TRANSCRIPTOMIC CHARACTERIZATION OF NOVEL FRUIT RIPENING PATHWAYS

By

SEANNA LOUISE HEWITT

A dissertation submitted in partial fulfillment of
the requirements for the degree of

DOCTOR OF PHILOSOPHY

WASHINGTON STATE UNIVERSITY
Program in Molecular Plant Sciences

To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of SEANNA LOUISE HEWITT find it satisfactory and recommend that it be accepted.

_____

Amit Dhingra, Ph.D., Chair

_____

Kate Evans, Ph.D.

_____

Anantharaman Kalyanaraman, Ph.D.

_____

N. Richard Knowles, Ph.D.

ACKNOWLEDGMENTS

I want to first thank my PhD advisor, Amit Dhingra, for providing invaluable mentorship and support both academically and personally. I would also like to thank the members of my committee—Kate Evans, Ananth Kalyanaraman, and Rick Knowles—for their guidance and support. The ARCS Seattle Chapter and NIH Protein Biotechnology Training Program provided funding the first several years of my graduate research and have provided a community of individuals who will remain in my professional network for years to come. BioBam Bioinformatics funded an international internship and provided me with access to genomics analysis software for the duration of my PhD program, for which I am most appreciative. The Pear Bureau Northwest, Blue Bird Growers (Wenatchee/Peshastin, WA), Blue Star Growers (Cashmere, WA) and Crunch Pak (Cashmere, WA) provided fruit and facility resources for ripening compound testing, without which this research would not be possible. I extend my gratitude to Scott Mattinson, for assistance with gas chromatography work, and to the Knowles lab, for use of their respiration system during the 2017 pear season. Furthermore, I want to recognize the students who assisted in the physiology aspects of this project—Grant Nelson, Coleman Schnebly, and Skylar Lynch—as well as my fellow lab mates and colleagues, past and present. Special thanks to Rick Sharpe, Karen Adams, Nathan Tarlyn, Ryan Christian, Bruce Williamson, Rishi Ghogare, Evan Stowe, Fabiola Ramirez and Elvir Tenic for being a part of this journey. Finally, I'd like to thank my family for their unconditional support and faith in my ability to succeed.

TRANSCRIPTOMIC CHARACTERIZATION OF NOVEL FRUIT RIPENING PATHWAYS

Abstract

by Seanna Louise Hewitt, Ph.D.
Washington State University
December 2019

Chair: Amit Dhingra

Ripening of climacteric fruits is characterized by a rise in respiration and concomitant burst of ethylene biosynthesis. While this general pattern is shared among climacteric fruits, there are a few exceptions. European pears (*Pyrus communis*) require a genetically pre-determined amount of cold exposure, called conditioning, to transition from System 1 (S1) to System 2 (S2) ethylene biosynthesis. Recent research from the lab has implicated pre-climacteric alternative oxidase activity in this transition. In addition to the need for cold exposure, European pear cultivars exhibit variability in response to ethylene receptor antagonist 1-methylcyclopropene (1-MCP). Unlike apples and other climacteric fruits, pears do not regain their ripening capacity following 1-MCP treatment despite the conditioning treatment. A chemical genomics study aimed at activating alternative oxidase in 1-MCP treated pears identified glyoxylic acid for its ability to override the metabolic blockage of ethylene biosynthesis caused by 1-MCP.

This dissertation characterizes a natural variation in climacteric ripening represented by European pear, by (1) Summarizing and distilling published information regarding the role of respiration in climacteric fruit. Many of the physiological and phenotypic changes that occur during ripening are attributed to ethylene, however the molecular and metabolic crosstalk between autocatalytic ethylene production and respiration are less understood. Results of recent work

implicate the alternative oxidase (AOX) respiratory pathway in crosstalk between ethylene, respiration, and other ripening-associated processes; (2) Conducting comparative physiological and transcriptomics analyses of two pear genotypes that require different durations of cold temperature exposure in order to ripen. Transcriptomic analysis revealed that cold temperature conditioning leads to heightened expression of vernalization-associated genes, homologs of which are necessary for other cold-dependent processes like seed germination and flowering. These genes may serve as regulatory points in the initiation of ripening in fruit that require cold conditioning; (3) Assessing the impact of glyoxylic acid in chemical induction of ripening in 1-MCP treated 'D'Anjou' pear fruit using physiological and transcriptomics approaches. Results demonstrate that glyoxylic acid effectively elicits physiological responses as well as significant changes in global gene expression, including that of AOX, in 1-MCP treated pear fruit that would be otherwise incapable of ripening normally.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 2

CHAPTER 3

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

ABBREVIATIONS

1-MCP, 1-Methylcyclopropene

ABA, Abscisic acid

ACC, 1-aminocyclopropane carboxylate

ACS, 1-aminocyclopropane carboxylate synthase

ACO, 1-aminocyclopropane carboxylate oxidase

AOX, Alternative Oxidase

ATP, Adenosine triphosphate

Cq, Quantification cycle value

CYTc, Cytochrome c pathway

DEC, Differentially expressed contig

GLA, Glyoxylic acid

GO, Gene ontology

H_2O_2, hydrogen peroxide

H_2S, Hydrogen sulfide

NMDS, Nonparametric multidimensional scaling

qRT-PCR, Quantitative reverse-transcriptase polymerase chain reaction

ROS, Reactive oxygen species

S2, System 1

S2, System 2

FOREWORD

With the global population projected to reach 9.7 billion by 2050, the need to adopt more sustainable agricultural practices has become critical if enough food is to be produced (Ehrlich & Harte, 2015). Every year, 1.6 billion tons of food goes to waste. This represents about one-third of the food that is produced for human consumption. Strikingly 45% of this waste can be attributed to losses in fruits and vegetables between the farm and the table (Buzby & Hyman, 2012). Unpredictable ripening of fruit is one of the leading causes of postharvest loss. Besides the need for increased production, feeding the world's growing population necessitates advances in postharvest technology, particularly with regards to postharvest preservation strategies (Office of Agriculture, 2013).

There are two well-established patterns of ripening: climacteric and non-climacteric. The ripening pattern of climacteric fruit is characterized by a dramatic increase in ethylene biosynthesis (S2 ethylene production) accompanied by a burst in respiration (CO_2 evolution). This is converse to the pattern observed in non-climacteric fruit, in which respiration and ethylene accumulation are maintained at basal levels during the ripening process. Regardless of ripening profile, the presence of ethylene seems to be essential (Blankenship & Dole, 2003; Burg & Burg, 1965; Barry & Giovannoni, 2007; Hiwasa-Tanase & Ezura, 2014). Because of their distinct ripening physiology, climacteric fruit can be harvested unripe, and continue to ripen off the tree. While the concept of two distinct ripening categories is simple in theory, the lines may be blurred in some species, with certain fruits displaying intermediate phenotypes (Paul, et al. 2012).

The ability to inhibit ripening is important at the commercial level, particularly considering the long distances that fruit is often transported nationally and internationally. 1-

methylcyclopropene (1-MCP), a compound which entered the commercial scene in 2002, has been used extensively to slow the ripening process in fruit and thereby impart a longer shelf life. 1-MCP works as an ethylene receptor antagonist, binding competitively to the receptors that would normally perceive the ripening hormone (Watkins, 2015; Watkins, 2006). In most fruit, this results in effectively delayed ripening—the eventual re-initiation of ripening is thought to occur as ethylene receptor proteins are turned over, although there is no supporting data for this hypothesis (Tatsuki et al., 2007).

Pyrus communis – a natural ripening variant

European pear (*Pyrus communis spp.)* is a nutritionally and economically valuable tree fruit both in the U.S. and throughout the world. It is one of the few types of fruit that can be tolerated by people with type II diabetes due to its low glycemic index (Reiland & Slavin, 2015). The pear industry in the Pacific Northwest brings in about $380 million annually (Northwest Horticultural Council, 2018). While the industry has enormous potential to grow, a primary challenge to this growth is that pear fruit ripens inconsistently and often does not achieve the desired buttery consistency, aromatics and flavor profile to meet consumer satisfaction standards (Serra et al., 2019). Thus, consumption of pears in the U.S. has remained stagnant. The unpredictability in this fruit's ripening profile makes it an interesting system in which to study ways that ripening can be better managed. Pear is climacteric, and, as previously described, ripening is characterized by a spike in respiration accompanied by a concomitant increase in ethylene evolution (Klee & Giovannoni, 2011; Alexander & Grierson, 2002). Despite its characterization as a climacteric fruit, there are two prominent aspects that distinguish pear from other fruits of this ripening profile. 1.) A pre-ripening period of cold temperature exposure is

required to initiate the transition from S1 to S2 ethylene biosynthesis (Hartmann et al., 1987) and 2.) Ripening in pear is inhibited indefinitely following treatment with 1-MCP.

Chilling requirement for ripening in pear fruit

All fruits are susceptible to chilling injury to some degree. As such, different species and genotypes have evolved cold temperature responses that alleviate stress and thereby mitigate cold-induced damage. Uniquely, pear fruit is not only able to avoid chilling injury at freezing temperatures but require long-term exposure to cold in order for normal ripening or S2 ethylene production to initiate. This conditioning period varies by pear genotype, with 'Bartlett' and 'D'Anjou' pear varieties representing the extremes of the chilling spectrum; 'Bartlett' pears require chilling for 15 days and 'Anjou', 60 days, at 0°C. 'Comice' pears represent an intermediate phenotype, with a chilling requirement of 30 days. In addition to genetic predetermination for duration, chilling time is affected by maturity of the fruit at harvest, with pears harvested at a higher maturity index requiring a less extensive period of cold conditioning, and vice versa (Sugar & Basile, 2009).

(Non)ripening of pear in the context of 1-MCP

1-MCP is used commercially to temporarily inhibit ripening and senescence in many perishable commodities, mainly climacteric fruits. Following application, many climacteric fruits eventually regain capacity to biosynthesize ethylene and ripen, with the benefit of an extended shelf-life. In pear, however, 1-MCP treatment indefinitely blocks ethylene perception and signaling, and endogenous S2 ethylene production and accelerated respiration associated with climacteric ripening reinitiate only sporadically, if at all (Argenta et al., 2016; Sugar & Einhorn,

2011; Villalobos-Acuna & Mitcham, 2008). In addition to virtually complete inhibition of ethylene biosynthesis in cold-conditioned pear following 1-MCP treatment, application of exogenous ethylene does little to affect the capacity of 1-MCP-treated pears to ripen (Argenta et al., 2016; Argenta et al., 2003). This phenomenon represents a unique biological anomaly in a well-established ripening paradigm.

Furthermore, it suggests that 1-MCP, which has been classically understood only in the context of its identity as an ethylene receptor antagonist, might exert additional metabolic consequences that prevent pear fruit from responding to cold conditioning. Recent research has lent support to the concept of differential effect of 1-MCP on ethylene biosynthetic pathways and signal transduction networks in different climacteric fruits, including peach and apple (Dal Cin et al., 2006; Watkins, 2015; Watkins, 2006). This exception in the paradigm to the ripening process presented by pear fruit challenges not only scientists, but also growers and consumers, since variation in cultivation, harvest time, and postharvest conditions makes ripening unpredictable. This unpredictability leads to millions of dollars in crop loss.

This dissertation addresses novel cold- and chemical-induced, ripening-associated pathways in climacteric fruit. European pear is used as the primary model for study. However, the results of the experiments detailed in the following pages are expected to translate to other fruit systems to gain better control over the timing and quality of ripening.

Caveats:

The fruits used in these experiments were acquired from different growing lots in the Wenatchee Valley each season, based on growers' availability. All fruit receiving 1-MCP

treatment was treated at the packinghouse prior to transport to WSU. While all fruit was harvested at physiological maturity, from a commercial standpoint, the position of fruit within the tree canopy prior to harvest is known to affect physiology, metabolic profile, and overall propensity to ripen to some degree (Serra et al., 2018; Rudell et al., 2017). Because of this natural variability, it was necessary to optimize the experimental designs over the course of several seasons and to ensure enough replication for statistical analysis. Much of the work to optimize experimental design and treatments has been omitted from the body of the dissertation and can be found in the supplementary information included.

Chapter 1—Beyond Ethylene: New Insights Regarding the Role of AOX in the Respiratory Climacteric

Fruits are classified either as climacteric or non-climacteric depending on how they respond to and synthesize ethylene during ripening. Long-established physiological models encompass broadly the genetic and hormonal regulatory mechanisms governing ripening in climacteric fruit, and ethylene has historically been the center of focus for such models. However, the connection between ethylene and climacteric respiration, another primary process at play during climacteric ripening, has not been investigated in great deal at the molecular level until recently. Results of studies in multiple systems, including tomato, mango, and pear, indicate that the AOX respiratory pathway may play an important role in mediating crosstalk between ethylene response, carbon metabolism, ATP production, and ROS signaling during climacteric ripening. Pears are particularly unique in that *AOX* expression peaks during the pre-climacteric phase, in comparison to other fruits, where elevated expression of *AOX* occurs after the S2 transition (Hendrickson et al., 2019; Dhingra & Hendrickson, 2017). Transcriptomic, metabolic, and

epigenetic analyses reveal new information regarding the interconnectedness of ripening metabolic pathways and the modulatory role of AOX in mediating crosstalk between these pathways. This information can be used to modify the classical, ethylene-centric physiological model. Understanding points at which ripening responses can be manipulated may reveal key, species- and genotype-specific targets for ripening regulation which extend beyond the classical model of ethylene biosynthesis and response.

Chapter 2— Evidence for the Involvement of Vernalization-related Genes in the Regulation of Cold-induced Ripening in 'D'Anjou' and 'Bartlett' Pear Fruit

The physiological responses of European pear to cold-temperature-induced ripening have been studied and documented in detail by tree fruit growers and postharvest biologists throughout the world. Despite an understanding of the temperature ranges and duration of chilling required for specific genotypes to ripen properly, little is understood with regards to why these requirements exist at the genetic level. A comparative transcriptomic approach was employed to investigate the response of ‘D’Anjou’ and ‘Bartlett’ pear genotypes at four different physiological stages during the cold conditioning process, and to test the hypothesis that *AOX* and other key cold-induced genes facilitate ripening. Differential expression, functional annotation, and GO enrichment analyses allowed for the identification of genes and associated ripening-related pathways of hormonal and environmental nature that are differentially expressed over time.

Interestingly, it was observed that vernalization-associated genes, which are important in other cold-induced processes, may be involved in the ripening of pear fruit. *AOX* was also induced pre-climacterically, lending support to its potential role in ripening mediated by conditioning. The information gained from this experiment provides insight into mechanisms of cold-induced

transcriptional regulation of ripening in European pear, as well as a unique comparative analysis of two genotypes with very different cold conditioning requirements.

Chapter 3— Physiological and transcriptomic analysis of glyoxylic acid-mediated ripening in 1-MCP treated 'D'Anjou' pear fruit

1-MCP treated 'D'Anjou' pears were treated with glyoxylic acid (GLA), and their resulting ripening responses were monitored to test the hypothesis that GLA treatment will activate AOX expression, thereby enabling override of 1-MCP inhibition and eliciting ripening responses. Physiological measurements, including established indicators of ripening (fruit firmness, ethylene evolution, and CO_2 evolution), and tissue samples were harvested post-GLA treatment. Transcriptomic and functional enrichment analyses revealed novel, ripening-associated genes, and pathways that are activated as a result of GLA application. Among the differentially expressed genes were those associated with cytochrome and alternative pathway respiration, organic acid metabolism, fatty acid metabolism, amino acid metabolism and ethylene-responsive pathways. These observations lend support to glyoxylic acid as a chemical stimulator of ripening and provide insight regarding how ripening blockage caused by 1-MCP may be circumvented at the metabolic level, thus opening avenues for more fine-tuned regulation of this process.

Taken together, this work explores the multifaceted aspects of ripening, questioning the classic, ethylene-centric model. While there is little question regarding the importance of ethylene in ripening, the ethylene response pathway does not operate in isolation. It is essential to look at the other factors and network of metabolic pathways that affect the S2 transition and ripening. Through synthesis of results of ongoing work to understand the mechanisms governing climacteric

ripening, using European pear as a study system, this work presents novel targets for ripening manipulation by which fruit shelf life and quality may be improved and waste can be mitigated.

REFERENCES

Alexander L, Grierson D, 2002. Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. *Journal of experimental botany* **53**, 2039-55.

Argenta LC, Fan X, Mattheis JP, 2003. Influence of 1-methylcyclopropene on ripening, storage life, and volatile production by d'Anjou cv. pear fruit. *Journal of agricultural and food chemistry* **51**, 3858-64.

Argenta LC, Mattheis JP, Fan X, Amarante CV, 2016. Managing 'Bartlett'pear fruit ripening with 1-methylcyclopropene reapplication during cold storage. *Postharvest Biology and Technology* **113**, 125-30.

Barry CS, Giovannoni JJ, 2007. Ethylene and fruit ripening. *Journal of Plant Growth Regulation* **26**, 143.

Blankenship SM, Dole JM, 2003. 1-Methylcyclopropene: a review. *Postharvest Biology and Technology* **28**, 1-25.

Burg SP, Burg EA, 1965. Ethylene action and the ripening of fruits: Ethylene influences the growth and development of plants and is the hormone which initiates fruit ripening. *Science* **148**, 1190-6.

Buzby JC, Hyman J, 2012. Total and per capita value of food loss in the United States. *Food Policy* **37**, 561-70.

Dal Cin V, Rizzini FM, Botton A, Tonutti P, 2006. The ethylene biosynthetic and signal transduction pathways are differently affected by 1-MCP in apple and peach fruit. *Postharvest Biology and Technology* **42**, 125-33.

Ehrlich PR, Harte J, 2015. Opinion: To feed the world in 2050 will require a global revolution. *Proceedings of the National Academy of Sciences* **112**, 14743-4.

Hartmann C, Drouet A, Morin F, 1987. Ethylene and ripening of apple, pear and cherry fruit. *Plant Physiology and Biochemistry (France)*.

Hiwasa-Tanase K, Ezura H, 2014. Climacteric and non-climacteric ripening. *Fruit Ripening, Physiology, Signalling and Genomics*, 1-14.

Klee HJ, Giovannoni JJ, 2011. Genetics and control of tomato fruit ripening and quality attributes. *Annual review of genetics* **45**, 41-59.

Northwest Horticultural Council, 2018. Pear Fact Sheet. In: Northwest Horticultural Council, ed.

Office of Agriculture B, And Textile Trade Affairs; Bureau of Economic and Business Affairs, 2013. Postharvest Loss Challenges Discussion Paper. In.: U.S. Department of State. (2015.)

Reiland H, Slavin J, 2015. Systematic review of pears and health. *Nutrition today* **50**, 301.

Rudell DR, Serra S, Sullivan N, Mattheis JP, Musacchi S, 2017. Survey of ‘d’Anjou’pear metabolic profile following harvest from different canopy positions and fruit tissues. *HortScience* **52**, 1501-10.

Serra S, Goke A, Diako C, Vixie B, Ross C, Musacchi S, 2019. Consumer perception of d'Anjou pear classified by dry matter at harvest using near-infrared spectroscopy. *International Journal of Food Science & Technology*.

Serra S, Sullivan N, Mattheis JP, Musacchi S, Rudell DR, 2018. Canopy attachment position influences metabolism and peel constituency of European pear fruit. *BMC Plant Biology* **18**, 364.

Sugar D, Basile SR, 2009. Low-temperature induction of ripening capacity in ‘Comice’and ‘Bosc’pears as influenced by fruit maturity. *Postharvest Biology and Technology* **51**, 278-80.

Sugar D, Einhorn TC, 2011. Conditioning temperature and harvest maturity influence induction of ripening capacity in ‘d’Anjou’pear fruit. *Postharvest Biology and Technology* **60**, 121-4.

Tatsuki M, Endo A, Ohkawa H, 2007. Influence of time from harvest to 1-MCP treatment on apple fruit quality and expression of genes for ethylene biosynthesis enzymes and ethylene receptors. *Postharvest Biology and Technology* **43**, 28-35.

Villalobos-Acuna M, Mitcham EJ, 2008. Ripening of European pears: the chilling dilemma. *Postharvest Biology and Technology* **49**, 187-200.

Watkins CB, 2006. The use of 1-methylcyclopropene (1-MCP) on fruits and vegetables. *Biotechnology Advances* **24**, 389-409.

Watkins CB, 2015. Advances in the use of 1-MCP. In. *Advances in postharvest fruit and vegetable technology*. CRC Press Boca Raton, FL, 117-45.

CHAPTER 1

Beyond Ethylene: New Insights Regarding the Role of AOX in the Respiratory Climacteric

Seanna Hewitt and Amit Dhingra

Molecular Plant Sciences, Department of Horticulture

Washington State University, Pullman WA 99164-614

Target Journal: Nature Horticulture

Abstract

Climacteric fruits are characterized by a dramatic increase in autocatalytic ethylene production, which is accompanied by a spike in respiration, at the onset of ripening. The change in the mode of ethylene production from autoinhibitory to auto-stimulatory is known as the system 1 (S1) to system 2 (S2) transition. Existing physiological models explain the basic and overarching genetic, hormonal, and transcriptional regulatory mechanisms governing the S1 to S2 transition of climacteric fruit. However, the links between ethylene and respiration, the two main factors that characterize the respiratory climacteric, have been largely overlooked at the molecular level. Results of recent studies indicate that the AOX respiratory pathway may play an important role in mediating cross talk between ethylene response, carbon metabolism, ATP production, and ROS signaling during climacteric ripening. New genomic, metabolic, and epigenetic information sheds light on the interconnectedness of ripening metabolic pathways, necessitating expanding the current, ethylene-centric physiological models. Understanding points at which ripening responses can be manipulated may reveal key, species- and cultivar-specific targets for regulation of ripening enabling superior strategies for reducing postharvest wastage.

Introduction

Ripening of fruit involves a symphony of transcriptionally and hormonally controlled processes that result in accumulation of sugars, reduction in acidity, development of aroma and nutritional profiles (Cherian et al., 2014). The ripening process has been under continual manipulation both as a result of natural selection for improved seed dispersal as well as human domestication via selection of desirable organoleptic properties (Liu et al., 2015a; Giovannoni, 2004). Fleshy fruits fall into one of two broadly defined ripening categories, climacteric and non-climacteric, based on the manner in which they respond to the phytohormone ethylene (Seymour et al., 2012). Non-climacteric fruit produce ethylene at basal levels throughout development and senescence. This mode of ethylene production is termed System 1 (S1) ethylene production. Non-climacteric fruits, including cherries, berries, and citrus, to name a few, are harvested ripe and do not exhibit increasing levels of ethylene production during ripening, although ripening may be accelerated through exogenous application of ethylene or ethylene-producing compounds such as Ethrel (Barry & Giovannoni, 2007; Barry et al., 2000; Chen et al., 2018). In contrast, ripening in climacteric fruits such as apple, pear, peach, avocado, mango and tomato, is characterized by a substantial increase in ethylene biosynthesis as fruit transitions from S1 to System 2 (S2) ethylene production accompanied by a concomitant burst of respiration (Figure 1.1) (Osorio et al., 2013; Chen et al., 2018). The synchronization of these two processes is known as the respiratory climacteric. Because of this distinct ripening physiology, climacteric fruit can be harvested unripe, and ripened off the tree or vine (Hiwasa-Tanase & Ezura, 2014; Seymour et al., 2013a). Following the respiratory climacteric, ripening proceeds rapidly and irreversibly, which presents additional challenges to the storage and preservation of climacteric fruit after harvest (Jogdand et al., 2017). While the concept of two distinct ripening categories is simple in theory, the reality is far more

complex, with certain fruits displaying intermediate phenotypes (Paul et al., 2012). Transcriptional and phytohormone regulation of ethylene-dependent ripening has been reviewed extensively (Cherian et al., 2014; Kumar et al., 2014; Karlova et al., 2014; Chen et al., 2018). In contrast to climacteric fruit however, the regulatory network involved in non-climacteric ripening has been much less studied. Nevertheless, it is known that abscisic acid, rather than ethylene, is essential in the control of ripening in non-climacteric fruits (Jia et al., 2016; Li et al., 2011). Results of studies in strawberry and tomato suggest that the split between climacteric and non-climacteric ripening responses may lie in the way that S-adenosyl-L-methionine (SAM) is preferentially utilized as a precursor to ethylene or as a substrate for polyamine biosynthesis, the latter of which is correlated with ABA associated gene expression in non-climacteric systems (Guo et al., 2018; Van de Poel et al., 2013). The underlying genetic factors of ripening of both climacteric and non-climacteric fruit have been explored in model systems, laying a foundation for evaluation of ripening processes in fruits exhibiting deviations. Not surprisingly, manipulation of environmental factors, genetic factors, and use of chemical inhibitors like 1-methylcyclopropene (1-MCP) to inhibit ripening result in developmental patterns that don't follow the classical model of ethylene response and signaling (Watkins, 2006; Watkins, 2015; Tatsuki et al., 2007; Chiriboga et al., 2013). Mechanisms for blockage and/or bypass of the concerted steps in classical ethylene biosynthesis are beginning to be elucidated as more studies examine variations in the classical paradigm of ripening via genetic manipulation or exogenous perturbation by temperature or chemicals (Klee & Giovannoni, 2011; Hewitt et al., 2019). As molecular biology, transcriptomics, and epigenetic analysis tools have rapidly advanced, new insights have been gained into some of the master regulators of ripening acting upstream of ethylene (Liu et al., 2015b; Giovannoni et al., 2017). This review briefly revisits the classical model of ripening before exploring points of variation from this model

that may result from physiological or chemical perturbations in metabolism, transcriptional regulatory elements, and epigenetic signatures regulating normal ethylene response in fruit. Understanding these variations may inform more efficient strategies to reduce postharvest waste while improving marketability of fruit.

Classical model for ethylene-dependent ripening

Fruits are of great economic and nutritional importance, so the way these plant organs become physiologically and horticulturally mature is of great interest to postharvest biologists. The simple, gaseous, phytohormone ethylene has long been described as *the* key player in ripening due to its clear effects on fruit organ development, maturation and stress response. Induction of the ripening process may be achieved through both endogenous and exogenous stimulation of ethylene receptors and elicit a number of downstream responses as a result of ethylene signal transduction (Cherian et al., 2014). The centrality of ethylene to the ripening process has made the components of ethylene biosynthesis and transduction primary targets for ripening control (Liu et al., 2015a; Mattoo & White, 2018). Tomato (*Solanum lycopersicum*), is the most extensively studied model system for climacteric fruit ripening as well as for understanding points at which genetic mutations in the ripening pathway result in various non-ripening phenotypes (Barry & Giovannoni, 2007; Klee & Giovannoni, 2011). The classical model of climacteric ripening in fleshy fruit involves a feed forward cycle of ethylene biosynthesis, signaling, and response. Ethylene is produced endogenously in the plant via the methionine cycle, or Yang cycle (Yang & Hoffman, 1984). In addition to playing a fundamental role in the biosynthesis of ethylene, the Yang cycle allows for the reutilization of the sulfur-containing amino acid methionine for further biosynthesis of ethylene as well as for other metabolic processes. The first committed step in

ethylene biosynthesis is the conversion of L-methionine into S-adenosyl-L-methionine (SAM). SAM is then converted into 5'-methylthioadenosine (MTA) and 1-aminocyclopropane carboxylate (ACC) via ACC synthase (ACS). ACC is subsequently converted into ethylene by 1-aminocyclopropane carboxylate oxidase (ACO). Through a series of steps, MTA is converted back into methionine, allowing for continuation of the cycle without additional input of the amino acid (Yang & Hoffman, 1984). The catalysis of ethylene production by the enzyme 1-aminocyclepropane carboxylate synthase (ACS) is thought to be the rate limiting step in ethylene biosynthesis in fruits, making it a critical point in the S1-S2 transition. While in most fruit, the S1-S2 transition is seamless, European pear (*Pyrus communis*) requires a pre-ripening period where the fruit is exposed to a genetically pre-determined amount of cold to activate the S2 autocatalytic ethylene biosynthesis (Villalobos-Acuna & Mitcham, 2008; Hendrickson et al., 2019). The process of cold temperature exposure is called 'conditioning', which then triggers the fruit to ripen (Hartmann et al., 1987) (Figure 1.1).

The roles of ACS and ACO genes in ethylene biosynthesis and ripening of climacteric fruit, both in model and non-model systems, have been well established; transcripts of both have been shown to increase in expression throughout the ripening climacteric (Pech et al., 2008, Alexander & Grierson, 2002). Following biosynthesis, ethylene is perceived by a family of ER membrane-bound ethylene receptor (ETR) family proteins (Chen et al., 2018). Proper ETR function is dependent upon the activity of RAN1, which utilizes a copper ion cofactor, and CONSTITUITIVE-TRIPLE-RESPONSE 1 (CTR1)which mediates transduction of the ethylene signal via ethylene insensitive 2 (EIN2) and ethylene insensitive-like (EIL) family proteins (Alexander & Grierson, 2002; Guo & Ecker, 2004; Binder et al., 2010; Qiao et al., 2012). Receptor function has also been shown to involve association with reversion-to-ethylene-sensitivity 1

(RTE1), a negative regulator of ethylene response, and cytochrome b5 proteins (Resnick et al., 2008; Deshpande et al., 2017; Xu et al., 2015; Barry & Giovannoni, 2007). Following successful perception of ethylene, the hormone signal is transduced via a series of messengers to the nucleus where ethylene responsive transcription factors activate downstream ripening-associated genes involved in cell wall softening, and starch to sugar conversion (Figure 1.2) (Seymour et al., 2012, Osorio et al., 2013, Cherian et al., 2014). Recent advances in genome editing have led to questioning of classic understanding of upstream transcriptional regulation of ripening (Ito et al., 2017), and have provided opportunities for targeted manipulation of genes associated with upstream transcriptional regulation of ripening, fruit texture, photoperiodic response, and post-transcriptional regulation (Martín-Pizarro & Posé, 2018). While ethylene is clearly important in ripening, this pathway does not operate in isolation. Understanding the way that other key pathways may interact with ethylene biosynthesis and response during ripening will lend important insight into how control of ripening in various fruits can be fine-tuned to increase predictability and marketability.

Involvement of AOX in fruit ripening

A major aspect of climacteric ripening that has been extensively documented in terms of physiology, but which is often overlooked at the molecular level, is respiration. As respiration has a large impact on postharvest fruit quality correlating directly with senescence, greater examination of the genetic underpinnings of the respiratory climacteric is needed. In climacteric fruits, respiratory rise occurs prior to the S1-S2 ethylene transition. An initially gradual increase in carbon dioxide evolution is followed by a heightened burst in respiratory activity during the ripening climacteric (Colombié et al., 2017; Hiwasa-Tanase & Ezura, 2014).

Climacteric respiration represents the combined activity of several mitochondrial pathways that differentially direct electron transport, leading to several possible energetic fates. The first is the cytochrome c (CYTc) pathway. CYTc operates as a result of a proton gradient generated in the mitochondrial intermembrane space and concludes in the production of cellular energy currency via ATP synthase. In plants, CYTc activity affects the synthesis of antioxidant compounds and influences cellular detoxification (Welchen & Gonzalez, 2016). At times when cellular respiratory demands are high, and CYTc is at maximum capacity, the alternative oxidase (AOX) pathway provides a secondary avenue for electron flux. Unlike CYTc, AOX is insensitive to cyanide-containing compounds, allowing for viability when normal respiratory activity is inhibited (Rogov & Zvyagilskaya, 2015). AOX also prevents overreduction of the electron transport chain when electron flux is high through CYTc pathway and can initiate a retrograde message to the nucleus to signal stress in the presence of reactive oxygen species (ROS) (LI et al., 2013). Because of this, AOX activity has been used both as an indicator of stress and as a metric to infer the energetic and metabolic status of plant biological systems during development (Saha et al., 2016).

Transition from S1-S2 ethylene biosynthesis involves numerous metabolic changes that require a great deal of regulation and feedback mechanisms to ensure that ripening occurs properly. There is increasing evidence supporting a role of AOX in the modulation of respiration at various stages around the time of climacteric via induction of S2 ethylene, which thereby influences development of ripening-associated phenotypes downstream (Perotti et al., 2014; Hendrickson et al., 2019; Xu et al., 2012; Ng et al., 2014).

Activation of AOX-mediated ripening by external stimuli

In a number of plant systems, AOX is activated as a result of cold temperatures, a strategy utilized to reduce chilling injury (Carvajal et al., 2015; Aghdam, 2013). Recently, it has been

demonstrated that completion of cold conditioning in European pear coincided with pre-climacteric maxima in AOX transcript accumulation (Hendrickson et al., 2019). Exploiting this natural phenomenon could allow for development of ripening induction strategies in fruits, such as European pear that require cold conditioning for ripening. Furthermore, knowledge of how pre-conditioning with cold temperatures can mitigate chilling injury via AOX stimulation may allow for improved management practices of mango, avocado, banana, zucchini and other temperature sensitive fruits during storage (Valenzuela et al., 2017; Aghdam, 2013; Carvajal et al., 2015; Lederman et al., 1997; Luo et al., 2015). Recent studies provide new insights into the role of AOX in fruit ripening, and how exogenous stimulation (e.g. chemical or temperature) can be utilized to intentionally modulate ripening responses (Dhingra et al., 2017; Dhingra & Hendrickson, 2017; Hendrickson et al., 2019; Hewitt et al., 2019). Results of physiology and gene expression studies of cold conditioned pear fruit suggest that pre-climacteric activity of AOX facilitating the S1-S2 transition directly impacts the ripening process in these fruit (Hendrickson et al., 2019; Dhingra & Hendrickson, 2017; Hewitt et al., 2019). Furthermore, exogenous chemical manipulation of AOX activity with hydrogen sulfide and glyoxylic acid to modulate ripening in pre-climacteric fruit has also been demonstrated (Dhingra & Hendrickson, 2017; Hewitt et al., 2019). Transcriptomic characterization of expressed genes in response to cold conditioning and to glyoxylic acid application in pear fruit has provided insight into novel ripening-associated pathways and support the putative roles of the AOX alternative respiratory pathway and the glyoxylate cycle in the larger ripening network (Hewitt & Dhingra, 2019; Hewitt et al., 2019).

It has been previously demonstrated that organic acids pyruvate and glyoxylic acid directly activate AOX in Arabidopsis through interaction with two cysteine residues that gate the protein (Umbach et al., 2006). Recent research further elucidates the role of glyoxylic acid in AOX and

ripening stimulation through activation of numerous interconnected pathways including: glyoxylate cycle, TCA cycle, fatty acid metabolism, glycolysis, and gluconeogenesis (Hewitt & Dhingra, 2019). In tomato, four isoforms of AOX have been identified, with AOX1 isoform expressed in a fruit specific manner (Holtzapffel et al., 2002; Fung et al., 2006). There is now evidence for activation and differential regulation of AOX protein isoforms and TCA cycle intermediates, as the five homologues present in Arabidopsis demonstrate differential activation as a result of application of TCA cycle metabolites (Selinski et al., 2018). The results of this study provide information necessary to develop testing "cocktails" of TCA cycle intermediates that could result in optimal activation of alternative respiration in the context of fruit ripening regulation when applied exogenously to pre-climacteric fruit postharvest. Beyond intermediates of primary respiratory metabolism, additional compounds have been shown to enhance the activation of AOX when applied exogenously in appropriate dosages. Hydrogen sulfide (H_2S), though a known phytotoxin, in miniscule doses may serve to enhance alternative pathway respiration and to inhibit ROS production (Hu et al., 2012; Li et al., 2016; Luo et al., 2015; Ziogas et al., 2018). It has also been used as a postharvest processing preservation strategy to reduce oxidative browning and fungal growth in fresh cut sliced pears (Hu et al., 2014). Physiological and gene expression studies conducted on 'D'Anjou' and 'Bartlett' pear fruit demonstrated that application of low doses of H_2S elicited a pronounced ripening response (Dhingra & Hendrickson, 2017). In addition to sulfur, AOX has been shown to be induced via the stress hormones salicylic acid (SA) and jasmonic acid (JA) which play a critical role in metabolic adjustments under stress conditions (Leng et al., 2013; Geigenberger and Fernie, 2014; Gakière et al., 2018a). Exogenous application of methyl jasmonate and methyl salicylate resulted in increased resistance to chilling injury via activation of AOX (Fung et al., 2006; Wang et al., 2015). These findings reveal

interesting insights into chemical and hormonal events that operate in an ethylene-independent space during climacteric ripening as well as how ripening can be better regulated as a result of this information.

Molecular and metabolic links between respiration and ethylene

Clearly, respiration and ethylene are physiologically correlated during climacteric ripening. Pre-climacteric rise in respiration occurs prior to the ethylene spike, but blocking ethylene prevents respiration from increasing further. In tomato, 1-MCP treatment reduces transcript levels of AOX1a (Xu et al., 2012). The inhibition of ethylene response and maintenance of respiration at low levels by 1-MCP indicates a crosstalk between ethylene and AOX at the molecular level.

Results of several studies point towards direct crosstalk between ethylene, ROS signaling, and alternative respiration in response to stress (Sewelam et al., 2016). Ethylene signaling most often occurs as a stress response. As such, it is accompanied by generation of reactive oxygen species. ROS have historically been thought of in terms of their toxicity to plants in high concentrations, however the critical roles they play in signaling and crosstalk between pathways has become clearer only recently (El-Maarouf-Bouteau et al., 2015). In Arabidopsis, ethylene has been shown to activate AOX through signaling by hydrogen peroxide (H_2O_2), a form of ROS, in response to cold temperatures (Wang et al., 2010; Wang et al., 2012). Conversely, AOX-mediated generation of ROS is thought to be responsible for retrograde signaling to the nucleus communicating the redox state of the mitochondria, thereby eliciting antioxidative responses and alteration in metabolic processes (McDonald & Vanlerberghe, 2018). Cold signals perceived by AOX are likely initiated via activation of membrane bound respiratory burst oxidase (NADPH

oxidase) homologs. These NADPH oxidases produce O_2^-, thereby triggering additional downstream ROS activated processes (Suzuki et al., 2011) (Figure 1.3)

In addition to ROS signaling, activity of AOX and biosynthesis of ethylene are directly dependent upon flux through CYTc and the availability of ATP. RNA interference studies in tomato reveal a modulatory role of *AOX* in ethylene production, as ACS4 activity in *AOX-RNAi* plants is significantly lower than in wildtype (WT) plants (Xu et al., 2012). Reduced activity of ethylene biosynthetic enzymes when *AOX* is silenced could be due to a decrease in precursors for ethylene production. For example, the methionine cycle, and therefore ethylene biosynthesis, is dependent upon ATP generation via respiration. Specifically, methionine is converted to the immediate precursor to ACC, S-adenosyl-L-methionine (SAM), in an ATP dependent reaction catalyzed by SAM synthetase (Yang & Hoffman, 1984). During ripening, AOX may allow for heightened carbon flux through glycolysis and the TCA cycle; prevention of overreduction of the ubiquinone pool in combination with increased carbon turnover results in the production of large amounts of ATP that can be used for S2 ethylene and other ripening-associated metabolic processes. Taken together, these results indicate that the regulatory effects of ethylene and AOX go both ways. AOX produces ATP and elicits retrograde signaling to nucleus via ROS. Ethylene signaling may be responsible for initial AOX activity, but the activity of both thereon out is self-perpetuating by means of an auto stimulatory feedback loop (Figure 1.3). Furthermore, it is possible that external perturbations resulting in increased AOX activity prior to the S2 transition, has a vacuum effect by causing under reduction of the mitochondrial electron transport chain, forcing the glycolytic pathway and TCA cycle into action to deliver more reducing power, thereby initiating CYTc respiratory activity. Understanding regulation of the respiratory climacteric and

how cross talk between ethylene and AOX is facilitated may require a look at the transcriptional regulators of these responses.

