±0.1	2.8±0.2	4.1±0.3
CP										
Weeks	-9	-8	-7	-6	-5	-4	-3	-2	-1	0
DAFB	128	135	142	149	156	163	170	177	184	191
IEC (ppm)	0	0.03±0.04	0	0	0.06±0.06	0.06±0.01	0	0.03±0.02	0.06±0.01	0.16±0.05
Firmness (lbs)	21.5±0.4	20.8±0.5	20.6±0.5	19.8±0.4	19.8±0.3	18.9±0.3	18.9±0.3	18.6±0.3	18.5±0.3	18.0±0.3
Starch Index	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.1±0.0	1.2±0.1	1.4±0.0	2.2±0.1	2.8±0.2

Values of IEC concentration, fruit firmness (Ibs) and starch pattern index (SPI, based on 1-6 scale) of weekly fruit samples represent the average of 15 apples with standard error.

4. Expression profiling of an auxin transporter encoding gene

Due to its unique weak acid character, auxin (indolyl-3-actic acid) requires specific carrier proteins or transporters on the plasma membrane for its distribution and function. In the apple fruit cortex, the transcript of an auxin transporter gene, denoted as *MdPIN1-1*, was readily detected seven to nine weeks before physiological maturity (Fig. 2). The peak expression of *MdPIN1-1* was observed around week -4 in all these cultivars, which corresponded approximately with the timing of *MdACS3* activation in most apple cultivars (see Fig. 1). Some subtle variations of the *MdPIN1-1* expression were identified among early, mid- and late-season cultivars. In early-season cultivar ST a secondary peak of expression was evident at week -1; in both mid-season cultivars JZ and GD a slight down-regulation was observed after week -4. In late-season cultivar CP, the peak transcript abundance was also occurred at week -4 though the overall expression level is low. At any specific time, for example September 21, the expression levels or transcript abundances varied greatly between cultivars, although the values of DAFB were approximately same. The differences at *MdPIN1-1* expression could be one of the important factors that differentiate the timing of ethylene biosynthesis pathway activation, putatively through *MdACS3*, and therefore the rate of maturation and ripening.

