USING MOLECULAR TOOLS TO PREDICT RIPENING CAPACITY OF 'BARTLETT' PEARS

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SUMMARY

Freshly harvested early-season 'Bartlett' pears often ripen unevenly and fail to achieve acceptable quality. The main objective of this project is to develop a reliable method to predict the variable ripening behavior of early-season pears. To induce different ripening capacity in 'Bartlett' pear, three experiments were designed: Experiment I based on different fruit maturity, Experiment II based on different temperature conditioning treatments, Experiment III based on different plant growth regulators. The fruit from these treatments were evaluated for their physiological properties (firmness, color, and ethylene production) to determine ripening capacity (e.g. firmness reaches 3-4 lbs. after 6 days at 68°F). RNA-sequencing data was generated from samples collected from Exp I and II and expression levels of several genes were validated. Functional analysis on RNA sequencing showed that in addition to ethylene, auxin and jasmonic acid may play important roles in the regulation of pear ripening. Genes associated with auxin and ethylene metabolism and transcription factors are potential candidates to be biomarkers to predict ripening capacity. Future work includes further molecular analysis on fruit from samples collected from different postharvest treatments, harvest seasons, and growth regions to validate the role of candidate genes.

INTRODUCTION

Freshly harvested early-season 'Bartlett' pears often ripen unevenly, and fail to achieve acceptable color, texture and flavor. This resistance to ripening at ambient temperature immediately after harvest is associated with low concentrations of ethylene in fruit tissues. While treatment with exogenous ethylene and/or chilling temperatures can stimulate ethylene production to initiate ripening, it is not always practiced given the rush to deliver early-season pears to the market. At present, there is no reliable method to predict the variable ripening behavior of early-season pears. In addition, 'Bartlett' pear fruit response to SmartFresh^{TM} is variable from season to season and by harvest date. The variability appears to be partially due to production of ethylene by the pear fruit during treatment. However, there may be other factors inherent to more and less mature pear fruit that influence the fruit's response to SmartFresh. The availability of modern molecular tools such as gene sequencing provides an exciting opportunity to

rapidly ‘mine’ the pear genome to look for markers of ripening competence. RNA - sequencing has helped to narrow our search to select candidate genes or proteins with potential to rapidly and accurately predict ripening behavior and responses of ‘Bartlett’ pear fruit.

OBJECTIVES

1. Identify promising candidate genes as biomarkers of fruit ripening capacity.
2. Determine the reliability of candidate genes to predict ripening capacity in fruit from different districts and in response to postharvest treatments.
3. Understand cold-induced genes associated with the regulation of pear ripening.
4. Understand the effect of some plant growth regulators (jasmonic acid - JA, abscisic acid - ABA, and indole-3-acetic acid - IAA) independent and dependent with ethylene in the regulation of pear ripening.

MATERIALS AND METHODS

Our rationale was to identify changes in key physiological and molecular processes that were closely associated with the onset of ripening capacity. Three experiments (Exp. I, II, and III) were completed to assess the influence of fruit maturity, temperature conditioning treatments, and some plant growth regulators on the development of ripening capacity. The physiological properties of fruit (firmness, color, and ethylene production) were determined in these experiments. Fruit peel samples were collected for molecular analysis. The procedures were described in detail in our 2013 report to the California Pear Advisory Board and are outlined briefly below.

EXPERIMENT I. Ripening capacity of fruit induced by development on the tree

Plant materials, treatments, and physiological evaluation: ‘Bartlett’ pear fruit were harvested from five representative trees in a commercial orchard near Walnut Grove, California. Fruit were randomly picked from the trees every 6-7 days for 5 weeks to capture different stages of development. Peel samples were collected from 12 representative fruit on each harvest date for molecular analysis. All remaining fruit were randomly assigned to treatments for assessing ripening capacity. Fruit were enclosed in 5 gallon glass jars and exposed to 0 or 100 ppm ethylene gas in flowing air streams for 24 hours at 68°F. After treatment, the fruit were held at 68°F and 90% relative humidity for 14 days for ripening capacity evaluation. Fruit weight, diameter, firmness, skin color, soluble solid content, internal ethylene concentration, ethylene production and respiration rate were measured at harvest or during ripening.