Transcriptional regulation modulates both ethylene and respiratory responses

Within the last decade, the importance of transcriptional regulation of ripening response has become more evident. During ripening, signals from upstream transcription factors (which may be activated by environmental or intrinsic triggers) facilitate a cascade of downstream signaling activity (Cherian et al., 2014). In fruits, this signaling activity leads to increased respiration, cell wall softening, and changes in production of pigments, volatiles, starch and sugar content, and phytonutrient metabolite content—these processes are all characteristic of ripening, with respiration and biosynthesis of ethylene particularly relevant to climacteric ripening (Karlova et al., 2014; Seymour et al., 2013b). Among some of the most important transcriptional regulators during ripening are RIN, CNR, TAG1, TAGL1, FUL1, NOR; all of these are involved in a complex and interconnected regulatory network that ultimately leads to fruit ripening and the aforementioned ripening-associated qualities (Figure 1.4). Several studies examining loss of function of some of the most critical of these transcription factors have led insight into the way in which they serve to activate ripening-associated genes (Seymour et al., 2013b).

Because of its resultant complete inhibition of ripening in tomato, the *rin* mutation has become one of the most iconic ripening-associated mutations in studies of climacteric fruit. *Rin* mutant tomatoes fail to mature beyond the green-ripe stage and do not exhibit the characteristic ripening climacteric of wild type fruit (Vrebalov et al., 2002). Commercial varieties of tomato, heterozygous for the *rin* mutation have been introduced to the market and have displayed increased shelf life with little noticeable alteration to the desired flavor profile (Garg et al., 2008). Chromatin

immunoprecipitation studies have revealed several direct targets of *RIN*, including the ethylene biosynthesizing enzyme 1-aminocyclopropene carboxylate oxidase 4 (ACO4) and the α-galacturonase (α-gal), an enzyme-associated with cell wall breakdown and fruit softening (Fujisawa et al., 2012; Martel et al., 2011). It has been demonstrated recently that α-galactosidase and ACO4 genes have RIN protein binding sites (CArG box) in their promoter regions. Furthermore, RIN protein has been shown to bind to sites in the promoter of ACS2 (Fujisawa et al., 2013). It has also been demonstrated that RIN binding is in concordance with demethylation of these promoter regions (Li et al., 2017). Expression of ACS1 and ACO1 in apple are greatly decreased when expression of various *RIN-like* MADS-box genes is downregulated or silenced (Ireland et al., 2013). Bisulfite sequencing studies revealed that binding sites in the promoter regions of known transcriptional targets of RIN were found to be demethylated suggesting that demethylation is necessary for RIN binding and development (Zhong et al. 2013). Treatment with the methyltransferase inhibitor 5-azacytidine resulted in fruit that ripened prematurely, further lending support to demethylation of binding sites as a trigger for RIN activated ripening (Zhong et al., 2013; Liu et al., 2015a). While the *rin* mutant was classically understood as a loss of function mutant, recent work suggests that it is actually a gain-of-function mutant that produces a protein that actively represses ripening (Ito et al., 2017). Regardless, it is clear that when RIN is perturbed, ripening does not proceed to completion. Tomato Agamous 1 (TAG1), Tomato Agamous-like 1 (TAGL1), and MADS-box transcription factors Fruitful 1 and 2 can form complexes with RIN. Mutation of these regulatory factors also results in decreased ripening capacity, and transgenic repression of TAGL1 results in fruit with similar non-ripening phenotypes (Itkin et al., 2009; Garceau et al., 2017). Colorless non-ripening (CNR) transcription factor is a squamosa promoter binding-like protein, which appears to be necessary for RIN to bind to promoters (Martel et al.,

2011; Zhong et al., 2013). With a hypermethylated, heritable promoter that results in reduced transcriptional activity, CNR is a unique example of an epiallele (Seymour et al., 2013b). Thus, the CNR transcription factor lends evidence for a role of epigenetics in critical developmental transitions such as those that occur during S1-S2 ethylene production and ripening. Another TF among the core set of regulatory elements is the NAC-domain containing protein at the tomato non-ripening (NOR) locus. NOR mutants fail to ripen in a physiologically similar manner to RIN and TAGL1 mutants. NOR acts upstream of ethylene biosynthesis and, like RIN, appears to bind to promotor regions of genes involved in ethylene biosynthesis, thereby positively regulating ripening (Gao et al., 2018) (Figure 1.4). It is unclear whether RIN and NOR interact with one another to stimulate ripening in conjunction. Considering increasing understanding of its regulatory role in ripening, NOR has been a recent target for improving shelf life in tomato fruit (Nguyen & Sim, 2017).

The availability of many of these mutants in tomato, allows for study of consequences of ripening perturbation at the regulatory level. Many studies have demonstrated the detrimental effects of mutations in these key transcriptional regulators on ethylene production and signaling. Recently, the role of AOX has been investigated in NOR, CNR, and RIN mutant fruit (Xu et al., 2012; Manning et al., 2006; Perotti et al., 2014). Interestingly, AOX activity elicits differential effects in these mutants, and expression of RIN, CNR, and the ethylene receptor Never Ripe (NR) has been observed in fruit in which *AOX* is silenced. When *AOX* was inhibited via RNA interference, the expression of these transcriptional regulators decreased (Xu et al., 2012). This is interesting, as CNR acts upstream of ethylene biosynthesis and the NR receptor acts downstream; this observation suggests that AOX plays a yet uncharacterized role in ripening mediated by

transcriptional regulators that affect ethylene biosynthesis, signal transduction, and response (Seymour et al., 2013b; Giovannoni, 2004; Hewitt et al., 2019).

Epigenetic and epigenomic factors in regulation of ripening

Epigenetics refers to the heritable modifications of the genome beyond the physical nucleotide sequence, including DNA methylation and modifications to histone proteins, while epigenomics refers to all modifications, regardless of heritability (Giovannoni et al., 2017). One of the most commonly studied forms of epigenetic modification is DNA methylation. Methylation status is in constant flux due to the changing environment; therefore, condition specific methylation status may be used to infer stress condition, ripening competency, and developmental progress among other things.

Temperature is known to be a major factor in alteration of methylation status, and conditioning of fruit requiring chilling to ripen, or to avoid chilling injury, could affect the methylation of promoter regions of key ripening related genes and regulatory transcription factors. With more tools for epigenetic and epigenomic analyses available, including bisulfite sequencing and PacBio long read sequencing, new insights are being gained into the impact of epigenetic signatures on development and senescence of fruit. Understanding how the epigenome governs downstream transcriptional regulation and response is critical to better understanding ripening and senescence.

Chemically induced demethylation of tomato fruit using 5-azacytidine results in early ripening of fruit. This finding indicates that alteration of methylation status is one of the first steps in regulation of downstream processes associated with ripening. Recent transcriptomic and gene ontology enrichment analysis of cold conditioned ‘D’Anjou’ and ‘Bartlett’ pear fruit suggests that both methylation and chromatin modifications may be important for activation of vernalization-

associated genes and ripening-associated transcriptional elements (Hewitt et al., 2019). Interestingly, these two cultivars appear to differentially engage expression of vernalization genes VRN1 and VIN3, which could justify the need for different cold exposure time in different cultivars (Hewitt et al., 2019). Studying the epigenomic status in light of mutations to key transcription factors, mentioned previously, illuminates the way that DNA methylation and histone modifications in genetic regulatory elements serve to modulate certain aspects of ripening early on in development. The breadth of factors that may contribute to alterations in the epigenome is still being elucidated. Beyond abiotic influences, it is possible that some signal from mature seeds is originally what signals the onset of ripening progression. This hypothesis is supported by the recent characterization of enzymes responsible for removing epigenetic signatures to DNA or histones, such as the recently characterized DEMETER-like DNA demethylase gene *SlDML2* in tomato (Liu et al., 2015b). These proteins are particularly highly expressed in the locular tissue surrounding mature seeds in fruit.

Opportunities to understand the molecular basis of ripening with omics resources

The ever-growing fields of genomics, transcriptomics, and epigenomics offer high throughput strategies for extrapolation of important biological function and expression information from large datasets in both pairwise and multifactorial time course experiments (Morozova & Marra, 2008; Banerjee et al., 2019). Use of such next generation sequencing technologies allows for understanding the array of diverse transcriptional responses, interaction of associated pathways, functional implications of the S1-S2 transition, and interdependent roles of ethylene and respiration in ripening (Nham et al., 2017; Hewitt & Dhingra, 2019; Hewitt et al., 2019). The relatively new ability to examine plant epigenomes, and most recently the “ripenome” at the level of single nucleotide bases reveals further avenues for understanding epigenetic regulators of the

transition from pre-climacteric to climacteric, and the subsequent development of species-specific ripening phenotypes (Giovannoni et al., 2017). Such approaches used independently or in conjunction, will facilitate identification of candidate regulators of important ripening and fruit quality-associated characteristics in fruits.

Conclusion

The interplay of ethylene (biosynthesis, signaling and response) and respiration (CYTc and AOX) have been extensively characterized at the physiological level. Studies conducted within the last several years provide new insights with respect to the connection of these two critical pathways, hallmarks of the respiration climacteric, at the molecular and metabolic level. *AOX* activity begins to increase during the pre-climacteric phase, prior to the S1-S2 ethylene transition, particularly in the context of cold temperature conditioning or other external stimuli. This activity may be accompanied by the accumulation of ROS as the CYTc pathway capacity reaches maximum. At the onset of S2 ethylene stimulation of both ethylene response and alternative pathway respiration appear to be interdependent, with ethylene biosynthesis requiring ATP generated via respiration and activity of respiratory pathways modulated by ethylene responsive transcription factors in the nucleus, as evidenced by the inhibition of ethylene production and respiration by the ethylene receptor antagonist 1-MCP. The commencement of both ethylene and respiration-associated processes is likely due to upstream transcriptional and epigenetic regulators, as well as the signaling activity of ROS generated during increased respiration. Mutants of master transcriptional regulators in tomato have provided a means for the study of their effects on both ethylene production and respiration during ripening. Furthermore, studies have investigated the effects of temperature and chemical manipulation of AOX with the aim to understand ways in

which the timing of ripening can be controlled. AOX and related processes represent a new frontier in regulating postharvest ripening and therefore better management to reduce fruit wastage and development of postharvest management strategies in different types of fruits.

REFERENCES

Aghdam MS, 2013. Role of alternative oxidase in postharvest stress of fruit and vegetables: Chilling injury. *African Journal of Biotechnology* **12**, 7009-16.

Alexander L, Grierson D, 2002. Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. *Journal of experimental botany* **53**, 2039-55.

Banerjee R, Kumar GV, Kumar SJ, 2019. *Omics-based Approaches in Plant Biotechnology*. John Wiley & Sons.

Barry CS, Giovannoni JJ, 2007. Ethylene and fruit ripening. *Journal of Plant Growth Regulation* **26**, 143.

Barry CS, Llop-Tous MI, Grierson D, 2000. The regulation of 1-aminocyclopropane-1-carboxylic acid synthase gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato. *Plant Physiology* **123**, 979-86.

Binder BM, Rodríguez FI, Bleecker AB, 2010. The copper transporter RAN1 is essential for biogenesis of ethylene receptors in Arabidopsis. *Journal of Biological Chemistry* **285**, 37263-70.

Carvajal F, Palma F, Jamilena M, Garrido D, 2015. Preconditioning treatment induces chilling tolerance in zucchini fruit improving different physiological mechanisms against cold injury. *Annals of Applied Biology* **166**, 340-54.

Chen Y, Grimplet J, David K, *et al.*, 2018. Ethylene receptors and related proteins in climacteric and non-climacteric fruits. *Plant Science* **276**, 63-72.

Cherian S, Figueroa CR, Nair H, 2014. 'Movers and shakers' in the regulation of fruit ripening: a cross-dissection of climacteric versus non-climacteric fruit. *Journal of experimental botany* **65**, 4705-22.

Chiriboga M-A, Saladié M, Bordonaba JG, Recasens I, Garcia-Mas J, Larrigaudière C, 2013. Effect of cold storage and 1-MCP treatment on ethylene perception, signalling and synthesis: Influence on the development of the evergreen behaviour in 'Conference'pears. *Postharvest Biology and Technology* **86**, 212-20.

Colombié S, Beauvoit B, Nazaret C, *et al.*, 2017. Respiration climacteric in tomato fruits elucidated by constraint-based modelling. *New Phytologist* **213**, 1726-39.

Deshpande S, James A, Franklin CH, Leach LJ, Taramonli S, Yang J. An RNA-Seq Bioinformatics Pipeline for Data Processing of Arabidopsis Thaliana Datasets. *Proceedings of the Proceedings of the International Conference on Bioinformatics Research and Applications 2017, 2017*: ACM, 1-8.

Dhingra A, Hendrickson C, 2017. Control of ripening and senescence in pre-harvest and post-harvest plants and plant materials. In.: Google Patents.

Dhingra A, Hendrickson C, Hewitt S, 2017. Control of ripening and senescence in pre-harvest and post-harvest plants and plant materials by manipulating alternative oxidase activity. In.: Google Patents.

El-Maarouf-Bouteau H, Sajjad Y, Bazin J, *et al.*, 2015. Reactive oxygen species, abscisic acid and ethylene interact to regulate sunflower seed germination. *Plant, Cell & Environment* **38**, 364-74.

Fujisawa M, Nakano T, Shima Y, Ito Y, 2013. A large-scale identification of direct targets of the tomato MADS box transcription factor RIPENING INHIBITOR reveals the regulation of fruit ripening. *The Plant Cell* **25**, 371-86.

Fujisawa M, Shima Y, Higuchi N, *et al.*, 2012. Direct targets of the tomato-ripening regulator RIN identified by transcriptome and chromatin immunoprecipitation analyses. *Planta* **235**, 1107-22.

Fung RW, Wang CY, Smith DL, Gross KC, Tao Y, Tian M, 2006. Characterization of alternative oxidase (AOX) gene expression in response to methyl salicylate and methyl jasmonate pre-treatment and low temperature in tomatoes. *Journal of plant physiology* **163**, 1049-60.

Gao Y, Wei W, Zhao X, *et al.*, 2018. A NAC transcription factor, NOR-like1, is a new positive regulator of tomato fruit ripening. *Horticulture research* **5**.

Garceau DC, Batson MK, Pan IL, 2017. Variations on a theme in fruit development: the PLE lineage of MADS-box genes in tomato (TAGL1) and other species. *Planta* **246**, 313-21.

Garg N, Cheema D, Dhatt A, 2008. Utilization of rin, nor, and alc alleles to extend tomato fruit availability. *International journal of vegetable science* **14**, 41-54.

Giovannoni J, Nguyen C, Ampofo B, Zhong S, Fei Z, 2017. The epigenome and transcriptional dynamics of fruit ripening. *Annual Review of Plant Biology* **68**, 61-84.

Giovannoni JJ, 2004. Genetic regulation of fruit development and ripening. *The Plant Cell* **16**, S170-S80.

Guo H, Ecker JR, 2004. The ethylene signaling pathway: new insights. *Current Opinion in Plant Biology* **7**, 40-9.

Guo J, Wang S, Yu X, *et al.*, 2018. Polyamines Regulate Strawberry Fruit Ripening by Abscisic Acid, Auxin, and Ethylene. *Plant Physiology* **177**, 339-51.

Hartmann C, Drouet A, Morin F, 1987. Ethylene and ripening of apple, pear and cherry fruit. *Plant Physiology and Biochemistry (France)*.

Hendrickson C, Hewitt S, Swanson ME, Einhorn T, Dhingra A, 2019. Evidence for pre-climacteric activation of AOX transcription during cold-induced conditioning to ripen in European pear (Pyrus communis L.). *bioRxiv*, 755686.

Hewitt S, Dhingra A, 2019. Glyoxylic acid overcomes 1-MCP induced blockage of fruit ripening in Pyrus communis L. var. ‘D’Anjou. *Pre-publication*.

Hewitt S, Hendrickson C, Dhingra A, 2019. Vernalization-related genes regulate cold-induced ripening in 'D'Anjou' and 'Bartlett' pear fruit. *Pre-publication*.

Hiwasa-Tanase K, Ezura H, 2014. Climacteric and non-climacteric ripening. *Fruit Ripening, Physiology, Signalling and Genomics*, 1-14.

Holtzapffel RC, Finnegan PM, Millar AH, Badger MR, Day DA, 2002. Mitochondrial protein expression in tomato fruit during on-vine ripening and cold storage. *Functional Plant Biology* **29**, 827-34.

Hu K-D, Wang Q, Hu L-Y, *et al.*, 2014. Hydrogen sulfide prolongs postharvest storage of fresh-cut pears (Pyrus pyrifolia) by alleviation of oxidative damage and inhibition of fungal growth. *PLoS ONE* **9**, e85524.

Hu L-Y, Hu S-L, Wu J, *et al.*, 2012. Hydrogen sulfide prolongs postharvest shelf life of strawberry and plays an antioxidative role in fruits. *Journal of agricultural and food chemistry* **60**, 8684-93.

Ireland HS, Yao JL, Tomes S, *et al.*, 2013. Apple SEPALLATA1/2-like genes control fruit flesh development and ripening. *The Plant Journal* **73**, 1044-56.

Itkin M, Seybold H, Breitel D, Rogachev I, Meir S, Aharoni A, 2009. TOMATO AGAMOUS-LIKE 1 is a component of the fruit ripening regulatory network. *The Plant Journal* **60**, 1081-95.

Ito Y, Nishizawa-Yokoi A, Endo M, *et al.*, 2017. Re-evaluation of the rin mutation and the role of RIN in the induction of tomato ripening. *Nature plants* **3**, 866.

Jia H, Jiu S, Zhang C, et al., 2016. Abscisic acid and sucrose regulate tomato and strawberry fruit ripening through the abscisic acid-stress-ripening transcription factor. Plant Biotechnology

Jogdand S, Bhat S, Misra K, Kshirsagar A, Lal R, 2017. New promising molecules for ethylene management in fruit crops, 1-MCP and nitric oxide: A review. *IJCS* **5**, 434-41.

Karlova R, Chapman N, David K, Angenent GC, Seymour GB, De Maagd RA, 2014. Transcriptional control of fleshy fruit development and ripening. *Journal of experimental botany* **65**, 4527-41.

Klee HJ, Giovannoni JJ, 2011. Genetics and Control of Tomato Fruit Ripening and Quality Attributes. *Annual review of genetics* **45**, 41-59.

Kumar R, Khurana A, Sharma AK, 2014. Role of plant hormones and their interplay in development and ripening of fleshy fruits. *Journal of experimental botany* **65**, 4561-75.

Lederman IE, Zauberman G, Weksler A, Rot I, Fuchs Y, 1997. Ethylene-forming capacity during cold storage and chilling injury development in 'Keitt'mango fruit. *Postharvest Biology and Technology* **10**, 107-12.

Li C, Jia H, Chai Y, Shen Y, 2011. Abscisic acid perception and signaling transduction in strawberry: a model for non-climacteric fruit ripening. Plant signaling & behavior 6, 1950-3.

Li CR, Liang DD, Li J, *et al.*, 2013. Unravelling mitochondrial retrograde regulation in the abiotic stress induction of rice ALTERNATIVE OXIDASE 1 genes. *Plant, Cell & Environment* **36**, 775-88.

Li D, Limwachiranon J, Li L, Du R, Luo Z, 2016. Involvement of energy metabolism to chilling tolerance induced by hydrogen sulfide in cold-stored banana fruit. *Food chemistry* **208**, 272-8.

Li L, Wang X, Zhang X, Guo M, Liu T, 2017. Unraveling the target genes of rin transcription factor during tomato fruit ripening and softening. *Journal of the Science of Food and Agriculture* **97**, 991-1000.

Liu M, Pirrello J, Chervin C, Roustan J-P, Bouzayen M, 2015a. Ethylene control of fruit ripening: revisiting the complex network of transcriptional regulation. *Plant Physiology* **169**, 2380-90.

Liu R, How-Kit A, Stammitti L, *et al.*, 2015b. A DEMETER-like DNA demethylase governs tomato fruit ripening. *Proceedings of the National Academy of Sciences* **112**, 10804-9.

Luo Z, Li D, Du R, Mou W, 2015. Hydrogen sulfide alleviates chilling injury of banana fruit by enhanced antioxidant system and proline content. *Scientia horticulturae* **183**, 144-51.

Manning K, Tör M, Poole M, *et al.*, 2006. A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. *Nature genetics* **38**, 948.

Martel C, Vrebalov J, Tafelmeyer P, Giovannoni JJ, 2011. The tomato MADS-box transcription factor RIPENING INHIBITOR interacts with promoters involved in numerous ripening processes in a COLORLESS NONRIPENING-dependent manner. *Plant Physiology* **157**, 1568-79.

Martín-Pizarro C, Posé D, 2018. Genome Editing as a Tool for Fruit Ripening Manipulation. *Frontiers in Plant Science* **9**.

Mattoo AK, White WB, 2018. Regulation of ethylene biosynthesis. In. *The plant hormone ethylene.* CRC Press, 21-42.

Mcdonald AE, Vanlerberghe GC, 2018. The organization and control of plant mitochondrial metabolism. *Annual Plant Reviews online*, 290-324.

Morozova O, Marra MA, 2008. Applications of next-generation sequencing technologies in functional genomics. *Genomics* **92**.

Ng S, De Clercq I, Van Aken O, *et al.*, 2014. Anterograde and retrograde regulation of nuclear genes encoding mitochondrial proteins during growth, development, and stress. *Molecular plant* **7**, 1075-93.

Nguyen TT, Sim S-C, 2017. Development of a Gene-based Marker for the non-ripening (nor) Gene in Cultivated Tomato. *한국원예학회 학술발표요지*, 110-.

Nham NT, Willits N, Zakharov F, Mitcham EJ, 2017. A model to predict ripening capacity of 'Bartlett'pears (Pyrus communis L.) based on relative expression of genes associated with the ethylene pathway. *Postharvest Biology and Technology* **128**, 138-43.

Osorio S, Scossa F, Fernie A, 2013. Molecular regulation of fruit ripening. *Frontiers in Plant Science* **4**.

Paul V, Pandey R, Srivastava G, 2012. *The fading distinctions between classical patterns of ripening in climacteric and non-climacteric fruit and the ubiquity of ethylene-An overview*.

Perotti VE, Moreno AS, Podestá FE, 2014. Physiological aspects of fruit ripening: the mitochondrial connection. *Mitochondrion* **17**, 1-6.

Qiao H, Shen Z, Huang S-SC, *et al.*, 2012. Processing and subcellular trafficking of ER-tethered EIN2 control response to ethylene gas. *Science* **338**, 390-3.

Resnick JS, Rivarola M, Chang C, 2008. Involvement of RTE1 in conformational changes promoting ETR1 ethylene receptor signaling in Arabidopsis. *The Plant Journal* **56**, 423-31.

Rogov A, Zvyagilskaya R, 2015. Physiological role of alternative oxidase (from yeasts to plants). *Biochemistry (Moscow)* **80**, 400-7.

Saha B, Borovskii G, Panda SK, 2016. Alternative oxidase and plant stress tolerance. *Plant signaling & behavior* **11**, e1256530.

Selinski J, Hartmann A, Deckers-Hebestreit G, Day DA, Whelan J, Scheibe R, 2018. Alternative oxidase isoforms are differentially activated by tricarboxylic acid cycle intermediates. *Plant Physiology* **176**, 1423-32.

Sewelam N, Kazan K, Schenk PM, 2016. Global plant stress signaling: reactive oxygen species at the cross-road. *Frontiers in Plant Science* **7**, 187.

Seymour GB, Chapman NH, Chew BL, Rose JK, 2013a. Regulation of ripening and opportunities for control in tomato and other fruits. *Plant Biotechnology Journal* **11**, 269-78.

Seymour GB, Taylor JE, Tucker GA, 2012. *Biochemistry of fruit ripening*. Springer Science & Business Media.

Seymour GB, Tucker GA, Poole M, Giovann, 2013b. *The molecular biology and biochemistry of fruit ripening*. Wiley Online Library.

Suzuki N, Miller G, Morales J, Shulaev V, Torres MA, Mittler R, 2011. Respiratory burst oxidases: the engines of ROS signaling. *Current Opinion in Plant Biology* **14**, 691-9.

Sugar, D, & Einhorn, T, 2011. Conditioning temperature and harvest maturity influence induction of ripening capacity in ‘d’Anjou’pear fruit. *Postharvest Biology and Technology,* **60,** 121-124.

Tatsuki M, Endo A, Ohkawa H, 2007. Influence of time from harvest to 1-MCP treatment on apple fruit quality and expression of genes for ethylene biosynthesis enzymes and ethylene receptors. *Postharvest Biology and Technology* **43**, 28-35.

Umbach AL, Ng VS, Siedow JN, 2006. Regulation of plant alternative oxidase activity: A tale of two cysteines. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1757**, 135-42.

Valenzuela JL, Manzano S, Palma F, Carvajal F, Garrido D, Jamilena M, 2017. Oxidative stress associated with chilling injury in immature fruit: postharvest technological and biotechnological solutions. *International journal of molecular sciences* **18**, 1467.

Van De Poel B, Bulens I, Oppermann Y, *et al.*, 2013. S-adenosyl-l-methionine usage during climacteric ripening of tomato in relation to ethylene and polyamine biosynthesis and transmethylation capacity. *Physiologia plantarum* **148**, 176-88.

Villalobos-Acuna M, Mitcham EJ, 2008. Ripening of European pears: the chilling dilemma. *Postharvest Biology and Technology* **49**, 187-200.

Vrebalov J, Ruezinsky D, Padmanabhan V, *et al.*, 2002. A MADS-box gene necessary for fruit ripening at the tomato ripening-inhibitor (rin) locus. *Science* **296**, 343-6.

Wang H, Huang J, Liang X, Bi Y, 2012. Involvement of hydrogen peroxide, calcium, and ethylene in the induction of the alternative pathway in chilling-stressed Arabidopsis callus. *Planta* **235**, 53-67.

Wang H, Liang X, Huang J, *et al.*, 2010. Involvement of ethylene and hydrogen peroxide in induction of alternative respiratory pathway in salt-treated Arabidopsis calluses. *Plant and Cell Physiology* **51**, 1754-65.

Wang L, Baldwin EA, Plotto A, *et al.*, 2015. Effect of methyl salicylate and methyl jasmonate pre-treatment on the volatile profile in tomato fruit subjected to chilling temperature. *Postharvest Biology and Technology* **108**, 28-38.

Watkins CB, 2006. The use of 1-methylcyclopropene (1-MCP) on fruits and vegetables. *Biotechnology Advances* **24**, 389-409.

Watkins CB, 2015. Advances in the use of 1-MCP. In. *Advances in postharvest fruit and vegetable technology*. CRC Press Boca Raton, FL, 117-45.

Welchen E, Gonzalez DH, 2016. Cytochrome c, a hub linking energy, redox, stress and signaling pathways in mitochondria and other cell compartments. *Physiologia plantarum* **157**, 310-21.

Xu C, Zhou X, Wen C-K, 2015. HYPER RECOMBINATION1 of the THO/TREX complex plays a role in controlling transcription of the REVERSION-TO-ETHYLENE SENSITIVITY1 gene in Arabidopsis. *PLoS genetics* **11**, e1004956.

Xu F, Yuan S, Zhang D-W, Lv X, Lin H-H, 2012. The role of alternative oxidase in tomato fruit ripening and its regulatory interaction with ethylene. *Journal of experimental botany* **63**, 5705-16.

Yang SF, Hoffman NE, 1984. Ethylene biosynthesis and its regulation in higher plants. *Annual review of plant physiology* **35**, 155-89.

Zhong S, Fei Z, Chen Y-R, *et al.*, 2013. Single-base resolution methylomes of tomato fruit development reveal epigenome modifications associated with ripening. *Nat Biotech* **31**, 154-9.

Ziogas V, Molassiotis A, Fotopoulos V, Tanou G, 2018. Hydrogen sulfide: A potent tool in postharvest fruit biology and possible mechanism of action. *Frontiers in Plant Science* **9**.

FIGURES

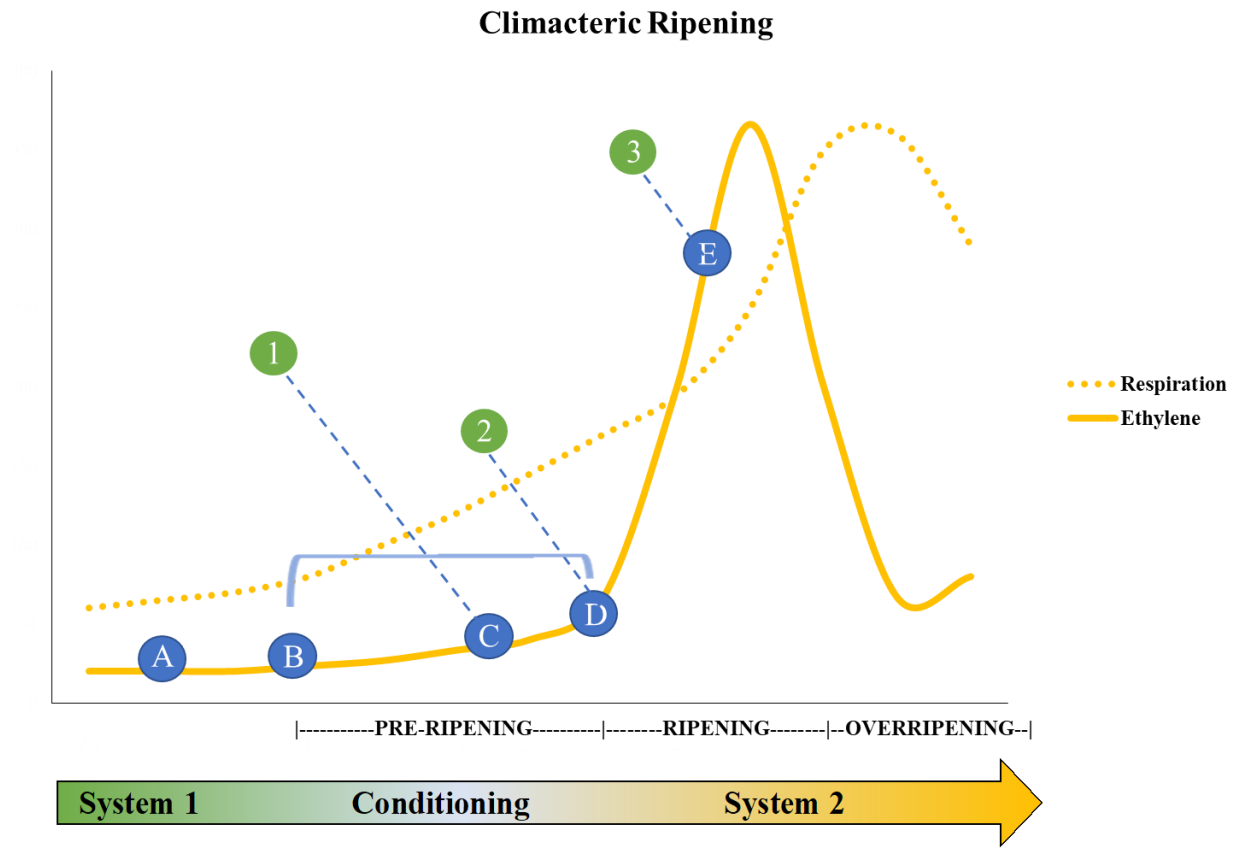

Figure 1.1 Ripening of climacteric fruit. Numbers refer to degree of responsiveness to ethylene: (1) S1 ethylene production, (2) Start of S2 ethylene production; (3) Increased ethylene response due to autocatalytic synthesis and post-transcriptional effects of ethylene. Letters represent critical points of fruit development: (A) Fruit tissues begin to become sensitive to ethylene; (B) Fruit reaches physiological maturity (seeds are mature); (C) ethylene synthesis reaches level 1; (D) Ethylene synthesis reaches level 2; (E) Ethylene synthesis reaches level 3. Respiration begins to increase during the pre-ripening phase and is amplified greatly after ethylene sensitivity is highest. Some fruits require pre-climacteric cold conditioning at 0-10°C (indicated by the light blue, solid line) to become competent to ripen (Adapted from Hartmann et al., 1987).

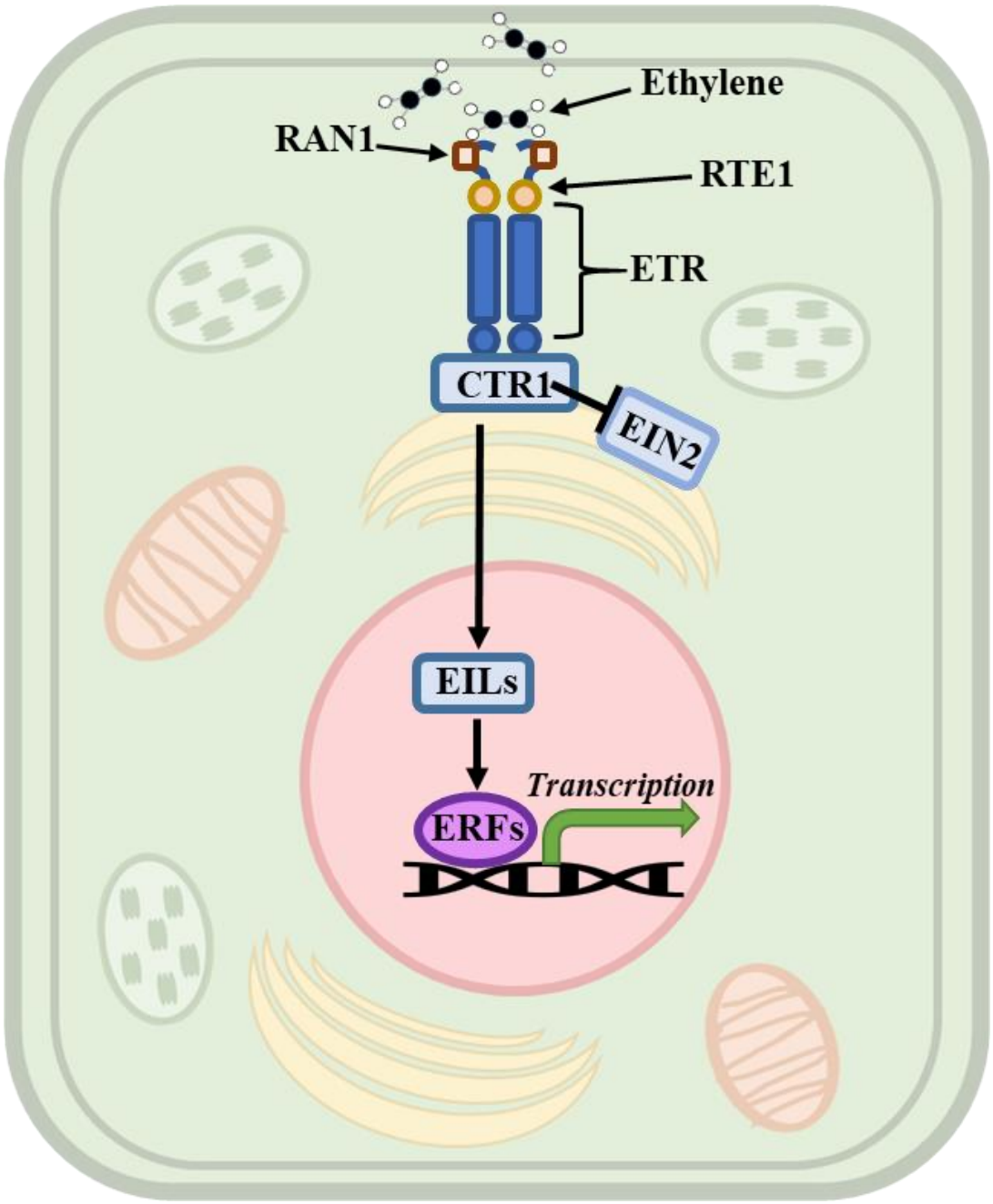

Figure 1.2 Main components of classical ethylene perception and signaling and cellular location. Ethylene is perceived by ETRs in conjunction with RTE and CTR1. Activation of CTR1 leads to initiation of ethylene responses and inhibition of EIN2, a negative regulator of ethylene signaling. Signal cascades mediated by EILs and ending with translocation of ERFs to the nucleus result in transcriptional activation of ethylene responses.

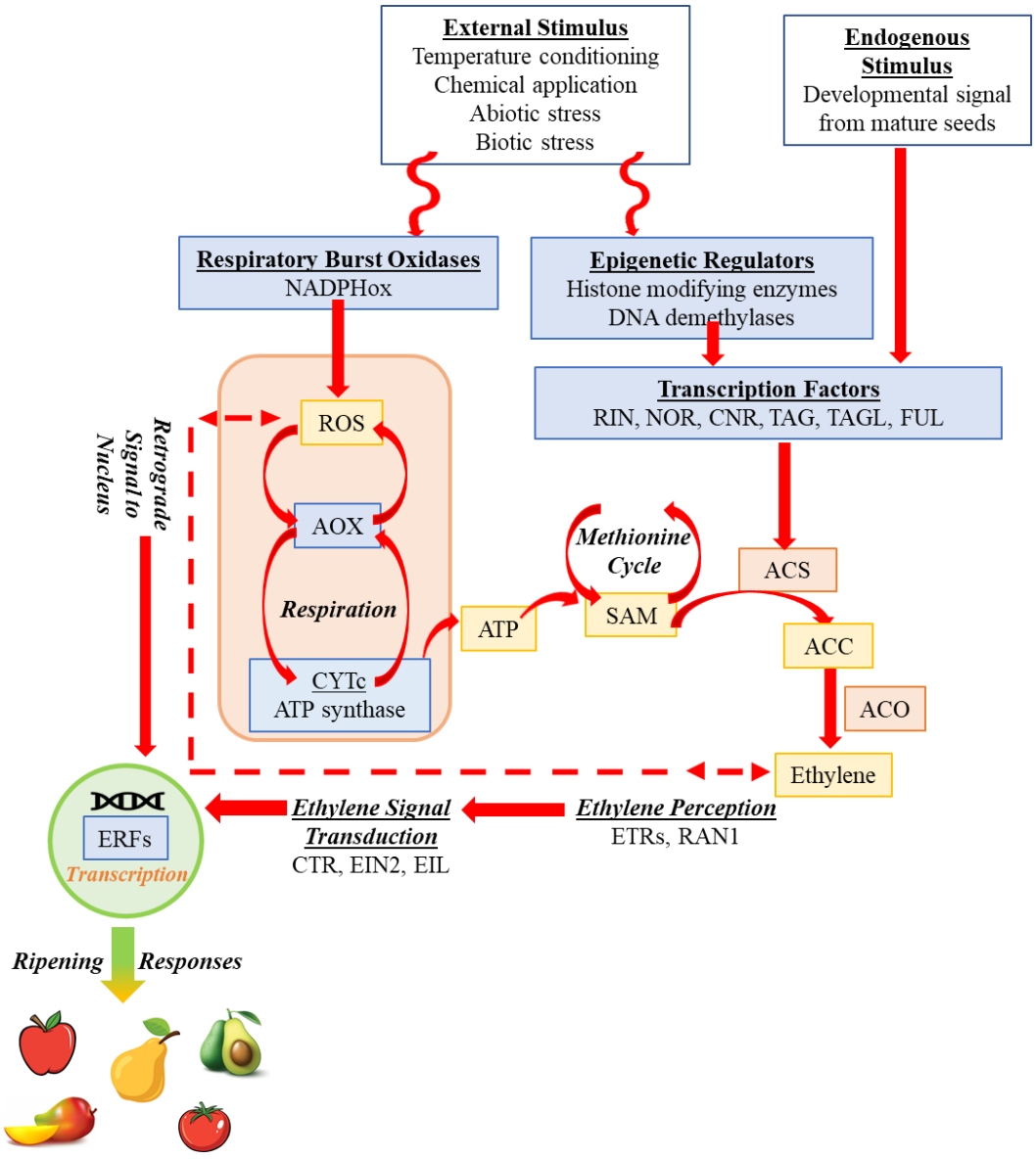

Figure 1.3 Proposed crosstalk between ethylene and respiration-associated pathways. External stimulus, such as cold or chemical application, induces respiratory burst oxidases and epigenetic regulators of ripening. NADPHox initiates ROS mediated activation of AOX and CYTc pathways. Activation of transcriptional regulators allows for facilitation of downstream ethylene responses. AOX may mediate additional crosstalk with ethylene-associated pathways via retrograde signaling to the nucleus based upon cellular redox state and presence of ROS.