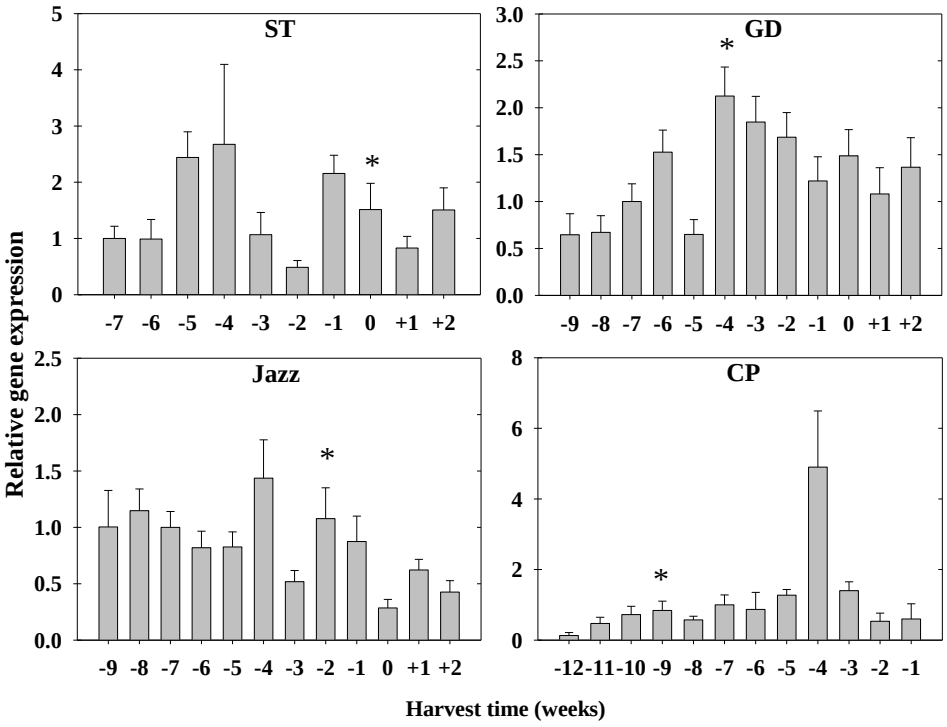

Fig. 2. Expression profile of auxin efflux carrier protein gene, *MdPIN1-1*, during apple fruit maturation and ripening. ST: 'SweeTango', GD: 'Golden Delicious', Jazz: 'Jazz', CP: 'Cripps Pink'. Y axis indicates the fold change of relative gene expression level; X axis indicates week prior to or after physiological maturity (week=0) when fruit were harvested. Each data point represents the means of four replicates with standard error. For better comparison of gene expression levels among cultivars, one comparable Ct value from each cultivar was designated as calibrator for relative gene expression analysis within the sample series for a cultivar. For *MdPIN1-1* gene, week -7 samples for all four cultivars were used as calibrator for relative gene expression analysis.

5. Auxin homeostasis gene

GH3 proteins in plant tissues control free or biologically active IAA levels by deactivating IAA. A previously identified apple GH3.5 gene had down-regulated expression patterns as fruit maturity progressed in all four cultivars studied (Fig. 3). In early-season cultivar ST, stronger reductions in transcript levels were observed from week -6 to week -5, and week -3 to week -2; similar patterns were also observed in GD. Overall, considerably higher transcript levels were detected in early stages in all cultivars studied, but more dynamic changes in GH3.5 transcript abundance were found in both JZ and CP as fruit maturity increased.

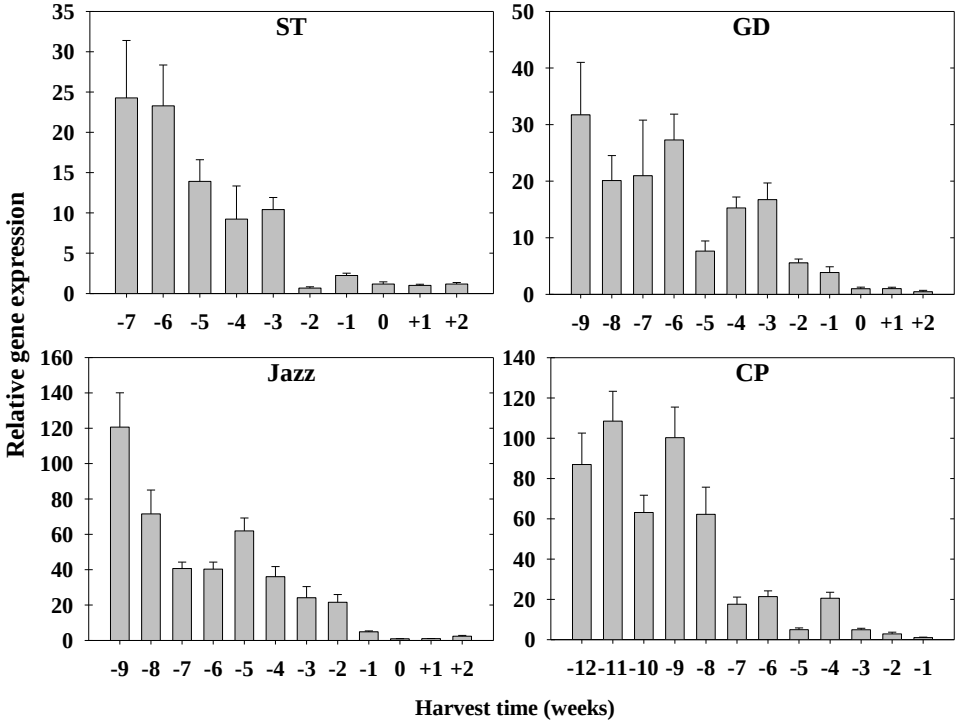

Fig. 3. Expression profiles of an *MdGH3.5* genes during apple fruit maturation and ripening. ST: 'SweeTango', GD: 'Golden Delicious', Jazz: 'Jazz', CP: 'Cripps Pink'. Number on Y axis indicate the fold change of relative gene expression level; number on X axis indicate the weeks when samples were harvest and "-" means week before physiological maturity and "+" means week after physiological maturity. Each data point represents the means of four replicates with standard error.
For better comparison of gene expression levels among cultivars, one comparable Ct value from each cultivar was designated as calibrator for relative gene expression analysis within the sample series for a cultivar. For *MdGH3.5* gene, week +1 for ST, GD and Jazz, and week -1 for CP were used as calibrator for relative gene expression analysis.

