Report to California Pear Advisory Board December 2013

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 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, two experiments were designed: Experiment I based on different fruit maturity, Experiment II based on different temperature conditioning treatments. The fruit from these treatments were evaluated for their physiological properties (firmness, color, and ethylene production) to determine ripening capacity (e.g. firmness reaches 3-4lbs after 6 days at 68°F). Samples collected during fruit development, and after temperature treatments were used for RNA-sequencing. The data obtained from RNA - seq were validated. Preliminary functional analysis showed that in addition to ethylene, auxin and jasmonic acid may play important roles in the regulation of ripening during fruit development and temperature conditioning, respectively. Genes related to softening and growth regulators are potential candidates to be biomarkers to predict ripening capacity. Future work includes further molecular analysis to understand the interaction among hormones associated with ripening and more validation on fruit from different harvest seasons, growth regions, and pear cultivars.

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. 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. Identify ethylene independent and cold induced genes which regulate ripening development.

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. Two experiments (Exp.I and II) were completed to assess the influence of fruit maturity and temperature conditioning treatments 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 also collected for molecular analysis. The procedures were described in detail in our 2012 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: 'Bartlett' pear fruit were harvested from three 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.

Treatments and fruit physiological evaluation: 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.

The samples from 2011 were sent for RNA-sequencing. Samples from similar experiment in 2013 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 same ripening capacity and changed with changes in ripening capacity.

EXPERIMENT II. Ripening capacity of fruit in response to different temperature treatments

Plant materials:

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. Following temperature conditioning, 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 temperature conditioning treatment were used for molecular analysis.

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 alone and independent of ethylene effects. The samples from Retain and SmartFresh treatments, with or without temperature conditioning, were also used for validation.

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 below. Downstream analysis of the sequencing provided a gene collection with their change in expression between samples. The significant increase and decrease in expression indicates the essential contribution of these genes to ripening capacity development.

RNA extraction: RNA was isolated from frozen fruit skin using the Qiagen RNeasy Plant Mini Kit® according to the manufacturer's instruction but with some modification.

RNA sequencing, transcriptome assembly and virtual gene expression values: RNA samples were submitted to Illumina Ultra High Throughput Sequencing (Illumina, Inc., San Diego, CA, USA) in the Genome Center at UCDavis (http://dnatech.genomecenter.ucdavis.edu/uhtsequencing.html).

Raw data, which are millions of sequences of 100 nucleotides, from the RNA sequencing analysis were checked for quality and assembled into longer fragments using Trinity (http://trinityrnaseq.sourceforge.net/). Counts of these virtual genes were then calculated using RSEM (http://deweylab.biostat.wisc.edu/rsem/README.html). The collection of these genes with their abundance provides us information about how the genes are expressed in large-scale (called transcriptome) during ripening capacity development of pear fruit.

Quality validation of virtual transcriptome: The virtual genetic fragments were actually assembled by a computer program. To test the reliability of this virtual approach, these sequences are aligned against the available Asian pear genome (*P. bretschneideri*

Rehd. cv. Dangshansuli) (http://peargenome.njau.edu.cn:8004/default.asp?d=1&m=1) using Gmap (http://research-pub.gene.com/gmap/) and European pear (*Pyrus communis*) genetic database (however this is very small and has only 3072 nucleotide sequences) using BioEdit (http://www.mbio.ncsu.edu/bioedit/page2.html). Furthermore, all the sequences obtained from the transcriptome assembly were compared with non-redundant, plant and Arabidopsis databases to determine a likely function of these sequences.

Differential expression analysis: The genes were analyzed for differences in expression between pear samples with different capacity to ripen using the EBSeq package http://www.biostat.wisc.edu/~kendzior/EBSEQ/. Quantitative PCR (qPCR), the typical method to validate the gene expression change obtained from the RNA sequencing approach was also processed to validate the relative fold change of genes of interest in treatment to control, which have been obtained from RNA-seq. After checking the consistency among different internal control genes (data not shown), EF1alpha was used as an internal control gene for both experiments.