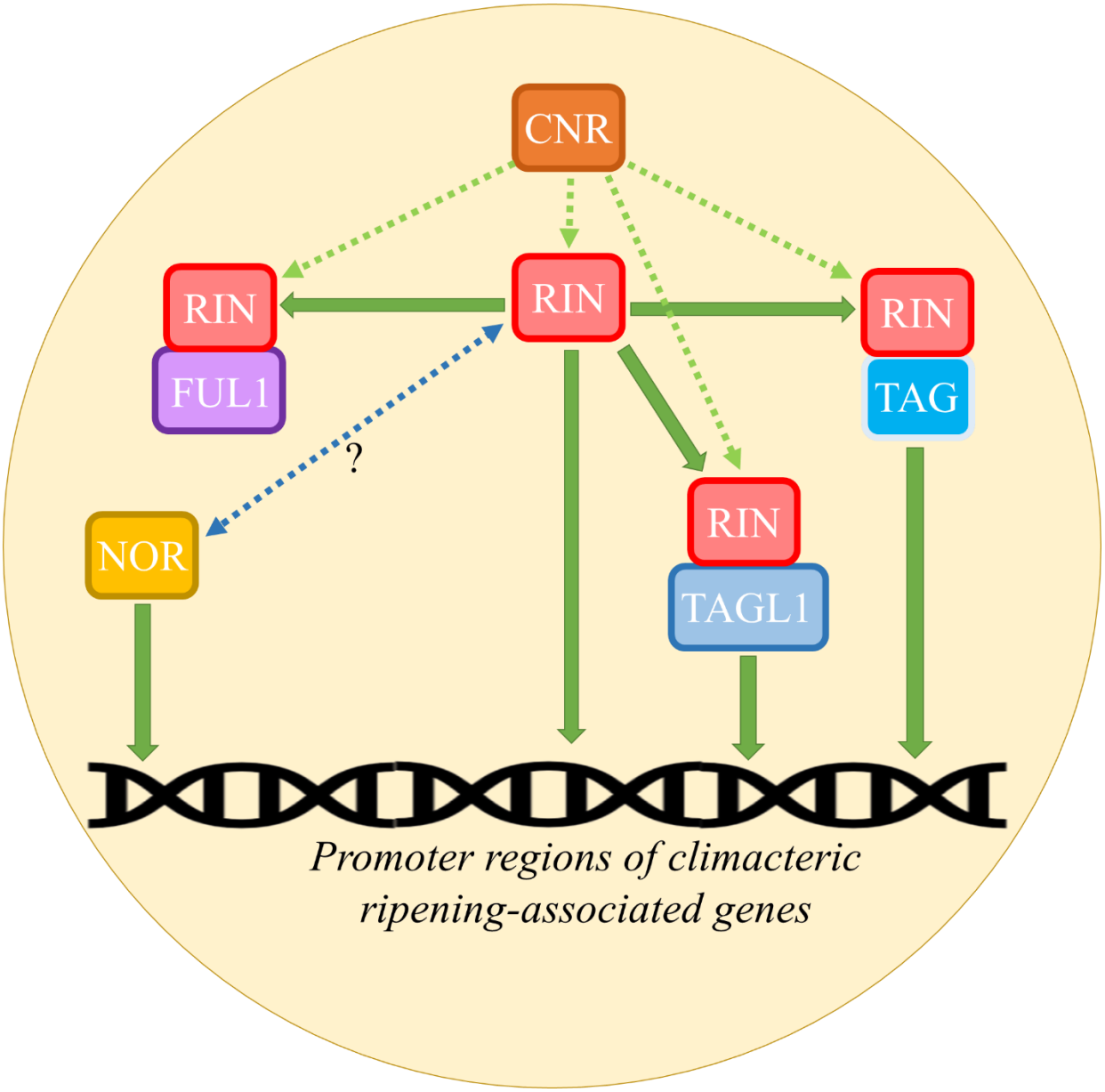

Figure 1.4 Transcriptional activation of ripening-associated genes. RIN may act independently or in complexes with FUL1, TAG, and TAGL1 transcription factors to activate ripening. Dashed green arrows indicate that direct vs. indirect interaction between RIN and CNR is yet to be determined. Dashed blue arrow indicates that interaction between RIN and NOR is unclear.

CHAPTER TWO

Evidence for the Involvement of Vernalization-related Genes in the Regulation of Cold-induced Ripening in 'D'Anjou' and 'Bartlett' Pear Fruit

Seanna Hewitt[1,3], Christopher A. Hendrickson[2], and Amit Dhingra[1,3*]

1 Molecular Plant Sciences, Washington State University, Pullman, Washington

2 National University, San Diego, California

3 Department of Horticulture, Washington State University, Pullman, Washington

*Corresponding author: adhingra@wsu.edu

Target Journal: Nature Scientific Reports

Abstract

European pear (*Pyrus communis L.*) cultivars require a genetically pre-determined duration of cold-temperature exposure to induce autocatalytic ethylene biosynthesis and subsequent fruit ripening. The physiological responses of pear to cold-temperature-induced ripening have been well characterized, but the molecular mechanisms underlying this phenomenon are still being elucidated. This study employed established cold temperature conditioning treatments for ripening of two pear cultivars, 'D'Anjou' and 'Bartlett'. Using a transcriptomic approach, global gene expression responses of each cultivar were assessed at four different developmental stages during the cold conditioning process. Differential expression, functional annotation, and gene ontology

enrichment analyses facilitated identification of cold-induced, vernalization-related genes and repressors of endodormancy release that have not previously been described in fruit during the ripening transition. The resulting data provide insight into mechanisms of cold-induced transcriptional regulation of ripening in European pear, as well as a unique comparative analysis of two cultivars with very different cold conditioning requirements.

Introduction

Pear (*Pyrus spp.*) is an economically important and nutritionally valuable tree fruit genus worldwide. European pear (*Pyrus communis L.*) cultivars are among the most widespread, commercially grown Pyrus members, and are cultivated in Europe, North America, South America, Africa, and Australia (Xie et al., 2013). Along with apples, bananas, peaches, tomatoes, mangoes, and avocados, European pear is classified as climacteric in its ripening profile. Ripening in most climacteric fruits involves a seamless transition between system 1 (S1) and system 2 (S2) ethylene production. This is the point at which regulation of ethylene synthesis changes from autoinhibitory to auto-stimulatory (Seymour et al., 2012; Barry et al., 2000). Increased ethylene biosynthesis during climacteric ripening is accompanied by a concomitant spike in respiration (Seymour et al., 2013; Klee & Giovannoni, 2011; Alexander & Grierson, 2002). A prominent aspect that distinguishes European pear and Chinese white pear (*Pyrus bretschneideri* Rehder.) from most other climacteric fruits is that these species have a pre-ripening period during which they require a specific amount of cold exposure in order to transition from S1 to S2 ethylene production (Hartmann, 1987; Hewitt & Dhingra, 2019). The process of cold exposure is called 'conditioning' and accumulation of chilling hours necessary for this transition, and therefore ripening initiation, is referred to as the 'chilling requirement' (Sugar & Einhorn, 2011).

The chilling requirement for ripening varies by cultivar. 'Bartlett' pears require 15 days of chilling, 'Comice' require 30, and 'D'Anjou' require 60 days of chilling at 0°C. 'Passe Crassane' pears lie at the extreme end of the conditioning spectrum, with a requirement of 90 days of chilling at 0°C to ripen. However, the duration of chilling may be manipulated by increasing the temperature at which conditioning is conducted, with an appropriate temperature range of 0-15°C

(Sugar & Einhorn, 2011). In addition to genetic predetermination for chilling duration, chilling time is affected by maturity of the fruit at harvest, with pears harvested at a greater maturity index requiring a less extensive period of cold conditioning, and vice versa (Sugar & Basile, 2009).

Physiological studies have characterized the conditioning requirements for a range of European pear cultivars under defined conditioning temperatures, exogenous ethylene application regimes, and other pre-harvest treatments (Sugar & Einhorn, 2011; Villalobos-Acuna & Mitcham, 2008; Chiriboga et al., 2013; Sugar & Basile, 2009). While exogenous ethylene treatment reduces cold conditioning needs, in most European pear cultivars it does not entirely compensate for the need of cold conditioning. This indicates that cold-dependent mechanisms are partly responsible for regulating the development of ripening competency, which in turn impacts the quality and marketability of pear fruit. Interestingly, in contrast to *P. communis* and *P. bretschneideri*, many Japanese pear (*Pyrus pyrifolia L.*) varieties have no conditioning requirements and are also regarded as non-climacteric fruits because they do not display the characteristic S1 to S2 transition during ripening (Oraguzie et al., 2010). Additionally, 'Bosc', unique from other European pear cultivars, acquires competency for ripening with exogenous ethylene only, needing no chilling to ripen (Sugar & Einhorn, 2011).

Requirement of cold exposure in pear to induce ripening is reminiscent of other natural cold temperature-dependent developmental phenomena, such as the vernalization and stratification that are needed for flowering and seed germination, respectively. The genes that regulate vernalization and stratification have been well-studied in model organisms, including Arabidopsis, wheat, and barley (Millar et al., 2006; Yan et al., 2003; Levy et al., 2002); however, similar gene homologues have not yet been reported in cold-induced fruit ripening. With respect to flowering,

the process of developmental initiation following an environmentally governed dormant state is known as endodormancy release (Niu et al., 2016). Thus far, endodormancy release has not been used to characterize ripening after chilling, although the two processes share many similarities with regards to the timing and environmental nature of cold required, suggesting that similar genetic and regulatory mechanisms may govern these processes. Various forms of the chilling requirement for ripening have been described in avocado and mango, although to a lesser degree than in pear, as the former are more prone to chilling injury (Bower et al., 2002; Lederman et al., 1997).

Few recent studies have utilized a transcriptomics approach to characterize the molecular underpinnings of cold-induced S1 to S2 transition in Pear. There is a complex interaction of genes involved in regulating phytohormones, secondary messengers, signaling pathways, respiration and chromatin modification that underlie cold-induced progression of ripening (Saavedra et al., 2016; Han et al., 2016; Nham et al., 2017). Genes associated with phytohormones such as abscisic acid (ABA), auxin, and jasmonic acid along with transcription factors were implicated in low-temperature-mediated enhancement of ripening in 'Bartlett' (Nham et al., 2017). In 'Passe Crassane' the impact of low temperature (LT) induced ethylene and exogenous ethylene treatments were evaluated using a transcriptomics approach (Mitalo et al., 2019). It was observed that the expression of a subset of the low temperature-induced differentially expressed genes was disrupted by 1-MCP treatment indicating that they were regulated by LT-induced ethylene. It was also reported that several transcription factors were unaffected by 1-MCP treatment, implying that they were under the control of LT alone (Mitalo et al., 2019). Recent work quantifying expression of key genes representing ripening-related metabolic pathways in 'D'Anjou' and 'Bartlett' pear cultivars during the process of cold-conditioning, revealed an increased alternative oxidase (AOX)

expression prior to the onset of the ripening climacteric. This novel finding suggests that AOX may play an important role in the achievement of ripening competency (Hendrickson et al., 2019) and may be necessary for the onset of the ripening climacteric in pear and other chilling dependent fruit. The Alternative Oxidase (AOX) respiratory pathway is known to play a role in cold stress mediation and response in many plant systems including cold temperature induced activation of respiration in potatoes, cell expansion and elongation in cotton, and mitigation of chilling injury in tomato and chickpea (Saha et al., 2016; Erdal et al., 2015; Wang et al., 2015). In many plant systems, AOX activity serves to maintain carbon metabolism homeostasis, cellular redox state, and ROS homeostasis during development (Szal & Rychter, 2016).

Based on our previous work (Hendrickson et al., 2019), a time course RNAseq analysis was performed in this study using 'Bartlett' and 'D'Anjou' fruit to evaluate the hypothesis that AOX and other key cold-induced genes facilitate ripening, and that the fruit from the two cultivars utilize different set of genes during cold conditioning. Key genes and networks involved in cold-induced, ripening-associated biochemical pathways were identified.

Results and Discussion

Fruit Firmness

Tissues from the fruit used in Hendrickson et al., 2019 were utilized for RNAseq analysis conducted in this study. Cold conditioning of the fruit at 10°C resulted in a reduction of fruit firmness in both 'Bartlett' and 'D'Anjou' cultivars as demonstrated (see Figure 2: Hendrickson et al., 2019). For both cultivars, fruit softening accelerated once the fruit was transferred to 20°C. The rate of softening was more rapid for 'Bartlett' than 'D'Anjou'.

RNAseq Assembly Analysis

RNAseq assembly generated 140077 contigs (Supplementary File 2.1). In the OmicsBox suite, the maSigPro R package was used to conduct time course differential expression analyses for both cultivars. 17,711 differentially expressed contigs ($p<0.05$) were identified for 'D'Anjou', with 7,174 of these contigs exhibiting significant linear or quadratic trends over time ($R>0.8$). In 'Bartlett' 31,481 contigs were identified as being differentially expressed, with 7,174 contigs exhibiting significant quadratic or linear trends over time ($R>0.8$) (Supplementary File 2.2). Similarities and differences in expression trends of contigs of interest between 'D'Anjou' and 'Bartlett', as well as expression patterns of differentially expressed contigs (DECs) associated with ethylene and phytohormone metabolism, abscisic acid metabolism, TCA cycle, respiration, were assessed. Additionally, in order to better understand the mechanisms underlying the chilling requirement for ripening in *Pyrus*, the expression of genes associated with: vernalization, flowering, dormancy, and other processes directly induced by cold/chilling were observed. Pre-climacteric expression of *AOX1* peaked during conditioning prior to onset of ripening (Figure 2.1), supporting the hypothesis. Additional key genes, including those that mediate vernalization and endodormancy release were also observed to be differentially expressed. Detailed analysis of RNAseq results and enriched gene ontologies related to phytohormone metabolism and cold-response pathways are discussed in detail in the following sections.

Alternative Oxidase

Confirming previous observation, pre-climacteric activation of alternative respiratory pathway transcription was observed during conditioning. Two DECs corresponding to mitochondrial ubiquinol oxidases, homologs of AOX1, displayed an increase in expression trend

consistent with a preceding report where pre-climacteric increase in *AOX1* was observed as the fruit completed its conditioning requirement (Hendrickson et al., 2019) (Figure 2.1). *AOX1* gene expression has been reported in many fruit systems, however mostly at climacteric or post-climacteric stages, with *AOX* isoforms displaying responses to a broad range of stresses. The different expression patterns of the two *AOX* contigs corresponding to 'D'Anjou' and 'Bartlett' suggests that *AOX* isoforms differentially regulate responses in different genetic backgrounds. The variable actions of AOX homologues on biological processes has been previously observed in Arabidopsis and tomato (Selinski et al., 2018; Holtzapffel et al., 2002). Knock-down of *AOX* in tomato delayed ripening, indicating a regulatory role of AOX in fundamental processes like ethylene response. Furthermore, overexpression of *AOX* in tomato alleviated some of the inhibitory effects of 1-MCP on ripening (Xu et al., 2012a). In European pear, respiratory partitioning into the alternative pathway may impact S2 ethylene biosynthesis, the climacteric respiration peak, and consequent ripening-related trait development, independent of prior ethylene sensitivity (Perotti et al., 2014; Xu et al., 2012b; Hendrickson et al., 2019).

Ethylene

For both cultivars, abundance of many transcripts associated with ethylene biosynthesis, perception, and signaling increased throughout the conditioning and ripening period in agreement with similar previous studies (Nham et al., 2017; Mitalo et al., 2019). *ACO1* and *ACS1*, were significantly differentially expressed and increased in expression throughout the duration of the conditioning time course in both 'D'Anjou' and 'Bartlett', as did *RAN1*, which delivers a copper ion that is necessary for ethylene to bind to its receptors (Villalobos-Acuña et al., 2010; Villalobos-Acuna & Mitcham, 2008; Alba et al., 2005; Liu et al., 2015).

Of particular interest in this study was expression pattern of ethylene repressors in response to cold. Consistent with results of recent studies in pear, a *MYB1R* transcription factor, a repressor of ethylene and ripening responses, decreased in expression once ripening competency was reached (Nham et al., 2017; Nham, 2016) (Figure 2.2). Furthermore, brassinosteroid-associated *Brassinazole-Resistant 1* (*BZR1*) and chromatin modification-associated *Multicopy Suppressor of IRA4* (*MSI4*), which have been shown to repress ethylene responses in banana and tomato, respectively, displayed different expression trends in the two cultivars. *BZR1* increased over time in 'D'Anjou' and decreased over time in 'Bartlett' (Guo et al., 2019) (Figure 2.3). These observations may provide further insight into the cultivar specific nature of ripening in pear, as 'D'Anjou' is known to be inherently more recalcitrant to ripening (Xie et al., 2014). The different ripening trajectories apparent from analysis in this study are congruent with the different vectors followed by the two cultivars in the recent study, which used NMDS analysis to evaluate the relationship between ripening and genes related to the process (Hendrickson et al., 2019). The different expression patterns of ethylene signaling genes during ripening suggest a more pronounced ethylene response in the 'Bartlett' cultivar, which may be associated with the shorter conditioning time, but perhaps a more complex and a different system of regulation in 'D'Anjou'.

Abscisic Acid (ABA)

ABA is well-established as a key regulator of timing of endodormancy release in both model and non-model organisms, such as pear (Huang et al., 2014; Bai et al., 2013). ABA-related DECs that displayed a similar increase in expression patterns over time for both cultivars included *9-cis-epoxycarotenoid dioxygenase* (*NCED1*), which catalyzes the first step in ABA biosynthesis and regulates some genes associated with cell wall degradation during ripening (Osorio et al.,

2013); *abscisic acid 8'-hydroxylase 2* (*CYP707A*), which is important for regulating seed dormancy and germination in Arabidopsis, accumulating over the course of seed maturation and resulting in the breakdown of ABA (Okamoto et al., 2006) (Figure 2.4). CYP707A has also been shown to inhibit the expression of *NCED-like* genes, thereby reducing ABA biosynthesis in strawberry and tomato (Jia et al., 2016). *Abscisic-Aldehyde Oxidase* (*AAO*) related transcript displayed variable expression between the two cultivars (Figure 2.4). The concomitant increase in transcripts encoding these antagonistic enzymes suggests that increased ABA synthesis is paralleled by a simultaneous increase in the ABA degradation in a tug-of-war between endodormancy maintenance and release, where the latter is favored only when sufficient chilling has occurred.

In contrast to DECs displaying a continual increase in expression over time, *Abscisic Acid Insensitive 5* (*ABI5*) displayed decreased expression in both cultivars during the second half of conditioning and ripening period. This gene is a negative regulator of flowering in Arabidopsis (Shu et al., 2015) and therefore may play a similar role in negative regulation of other endodormancy-associated processes including fruit ripening.

Sulfur metabolism

Sulfur containing compounds, including hydrogen sulfide (H_2S), enhance alternative pathway respiration and inhibit ROS production in fruits (Hu et al., 2012, Li et al., 2016; Luo et al., 2015; Ziogas et al., 2018). Such compounds have also been used in fruit processing as a preservation strategy to reduce oxidative browning (Hu et al., 2014), and low dose applications of H_2S elicit a pronounced ripening response in pear fruit (Dhingra & Hendrickson, 2017). The sulfur metabolism genes, *ATP sulfurylase* and *ATP sulfurylase 2*, were highly expressed only in

‘Bartlett’, with transcript abundance increasing over the course of conditioning and ripening (Figure 2.5). It is possible, given these results, that sulfur metabolism genes play a role in ripening in a cultivar specific manner.

Cold and temperature stress-induced processes

In addition to phytohormone-associated genes, several genes and gene families that have previously been implicated in cold-induced endodormancy release and vernalization processes displayed similar expression patterns over time. Those increasing continually during cold conditioning for both cultivars included *Early Flowering 3* (*EF3*), which has been shown to maintains the circadian clock in a temperature-dependent manner in barley and Arabidopsis (Ford et al., 2016) (Figure 2.6). Upregulation of this regulatory gene in pear fruit exposed to chilling may result in induction of genes associated with release of endodormancy. *Polycomb group embryonic flower 2-like isoform x1* (*EMF2*) decreased continually in expression during conditioning and ripening in ‘Bartlett’. In Arabidopsis, loss of function of EMF2 causes direct initiation of flowering, causing a bypass of vegetative shoot growth (Yoshida et al., 2001). The isoform present in ‘Bartlett’ may play a similar role in modulating initiation of ripening (Figure 2.6).

Furthermore, a DEC corresponding to a BRCA1 homolog increased during conditioning. The *BRCA1* gene has homologues in humans, and BRCA mutations are most often associated with increased cancer susceptibility; however, the primary function of the gene is DNA damage repair and chromatin remodeling, and BRCA1 has been shown to play a similar reparatory role in plants (Jiao et al., 2016; Trapp et al., 2011). It is possible that BRCA1 in fruit, might play a role in mediating temperature-induced stress damage to DNA during cold conditioning. In addition to *BRCA1*, *Next-to-BRCA* (*NBR1*) displayed increasing expression during conditioning and ripening

for both cultivars (Figure 2.7). NBR1 plays a role in heat stress tolerance in Arabidopsis (Zhou et al., 2014), although little work has been done to study its effects in other plants.

The vernalization-associated gene *VRN1*, has been characterized with regards to flowering time in both model and non-model species, specifically in the context of cold (Rampey et al., 2004; Levy et al., 2002). In cereal grains transcriptional activation of *VRN1* after prolonged chilling results in accelerated flowering (Oliver et al., 2013). Interestingly, *VRN1* was significantly differentially expressed over time in 'Bartlett' during the accumulation of chilling hours, while expression levels remained low in 'D'Anjou' throughout the time course (Figure 2.8). In 'Bartlett' the observed patterns of expression of *VRN1* during conditioning are consistent with a previously described model in wheat, in which increased accumulation of *VRN1* transcripts correlates with a decreased repression of endodormancy release, primarily via repression of FLC-like genes and other developmental repressors (Trevaskis, 2010; Trevaskis et al., 2003).

Another vernalization-associated gene, *VIN3* isoform x1, which is associated with temperature-mediated epigenetic regulation of endodormancy repressors (Sung & Amasino, 2004), displayed increasing expression in both cultivars during the cold conditioning period (Figure 2.8). As VIN3 and VRN1 are both cold-induced repressors of endodormancy release and are expressed differently in 'D'Anjou' and 'Bartlett', the opposite, genotypic-specific expression of these two DECs suggests that cold induced ripening might occur via two different vernalization-associated pathways, and may influence the duration of cold-requirement in different pear cultivars.

Working in an antagonistic manner to VRN1 and VIN3, which downregulate repressors of endodormancy release, are *FRIGIDA 4* and *CONSTANS-like* genes. Increased expression and

activity of *FRIGIDA 4* results in increased activity of repressors of endodormancy release, such as *Flowering Locus C* (*FLC*), in Arabidopsis (Michaels & Amasino, 2001) and blueberry (Song, 2018). *FRIGIDA 4* decreased significantly in expression throughout conditioning in 'Bartlett', suggesting that its downregulation may correspond to decreased activation of ripening repressors in a role homologous to regulation of flowering time (Figure 2.9).

In addition to *FRIGIDA 4*, overexpression of *CONSTANS-like 9* has been shown to delay flowering and regulate the circadian clock by repressing *CONSTANS* and Flowering Locus T (*FT)* gene expression in photoperiod sensitive plants, like Arabidopsis (Cheng & Wang, 2005). Recently, however, *CONSTANS-like* gene families have been shown to display distinct, tissue-specific patterns of expression in banana fruit and pulp, in addition to other tissues (Chaurasia et al., 2016), suggesting that *CONSTANS-like* gene family members play a role not only in flower development, but also, fruit development and senescence. *CONSTANS-like* 9, 5, 6, 13, and 14 were all differentially expressed. Overall, the *CONSTANS-like* genes displayed a similar decreasing expression trend in both cultivars throughout the conditioning period (Figure 2.9). As with *FRIGIDA 4*, this decrease suggests that the repressive role of these genes with regards to process of endodormancy release is downregulated during cold conditioning, thereby promoting ripening.

Functional Enrichment Analysis

Shared overrepresented terms included 'cold acclimation', dormancy-related ('embryo development ending in seed dormancy', 'flowering'), hormone signaling ('ethylene-activated signaling pathway', 'ABA-activated signaling pathway', 'auxin-activated signaling pathway'), hormone biosynthesis ('jasmonic acid biosynthetic process', 'salicylic acid biosynthetic process', 'brassinosteroid biosynthetic process'), and respiration-associated processes ('ATP synthesis

coupled proton transport', 'electron transfer activity') (Figure 2.10). The shared overrepresentation of cold- and dormancy-related GO terms lends support to mediation of cold-induced ripening responses by vernalization-associated genes, in conjunction with downregulation of repressors of endodormancy release. Additionally, the presence of many enriched phytohormone-related ontologies lends support to the concept of interacting networks of phytohormonal crosstalk that serve to mediate ripening and mitigate chilling injury (Nahm et al., 2017; Wang et al., 2015, Gray, 2004). This lends support to the importance of auxin biosynthesis and metabolism during the ripening process in *Pyrus*. Finally, enriched ontologies associated with mitochondrial respiration, which implicate a high rate of ATP production, provide further evidence that AOX1 alternative respiratory activity is needed to alleviate some of the stress on the cytochrome respiratory pathway during ripening (Vishwakarma et al., 2015). While shared enriched GO terms lend insight into conserved biological basis for cold-conditioning mediated ripening, enriched ontologies unique to 'D'Anjou' or 'Bartlett' provide information regarding the cultivar-specific ripening responses.

'D'Anjou' pears are genetically programmed to require a longer conditioning time to ripen than 'Bartlett' pears (60 versus 15 days of conditioning at 0°C). Overrepresented GO terms unique to 'D'Anjou' associated with chilling-induced endodormancy release included 'seed germination' and 'methylation', while for 'Bartlett' such terms included 'vernalization response', 'regulation of seed germination' (Figures 2.11-2.12). Regulation of vernalization sensitive genes, including homologs of those governing timing of seedling germination in many crops, is highly dependent on methylation status and other epigenetic modifications, suggesting that such genes may play a larger role in the ripening of 'D'Anjou'. Further investigation is needed of the effects of external abiotic factors like chilling on the epigenome of fruit undergoing developmental transitions such as a shift to ripening. The overrepresentation of terms associated with respiration and senescence

are expected, as such processes are characteristic of ripening and the terminal stages of fruit development. In ‘D’Anjou’, the terms ‘aerobic respiration’ and ‘aging’ were overrepresented, while in ‘Bartlett’, TCA cycle-associated terms (‘tricarboxylic acid cycle’, ‘malate metabolic process’, ‘malate dehydrogenase activity’, ‘isocitrate metabolic process’) were overrepresented (Figures 2.11-2.12). Differential overrepresentation of aerobic respiration and TCA cycle metabolism GO terms in the two cultivars suggest that these processes are under cultivar-specific regulation. Interestingly, enrichment of terms associated with production of protective boundary layers (‘cutin biosynthetic process’, ‘cuticle development’) may represent a genetically programmed stress management strategy considering long conditioning requirements. Development of such barriers could mitigate the occurrence of chilling injury while ‘D’Anjou’ fruits accumulate the required chilling hours (Figure 2.11). In ‘Bartlett’, ‘sulfur assimilation’ and ‘methionine metabolic process’ were enriched. This is interesting because the Yang cycle, which recycles the sulfur containing amino acid methionine, also feeds into the production of the ethylene biosynthetic precursor ACC. Increased sulfur metabolic capacity in ‘Bartlett’ may in turn correspond to higher methionine cycling capacity, and therefore production of ethylene for this cultivar. Based on this finding, and that of the DE analysis, in which ATP sulfurylases were highly expressed during conditioning and ripening in ‘Bartlett,’ it is possible that cold conditioning directly or indirectly induces sulfur metabolism, thereby inducing ethylene biosynthesis and downstream processes. This is the case for soybean, in which ATP-sulfurylase is induced by cold and catalyzes activation of sulfate (Phartiyal et al., 2006; Prioretti et al., 2014). The GO analysis results implicate cold temperature induction of numerous metabolic pathways, many of which operate upstream or independently of ethylene. This observation aligns with a recent study in cold

conditioned pear fruit that demonstrated that LT induces expression of both ethylene-dependent and independent genes affecting ripening (Mitalo et al., 2019).

To summarize, the ontology enrichment results provide a global overview with regards to some of the overarching processes responsible for cold-induced, ripening induction in pear. These results lend credibility to the role of vernalization-associated genes, and their potential role in influencing the duration of cold required for conditioning for fruit ripening.

Conclusion

In this study, time-course differential expression analysis, functional annotation and GO enrichment methods were used to identify candidate genes and gene networks associated with the chilling requirement for ripening in pear. The results agree with previously reported expression patterns of known ripening-related genes during achievement of ripening competency, specifically genes associated with ethylene biosynthesis and phytohormonal crosstalk. A novel outcome of this study was that differentially expressed cold-responsive, vernalization-associated genes may play a role in the ripening of European pear. While described in other systems, these genes have not yet been characterized with respect to their role in climacteric ripening.

Notably, *AOX* expression results are consistent with our recent work in pear, providing support for the possible role of cold-induced AOX activity in the achievement of ripening competency. AOX has been described previously in the context of cold stress response and ROS mediation, and more recently in pre-climacteric S2-S2 transitionary phase (Vishwakarma et al., 2015, Dhingra & Hendrickson, 2017, Hendrickson et al., 2019). Its expression and activity may be linked to or activated in conjunction with vernalization-associated genes via ROS as a response

to cold temperatures (Figure 2.13). Further studies are needed to elucidate the precise connections, but it is clear based on expression data that these transcripts share a similar response to cold conditioning in pear fruit.

Based on these findings, the mechanism by which vernalization-associated genes may mediate cold-induced ripening may manifest as follows: Cold temperature stimulates VIN3 or VRN1 in a cultivar dependent manner. These, in turn inhibit fruit-tissue specific repressors of endodormancy release (CONSTANS-like 9, FRIGIDA 4, dormancy-associated MADS-box genes, and ERF2). Inhibition of repressors of endodormancy release, such as BZR1 and MSI4 and ABA precursors, via VRN1 and VIN3 pathways allows for activation of ripening-specific transcription factors, such as MYB1R1 and others, which may regulate autocatalytic ethylene production during conditioning (Nham et al., 2017). ROS induced by cold temperatures may concurrently serve to activate AOX and ethylene response factors (Saha et al., 2016; Oracz et al., 2009). The normal ripening climacteric, characterized by the conversion of ACC to ethylene by ACS, commences following transcription factor-mediated activation. Ethylene biosynthesis and response results in activation of downstream ripening processes (Figure 2.13).

Results of this study provide new information with regards to vernalization and cold response-associated genes that are differentially expressed over time during conditioning and subsequent ripening. Many of the genes which have been identified as potential regulators of chilling-induced ripening in pear fruit represent members of diverse gene families. Furthermore, several studies have previously indicated the diversification and neofunctionalization of *VRN*, *VIN*, *CONSTANS-like* gene families among others, in a multitude of plant tissues, including roots, shoots, leaves, apical meristems, buds, and flowers (Jiménez et al., 2009; Chaurasia et al., 2016).

Here we provide evidence suggesting that members of these gene families have diversified to play similar roles in chilling-dependent fruit ripening. These findings lend support to the idea of chilling-induced ripening as a process of endodormancy release that might explain the underlying basis of different chilling requirement across different cultivars.

Materials and Methods

Experimental Design

The experimental design was similar as previously reported (Hendrickson et al., 2019). Briefly, 'Bartlett' and 'D'Anjou' pear fruit were obtained from Blue Star Growers (Cashmere, Washington). During the time between harvest and acquisition (5 days), the pear fruit was maintained in temporary storage at 1°C. 'Bartlett' fruit had a mean firmness of 76.2 N, and 13.40 °Brix and 'D'Anjou' fruit had a mean firmness of 53.5 N, and 12.66 °Brix at initiation of the experiment. Ripening of 'Bartlett' requires 15 days of cold conditioning, while 'D'Anjou' typically requires 60 days of -1°C to attain ripening competency (Sugar et al., 2009; Villalobos-Acuna & Mitcham, 2008). The duration of cold conditioning, however, is reduced when conditioning temperatures are increased to 10°C (Sugar & Einhorn, 2011). Pears were divided into equal replicate groups and then placed into storage at 10°C for conditioning (Sugar & Einhorn, 2011). After the conditioning period (Figure 2.14), the fruit was transferred to 180-liter flow-through respiration chambers held at 20°C for seven days. The flow rate of the chambers was maintained at 5.0 ml/min with compressed air. Fruit was evaluated at four physiological time points: at 0% conditioned, 50% conditioned, and 100% conditioned and 100% ripened, which comprised 7 days after completion of conditioning. The pears that were allowed to accumulate the required chilling hours for ripening were transferred to 20°C and were then sampled at the 100%

ripened time point (Figure 2.14). These time points were similar to previously utilized physiological stages determined during conditioning (Hendrickson et al., 2019).

Fruit firmness measurements and tissue sampling

Firmness was measured for 10 replicate fruit at each sampling time point. A GS-14 Fruit Texture Analyzer (GÜSS Instruments, South Africa) equipped with an 8.0 mm probe set at 5.0 mm flesh penetration was used to measure firmness at two equidistant points around the equatorial region of each fruit after removal of the peel. Firmness data were assessed using ANOVA, following the statistical approaches described previously (Zucoloto et al., 2016; Val et al., 2008).

RNA Extraction

Peel tissue used in this study was the same as that used for the qRT-PCR study, published recently (Hendrickson et al., 2019). Briefly, peel tissue was obtained from a 1 cm wide equatorial region of 3 randomly sampled fruit of each cultivar and at each conditioning time point, flash frozen in liquid nitrogen, pooled for each treatment/time point, and then ground using a SPEX Freezer/Mill 6870 (Metuchen, NJ USA). Total RNA was extracted from pulverized 'D'Anjou' and 'Bartlett' peel tissue for each of the four technical replicates at 0% conditioned, 50% conditioned, 100% conditioned, and 100% ripened time points following the methods of (Gasic et al., 2004). Contaminating genomic DNA was removed with DNaseI per manufacturer instructions (NEB, Ipswich, MA USA). RNA was quality checked using a denaturing gel and BioAnalyzer 2100 (Agilent, CA, USA) and was quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Illumina Sequencing

cDNA libraries were qualified and quantified using a Life Technologies Qubit Fluorometer (Carlsbad, CA) as well as an Agilent 2100 Bioanalyzer (Santa Clara, CA). The cDNA libraries prepared from the extracted RNA were sequenced on an Illumina Hi Seq 4000 platform as 2x100 paired end reads. The Illumina TruSeq RNA Sample Preparation v2 kit (San Diego, CA) was used to generate the final library molecules, and the Ailine Biosciences' (Woburn, MA) DNA SizeSelector-I bead protocol was used to filter for library molecules of >450 base pairs.

Transcriptome Assembly

Transcriptome assembly was performed as reported previously (Sharpe et al., 2019; Biesla et al., 2018). The 2x100 paired end fastq files generated using Illumina HiSeq 2000 were input into the CLC Bio Genomics Workbench (ver 6.0.1) (Aarhus, Denmark) for pre-processing and assembly. The CLC Create Sequencing QC report tool was used to assess quality. The CLC Trim Sequence process was used to trim quality scores with a limit of 0.001, corresponding to a Phred value of 30. Ambiguous nucleotides were trimmed, and the 13 5' terminal nucleotides removed. Reads below length 34 were discarded. Overlapping pairs were merged using the 'Merge Overlapping Pairs' tool, and a subsequent de novo assembly was performed with all datasets. Parameters used in the assembly are as follows: Map reads back to contigs = TRUE, Mismatch cost = 2, Insertion cost = 3, Deletion cost = 0.4, Similarity Fraction = 0.95, Global Alignment = TRUE, Minimum contig length = 200, Update contigs = true, Auto-detect paired distances = TRUE, Create list of un-mapped reads = TRUE, Perform scaffolding = TRUE. The de novo assembly resulted in the production of 140,077 contiguous sequences (contigs). Contigs with less than 2x coverage and those less than 200bp in length were eliminated. For each individual dataset

(treatment/replicate) the original, non-trimmed reads were mapped back to the master assembly subset. Default parameters were used, except for the length fraction, which was set to 0.5, and the similarity fraction, which was set to 0.9. Mapping resulted in the generation of individual treatment sample reads per contig. The master transcriptome was exported as a fasta file for functional annotation and the read counts for each dataset were exported for normalization with the Reads Per Kilobase per Million reads (RPKM) method (Mortazavi et al., 2008).

Functional Annotation with Blast2GO

The master transcriptome fasta produced from the Illumina assembly was imported into OmicsBox (BioBam Bioinformatics S.L., Valencia, Spain) for functional annotation of expressed contigs using the Blast2GO feature (Conesa et al., 2005). Contig sequences were identified by a blastx alignment against the NCBI 'Viridiplantae' database with and e-value specification of 10.0E-3. Gene ontology (GO) annotation was assigned using the 'Mapping' and 'Annotation' features. Expression analysis was limited to the consensus sequence for each contig, and therefore in this paper we do not distinguish between specific alleles, highly similar gene family members. This is due to assembler constraints (T O'Neil & Emrich, 2013).