Gene expression profiling and validation: The samples from 2011 were sent for RNA-sequencing. Samples from similar experiment in 2013 and 2014 were used for validation. In other words, we checked to see if the expression levels of the candidate

genes were similar among the samples of similar ripening capacity and changed with changes in ripening capacity as in 2011.

EXPERIMENT II. Ripening capacity of fruit in response to different temperature treatments, dependent or independent of cold-induced pathways.

Plant materials, treatments, and physiological evaluation:
Season 2010: Early season 'Bartlett' pears were harvested from commercial orchards in Sacramento and Lake Counties of California. Fruit were conditioned at 32 and 50°F for 0, 2, 5, 8, 11, and 14 days.
Season 2012: Experiment was repeated for validation.
Season 2013: In addition to the similar experiment designed in 2010 and 2012, Retain and SmartFresh treatments were applied after harvest to block ethylene pathways before cold conditioning. This was done to identify genes associated with ripening regulation induced by chilling temperatures and independent of ethylene effects.
Season 2014: Experiment of 2013 was repeated but only SmartFresh was applied to block the ethylene pathways before cold conditioning.

For all seasons, following temperature conditioning and/or postharvest Retain or SmartFresh, fruit were transferred to 68°F for evaluation of ripening capacity as evidenced by changes in fruit firmness, skin color, and ethylene production. Peel samples collected from fruit at harvest and at the completion of each treatment were used for molecular analysis.

Gene expression profiling and validation: Three samples from 2010 including Control (fruit at harvest), 32°F for 14 days, and 50°F for 5 days, were submitted for RNA sequencing. The samples from postharvest Retain and SmartFresh treatments, with or without temperature conditioning, of fruit collected in Sacramento and Lake County in the 2012, 2013, and 2014, were used for validation of candidate genes to predict ripening capacity. The SmartFresh treatments in 2013 and 2014 also help in understanding cold-induced genes during ripening capacity development.

EXPERIMENT III. Ripening capacity of fruit in response to different plant growth regulators, dependent or independent of ethylene pathways.

Plant materials, treatments and physiological evaluation:
Season 2014: Early season 'Bartlett' pears were harvested from commercial orchards in Sacramento and Lake Counties of California. Fruit were separated into two groups. Group 1 was treated with MeJA, ABA, and IAA. Group 2 was treated with SmartFresh to block the ethylene pathways, and then MeJA, ABA, and IAA. Following the treatments, fruit were transferred to 68°F for evaluation of ripening capacity as evidenced by changes in fruit firmness, skin color, and ethylene production.

Gene expression evaluation: Peel samples collected from fruit at harvest and at the completion of each treatment were used for molecular analysis. The expression of genes associated with plant growth regulators will soon be assessed to understand the function of these genes in ripening capacity of pear fruit, dependent or independent of ethylene pathways.

MOLECULAR ANALYSIS With a view to narrowing our search to select candidate genes or proteins exclusively associated with ripening capacity in 'Bartlett' pears, we determined the relative abundance of each gene via RNA sequencing. Briefly, RNA was extracted from the peel samples and sequenced as described in the 2013 report and briefly stated below. Downstream analysis of the sequencing provided a collection of genes with their change in expression between samples. A significant increase or decrease in expression associated with changes in pear ripening indicates the essential contribution of these genes to ripening capacity development.

RNA extraction and gene expression evaluation: Total RNA was isolated from frozen peel tissue. Expressions of genes of interest were determined through quantitative PCR.

RNA sequencing, gene expression profile, and validation: RNA samples were submitted for sequencing at the Genome Center at UCDavis. Virtual genes and their count were established based on fragments of 100 nucleotides collected from RNA sequencing. This provides gene expression profiles during ripening capacity development of pear fruit.

