'Bartlett' pear fruit (*Pyrus communis* L.) ripening regulation by low temperatures involves genes associated with jasmonic acid, cold response, and transcription factors

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Abstract

Low temperature (LT) treatments enhance ethylene production and ripening rate in the European pear (*Pyrus communis* L.). However, the underlying molecular mechanisms are not well understood. This study aims to identify genes responsible for ripening enhancement by LT. To this end, the transcriptome of 'Bartlett' pears treated with LT (0 °C or 10 °C for up to 14 d), which results in faster ripening, and control pears without conditioning treatment was analyzed. LT conditioned pears reached eating firmness (18 N) in 6 d while control pears took about 12 d when left to ripen at 20 °C. We identified 8,536 differentially expressed (DE) genes between the 0 °C-treated and control fruit, and 7,938 DE genes between the 10°C-treated and control fruit. In an attempt to differentiate temperature-induced vs. ethylene-responsive pathways, we also monitored gene expression in fruit sequentially treated with 1-MCP then exposed to low temperature. This analysis revealed that genes associated with jasmonic acid biosynthesis and signaling, as well as the transcription factors TCP9a, TCP9b, CBF1, CBF4, AGL24,

MYB1R1, and HsfB2b could be involved in the LT-mediated enhancement of ripening independently or upstream of ethylene.

Keywords

European pears; Ripening; Ethylene; Jasmonate; Low temperature; Transcription factor.

Abbreviations

1-MCP, 1-methylcyclopropene; ACO, 1-aminoacyl cyclopropane-1-carboxylic acid oxidase; ACS, 1-aminoacyl cyclopropane-1-carboxylic acid synthase; AGL, Agamous-like; AOC, allene oxide cyclase; AOS, allene oxide synthase; bHLH, basic helix-loop-helix; CaLB, calcium-dependent lipid protein; CAP, cold-acclimation protein; CBF, C-repeat binding factor; CDF, cycling DOF factor; CNR, colorless non ripening; COR, cold regulated; CRT/DRE, C-repeat/dehydration-responsive element; DAM, dormancy-associated mads-box; DE, differentially expressed; DOF, DNA binding with one finger; DREB: dehydration response element binding factor; EBF1, ethylene insensitive 3-binding f-box; EIN, ethylene-insensitive; ERS, ethylene response sensor; ETR, ethylene receptor; FC, fold change; HSF, heat shock factor; hsp, heat shock protein; ICE, inducer of CBF expression; JA: jasmonic acid; JAZ: jasmonate-zim-domain protein; LOX2: LIPOXYGENASE2; MeJA: methyl jasmonate; PDJ, n-propyl dihydrojasmonate; PG, polygalacturonase; RIN, ripening-inhibitor; RSEM, RNA-Seq by Expectation Maximization; TF, transcription factor; WRST, Wilcoxon rank sum test.

1. Introduction

Ethylene is widely recognized as an important signal molecule regulating fruit ripening [1-3]. The underlying molecular mechanisms of ethylene action have been studied during fruit development and ripening [3-7]. In tomato, a model organism to study fruit ripening, it is proposed that the TF CNR and

RIN activate the onset of ripening by inducing signaling pathways that control many ripening-related events, including fruit softening, starch degradation to sugars, aroma volatile production, and pigment accumulation [8-10]. However, the role of other TFs besides CNR and RIN in tomato, as well as transcriptional regulation of fruit ripening in other fruit-producing species, has received less attention.

Jasmonic acid was suggested to be involved in early stages of ripening by triggering ethylene production [11, 12]. However, the role of JA in fruit ripening is still unclear, as conflicting reports in the literature show an inhibiting or accelerating effect depending on the fruit species and stage of maturity [12-14]. Cross-talk between the JA and ethylene signal transduction networks has been suggested, with the interaction of JAZ with EIN3 (a key player in the ethylene signal transduction cascade) following *Arabidopsis* exposure to necrotrophic pathogens [15]. However, how JA regulates fruit development and ripening at molecular levels is not understood.

Low temperature (LT) treatments have been reported to hasten ripening in different fruit such as 'Granny Smith' apple, 'Hayward' kiwifruit, and European pears [16-18]. In European pears, LT increased activities of ACS and ACO, two key enzymes in ethylene biosynthesis, accelerated ethylene production rate, and consequently promoted faster fruit ripening [18, 19]. Within the LT range typically used in the trade, intermediate temperatures (5 or 10 °C) can stimulate an earlier climacteric peak and more rapid ripening than lower temperatures (-1 or 0 °C) [18, 20, 21].

