FINAL PROJECT REPORT

Project Title: Physiological genomics of pear ripening

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Other funding sources: *None*

Budget History:

Budget 1

Organization Name: Washington State University **Contract Administrator:** ML Bricker

Telephone: 509 335 7667 **Email address:** mdesros@wsu.edu

Item	2010	2011	2012
Salaries[1]	29,255	30,426	31,643
Benefits			
Wages	6,500	6,760	7,030
Benefits	310	322	335
Equipment			
Supplies	6000	7000	7000
Travel	2000	1,000	2,000
Miscellaneous – 454 sequencing		11,000	
Total	$40,065	$56,508	$48,008

Footnotes: [1] **Salaries for agriculture research assistant for performing physiological and genomic profiling and all molecular work. The increase in salaries for years two and three reflects a 4 % rate increase.**

Budget 2

Organization Name: OSU-MCAREC **Contract Administrator:** Dorothy Beaton

Telephone: 541 737 3228 **Email address:** dorothy.beaton@oregonstate.edu

Item	2010	2011	2012
Salaries[1]	4,140	4,306	4,478
Benefits[2]	2,857	2,971	3,089
Wages			
Benefits			
Equipment			
Supplies	1,000	1,000	1,000
Travel			
Miscellaneous			
Total	$7,997	$8,277	$8,567

Footnotes: [1] **Salary is based upon as 0.15 FTE Technician for harvest, cold storage and ethylene room maintenance, fruit quality attribute measurements, and data management. The increase in salaries for years two and three reflects a 4 % rate increase.** [2] **OPE rate is 69 %. Supplies largely include overnight shipping costs.**

OBJECTIVES

This project was aimed at identify the genetic underpinnings of the chilling-requirement for ripening in European pear varieties and establish information for short and long term improvement of pear fruit quality.

With a range of variability in conditioning requirements among PNW pear varieties, identifying genetic causes of chilling-induced ripening and System 2 ethylene production will provide the foundational knowledge required for physiological management in the short term and in the future use breeding for adequate variety development. The physiological conditioning model implemented in the lab (Figure 1A, 1B, 1C), has established a reliable system for pear research at the physiological and genetic level. This infrastructure, although archaic, will be employed for further pear-focused research at WSU, including another ongoing project focusing on physiogenomics of 1-MCP use in pear.