The virtual genetic fragments were actually assembled by a computer program. To test the reliability of this virtual approach, these sequences were compared to the available Asian pear genome (*P. bretschneideri Rehd.* cv. Dangshansuli) and the newly released European pear genome (*Pyrus communis*). Furthermore, all the sequences were compared with non-redundant, plant and Arabidopsis databases to determine a likely function of these sequences. The expression values of genes of interest were validated using quantitative PCR.

Differential expression analysis: The genes were statistically analyzed for differences in expression between pear samples with different capacity to ripen.

Functional analysis: The function of differentially expressed genes was then classified into closely related groups using Mercator-Mapman (http://mapman.gabipd.org/web/guest/ app/mercator). This analysis permits an understanding of the mechanism of ripening capacity development and identifies gene families that play essential roles in regulating pear ripening.

RESULTS OF EXP. I - FRUIT RIPENING DEVELOPMENT ON THE TREE

EXP. I POSTHARVEST PHYSIOLOGICAL CHARACTERISTICS

The physiological response of fruit harvested at different stages of maturity in 2011 are described in our 2012 Report to the Pear Advisory Board and are summarized as follows. Fruit harvested at stages S1 to S3 (1 to 3 weeks before the first commercial harvest, failed to ripen in 14 days at 68°F even when exposed to 100 ppm ethylene (Fig. 1, 2011). Fruit gradually developed a capacity to ripen in response to ethylene treatment by the third harvest S3, 1 week prior to the first commercial harvest. In the absence of external ethylene, ripening capacity was slower to fully develop in all fruit. Only the fruit harvested at S4 and S5 ripened to acceptable quality without ethylene treatment. RNA that was extracted from pear tissues at S1, 2, 3 and 4 were sent for sequencing.

The capacity to ripen with ethylene treatment of fruit from 2013 developed 3 weeks before the first day of commercial harvest (Fig. 1). The capacity to ripen without ethylene treatment of 2014 season developed one week after the 1st day of commercial harvest. These data imply the complication of fruit response from season to season. The nature of variance in this response actually provides us an opportunity to validate candidate genes that have been identified in 2011.

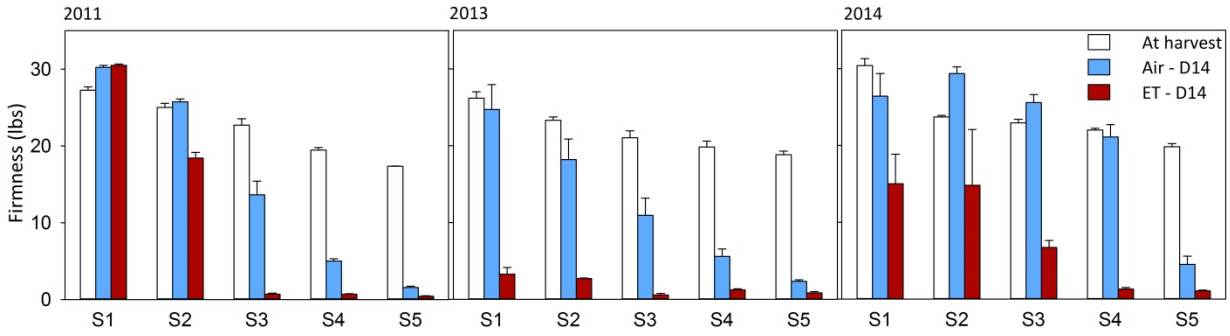


Figure 1: Fruit firmness at different developmental stages and following 14 days of ripening at 68°F with (ET-D14) or without (Air-D14) ethylene treatment of season 2011, 2013, and 2014.

RESULTS: VALIDATION OF THE GENE EXPRESSION PROFILE

(Note: This part is presented for both Exp. I and II.)

Identification: The RNA sequencing approach generated 68,067 longer fragments called virtual genes (length of 201 – 18,868 nucleotides, mean length of 917). Mapping with the available European pear genome identified 11.81% possible non-gene structure sequences. The putative functions of 41.5% of the genes were identified based on the sequence comparison with plant databases.