6. Tissue-specific expression of auxin transporter genes and ethylene biosynthesis genes

The temporal and spatial expression features, i.e. in different tissues of seed, core, cortex and peel and along the maturation processes, of auxin transporter genes *MdPIN1-1* and a pre-climacteric ethylene biosynthesis gene *MdACS3* were examined using mid-season cultivar 'Jazz' (Fig. 4). For *MdPIN1-1*, a stronger expression was observed in seed and core tissues and a relatively weak expression was found in the cortex and peel. For *MdACS3*, a strong expression was observed in core and cortex but

no expression was detected in seed tissues. This expression pattern is correlated to the starch degradation process observed in maturing fruit. This data supports a scenario of auxin-ethylene interaction that the developing seed is a primary source of auxin which is transported to core and cortex; auxin triggers the expression of *MdACS3* initially in the core, then in cortex and finally in peel as auxin is transported to these tissues. As the result of *MdACS3* expression and internal ethylene accumulation, *MdACS1* expression begins and sustained ethylene production promoting fruit ripening occurs. If confirmed, genetic differences in the capacity and rate of auxin metabolism (biosynthesis, transport and homeostasis) could determine the timing of ethylene biosynthesis pathway activation as illustrated in Figure 5 of proposed model on the roles of auxin and its interaction with ethylene biosynthesis pathway in apple fruit.