Low temperature exposure has been associated with a process called "cold acclimation" in plants grown in temperate regions [22] or "chilling injury" in tropical and subtropical plants [23]. Cold stress causes rigidification of plasma membranes that promotes the opening of Ca^{2+} channels and leads to an increase in cytosolic Ca^{2+} levels; this, in turn, leads to the activation of protein kinases and other signaling cascades that then activate the TFs ICE1 and ICE1-like [24]. ICE1 and ICE1-like then activate *CBF* TFs that regulate the expression of *COR* genes by binding to their CRT/DRE promoter region [22, 24]. LT can cause chilling injury, such as 2 - 8 °C in peach, resulting in modification in cell membrane, increases

in reactive oxygen species, and down-regulation of ethylene biosynthesis [25, 26]. The plant responses to LT have typically been studied in vegetative tissues or in chilling sensitive fruit. The early molecular events, when fruit start to sense the LT and their ripening rates are therefore enhanced (other than ethylene-related signaling pathways), are not well understood.

Recent research provides some evidence of the connections between the LT-induced pathways, the JA and the ethylene biosynthesis and signaling pathways, as well as fruit ripening. The ICE-CBF cascade in *Arabidopsis* was shown to be positively regulated by JA [27]. *CBF1* was up-regulated by ethylene treatment in tomato fruit [28]. Both apple *CBF2* and EIN3-like TFs were shown to regulate *PG1*, encoding a cell wall hydrolase activated upon fruit ripening [29]. In addition, the TCP family, which has primarily been associated with the regulation of plant growth and development, has some members that have been shown to be involved in LT acclimation of 4 week-old seedlings in *Chrysanthemum nankingense* [30]. Intriguingly, several members of the tomato TCP family, including *SITCP12*, *SITCP15* and *SITCP18*, were suggested to play a role during fruit ripening [31].

Using RNA-seq to analyze the transcriptome of 'Bartlett' pears, the most popular European pear variety in California, this study was conducted to investigate the effect of LT treatments (0 °C or 10 °C) on fruit ripening. We hope to untangle the roles of and potential interactions between JA, ethylene, and LT-induced pathways controlling pear ripening. We examined the effect of LT alone on expression of selected genes to clarify if they act independently or potentially upstream of ethylene. Additionally, the potential role of selected TFs in regulating LT-associated ripening enhancement is discussed.

2. Materials and methods

2.1. Plant materials and ripening evaluation

'Bartlett' pear fruit, 80 N firmness and 65 mm average diameter, were harvested from Sacramento (2010) or Lake County (2013), California and transported to the Postharvest Pilot Plant at the University

of California, Davis. Fruit were sorted to remove sunburned and damaged fruit before being randomly assigned to treatments.

In 2010 and 2013, LT experiments were conducted to induce ripening at different rates (Sup. File 1).

In both 2010 and 2013, fruit after sorting were treated at 0 °C or 10 °C up to 14 d. The shortest

treatment that induced fruit with full ripening capacity (fruit firmness $\leq 20\text{N}$ within 6 d after transfer to

20 °C) were selected for further evaluation (data not shown). In 2010, fruit were LT treated at 0 °C for 14

d or 10 °C for 5 d. In 2013, fruit were divided into two groups; one group was treated with LT (0 °C for 8

d or 10 °C for 3 d), the other group was treated with $1\ \mu\text{L L}^{-1}$ 1-MCP for 24 h at 20 °C before LT exposure.

This high concentration of 1-MCP was applied in order to examine the effect of temperature alone on

genes of interest when the ethylene pathway was strongly inhibited. The control in both years received

no cold treatment.

After the treatments in both 2010 and 2013, fruit were transferred to 20 °C (90 % RH) for ripening.

Fruit ripening was evaluated based on flesh firmness and ethylene production rate. Flesh firmness was

determined as the resistance to 9 mm penetration with an 8 mm-diameter probe using a Fruit Texture

Analyzer (Güss, Strand, South Africa) on two opposite sides of the fruit after the peel was removed; data

were collected on three biological replicates with six fruit per replicate. Ethylene concentration was

measured according to the method described by Villalobos-Acuna et al. [32]. Briefly, headspace gas

samples from 6 fruit per replicate were collected and injected into a gas chromatograph fitted with a

flame ionization detector (Series 400 AGC, Hach-Carle Co, CA).