Differential Expression Analysis

An Excel file was prepared containing 'D'Anjou' and 'Bartlett' RPKM data for each contig, treatment, and replicate. The data was imported into OmicsBox as a count table for differential expression analysis, which employs the maSigPro R package (Nueda et al., 2014). An additional experimental design matrix was imported which dictated the number of time points and replicates (Supplementary File 2.3). The level of FDR control was set to 0.05, resulting in

identification of significant genes. A stepwise regression was employed to model the data and then generate a list of all genes displaying significant linear or quadratic trends over the cold conditioning time course (R>0.8) (Nueda et al., 2014) (Supplementary File 2.2).

GO Enrichment Analysis

OmicsBox gene ontology (GO) enrichment analysis utilizing the Fisher's Exact Test was employed (Conesa et al. 2005). Due to many enriched GO terms, the resulting terms were reduced to only the most specific ontologies ($p<0.00001$). Ontologies shared between 'D' Anjou' and 'Bartlett' and unique to each cultivar were identified. A complete list of shared and unique GO terms can be found in Supplementary File 2.4.

qRT-PCR validation

qRT-PCR was performed as reported earlier (Hendrickson et al., 2019). Briefly, RNA samples were treated with DNAseI to eliminate any DNA contamination according to the manufacturer's methods (NEB, Ipswich, MA USA), prior to cDNA synthesis. RNA concentration was determined for each sample using a Nanodrop ND-8000 (ThermoFisher, MA, USA). RNA quality was verified using a denaturing gel and BioAnalyzer 2100 (Agilent, CA USA). For each sample, 500 ng of total RNA was used to generate first strand cDNA using the Invitrogen VILO kit (Life Technologies, Carlsbad, CA USA). Each cDNA preparation was quantified, and the mean concentration calculated from eight replicate quantification measurements, recorded using a NanoDrop8000 (Thermo Fisher Scientific, Waltham, MA). The samples were diluted to a final concentration of 50 ng/uL. Initial qRT-PCR technical replicate reactions were prepared for each of the 90 selected genes using the iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA).

Primers for quantitative reverse transcriptase PCR (qRT-PCR) were designed from Pyrus ESTs or sequences derived from *Malus × domestica* transcripts among various hormonal and environmental signaling pathways. 500ng RNA for each sample (same as used for RNAseq) was used to generate 1st strand cDNA using the Invitrogen VILO kit (Life Technologies, Carlsbad, CA USA). cDNA preparations were then diluted to 50ng/uL. qRT-PCR technical replicate reactions were prepared for each of the genes using the iTAq Universal SYBR Green Supermix with ROX reference dye (BioRad, Hercules, CA) per the manufacturer's protocols with 100ng of template cDNA. In a Strategene MX3005P, the following thermocycle profile was used: 95°C initial disassociation for 150s followed by 50 amplification cycles (95°C for 30s, 60°C for 30s, and 72°C for 30s) and a final, single cycle phase to generate a dissociation curve (95°C for 150s, 95°C for 30s, and 60°C for 30s).Using the LinRegPCR tool, we calculated the Cq values for each reaction (Ruijter et al. 2009; Ramakers et al. 2003) (Supplementary File 2.5).

Acknowledgements

The authors thank Blue Bird Growers (Peshastin, WA) and Blue Star Growers (Cashmere, WA) for providing pears for conditioning experiments and to Scott Mattinson for assistance in maintenance of the experimental infrastructure. Work in the Dhingra lab was supported in part by Washington State University Agriculture Center Research Hatch Grant WNP00011 and grant funding from Pear Bureau NW to AD. SLH acknowledges the support received from ARCS Seattle Chapter and National Institutes of Health/National Institute of General Medical Sciences through an institutional training grant award T32-GM008336. The contents of this work are solely the responsibility of the authors and do not necessarily represent the official views of the NIGMS or NIH.

REFERENCES

Alba R, Payton P, Fei Z, *et al.*, 2005. Transcriptome and selected metabolite analyses reveal multiple points of ethylene control during tomato fruit development. *The Plant Cell* **17**, 2954-65.

Alexander L, Grierson D, 2002. Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. *Journal of experimental botany* **53**, 2039-55.

Bai G, Yang D-H, Zhao Y, *et al.*, 2013. Interactions between soybean ABA receptors and type 2C protein phosphatases. *Plant molecular biology* **83**, 651-64.

Barry CS, Llop-Tous MI, Grierson D, 2000. The regulation of 1-aminocyclopropane-1-carboxylic acid synthase gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato. *Plant Physiology* **123**, 979-86.

Bower J, Dennison M, Fowler K. Avocado and mango cold storage damage as related to water loss control. *Proceedings of the XXVI International Horticultural Congress: Issues and Advances in Postharvest Horticulture 628, 2002*, 401-6.

Chaurasia AK, Patil HB, Azeez A, *et al.*, 2016. Molecular characterization of CONSTANS-Like (COL) genes in banana (Musa acuminata L. AAA Group, cv. Grand Nain). *Physiology and Molecular Biology of Plants* **22**, 1-15.

Cheng XF, Wang ZY, 2005. Overexpression of COL9, a CONSTANS-LIKE gene, delays flowering by reducing expression of CO and FT in Arabidopsis thaliana. *The Plant Journal* **43**, 758-68.

Chiriboga M-A, Saladié M, Bordonaba JG, Recasens I, Garcia-Mas J, Larrigaudière C, 2013. Effect of cold storage and 1-MCP treatment on ethylene perception, signalling and synthesis: Influence on the development of the evergreen behaviour in 'Conference'pears. *Postharvest Biology and Technology* **86**, 212-20.

Conesa A, Götz S, Garcia-Gomez JM, Terol J, Talon M, Robles M, 2005. Blast2GO:a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**.

Dhingra A, Hendrickson C, 2017. Control of ripening and senescence in pre-harvest and postharvest plants and plant materials. In.: Google Patents.

Dhingra A, Hendrickson C, Hewitt S, 2017. Control of ripening and senescence in pre-harvest and postharvest plants and plant materials by manipulating alternative oxidase activity. In.: Google Patents.

Erdal S, Genisel M, Turk H, Dumlupinar R, Demir Y, 2015. Modulation of alternative oxidase to enhance tolerance against cold stress of chickpea by chemical treatments. *Journal of plant physiology* **175**, 95-101.

Ford B, Deng W, Clausen J, *et al.*, 2016. Barley (Hordeum vulgare) circadian clock genes can respond rapidly to temperature in an EARLY FLOWERING 3 -dependent manner. *Journal of experimental botany* **67**, 5517-28.

Gasic K, Hernandez A, Korban S, 2004. RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. *Plant Molecular Biology Reporter* **22**, 437-8.

Gray WM, 2004. Hormonal regulation of plant growth and development. *PLoS biology* **2**, E311-E.

Guo YF, Shan W, Liang SM, *et al.*, 2019. MaBZR1/2 act as transcriptional repressors of ethylene biosynthetic genes in banana fruit. *Physiologia plantarum* **165**, 555-68.

Han Y-C, Kuang J-F, Chen J-Y, *et al.*, 2016. Banana transcription factor MaERF11 recruits histone deacetylase MaHDA1 and represses the expression of MaACO1 and expansins during fruit ripening. *Plant Physiology* **171**, 1070-84.

Hendrickson C, Hewitt S, Swanson ME, Einhorn T, Dhingra A, 2019. Evidence for pre-climacteric activation of AOX transcription during cold-induced conditioning to ripen in European pear (Pyrus communis L.). *bioRxiv*, 755686.

Hewitt S, Dhingra, A, 2019. Beyond Ethylene: New Insights Regarding the Role of AOX in the Respiratory Climacteric. *Pre-publication.*

Holtzapffel RC, Finnegan PM, Millar AH, Badger MR, Day DA, 2002. Mitochondrial protein expression in tomato fruit during on-vine ripening and cold storage. *Functional Plant Biology* **29**, 827-34.

Hu K-D, Wang Q, Hu L-Y, *et al.*, 2014. Hydrogen sulfide prolongs postharvest storage of fresh-cut pears (Pyrus pyrifolia) by alleviation of oxidative damage and inhibition of fungal growth. *PLoS ONE* **9**, e85524.

Hu L-Y, Hu S-L, Wu J, *et al.*, 2012. Hydrogen sulfide prolongs postharvest shelf life of strawberry and plays an antioxidative role in fruits. *Journal of agricultural and food chemistry* **60**, 8684-93.

Huang G, Li T, Li X, *et al.*, 2014. Comparative transcriptome analysis of climacteric fruit of Chinese pear (Pyrus ussuriensis) reveals new insights into fruit ripening. *PLoS ONE* **9**, e107562.

Jia H, Jiu S, Zhang C, *et al.*, 2016. Abscisic acid and sucrose regulate tomato and strawberry fruit ripening through the abscisic acid-stress-ripening transcription factor. *Plant Biotechnology Journal* **14**, 2045-65.

Jiao Y, Zhang Y, Zhu YX, 2016. Recent advances in the research for the homolog of breast cancer associated gene AtROW1 in higher plants. *Sci China Life Sci* **59**, 825-31.

Jiménez S, Lawton-Rauh AL, Reighard GL, Abbott AG, Bielenberg DG, 2009. Phylogenetic analysis and molecular evolution of the dormancy associated MADS-box genes from peach. *BMC Plant Biology* **9**, 81.

Klee HJ, Giovannoni JJ, 2011. Genetics and control of tomato fruit ripening and quality attributes. *Annual review of genetics* **45**, 41-59.

Lederman IE, Zauberman G, Weksler A, Rot I, Fuchs Y, 1997. Ethylene-forming capacity during cold storage and chilling injury development in 'Keitt'mango fruit. *Postharvest Biology and Technology* **10**, 107-12.

Levy YY, Mesnage S, Mylne JS, Gendall AR, Dean C, 2002. Multiple roles of Arabidopsis VRN1 in vernalization and flowering time control. *Science* **297**, 243-6.

Li D, Limwachiranon J, Li L, Du R, Luo Z, 2016. Involvement of energy metabolism to chilling tolerance induced by hydrogen sulfide in cold-stored banana fruit. *Food chemistry* **208**, 272-8.

Liu M, Pirrello J, Chervin C, Roustan J-P, Bouzayen M, 2015. Ethylene control of fruit ripening: revisiting the complex network of transcriptional regulation. *Plant Physiology* **169**, 2380-90.

Luo Z, Li D, Du R, Mou W, 2015. Hydrogen sulfide alleviates chilling injury of banana fruit by enhanced antioxidant system and proline content. *Scientia horticulturae* **183**, 144-51.

Michaels SD, Amasino RM, 2001. Loss of FLOWERING LOCUS C activity eliminates the late-flowering phenotype of FRIGIDA and autonomous pathway mutations but not responsiveness to vernalization. *The Plant Cell* **13**, 935-41.

Millar AA, Jacobsen JV, Ross JJ, *et al.*, 2006. Seed dormancy and ABA metabolism in Arabidopsis and barley: the role of ABA 8′-hydroxylase. *The Plant Journal* **45**, 942-54.

Mitalo OW, Tosa Y, Tokiwa S, *et al.*, 2019. 'Passe Crassane'pear fruit (Pyrus communis L.) ripening: Revisiting the role of low temperature via integrated physiological and transcriptome analysis. *Postharvest Biology and Technology* **158**, 110949.

Mortazavi A, Williams BA, Mccue K, Schaeffer L, Wold B, 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* **5**.

Nham NT, 2016. Investigation of the Molecular Mechanisms Regulating the Developmentof Ripening Capacity in European Pears (Pyrus communis L. cv Bartlett): University of California, Davis.

Nham NT, Macnish AJ, Zakharov F, Mitcham EJ, 2017. 'Bartlett' pear fruit (Pyrus communis L.) ripening regulation by low temperatures involves genes associated with jasmonic acid, cold response, and transcription factors. *Plant Science* **260**, 8-18.

Niu Q, Li J, Cai D, *et al.*, 2016. Dormancy-associated MADS-box genes and microRNAs jointly control dormancy transition in pear (Pyrus pyrifolia white pear group) flower bud. *Journal of experimental botany* **67**, 239-57.

Nueda MJ, Tarazona S, Conesa A, 2014. Next maSigPro: updating maSigPro bioconductor package for RNA-seq time series. *Bioinformatics*, btu333.

Okamoto M, Kuwahara A, Seo M, *et al.*, 2006. CYP707A1 and CYP707A2, which encode abscisic acid 8′-hydroxylases, are indispensable for proper control of seed dormancy and germination in Arabidopsis. *Plant Physiology* **141**, 97-107.

Oliver SN, Deng W, Casao MC, Trevaskis B, 2013. Low temperatures induce rapid changes in chromatin state and transcript levels of the cereal VERNALIZATION1 gene. *Journal of experimental botany*, ert095.

Oracz K, El-Maarouf-Bouteau H, Kranner I, Bogatek R, Corbineau F, Bailly C, 2009. The Mechanisms Involved in Seed Dormancy Alleviation by Hydrogen Cyanide Unravel the Role of Reactive Oxygen Species as Key Factors of Cellular Signaling during Germination. *Plant Physiology* **150**, 494-505.

Oraguzie N, Whitworth C, Brewer L, *et al.*, 2010. Relationships of PpACS1 and PpACS2 genotypes, internal ethylene concentration and fruit softening in European (Pyrus communis) and Japanese (Pyrus pyrifolia) pears during cold air storage. *Plant breeding* **129**, 219-26.

Osorio S, Scossa F, Fernie A, 2013. Molecular regulation of fruit ripening. *Frontiers in Plant Science* **4**.

Perotti VE, Moreno AS, Podestá FE, 2014. Physiological aspects of fruit ripening: the mitochondrial connection. *Mitochondrion* **17**, 1-6.

Phartiyal P, Kim W-S, Cahoon RE, Jez JM, Krishnan HB, 2006. Soybean ATP sulfurylase, a homodimeric enzyme involved in sulfur assimilation, is abundantly expressed in roots and induced by cold treatment. *Archives of Biochemistry and Biophysics* **450**, 20-9.

Prioretti L, Gontero B, Hell R, Giordano M, 2014. Diversity and regulation of ATP sulfurylase in photosynthetic organisms. *Frontiers in Plant Science* **5**, 597-.

Rampey RA, Leclere S, Kowalczyk M, Ljung K, Sandberg G, Bartel B, 2004. A family of auxin-conjugate hydrolases that contributes to free indole-3-acetic acid levels during Arabidopsis germination. *Plant Physiology* **135**, 978-88.

Saavedra GM, Figueroa NE, Poblete LA, Cherian S, Figueroa CR, 2016. Effects of preharvest applications of methyl jasmonate and chitosan on postharvest decay, quality and chemical attributes of Fragaria chiloensis fruit. *Food chemistry* **190**, 448-53.

Saha B, Borovskii G, Panda SK, 2016. Alternative oxidase and plant stress tolerance. *Plant Signaling & Behavior* **11**, e1256530.

Selinski J, Hartmann A, Deckers-Hebestreit G, Day DA, Whelan J, Scheibe R, 2018. Alternative oxidase isoforms are differentially activated by tricarboxylic acid cycle intermediates. *Plant Physiology* **176**, 1423-32.

Seymour GB, Chapman NH, Chew BL, Rose JK, 2013. Regulation of ripening and opportunities for control in tomato and other fruits. *Plant Biotechnology Journal* **11**, 269-78.

Seymour GB, Taylor JE, Tucker GA, 2012. *Biochemistry of fruit ripening*. Springer Science & Business Media.

Shu K, Chen Q, Wu Y, *et al.*, 2015. ABSCISIC ACID-INSENSITIVE 4 negatively regulates flowering through directly promoting Arabidopsis FLOWERING LOCUS C transcription. *Journal of experimental botany* **67**, 195-205.

Song G-Q, 2018. Comparative transcriptome analysis of nonchilled, chilled, and late-pink bud reveals flowering pathway genes involved in chilling-mediated flowering in blueberry. *BMC Plant Biology* **v. 18**, pp. 98--2018 v.18 no.1.

Sugar D, Basile SR, 2009. Low-temperature induction of ripening capacity in 'Comice'and 'Bosc'pears as influenced by fruit maturity. *Postharvest Biology and Technology* **51**, 278-80.

Sugar D, Einhorn TC, 2011. Conditioning temperature and harvest maturity influence induction of ripening capacity in ‘d’Anjou’pear fruit. *Postharvest Biology and Technology* **60**, 121-4.

Sugar D, Mitcham E, Kupferman G, 2009. Rethinking the chill requirement for pear ripening. In. *Good Fruit Grower*. https://www.goodfruit.com/rethinking-the-chill-requirement-for-pear-ripening/: Washington State Fruit Commission.

Sung S, Amasino RM, 2004. Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. *Nature* **427**, 159-64.

Szal B, Rychter A, 2016. Alternative oxidase-never ending story. *Postepy biochemii* **62**, 138-48.

T O’neil S, Emrich SJ, 2013. Assessing De Novo transcriptome assembly metrics for consistency and utility. *BMC Genomics* **14**, 465.

Trapp O, Seeliger K, Puchta H, 2011. Homologs of breast cancer genes in plants. *Frontiers in Plant Science* **2**, 19.

Trevaskis B, 2010. The central role of the VERNALIZATION1 gene in the vernalization response of cereals. *Functional Plant Biology* **37**, 479-87.

Trevaskis B, Bagnall DJ, Ellis MH, Peacock WJ, Dennis ES, 2003. MADS box genes control vernalization-induced flowering in cereals. *Proceedings of the National Academy of Sciences* **100**, 13099-104.

Val J, Monge E, Risco D, Blanco A, 2008. Effect of Pre-Harvest Calcium Sprays on Calcium Concentrations in the Skin and Flesh of Apples. *Journal of Plant Nutrition* **31**, 1889-905.

Villalobos-Acuna M, Mitcham EJ, 2008. Ripening of European pears: the chilling dilemma. *Postharvest Biology and Technology* **49**, 187-200.

Villalobos-Acuña MG, Biasi WV, Flores S, Mitcham EJ, Elkins RB, Willits NH, 2010. Preharvest application of 1-methylcyclopropene influences fruit drop and storage potential of 'bartlett'pears. *HortScience* **45**, 610-6.

Vishwakarma A, Tetali SD, Selinski J, Scheibe R, Padmasree K, 2015. Importance of the alternative oxidase (AOX) pathway in regulating cellular redox and ROS homeostasis to optimize photosynthesis during restriction of the cytochrome oxidase pathway in Arabidopsis thaliana. *Annals of Botany* **116**, 555-69.

Wang L, Baldwin EA, Plotto A, *et al.*, 2015. Effect of methyl salicylate and methyl jasmonate pre-treatment on the volatile profile in tomato fruit subjected to chilling temperature. *Postharvest Biology and Technology* **108**, 28-38.

Xie M, Huang Y, Zhang Y, *et al.*, 2013. Transcriptome profiling of fruit development and maturation in Chinese white pear (Pyrus bretschneideri Rehd). *BMC Genomics* **14**, 1-20.

Xie X, Song J, Wang Y, Sugar D, 2014. Ethylene synthesis, ripening capacity, and superficial scald inhibition in 1-MCP treated 'd'Anjou'pears are affected by storage temperature. *Postharvest Biology and Technology* **97**, 1-10.

Xu F, Yuan S, Zhang D-W, Lv X, Lin H-H, 2012a. The role of alternative oxidase in tomato fruit ripening and its regulatory interaction with ethylene. *J Exp Bot* **63**, 5707-16.

Xu F, Yuan S, Zhang DW, Lv X, Lin HH, 2012b. The role of alternative oxidase in tomato fruit ripening and its regulatory interaction with ethylene. *Journal of Experimental Botany* **63**, 5705-16.

Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J, 2003. Positional cloning of the wheat vernalization gene VRN1. *Proceedings of the National Academy of Sciences* **100**, 6263-8.

Yoshida N, Yanai Y, Chen L, *et al.*, 2001. EMBRYONIC FLOWER2, a novel polycomb group protein homolog, mediates shoot development and flowering in Arabidopsis. *The Plant Cell* **13**, 2471-81.

Zhou J, Zhang Y, Qi J, *et al.*, 2014. E3 Ubiquitin Ligase CHIP and NBR1-Mediated Selective Autophagy Protect Additively against Proteotoxicity in Plant Stress Responses. *PLoS genetics* **10**, e1004116.

Ziogas V, Molassiotis A, Fotopoulos V, Tanou G, 2018. Hydrogen sulfide: A potent tool in postharvest fruit biology and possible mechanism of action. *Frontiers in Plant Science* **9**.

Zucoloto M, Antoniolli LR, Squeira DL, Czermainski ABC, Salomao LCC, 2016. Conditioning temperature for inducing uniform ripening of 'Abate Fetel' pears. *Revista Ciência Agronômica* **47**, 344-50.

Table 2.1 Summary of differentially expressed contigs. Information includes general role or associated pathway, full and abbreviated names, contig number (corresponding to sequences, annotations, and expression values in Supplementary Files 2.1 and 2.2), length, and indication of significant differential expression and/or significant expression trends.

General Role	Gene/Contig Name	Abbreviation	Contig #	Contig Length (bp)	DE 'D'Anjou' (p<0.05)	DE 'Bartlett' (p<0.05)	Significant Trend 'D'Anjou '(R>0.8)	Significant Trend 'Bartlett' (R>0.8)
Ethylene biosynthesis	1-aminocyclopropane-1-carboxylate oxidase 1	ACO1	24220	516	Yes	Yes	Linear, Quadratic	
Ethylene biosynthesis	1-aminocyclopropane-1-carboxylate synthase 1	ACS1	45750	1929	No	Yes		
Ethylene perception	constituitive-triple-response 1, isoform X2	CTR1 x2	2886	2978	Yes	Yes		Linear, Quadratic
Ethylene perception	Copper-transporting ATPase RAN1-like	RAN1-like	36171	366	No	Yes		
Ethylene perception	Reversion-to-ethylene-sensitivity 1	RTE1	4369	954	Yes	Yes	Linear	
Ethylene regulatory	Brassinazole-resistant 1 homolog 4-like	BZR1 4-like	42873	538	Yes	Yes		
Ethylene regulatory	Multicopy Suppressor of IRA4	MSI4	20932	1494	No	Yes		
Ethylene regulatory	Transcription factor MYB1R1	MYB1R1	17108	1358	Yes	Yes		
Ethylene response	Ethylene responsive transcription factor ERF060-like	ERF060-like	22945	388	Yes	Yes	Linear	
ABA metabolism	abscisic-aldehyde oxidase-like	AAO	23005	1294	No	Yes		
ABA metabolism	abscisic acid insensitive 5-7	ABI5-7	9409	573	Yes	Yes	Linear, Quadratic	
ABA metabolism	abscisic acid 8'-hydroxylase 2	CYP707A	40225	1904	Yes	Yes		
ABA metabolism	9-cis-epoxycarotenoid dioxygenase	NCED1	8780	796	Yes	Yes	Linear, Quadratic	Linear, Quadratic
Respiration	mitochondrial ubiquinol oxidase	AOX1-1	57573	1434	Yes	Yes		
Respiration	mitochondrial ubiquinol oxidase-like	AOX1-2	45965	1563	Yes	Yes		
Sulfur metabolism	ATP sulfurylase 2	ATP sulfurylase 2	3305	2207	Yes	Yes		Linear, Quadratic
Sulfur metabolism	ATP sulfurylase 2, chloroplastic	ATP sulfurylase 2, chloroplastic	41025	211	Yes	Yes		
DNA repair	Breast Cancer Susceptibility Associated 1 homolog	BRCA1 homolog	25664	2219	Yes	Yes	Linear, Quadratic	
DNA repair	Next to BRCA1 1	Next to BRCA1 1	11190	1329	Yes	Yes		
Dormancy/Vernalization	Dormancy-associated MADS-box transcription factor	DAM	6262	537	Yes	Yes		
Dormancy/Vernalization	EARLY FLOWERING 3-like	EARLY FLOWERING 3-like	35358	1263	Yes	Yes	Linear	
Dormancy/Vernalization	Ethylene insensitive-like 3	EIN3-like 3	2217	2217	Yes	Yes	Linear, Quadratic	Linear
Dormancy/Vernalization	polycomb group EMBRYONIC FLOWER 2-like isoform X1	EMBRYONIC FLOWER 2-like isoform X1	25290	696	No	Yes		
Dormancy/Vernalization	Vernalization insensitive 3 2 isoform x2	VIN3 2 isoform x2	11789	2339	Yes	Yes	Linear, Quadratic	Linear
Dormancy/Vernalization	B3 domain-containing transcription factor VRN1-like	VRN1-like	567	1669	No	Yes		Linear, Quadratic
Repression of dormancy release	Zinc finger CONSTANS-LIKE 14-like	COL14-like	25134	1886	Yes	Yes	Linear	Linear
Repression of dormancy release	Zinc finger CONSTANS-LIKE 5-like	COL5-like	5185	1744	Yes	Yes		
Repression of dormancy release	Zinc finger CONSTANS-LIKE 9-like	COL9	24463	2475	Yes	Yes		Linear
Repression of dormancy release	FRIGIDA 4a	FRIGIDA 4a	3425	1116	No	Yes		Linear

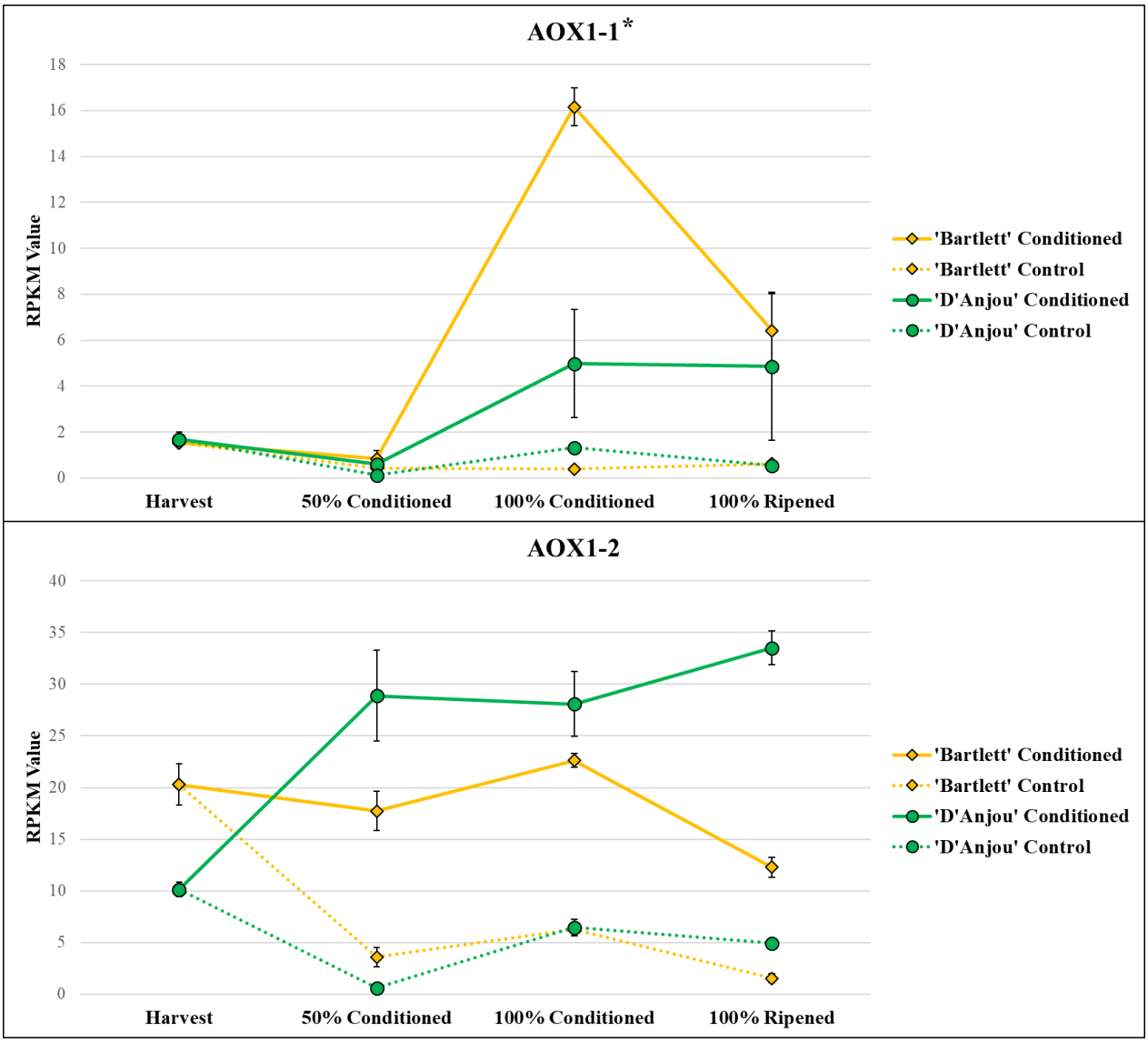

Figure 2.1 Two homologues of mitochondrial *AOX1* were found to be differentially expressed ($p>0.05$). Asterisk indicates significant differential expression over time in 'Bartlett', but not in 'D'Anjou. Significant linear and quadratic trends ($R>0.8$) displayed by genes can be seen in Table 2.1.

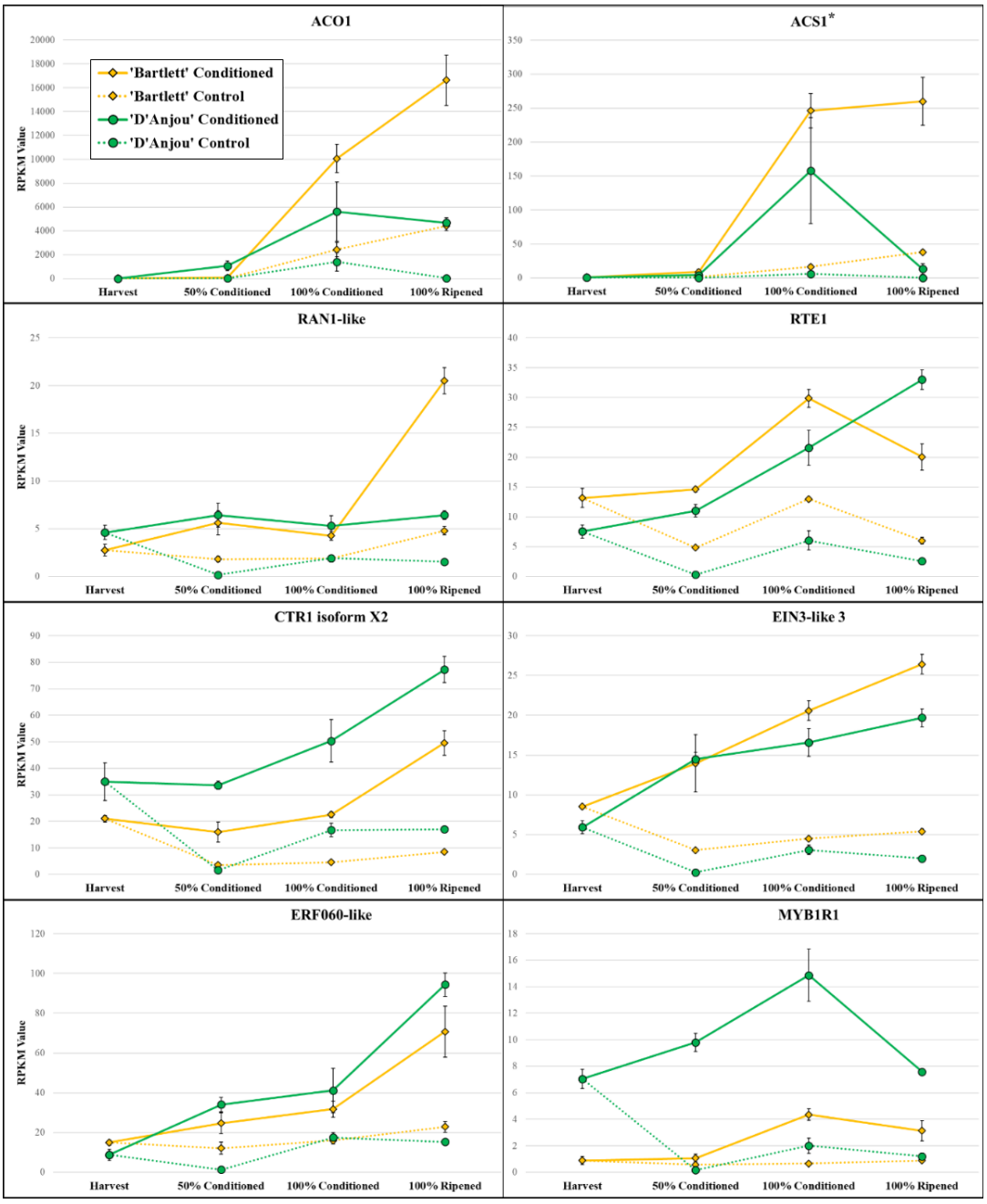

Figure 2.2 Transcript abundance for differentially expressed ethylene-associated contigs. Asterisk indicates significant differential expression over time in conditioned ‘Bartlett’ but not conditioned ‘D’Anjou’. Significant linear and quadratic trends (R>0.8) displayed by genes can be seen in Table 2.1.

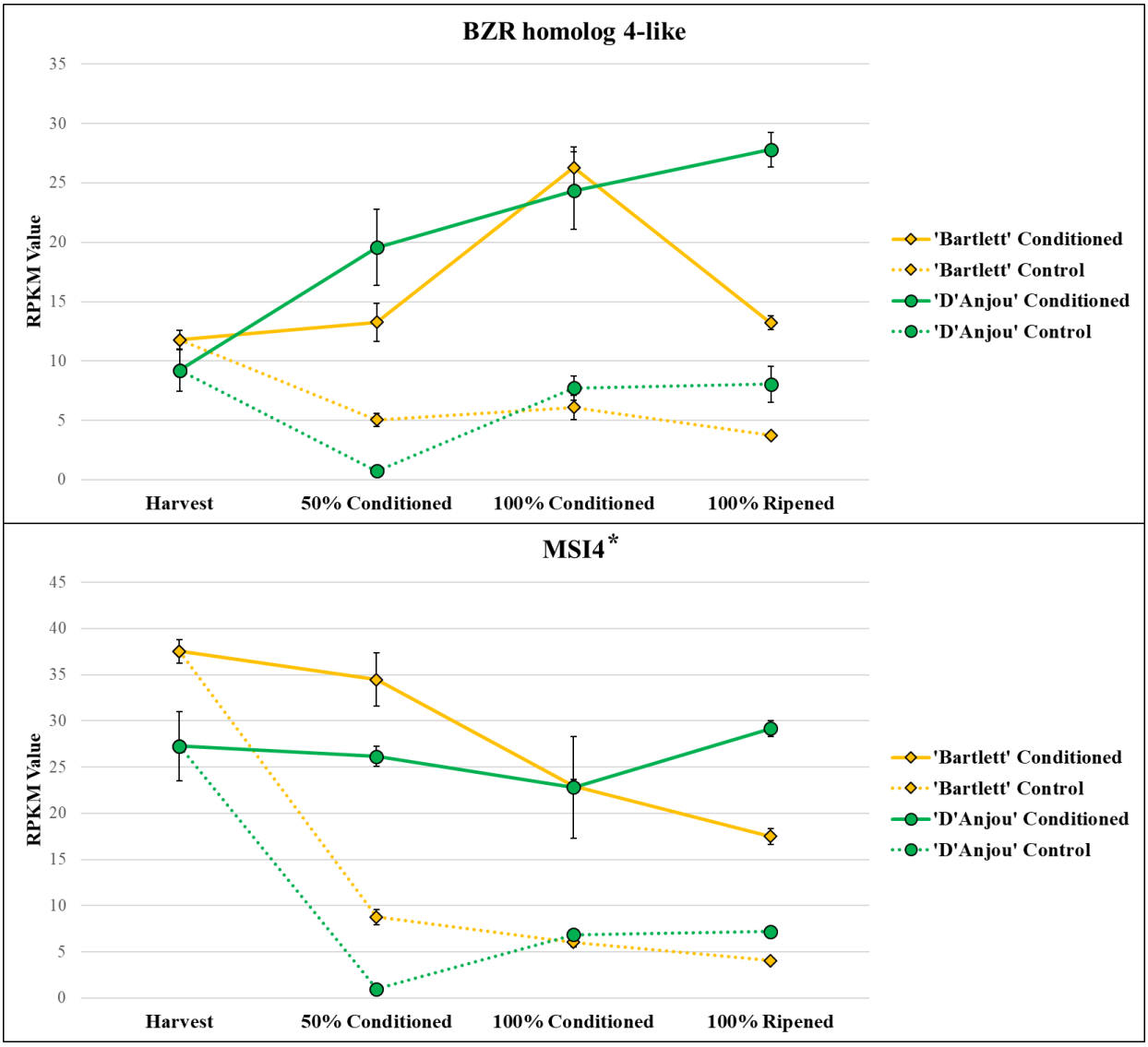

Figure 2.3 Transcript abundance for differentially expressed ethylene biosynthesis regulatory genes *BZR1* and *MSI4* during conditioning and ripening for 'D'Anjou' and 'Bartlett' cultivars ($p<0.05$). Asterisk indicates significant differential expression over time in conditioned 'Bartlett' but not in conditioned 'D'Anjou'. Significant linear and quadratic trends ($R>0.8$) displayed by genes can be seen in Table 2.1.