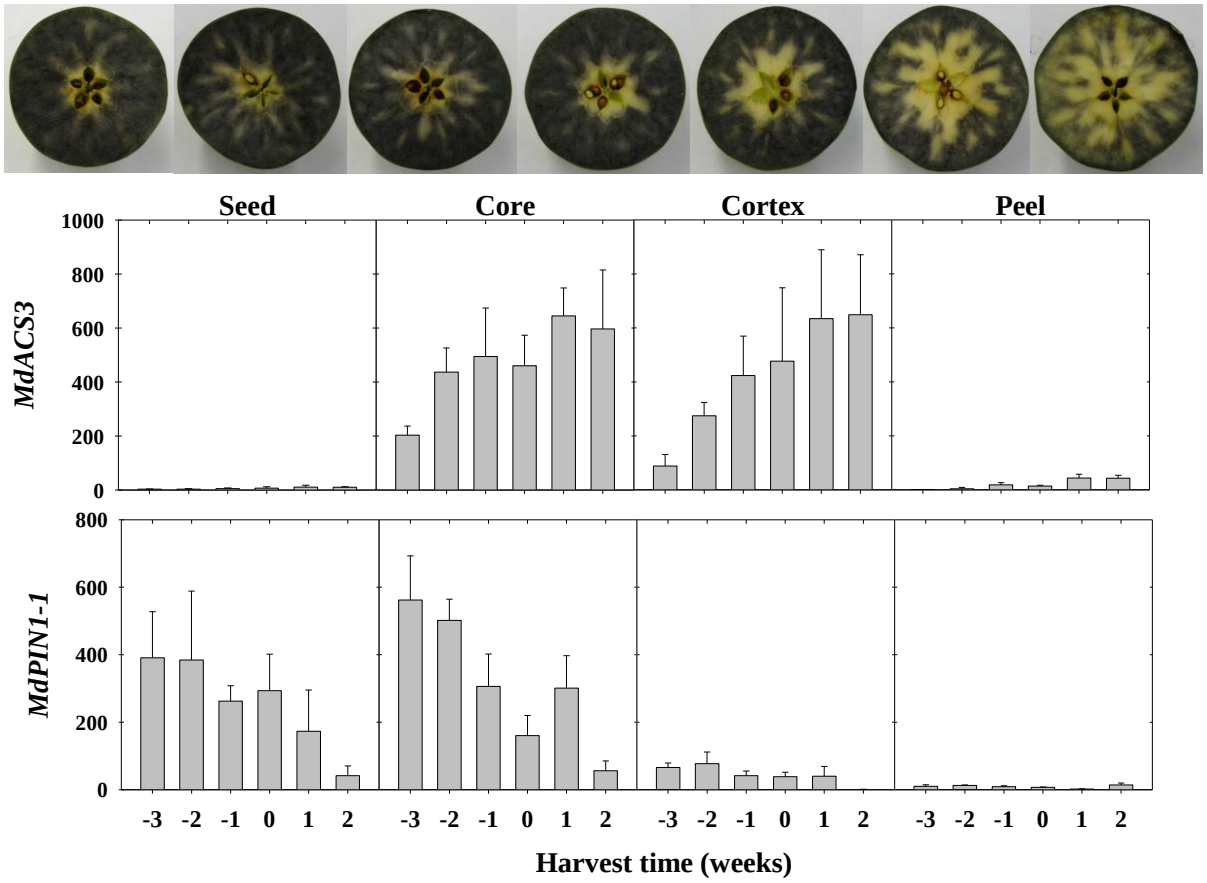

Fig. 4. Center-initiated ripening patterns as suggested by the starch clearing is correlated with "from center to out-layer" gene activities for both auxin transporting function *MdPIN1-1*and ethylene biosynthesis gene *MdACS3* in four different 'Jazz' apple tissues during apple maturation and ripening. The number indicated the weekly samples with week 0 as physiological maturity; "-" means before and "+" means after week 0.

7. A simplified model to illustrate the interaction between ethylene and auxin

While apple fruit ripening is likely not solely regulated by ethylene, little is known regarding other molecular mechanisms regulating fruit ripening and quality attributes, especially those with plant hormone interactions. For example, all apple genotypes produce ethylene during ripening but production duration and strength vary with cultivar. The very basic question is what is behind the

timely activation and the strength of ethylene production? The results from this study indicate auxin may function to activate ethylene biosynthesis pathway, and therefore regulate onset of apple fruit ripening time or the ripening season.

