Using Molecular Tools To Predict Ripening Capacity Of ‘Bartlett’ Pears

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ABSTRACT

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 capable of predicting the variable ripening behavior in early-season pears. To induce different ripening capacity in 'Bartlett' pears, three experiments were designed: Experiment I based on different fruit maturity, Experiment II based on different temperature conditioning treatments, and Experiment III based on different plant growth regulators. The main results of the projects are presented below:

1. Gene expression profiles associated with the development of ripening capacity during fruit growth and development, as well as by cold conditioning, were characterized.
2. Functional analysis of RNA-sequencing data from samples collected during fruit growth and development suggested that auxin may be essential in regulating the transition of pear fruit from being ethylene-unresponsive to ethylene-responsive, which then resulted in fruit softening. Cell wall genes and transcription factors (a gene that regulates many other genes) associated with the fruit’s responsiveness to exogenous ethylene application or the capacity to produce ethylene and ripen were also identified.
3. Functional analysis of RNA-sequencing data from cold-conditioned pear fruit samples identified genes related with jasmonic acid metabolism, cold-induced pathways, and transcription factors. Using SmartFresh (1-MCP) treatments, which inhibited ethylene production and delayed fruit ripening, we determined which of these genes were dependent on ethylene and which acted independently of ethylene.
4. The effects of plant growth regulators on the development of ripening capacity were determined and validated. Jasmonic acid inhibits ripening while auxin and abscisic acid facilitate ripening.
5. A model to predict pear fruit ripening capacity, based on gene expression at harvest, was established for ACO, a gene encoding an ethylene synthesis

enzyme. Two ethylene-related genes (ETR2 and EBF1) were also found to potentially predict ripening capacity in fruit treated with 1-MCP.

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™ 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 (Jasmomic acid - JA, abscisic acid - ABA, and auxin/indole-3-acetic acid - IAA) independent and dependent of ethylene in the regulation of pear ripening.

PROCEDURES

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 and 2014 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 the trees every 6-7 days for 5 weeks to capture different stages of development. Fruit were 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 firmness, ethylene production (and other characteristics such as skin color and soluble solids content) 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.

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 County of California. Fruit were conditioned at 32, 41, 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 (1-MCP) treatments were applied to another set of fruit after harvest to block ethylene pathways before cold conditioning. The purpose of Retain and SmartFresh (1ppm 1-MCP) is to block the ethylene pathway 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 application, 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 pear samples from postharvest application of Retain and SmartFresh, with or without temperature conditioning, 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 methyl jasmonate (MeJA), ABA, and IAA. Group 2 was treated with SmartFresh (1ppm 1-MCP) to block the ethylene pathways, and then MeJA, ABA, and IAA. A high 1-MCP concentration was applied to isolate the effect of these plant growth regulators from the effect of ethylene. 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.
Season 2015: Experiment of 2014 was repeated for validation.

Gene expression evaluation: Peel samples collected from fruit at harvest and at the completion of each treatment were used for molecular analysis.

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.

Differential expression analysis: The genes were statistically analyzed for differences in expression between pear samples with different capacity to ripen. A significant increase or decrease in expression associated with changes in pear ripening indicates the essential contribution of these genes to ripening capacity development.

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

This study was recently published in BMC Genomics, open access journal: *NT Nham, ST de Freitas, AJ Macnish, KM Carr, Tkietikul, AJ Guilatco , C-Z Jiang, F Zakharov, EJ Mitcham.* ***A transcriptome approach towards understanding the development of ripening capacity in 'Bartlett' pears (Pyrus communis L.)*** *BMC Genomics 10/2015; 16(1):762. DOI:10.1186/s12864-015-1939-9*
http://www.biomedcentral.com/1471-2164/16/762

The objective of Exp. I was to characterize changes in ripening capacity during fruit maturation and transcription profiles leading to attainment of ripening capacity. Results were presented in the 2014 report. We only summarize the main results of Exp. I in this report to set the stage for additional results presented.