Quantification: The expression levels of several genes associated with plant growth regulators and cell walls were validated using qPCR. The high correlation values (R^2 = 0.95 in Exp I, R^2= 0.98 in Exp II) in the regression analyses between fold changes

gained from RNA sequencing and qPCR indicates the reliability of expression values obtained from RNA sequencing (graphs not shown).

EXP. I DIFFERENTIAL GENE EXPRESSION ANALYSIS and FUNCTIONAL CLASSIFICATION ANALYSIS

We have found ~7000 genes that were significantly different across the four maturity stages. Based on their expression levels, genes were grouped into 12 clusters (Fig. 2A). The functional classification gave us an overview of the dominant groups in each cluster (Fig. 2B). Among these clusters, cluster K6 and K8 showed high gene expression at S3 and statistical test showed that Aux/IAA (Auxin/indole-3-acetic acid)-associated genes were represented in these groups. This suggests that auxin may play an important role in the transition of pear fruit from insensitive to sensitive to ethylene.

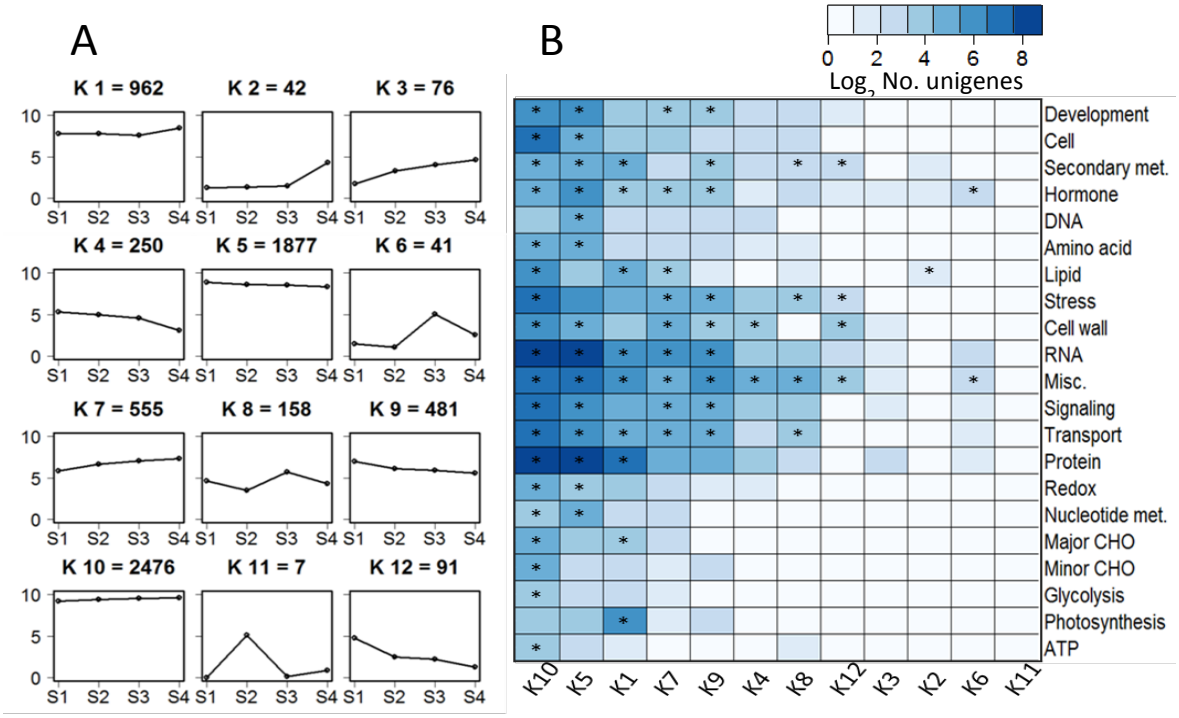


Figure 2: A. Clusters of differentially expressed genes among four stages. The y-axis is the base-2 logarithm of the mean gene counts. The number of genes in each cluster is indicated. B. The functional classification of all clusters. Clusters from left to right: from the most abundant K10 to the least abundant K11. * indicates statistically represented categories (p-value≤0.05).