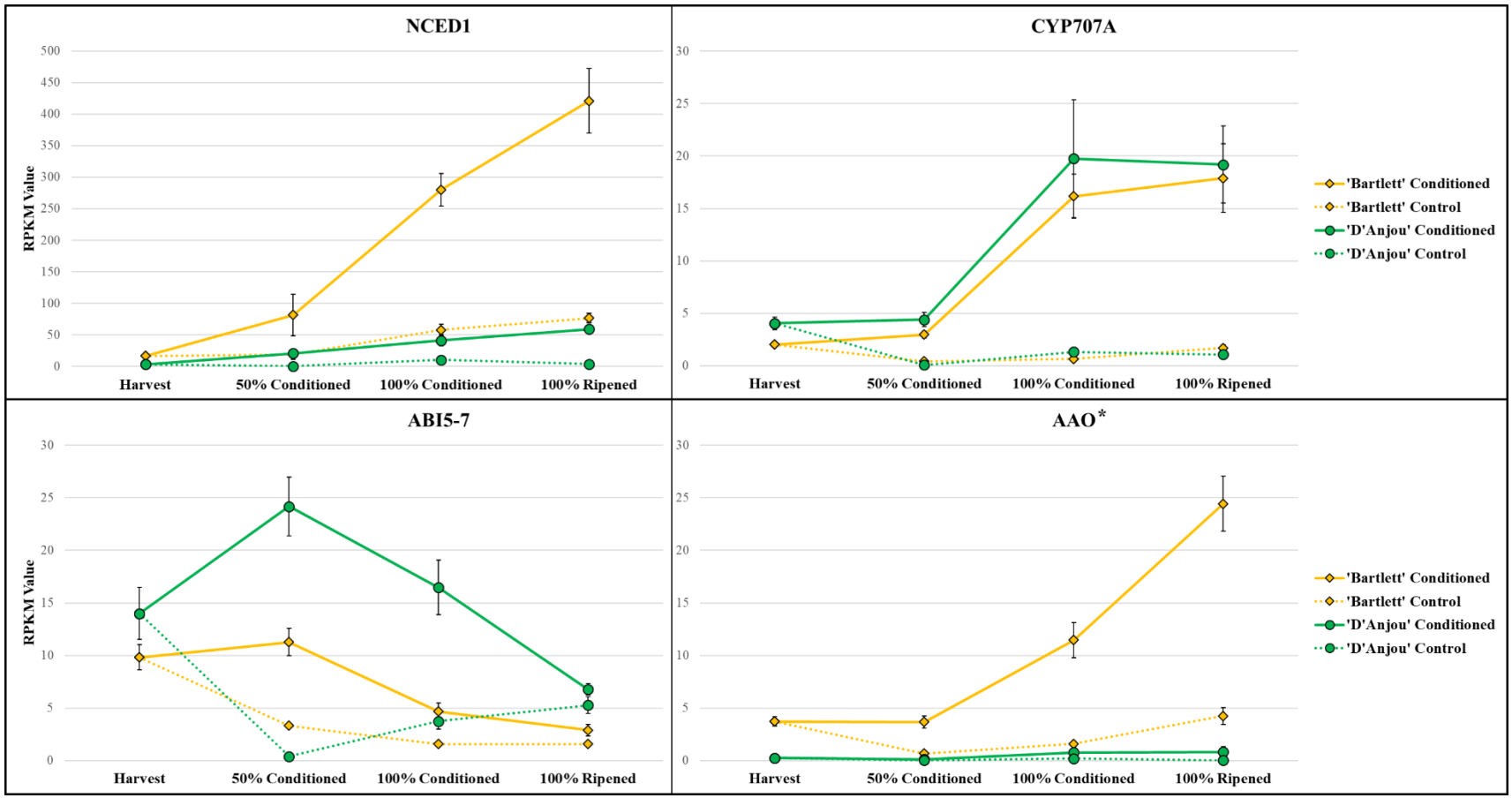

Figure 2.4 Transcript abundance for differentially expressed ABA-associated contigs ($p<0.05$). Asterisk indicates significant differential expression over time in conditioned 'Bartlett', but not in conditioned 'D'Anjou'. Significant linear and quadratic trends ($R>0.8$) displayed by genes can be seen in Table 2.1.

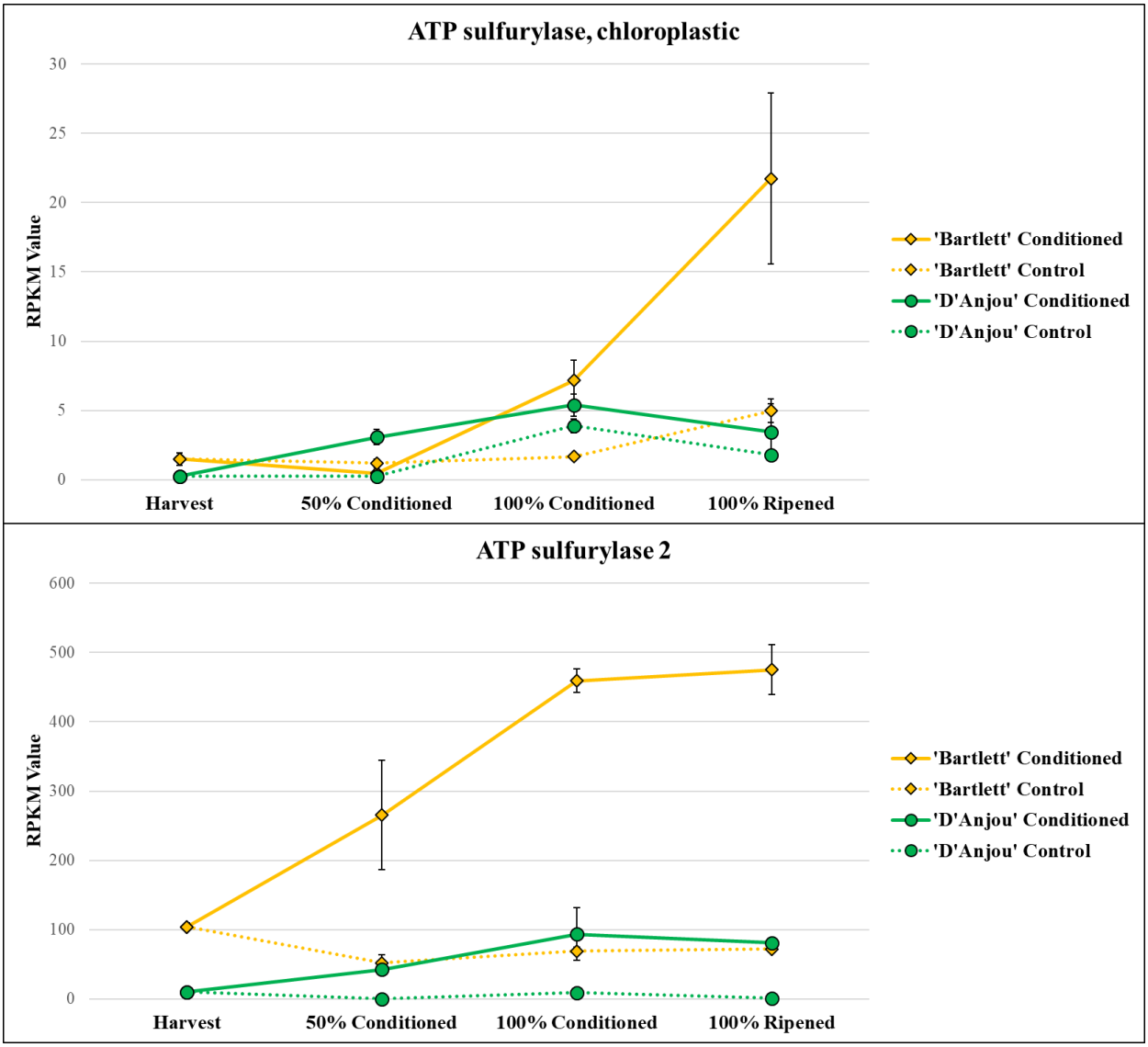

Figure 2.5 Transcript abundance of sulfur metabolism-associated contigs during conditioning and ripening for ‘D’Anjou’ and ‘Bartlett’ cultivars ($p<0.05$). Significant linear and quadratic trends ($R>0.8$) displayed by genes can be seen in Table 2.1.

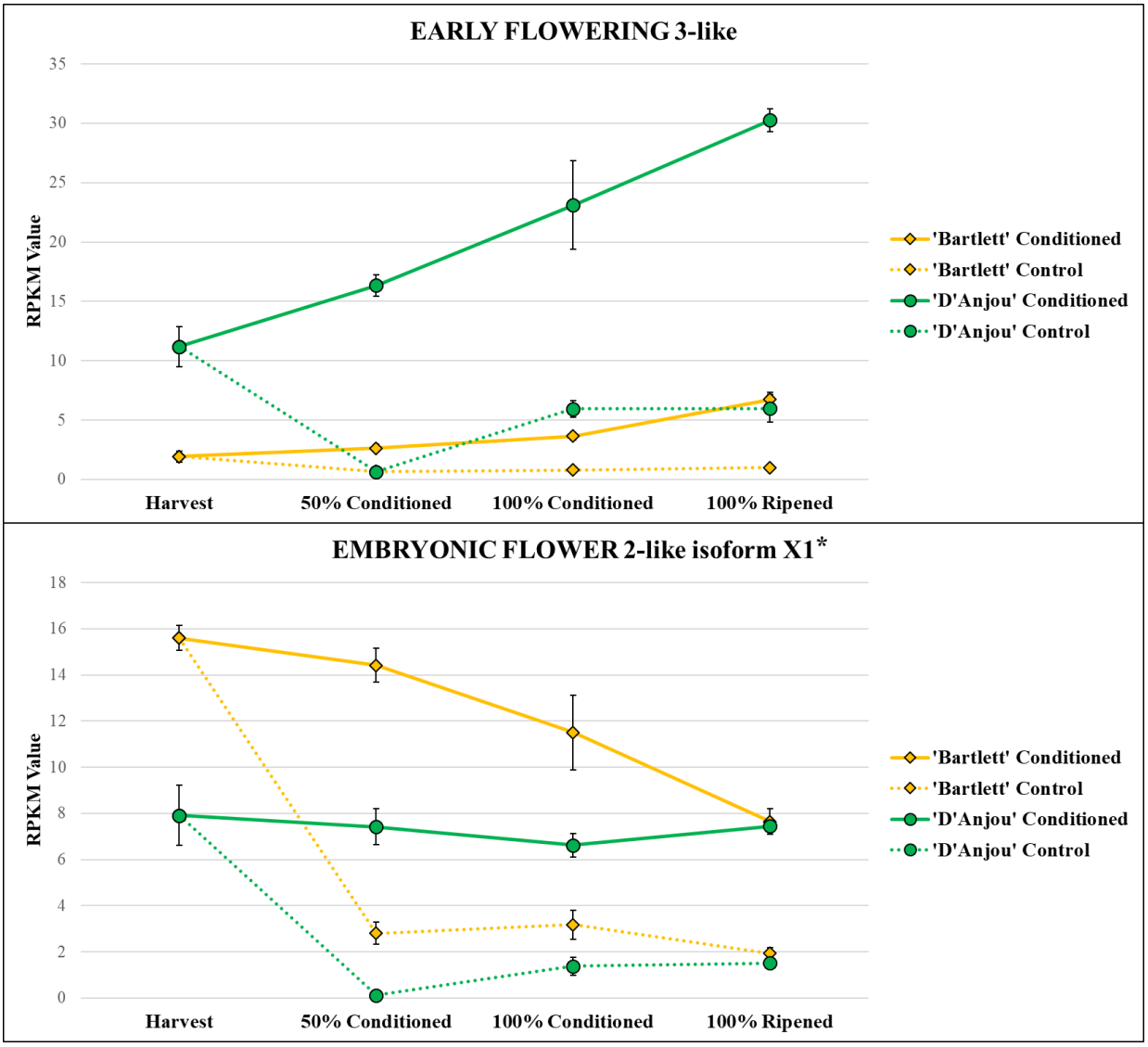

Figure 2.6 Transcript abundance of differentially expressed flowering-associated genes which may also play a role in regulation of chilling induced ripening in pear fruit. Asterisk indicates significant differential expression over time in conditioned 'Bartlett', but not in conditioned 'D'Anjou'. Significant linear and quadratic trends (R>0.8) displayed by genes can be seen in Table 2.1.

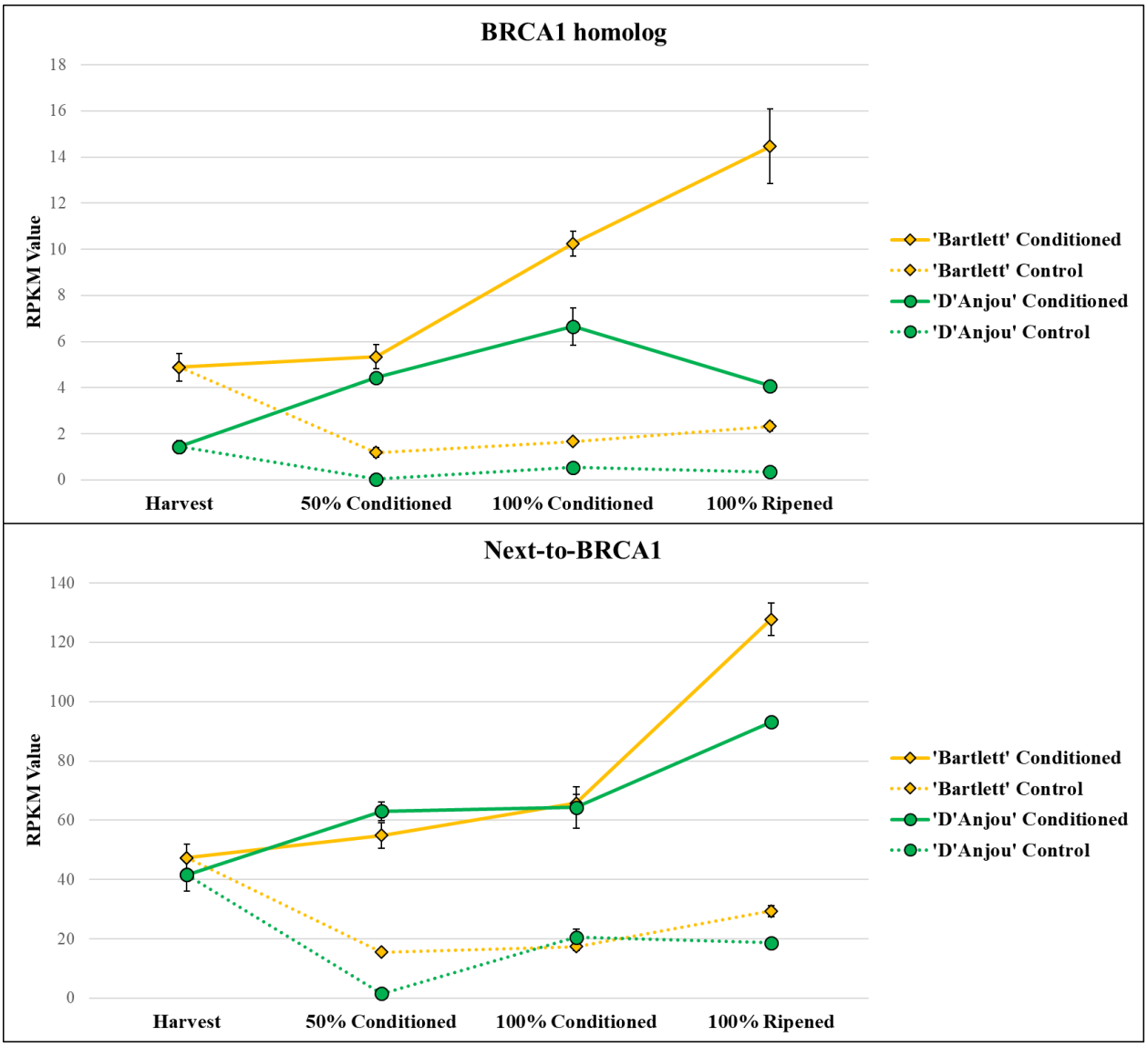

Figure 2.7 Differentially expressed DNA damage repair and stress responsive *BRCA1* and *Next to BRCA1* DECs shared between 'D'Anjou' and 'Bartlett' pears ($p>0.05$). Significant linear and quadratic trends ($R>0.8$) displayed by genes can be seen in Table 2.1.

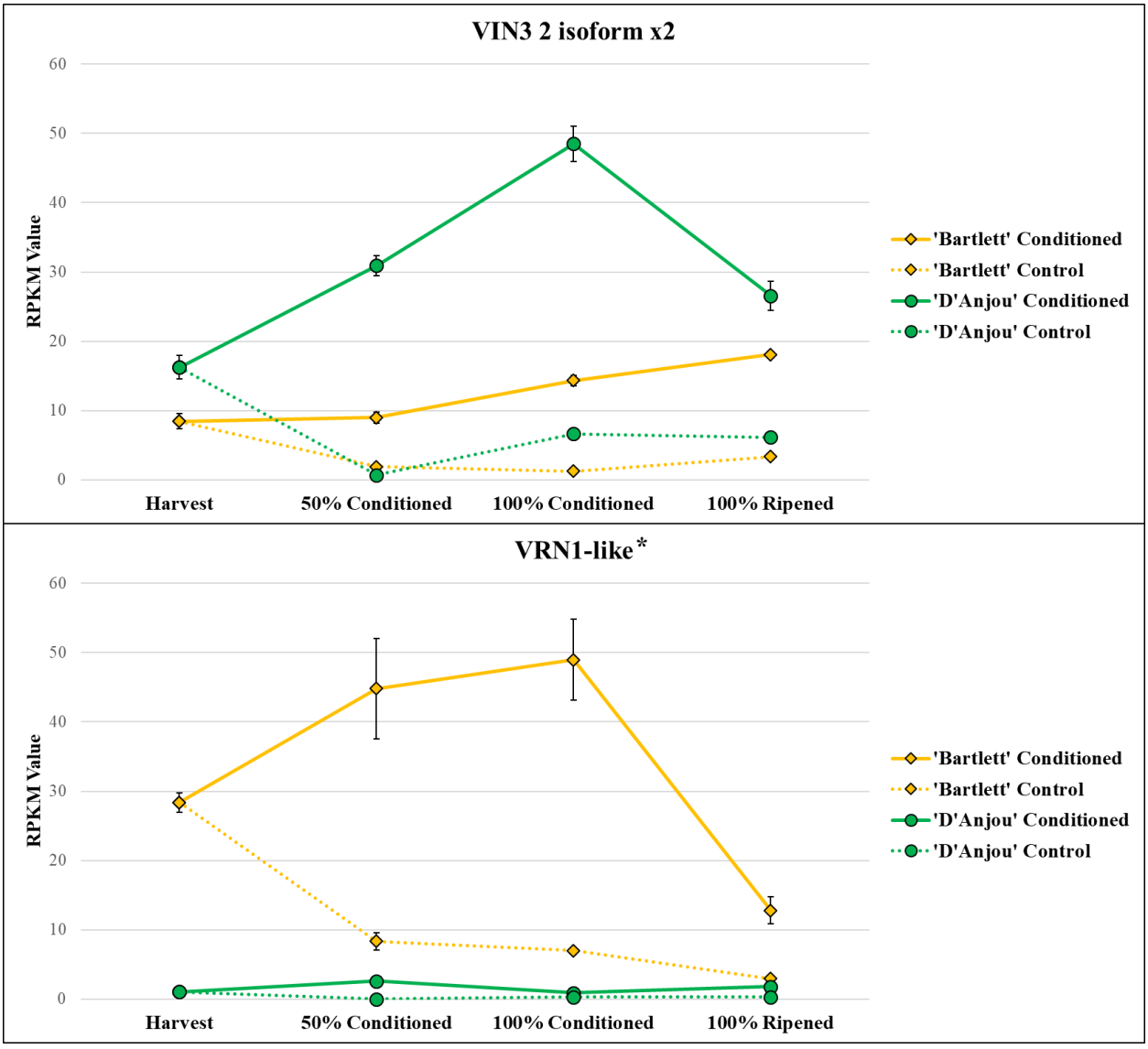

Figure 2.8 Differentially expressed vernalization-associated genes *VIN3* and *VRN1* ($p>0.05$). Asterisk indicates significant differential expression over time in conditioned 'Bartlett' but not in conditioned 'D'Anjou'. Significant linear and quadratic trends ($R>0.8$) displayed by genes can be seen in Table 2.1.

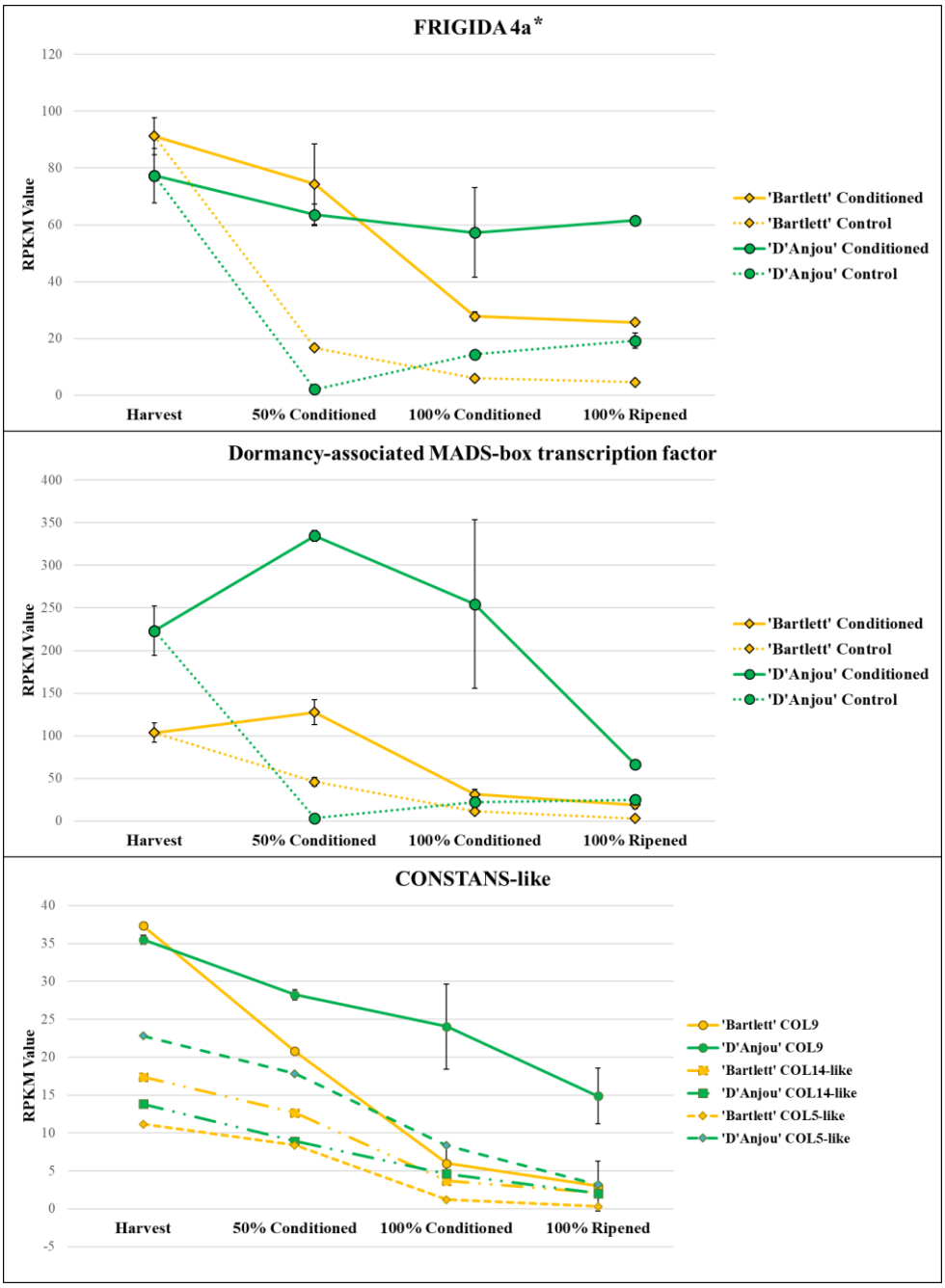

Figure 2.9 Differentially expressed endodormancy release-repressing genes FRIGIDA 4a, dormancy-associated MADS-box transcription factor, and CONSTANS-like over conditioning time course ($p>0.05$). Significant linear and quadratic trends ($R>0.8$) displayed by genes can be seen in Table 2.1.

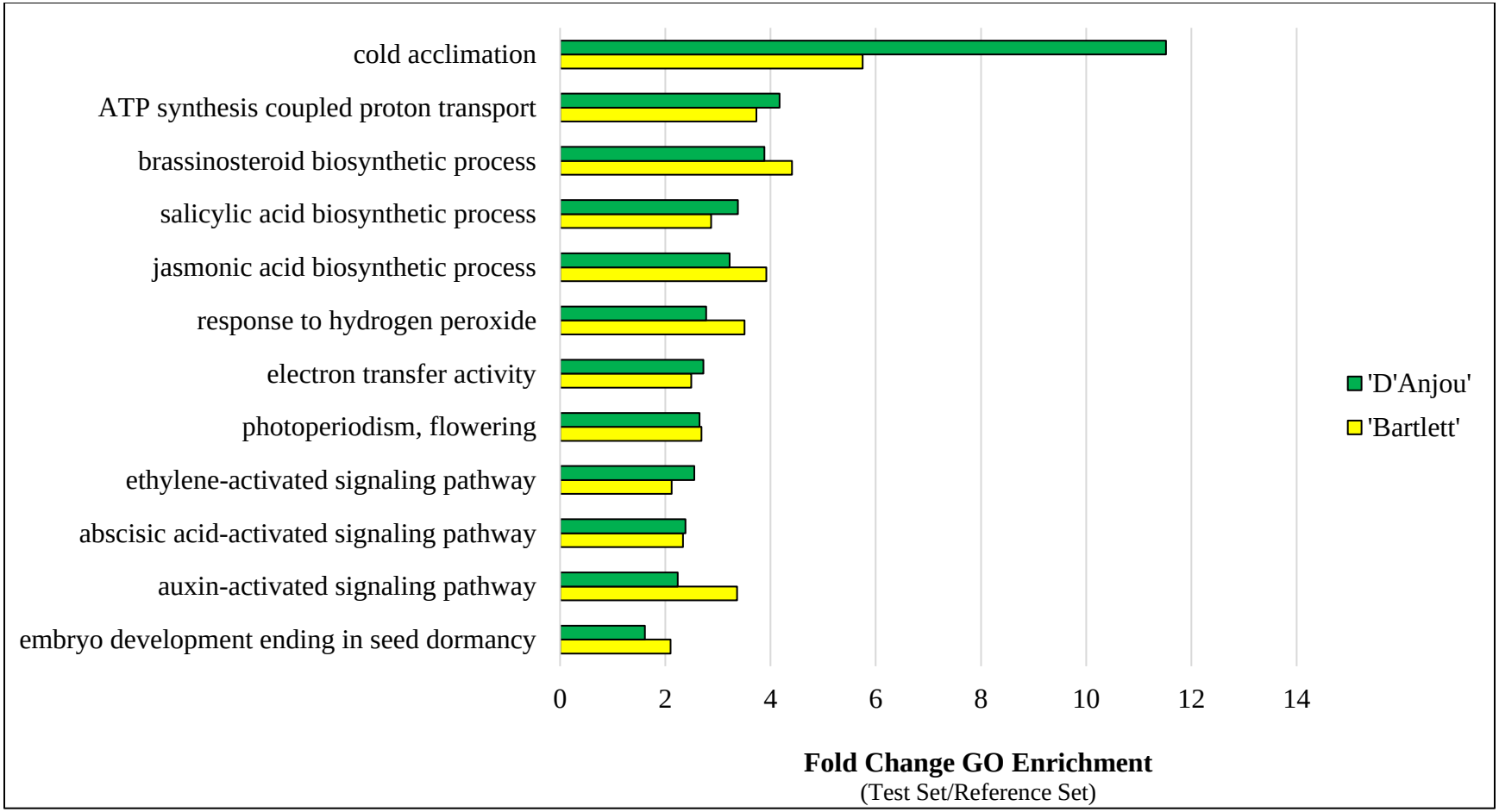

Figure 2.10 Selection of overrepresented GO terms shared between 'D'Anjou' and 'Bartlett' cultivars and identified using the OmicsBox enrichment analysis feature based on the Fisher's Exact Test ($p<0.00001$). Additional values can be seen in Supplementary File 2.4.

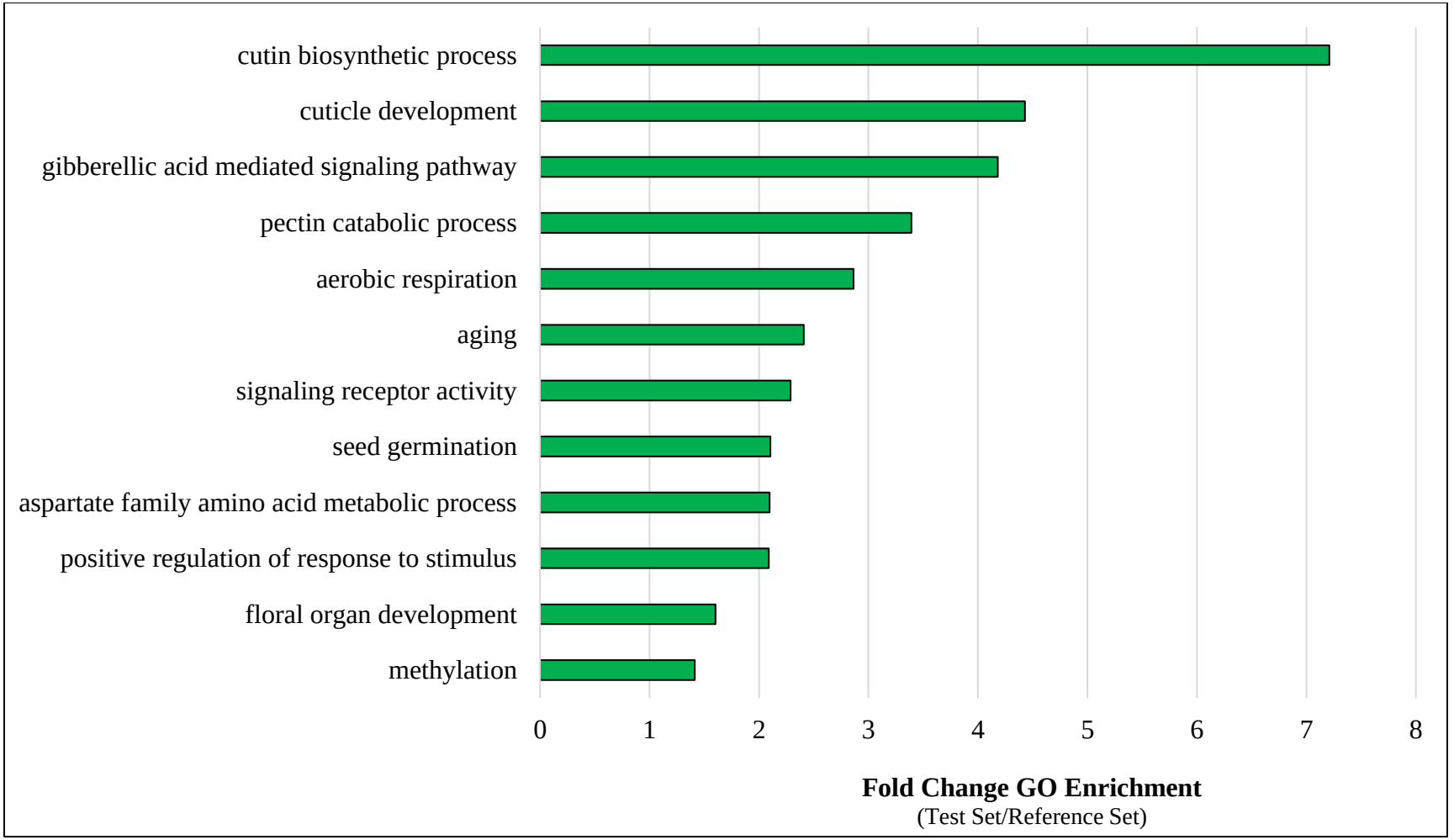

Figure 2.11 Selection of unique overrepresented GO terms in cold conditioned 'D'Anjou' fruit. Ontologies were reduced to the most specific terms ($p<0.00001$). Additional values can be seen in Supplementary File 2.4.

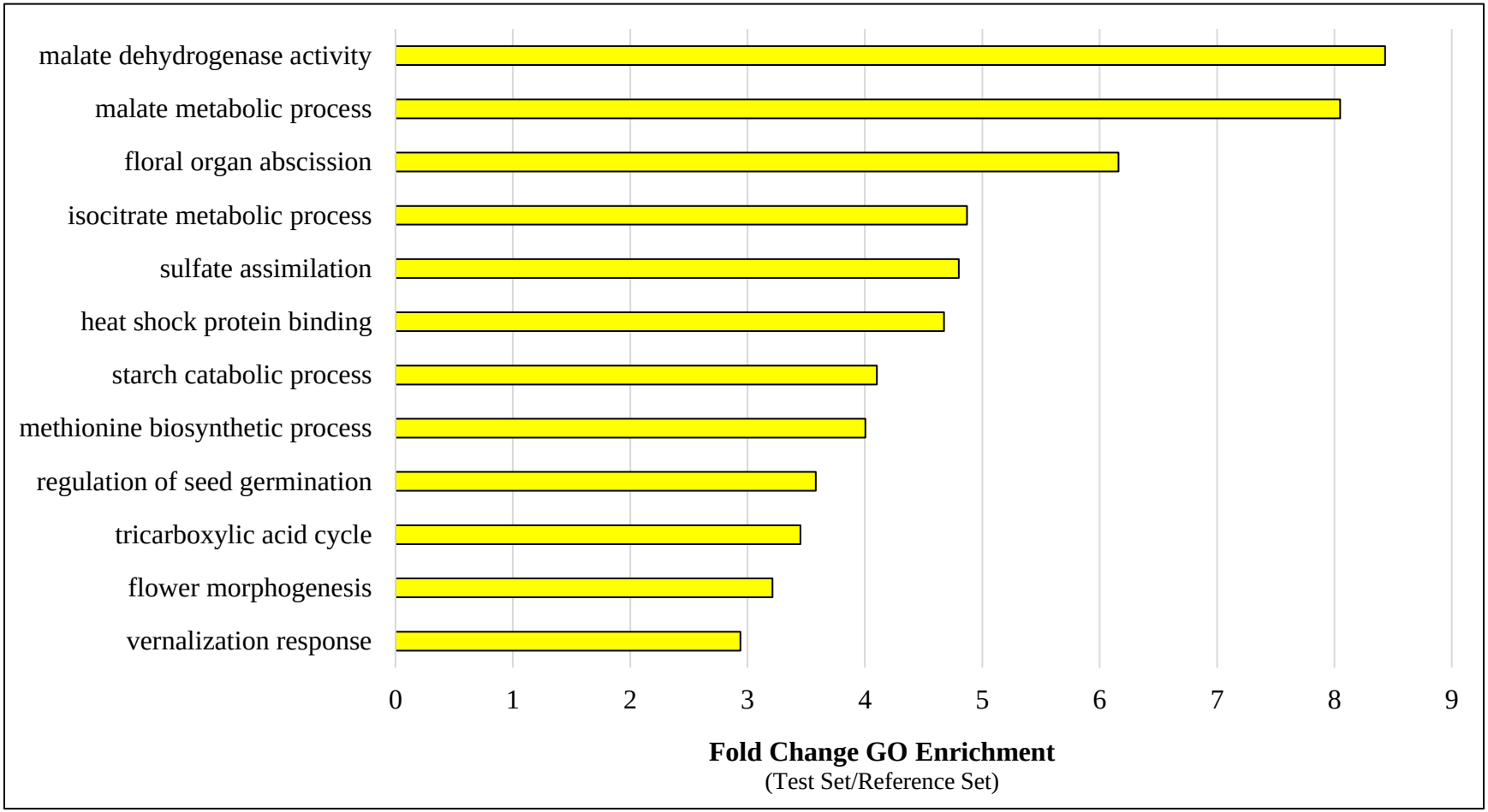

Figure 2.12 Selection of unique overrepresented GO terms in cold conditioned 'Bartlett' fruit. Ontologies were reduced to the most specific terms ($p<0.00001$). Additional values can be seen in Supplementary File 2.4.

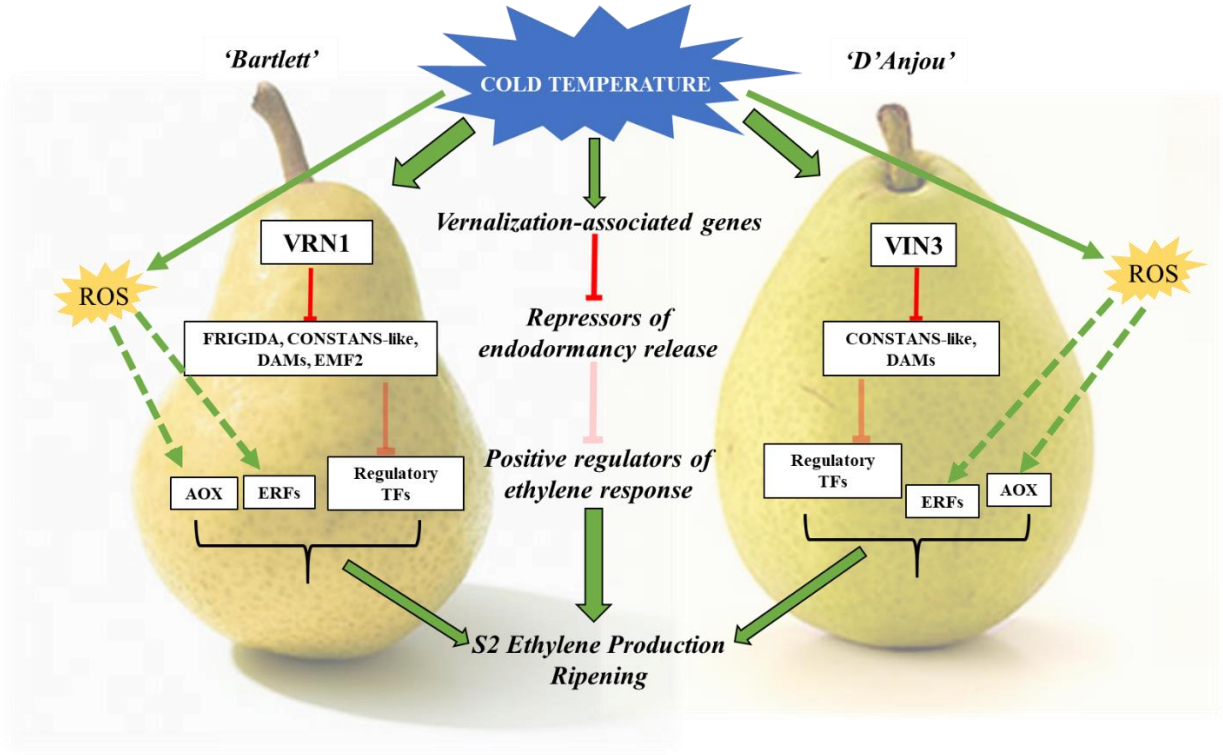

Figure 2.13 Model describing a possible mechanism by which vernalization-associated genes may mediate cold-induced ripening. Cold temperature stimulates VRN1/VIN3. Vernalization genes, in turn inhibit repressors of endodormancy release—FRIGIDA, CONSTANS-like, DAMs, EMF2. Downregulation of these repressors allows for transcriptional activation of ethylene response. Cold also triggers ROS signaling, leading to activation of ERFs (Oracz et al., 2009) and AOX (Saha et al., 2016). S2 ethylene production is triggered and ripening commences.