The softening response of pear fruit held for 14 days at 68 °F after harvest depended on their maturity. We identified four maturity stages: S1-failed to soften and S2- displayed partial softening (with or without ethylene treatment); S3 - able to soften following ethylene treatment; and S4 - able to soften without ethylene treatment. High expression of genes putatively encoding pectin degradation enzymes in the S1-S2 transition suggests pectic oligomers may be involved as early signals triggering the transition to responsiveness to ethylene in pear fruit. Moreover, the co-expression of these genes with *Expansin* genes suggests their collaboration in modifying cell wall polysaccharide networks that are required for fruit growth. Cluster analysis revealed that auxin signaling associated transcripts were enriched in cluster K6 that showed the highest gene expression at S3. *AP2/EREBP* (*APETALA 2/ethylene response element binding protein*) and *bHLH* (*basic helix-loop-helix*) transcripts were enriched in all three transitions S1-S2, S2-S3, and S3-S4. Several members of Aux/IAA (Auxin/indole-3-acetic acid), ARF (Auxin response factors), and WRKY appeared to play an important role in orchestrating the S2-S3 transition, when fruit became capable of ripening if ethylene was added. A summary of the proposed mechanisms regulating ripening capacity in Bartlett pears is shown in Figure 1.

In conclusion, we identified maturity stages associated with the development of ripening capacity in ‘Bartlett’ pear, and described the transcription profile of fruit at these stages. Our findings suggest that auxin is essential in regulating the transition of pear fruit from being ethylene-unresponsive (S2) to ethylene-responsive (S3), resulting in fruit softening. The transcriptome will be helpful for future studies about specific developmental pathways regulating the transition to ripening.