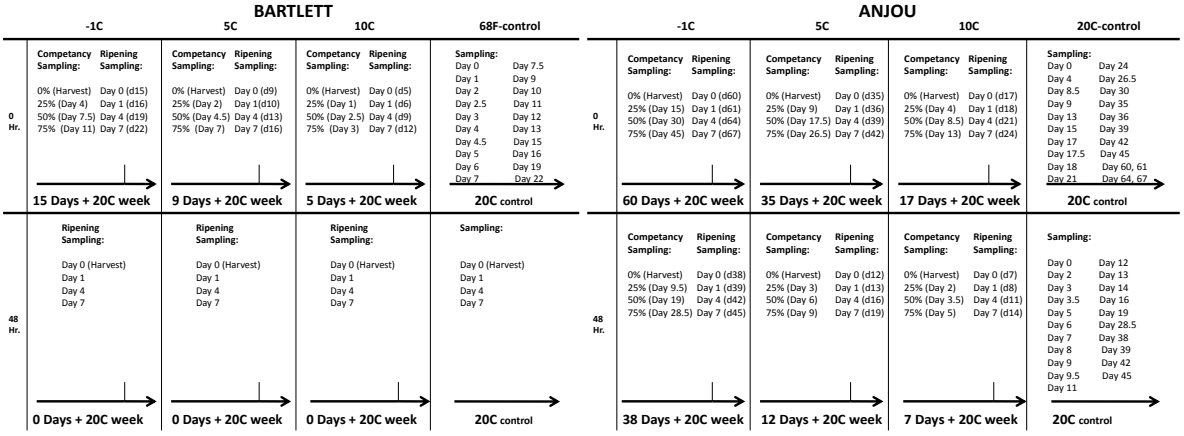

Figure 1. **(Top left)** Bartlett treatment and sampling scheme following conditioning protocols of Sugar and Kupferman. 1,920 fruit were divided into 8 groups of 24 each. These were subjected to one of 6 treatments, with 2 groups held at a constant 20°C. **(Top right)** D'D'Anjou treatment and sampling scheme following the conditioning protocols of Sugar and Kupferman. 1,920 fruit were divided into 8 groups of 240 each, then treated in the same manner as described for Bartlett. **(Lower left)** Flow-through respiration chambers inside climate-controlled room during D'Anjou conditioning. Where applicable, ethylene was injected into the system through a port on the rear of each chamber, to a concentration of 100 ppm (verified by gas chromatography). Outflow was set at 5 ml/min.

Objectives of this project were:

1. (Year1) Test the correlated activity of all ethylene, ripening-related and proposed regulatory genes along with the proposed cold-induced ripening master switch gene.

In this approach, peel tissue was sampled at regular intervals during conditioning, and subsequent ripening from Bartlett, Comice, and D'Anjou varieties. This work was performed both at OSU-MCAREC (Comice) and WSU-Pullman (Bartlett and D'Anjou). RNA, representing the active genes in the tissue, was then isolated from this tissue and used for quantitative real-time PCR (qPCR) analysis. This robust technique allows quantitative comparison of individual gene activity levels, and can help identify correlations between physiological processes and individual genes. In our work, we analyzed expression of 90 candidate ripening and System 2 ethylene biosynthetic regulatory genes that correspond to 6 major hormone and stress signaling pathways in pear. All genes examined via qPCR were related to one or more of the major regulatory pathways reported to control the onset of climacteric ripening in pear (Figure 2). These include a novel cold-induced gene (MIP, membrane integral protein identified in our lab from previous experiments) in cells and may play a critical role in integrating many of the signals reported to be involved in induction of climacteric ripening, and System 2 ethylene production in fruits. Also included are genes of a pathway which has been targeted in stimulation of ripening in 1-MCP treated fruit, under work for a related project. At the time of submission of this report, technical replicates of PCR tests are being performed for comprehensive data analysis and subsequent publication.

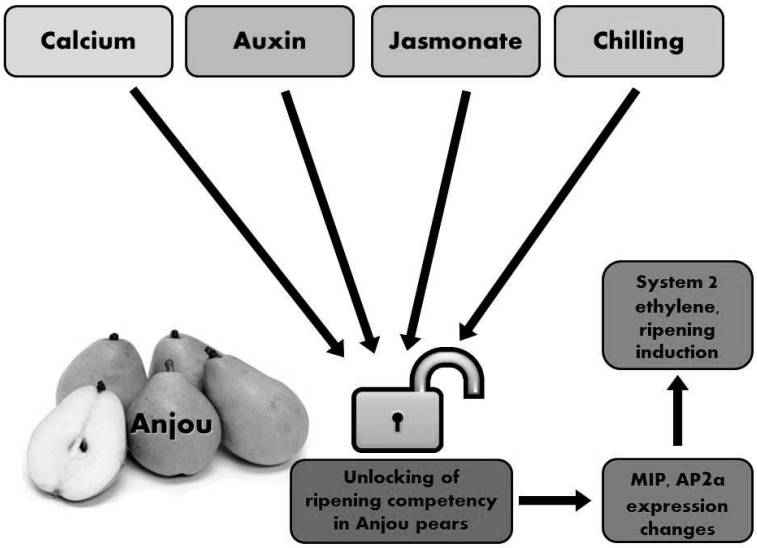

Figure 2. Model of physiological factors implicated in chilling-induced ripening and System 2 ethylene production in pear. Over 90 genes corresponding to these pathways were evaluated in this study providing a comprehensive insight into cold-induced ripening in pears.