K-means cluster analysis: This allows us to determine particular trends of gene expression during different stages of fruit development. The number of clustered and genes in each clustered were identified by using Figures of Merit application embedded in MEV (http://www.tm4.org/mev/.) and the package amap in R (http://cran.r-project.org/web/packages/amap/index.html), respectively.

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). These analyses permit an understanding of the mechanism of ripening capacity development and identify gene families that play essential roles in regulating pear ripening.

Validation in samples from different seasons and growing regions: This work is to check the consistency in the response of genes of interest that have been identified from RNA-seq data through different seasons and different growth areas. Samples were collected in 2012 and 2013 in orchards located in Sacramento and Lake Counties.

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 5 ripened to acceptable quality without ethylene

treatment. RNA that was extracted from pear tissues S1, 2, 3 and 4 were sent for sequencing.
Interestingly, fruit harvested from 2013 season developed the capacity to ripen with ethylene treatment right at 3 weeks before commercial harvest (Fig.1, 2013). 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 which will be explained later in this report.

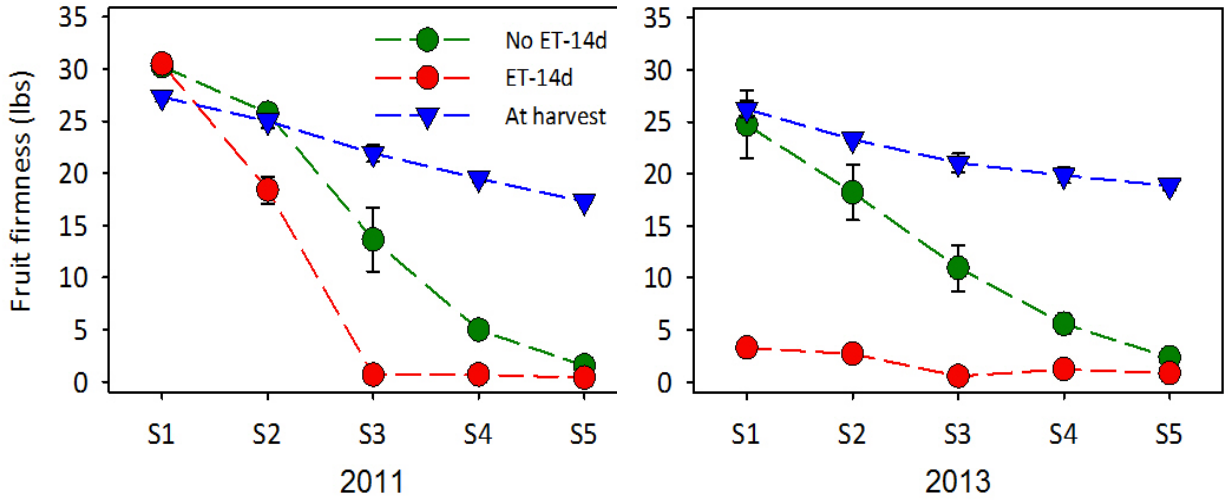


Figure 1: Fruit firmness at different developmental stages and following 14 days of ripening at 68°F with (ET-14d) or without (No ET-14d) ethylene treatment. Commercial harvest happened at S4 in 2011 (left) and 2013 (right)

EXP. I VALIDATION OF TRANSCRIPTOME
***(Note:** This part is presented for both Exp. I and II.)*
Identification: To achieve better results, the short fragments (length of 100 nucleotides) generated from RNA sequencing from both Exp. I (samples collected in 2011) and II (samples collected in 2010) were pooled together for assembly which generated 68,067 longer fragments called virtual genes (length of 201 – 18,868 nucleotides, mean length of 917). To check the assembly quality, these genes were aligned against published genetic databases of Asian and European pear. Mapping with the available Asian pear genome identified 11.81% possible non-gene structure sequences. Of the sequences that corresponded to ethylene pathways, 93-98% of the virtual fragments from 'Bartlett' pears were identical to previously isolated sequences. In addition, putative function of 37.4% and 41.5% of virtual sequences were identified based on the alignment with Arabidopsis and plant databases, respectively. The results have confirmed the reliability of this sequencing technology and assembly methods.