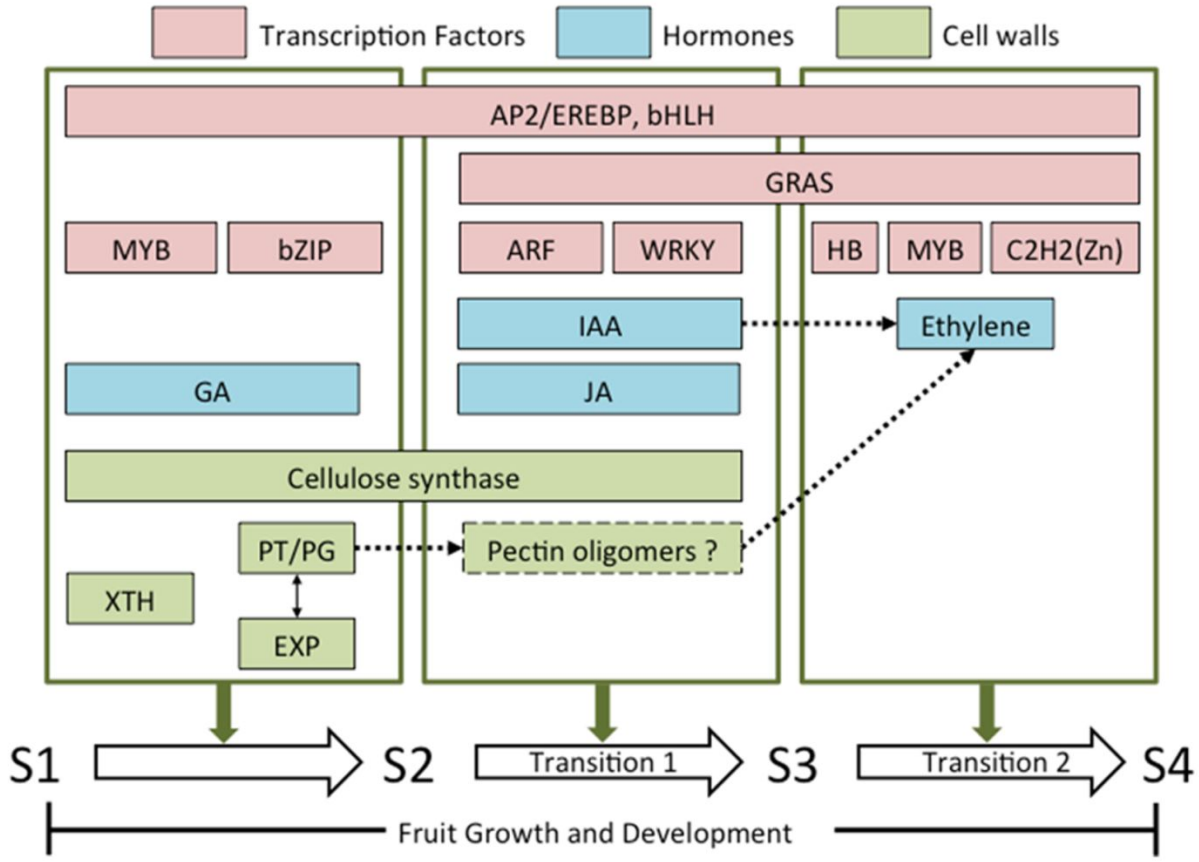

Fig. 1: Proposed mechanisms regulating ripening capacity development during the final stages of pear fruit growth. Transition 1: Fruit develop ripening capacity when treated with ethylene; Transition 2: Fruit develop ability to ripen without ethylene treatment. *AP2/EREBP: APETALA 2/ethylene response element binding protein, bHLH: basic helix-loop-helix, bZIP: basic region/leucine zipper, ARF: Auxin response factors, HB: homeobox, C2H2(Zn): Cys_2His_2 Zinc finger; GA: Gibberellin, JA: Jasmonic acid, IAA: Auxin/indole-3-acetic acid; XTH :Xyloglucan endotransglucosylase/hydrolase , PT/PG: pectin lyase/pectate lyase/polygalacturonase, Exp: Expansin.*

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

EXP. IIA: COLD CONDITIONING TREATMENTS INDUCING FRUIT WITH DIFFERENT RIPENING CAPACITY

The purpose of cold conditioning treatments is to trigger fruit to ripen rapidly and uniformly. In 2010, the control- fruit without any cold treatment took about 12 days to

ripen while treatment at 32 °F for 14 days and 50 °F for 5 days was able to induce full ripening capacity - fruit reached firmness of approximately 4 lbs in 6 days (Fig. 2). RNA collected from these three samples was subjected to RNA-sequencing. In 2013, less time in the cold was required to induce full ripening capacity (Fig. 2). Fruit without any cold treatments took merely 9 days to ripen, while treatments of 32°F for 7 days and 50°F for 3 days induced full ripening capacity (Fig. 2).

Fig. 2: Firmness and ethylene production rate of selected cold-conditioned treatments during ripening at 68 °F. The 1ppm 1-MCP treatment in 2013 is to eliminate the effects of ethylene on these fruit and therefore tease out responses due to cold that are NOT related to ethylene.

EXP. IIB: EXAMINING FUNCTIONS OF GENES ASSOCIATED WITH JASMONIC ACID METABOLISM, COLD-INDUCED PATHWAYS, AND TRANSCRIPTION FACTORS

From the data obtained from the RNA-sequencing on cold-conditioned samples, genes with functions of interest and showing high up-regulation or down-regulation (increases and decreases in expression) in the cold conditioning treatments were selected for

further analysis. In this report, we presented a few genes among the many genes that we have examined.

Table 1: Selected genes associated with ethylene and jasmonic acid, cold-induced pathways and transcription factors (Log2FC RNA-Seq: Log 2 fold change from RNA sequencing data)

Gene ID	Function	Log2FC RNA-Seq		Group
		32F/Ctrl	50F/Ctrl	
PcM_59277	ACO (ethylene biosynthesis)	2.29	3.29	Ethylene
PcM_61151	ETR2 (ethylene receptor)	2.32	3.28	
PcM_57563	ERS1a (ethylene receptor)	1.22	2.26	
PcM_60321	EIN3-binding f-box protein (EBF1)	1.26	2.44	
PcM_40167	allene oxide cyclase (AOC1)	-1.97	-1.92	Jasmonic acid
PcM_36557	allene oxide cyclase (AOC2)	-4.30	-3.50	
PcM_44588	allene oxide synthase (AOS)	-2.64	-2.36	
PcM_40485	Calcium-dependent lipid-binding (CaLB domain)	-1.68	-1.07	Cold-related
PcM_51546	Calcium-dependent lipid-binding (CaLB domain)	-1.47	-0.98	
PcM_67226	CBF1 (C-repeat binding factors)	-6.78	NA	
PcM_49115	CBF4 (C-repeat binding factors)	-7.64	-2.74	
PcM_35299	AGL-24 (Agamous - like 24)	2.25	2.26	Transcription factors
PcM_53369	Winged-helix DNA-binding TF	3.35	2.38	
PcM_46371	TCP9a	1.84	2.48	
PcM_37893	TCP9b	-3.71	-2.94	

