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A model to predict ripening capacity of 'Bartlett' pears (*Pyrus communis* L.) based on relative expression of genes associated with the ethylene pathway

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ABSTRACT

Resistance to ripening in European pears depends on various preharvest and postharvest factors. Exposure to cold temperatures has been demonstrated to condition pears to produce endogenous ethylene and ripen. Mature green 'Bartlett' pears were exposed to 0, 5, or 10 °C for 2–14 d to induce different rates of ripening. At higher temperatures, expression of ethylene biosynthesis genes (*ACS1a* and *ACO*) and ethylene receptor genes (*ETR2*, *ERS1a*, and *ETR1a*) increased, while *CTR1* expression decreased. Multiple linear regressions between relative expression of these genes after 0 d and fruit firmness after 6 d during ripening at 20 °C were conducted. Using K-fold cross validation and conventional validation, it appears that expression of *ACO* could be utilized as an early predictor of pear ripening capacity.

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1. Introduction

European pears develop a buttery and juicy texture when ripened off the tree (Murayama et al., 1998). However, most European pear fruit (*Pyrus communis* L.) have some resistance to ripening that can induce non-uniform ripening after harvest (Villalobos-Acuna and Mitcham, 2008). Although 'Bartlett' pears are less resistant to ripening compared to winter pears such as 'Comice' and 'd'Anjou' (Villalobos-Acuna and Mitcham, 2008), early season 'Bartlett' pears usually fail to ripen uniformly (Hansen, 1939) and sometimes stay > 80 N after 9 d (Agar et al., 2000b). Ethylene treatment (e.g., 100 μL L^{-1} at 20 °C for 24 h) can be used to induce and speed up the ripening process (Agar et al., 2000a; Agar et al., 2000b; Hansen, 1939; Wang et al., 1971). This treatment can be replaced by low temperature exposure (i.e., −1 to 10 °C) (Villalobos-Acuna and Mitcham, 2008). In this low temperature range, intermediate temperatures (5–10 °C) have been reported to induce faster ripening more quickly than lower temperatures (−1 to 0 °C) (Agar et al., 2000b; Sugar and Basile, 2009).

Cold treatments hasten fruit ripening in European pears by triggering the ethylene pathway (Blankenship and Richardson, 1985; Villalobos-Acuna and Mitcham, 2008). Enzyme activities of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO), as well as ethylene production of 'Passe-Crassane' pears increased after three months at 0 °C (Lelievre et al., 1997). Among multiple *ACS* genes in 'Passe-Crassane' pears, only *Pc-ACS1a* showed an increase in expression after 80 d at 0 °C (El-Sharkawy et al., 2004); the ethylene receptor *Pc-ETR1a* transcript was also up-regulated at 0 °C (El-Sharkawy et al., 2003). In 'Bartlett' pears, ACS and ACO enzyme activities increased during ripening at 20 °C after 5 or 10 °C conditioning treatments; however significant increases in these enzyme activities were only observed following a few days of ripening at 20 °C after the fruit had been transferred from the cold treatments (Agar et al., 2000b).

The expression of genes associated with ethylene biosynthesis and signal transduction has not been assessed following cold exposure in 'Bartlett' pears. This study was conducted to examine the expression of genes associated with ethylene biosynthesis (*ACS1a*, *ACO*) and signal transduction (*ETR1a*, *ETR2*, *ERS1a*, and *CTR1*), comparing the effect of 0 °C and intermediate temperature (5 and 10 °C) treatments. Additionally, given that current maturity indices such as fruit firmness and skin color are unreliable indicators of pear fruit ripening rate (Agar et al., 2000a), we also investigated if the expression of ethylene-associated genes can be used as an early predictor of fruit ripening capacity. In this study, we define full ripening capacity as the ability of pear fruit to soften to ≤ 20 N after 6 d at 20 °C.

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2. Materials and methods

2.1. Plant materials

Mature green early season 'Bartlett' pear fruit, average 82 N firmness and 65 mm average diameter were harvested in Sacramento County, California at the initiation of commercial harvest (July 19, 2010). Pear fruit were sorted to remove sunburned, damaged or defective fruit, and randomized before assignment to temperature treatments. Fruit were cold-treated at 0, 5, or 10 °C for 2, 5, 8, 11, or 14 d (14 d treatment was used only at 0 °C) (Table 1). Similar temperature conditioning experiments with 0 and 10 °C were repeated in 2012, 2013, and 2014 (Table 1). In 2012 and 2013, separate sets of fruit were also treated with 100 μL L^{-1} ethylene at 20 °C for 12, 18, or 24 h (Table 1). The control received no cold or ethylene exposure. After treatment, fruit were then transferred to 20 °C and 90% relative humidity for ripening. Each measurement described below was obtained from three biological replicates with six fruit per replicate.

2.2. Ripening evaluation

The rate of fruit softening at 20 °C was monitored by measuring firmness on opposite sides of each fruit after removing a section of skin after 0, 3, 6, and 9 d, using a Gűss FTA Penetrometer fitted with an 8 mm probe (Gűss, Strand, Western Cape, South Africa). Ethylene production rate was determined on headspace gases produced by six fruit per replicate after 0, 2, 4, 6, 8, and 10 d, using flame ionization gas chromatography (Model Carle AGC-211, EG&G Chandler Engineering, Tulsa, OK) as described by Villalobos-Acuna et al. (2010).

