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 development on the tree (different harvest time), Experiment II based on different temperature treatments after harvest (32 and 50°F). 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 after harvest (Exp. I), and after temperature treatments (Exp II) were used for RNA-sequencing. The preliminary results showed that there are some potential candidates to detect ripening capacity such as green-ripen gene. A more thorough analysis, including a time course analysis for Exp. 1 and quantitative-PCR, will be made to confirm the expression of marker genes. Validation with fruit from different locations and maturities, and following different postharvest treatments will be carried out to be confident about these markers ability to predict fruit response.

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, and its precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) in fruit tissues. While treatment with exogenous ethylene and/or chilling temperatures can stimulate production of ACC and ethylene 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 is variable from season to season. The variability appears to be due, in part, to production of ethylene by the pear fruit during treatment. However, there may been 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 DNA sequencing provides an exciting opportunity to rapidly 'mine' the pear genome to look for markers of ripening competence. Two years ago, we began an analysis of the gene expression of pear fruit with and without the ability to ripen with a goal to identify marker genes that can predict fruit capacity to ripen. We are continuing to analyze and identify these genetic sequences with a view to narrowing our search to select candidate genes or proteins with potential to rapidly and accurately predict ripening responses of 'Bartlett' fruit.

OBJECTIVES

1. Determine the relative changes in gene expression to help select the best candidates to predict ripening capacity.

2. Identify promising candidate genes as markers of fruit ripening capacity.

3. Determine the reliability of candidate genes to predict ripening capacity in fruit from different districts and in response to postharvest treatments.

MATERIALS AND METHODS

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. Our rationale was to identify changes in key physiological and molecular processes that were closely associated with the onset of ripening capacity. This approach will lead to a deeper understanding of pear ripening capacity development and ultimately aid the selection of molecular markers to predict ripening capacity.

EXPERIMENT I. Ripening capacity of fruit induced by development on the tree
The procedures were described in detail in our 2011 report to the California Pear Advisory Board and are outlined briefly below.

Plant materials: 'Bartlett' pear fruit were harvested from three representative trees in a commercial orchard near Walnut Grove, California. Fruit were picked at random every 6-7 days for 5 weeks to capture different stages of development (Figure 1). Fruit harvested at week 4 coincided with the first commercial harvest of the season.
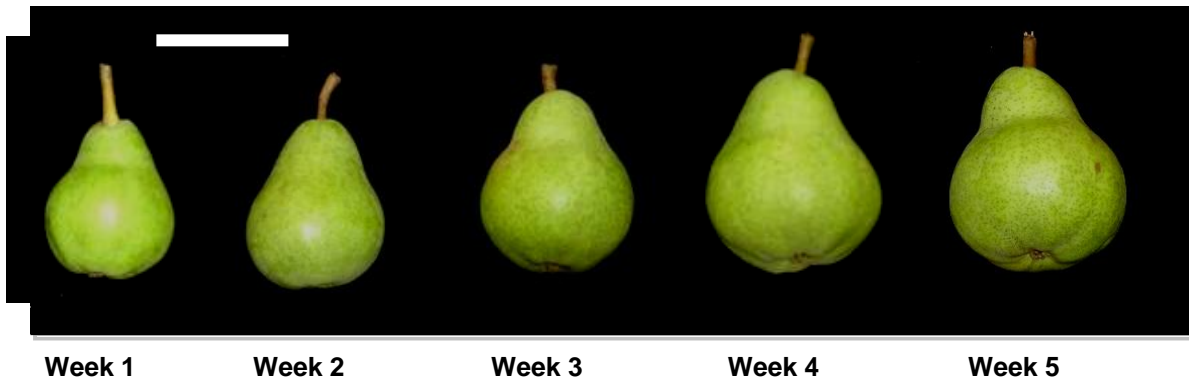

| Week 1 | Week 2 | Week 3 | Week 4 | Week 5 |

Figure 1. 'Bartlett' fruit immediately after harvest (The horizontal bar represents 2 inches.)