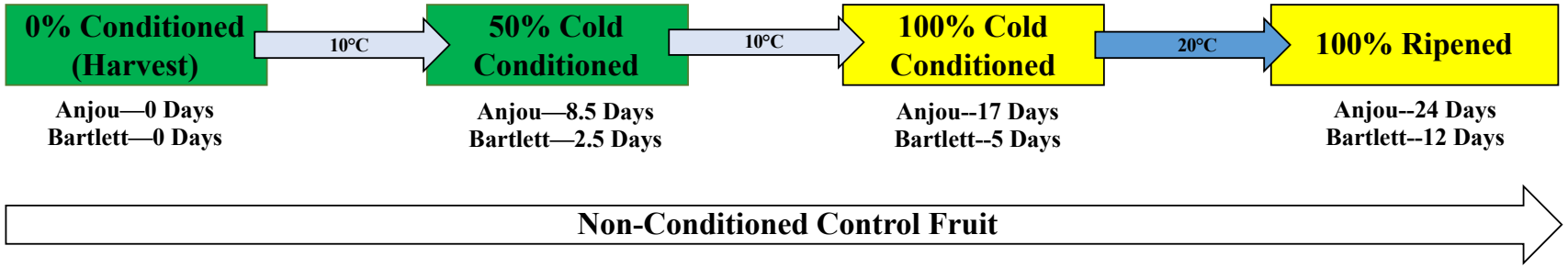

Figure 2.14 Conditioning time course schematic used for 'D'Anjou' and 'Bartlett'. Conditioning times were determined based on the established, cultivar-specific chilling conditions required for achievement of competency and ripening.

SUPPLEMENTARY FILES

Supplementary File 2.1 Annotated master assembly fasta for cold conditioned ‘D’Anjou’ and ‘Bartlett’ pear fruit.

Supplementary File 2.2 Mean RPKM values, standard error, and time course differential expression information for cold conditioned ‘D’Anjou’ and ‘Bartlett’ pear fruit.

Supplementary File 2.3 RPKM data files and experimental design files used for MaSigPro time-series differential expression analysis in OmicsBox.

Supplementary File 2.4 All shared and unique enriched gene ontologies for cold conditioned ‘D’Anjou’ and ‘Bartlett’ pear fruit.

Supplementary File 2.5 Quantitative RT-PCR validation with calculated expression values.

CHAPTER THREE

Glyoxylic acid overcomes 1-MCP-induced blockage of fruit ripening in *Pyrus communis L.* var. 'D'Anjou'

Seanna Hewitt[1,2] and Amit Dhingra[1,2*]

[1]-Molecular Plant Sciences Graduate Program, Washington State University, Pullman, WA

[2]-Department of Horticulture, Washington State University, Pullman, WA

*Author to whom correspondence should be addressed: adhingra@wsu.edu

Target Journal: Nature Scientific Reports

Abstract

The ripening response of 1-MCP treated 'D'Anjou' pear fruits treated with glyoxylic acid was observed. Physiological measurements were taken in time course and were paralleled by transcriptomic analysis to assess the combined effects of chemical stimulation of ripening. Accelerated ripening—indicated by decreased fruit firmness, onset of S1-S2 ethylene transition, and changes in expression of critical ripening related genes—was observed in the glyoxylic acid treated pears in comparison with control fruit. Transcriptomic and functional enrichment analyses revealed genes and ontologies implicated in glyoxylic acid mediated ripening, including alternative oxidase, TCA cycle, oxylipin metabolism, amino acid metabolism, organic acid metabolism, and ethylene responsive pathways. These observations implicate the glyoxylate cycle as a metabolic hub linking multiple pathways during ripening, lending support to glyoxylic acid as a chemical stimulator of ripening. The results provide information regarding how blockage

caused by 1-MCP may be circumvented at the metabolic level, thus opening avenues for more fine-tuned regulation of ripening in pear and possibly other fruit.

Introduction

Every year, 1.6 billion tons of food goes to waste. This is about one third of the food that is produced for human consumption (Buzby & Hyman, 2012). Unpredictable ripening of fruit is one of the main causes of loss after harvest. This is particularly true of climacteric fruits, which are characterized by a spike in ethylene biosynthesis, known as S2 ethylene production, and a concomitant burst in respiration at the onset of ripening. The ethylene receptor antagonist 1-methylcyclopropene (1-MCP) is used to impart a longer shelf life by limiting the ethylene perception and activation of downstream ripening responses (Watkins, 2006; Watkins, 2015; Sisler & Serek, 1997).

Uniquely, in European pear fruit (*Pyrus communis*), 1-MCP treatment may irreversibly inhibit endogenous or system 2 ethylene production and the respiratory climacteric (Villalobos-Acuna et al. 2008; Villalobos-Acuna et al., 2011). Furthermore, exogenous ethylene application does little to affect the capacity of 1-MCP-treated pears to ripen (Argenta et al., 2003; Xie et al., 2016). This observation suggests that 1-MCP, which has been classically understood only in the context of its identity as an ethylene receptor antagonist (Tatsuki et al., 2007), seems to exert additional metabolic consequences. This presents a challenge to the U.S. pear industry, as 1-MCP pear fruit fail to ripen properly and do not achieve the desired buttery consistency, aromatics and flavor profile to meet consumer satisfaction standards (Serra et al., 2019). Recent research has lent support to the concept of differential effect of 1-MCP on ethylene biosynthetic pathways and signal transduction networks in different climacteric fruits, including peach, pear, apple and tomato (Hao et al., 2018; Mata et al., 2018; Dal Cin et al., 2006). Attempts to metabolically reverse the

ethylene antagonism of 1-MCP in pear and other climacteric fruit have not been reported previously.

In order to ripen, European pears require a genetically pre-determined amount of cold temperature exposure, known as conditioning (Hartmann et al., 1987). Recent research revealed that AOX1, the key protein in the cyanide resistant alternative respiratory pathway, is elevated significantly in transcript expression during the pre-climacteric stages of cold conditioning in 'D'Anjou' and 'Bartlett' pear (Hendrickson et al. 2019). Climacteric bursts in respiration have been observed in mango and apple fruit and are attributed in part to enhanced post-climacteric AOX activity (Considine et al., 2001; Duque & Arrabaça, 1999). It has been proposed that AOX and cytochrome pathway activity may occur simultaneously in the ripening process, with the alternative respiratory pathway playing a greater role in the senescent processes following the climacteric burst (Duque & Arrabaça, 1999; Considine et al., 2001).

Based on the recent findings in pear, it appears that AOX may play a role in the pre-climacteric stage in fruits that require conditioning in order to ripen. Chemical genomics approaches targeting AOX for pre-climacteric ripening stimulation identified glyoxylic acid (GLA), the key metabolic intermediate in the glyoxylate cycle, as an activator ripening in 1-MCP treated pear fruit (Dhingra et al., 2017). The glyoxylate cycle is a shortcut in the TCA cycle used by bacteria and plants for gluconeogenesis and carbohydrate synthesis via β-oxidation of fatty acids to Ac-CoA (Eastmond & Graham, 2001; Graham et al., 1994; Penfield et al., 2018). Thus far, the role of the glyoxylate cycle in ripening is largely unexplored, with no studies of metabolic override of 1-MCP ripening blockage reported in present literature.

In this study, the hypothesis that GLA treatment will activate AOX expression and facilitate identification of additional significant genes and gene networks that enable override of 1-MCP blockage of ripening was evaluated. Established indicators of ripening, including fruit firmness, total soluble solids, and internal ethylene production along with RNAseq were analyzed over the course of ripening post-GLA treatment.

Results and Discussion

Glyoxylic acid treatment resulted in significant decrease in firmness ($p<0.05$) of 1-MCP treated 'D'Anjou' in comparison with the control in each of the three experiments conducted in 2018 (Figure 3.1a-c). Likewise, internal ethylene peaked significantly ($p<0.05$) in the GLA-treated pears, a response characteristic of the S1-S2 ethylene transition and the ripening climacteric (Figures 3.2a-c). Brix did not change significantly for either the treated or control pears throughout the duration of the experiment (3.3a-b). HPLC mean glucose and fructose values were elevated in the 3% GLA treated fruit in comparison with the control despite lack of statistically significant difference between treatment and control groups. Furthermore, analysis of organic acids revealed increased mean malic acid (though not statistically significant) and highly increased citric acid production ($p<0.05$) in the treated fruit vs the control fruit throughout the ripening time course (Figure 3.4). Increase in citric acid implicates activation of TCA cycle and respiration upon GLA treatment. Fruit tissues, including peel and flesh, sampled from the 3% GLA treated fruits and from the control fruits were used for a time-course transcriptomic analysis. Visual observations before and after treatment are shown in Figure 3.5.

The duration that each experimental group of pear fruit was held in controlled atmosphere (CA) storage prior to GLA application must also be considered. For delaying ripening of pear fruit,

2-3% oxygen and 0-1% carbon dioxide are the established ranges for CA storage (Argenta et al., 2003, Kader, 1980), and length of time in CA could impact the fruit's propensity to ripen. Observations in this study indicated that the overall ripening time course, both in GLA-treated fruit and in control fruit, was shorter the longer the fruit had been in CA storage prior to the start of an experiment. This phenomenon necessitated modified sampling timepoints for each experiment described in the methods section. Analysis of experiments conducted in 2017 that led to optimization of GLA treatment strategy can be found in Supplementary File 3.1.

Transcriptomics

RNAseq assembly generated 148,946 contigs (Supplementary Files 3.2 and 3.3). Using the time course differential expression analysis feature in OmicsBox, 5,912 contigs were identified as being differentially expressed over time in the 3% GLA-treated fruit in comparison with the control in all three ripening experiments (Supplementary File 3.4). To better understand the mechanisms underlying GLA induction of pear fruit ripening, the expression of genes/contigs associated with TCA cycle, glyoxylate cycle, lipid metabolism, AOX and mitochondrial electron transport, sugar/starch breakdown, and organic acid metabolism was analyzed. Among the differentially expressed contigs (DECs) were ethylene metabolism-associated contigs and amino acid precursors, numerous ethylene response factors (*ERFs*), a pear *AOX* homolog (ubiquinol oxidase), TCA cycle and glyoxylate cycle-associated contigs, and lipid metabolism-associated contigs. Many of the identified DECs displayed heightened expression in the 3% GLA-treated fruit in comparison with the control fruit at the 'Unripe' stage, which was sampled immediately following the 16 hour GLA humidification treatment, indicating that this set of DECs directly responded to the application of GLA, but decreased in expression throughout the time course. Such

genes with significantly heightened expression in the GLA- treated fruit immediately following treatment included: *AOX1*, several *ERF* transcripts, *RAN1*, isocitrate dehydrogenase, lipoxygenase, and *AOS1*. DECs which displayed delayed transcriptional responses, which became evident at the '50% Ripened' to '100% Ripened' included: ethylene biosynthetic enzymes, ACS and ACO, and citrate synthase, which displayed heightened expression in the treatment group in later stages of ripening.

Glyoxylic acid activation of AOX

Alterative oxidase (AOX) has been implicated previously in ethylene biosynthetic processes, the respiratory climacteric and ripening. Immediately following treatment with GLA, *AOX* transcript abundance was higher, with its expression decreasing over time in both treatment and control 1-MCP fruit but remaining consistently higher in the GLA treatment group (Figure 3.6). This expression trend is reminiscent of the pre-climacteric maxima of AOX transcription in non-1-MCP treated pear fruit that had undergone full conditioning (Hendrickson et al., 2019; Hewitt et al., 2019a). AOX pathway activity has been shown to contribute to the respiratory climacteric during ripening in tomato, papaya, and mango (Xu et al., 2012; Perotti et al., 2014; Oliveira et al., 2015; Considine et al., 2001). While GLA activated *AOX*, the results of this transcriptomic analysis suggest that AOX activity in 1-MCP treated pear fruit is potentially regulated in a feed-forward manner as a result of carbon metabolism upstream (Cherian et al., 2014; Xu et al., 2012; Perotti et al., 2014). This alternative respiratory stimulation may partially account for the enhanced respiratory response and ripening of 1-MCP treated pear (Dhingra et al., 2017).

Gluconeogenesis and glycolytic flux balance

The glyoxylate cycle is directly linked to the production of glucose via gluconeogenesis in certain biological contexts such as fatty acid to sugar conversion during seedling germination (Eastmond & Graham, 2001). Previously, study of gluconeogenic enzymes in tomato and peach revealed that *PEPCK* abundance increases during ripening of these fruit (Famiani et al., 2016). It has also been demonstrated that malate and citrate accumulate throughout fruit development and may be utilized both as substrates for respiration in the TCA cycle, as well as for gluconeogenesis during fruit ripening; however, 1-MCP slows the rate of organic acid to sugar conversion via gluconeogenesis which thereby delays ripening (Liu et al., 2016; Etienne et al., 2013; Beaudry et al., 1989). Thus, it was of interest to examine expression of genes in the gluconeogenic pathway in addition to the glycolytic pathway.

The control 1-MCP fruit in this experiment displayed stable mean expression levels of *PEPCK* over the time course. In GLA treated 1-MCP fruit, mean expression levels of *PEPCK* were higher than in the control immediately following treatment, and gradually decreased over the time course, ending with very low expression levels in comparison with the control. *Fructose 1,6 bisphosphatase* (*FBPase*), which encodes an enzyme that plays an additional key role in gluconeogenic pathway, displayed elevated expression levels in the control versus the treatment throughout the duration of the experimental time course (Figure 3.7). Conversely, *ATP-dependent 6-phosphofructokinase 3-like* (*PFK-like*) DEC exhibited dramatically elevated expression in the GLA-treated fruit immediately following treatment in comparison with the control fruit and decreased over time. PFK catalyzes the first committed step of glycolysis, which leads to the formation of ATP and pyruvate (Figure 3.7). The ATP generated through this process can catalyze other ripening related processes.

Taken together, these results suggest that in GLA-treated 1-MCP pear fruit, flux through the gluconeogenic pathway results in additional allocation of carbon-based metabolites to the TCA cycle, potentially leading to overreduction of the mitochondrial electron transport chain, thereby necessitating increased AOX activity.

Fatty Acid Metabolism and Oxylipins

The glyoxylate cycle has an important role in β-oxidation of fatty acid under certain biological conditions, seedling germination being one of them, in which production of sugar from other organic substrates is of developmental importance (Cornah et al., 2004; Eastmond & Graham, 2001). Addition of GLA could potentially induce multiple metabolic pathways, thereby facilitating a link between fatty acid metabolism and GLA-induced ripening.

Fatty acid oxidation capacity typically is at its highest in pre-climacteric fruit and in fruit entering the climacteric stage (Baqui et al., 1977). Furthermore, a shift from mitochondrial oxidation of fatty acids to TCA cycle intermediates occurs in the climacteric transition (S1-S2 ethylene biosynthesis) (Baqui et al., 1977). In mango, addition of glyoxylate stimulated oxidation of fatty acids occurs in a concentration dependent manner; it appears the same may be occurring in GLA treated 1-MCP pear fruit.

Oxidation of unsaturated fatty acids results in the generation of a class of lipophilic signaling molecules called oxylipins. Initial synthesis of these molecules occurs via conversion of polyunsaturated fatty acids to fatty acid hyperperoxides via lipoxygenase (LOX) (Seymour et al., 2012). The phytohormone jasmonic acid (JA) is among the most well characterized oxylipins, as jasmonates, together with ethylene, are believed to play a role in the early stages of climacteric

ripening (Fan et al., 1998; Mosblech et al., 2009). Methyl jasmonate increases postharvest shelf life in Chilean strawberry (*Fragaria chiloensis*) by modulating the soluble solid to TA ratios as validated by assessments of quality chemical attributes and decay incidence (Saavedra et al., 2016). JA has been shown to negatively regulate ripening in 'Bartlett' pears and is suggested to do so by working either independently or upstream of ethylene biosynthesis (Argenta et al., 2016). The first specific enzyme, and rate limiting point in JA biosynthesis is allene oxide synthase (AOS) (Haga & Iino, 2004). Expression of AOS was significantly elevated in the GLA-treated fruit in comparison with the control 1-MCP fruit immediately following treatment, suggesting an increased flux through the JA biosynthesis pathway, which could potentially aid in further stimulation of an ethylene response in the treated fruit (Figure 3.8). In addition to AOS, several lipoxygenases were also significantly elevated in expression in the treatment versus the control fruit (some at the beginning and others at the end of the time course), indicating the commitment of fatty acid breakdown products to oxylipin metabolism (Figure 3.8). Based on these observations, it appears that fatty acid metabolism contributes both to a hormonal response and to increased pools of carbon-metabolites available for TCA cycle, thereby contributing to the multifaceted ripening response stimulated by GLA.

TCA and glyoxylate cycle metabolism

The glyoxylate cycle is primarily compartmentalized in the peroxisome, however, two of the five enzymes that comprise the cycle are cytosolic (gCS, gICL, gMS, ctAC, ctMDH). Malate is an intermediate in both glyoxylate and mitochondrial TCA cycles and has been suggested to play a role as an important regulatory metabolite during ripening (Seymour et al., 2013). This important organic acid is involved in many other processes besides the glyoxylate cycle and

respiratory metabolism, and therefore, membrane transport is required to shuttle this substrate into other subcellular compartments (Pracharoenwattana & Smith, 2008). Glyoxylate cycle enzyme encoding malate synthase gene expression has been detected specifically in ripening tissue, but this expression varied by ripening stage and has been suggested that expression changes could be stimulated by exogenous ethylene (Pistelli et al., 1996; Mateos et al., 2003). Alteration in the supply of malate has been shown to affect postharvest storage quality (Centeno et al., 2011). Like malate, citrate is an intermediate in both TCA and glyoxylate cycles that accumulates in the pre-climacteric stages and is metabolized during ripening and is one of the most prevalent metabolites contributing to flavor and titratable acidity during fruit development (Baqui et al., 1977). Contigs corresponding to glyoxysomal and mitochondrial citrate synthase enzymes differed significantly in expression over time in GLA-treated 1-MCP fruit versus the control 1-MCP fruit, with increased expression of both observed in the treatment. Additional contigs corresponding to TCA cycle enzymes fumarate hydratase, isocitrate dehydrogenase were also elevated in expression in GLA-treated fruit (Figure 3.9). It implicated, based on the expression of TCA/glyoxylate cycle genes that GLA could directly affect the flux through these pathways.

Glyoxylic acid link to ethylene biosynthesis

Methionine cycling, and therefore ethylene production is a process that operates concomitantly with respiration and is co-dependent upon the TCA/glyoxylate cycle compound, oxaloacetate. In addition to serving as a substrate for the formation of citrate, OAA may be converted into intermediates for other metabolic processes (Baur & Yang, 1972). The link between the glyoxylate and TCA cycle and ethylene begin with the conversion of OAA to aspartate via aspartate aminotransferase (AAT) or by bifunctional aspartate/prephenate aminotransferase (PT-

AAT/PAT). Aspartate is then converted to homoserine, cystathionine, homocysteine, and then to methionine. Methionine is converted to S-adenosyl-L methionine catalyzed by SAM synthetase, forming the precursor to ACC and ethylene biosynthesis. The enzymes involved in these conversions depend on the co-factor pyridoxal-5'-phosphate (PLP), the production of which is catalyzed by pyridoxine synthases (PDX1 and PDX2) (Le Deunff, 2019; Burstenbinder & Sauter, 2012). The heightened expression of genes in the path from OAA to the production of ACC in ethylene biosynthesis in the GLA-treated fruit in comparison with the control 1-MCP fruit suggest that the ripening compound elicits effects that upregulate this pathway via a yet uncharacterized mechanism. *AAT* expression was similar in the treatment and control at the experimental start and end points; however, its expression was significantly higher at the 50% Ripened stage (Figure 3.10). *AAT/PAT* expression was highest in the GLA-treated fruit immediately following treatment, decreasing throughout the time course, but all the while remaining expressed at higher levels than the control fruit. *CγS* expression remained highest in the GLA treatment over time than the control fruit. The expression pattern of *AAT/PAT*, *CγS*, and *SAM* as well as the cofactor producing *PDX2*, followed a nearly identical pattern, with highest expression immediately following treatment and decreasing expression throughout the time course (Figure 3.10). This methionine may feed directly into the methionine cycle, thereby resulting in increased ethylene production when OAA is produced abundantly. The observed changes in expression patterns suggest that increased flux through the glyoxylate/TCA cycles leads to increased production of OAA, some of which is shunted into the methionine biosynthesis pathway and can be consequently converted to ethylene (Figure 3.10). Methionine cycling is an ATP-dependent process and in situations where respiration is interrupted, as is in the case of 1-MCP treatment, this fundamental process cannot occur.

Activation of the glyoxylate cycle in fruit subjected to respiratory inhibition is the potential source for the ATP necessary for continued methionine cycling and, therefore, ethylene biosynthesis.

Significant changes in the expression of DECs associated with ethylene biosynthesis, perception, and signaling were identified throughout the ripening time course following GLA treatment, including those corresponding to ethylene biosynthetic genes *ACO1* and *ACS1*, ethylene perception-associated *RAN1* and *CTR1* (Figure 3.11), and numerous ethylene response factors (Figure 3.12).

Functional Enrichment Analysis

GO enrichment analysis was conducted using the differentially expressed contigs in the 3% GLA treatment group versus the control group as the test set and the annotated master assembly as the reference set, and the resulting enriched ontologies were reduced to most specific (FDR<0.05). The results provide complementary information to the differential expression analysis, with many of the over and underrepresented terms corresponding to biological processes and molecular functions associated with known and novel ripening related pathways (Figure 3.13).

Overrepresented ontologies in the 3% GLA-treated fruit included: 'fruit ripening' and 'response to chemical'. These are expected terms, considering that physiological changes indicative of ripening in response to chemical treatment were observed in the treated fruit. Additional overrepresented GO terms included those associated with phytohormone metabolism ('1-aminocyclopropane-1-carboxylate biosynthetic process', 'hormone metabolic process', and 'regulation of hormone levels'), 'aspartate family amino acid biosynthetic process', metabolism of sulfur compounds ('sulfate transport, 'sulfur compound metabolic process'), organic acid

metabolism (‘carboxylic acid catabolic process’, ‘oxoacid metabolic process’), lipid metabolism (‘lipid metabolic process’, ‘oxylipin biosynthetic process’). These findings coincide with the results of the differential expression analysis where contigs associated with ethylene biosynthesis and signaling, organic acid metabolism, and lipid metabolism displayed significant differential expression (Figures 3.8, 3.9, 3.11, and 3.12), providing support for crosstalk between these seemingly independent pathways during GLA-mediated ripening. Furthermore, results of this analysis again implicate AOX as an important factor in ripening. Sulfur metabolism-associated terms were overrepresented, and sulfur has previously been implicated in the pre-climacteric activation of the AOX respiratory pathway and ripening in cold-conditioned pear fruit (Dhingra & Hendrickson, 2017). Interestingly, ‘response to cold’, ‘cellular oxidant detoxification’ and ‘antioxidant activity’ were also enriched. While these experiments were conducted at ambient temperature, cold conditioning is required for pear fruit to ripen (Villalobos-Acuna & Mitcham, 2008), and recent studies of cold-induced ripening of pear implicate the involvement of AOX for crosstalk with ethylene and scavenging of reactive oxygen species (ROS), thereby alleviating oxidative damage under external stress conditions (Hendrickson et al., 2019; Hewitt et al., 2019). Enrichment of these ontologies may indicate that similar mechanisms are activated in both cold and GLA- induced ripening, with both instances involving AOX activity. It raises an interesting possibility that GLA might be able to, in part, replace the requirement of cold conditioning in pear.

Underrepresented GO terms include those associated with maintenance of DNA and chromosomal integrity and organization (‘DNA-dependent DNA replication’, ‘DNA repair’, ‘DNA conformation change’, ‘chromosome modification’, ‘chromatin organization’, and ‘histone modification’) (Figure 3.13). The decreased representation of these functions in the GLA-treated test set, which exhibited physiological and transcriptomic indications of more rapid senescence,

indicates that compositional organization decreases, and cellular entropy increases as ripening progresses. These processes are directly correlated to cell wall softening, and other processes of decompartmentalization in the ripening fruit. Backtracking from the GO enrichment data to corresponding differentially expressed genes may provide additional targets for ripening regulation in a number of related pathways.

Glyoxylic acid as a metabolic hub

The findings of this study suggest that the propensity of GLA to stimulate a ripening response in pear may result from the centrality of the glyoxylate metabolism to several critical biochemical pathways, as evidenced by the elevation in expression of genes encoding critical rate limiting enzymes in glycolysis, fatty acid metabolism, aspartate/cysteine/methionine metabolism, TCA cycle, and the AOX respiratory pathway immediately following GLA treatment (Figures 3.14-3.16). Interestingly, while such closely associated processes with the glyoxylate cycle appear to be stimulated upon GLA administration, differential expression of the unique glyoxylate cycle enzymes malate synthase and isocitrate lyase was not observed. This finding necessitates further protein expression-based work but could also indicate that following application, GLA is being directly converted into other substrates that can be used for respiration and ethylene biosynthesis—testing the effects of continual administration of GLA at low levels throughout a designated ripening period could shed light on this hypothesis. Such an approach has already been successfully employed with other compounds, including 1-MCP (Villalobos-Acuña et al., 2011). Additionally, investigating the potential stimulatory effects of exogenously applied TCA and glyoxylate cycle intermediates would add further insight to the understanding of the way in which

these pathways and their metabolic intermediates affect critical developmental processes like ripening.

Conclusion

Transcriptome wide gene expression analysis allowed for identification of interacting networks of genes and pathways that are involved in GLA-mediated activation of AOX and the metabolic override of ripening blockage in pear caused by 1-MCP. Based on prior knowledge of the glyoxylate cycle, its intermediates, and its regulation, it is hypothesized that its mode of action in ripening induction occurs via one or more of the following processes: direct activation of AOX, induction of fatty acid oxidation, provision of organic acid as respiratory substrate, indirect TCA cycle stimulation, or induction of gluconeogenesis (Umbach et al., 2006; Baqui et al., 1977; Cornah et al., 2004; Igamberdiev & Eprintsev, 2016; Saltveit, 2019; Osorio et al., 2013). Results of time course differential expression analysis of GLA-treated 'D'Anjou' 1-MCP pear fruit compared to control 1-MCP fruit presented here lend support to this hypothesis and have provided additional molecular targets for fine-tuned ripening regulation of European pear and other climacteric fruits subjected to 1-MCP treatment.

Ripening in climacteric fruit has long been attributed to biosynthesis of ethylene. It is clear, however, that the nuances of ripening extend far beyond ethylene production and respiration. Due to complex requirements for S1-S2 transition in European pear, the fruit may serve as a useful system in which to explore the vagaries of the ripening process (Hewitt & Dhingra, 2019). This study addresses a knowledge gap in the ripening process in pear, epitomized by the inability of 1-MCP treated fruit to regain ripening capacity naturally and paralleled with the discovery that GLA can overcome the 1-MCP blockage. Exploring the potential role of glyoxylate as a metabolic hub,

its link to ethylene biosynthesis, fatty acid metabolism, glycolysis, and activation of the AOX respiratory pathway in the context of 1-MCP inhibited ripening, provides further insight into mechanism of ripening in European pear and other fruit. It also enriches understanding regarding how ripening control may be better achieved through 1-MCP inhibition and subsequent stimulation of ripening using natural metabolic intermediates.

The role of GLA in stimulation of ripening and the potential genes and pathways implicated in this process provide additional molecular targets for fine-tuned regulation of ripening, and contribute to a body of work in which genetic markers associated with sugars, acids, volatiles, and ripening in general are being mapped (Tieman et al., 2017; Martín-Pizarro & Posé, 2018). In addition to molecular, biochemical, and gene expression-based work conducted to better understand the genetic factors underlying GLA mode of action in 1-MCP treated pear fruit, collaborations with industry partners in the Pacific Northwest have allowed for testing of this ripening technology at a highly applied scale. As GLA is a natural plant metabolite and has previously been used as commercial food preservative (Hendrickson, 2014; Postma & Erickson, 2005), it is feasible to introduce it as a ripening tool in the tree fruit industry. Translation of this ripening tool set—1-MCP inhibition and GLA activation—to other crops in the future, is expected to provide the opportunity to inhibit ripening at harvest, store fruit for the desired duration or ship it to domestic or international locations, and reactivate ripening in a planned and predictable manner, thereby improving consumer satisfaction and ultimately improving postharvest sustainability by reducing the waste associated with unpredictable ripening of fruit.

Materials and Methods

Acquisition of 'D'Anjou' pear fruit

Mature, late season ‘D’Anjou’ pears were obtained from Blue Star Growers (Cashmere, WA) in the Winter/Spring of 2018. Prior to acquisition, pears had been treated with 130ppm 1-MCP and retained in controlled atmosphere storage at 5°C. Following acquisition, pears were transferred to 1°C of the WSU Johnson hall postharvest facility’s controlled atmosphere room for approximately one week prior to initiation of experiments.

Ultrasonic humidification of ripening compound treatment solutions

Pears were transferred to non-airtight plexiglass tanks (dimensions 40cm x 58cm x 66cm) fitted with an inlet for a tube connected to a Crane Ultrasonic humidifier (Crane USA). The bottom of each humidifier was lined with baker’s drying racks to allow for excess treatment liquid to drip to the bottom of the chamber during the humidification period. Humidifiers were loaded with treatment and control solutions of 3% GLA and deionized water, respectively. Depending on the number of samples to be taken throughout the duration of the experiment, between 32 and 64 pears were treated in each chamber. The pears were treated for a total of 16 hours overnight with their designated ripening compound or control solution.

Tissue sampling

It was necessary to classify sampling points to be used for sequencing based on percent ripeness. This method of sample point determination has been previously employed in ripening studies (Hendrickson, 2014). Fruit was classified as ‘Unripe’ on Day 1, immediately upon removal from GLA or control humidification chambers, when firmness between 11-13 lbf (48.9-57.8 N). The classification as ‘50% Ripened’ was given to fruit at the sample point where average firmness of 3% GLA-treated fruit was between 5-7 lbf (22.2-31.1 N). The fruit were classified as

‘100% Ripened’ when the firmness of the 3% GLA-treated fruit were between 1-3 lbf (4.4-13.3N) (Figure 3.14). Sample analysis stages corresponding to 0, 50, and 100% Ripened for Experiment 1 (January-February 2018) were determined to be Day 1, Day 7 and Day 14; Experiment 2 (February 2018) Day 1, Day 4, and Day 10; and Experiment 3 (June 2019) Day 1, Day 4 and Day 7.

In each experiment, peel tissue was sampled from a 1 cm wide equatorial region of each of 4 fruit per treatment and time point, pooled, flash frozen in liquid nitrogen, and ground using a SPEX Freezer/Mill 6870 (Metuchen, NJ USA). Tissues were stored at -80°C prior to RNA extraction and HPLC analysis.

Monitoring of carbon dioxide evolution

Following 16-hour humidification treatment, GLA-treated and control pears were weighed and sealed into continuous air flow chambers (four replicates of 4 fruit per replicate), which sample CO_2 at 4-hour intervals. Fruit respiration rates were determined by calculating mean CO_2 evolution per kilogram of fruit at each sample time using the previously described methods and sampling system (Zommick et al., 2014).

Internal ethylene gas chromatographic measurements

Ethylene gas was vacuum extracted from the inside of the fruit using a vacuum aspirator as described in Beyer & Morgan, 1970. One quarter of four separate pear fruit per treatment was sliced into 4-5 pieces and then placed into an inverted funnel submerged at the base in previously de-gassed deionized water (Figure 3.15). Headspace air was removed through a septum in the top of the funnel. Subsequently, the vacuum chamber was sealed, and the aspirator used to extract the

gas from within the fruit. 0.5mL headspace gas that collected in the neck of the inverted funnel was extracted in triplicate with 1mL hypodermic syringes.

Gas samples (2 replicates per treatment, 1/4 pear sections from 4 fruits per replicate) were injected into a HP 5890A gas chromatograph (Agilent, Avondale, PA, USA) with a flame ionization detector (FID) connected to a 0.53 mm x 15 m GS-Q-PLOT column (Agilent) to measure the ethylene composition of the extracted gas. Ethylene gas composition measurements were repeated every three days throughout each experimental time course. Mean and standard error for ethylene evolution were calculated for each sample time point.

Firmness measurements

Firmness measurements were collected at each time point using four replicate pears from each treatment group. A GS-14 Fruit Texture Analyzer (GÜSS Instruments, South Africa) with an attached 8.0 mm probe set at 5.0 mm flesh penetration was used to measure firmness at 3 equidistant points around the equatorial region of each fruit following peel removal. Mean firmness value for each fruit was used for the final data assessment.

°*Brix*

Soluble solid content was measured at each sample point by pooling approximately 0.5mL extracted juice from four replicate fruit per treatment/control and quantifying °*Brix* using a handheld refractometer.

Statistical analysis of physiological data

Analysis of variance (ANOVA) was conducted for CO_2, ethylene, firmness, and °Brix data within and across each of the experiments using SAS® University Edition (SAS Institute Inc., Cary, NC) with time, and GLA as treatments. Standard error for carbon dioxide evolution was plotted in 3-day intervals, although measurements of CO_2 were taken every 4 hours throughout the experiment.

RNA Extraction and Sequencing

Total RNA was extracted from pulverized 'D'Anjou' and 'Bartlett' peel tissue for each of the three technical replicates at 'Unripe', '50% Ripened', and '100% Ripened' stages following fruit tissue specific DEPC-CTAB protocol (Gasic et al., 2004). RNA was quality checked on an agarose gel and was quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Following quality validation and quantification using a Life Technologies Qubit Fluorometer (Carlsbad, CA) and Agilent Bioanalyzer (Santa Clara, CA), RNA libraries were sequenced by BGI Hong Kong Tech Solution NGS Lab on an Illumina HiSeq 4000 platform as 2x100 paired end reads.

Transcriptome Assembly

The 2x100 paired end fastq files generated using Illumina HiSeq 4000 were input into the CLC Bio Genomics Workbench (ver 6.0.1) (Aarhus, Denmark) for pre-processing and assembly. The CLC Create Sequencing QC report tool was used to assess quality. The CLC Trim Sequence process was used to trim quality scores with a limit of 0.001, corresponding to a Phred value of 30. Ambiguous nucleotides were trimmed, and the 13 5' terminal nucleotides removed. Reads below length 34 were discarded. Overlapping pairs were merged using the 'Merge Overlapping

Pairs' tool, and a subsequent de novo assembly was performed with all datasets. Parameters used in the assembly are as follows: Map reads back to contigs = TRUE, Mismatch cost = 2, Insertion cost = 3, Deletion cost = 0.4, Similarity Fraction = 0.95, Global Alignment = TRUE, Minimum contig length = 200, Update contigs = TRUE, Auto-detect paired distances = TRUE, Create list of un-mapped reads = TRUE, Perform scaffolding = TRUE. The de novo assembly resulted in the production of 148,946 contiguous sequences (contigs). Contigs with less than 2x coverage and those less than 200bp in length were eliminated. For each individual dataset (treatment/replicate) the original, non-trimmed reads were mapped back to the master assembly subset. Default parameters were used, except for the length fraction, which was set to 0.5, and the similarity fraction, which was set to 0.9. Mapping resulted in the generation of individual treatment sample reads per contig. The master transcriptome was exported as a fasta file for functional annotation and the read counts for each dataset were exported for normalization with the Reads Per Kilobase per Million reads (RPKM) method (Mortazavi et al., 2008) (Supplementary File 3.2).

Functional annotation

The master transcriptome fasta produced from the Illumina assembly was imported into OmicsBox 1.1.135 (BioBam Bioinformatics S.L., Valencia, Spain) for functional annotation of expressed contigs. Contig sequences were identified by a blastx alignment against the NCBI 'Viridiplantae' database with and e-value specification of 10.0E-3. Gene ontology (GO) annotation was assigned using the 'Mapping' and 'Annotation' features using default parameters to generate a functionally annotated master assembly (Conesa et al., 2005) (Supplementary File 3.3).

Differential expression analysis

Temporally differentially expressed genes were identified using the time course, multi-series differential expression feature in the OmicsBox suite, which employs the maSigPro R package. FDR cutoff value was set to 0.05. The statistical analysis ensured that genes that did not meet the assumption of equal variances were eliminated from the analysis, which was particularly important given that the three experiments were performed at different times throughout the 2018 season. The DECs and expression values were matched with their corresponding functional annotations (Supplementary File 3.4).

GO enrichment analysis

Gene ontology (GO) enrichment analysis was conducted to determine over and underrepresented biological processes, molecular functions, and cellular components among the differentially expressed sequences using the OmicsBox Enrichment Analysis (Fisher's Exact Test) function (Conesa et al., 2005) (Supplementary File 3.5). The annotated master transcriptome was used as the reference dataset, and the set of genes identified as differentially expressed over time in the treatment group versus the control group was used as the test dataset.

qRT-PCR Validation

Primers for quantitative reverse transcriptase PCR (qRT-PCR) targeting seven genes in ripening related pathways which were differentially expressed in the RNAseq results were designed using the NCBI Primer-BLAST tool (Ye et al., 2012) (Supplementary File 3.6). 200ng RNA for each sample was used to generate 1st strand cDNA using the Invitrogen VILO kit (Life Technologies, Carlsbad, CA USA). cDNA preparations were then diluted to 20ng/uL. Final library

concentrations were quantified using a Qubit fluorometer (Carlsbad, CA). qRT-PCR technical replicate reactions were prepared for each of the gene targets using the iTAQ Universal SYBR Green Supermix with ROX reference dye (BioRad, Hercules, CA) per the manufacturer's protocols with 20ng of template cDNA. In a Strategene MX3005P, the following thermocycle profile was used: 95°C initial disassociation for 2:30 minutes followed by 50 amplification cycles (95°C for 30s, 60°C for 30s, and 72°C for 30s) and a final, single cycle phase to generate a dissociation curve (95°C for 30s, 57°C for 30s, and 72°C for 30s). The LinRegPCR tool was used to calculate the Cq values for each reaction (Ruijter et al. 2009; Ramakers et al. 2003) (Supplementary File 3.6). Cq values which were calculated from efficiency scores below 1.80 or 2.20 were considered sufficiently low in confidence and were deemed unacceptable and were omitted from the analysis.

Acknowledgements

The authors thank Blue Bird Growers (Cashmere, WA) for providing pears for conditioning experiments and to Scott Mattinson for assistance with the gas chromatography work. Work in the Dhingra lab was supported in part by Washington State University Agriculture Center Research Hatch Grant WNP00011 and grant funding from Pear Bureau NW to AD. SLH acknowledges the support received from ARCS Seattle Chapter and National Institutes of Health/National Institute of General Medical Sciences through an institutional training grant award T32-GM008336. The contents of this work are solely the responsibility of the authors and do not necessarily represent the official views of the NIGMS or NIH.

REFERENCES

Argenta LC, Fan X, Mattheis JP, 2003. Influence of 1-methylcyclopropene on ripening, storage life, and volatile production by d'Anjou cv. pear fruit. *Journal of agricultural and food chemistry* **51**, 3858-64.

Argenta LC, Mattheis JP, Fan X, Amarante CV, 2016. Managing 'Bartlett'pear fruit ripening with 1-methylcyclopropene reapplication during cold storage. *Postharvest Biology and Technology* **113**, 125-30.

Baqui SM, Mattoo AK, Modi VV, 1977. Glyoxylate metabolism and fatty acid oxidation in mango fruit during development and ripening. *Phytochemistry* **16**, 51-4.

Baur AH, Yang SF, 1972. Methionine metabolism in apple tissue in relation to ethylene biosynthesis. *Phytochemistry* **11**.