2. (Year 2 and 3) Establish a relationship between ripening in winter pear and activity of the master switch regulator gene(s):

Tissues collected in Objective 1 were also be subjected to a gene-level comparative analysis to identify other genes involved in this phenomenon during ripening inductive conditions with ethylene and cold treatment. Among the genes tested, we applied statistical tests to determine which genes exhibited differential activity between Bartlett and D'Anjou samples during conditioning and ripening (Figure 3A, 3B, 3C below). Genes identified through this rigorous test serve as high-confidence elements in the overall regulatory mechanism governing the onset of ripening in conditioned pear fruit. Progress on this has yielded perhaps the most robust set of target genes involved in the chilling requirement for climacteric ripening and System 2 ethylene induction in pear available. Additionally, determination of when these genes are actively expressed in the fruit can offer in-field clues to growers toward fruit maturity and potential harvest windows.

3. (Year 2 and 3) Establish genetic diversity of the cold-induced ripening genes in pears:

Among the genes tested in Objectives 1 and 2, were the 1-aminocyclocarboxylic acid synthase (ACS) genes ACS1 and ACS2, which catalyze production of the immediate ethylene precursor, prior to and during the System 1-to-System 2 ethylene biosynthetic transition in pear (El-Sharkawy et al., 2004). This approach sought to isolate and sequence the unique MIP1, ACS1 and ACS2 sequences among PNW-specific varieties. This technique is useful in generating foundational knowledge to appropriately catalog ripening behavior and use by breeders to screen for desirable phenotypes during future pear improvement efforts.

SIGNIFICANT FINDINGS

Significant findings for Objective 1

- Auxin, jasmonate, ethylene, calcium, and a variety of cold-signaling pathway genes appear to be closely involved in chilling induced ripening and System 2 ethylene production. Many of these genes have powerful effects on the activity of several other genes, which in turn confers the traits of a ripened climacteric fruit, such as a 'burst' in ethylene production.
- Much of the same genetic machinery in other climacteric fruits is present in pear, aiding identification of novel genes which are not present, or whose activity is different than that reported in other species.
- The master-switch ripening regulatory gene (MIP) appears to be more heavily expressed in D'Anjou then in Bartlett fruit, where it may serve to suppress the activity of ripening-related genes.

Significant findings for Objective 2

- Statistical analysis of gene expression data identified nearly 20 of the 90 genes to be differentially expressed during the course of fruit conditioning and ripening (between Bartlett and D'Anjou). These represent candidate genes regulating activity of the proposed master-switch gene (MIP) and numerous downstream ripening-associated genes, including those associated with System 2 'burst' of ethylene production.
- Overall, there is ample evidence showing fruit of each variety respond differentially to calcium, auxin, jasmonate, and abscisic acid (ABA) in early stages of conditioning at the gene level. This work identifies an important phenological window where manipulation of ripening can be tested in future work.
- Some differentially active genes (between Bartlett and D'Anjou) illustrate completely novel avenues of research in ripening regulation in tree fruit.

Significant findings for Objective 3

- Established full gene sequence for D'Anjou and Bartlett MIP master-switch gene.
- ACS 1A/B and ACS2A/B genes have been amplified from D'Anjou and Bartlett.
- Work to establish the genetic diversity information is ongoing and expected to be complete by April 2013.

RESULTS & DISCUSSION

The following table summarizes the progress and milestones achieved for each objective outlined in the project.

Time Frame	*Objectives*	*Progress*	*Milestones*
January 2010 – December 2012	1. Test activity of chilling and ripening-related genes in conditioning pear fruit	Over 90 genes of 6 major hormone and stress signaling pathways examined using quantitative real-time PCR. Performing tests in triplicate which are expected to be complete by April 2013.	Completed the first comprehensive examination of activity among genes in pathways implicated in controlling chilling-induced ripening.
	2. Correlate expression of master-switch regulator to ripening in winter pear	Completed statistical analysis of gene activity. Identified set of about 20 candidate genes differentially expressed among winter pear regulating chilling-induced ripening.	Identified cold, auxin, and calcium signaling pathway members as candidate genes for the differential conditioning requirement between Bartlett and D'Anjou.
	3. Establish genetic diversity of cold-induced ripening genes in pear varieties.	Genes have been amplified. They are currently being cloned and being sequenced.	Expect to complete this aspect by April 2013.