2.2. RNA extraction

Skin and flesh tissues peeled from two opposite sides of 6 fruit were frozen in liquid N_2 for RNA

analysis. The control samples were collected within 24 h of harvest; LT-treated samples were collected 3

h after removal from the cold treatments of 0 °C and 10 °C. Total RNA was isolated from 0.5 g ground

tissue, using the method from the Qiagen RNeasy Plant Mini Kit (Qiagen, Netherlands) that was described by Nham et al. [33]. DNase I (Roche, Switzerland) was used to remove genomic DNA contamination from the total RNA. The RNA concentration was quantified based on OD₂₆₀ nm on a NanoDrop Spectrophotometer (Thermo Fisher Scientific, MA); the RNA quality was verified by examining the ratio OD₂₆₀ nm/OD₂₈₀ nm.

2.3. RNA-seq, de novo assembly, differential expression analysis, and functional annotation analysis

RNA-seq of nine samples from 2010 (two LT treatments and one control), three biological replicates each and *de novo* assembly were completed as described in our previous publication [33]. Briefly, library and Illumina sequencing were completed by UC Davis DNA Technologies Core (<http://dnatech.genomecenter.ucdavis.edu/>). The *de novo* assembly was performed on Trinity (ver. trinityrnaseq_r2012-06-08) on reads of 21 samples [34] (12 samples were used from our previous study [34] and 9 samples were collected for the current study) and reads were mapped using Bowtie [35]. Putative function of genes was identified by using BLASTX (v2.2.26+) to TAIR10 and Refseq v54 databases [36]. Differential expression analysis was carried out between the two LT treatments and the control using EBSeq package (v1.1.6) with False Discovery Rate of 0.05 [37] using a similar method as described in our previous study [33]. Mapman functions of transcripts were assigned by Mercator with a blast cut-off of 50 [38]. Significant functional groups in DE genes were examined by WRST (Benjamin Hochberg correction). Log₂FC of RNA-Seq was the logarithm-base-2 of the ratio RSEM count in each temperature treatment/RSEM count in the control.

2.4. Quantitative PCR

The method used was similar to that reported in our previous study [33]. Briefly, cDNA was synthesized from 1 µg total RNA, using Superscript™ III First Strand Synthesis Systems for RT-PCR

Systems (Invitrogen, CA). Gene expression of two technical replicates was examined on a 7300 Real Time PCR System (Applied Biosystems, CA) using SYBR Green PCR Master Mix provided by the company. *Tubulin1* was chosen as the housekeeping gene after testing with *18s*, *26s*, *beta-actin*, and *ef1alpha*. Primers were designed using Primer 3 for 25 selected genes as shown in Sup. File 2 [39]. The FC of qPCR was calculated as $\Delta\Delta Ct$ [40].

2.5. Experiment design and analysis of variance

The experiment was a completely randomized factorial design. Using SAS 9.3 (SAS Institute, NC), the gene expression data were checked for homogeneity of variance and logarithm-base-2 transformed before ANOVA and means separation (Tukey's test or LSD - Least significant difference, $p\text{-value} \leq 0.05$).

2.6. Script availability

The reads produced in this study were deposited at GenBank Short Read Archive SRP090677 with BioSamples SAMN05845018-SAMN05845026, under BioProject PRJNA344800. The reads were aligned to the published *de novo* transcriptome GBXL000000000 [33].

3. Results

3.1. Effect of LT treatments on the rate of ripening in 'Bartlett' pear

LT treatments carried out in 2010 and 2013 hastened the rate of ripening: fruit reached a firmness of approximately 20 N within 6 d, although fruit from the 2010 harvest were more "resistant" to ripening than fruit from 2013 (Fig. 1). Indeed, the 2010 control fruit without any LT treatments took about 12 d to ripen, while treatments of 0 °C for 14 d and 10 °C for 5 d resulted in full ripening after 6 d at 20 °C (Fig. 1). In 2013, however, fruit without any LT treatments took merely 9 d to ripen, and shorter LT treatments (0 °C for 8 d and 10 °C for 3 d) allowed fruit to ripen within 6 d at 20 °C. RNA extracted from

the fruit at the end of the LT treatment period (i.e., “D0” during ripening at 20 °C), and from control fruit at the start of the ripening period (also “D0” at 20 °C) was submitted for RNA-seq analysis for DE genes screening. Treatment with 1-MCP in 2013 prevented fruit softening for the duration of the experiment (up to 9 d); fruit firmness was approximately 80 N after 6 d at 20 °C (Fig. 1). Furthermore, ethylene production was barely detectable in these fruit (Fig. 1). It appears that 1-MCP treatment effectively inhibited the ripening process; we therefore used the 1-MCP-then-LT fruit to examine molecular response that may act upstream or independently of the ethylene pathway.