Ethylene genes: All of the examined ethylene-related genes were up-regulated by cold treatments (Table 1). In the samples collected in 2013, *ACO* transcript showed a higher

abundance in fruit conditioned with low temperature; however, *ERS1a* was up-regulated only in the 50 °F treatment, *ETR2* showed no changes, and *EBF1* was up-regulated in the 32 °F treatment (Fig. 3). When 1-MCP was applied (which blocks fruit response to 1-MCP), expression of all ethylene genes was significantly decreased (Fig. 3). The minimal ethylene production rates and the significant down-regulation of ethylene biosynthesis and signaling genes in the 1-MCP treated fruit indicate the effectiveness of 1-MCP treatments in inhibition of the ethylene pathway. This suggests the 1-MCP-then-cold treated fruit (8D in 32 °F and 3D in 50 °F) in 2013 can serve as samples to investigate the function of genes of interest, independent or dependent of ethylene.

Jasmonic acid genes: Genes associated with JA synthesis identified in the RNA-sequencing data were down-regulated (Table 1). The results from the 2013 experiment showed that these genes maintained lower transcript abundance in the cold treated samples compared to the control, both in fruit treated and not treated with 1-MCP (Fig. 3). Therefore, we postulate that low temperatures contribute to the decrease in JA and this likely assists pears to develop ripening capacity independent of ethylene.

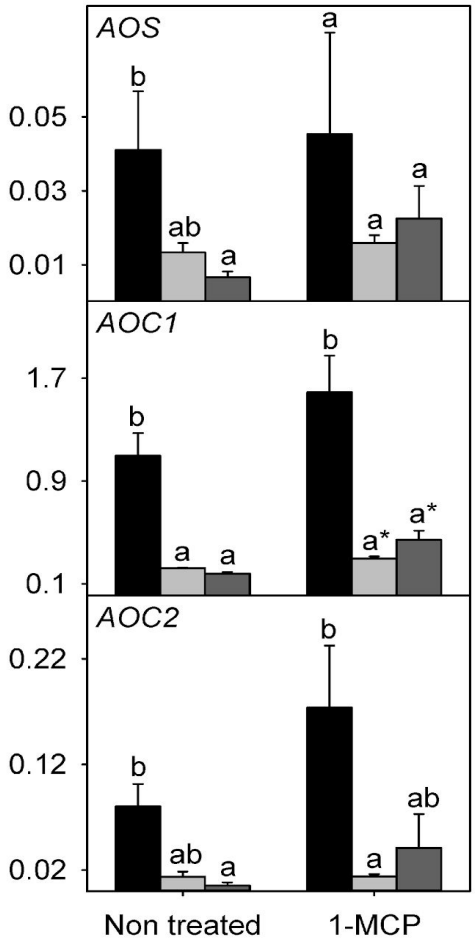

Fig. 3: Relative expression of ethylene (left) and jasmonic acid (right) genes after fruit were transferred from the cold treatments of 32 °F and 50 °F in pears pretreated with 1ppm 1-methylcyclopropene (1-MCP) treated and not. Different letters indicate significant differences between the temperature treatments via ANOVA; * indicates significant differences between with and without 1-MCP (Tukey's test, p-value ≤ 0.05).

Cold-induced genes: The calcium signal transduction pathway is important in regulating responses to low temperatures (Thomashow 1999). *CaLB* was found to be a negative regulator (more CaLB protein reduced the ability of the fruit to tolerate stress response) of abiotic stress response including salt and drought stress in Arabidopsis (De Silva et al., 2011). Two *calcium-dependent lipid protein* (*CaLB*) transcripts were down-regulated by our two cold conditioning treatments, with a greater reduction at 32 °F than 50 °F. PcM_51546 and PcM_40485, which were named *CaLB1* and *CaLB2* were down-regulated in both 1-MCP treated and non-treated samples (Fig. 4). The down regulation of *CaLBs* by cold conditioning treatments in the present study suggests that these genes may also negatively regulate cold stress.

Furthermore, cold-treated samples of 2010 showed a large decrease in transcript abundance of *C-binding factors* (*CBFs*), a transcription factor family regulating cold-induced pathways, with a greater decrease at 32 °F than at 50 °F (Table 1). Expression of *CBF1* and *CBF4* decreased in the 1-MCP-treated sample (Fig. 4) indicating it decreases independently of ethylene effects. The down-regulation of these *CBFs* in our study does not agree with what has been found in cold treatments in other species. Interestingly, among *CBF* genes, CBF2 protein acted as a negative regulator (more protein reduced the gene expression of target genes) of *CBF1* and *CBF3* expression to control the proper induction of genes associated with freezing tolerance (Novillo et al., 2003). It is possible that *CBF1* and *CBF4* in pears may be negative regulators of the development of ripening capacity during cold conditioning and during ripening at 68 °F. It is unclear to us why the expression of these genes significantly decreased in 1-MCP treated fruit left at 68 °F (Fig. 4).