Overall, results illustrate numerous gene-level and physiological differences between conditioning Bartlett and D'Anjou fruit. The experimental infrastructure utilized and implemented at WSU-Pullman was effective in providing a physiological model of conditioning in Bartlett and D'Anjou pear.

1. *Gene expression analysis*: We based our gene analysis on previous research on physiological models that trigger ripening and System 2 ethylene induction pear and numerous other climacteric fruits. Overall results illustrate numerous gene-level and physiological differences between conditioning of Bartlett and D'Anjou fruit. At equal stages of conditioning and ripening, significant differences in gene activity are seen in members of the cold-signaling pathway (Figure 3A, 3B). Figure 3 is a heat map that indicates relative expression based on color. (Pardon the grayscale presentation in the written report). The different of individual gene activity is clearly visible amongst comparable samples from Bartlett and D'Anjou. Typically these genes have powerful downstream effects including activation of genes from nearly all other pathways probed in this work, including those of ABA, ethylene, calcium, and general stress responses. This suggests critical differences in varietal capacity to respond to prolonged chilling exposure during conditioning. Similar differences are seen for auxin-signaling pathway genes in the fully conditioned and fully ripened samples. With internal auxin accumulation being one of the primary physiological clues preceding the onset of ripening and System 2 ethylene production in chilling-dependent tree fruits (El-Sharkawy et al., 2008; El-Sharkawy et al. 2010), these differences also suggest powerful differences in the fruits' capacity to produce and respond to auxin, which could obstruct ripening progression. There is likely a relationship between the altered ability (between Bartlett and D'Anjou) of the fruit to respond to cold, and the accumulation of auxin in the fruit. Characterizing this relationship in greater detail will be a subject for future research efforts in the lab. Such work will need to include an in depth examination of genetic variability in sequence and regulation of genes comprising these pathways. The recently published apple (Velasco et al., 2010), Chinese pear (Wu et al., 2012) and European pear (Dhingra lab) genomes may help in this regard, highlighting the importance of foundational genomics resources. Interestingly, this work showed many aspects of ethylene-signaling which are similarly active during progression of conditioning and ripening, supporting the presence of an ethylene-independent but cold and/or auxin-dependent mechanism underlying differences in fruit competency for ripening as they undergo conditioning treatments. Finally, this work has yielded the first reported instance of differential expression among genes which confer signaling ability to ethylene receptors.