3.2. Transcriptome: Differential expression analysis

Two pairwise analyses were carried out to find significantly up- and down-regulated transcripts in the 0 °C and 10 °C treatments, compared to the control (Fig. 2A). There were 8,536 and 7,938 DE genes between 0 °C treatment and the control and between 10 °C treatment and the control, respectively. More genes were down-regulated than up-regulated by both LT treatments. Additionally, there was a strong correlation (R^2 of 0.743) between the log2FC for the 0 °C and 10 °C treatments compared to the control (with values ≥ 1 or ≤ -1) suggesting high similarity in gene expression between the two temperatures (Fig. 2B). The strong correlation ($R^2 = 0.896$) between the FCs by qPCR and by RNA-seq confirms the reliability of the RNA-seq gene expression values (Fig. 3).

3.3. Transcriptome: Overview of Mapman/Blast2GO functional annotation

Using Mapman, the putative functions of DE genes were classified into functional groups (Sup. File 3). The most common functional groups were Protein (22%), RNA (18%) and Signaling (8%); the Stress and Hormone Metabolism groups were also abundant in the DE gene lists with 5% and 3%, respectively. The number of DE genes in pear tissues exposed to 0 °C and 10 °C compared to control pears was also calculated and analyzed for the enrichment of functional groups (Sup. File 4). The Stress group (both

biotic and abiotic) was enriched at 0 °C but not at 10 °C. Additionally, the Transcription Factor group, including AP2/EREBP, C2C2(Zn) CO-like, DOF, and WRKY, as well as the Protein Posttranslational Modification, Protein Degradation, and Signalling groups were enriched in both treatments.

3.4. Transcripts associated with ethylene biosynthesis and signalling

To investigate the ethylene pathway during fruit ripening and in 1-MCP-then-LT treated samples, we further examined four genes: *ACO*, *ETR2*, *ERS1a*, and *EBF1*, which had been identified as significantly up-regulated following LT treatments in the transcriptome data (Table 1). In 2013, the increase in expression of *ACO* by LT was maintained, but *ERS1a* was up-regulated only in the 10 °C treatment, *EBF1* was up-regulated in the 0 °C treatment, and *ETR2* expression showed no changes (Fig. 4). In contrast, when 1-MCP was applied before LT treatments, expression of these genes significantly decreased (Fig. 4). During ripening of control fruit at 20 °C, expression of these genes increased (Fig. 5).

3.5. Transcripts associated with jasmonates

Most of the genes associated with JA biosynthesis and signaling identified in the RNA-seq data from the 2010 experiment were down-regulated by LT treatment. These genes encode JA biosynthesis enzymes (*AOS*, *AOC1*, and *AOC2*) (Table 1), as well as *LOX2*, four JA signaling proteins *JAZs* and a JA-induced protein (data not shown). Quantitative PCR revealed that transcript abundance of *AOS*, *AOC1*, and *AOC2* was also lower in LT treatments compared to the control in the 2013 experiment (Fig. 4), both with and without 1-MCP treatment. In control fruit not exposed to LT, expression of these genes decreased significantly on day 6 (Fig. 5), when the softening rate became more rapid (Fig. 1).

3.6. Transcripts associated with LT-induced pathways

Seven genes associated with LT-induced pathways, which were highly up- or down-regulated in RNA-seq results, were selected to examine their transcriptional changes following treatment with 1-MCP and/or LT and during non-LT-induced fruit ripening. Calcium signal transduction is important in regulating responses to LTs [22, 24]. *CaLB* acts as a negative regulator of abiotic stress response, including salt and drought stress [41]. In the current study, putative *CaLB* transcripts (PcM_51546 and PcM_40485) were down-regulated by the two LT treatments (Table 1, Fig. 4). These genes were also down-regulated by LT in 1-MCP-treated fruit (Fig. 4). However, they showed no changes or were slightly up-regulated during ripening of control fruit (Fig. 5).

Another cold-associated gene *COR413* (PcM_27854) showed inconsistent expression patterns in LT treatments between the 2010 and 2013 experiments. While expression of putative *COR413* decreased at 0 °C and increased at 10 °C in 2010 (Table 1), the gene was down-regulated in both LT-treated fruit in 2013, although the decrease was greater at 0 °C than 10 °C (Fig. 4). Meanwhile, putative *CAP160* (PcM_58422), a gene encoding a protein linked with cold acclimation [42, 43], was highly down-regulated ($\log_2FC = -4.89$) at 10 °C and showed no significant change at 0 °C in 2010 (Table 1), but was up-regulated by LT in 2013 samples, especially when fruit were treated with 1-MCP (Fig. 4).