Fig. 4: Relative expression of cold-related genes after fruit were transferred from the cold treatments at 32 °F and 50 °F in fruit pretreated and not treated with 1-methylcyclopropene (1-MCP). Different letters indicate significant differences between the temperature treatments via ANOVA; * indicates significant differences between fruit treated and not treated with 1-MCP (Tukey's test, p-value ≤ 0.05).

Transcription factors: A majority of research on TCPs has been done on leaf tissues; this new transcription factor family was reported to regulate cell proliferation, cell differentiation, leaf development, and lateral branching (Luo et al., 1996; Kosugi and Ohashi, 1997; Doebley et al., 1997). Our data showed that PcM_46371, a *TCP9a,* was up-regulated by cold conditioning (Table 1). The 2013 data confirmed the increase in this transcript by cold treatments (Fig. 5). When the ethylene pathway was blocked with 1-MCP, transcript abundance of *TCP9a* still increased, but to a lesser extent. In contrast, PcM_37893 (*TCP9b*) was down-regulated in cold treatments. Down-regulation of *TCP9b* by cold was confirmed in 2013 (Fig. 5). According to these results, we suggest that both *TCP9a* and *TCP9b* can play important roles in regulating the development of ripening capacity in low temperature treatments.

One component of the fruit ripening regulatory network in tomato is AGAMOUS-LIKE 1 (TAGL1) (Itkin et al., 2009). Our RNA-sequencing data showed that *AGAMOUS-24* (PcM_35299) transcripts are among the highest up-regulated by cold conditioning (Table 1). The validation in 2013 confirmed the up-regulation of this gene by cold treatments (Fig. 5). In the fruit treated with 1-MCP, the gene expression in fruit treated at 32 °F and 50 °F also increased (Fig. 5). The results suggest an important function of AGL24 in the development of pear ripening capacity.

Fig. 5: Relative expression of transcription factors after fruit were transferred from cold treatments at 32 °F and 50 °F in fruit treated and not treated with

1-methylcyclopropene (1-MCP). Different letters indicate significant differences between the temperature treatments via ANOVA; * indicates significant differences between fruit treated with 1-MCP and not treated (Tukey's test, p-value ≤ 0.05).

EXP. II: EXPRESSION OF GENES ASSOCIATED WITH ETHYLENE PATHWAYS AND THE PREDICTION OF RIPENING CAPACITY BASED ON ACO EXPRESSION

In 2010, we used temperature conditioning (32, 41 and 50 °F) to induce different levels of ripening capacity in 'Bartlett' pears. Ripening capacity developed faster when the conditioning temperature increased from 32 to 50 °F (Fig. 6). Without cold conditioning, the control fruit, which were ripened immediately at 68 °F after harvest, softened to 3.6 lbs after 12 days (Fig. 6A). Fruit slowly developed ripening capacity during storage at 32 °F; after 14 days at 32 °F fruit softened to 3.9 lbs by day 6 (D6) of ripening (Fig. 6E). Conditioning the fruit for 5 days at 41 or 50 °F promoted full ripening with fruit firmness after 6 days of ripening at 3.2 and 2.3 lbs, respectively (Fig. 6B). In addition, higher and earlier peaks in ethylene production rate were observed in the fruit that softened more quickly (Fig. 6F-J).