Furthermore, in the hormone functional group, the highest number of DE unigenes was associated with auxin (Fig. 3). The largest changes in expression level across stage transition were observed for auxin-related genes (data not shown), highlighting the potential role of this hormone in regulating developmental processes leading to the attainment of ripening capacity.

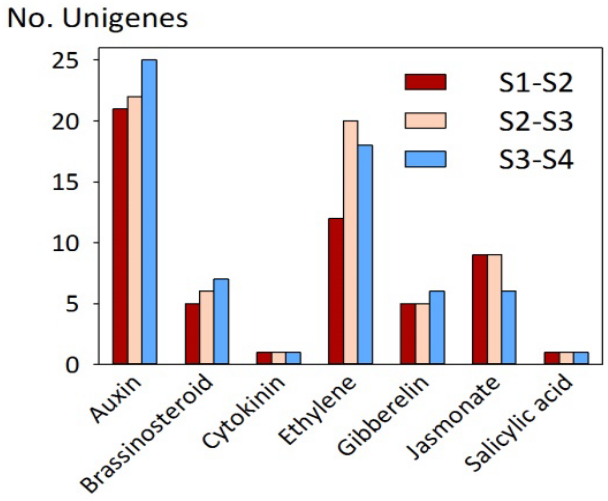


Figure 3: The number of genes related with plant growth regulators in three transitions: S1-S2, S2-S3, and S3-S4.

EXP. I: IDENTIFICATION OF CANDIDATES GENES AND VALIDATION

Twelve candidate genes were chosen from RNA sequencing data based on their functions, which were associated with plant growth regulators, transcription factors, and cell walls, and their expression values, which constantly increased from S1 to S4 in 2011 samples. However, for all of these candidates, we have thus far been unsuccessful in seeing one that induced a similar expression pattern in 2013 samples (data not shown). This implies the complexity of molecular pathways in the development of ripening capacity at different maturity stages. Future research work will include more validation of other candidates and coordination of candidate genes.

RESULTS OF EXP. II - FRUIT RIPENING DEVELOPMENT INDUCED BY TEMPERATURE CONDITIONING

EXP. II: POSTHARVEST PHYSIOLOGICAL CHARACTERISTICS

The purpose of low temperature treatments is triggering fruit to ripen rapidly and uniformly. As described in our 2013 report, fruit harvested at 18.0 lbs. and ripened immediately after harvest only softened to 16.9 lbs. after 6 days and ripened unevenly after 11 days at 68°F (Figure 4). Treatment at 32°F for 14 days allowed fruit to soften to 3.9 lbs after 6 days at 68°F, while treatment at 50°F for 5 days allowed fruit to soften to 2.3 lbs after 6 days at 68°F. Skin samples at harvest or immediately following the cold treatments, when the fruit firmness was still at 18-19 lbs. were used for RNA-seq analysis of gene expression.