DREBs belong to the ERF TF family and consist of two subclasses, DREB1/CBF and DREB2. In *Arabidopsis*, expression of *DREB1s/CBFs* was triggered by LT while *DREB2s* were regulated by dehydration [44, 45]. According to BLAST results, PcM_49115 and PcM_67226 are putative *CBF4* and *CBF1* (members of *DREB1/CBF*), respectively, and PcM_53561 is a putative *DREB2* family member. Transcriptome analysis indicated that the expression of these *DREB* family genes decreased in response to both LT treatments, with a greater decrease following the 0 °C treatment compared to 10 °C (Table 1). Down-regulation of all three examined *DREB* genes also occurred in all LT-treated samples compared to control fruit from the 2013 experiment, regardless of 1-MCP application (Fig. 4). In addition, the

expression of *CBF1* and *CBF4* decreased in the control (non-LT) fruit treated with 1-MCP compared to the control (non-LT) untreated fruit (Fig. 4). Down-regulation of the two putative *CBFs* was also observed on day 6 during ripening of control, non-LT treated fruit (Fig. 5). In contrast, transcript abundance of the putative *DREB2* gene was unaffected by 1-MCP treatment in control (non-LT) fruit, and showed no significant change over the ripening period in control (non-LT) fruit (Fig. 5).

3.7. Transcripts associated with transcription factors

One of the objectives of this study was to identify transcriptional regulators that act independently or upstream of the ethylene pathway to regulate ripening. Transcriptome analysis allowed the global study of TF expression in response to LT treatments, compared to non-LT treated fruit. A significant number of DE genes were classified as TF (8.5 % in the 0 °C-treated fruit and 8.1 % in the 10 °C-treated fruit). Of all TF identified in the transcriptome, 40.6 % and 36 % were DE in fruit treated at 0 °C and 10 °C, respectively (data not shown). These DE genes represented a wide range of TF families, among which bHLH, MYB, AP2/EREBP, HB, C2H2(Zn), and WRKY had the highest numbers of DE members (Fig. 6). Low temperature treatments induced a higher number of down-regulated members than up-regulated, such as in the TF family of bHLH, AP2/EREBP, and WRKY (Fig. 6). Overall, both LT treatments (0 °C and 10 °C) resulted in similar patterns of differential expression between treated and control fruit (Fig. 6).

In addition to LT-associated TFs that have been discussed earlier, eleven highly up- or down-regulated TFs were selected for analysis in 1-MCP treated fruit and ripening samples (Table 1, Fig. 7 and 8). Of these eleven TFs, six (*A6B*, *CDF2*, *bHLH135*, *TCP4*, *TCP14* and *TCP20*) had a different pattern of expression following LT treatments in the 2013 compared to the 2010 experiments (Fig. 8). In contrast, the expression of putative *TCP9a* (PcM_46371) consistently increased following LT treatments in both experiments (Table 1, Fig. 7). When the ethylene signal transduction pathway was blocked by 1-MCP, transcript abundance of *TCP9a* also increased, but to a lesser extent (Fig. 7). *TCP9a* expression was also

strongly up-regulated by day 3 in non-LT treated ripening fruit (Fig. 8). In contrast, *TCP9b* expression strongly decreased after LT treatments regardless of previous 1-MCP treatment (Fig. 7), and also decreased in control (non-LT) fruit during ripening at day 6 (Fig. 8).

The expression of *AGL24* (PcM_35299) and *HsfB2b* (PcM_53369) TFs consistently increased after LT treatments (Table 1, Fig. 7). This increase by LT treatments was also observed in fruit treated with 1-MCP prior to 0 °C and 10 °C treatments (Fig. 8), and in non LT-treated control fruit during the ripening process (Fig. 8). Finally, the expression of a putative *MYB1R1* (PcM_55822) TF was generally down-regulated following LT treatments, regardless of 1-MCP treatment (Table 1, Fig. 8), and down-regulation of this gene was also observed during ripening of control fruit (Fig. 8).