Quantification: The number of fold changes (FC) of genes of interest in Exp. I and Exp. II were validated by using qPCR. These genes of interest are transcription factors associated to growth regulators and cell walls. The high correlation values (R^2 = 0.95 in

Exp I, R^2= 0.98) in the regression analyses between FC gained from RNA sequencing and FC gained from qPCR in Exp. I and Exp. II indicates the reliability of expression values obtained from RNA sequencing (graphs not shown).

EXP. I PAIRWISE DIFFERENTIAL GENE EXPRESSION ANALYSIS

Genes that are significantly expressed (differentially expressed genes = DE genes) between samples of pears with varying ripening capacity were identified.
Table 1 shows that as the fruit develop further on the tree, there are greater changes in gene expression. The number of DE genes increase from 2505 to 4785 DE at stage S2 to S4 compared to S1. There are more genes involved in the transition S3-S4 and S1-S2 than the earlier S2-S3.

Table 1: Number of DE genes between two developmental stages.

	S1	S2	S3	S4
S1	X	2505	3397	4785
S2	2505	X	2037	3105
S3	3397	2037	X	2805
S4	4785	3105	2805	X

EXP. I PRELIMINARY FUNCTIONAL ANALYSIS

The preliminary functional analysis was processed based on likely functions of DE genes in both experiments. This analysis provides us general ideas about the transcription profile during ripening capacity development, either on trees or by temperature conditioning.

Differential expression analysis on four different stages of development revealed 7016 DE genes. These genes were grouped into 12 clusters (Table 2 and Fig. 6). The highest number of genes belong to clusters in which gene expression continuously increases (C10 and C7) or decreases (C5, C9, and C4) from fruit harvest S1 to S4. As with the physiological data, at S3, when the fruit develop the ability to respond to ethylene (ripen with ethylene treatment and do not ripen without ethylene),Cluster C1 may contain genes that are significant to the developmental transition between fruit that can ripen with ethylene treatment (S3) as compared with fruit that cannot ripen regardless (S1) due to their significant decrease in expression value at this stage. Genes in the group C10 and C7 might be good candidates to detect ripening capacity.

Table 2: Number of genes in each cluster

Cluster	No. genes	Percentage
C10	2476	35.29
C5	1877	26.75
C1	962	13.71
C7	555	7.91
C9	481	6.86
C4	250	3.56
C8	158	2.25
C12	91	1.30
C3	76	1.08
C2	42	0.60
C6	41	0.58
C11	7	0.10

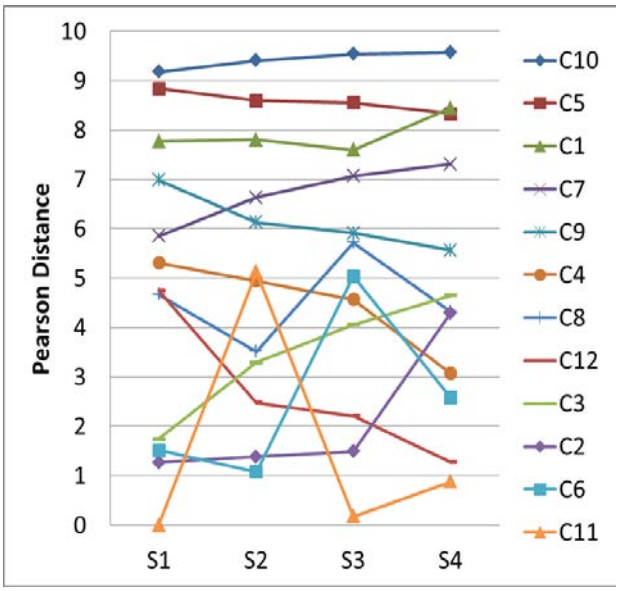


Figure 2: Gene expression pattern of 12 clusters

Using Mercator – Mapman, DE genes were classified into different functional groups such as cell wall, growth regulators, photosynthesis, transcription factors and stress. The number of genes in the each category was then calculated and some of them are presented in Fig. 7. A high number of genes were related to auxin and ethylene within the growth regulator group. The abundance of WRKY and Aux/IAA (auxin associated) transcription factor families in DE genes at the transition S2-S3, when the fruit developed perception to ethylene, indicate that these two families may play essential roles for the fruit response to ethylene.