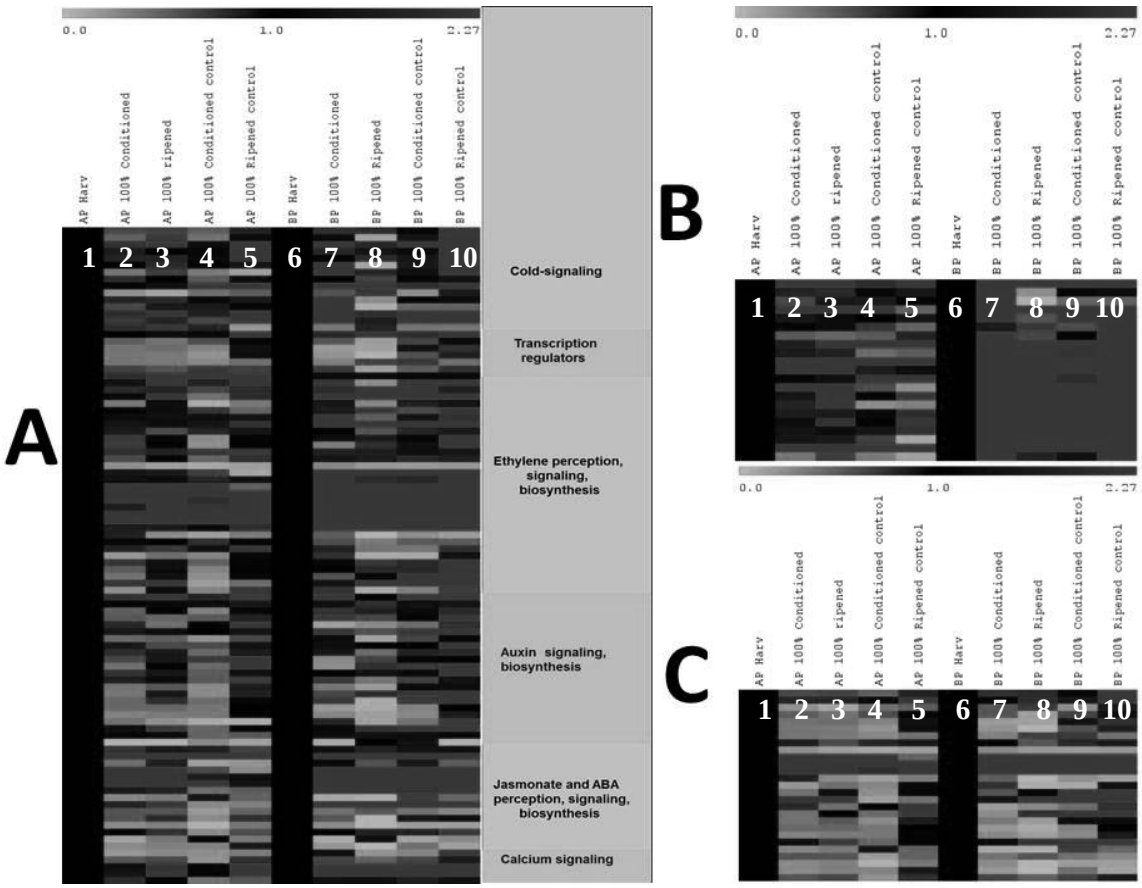

Figure 3. **(A)** Overall heat map indicating relative gene activity among 6 pathways containing selected genes regulating chilling-induced ripening and System 2 ethylene production in harvested D'Anjou and Bartlett (lanes 1 and 6, respectively), fully conditioned D'Anjou and Bartlett (lanes 2 and 7, respectively), fully ripened D'Anjou and Bartlett (lanes 3 and 8 respectively), unconditioned D'Anjou and Bartlett (lanes 4 and 9, respectively), and unconditioned D'Anjou and Bartlett controls at (conditioned) ripening dates (lanes 5 and 10, respectively). Darker colors represent higher activity, lighter colors represent lesser activity (relative to harvest date standards). Data represents quantitative real-time PCR C_t-values after 2-log transformation. **(B)** Genes exhibiting significantly differential expression (between D'Anjou and Bartlett during fruit conditioning and ripening) by variety using the Significance Analysis for Microarray (SAM). **(C)** Genes exhibiting differential expression by variety during ripening progression using the Bayesian Estimation of Temporal Regulation. The SAM approach clearly illustrates significant divergence in gene activity at equal conditioning stages in comparison of Bartlett and D'Anjou fruit. Heatmaps were generated using the MultiExperiment

2. *Correlate expression of ripening-regulatory genes to ripening in winter pear*:

Flesh softening correlated well with observed gene activity of ripening-related genes, with increases in expression of late-stage ethylene production genes corresponding with significant reductions in flesh firmness in both varieties, consistent with the results of Sugar and Kupferman (Figure 4). The cold-requirement can be supplemented with warmer conditioning temperatures, and ethylene to produce ripening competent fruit. Ethylene treatment of Bartlett fruit reduces the time required to reach marketable firmness- with only a 7 day treatment at 20°C needed. Interestingly, ungassed fruit stored at 10°C appear to soften more rapidly than fruit held at -1°C, illustrating ethylene-independent mechanisms at work in pear ripening. However, after over a month at -1°C, D'Anjou fruit retained most of its firmness. In Bartlett, a clearly decreasing flesh firmness is already apparent in the ungassed (no exogenous ethylene during conditioning) 10°C-stored fruit. Gene expression analysis in

these same sample tissues illustrates clear differences at this early stage. Results shown here demonstrate the powerful and rapid effects these genes may have in regulating the ripening process.