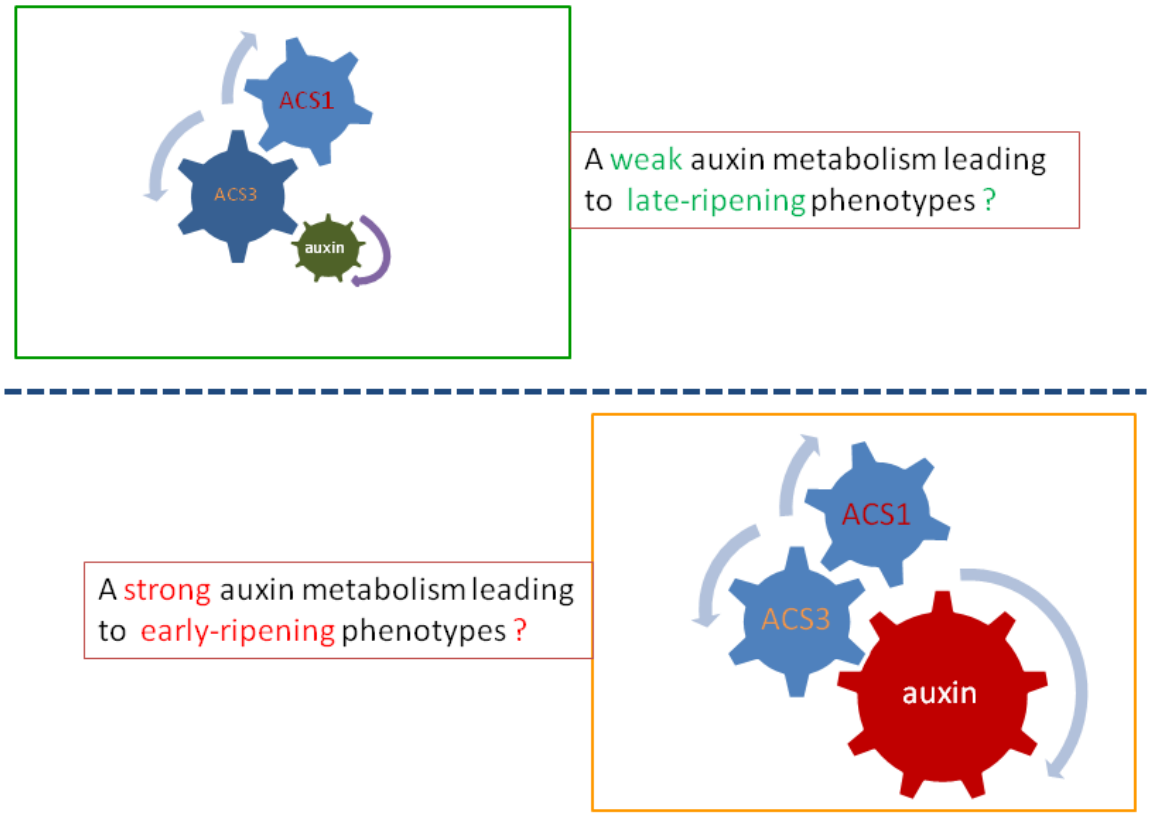

Fig. 5. A simple model describing the relationship between auxin metabolism and ethylene biosynthesis pathway in maturing apple fruit

8. What this study means to the tree fruit industry?

From current analysis, several candidate genes, especially those genes regulating auxin metabolism, showed strong association with the apple fruit ripening season. Understanding the molecular regulation of auxin in apple fruit could potentially generate innovative technology for managing pre-harvest maturation process and postharvest fruit quality. Elucidating the specific mechanism of plant hormone interactions and their roles on cultivar-specific fruit quality and maturation/ripening patterns is an essential step in developing useful molecular breeding tools, such as gene-specific "functional molecular markers", for improved precision and efficiency in the apple breeding processes.

EXECUTIVE SUMMARY

Apple cultivars exhibit variation in maturation and ripening physiology and fruit quality traits. The ripening season of apple vary up to 3 months even within elite cultivars. In a breeding segregation population, depending on the parent combination, up to 8-10% of the individuals are those which are unable to fully ripen in North Central Washington State. The phenotype of the apple ripening date/season is a simple but economically important horticultural trait, which can also substantially influence at-harvest quality and postharvest storability. Understanding the molecular regulation for this trait is critical for breeding locally-adapted new apple cultivars. Ethylene has been well-established for its regulating roles in apple fruit ripening; but ethylene may not account for every aspect of fruit ripening and quality. This study was built on two previous experiments in our lab: 1. The transcriptomics study of fruit ripening using a gene chip (microarray) containing 24,000 apple genes: among genes identified, those functioning in metabolism and response of auxin were a primary group of differentially regulated genes. Therefore, it was proposed that auxin metabolism plays a key role in the timely activation of the ethylene production and ripening season for an apple cultivar; 2. An earlier study on a pre-climacteric ethylene biosynthesis gene *MdACS3*: the activation of *MdACS3* was observed around 4 weeks prior to onset of climacteric ethylene production regardless of the actual ripening season. The primary goal of the current study was to get insight into the relationships between auxin metabolism and the timing of ethylene biosynthesis initiation in maturing apple fruit.

Among siblings of 'Honeycrisp' x 'Cripps Pink' progeny, several candidate genes showed strong correlation between apple fruit ripening and quality phenotypes (fruit ripening time, firmness and size) and transcript abundance around physiological maturity. The genes functioning in auxin metabolism, specifically, auxin transport and auxin homeostasis regulation, were investigated for their roles in the activation of ethylene biosynthesis among four cultivars with distinct ripening seasons, 'SweeTango', 'Golden Delicious' 'Jazz' and 'Cripps Pink'. The results suggest that availability of biologically active auxin is a primary contributor to activate the ethylene pathway, and subsequently initiate the apple ripening process. The majority of candidate genes tested showed expression patterns during the fruit maturation and ripening consistent with this hypothesis. The tissue-specific expression patterns of the auxin transporter gene in seed, core, cortex and peel correspond with the "center originated" ethylene biosynthesis gene expression and starch degradation patterns in ripening fruit, adding evidence that auxin availability is essential for timely activation of the ethylene biosynthesis pathway. These results provided data for understanding the molecular aspects of apple ripening regulation in addition to ethylene. The results have been presented at several scientific symposiums, including Sixth Rosaceous Genomics Conference (Trento, Italy 2012); American Society of Horticulral Sciences (Miami, FL 2012) and Washington State Annual Horticultural meeting (Yakima, WA 2012). The data has been submitted for peer-reviewed publication.