General Processing: Peel samples were collected from 12 representative fruit at each harvest time and then stored at -80°C prior to RNA extraction and molecular analysis (see below). All remaining fruit were randomly assigned to treatments for assessing ripening capacity.

Treatments: 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 Evaluations: Fruit weight, diameter, firmness, skin color, soluble solids content, internal ethylene concentration, ethylene production and respiration rate were measured at harvest or during ripening.

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

Plant materials: 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 were collected

from fruit at harvest and at the completion of each postharvest treatment. These samples were then stored at -80°C prior to RNA extraction and molecular analysis (see below).

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. The RNA sequencing approach permits estimation of the number of copies of each genetic sequence in the sample. This provides an indication of the abundance and relative contribution of each gene to ripening capacity. Briefly, RNA was extracted from the peel samples and sequenced as described below. Genetic sequences showing a considerable (e.g. at least 2-fold) increase in expression during the development of ripening capacity were identified. Genes showing the greatest increase in expression will be selected as potential markers of ripening capacity and will be validated in additional experiments.

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 (RNA-Seq): To determine the expression values of up-regulated genes (increased expression into proteins), total 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).

Transcriptome assembly and contig/unigene counts: Raw data which are millions of sequences of 100 nucleotides from the RNA-seq analysis were checked for quality and assembled into longer fragments called "contigs" using Trinity (http://trinityrnaseq.sourceforge.net/). The contigs were then clustered into groups thought to represent the same gene, based on the similarity of the sequence. The contigs and gene counts were then calculated using RSEM (http://deweylab.biostat.wisc.edu/rsem/README.html). The collection of all contigs/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.

Differential expression analysis: The contigs and 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/. This package allows us to process either pairwise comparison or time course comparison.

Functional analysis: The contigs and genes are virtual genetic fragments created by computer assembly. These sequences are aligned against the available European pear (*Pyrus communis*) genetic database (even this has only 3072 nucleotide sequences) to test the reliability of this virtual approach, 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. 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.

RESULTS

The influence of fruit maturity and temperature conditioning treatments on ripening capacity

Exp. I: *Ripening capacity of fruit induced by development on the tree*
The physiological response of fruit harvested at different stages of maturity are described in the our 2011 Report to the Pear Advisory Board and are summarized as follows. Fruit harvested at week 1, 3 weeks before the first commercial harvest, failed to ripen in 14 days at 68°F even when exposed to 100 ppm ethylene (Figure 2). Fruit gradually developed a capacity to ripen in response to ethylene treatment by the third harvest, 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 weeks 4 and 5 ripened to
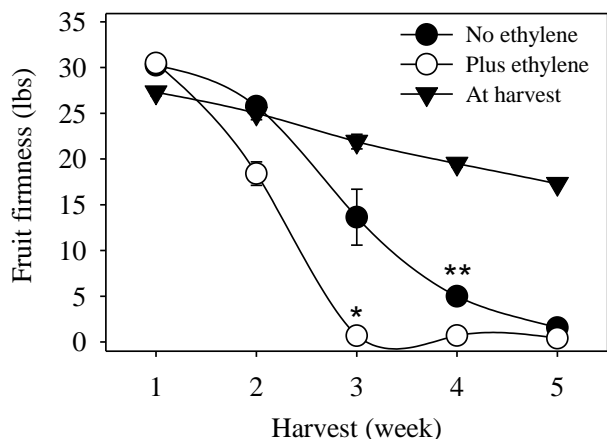
acceptable quality without ethylene treatment. RNA that was extracted from pear tissues sampled at harvest week 1, 2, 3 and 4 were sent for sequencing.

Figure 2. The firmness of 'Bartlett' pear fruit at harvest and following 14 days of ripening at 68°F with (Plus ethylene) or without (No ethylene) ethylene treatment. *Represents when fruit developed a capacity to ripen when exposed to ethylene. **Represents the first commercial harvest date.