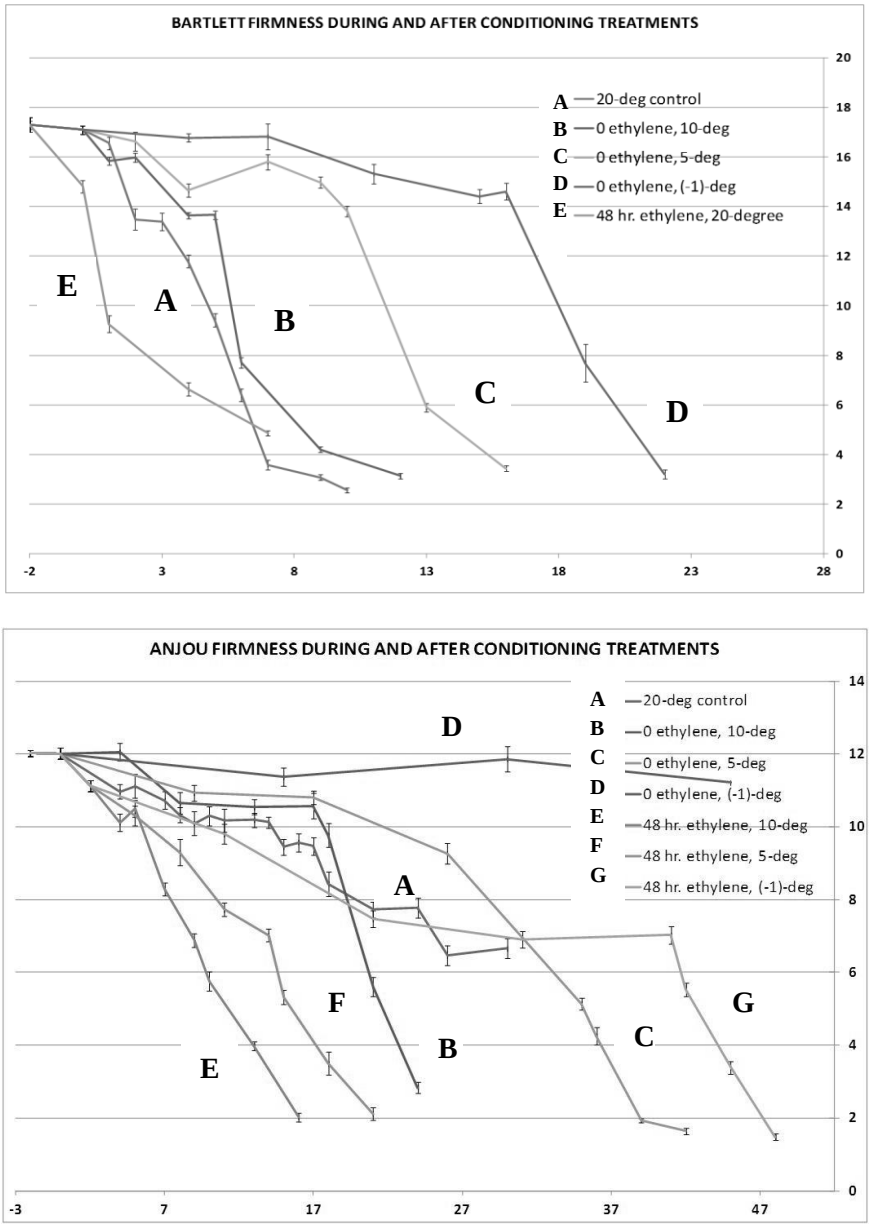

Figure 4. (Top) Flesh firmness in sampled conditioned (at -1, 5, and 10°C) and unconditioned (20°C-constant) Bartlett fruit in the presence and absence of use of a 48 hour 100 ppm ethylene dosing. (Bottom) Flesh firmness in sampled conditioned (at -1, 5, and 10°C) and unconditioned (20°C-constant) D'Anjou fruit in the presence and absence of use of a 48 hour 100 ppm ethylene dosing.