4. Discussion

European pear fruit are among a small number of fruit that respond to LT exposure with faster ripening. Using RNA-seq, genes that were up- or down-regulated in response to LT treatments were identified, including genes associated with biosynthesis and signaling of ethylene and JA, the LT-induced pathways, and transcriptional regulation. By examining changes in gene expression in response to LT in fruit pretreated with 1-MCP, we were able to identify genes that may be involved in the LT-induced enhancement of ripening independently or potentially upstream of ethylene. In addition, assessing transcript abundance of these genes during non-LT-enhanced fruit ripening at 20 °C helped to understand their general role in ripening processes.

4.1. LT-enhanced ripening rate and effect of 1-MCP

Cold treatments in 2010 and 2013 hastened the ripening of 'Bartlett' pears, although less time at LT was required to reach full ripeness (defined as 20 N firmness) in 2013. [The increase in ethylene biosynthesis and signal transduction genes after LT exposure agrees with findings in previous research](#)

with 'Bartlett' and other European pear cultivars [18], as well as in several other fruit including avocado, melon, and mango [25]. However, fruit that are chilling sensitive, including tomato and peach, ethylene genes were down-regulated after exposure to LT [46, 47]. Furthermore, the minimal ethylene production rates and the significant down-regulation of ethylene biosynthesis and signaling genes in the 1-MCP-then-LT treated fruit indicate the effectiveness of the 1-MCP treatments in inhibiting the ethylene signal transduction pathway. These results suggest that 1-MCP-then-LT treated fruit (8D at 0 °C or 3D at 10 °C) represent suitable samples to investigate gene expression responses due to LT conditioning, independent or upstream of ethylene signalling.

4.2. The involvement of JA-associated genes in LT conditioning

Jasmonic acid concentration has been shown to decrease as fruit of 'Golden Delicious' apple and 'La France' pears developed [48, 49]. In 'La France' pears, a decrease in trans- JA concentration occurred prior to ethylene evolution [49]. In 2007, Kondo et al. demonstrated that an application of n-propyl dihydrojasmonate (PDJ, a JA analog) at the climacteric stage of 'La France' pears decreased ACC synthase activity, as well as inhibited ethylene production and delayed fruit ripening [13]. In the present study, down-regulation of JA biosynthesis and signaling genes during ripening of control non-LT treated fruit strongly suggest JA involvement in pear ripening regulation. Recently, cross-talk between the ethylene and JA signal transduction pathways has been shown, via negative regulation of EIN3 by JAZ in Arabidopsis [15]. Our RNA-seq results showed that while JAZs were down-regulated, PcM_57850 (putative EIN3) was up-regulated (Log2FC = 1.02 and 0.91 in 0 °C and 10 °C, respectively; data not shown). Therefore, it is possible that JA may also regulate the ethylene pathway through EIN3 in pear fruit. Additionally, JA-biosynthesis transcripts were significantly reduced by LT treatments, even when the ethylene signal transduction pathway was blocked by 1-MCP. Thus, we postulate that LT exposure triggered a decrease in the expression of JA biosynthesis genes, which may lead to a reduction in JA

levels and consequently contribute to the enhancement of ripening in pears, independently or upstream of ethylene signaling.

4.3. Functions of LT-induced genes in the enhancement of ripening

The function of LT-induced genes in the enhancement of fruit ripening was also examined. In the current study, down regulation of *CaLBs* (negative regulators in the calcium signal transduction pathway) by LT in both fruit pretreated or not with 1-MCP suggests that these genes may negatively regulate the LT-induced signal transduction pathway in ‘Bartlett’ pears. Furthermore, according to the stable expression levels of these genes during fruit ripening without prior LT exposure, we postulate that these *CaLBs* may not be involved in non-LT-triggered ripening processes.

The expression patterns of other examined LT-associated genes, including *COR413* (PcM_27854) and *CAP160* (PcM_58422), were inconsistent between the 2010 and 2013 experiments. Higher transcript accumulation of *COR413* was recorded in cold-acclimated wheat (winter cultivar) than in non-acclimated wheat (spring cultivar) [50]. In spinach, expression of *CAP160* was increased by exposure to 5 °C for 1 to 7 d [43]. Our results suggest that *COR413* and *CAP160* may be not associated with ripening enhancement in pears.