Exp. II: *Ripening capacity of fruit induced by different temperature treatments*
The purpose of temperature treatments is triggering fruit to ripen rapidly and uniformly. Fruit harvested at 18.0 lbs and ripened immediately after harvest only softened to 16.9 lbs after 6 days (Trt. A) and ripened unevenly after 11 days at 68°F. Temperature conditioning treatments at 32°F and 50°F were able to induce different degrees of ripening capacity. Treatment at 32°F for 14 days (Trt. B) allowed fruit to soften to 3.9 lbs after 6 days at 68°F, while treatment at 50°F for 5 days (Trt. C) allowed fruit to soften to 2.3 lbs after 6 days at 68°F. Skin samples at harvest (Trt. A) or immediately following the cold treatments (Trt. B and C), when the fruit firmness was still at 18-19 lbs were used for RNA-seq analysis of gene expression.

Transcriptome assembly for pear ripening capacity
The raw data of RNA-seq from each experiment were pooled together for better assembly. The short fragments (length of 100 nucleotides) were assembled into longer ones. Following assembly, the virtual fragments were aligned against published European pear genetic databases. 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, our sequences were aligned against known sequences from the NCBI library to identify a putative function. The 'Bartlett' pear sequences were most closely matched with species such as *Vitis vinifera* (grape), *Glycine max* (soybean), and Arabidopsis – the species for which genome sequence data are available. This data confirmed the reliability of the new sequencing technology and assembly methods.

Preliminary Analysis of Gene Expression Data
NOTE: The following results are the initial analysis of gene expression data. Other approaches for data analysis are being tested to achieve meaningful and thorough conclusions.

Determine the relative changes in gene expression

The EBSeq package was utilized first to determine differences in the gene expression between samples of pears with varying capacity to ripen using pairwise comparisons. The results for each experiment are shown below.

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

Table 1. The number of contigs substantially up-regulated or down-regulated (with a change ≥ 2) in 'Bartlett' pears harvested at weeks 2, 3 and 4 relative to fruit harvested at week 1.

	Week 2	Week 3	Week 4
Up-regulated	212	363	479
Down-regulated	251	343	683

From this table we can see that as the fruit develop further on the tree, there are greater changes in gene expression, with many genes expressed more, but also many genes expressed less as the fruit developed ripening capacity.

Exp. II: Ripening capacity of fruit induced by different temperature treatments

Table 2. The number of contigs substantially up-regulated or down-regulated (with a change ≥ 2) in 'Bartlett' pears exposed to 32°F for 14 days (Trt. B) or 50°F for 6 days (Trt. C) relative to fruit at harvest (Trt. A) - the fruit without the capacity to ripen after 6 days in 68°F

	Trt B 32F for 14 days	Trt C 50C for 6 day
Up-regulated	1209	1090
Down-regulated	1731	1341

Identify promising candidate genes

From Exp. II, the genes showing the greatest changes in expression are considered strong candidates for molecular markers of ripening capacity. Our very preliminary analysis described one of these genes as a green-ripe protein and another was related to sorbitol breakdown. Data analysis will be continuing on the data for Exp. II. The approach for the data from Exp. I will be described under Future Work.