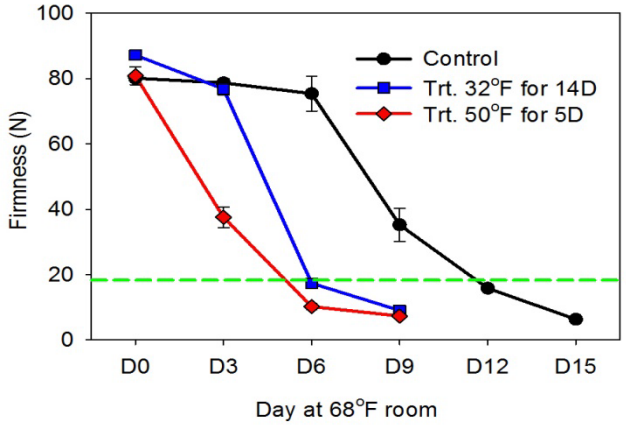
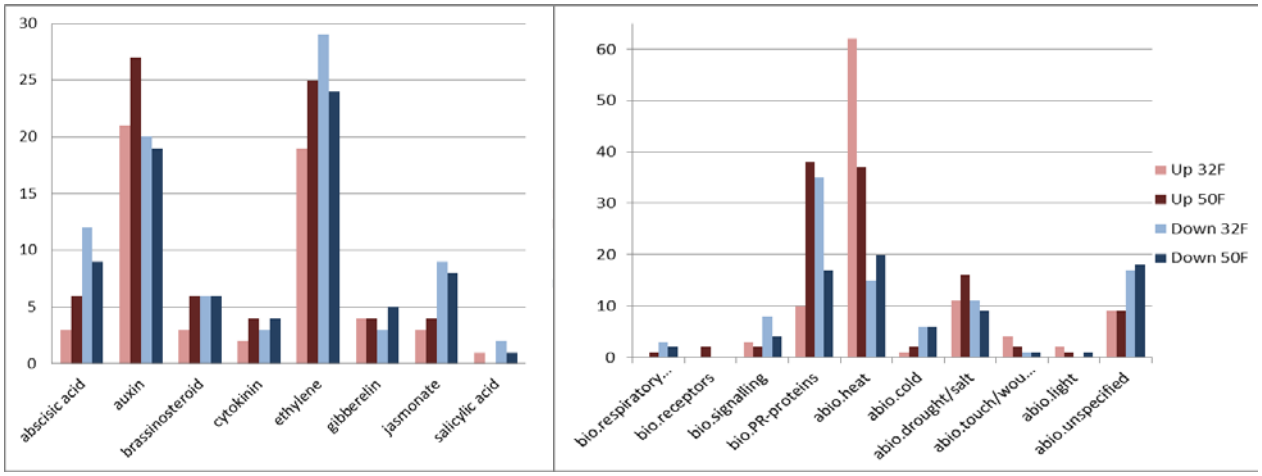


Figure 4: Firmness of fruit with or without temperature conditioning during ripening at 68°F (Sacramento, 2010). *The dashed green line indicates 4 lbs of firmness.*

EXP. II: PAIRWISE DIFFERENTIAL GENE EXPRESSION ANALYSIS and PRELIMINARY FUNCTIONAL ANALYSIS

Functional group classification was used to provide insight into the effect of different temperature conditioning treatments on ripening capacity. Genes with the greatest representation belonged to ethylene and auxin groups in the plant growth regulator group and to heat stress in the stress group (Fig. 5). By using this approach, the most abundant transcription factor groups, such as ethylene, (Ethylene-responsive element binding protein family and Basic Helix-Loop-Helix family) were also identified (graph not shown).



A **B**

Figure 5: Number of up-regulated (light and dark pink) and down-regulated (light and dark blue) genes in a) plant growth regulator, b) biotic and abiotic stress functional groups

Within the plant growth regulator group, most of the genes associated with jasmonic acid showed a reduction in expression after cold conditioning compared to the control. This suggested that jasmonic acid reduction may play an essential role in fruit ripening capacity development. We have completed physiological analysis of Experiment 3 to determine the effect of jasmonic acid, as well as other plant growth regulators including abscisic acid and auxin. This will be presented later in the Results of Exp. III.

In addition, most of the cold-induced genes observed did not show significant changes in gene expression levels. Nevertheless, we continue to examine the expression of these genes when the ethylene pathways are blocked (by SmartFresh treatment). We hope to find the connection between cold-induced pathways and ethylene pathways that play an important role in regulating the development of ripening capacity of 'Bartlett' pears during cold conditioning.

EXP. II: IDENTIFICATION OF CANDIDATES GENES AND VALIDATION

Exp. II was also repeated in 2012, 2013, and 2014. The validation results we have completed to date are presented in Table 3. From these results, similar changes in gene expression (comparable values in the fold changes) in several candidate genes were recorded among the years.