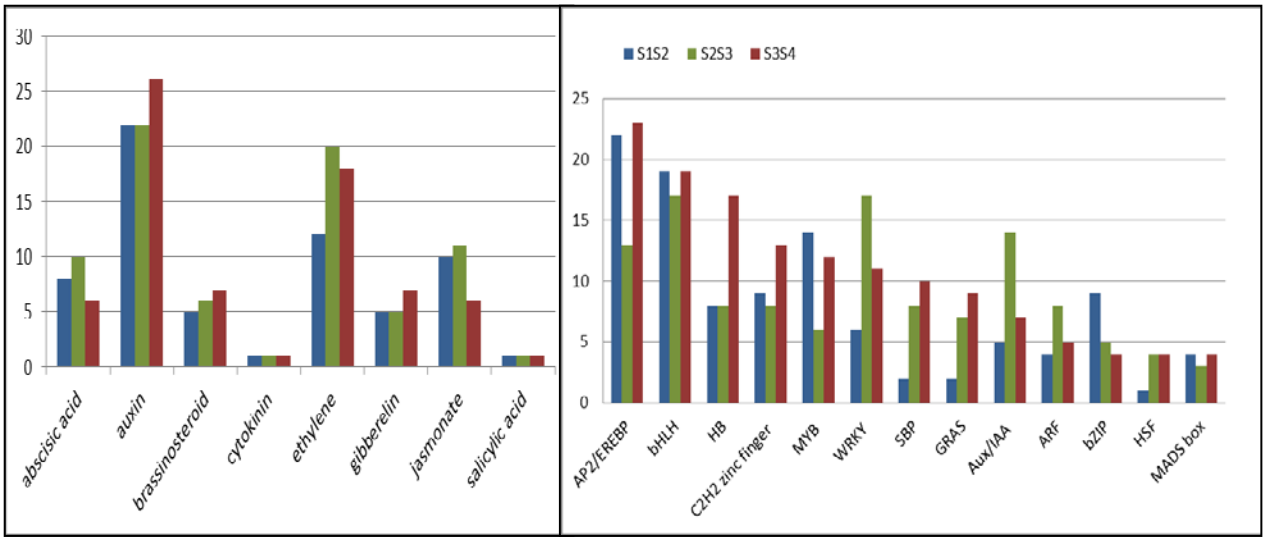


Figure 3: Number of DE genes between two adjacent stages of development (S1-S2, S2-S3, and S3-S4) in growth regulator (top) and transcription factor (bottom) groups in Mapman.

EXP. I: IDENTIFICATION OF CANDIDATES GENES AND VALIDATION

As stated previously in the report, Exp. I was repeated in 2013 and generated fruit with different response. Samples for validation were chosen based on the ability of the fruit to ripen with ethylene and without ethylene (S1 of 2013 is similar to S3 of 2011: ripen after 14 days at 68°F with ethylene treatment, S4 of 2013 is similar to S4 of 2011: ripen after 14 days at 68°F without ethylene treatment). Twelve candidate genes were chosen based on their functions, which are associated with growth regulator transcription factor and cell walls, and their expression values, which constantly increased from S1 to S4 in 2011 samples. These genes were quantified in 2013 samples. However, for all of these candidates, we have 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 2012 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.