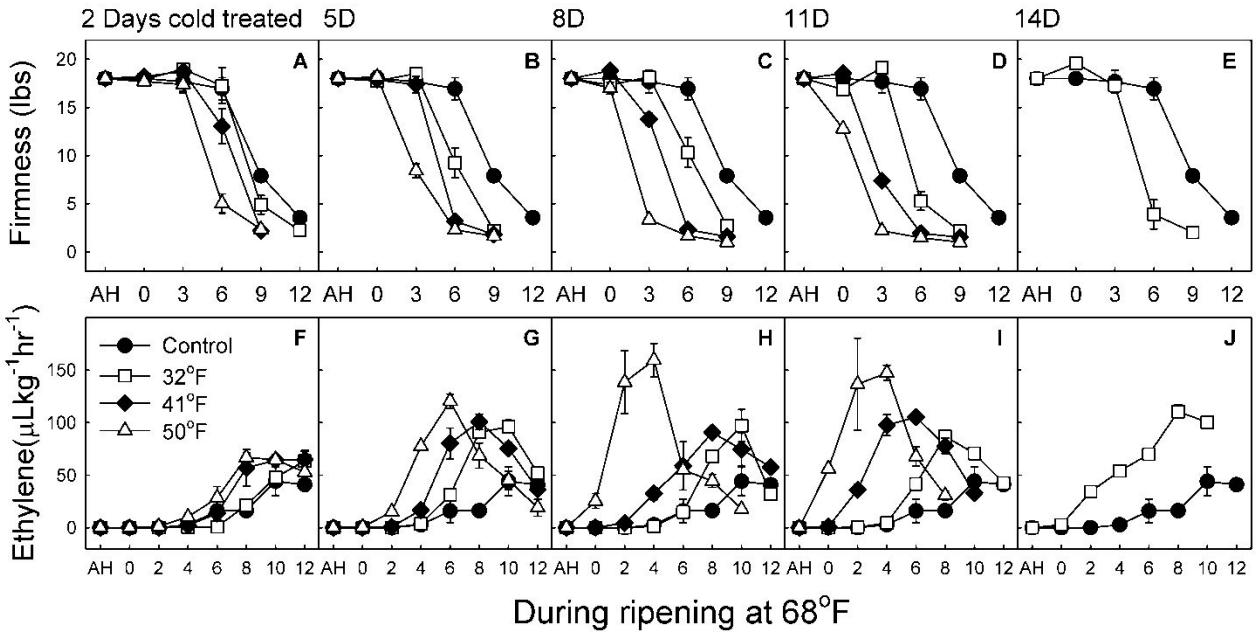

Fig. 6: Firmness and ethylene production at harvest (AH) and during ripening at 68 °F after cold treatment. Fruit were held at 32, 41, or 50 °F for 2, 5, 8, 11, or 14 days. Control: Fruit not subjected to cold treatment. Firmness was measured every 3 days; ethylene was measured every 2 days. Error bars indicate standard error of the means.

Linear correlations between expression of ethylene related genes (Pc-ACS1a, Pc-ACO, Pc-ETR1a, Pc-ETR2, Pc-ERS1a, and Pc-CTR1) after cold conditioning (D0) (when the flesh firmness was approximately 17 lbs) and fruit firmness after 6 days of ripening generated R^2 from 0.332 to 0.866 (Fig. 7); the highest correlation value was found in the

Pc-ACO analysis (R^2 = 0.886). Therefore, it appears promising to use the relative expression of *Pc-ACO* in unripe 'Bartlett' pears to predict the ability of fruit to ripen within 6 days at 68 °F. Over three years from 2012 to 2014, we conducted experiments using low temperatures and ethylene to develop ripening capacity in 'Bartlett' pear fruit. A prediction model based on *ACO* expression was developed based on 40 data points collected from these experiments (R^2 =0.633).