Beaudry RM, Severson RF, Black CC, Kays SJ, 1989. Banana ripening: implications of changes in glycolytic intermediate concentrations, glycolytic and gluconeogenic carbon flux, and fructose 2,6-bisphosphate concentration. *Plant Physiology* **91**, 1436-44.

Beyer EM, Morgan PW (1970). A method for determining the concentration of ethylene in the gas phase of vegetative plant tissues. *Plant Physiology* **46**, 352.

Burstenbinder K, Sauter M, 2012. Early events in the ethylene biosynthetic pathway—regulation of the pools of methionine and s-adenosyl-l-methionine. *Annual Plant Reviews, The Plant Hormone Ethylene* **44**, 22.

Buzby JC, Hyman J, 2012. Total and per capita value of food loss in the United States. *Food Policy* **37**, 561-70.

Centeno DC, Osorio S, Nunes-Nesi A, *et al.*, 2011. Malate Plays a Crucial Role in Starch Metabolism, Ripening, and Soluble Solid Content of Tomato Fruit and Affects Postharvest Softening. *The Plant Cell Online*.

Cherian S, Figueroa CR, Nair H, 2014. 'Movers and shakers' in the regulation of fruit ripening: a cross-dissection of climacteric versus non-climacteric fruit. *Journal of experimental botany* **65**, 4705-22.

Considine MJ, Daley DO, Whelan J, 2001. The Expression of Alternative Oxidase and Uncoupling Protein during Fruit Ripening in Mango. *Plant Physiology* **126**, 1619-29.

Cornah JE, Germain V Fau - Ward JL, Ward Jl Fau - Beale MH, Beale Mh Fau - Smith SM, Smith SM, 2004. Lipid utilization, gluconeogenesis, and seedling growth in Arabidopsis mutants lacking the glyoxylate cycle enzyme malate synthase.

Dal Cin V, Rizzini FM, Botton A, Tonutti P, 2006. The ethylene biosynthetic and signal transduction pathways are differently affected by 1-MCP in apple and peach fruit. *Postharvest Biology and Technology* **42**, 125-33.

Dhingra A, Hendrickson C, Hewitt S, 2017. Control of ripening and senescence in pre-harvest and postharvest plants and plant materials by manipulating alternative oxidase activity. In.: Google Patents.

Duque P, Arrabaça JD, 1999. Respiratory metabolism during cold storage of apple fruit. II. Alternative oxidase is induced at the climacteric. *Physiologia plantarum* **107**, 24-31.

Eastmond PJ, Graham IA, 2001. Re-examining the role of the glyoxylate cycle in oilseeds. *Trends in plant science* **6**, 72-8.

Etienne A, Génard M, Lobit P, Mbeguié-a-Mbéguié D, Bugaud C, 2013. What controls fleshy fruit acidity? A review of malate and citrate accumulation in fruit cells. *Journal of experimental botany* **64**, 1451-69.

Famiani F, Paoletti A, Battistelli A, *et al.*, 2016. Phosphoenolpyruvate carboxykinase, pyruvate orthophosphate dikinase and isocitrate lyase in both tomato fruits and leaves, and in the flesh of peach and some other fruits. *Journal of plant physiology* **202**, 34-44.

Fan X, Mattheis JP, Fellman JK, 1998. A role for jasmonates in climacteric fruit ripening. *Planta* **204**, 444-9.

Gasic K, Hernandez A, Korban S, 2004. RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. *Plant Molecular Biology Reporter* **22**, 437-8.

Graham IA, Denby KJ, Leaver CJ, 1994. Carbon Catabolite Repression Regulates Glyoxylate Cycle Gene Expression in Cucumber. *The Plant Cell Online* **6**, 761-72.

Haga K, Iino M, 2004. Phytochrome-mediated transcriptional up-regulation of ALLENE OXIDE SYNTHASE in rice seedlings. *Plant and Cell Physiology* **45**, 119-28.

Hao PP, Wang GM, Cheng HY, *et al.*, 2018. Transcriptome analysis unravels an ethylene response factor involved in regulating fruit ripening in pear. *Physiologia plantarum* **163**, 124-35.

Hartmann C, Drouet A, Morin F, 1987. Ethylene and ripening of apple, pear and cherry fruit. *Plant Physiology and Biochemistry (France)*.

Hendrickson C, 2014. Physiogenomics of Pear Ripening. Dissertation.

Hendrickson C, Hewitt S, Swanson ME, Einhorn T, Dhingra A, 2019. Evidence for pre-climacteric activation of AOX transcription during cold-induced conditioning to ripen in European pear (*Pyrus communis L.*). *bioRxiv*, 755686.

Hewitt S, Dhingra, A, 2019. Beyond Ethylene: New Insights Regarding the Role of AOX in the Respiratory Climacteric. *Pre-publication.*

Hewitt S, Hendrickson C, Dhingra A, 2019. Vernalization-related genes regulate cold-induced ripening in 'D'Anjou' and 'Bartlett' pear fruit. *Pre-publication*.

Igamberdiev AU, Eprintsev AT, 2016. Organic Acids: The Pools of Fixed Carbon Involved in Redox Regulation and Energy Balance in Higher Plants. *Frontiers in Plant Science* **7**.

Kader AA, 1980. Prevention of ripening in fruits by use of controlled atmospheres. *Food Technol* **34**, 51-4.

Le Deunff E, 2019. From Aspartate to Ethylene: Central Role of N, C, and S Shuttles by Aminotransferases During Biosynthesis. *Progress in Botany* **80**, 253.

Liu R, Wang Y, Qin G, Tian S, 2016. Molecular basis of 1-methylcyclopropene regulating organic acid metabolism in apple fruit during storage. *Postharvest Biology and Technology* **117**, 57-63.

Martín-Pizarro C, Posé D, 2018. Genome Editing as a Tool for Fruit Ripening Manipulation. *Frontiers in Plant Science* **9**.

Mata CI, Van De Poel B, Hertog ML, Tran D, Nicolai BM, 2018. Transcription analysis of the ethylene receptor and CTR genes in tomato: the effects of on and off-vine ripening and 1-MCP. *Postharvest Biology and Technology* **140**, 67-75.

Mateos RM, León AM, Sandalio LM, Gómez M, Luis A, Palma JM, 2003. Peroxisomes from pepper fruits (Capsicum annuum L.): purification, characterisation and antioxidant activity. *Journal of plant physiology* **160**, 1507-16.

Mortazavi A, Williams BA, Mccue K, Schaeffer L, Wold B, 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* **5**.

Mosblech A, Feussner I, Heilmann I, 2009. Oxylipins: structurally diverse metabolites from fatty acid oxidation. *Plant Physiology and Biochemistry* **47**, 511-7.

Oliveira M, Mazorra L, Souza A, *et al.*, 2015. Involvement of AOX and UCP pathways in the post-harvest ripening of papaya fruits. *Journal of plant physiology* **189**, 42-50.

Osorio S, Vallarino JG, Szecowka M, *et al.*, 2013. Alteration of the Interconversion of Pyruvate and Malate in the Plastid or Cytosol of Ripening Tomato Fruit Invokes Diverse Consequences on Sugar But Similar Effects on Cellular Organic Acid, Metabolism, and Transitory Starch Accumulation. *Plant Physiology* **161**, 628-43.

Penfield S, Pinfield-Wells H, Graham IA, 2018. Lipid metabolism in seed dormancy. *Annual Plant Reviews online*, 133-52.

Perotti VE, Moreno AS, Podestá FE, 2014. Physiological aspects of fruit ripening: the mitochondrial connection. *Mitochondrion* **17**, 1-6.

Pistelli L, Nieri B, Smith SM, Alpi A, De Bellis L, 1996. Glycoxylate cycle enzyme activities are induced in senescent pumpkin fruits. *Plant Science* **119**, 23-9.

Postma J, Erickson M, 2005. Food grade natural/organic method for treating food. In.: Google Patents.

Pracharoenwattana I, Smith SM, 2008. When is a peroxisome not a peroxisome? *Trends in plant science* **13**, 522-5.

Saavedra GM, Figueroa NE, Poblete LA, Cherian S, Figueroa CR, 2016. Effects of preharvest applications of methyl jasmonate and chitosan on postharvest decay, quality and chemical attributes of Fragaria chiloensis fruit. *Food chemistry* **190**, 448-53.

Saltveit ME, 2019. Chapter 4 - Respiratory Metabolism. In: Yahia EM, ed. *Postharvest Physiology and Biochemistry of Fruits and Vegetables.* Woodhead Publishing, 73-91.

Serra S, Goke A, Diako C, Vixie B, Ross C, Musacchi S, 2019. Consumer perception of d'Anjou pear classified by dry matter at harvest using near-infrared spectroscopy. *International Journal of Food Science & Technology*.

Seymour GB, Taylor JE, Tucker GA, 2012. *Biochemistry of fruit ripening*. Springer Science & Business Media.

Tatsuki M, Endo A, Ohkawa H, 2007. Influence of time from harvest to 1-MCP treatment on apple fruit quality and expression of genes for ethylene biosynthesis enzymes and ethylene receptors. *Postharvest Biology and Technology* **43**, 28-35.

Tieman D, Zhu G, Resende MF, *et al.*, 2017. A chemical genetic roadmap to improved tomato flavor. *Science* **355**, 391-4.

Umbach AL, Ng VS, Siedow JN, 2006. Regulation of plant alternative oxidase activity: A tale of two cysteines. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1757**, 135-42.

Villalobos-Acuna M, Mitcham EJ, 2008. Ripening of European pears: the chilling dilemma. *Postharvest Biology and Technology* **49**, 187-200.

Villalobos-Acuña MG, Biasi WV, Flores S, *et al.*, 2011. Effect of maturity and cold storage on ethylene biosynthesis and ripening in 'Bartlett' pears treated after harvest with 1-MCP. *Postharvest Biology and Technology* **59**, 1-9.

Watkins CB, 2006. The use of 1-methylcyclopropene (1-MCP) on fruits and vegetables. *Biotechnology Advances* **24**, 389-409.

Watkins CB, 2015. Advances in the use of 1-MCP. In. *Advances in postharvest fruit and vegetable technology*. CRC Press Boca Raton, FL, 117-45.

Xie X, Zhao J, Wang Y, 2016. Initiation of ripening capacity in 1-MCP treated green and red 'Anjou'pears and associated expression of genes related to ethylene biosynthesis and perception following cold storage and post-storage ethylene conditioning. *Postharvest Biology and Technology* **111**, 140-9.

Xu F, Yuan S, Zhang D-W, Lv X, Lin H-H, 2012. The role of alternative oxidase in tomato fruit ripening and its regulatory interaction with ethylene. *Journal of experimental botany* **63**, 5705-16.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL, 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**, 134.

Zommick DH, Knowles L, Knowles N (2014). Tuber respiratory profiles during low temperature sweetening (LTS) and reconditioning of LTS-resistant and susceptible potato (Solanum tuberosum L.) cultivars. *Postharvest biology and technology* **92**, 128-138.

Table 3.1 Summary of differentially expressed contigs discussed in the manuscript. Information includes associated pathway, full and abbreviated names, contig number (corresponding to sequences, annotations, and expression values in Supplementary File 3.4), length, and indication of significant trends.

Associated pathway	Gene/Contig Name	Abbreviation	Contig #	Contig Length (bp)	Significant Trend 3% Glyoxylic Acid (R>0.8)
Alternative respiration	Ubiquinol oxidase, mitochondrial-like	AOX1	22331	1156	
Aspartate, cysteine, methionine metabolism	Aspartate aminotransferase, cytoplasmic	cytAAT	18640	503	Linear, Quadratic
Aspartate, cysteine, methionine metabolism	Bifunctional aspartate aminotransferase	BiAAT	10407	850	
Aspartate, cysteine, methionine metabolism	SAM synthase 2	SAM synthase 2	2250	1734	
Aspartate, cysteine, methionine metabolism	Probable pyridoxal 5'-phosphate synthase subunit PDX2	PDX2	6815	1473	
Aspartate, cysteine, methionine metabolism	Cystathionine gamma-synthase 1, chloroplastic	CγS	9947	1598	
Ethylene biosynthesis	1-aminocyclopropane-1-carboxylate synthase	ACS	61428	1380	Linear
Ethylene biosynthesis	ACC oxidase	ACO	3326	595	
Ethylene perception	Copper-transporting ATPase RAN1	RAN1	20950	2399	Linear
Ethylene signaling/response	Ethylene-responsive transcription factor ERF073-like	ERF073-like	43790	243	
Ethylene signaling/response	ETHYLENE INSENSITIVE 3-like 1 protein	EIN3-like 1	3680	454	Linear, Quadratic
Ethylene signaling/response	Ethylene-responsive transcription factor 3-like	ERF 3-like	11186	1079	
Ethylene signaling/response	Ethylene-responsive transcription factor 4 isoform X1	ERF4 isoform x1	20105	297	
Ethylene signaling/response	Ethylene-responsive transcription factor 5-like	ERF5-like	54832	1214	Linear
Ethylene signaling/response	Ethylene-responsive transcription factor ABR1-like	ERF ABR1-like	7129	1416	
Ethylene signaling/response	Ethylene-responsive transcription factor ERF096-like	ERF096-like	35082	706	
Ethylene signaling/response	Serine/threonine-protein kinase CTR1 isoform X1	CTR1 isoform x1	20857	650	
Fatty Acid/Oxylipin metabolism	Lipoxygenase	LOX1	4033	2690	
Fatty Acid/Oxylipin metabolism	Allene oxide synthase	AOS	3632	1639	
Fatty Acid/Oxylipin metabolism	Allene oxide cyclase 4, chloroplastic	AOC4	2502	979	Quadratic
Fatty Acid/Oxylipin metabolism	Probable linoleate 9S-lipoxygenase 5	Linoleate 9S-LOX 5	15488	3275	Linear
Fatty Acid/Oxylipin metabolism	Linoleate 13S-lipoxygenase 3-1, chloroplastic-like	Linoleate 13S-LOX 3-1	10268	2980	
Glyoxylate cycle	Citrate synthase, glyoxysomal	gCS	3891	1784	
Glyxolysis/Gluconeogenesis	Phosphoenolpyruvate carboxylase kinase 1-like	PEPCK1-like	10747	754	
Glyxolysis/Gluconeogenesis	ATP-dependent 6-phosphofructokinase 3-like	PFK3-like	19400	904	
Glyxolysis/Gluconeogenesis	Fructose-1,6-bisphosphatase, cytosolic	F-1,6-Bpase	42519	255	Linear, Quadratic
TCA cycle	Fumarate hydratase 1, mitochondrial	mFH1	6653	845	Linear
TCA cycle	Isocitrate dehydrogenase [NADP]	IDH	7737	2131	
TCA/Glyoxylate cycle	Citrate synthase, mitochondrial	mCS	1515	418	
TCA/Glyoxylate cycle	Malate dehydrogenase	MDH	9623	2234	Linear

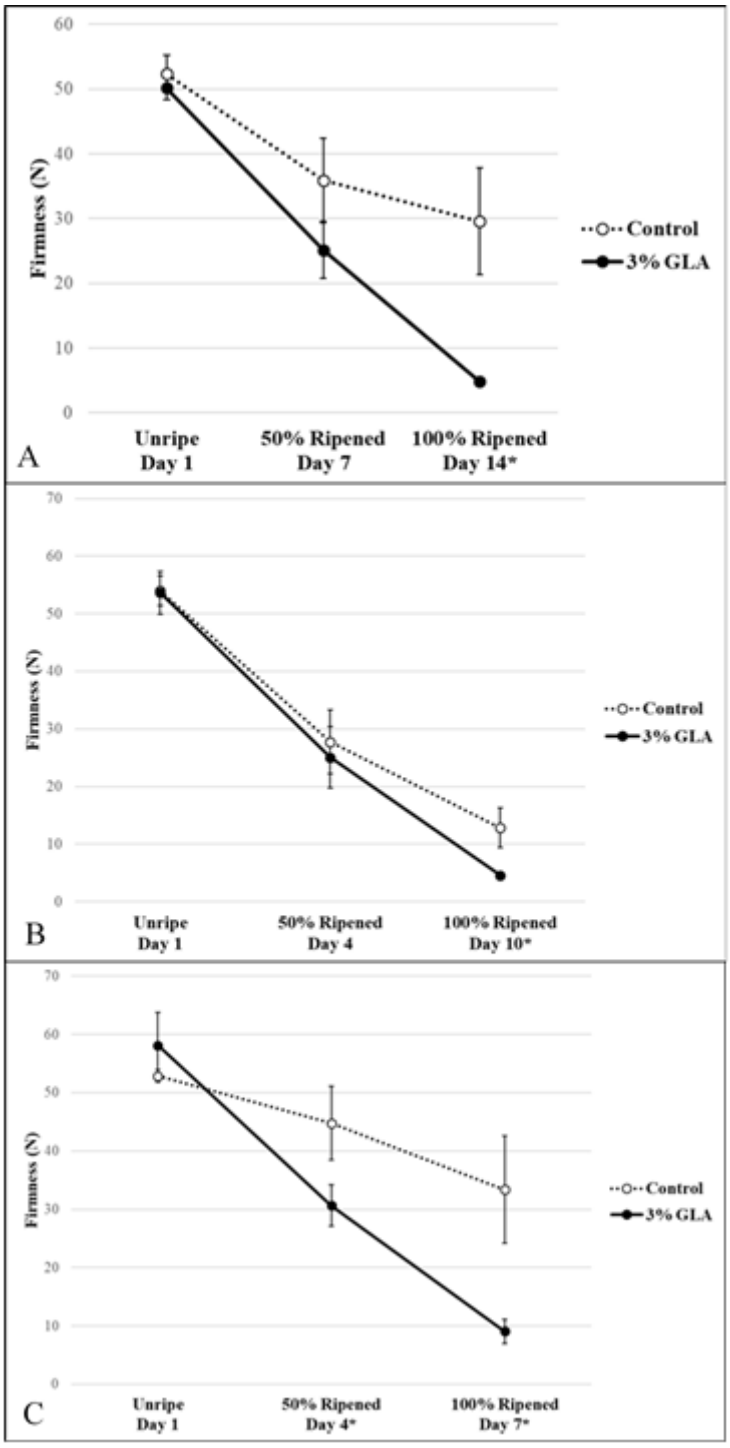

Figure 3.1 Firmness of 1-MCP treated 'D'Anjou pears following treatment with 3% GLA in January 2018 (A), February 2018 (B), and June 2018 (C). Asterisk indicates statistically significant difference at the respective time point ($p<0.05$).

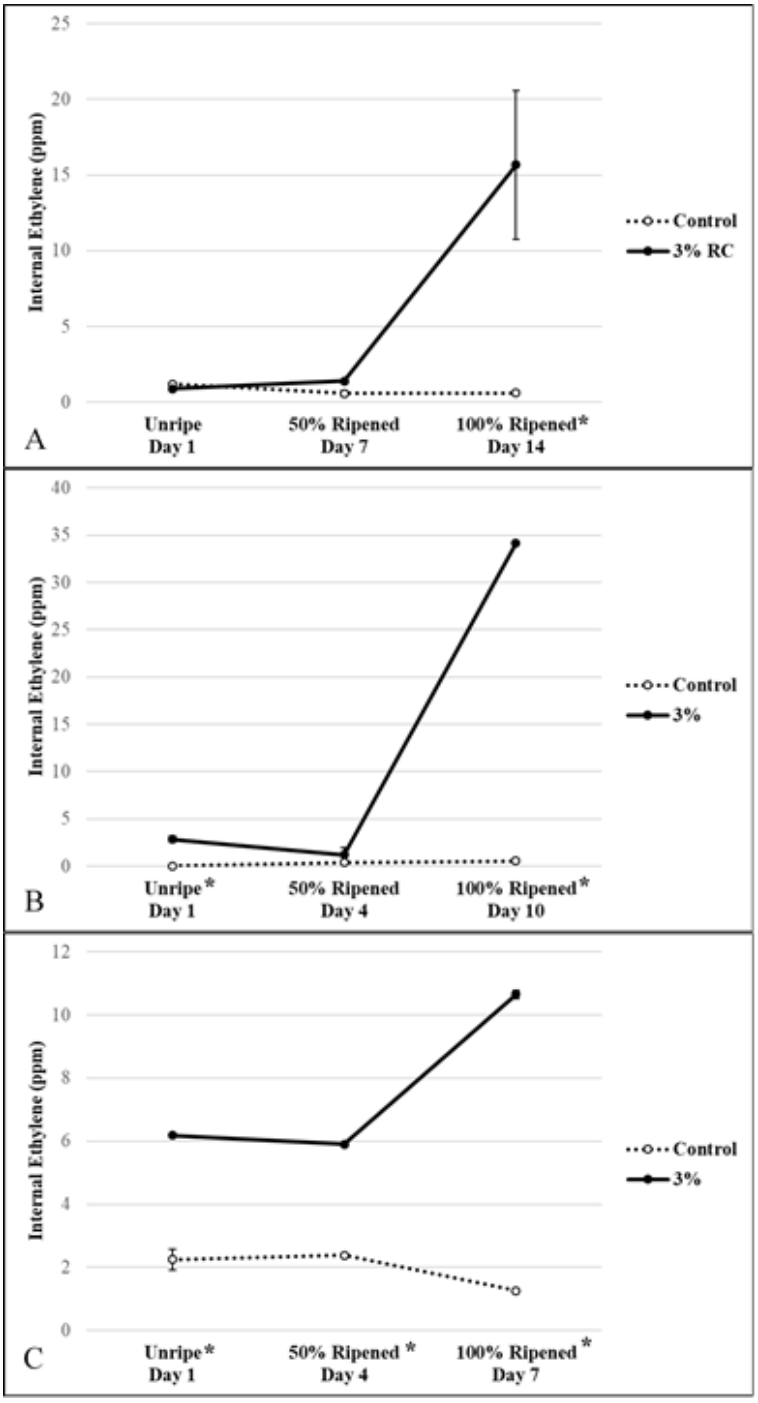

Figure 3.2 Internal ethylene evolution of 1-MCP treated 'D'Anjou' pears following treatment with 3% GLA in January 2018 (A), February 2018 (B), and June 2018 (C). Asterisks indicate statistically significant differences at the respective time points ($p<0.05$).

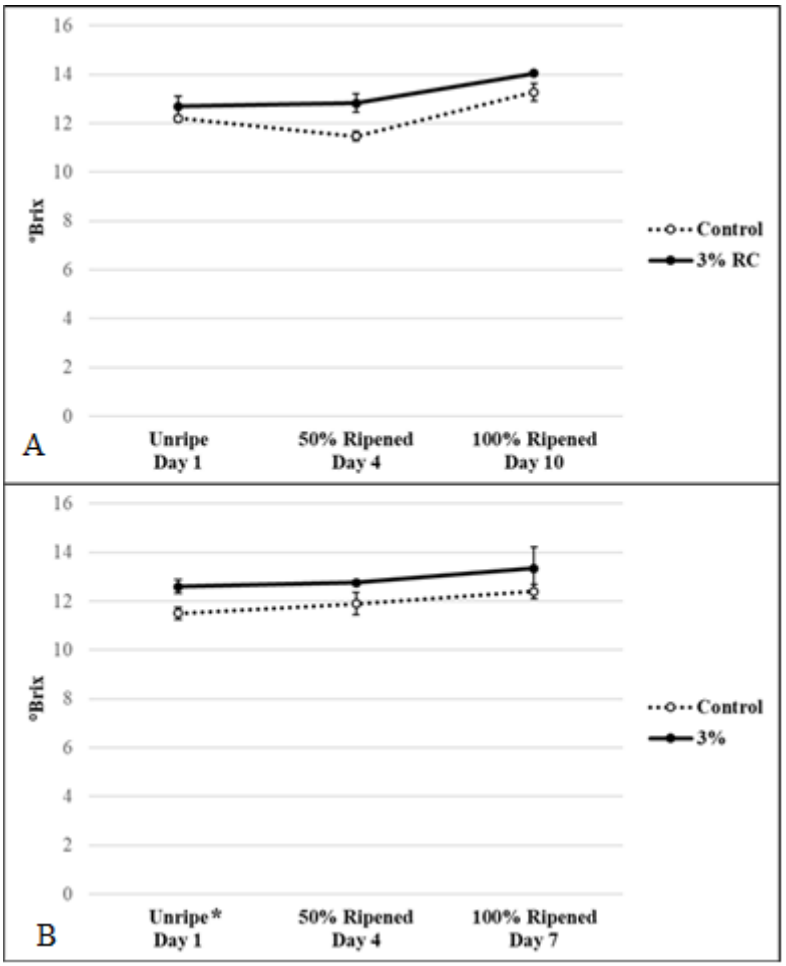

Figure 3.3 Soluble solid content of 1-MCP treated 'D'Anjou' pears following treatment with 3% GLA in February 2018 (A) and June 2018 (B). Asterisk indicates statistically significant difference at the respective time point ($p<0.05$).

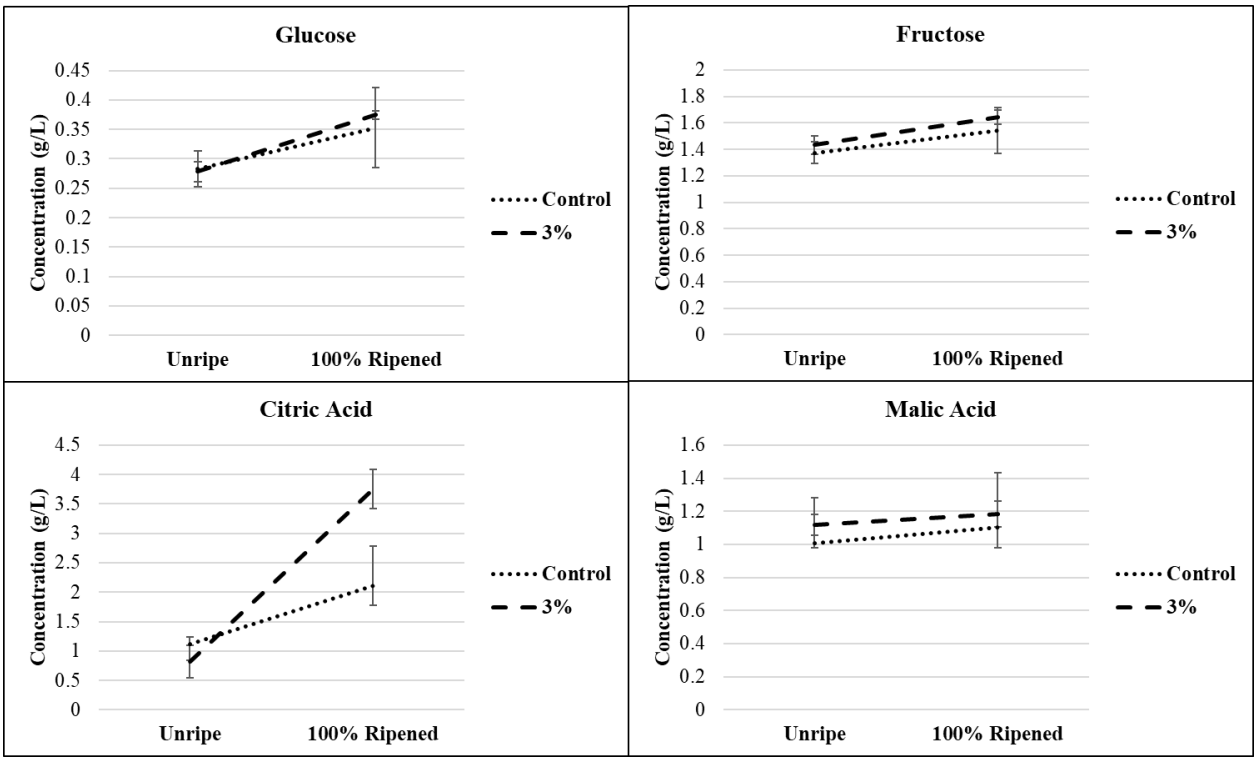

Figure 3.4 Mean HPLC profiles for glucose, fructose, citric acid, and malic acid for 2018 1-MCP 'D'Anjou' pears treated with 3% GLA solution ($p<0.05$).

Figure 3.5 Images taken on initial final days of a two-week GLA time course experiment. Left to right: untreated ‘D’Anjou’ fruit, day 1; untreated ‘D’Anjou’ fruit, day 14; 3% GLA-treated ‘D’Anjou’ fruit, day 14. Additional images can be seen in Supplementary File 3.1.

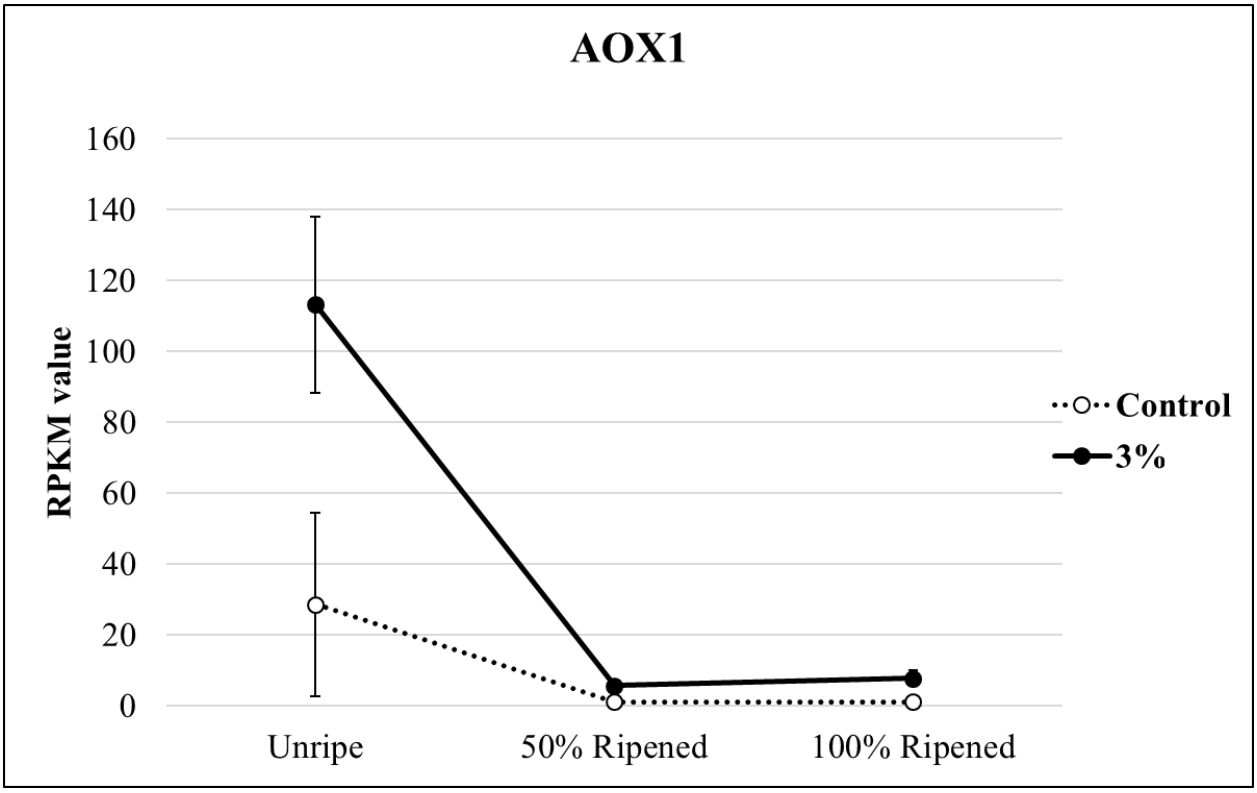

Figure 3.6 Expression of AOX1 homologue. AOX was significantly differentially expressed over time in the 3% GLA-treated fruit than in the control fruit ($p<0.05$).

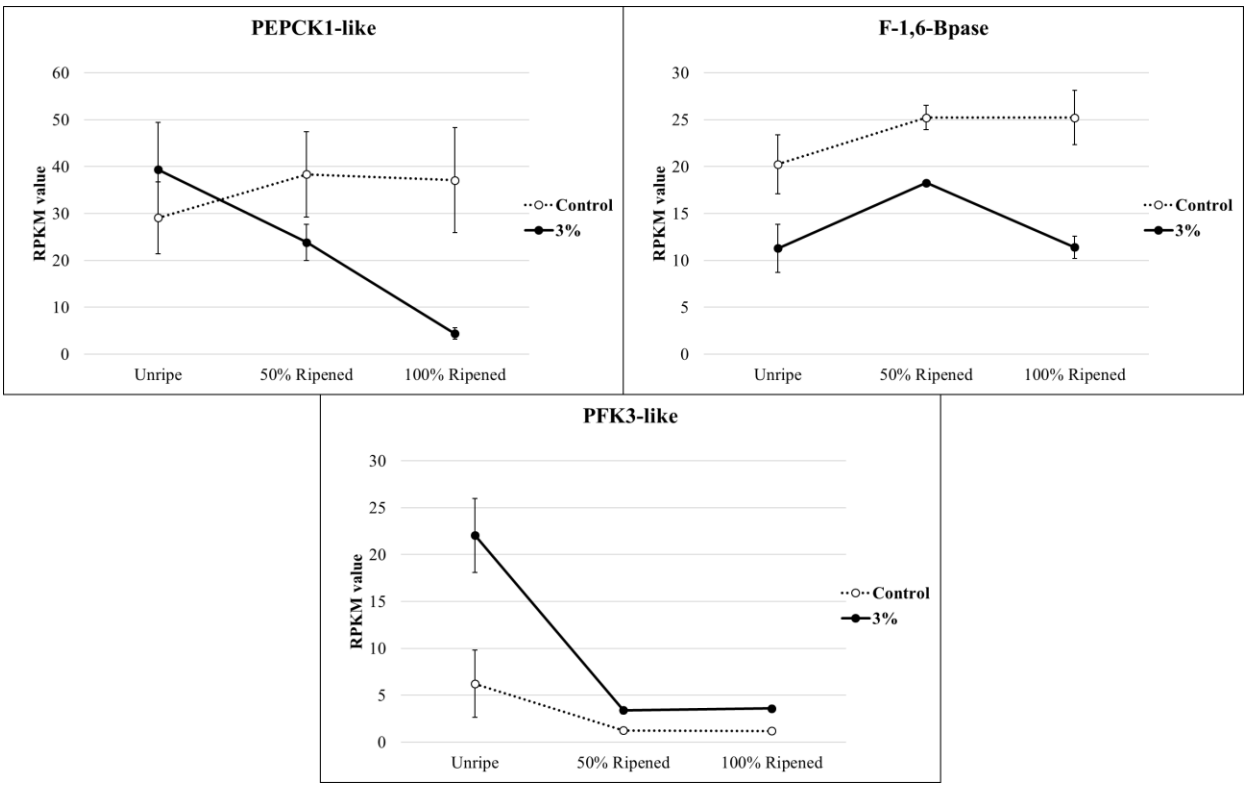

Figure 3.7 Expression of genes encoding rate limiting gluconeogenic (top) and glycolytic (bottom) enzymes. All genes pictured were significantly differentially expressed in the 3% GLA-treated fruit versus the control fruit over time ($p<0.05$).

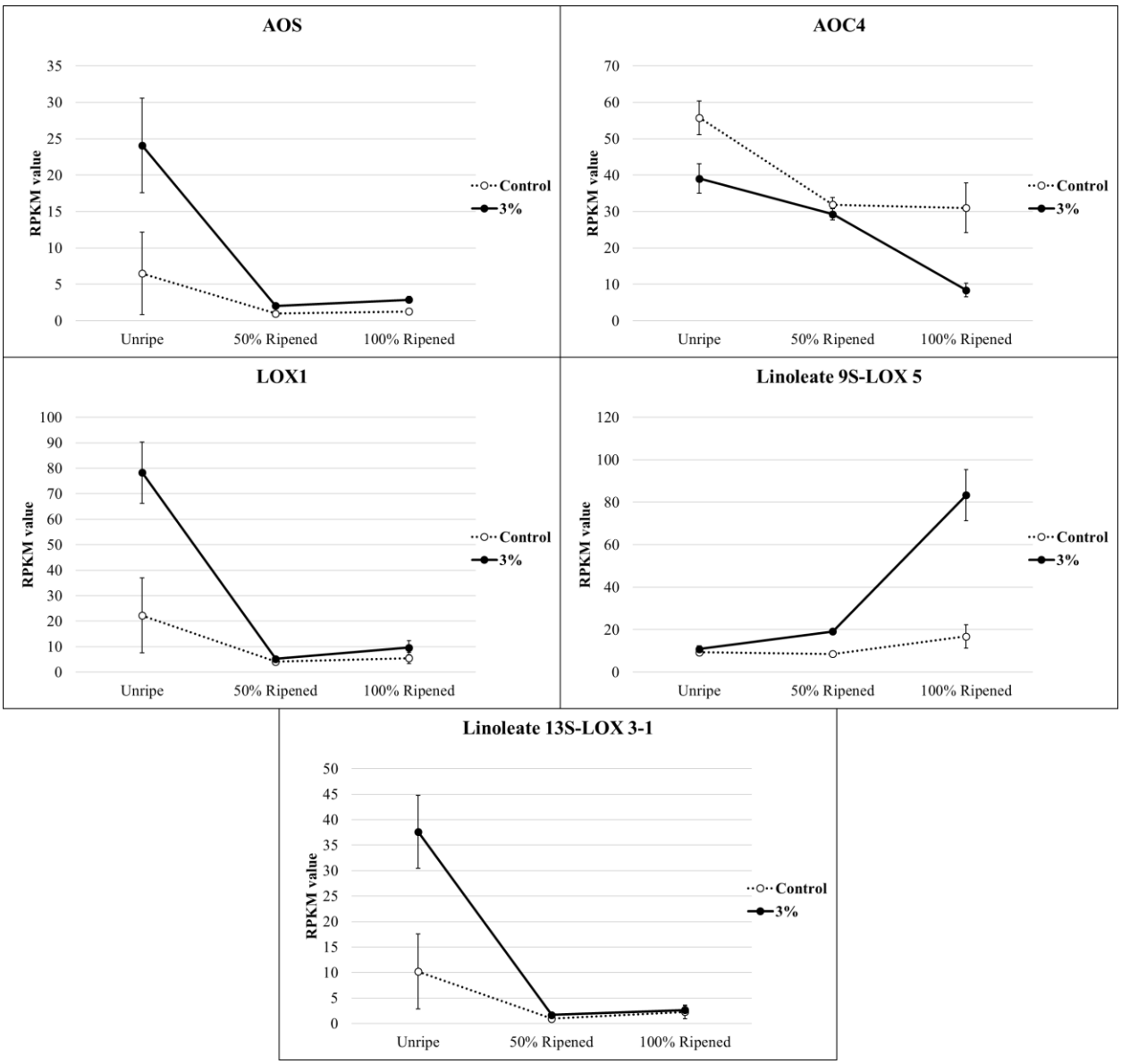

Figure 3.8 Genes encoding fatty acid and oxylipin metabolic enzymes that were significantly differentially expressed over time in the 3% GLA-treated fruit versus the control fruit ($p<0.05$).