Regarding the genes in the DREB1/CBF TF family, the down-regulation of *CBF4* (PcM_49115) and *CBF1* (PcM_67226) following LT treatments in our study appears to be in contrast to what has been previously found in other species. In Arabidopsis, an overexpression of *CBF4* activated genes that were involved in cold acclimation and drought adaptation; however, the expression of *CBF4* was increased by drought stress but not by LT [51]. *CBF1* was up-regulated within 24 hours in both tomato seedlings and fruit tissues treated with ethylene or LT [28]. Apple *CBF2*, which is the closest ortholog of the putative pear *CBF4* (PcM_49115), was up-regulated in cold-treated apple-cell cultures [29]. However, expression of this *CBF2* was unchanged during apple fruit development and after ethylene treatment, and was not

reported in cold-treated fruit [29]. One explanation for the decrease in *CBF* expression following LT treatments is through JA activity. As JA acts upstream of the ICE-CBF pathway [27], the decrease in JA-associated transcript abundance following LT treatments would result in down regulation of *CBF* expression, as was shown in our data. Therefore, we postulate that JA plays a regulatory role in the cold pathway associated with ripening in pears.

Liu et al. reported up-regulation of *DREB2A* and its homologs by dehydration [44], and Sakuma et al. showed that *DREB2A* regulated genes associated with both drought and heat stress [52]. However, there has been no evidence in the literature of down-regulation of *DREB2A* by abiotic stress, particularly by LT, as was observed with the putative *DREB2A* (PcM_53561) in our study. However, because the expression of this gene was unchanged in non-LT-treated fruit that are ripening, *DREB2A* may be involved in early LT responses that enhanced ripening, without being directly implicated in non-LT-triggered ripening.

4.4. Functions of genes encoding transcription factors in the LT-enhanced ripening

A high number of DE genes encoding TFs suggests that transcriptional regulation underlies the major programming events of ripening enhancement during LT treatments.

A few studies have shown the importance of AGAMOUS LIKE MADS-box TFs in fruit development and ripening. Higher expression of *TAGL1* (*TOMATO AGAMOUS-LIKE 1*) was found in ripening fruit and a *TAGL1*-loss-of-function mutant had lower transcript abundance of ripening-associated genes [53]. Tomato with ectopic expression of banana MADS7 had increased expression of ripening related genes such as *ACO1*, *ACS2*, and *PG* [54]. In the current study, up-regulation of putative *AGL24* (PcM_35299) by LT treatments and during ripening suggests the involvement of this gene in regulating ripening. In addition, the increase of this gene in 1-MCP-then-LT treated fruit reveals that LT alone had an effect on

357 this gene. This is in agreement with the finding in Arabidopsis that *AGL24* was up-regulated in seeds that
358 had been held at 2 °C up to 70 d [55].

359 The MYB TF superfamily plays regulatory roles in various developmental processes in plants [56].
360 Down-regulation of a putative *MYB1R1* (PcM_55822) by LT alone in the current study agrees with an
361 earlier finding in which *At1g70000*, the closest ortholog to *MYB1R1*, was down-regulated in an
362 Arabidopsis mutant that showed higher tolerance to cold [57]. However, the decrease of *MYB1R1*
363 expression by LT is in contrast with an increase of potato *MYB1R1* transcript abundance by cold stress
364 [58]. To our knowledge, there is no evidence in the literature of *MYB1R1* indicating any function in fruit
365 development. In our study, we showed that putative *MYB1R1* started to be down-regulated when the
366 fruit firmness began to decrease during ripening at 20 °C, as well as following LT treatments that
367 facilitate fruit ripening. We suggest that this TF may function as a negative regulator of ripening.
368 Heat stress transcription factors (Hsfs) play an important role in plant responses to environmental
369 stresses [59, 60]. In the current research, the up-regulation of a putative *HsfB2b* during ripening
370 suggests a role of this TF in regulating pear ripening. This is in agreement with the results in previous
371 research with tomato and Chinese grapevine, in which several members of Hsf family were shown to be
372 involved in fruit ripening [61, 62]. Moreover, apple *HsfB2a* was up-regulated during the late stage of
373 fruit growth, when fruit reached 20 mm in diameter [63]. In addition, tomato seedlings that had
374 overexpression of Arabidopsis *HsfA1b* exhibited an increase in chilling tolerance [64]. The increase of
375 *HsfB2b* by LT in 1-MCP and non-treated fruit in this study suggests that this TF is involved in LT-
376 associated ripening regulation.