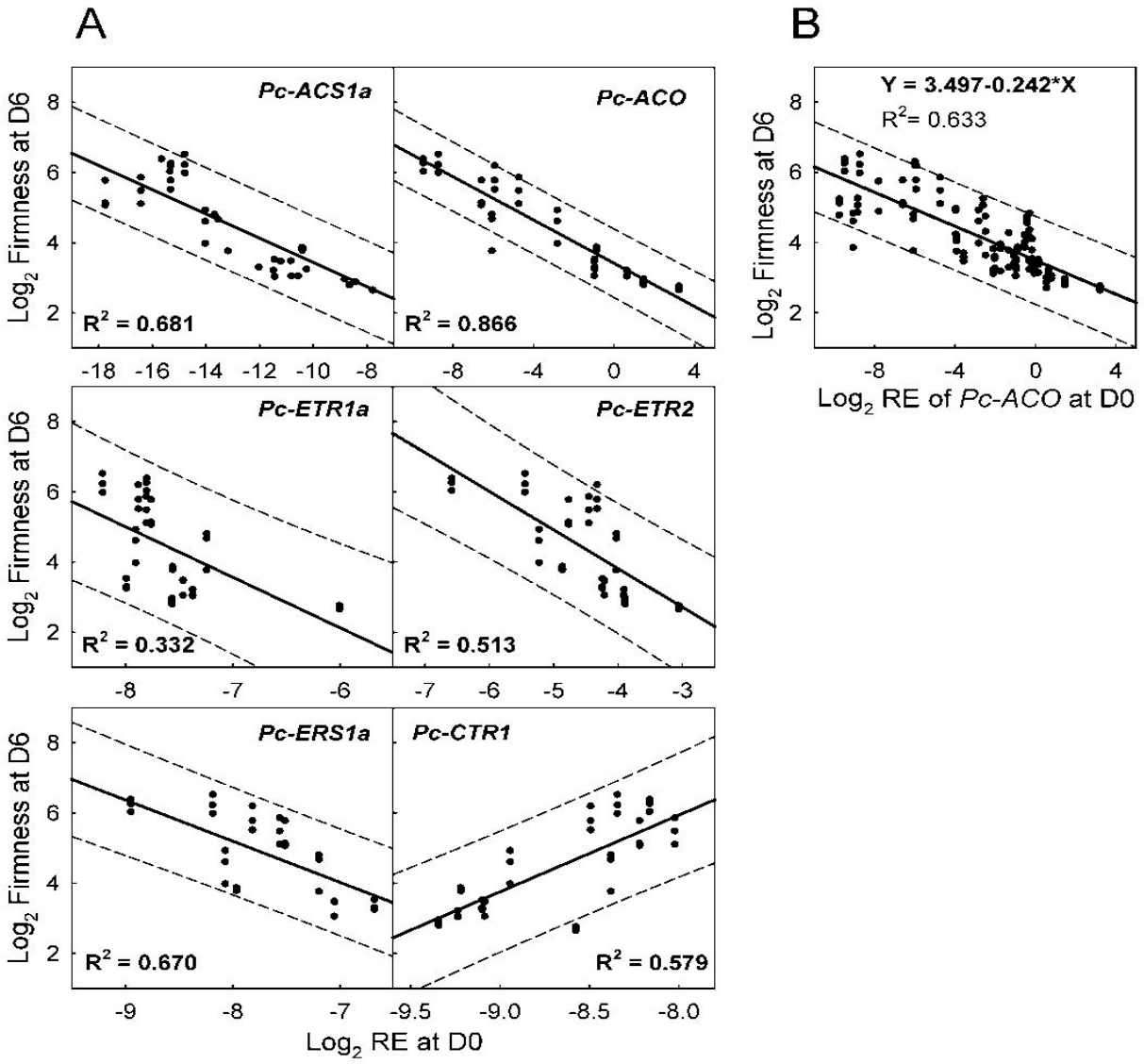

Fig. 7: Linear regression analysis of relative expression of genes associated with ethylene biosynthesis and signal transduction on day 0 of ripening and subsequent pear fruit firmness on day 6 of ripening at 68 °F (p-value < 0.001) A. 2010 Sacramento and B. 2010, 2012, 2013, and 2014 of Sacramento and Lake County combined. Dashed lines indicate prediction interval (95%).

However, it appears that ACO gene expression levels do not predict the ripening capacity of fruit that have been subjected to SmartFresh treatments (Table 2). For over 30 genes that have been tested, we have found a few genes such as ETR2 and EBF1 that may help to predict the ripening capacity of fruit from these treatments.

Table 2: Relative expression of ACO, ETR2 and EBF1 in the sample Lake 2013.

	Firmness at D6 (lbs)	Relative expression		
		ACO	ETR2	ERF1
Control	7.50	0.01	0.32	0.89
32F for 8 days	3.60	0.26	0.28	6.15
50F for 3 days	2.20	4.16	0.36	2.07
SmartFresh	18.4 (D15 13.3)	0.01	0.10	0.30
SmartFresh+32F for 8 days	19.10	0.11	0.07	0.55
SmartFresh+50F for 3 days	18.10	0.02	0.07	0.08

RESULTS OF EXP. III – EFFECTS OF PLANT GROWTH REGULATORS ON THE DEVELOPMENT OF FRUIT RIPENING

Our objective is to examine the crosstalk of ethylene with other plant growth regulators including JA, IAA, and ABA on the development of ripening capacity. We were also interested in the effect of these hormones on the *TCP* transcription factor family, a new transcription factor family not previously associated with fruit. Exp. III was completed in 2014 using fruit from Sacramento and Lake County and was repeated in 2015 with Sacramento fruit. The results from the experiments in both years showed that MeJA inhibited ripening while auxin and ABA facilitate ripening (Fig. 8). Moreover, gene expression of *TCPs* in the hormone treatments was evaluated, dependent and independent of ethylene (Fig. 9). For instance, TCP9a was down-regulated by MeJA, suggesting the involvement of JA in the regulation of the development of ripening capacity through this gene.