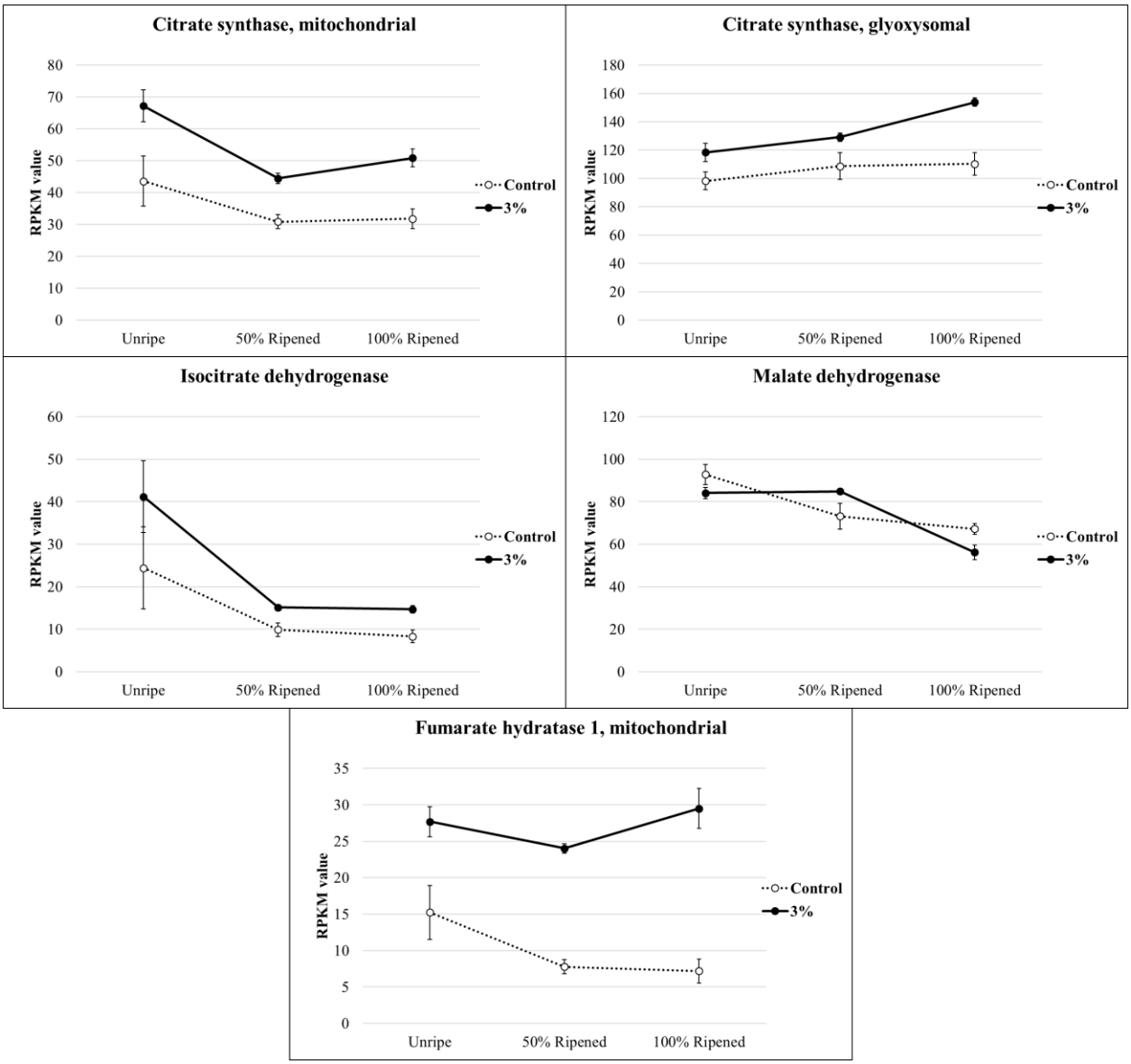

Figure 3.9 Genes encoding TCA/glyoxylate cycle enzymes that were significantly differentially expressed over time in the 3% GLA-treated fruit versus the control fruit ($p<0.05$).

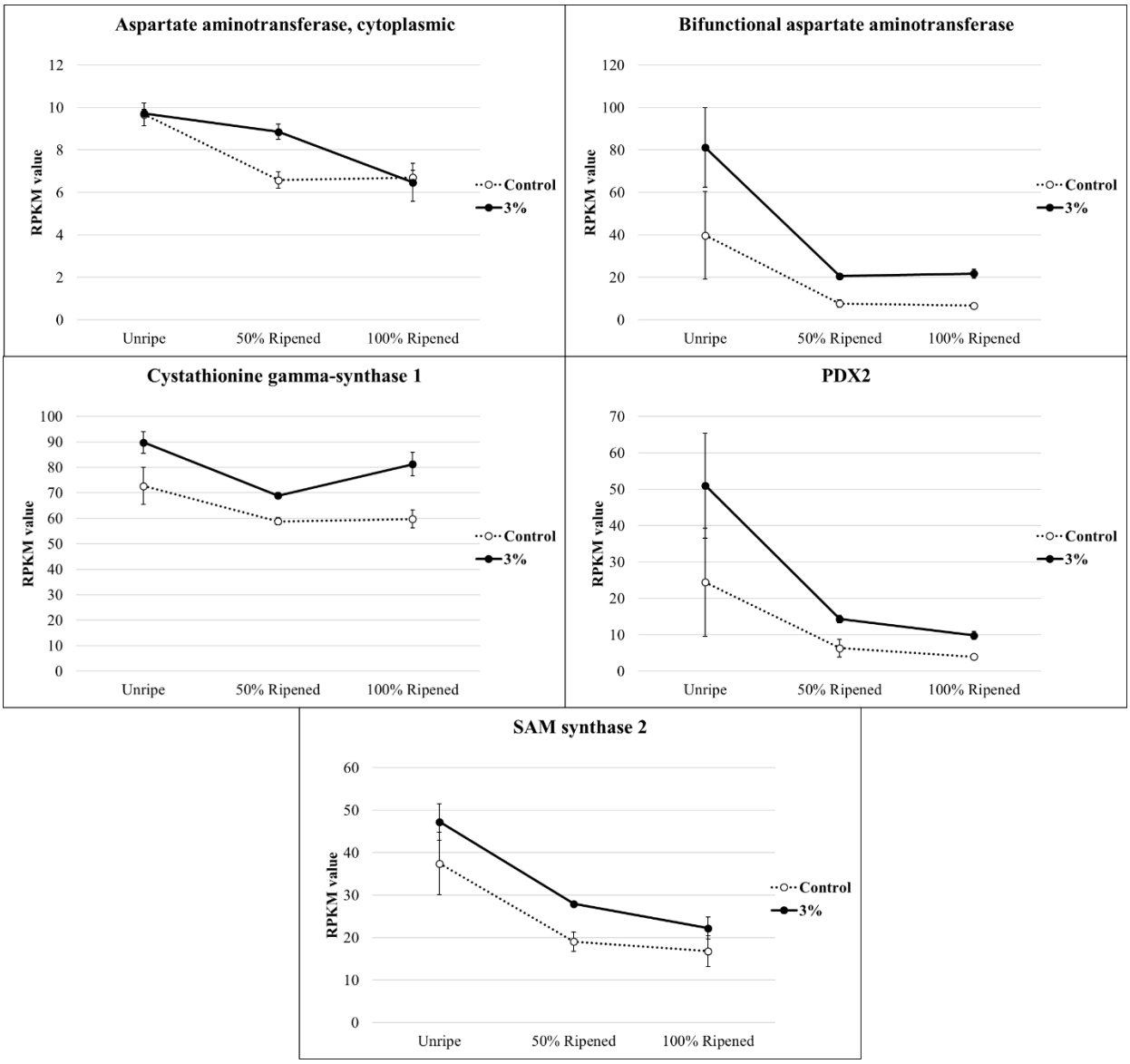

Figure 3.10 Genes differentially expressed over time in 3% GLA-treated fruit versus control fruit that are involved in metabolism of amino acids leading into precursors of ethylene biosynthesis ($p<0.05$).

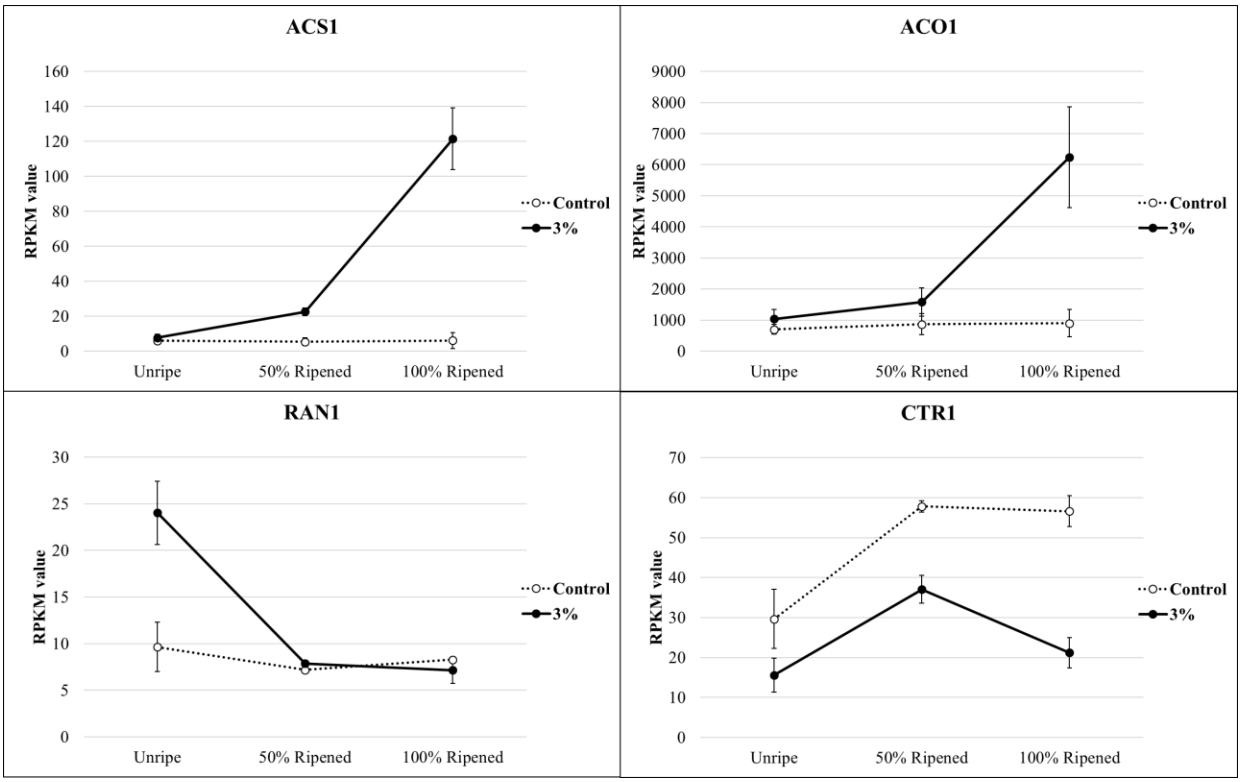

Figure 3.11 Expression of genes involved in ethylene biosynthesis and perception that were significantly differentially expressed over time in the treatment group versus the control ($p<0.05$).

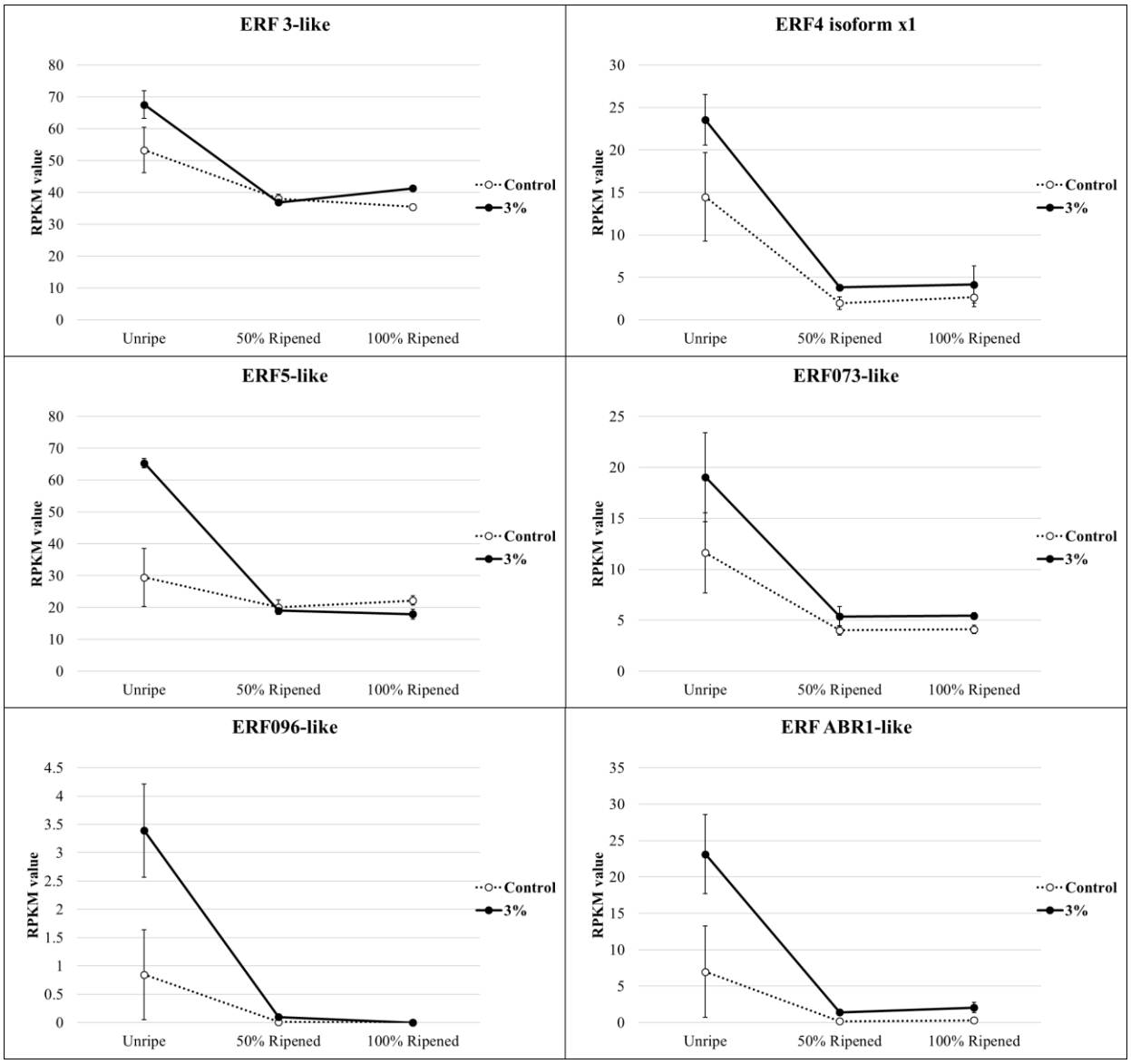

Figure 3.12 Expression of significantly differentially expressed ethylene responsive transcription factors in ‘D’Anjou’ pear fruit following 3% GLA treatment ($p<0.05$).

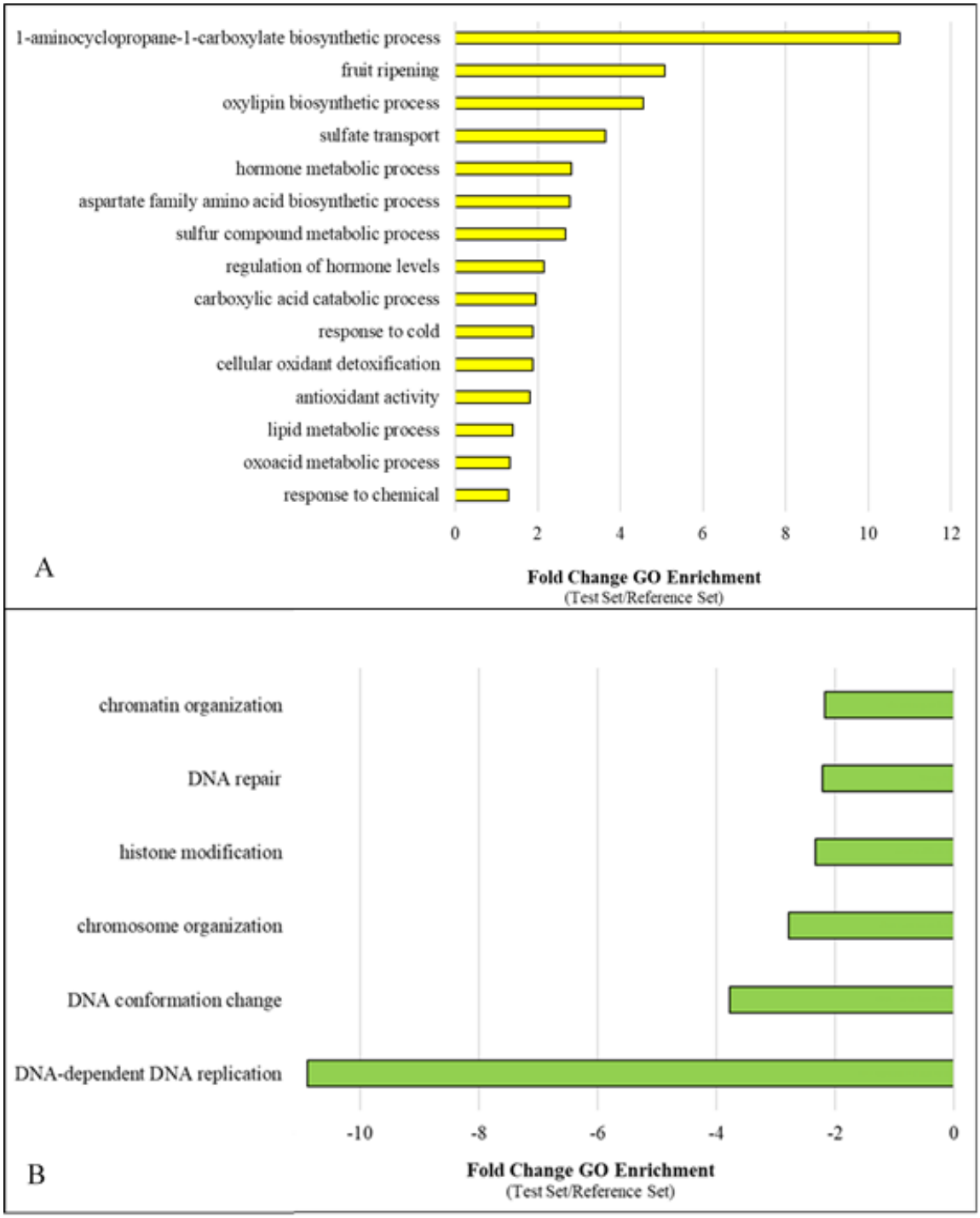

Figure 3.13 Overrepresented (A) and underrepresented (B) GO terms based on the results of Fisher's Exact Test with differentially expressed contigs in 3% GLA treatment set in comparison with annotated transcriptome (FDR<0.05).

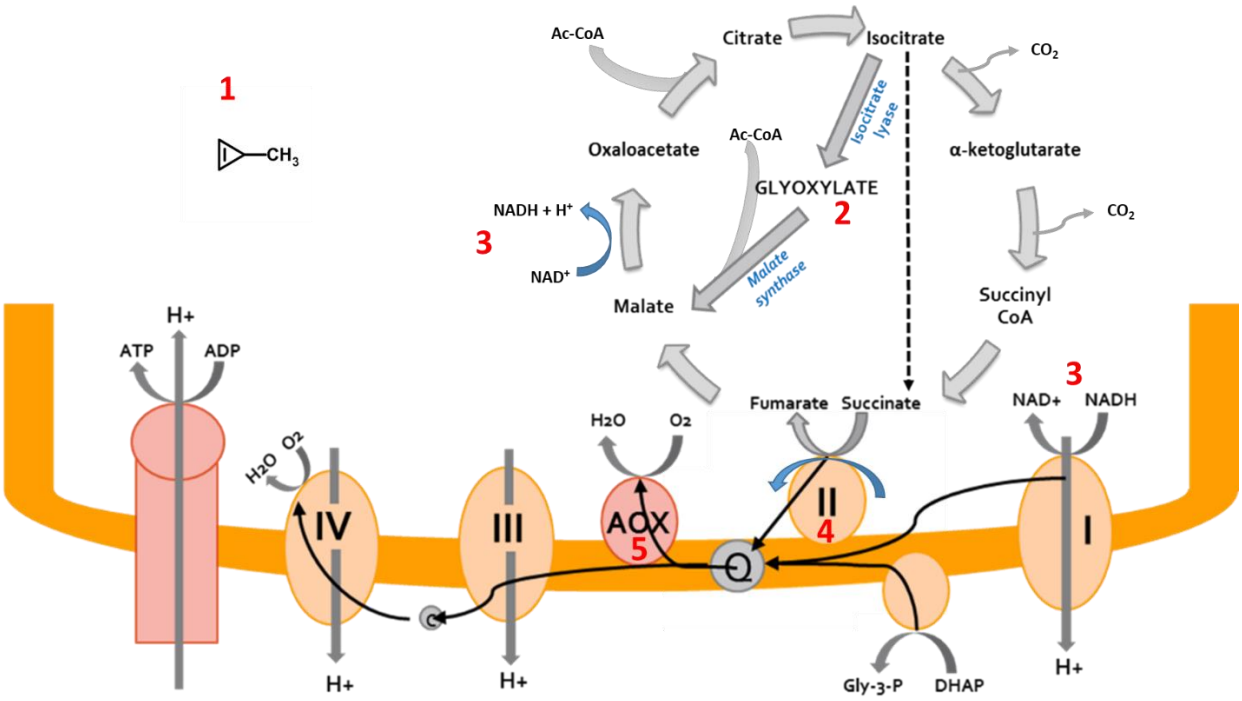

Figure 3.14 Proposed model for GLA activation of AOX activity. 1.) 1-MCP causes a respiratory block of the TCA cycle or an upstream pathway through an uncharacterized mechanism 2.) Addition of GLA increases pools of metabolic intermediates in the glyoxylate cycle, resulting in increased flux through the cycle. Production of malate and its subsequent conversion to OAA, perpetuates the glyoxylate cycle, producing intermediates shared by the TCA cycle 3.) The subsequent redox steps in the TCA cycle result in the transfer of high-energy electrons to NADH, which is transported to the cytochrome c pathway. This, in turn, creates the energy gradient that facilitates the generation of ATP 4.) Conversion of succinate to fumarate results in transfer of additional high energy electrons to complex II of the cytochrome respiratory pathway 5.) AOX is activated to shunt the excess electrons provided by glyoxylate cycle activity. This allows for the maintenance of the necessary reduced state of ubiquinone and may account for the re-initiation of ripening observed in GLA-treated pear fruit.

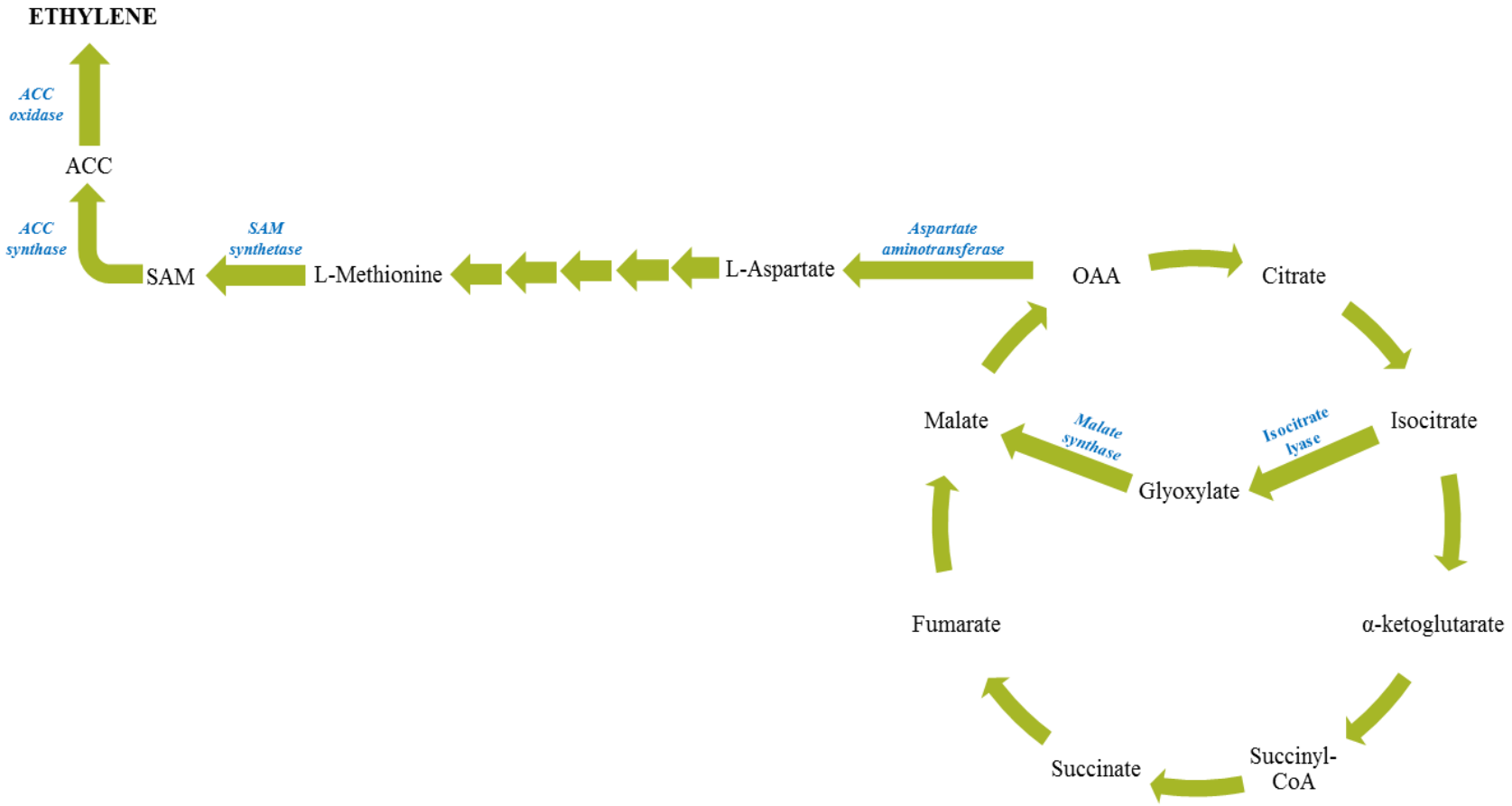

Figure 3.15 Working model for interconnectedness of the glyoxylate cycle and ethylene biosynthesis. OAA produced in TCA/Glyoxylate cycles can be converted to aspartate and then to methionine. Methionine can then be converted into ethylene.

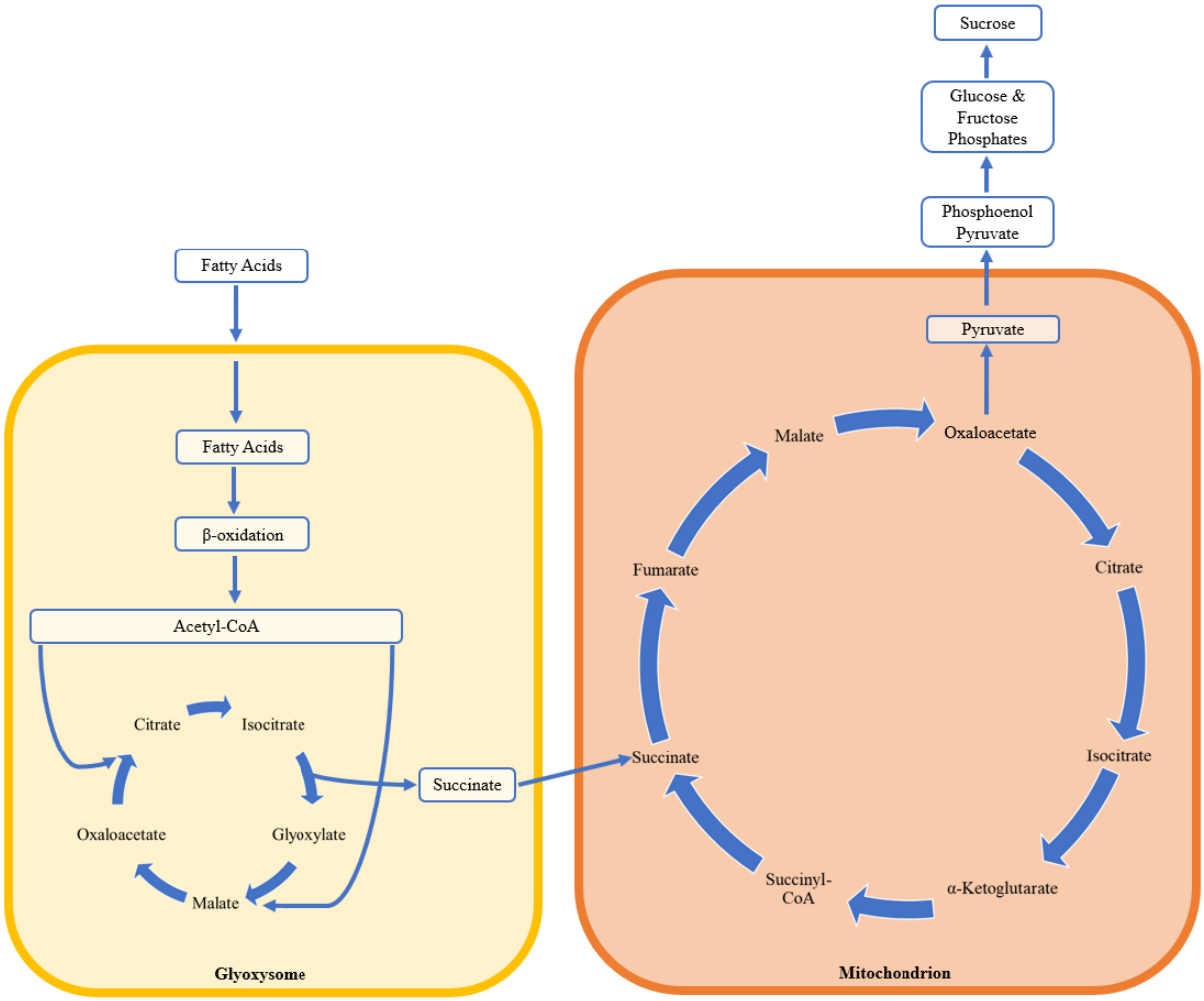

Figure 3.16 Glyoxylate cycle links fatty acid degradation, sugar accumulation, and TCA cycle intermediates.

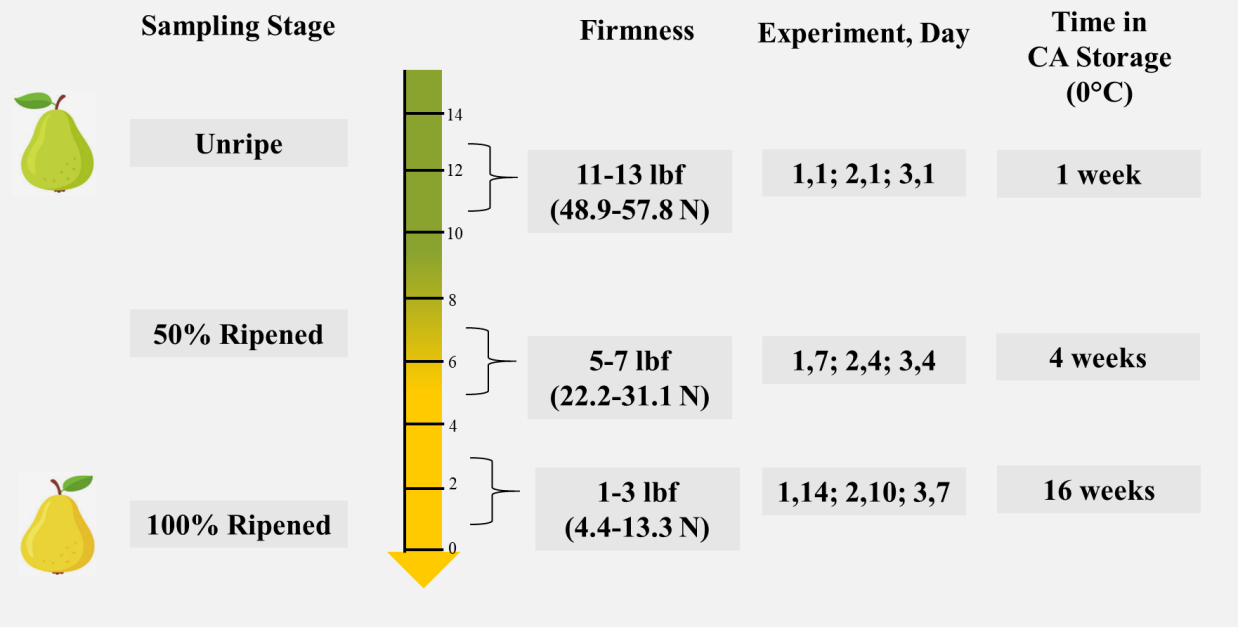

Figure 3.17 Sampling time course and experimental replicate specific information for 'D'Anjou' pear fruit used in each GLA experiment.

Figure 3.18 Left: Ultrasonic humidification chamber set up for ripening compound and control solution administration. Humidification is conducted over a period of 16 hours. Top right: Vacuum aspiration chamber used for internal ethylene gas extraction. Bottom right: Respiration monitoring system. Individual jars represent a single treatment and one of four technical replicates.

SUPPLEMENTARY FILES

Supplementary File 3.1 Additional GLA experiments and pH experiments from 2017-18 pear seasons.

Supplementary File 3.2 Master transcriptome assembly fasta file for GLA and control 'D'Anjou' pear fruit.

Supplementary File 3.3 Blasted, annotated, and mapped contigs from master assembly.

Supplementary File 3.4 Mean RPKM values, standard error, and time course differential expression information for GLA-treated and control 'D'Anjou' pear fruit.

Supplementary File 3.5 Enriched gene ontologies for GLA-treated pear fruit versus control pear fruit during experimental time course.

Supplementary File 3.6 Quantitative RT-PCR validation with LinRegPCR output and calculated expression values.

DISSERTATION SUMMARY AND FUTURE DIRECTIONS

This work approaches climacteric ripening as a highly multifaceted process, calling into question the classic, ethylene-centric model. There is no question that ethylene is important, but it is one piece of a larger, elegant network that is still being elucidated; it is important to look at the other factors in the network of metabolic pathways that affect the transition to S2 ethylene synthesis and ripening. The studies presented in this dissertation provide transcriptomic evidence that the alternative respiration plays a critical metabolic role in both cold- and chemical-induced ripening and may be involved in crosstalk with the ethylene biosynthetic pathway, as well as numerous other pathways. Furthermore, vernalization-associated genes are likely involved in the activation of the ripening climacteric in cold-conditioned pear fruit, and glyoxylic acid (GLA) can elicit ripening responses in 1-MCP treated fruit. By synthesizing ongoing work investigating the mechanisms governing climacteric ripening and related processes, and by using ripening-recalcitrant European pear as a system in which to study novel methods of climacteric induction, this work reveals new genetic targets for ripening manipulation by which fruit shelf life and quality may be improved, and waste can be mitigated.

Future work should include validating the gene expression results obtained in these studies with additional molecular techniques, since a primary criticism of transcriptomic analyses is that gene expression information doesn't always correlate directly with protein expression, enzyme activity, and resultant metabolite abundance. This work has laid the foundations for new experiments, already in the research pipeline, which will employ immunoblotting to measure expression of AOX and other proteins implicated in the climacteric transition in pear fruit. Furthermore, dynamic isotopic labeling experiments and/or radiolabeling of GLA will provide

information regarding precise impact of this compound on metabolic flux through the glyoxylate cycle and associated pathways, providing further support for the proposed model for GLA-mediated ripening. As the importance of epigenetic influences on gene expression has become better understood, and tools for analyzing signatures of the epigenome become more accessible, it will be pertinent to investigate the role of DNA methylation and histone modifications in cold-induced ripening. Such studies have been performed in other, non-fruit systems that require cold to initiate developmental processes (Banerjee et al., 2019; Thiebaut et al., 2019). Additional analyses need be done to complement the differential expression work and enrichment analyses conducted in the two transcriptome studies. For example, nonparametric multi-dimensional scaling (NMDS) at the whole transcriptome level, as demonstrated previously in our lab (Hendrickson et al., 2019), can facilitate connections between genotype, ripening phenotypes, and other factors in complex multifactorial experiments.

On the applied side, work will need to be done to improve the consistency of 1-MCP application when working with ripening inhibited fruit. Packinghouses typically treat with 130 ppb MCP, but there has been some question with regards to how uniform this application is. Recently, an avenue has been opened for collaboration with a company that could assist in application of 1-MCP to fruit for experimental purposes to minimize variation due to unequal application. Furthermore, regarding the utilization of GLA as a ripening compound, 3% solutions were effective in ripening fruit; however, at such high application concentrations, acidity of the treatment solution results in aesthetic damage to the fruit peel tissue. Future work to identify a pH at which GLA is biologically active, but at which damage to peel tissue is minimized, will be key in translating this technology to the market. Alternatively, precise application of 1-MCP at lower rates might help meter lower doses of GLA. Determination of phosphate or other buffers to be

used during treatment or use of lower concentrations of GLA in the presence of a surfactant could reduce damage.

The impacts of the present and future research extend far beyond the lab. Collaborations with tree fruit industry partners in the Pacific Northwest have opened doors for large-scale testing of new ripening technologies, including the use of GLA to stimulate ripening in 1-MCP treated whole and sliced fruit. Results of a recent consumer taste panel and willingness-to-pay study conducted in collaboration with the WSU School of Economic Sciences, Crunch Pak sliced fruit company (Cashmere, WA) and the Food Innovation Center at Oregon State University (Portland, OR) indicated that consumers are willing to pay a premium for high-quality, fresh, sliced pear fruit treated with ripening compound (GLA) in comparison with untreated fresh sliced pears (Ikiz et al., 2018). This is just one example of many direct applications of this research. The ultimate goal is to translate this ripening toolset to other fruit varieties in the future, as it provides the opportunity to inhibit ripening at harvest, store the fruit for however long is necessary, and reactivate ripening in a planned and predictable manner, thereby reducing the waste associated with unpredictable ripening.

REFERENCES

Banerjee R, Kumar GV, Kumar SJ, 2019. *Omics-based Approaches in Plant Biotechnology*. John Wiley & Sons.

Ikiz D, Gallardo RK, Dhingra A, Hewitt S, 2018. Assessing consumers' preferences and willingness to pay for novel sliced packed fresh pears: A latent class approach. *Agribusiness* **34**, 321-37.

Thiebaut F, Hemerly AS, Ferreira PCG, 2019. A role for epigenetic regulation in the adaptation and stress responses of non-model plants. *Frontiers in Plant Science* **10**.