3. Establish genetic diversity

Among differentially expressed genes during fruit conditioning and ripening, were the European pear System 1-to-System 2 ethylene production transition-specific (El-Sharkawy et al., 2004) genes ACS1 and ACS2. Prior work suggests only two allelic forms of these genes (ACS1a/1b, and ACS2a/2b). Cloning and sequencing of Bartlett and D'Anjou ACS1 and ACS2 genes is ongoing and is expected to be accomplished by April 2013.

References

1. Velasco et al., 2010. The genome of the domesticated apple. Nature Genetics. 42:833–839.

2. Wu et al., 2012. The genome of pear (*Pyrus bretschneideri* Rehd.). Genome Research. doi: 10.1101/gr.144311.112.

3. Dhingra 2012. The DH Pear Genome. http://genomics.wsu.edu/pear-research/ (accessed Jan 22, 2013).

3. El-Sharkawy et al., 2004. Differential regulation of ACC synthase genes in cold-dependent and -independent ripening in pear fruit. Plant, Cell and Environ. 27(10):1197–1210.

4. El-Sharkawy et al., 2008. Differential regulation of four members of the ACC synthase gene family in plum. J. Exp. Bot. 59(8):2009-2027.

5. El-Sharkawy et al., 2010. Regulation of two germin-like protein genes during plum fruit development. J. Exp. Bot. 61(6):1761-1770.

EXECUTIVE SUMMARY

The aim of this project was to produce the foundational gene-level knowledge required to better understand the underpinnings of the chilling-requirement for ripening and System 2 ethylene induction in PNW pear varieties. This information is critical for much-needed improvement of pear varieties to meet evolving market needs. Among these needs is greater control over the fruit ripening process. With unique ripening characteristics, PNW pear varieties require customized post-harvest management. Despite this, asynchronously ripened and damaged fruit can lead to unacceptably high amounts of unmarketable fruit progressing through the fruit production, storage, and transport and sale chain. Results of this project have identified hormone and stress-signaling pathways which respond differently through the course of fruit conditioning and ripening. Variability in sequences reported to be uniquely expressed near the onset of the ethylene 'burst' can be used to appropriately catalog varieties, use as predictors of ripening and serve as molecular markers in pear variety breeding efforts to select for desirable conditioning-requirement phenotypes. As a whole, this work established the foundation required for short and long-term improvement of pear fruit quality.

Summary of findings

This work has identified the control points in auxin, calcium and cold-signaling pathways in Bartlett and D'Anjou tissue during conditioning and ripening while also confirming the presence of much of the genetic elements common to climacteric fruits. Variability in gene sequences and their expression behavior in the two varieties can be useful in predicting conditioning levels in the short term to predict fruit quality. These genes can also serve as useful markers in gene-assisted selection to advance desirable conditioning requirements into progeny. We have arrived at these findings by establishing a robust physiological conditioning model following protocols of Kupferman and Sugar, and employing a gene-level analysis of the inherent differences in conditioning-requirements between PNW pear varieties. This approach allows direct interrogation of causal underpinnings of this complex phenomenon.

Future directions

This is one of the most comprehensive examinations of the genetic underpinnings of this unique ripening phenomenon in climacteric fruits performed to date. Identification of these candidate genes provides critical clues to understand how such genetically similar pear varieties can differ so greatly in their conditioning requirements. The MIP gene identified in the lab through this work may serve to integrate many of the phytohormone and environmental stress signals preceding the trigger of ripening and System 2 ethylene induction in pear. We will further explore the mechanism behind these interactions to understand which are the essential genetic differences responsible for impaired ripening in winter pear. This information can prove to be critical for post-harvest management of existing pear varieties.