2.3. Gene expression analysis

Gene expression analysis was conducted using a method from Nham et al. (2015). Peel tissues were collected from fruit at harvest (control) and from fruit that had been warmed to 20 °C for 3 h following the completion of each cold treatment or each ethylene treatment (0 d). Total RNA was isolated using the Qiagen RNeasy Plant Mini Kit (Qiagen, Netherlands) and treated with DNase I (Roche, Switzerland). RNA concentration and integrity were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, MA). cDNA was synthesized using 1 μg total RNA, using Superscript™ III First Strand Synthesis Systems for RT-PCR Systems (Invitrogen, CA). Relative gene expression was examined using SYBR Green PCR Master Mix and 7300 Real Time PCR System (Applied Biosystems, CA). *Ef1alpha* was chosen as the optimal internal control gene after testing with *18s*, *26s*, *beta-actin*, and *tubulin1*. Primers for tested genes (*ACS1a*, *ACO*, *ETR1a*, *ETR2*, *ERS1a*, and *CTR1*) were designed using Primer 3 (Untergasser et al., 2012) (Table 2). Relative expression (RE) was presented as $2^{-\Delta Ct}$.

Table 1
Summary of temperature and ethylene conditioning treatments applied over four years of study to enhance pear fruit's ability to ripen.

	Treatment			
	Low temperature			Ethylene
Year	0 °C	5 °C	10 °C	
2010	2, 5, 8, 11, or 14 d	2, 5, 8, or 11 d	2, 5, 8, or 11 d	NA
2012	5, 8, 11, 14, or 17 d	2, 5, 8, or 11 d	2, 5, 8, or 11 d	12, 18, or 24 h
2013	8, 11, or 14 d	NA	3, 5, or 7 d	12, 18, or 24 h
2014	3, 6, 9, 12, or 15 d	NA	3, 6, or 9 d	NA

NA: not applicable.

2.4. Experimental design, model building and validation

The experiment was a completely randomized factorial design. Linear regression analysis or multiple linear regression analysis was used to predict the firmness of individual replicates after 6 d during ripening from the average RE of one or multiple genes across three replicate trials. Two methods of model building and validation were conducted. In the first method, fruit firmness and gene expression of six genes from 2010 and 2014 data (57 data points) were combined for the model training and validation. K-fold cross-validation was carried out with k = 3 (38 randomized data points were used as the training set, 19 remaining data points formed the test set) and k = 19; each run was repeated 100 times. In the second method, 2010 data (36 data points) were used as the training set and 2014 data (19 data points) were utilized to validate. For the gene *ACO*, 69 data points from 2012 and 2013 were also used to validate. These regression and validation analyses were performed on R 2.15.0 (The R Core Development Team, 2013; RStudio i386-pc-mingw32/i386 platform). The Bootstrap package (https://cran.r-project.org/web/packages/bootstrap/index.html) was used for the K-fold cross-validation.

3. Results and discussion

3.1. Fruit ripening capacity development by low temperature conditioning

Ripening capacity developed faster when the conditioning temperature increased from 0 °C to 10 °C (Fig. 1). Without low temperature conditioning, the control fruit, which were transferred immediately to 20 °C after harvest, softened to 15.8 N after 12 d (Fig. 1A). Fruit slowly developed ripening capacity following exposure to 0 °C; after 14 d at 0 °C, fruit softened to 17.4 N after 6 d of ripening at 20 °C (Fig. 1E). Conditioning the fruit for 5 d at 5 or 10 °C promoted full ripening with fruit firmness of 14.2 and 10.2 N, respectively, after 6 d at 20 °C (Fig. 1B). In addition, higher and earlier peaks in ethylene production rate were observed in the fruit that softened more quickly (Fig. 1F–J). These results confirmed the effect of low temperatures on the development of fruit ripening capacity and ethylene production, which have been shown previously in 'Bartlett' (Agar et al., 2000b; Villalobos-Acuna and Mitcham, 2008) and in 'd'Anjou' (Sugar and Einhorn, 2011) pears.

3.2. Expression of genes encoding ethylene biosynthesis and signal transduction

To understand the effect of different cold temperature treatments on ethylene pathways, expression of *Pc-ACS1a*, *Pc-ACO*, *Pc-ETR1a*, *Pc-ERS1*, *Pc-ETR2*, and *Pc-CTR1* was examined on samples collected at harvest and after low temperature treatments (Fig. 2A). We also chose two treatments, 0 °C for 14 d and 10 °C for 5 d, which had induced full ripening capacity, to evaluate expression of these ethylene genes during ripening (Fig. 2B). Exposure to 10 °C for ≥5 d resulted in increased expression of ethylene biosynthesis genes (*Pc-ACS1a* and *Pc-ACO*) and there was a slight up-regulation of these genes following exposure to 5 °C for ≥5 d. These results are in agreement with previous research in 'Bartlett' pears where enzyme activities of ACS and ACO were higher following exposure to 10 °C than to 5 °C (Agar et al., 2000b). Transcript abundance of *Pc-ACS1a* remained low and unchanged following 0 °C exposure (Fig. 2A) and started to increase after 6 d of ripening at 20 °C following exposure to 0 °C for 14 d (Fig. 2B). Meanwhile, an increase in expression of *Pc-ACO* was recorded as early as after 11 d following 0 °C exposure (Fig. 2A).

In addition to ethylene biosynthesis genes, expression of ethylene receptors (*Pc-ETR1a*, *Pc-ETR2*, and *Pc-ERS1a*) were

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