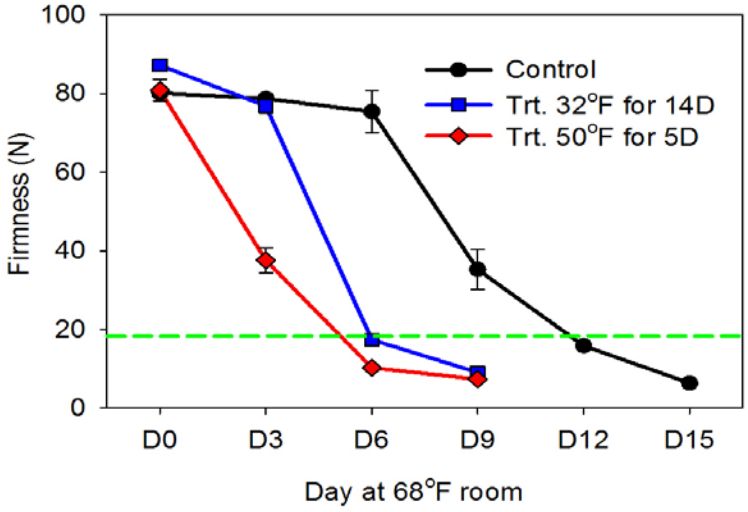


Figure 4: Firmness of fruit with or without temperature conditioning during ripening at 68°F (Sacramento, 2010). Green line indicates 4 lbs of firmness.

EXP. II: PAIRWISE DIFFERENTIAL GENE EXPRESSION ANALYSIS

The effect of temperature conditioning on gene expression was also examined. High numbers of shared differentially expressed (DE) genes (Fig. 3) and a significant correlation value (R^2 = 0.6851, P<.001) from regression analysis of expression fold change between 32°F - Control and 50°F - Control implies a high similarity at the molecular level in pathways regulating ripening capacity in two different temperature treatments. Candidates for ripening capacity are chosen based on their shared sequences with high expression values and their functions as transcription factors.

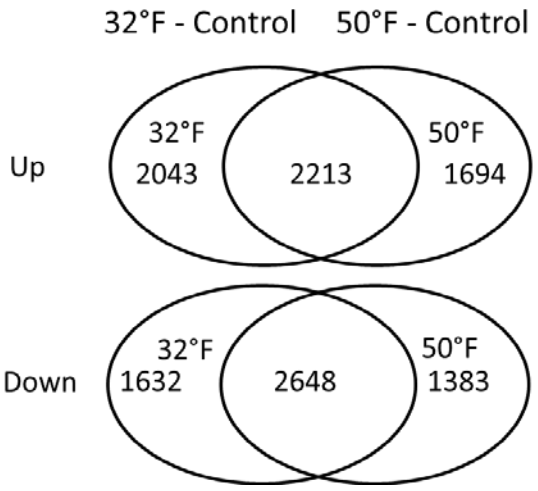
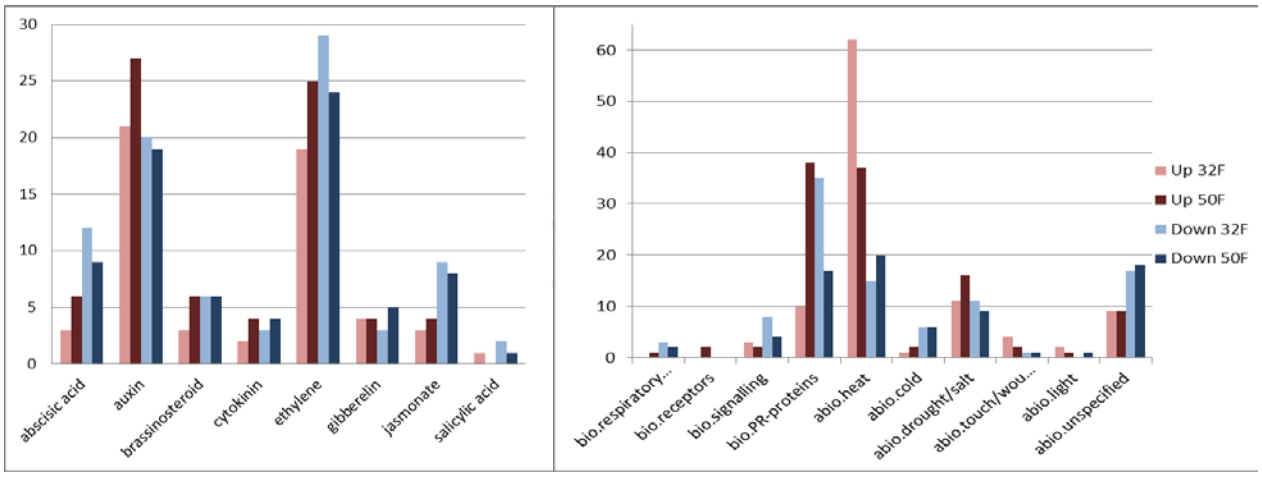