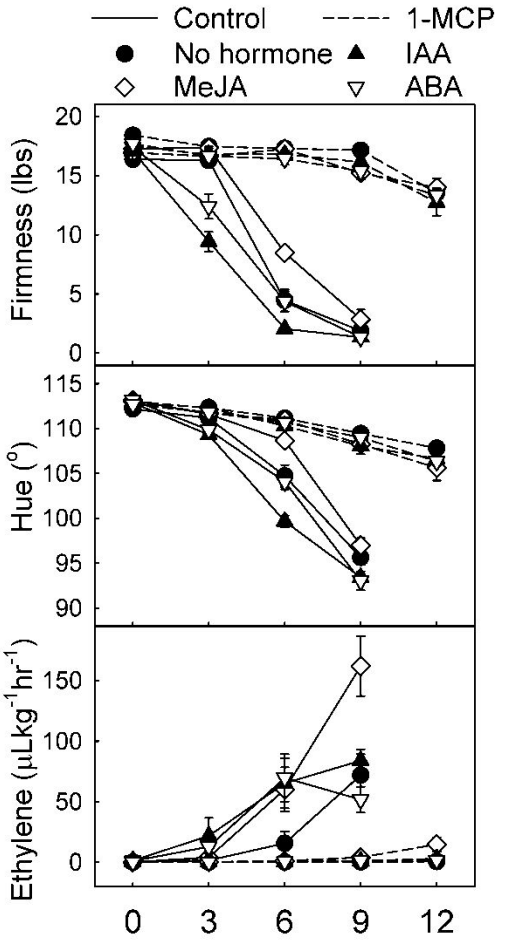

Fig. 8: Firmness, color, and ethylene production rate during fruit ripening following postharvest treatment with auxin (IAA), abscisic acid (ABA) or methyl

jasmonate (MeJA). One half of the fruit were treated with 1-MCP before the plant growth regulator treatment and the other half was not.

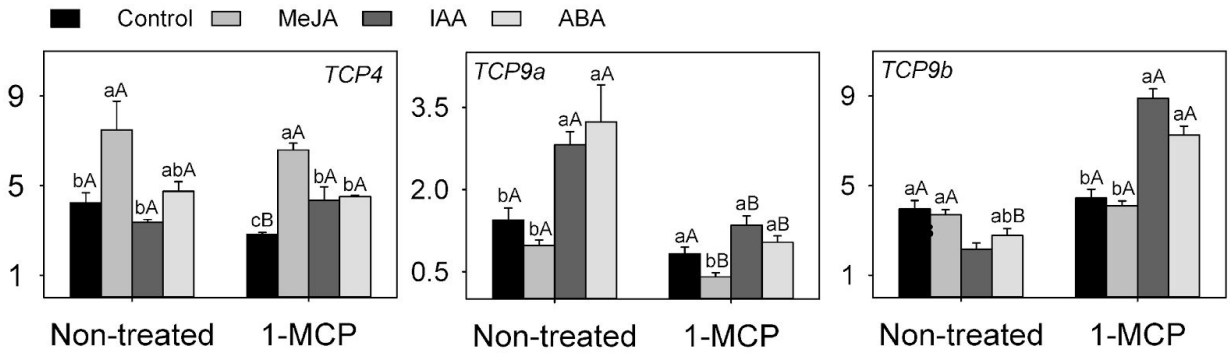

Fig. 9: Relative expression changes of three members of the TCP family (a new transcription factor family not previously associated with fruit) after the plant growth regulator treatments on fruit that have been treated with 1-MCP or not. Small letters indicate differences among different plant growth regulator treatments within non-treated or 1-MCP treated groups. Capital letters indicate differences between non-treated and 1-MCP treated in control or in any plant growth regulator treatment.