Genes associated with transcription factors (genes that control the expression of many other genes) and cold-induced pathways were validated in 2012 and 2013 samples. Among several genes related to cold-induced pathways and transcription factors that have been examined (Table 1), the cold-related gene CBF4 (this is also a transcription factor), and other transcription factors including TCP9b showed less significant changes with SmartFresh treatment. These genes might represent a node to connect cold-induced pathways and ethylene pathways. This would be beneficial for our understanding about the molecular mechanisms. The future approach can include finding the genes that are downstream of these transcription factors.

Table 1: Validation of gene expression of seven candidate genes in fruit from Lake County 2013.
Positive values indicate an increase in gene expression, negative values indicate a decrease in gene expression. Ctrl: Control

	Gene Expression Treatment/Control	EIN3 F-box	MAD-box	TCP9a	Dof zinc finger	TCP9b	CBF4	F-box	Zinc finger	ACO
Sac 2010	32^{o}F/Ctrl	1.18	2.10	1.63	-4.14	-3.75	-6.89	3.26	2.44	2.29
	50^{o}F/Ctrl	2.60	2.28	2.63	-5.44	-2.92	-4.59	4.97	2.91	3.29
Lake 2013	50^{o}F/Ctrl	0.98	1.39	1.94	-6.04	-3.49	-4.69	1.92	0.60	9.32
	SmartFresh & 50^{o}F/Ctrl	-1.65	3.51	1.65	-4.20	-2.61	-3.57	1.30	-1.95	2.54

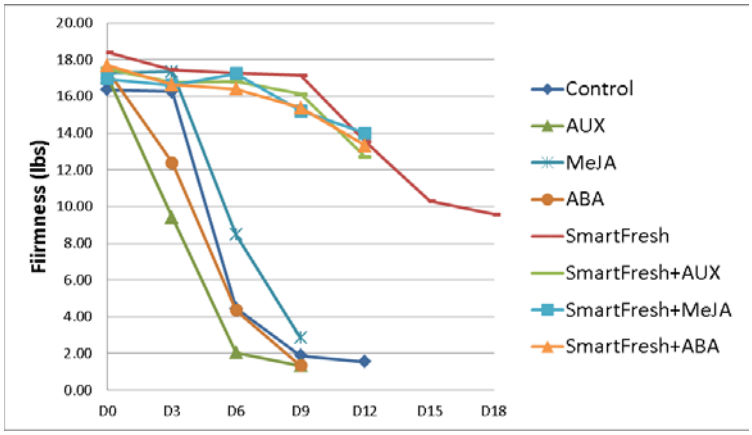
We have not yet been able to identify a gene candidate for a biomarker to predict ripening capacity. For all of the genes examined we have not found one gene that could be accurate for all of the postharvest treatments. However, we have observed that the gene expression of ACO could help us to estimate ripening capacity of fruit after cold

treatments and ethylene treatment, while EIN3 and Zinc finger can help to assist in checking if fruit would take a long time to ripen (about 2 weeks), similar to fruit from SmartFresh treatment.

The future research work is continuing validation of gene expression values in samples that were collected in 2014.

RESULTS OF EXP. III - FRUIT RIPENING DEVELOPMENT INDUCED BY PLANT GROWTH REGULATORS

Exp. III was completed in 2014 using fruit from Sacramento and Lake County. The results showed that MeJA inhibits ripening development while auxin and ABA facilitate ripening. However, when the fruit were treated with SmartFresh, the treatments of plant growth regulators made the fruit ripen faster than the SmartFresh control, but were not significantly different to each other. This suggests the interaction of MeJA and ethylene in the regulation of ripening development. The gene expression evaluation will help us to understand the crosstalk of these plant growth regulators in the development of ripening capacity.



Treatment	ANOVA
MeJA	a
Control	b
ABA	bc
Aux	c

Treatment	ANOVA
SmartFresh	a
SmartFresh+Aux	b
SmartFresh+ABA	b
SmartFresh+ MeJA	b

Figure 6: Firmness change of fruit during room storage after treated by plant growth regulators and/or SmartFresh. *The tables on the right show the statistic test for significant differences among treatments.*