Understanding the mechanism of ripening capacity development

At this time, only the data set from Exp. II (temperature treatments) has been analyzed. The results provide an insight into the effect of different ripening treatments on ripening capacity. Using Mercator – Mapman, contigs, which were differentially expressed (the potential candidate genes) were classified into different functional groups such as cell wall, hormones, photosynthesis, transcription factors and stress. The number of contigs in the same categories was calculated (Figures 4 and 5). Contigs with the greatest representation in hormone-related families were found to belong to ethylene and auxin groups. Additionally,15 contigs up-regulated in Trt. B – 32°F for 14 days (compared to Trt. A) were related to abiotic stress. High numbers of transcription factors were up-regulated during development of ripening capacity and were grouped to AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family, bHLH,Basic Helix-Loop-Helix family, C2C2(Zn) CO-like, Constans-like zinc finger family, and C2C2(Zn) DOF zinc finger family.
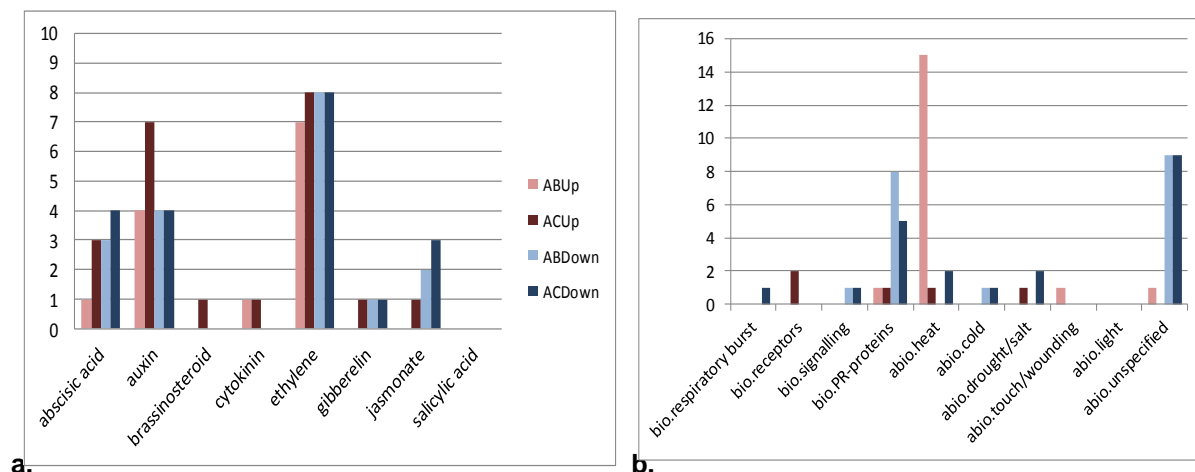

Figure 4. Number of up-regulated (light and dark pink) and down-regulated (light and dark blue) genes in each functional group: a) hormones, b) biotic and abiotic stress.
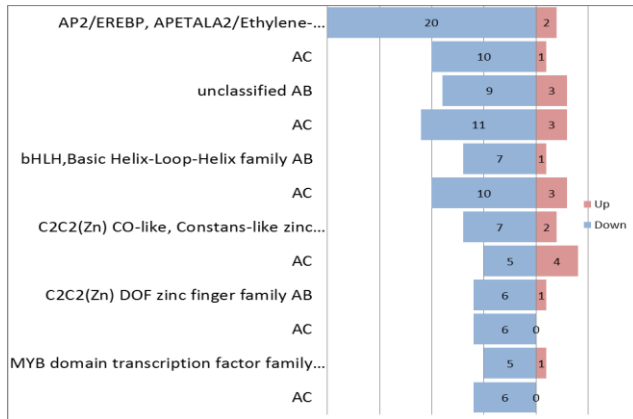
Figure 5. Number of transcription factor contigs up-regulated (light pink) and down-regulated (light blue) in two comparisons: AB (Trt. B and A), AC (Trt. C and A).

FUTURE WORK

From Exp. I, we plan to utilize a time course analysis to determine the expression pattern for the most promising genes with potential to be used as markers of ripening capacity. For example, genes that consistently increase with advancing harvest time represent potential candidates to detect ripening capacity. Moreover, shared candidate genes between the two experiments will be identified once the time course analysis of Exp. I is completed.

We will confirm the differential gene expression data obtained via RNA-seq by measuring the relative expression of the candidate genes using quantitative PCR, the typical method to validate the gene expression change obtained from the RNA-seq approach.

Furthermore, our objective is to determine the reliability of the selected candidate genes for their ability to precisely predict ripening capacity of fruit from the different growing districts and in response to various postharvest treatments. Once the most promising candidate genes have been selected, identified and validated by quantitative PCR, we will cross-check to determine if these genes can be relied on to predict ripening capacity for additional fruit from different growing districts. We will also evaluate the reliability of the candidate genes as markers of ripening capacity for fruit from the different growing districts and in response to various postharvest treatments (i.e. temperature conditioning, 1-methylcyclopropene; 1-MCP). Our core objective is to identify marker genes for a commercial detection kit for ripening competence.