Figure 5: *Number of up and down regulated genes in* 32°F *and* 50°F *treatments compared to Control*

EXP. II: PRELIMINARY FUNCTIONAL ANALYSIS

Mapman also was used to provide insight into the effect of different temperature conditioning treatments on ripening capacity. Genes with the greatest representation in were found to belong to ethylene and auxin groups in growth regulator group and heat stress in stress group (Fig. 8). By using this approach, most abundance 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 6. Number of up-regulated (light and dark pink) and down-regulated (light and dark blue) genes in a) growth regulator, b) biotic and abiotic stress functional groups

Interestingly, within the 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.

EXP. II: IDENTIFICATION OF CANDIDATES GENES AND VALIDATION

Exp. II was also repeated in 2012 and 2013. The validation results we have completed to date are present in Table 3. From these results, a similar change in gene expression (comparable values in the fold changes) in many candidate genes was recorded.

Table 3: Validation of gene expression of seven candidate genes in different seasons and different growth areas (blue color: 32°F, red color: 50°F)

		Pr23	Pr26	Pr28	Pr29	Pr31	Pr32	Pr33
Sacramento 2010	32°F - Control	2.10	1.63	0.79	1.85	-4.14	1.72	0.26
	50°F - Control	2.28	2.63	2.58	1.97	-5.44	2.03	1.18
Lake 2012	32°F - Control		2.19			-5.78		
	50°F - Control		2.86			-5.15		
Sacramento 2013	32°F - Control							
	10C - Control	1.81	1.95	2.34	1.10	-6.20	0.94	0.74
Lake 2013	32°F - Control		1.57					
	50°F - Control	1.39	1.94		1.15	-6.04	1.72	

In 2013 season, Retain and SmartFresh were utilized to block ethylene pathways and generate fruit resistant to ripening. These two chemicals both delayed softening of the fruit (data not shown). Future work will include determining expression values of our candidate genes in these samples. We hope to find genes down regulated in Retain and SmartFresh samples which imply resistance to ripening, compared to the control.

In addition to treatments with Retain or SmartFresh alone, temperature conditioning was added after these treatments in 2013 season. These combination treatments were used to determine if gene expression changes are ethylene-dependent and/or cold-dependent. From the validation, we identified genes coded Pr26 (and Pr31) which have increased (and decreased) fold changes in the effect of temperature, but less significant changes with Retain and SmartFresh. 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 which are downstream of these transcription factors.

Table 4: Validation of gene expression of five candidates in different postharvest treatments

		Pr23	Pr26	Pr29	Pr31	Pr32
Sacramento 2013	50°F - Control	1.81	1.95	1.10	-6.20	0.94
Sacramento 2013	(Retain+50°F) - Control	1.89	1.96	0.91	-5.68	0.02
Sacramento 2013	(1-MCP+50°F) - Control	3.19	1.37	1.61	-5.09	1.63
Lake 2013	50°F - Control	1.39	1.94	1.15	-6.04	1.72
Lake 2013	(Retain+50°F) - Control	1.52	1.88	1.07	-5.43	1.60
Lake 2013	(1-MCP+50°F) - Control	3.51	1.65	2.05	-4.20	3.52

The future research work is continuing validation of gene expression values in samples that have been collected in 2012 and 2013 and new samples that will be collected in 2014. We are also interested in deeper molecular analysis to understand growth regulator crosstalk and molecular networks of ripening development. The European pear genome that will be published soon will help us in validating the identity of our virtual genes and identifying critical genomic regions which contain multiple genes associated with ripening capacity development.