377 The majority of research on TCPs was conducted on leaf tissues; this TF family has been reported to
378 regulate cell proliferation, cell differentiation, leaf development, and lateral branching [65-67]. In one of
379 the few studies assessing the function of these TFs in fruit ripening, it was found that *SITCP12*, *SITCP15*,
380 and *SITCP18* were up-regulated during tomato ripening [31]. In the current study, both *TCP9a* and *TCP9b*

appear to play important roles, albeit in a contrasting manner, in regulating ripening following LT treatments and at ambient temperature. The functional antagonism of *TCP* genes has also been observed in *Arabidopsis*: while *TCP9* and *TCP20* served as negative regulators of *LOX2*, *TCP4* activated *LOX2* expression [68]. Therefore, we hypothesize that the coordinated up-regulation of *TCP9a* and down-regulation of *TCP9b* probably suppresses *LOX2* expression and this leads to down-regulation of JA-associated genes.

5. Conclusions

The transcriptome generated in this study and the follow-up expression analyses advanced our knowledge about the molecular mechanisms involved in ripening regulation by LT. Furthermore, by examining 1-MCP-then-LT treated fruit and fruit during ripening, we propose a molecular model regulating LT-induced ripening enhancement in ‘Bartlett’ pears (Fig. 9). In this model, *TCP9a* and *TCP9b* collaborate to regulate ripening via the JA biosynthesis and signaling pathways. The *LOX2*-mediated degradation of jasmonic acid could facilitate ripening independently of ethylene signaling or could lead to an increase in expression of *EIN3* in the ethylene pathway. Additionally, JA may regulate cold-associated TFs including *ICE1*, *CBF1*, and *CBF4* for ripening enhancement. Moreover, other transcription factors including *AGL24* and *HsfB2b* probably work as positive regulators while *MYB1R1* acts as a negative regulator of ripening. Future studies will identify targets of these transcription factors and confirm their involvement in both the LT- and the ethylene-signal transduction pathways.

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Figures

Fig. 1: Firmness and ethylene production rate of fruit of control and low temperature treated fruit. Each data point represents the mean of three biological replicates containing six pooled fruit each; error bars

indicate standard errors. Dashed line indicates eating firmness (18 N) of pear fruit. Vertical bars represent LSD (least significant difference) values for the treatment comparison on the given day (p-value ≤ 0.05).

Fig. 2: A. Number of shared and unique up- and down- regulated genes in 0 °C and 10 °C treatments, compared to the control. B. Regression analysis between log2 fold change (FC) (with values ≥ 1 or ≤ -1) of 1,798 differentially expressed genes of two treatments, 0 °C and 10 °C, compared to the control (p-value ≤ 0.0001).

Fig. 3: Regression analysis of relative expression (log2 fold change) between RNA-seq and quantitative PCR of 25 selected genes (p-value ≤ 0.0001).

Fig. 4: Relative expression of ethylene- (*ACO*, *ETR2*, *ERS1 α* , and *EBF1*), jasmonic acid- (*AOS*, *AOC1*, and *AOC2*), and low temperature- (*CaLB1*, *CaLB2*, *COR413*, *CAP160*, *CBF1*, *CBF4*, and *DREB2*) associated genes 3 h after transfer from the cold treatments of 0 °C and 10 °C in 1-methylcyclopropene (1-MCP) treated and non 1-MCP treated samples (2013 experiments). Bars represent mean values of three biological replicates containing six pooled fruit each; error bars indicate standard errors. Different letters indicate significant differences in ANOVA within 1-MCP treated or non-treated fruit; star (*) indicates significant differences between corresponding 1-MCP-treated and non-treated samples (Tukey's test, p-value ≤ 0.05).

Fig. 5: Relative expression of ethylene- (*ACO*, *ETR2*, *ERS1 α* , and *EBF1*), jasmonic acid- (*AOS*, *AOC1*, and *AOC2*), and low temperature- (*CaLB1*, *CaLB2*, *COR413*, *CAP160*, *CBF1*, *CBF4*, and *DREB2*) associated genes during ripening at 20 °C of the control fruit in 2010 (no-low temperature conditioning treatment).

Bars represent mean values of three biological replicates containing six pooled fruit each; error bars indicate standard errors. Different letters indicate significant differences in ANOVA (Tukey's test, p -value ≤ 0.05).

Fig. 6: Number of up- and down-regulated genes encoding transcription factors in 0 °C and 10 °C treatments, compared to the control identified in the RNA-seq data.

Fig. 7: Relative expression of genes encoding transcription factors 3 h after transfer from the cold treatments of 0 °C and 10 °C in 1-methylcyclopropene (1-MCP) treated and non 1-MCP treated samples (2013 experiments). Bars represent mean values of three biological replicates containing six pooled fruit each; error bars indicate standard errors. Different letters indicate significant differences in ANOVA within 1-MCP-treated or non-treated fruit; star (*) indicates significant differences between 1-MCP-treated and non-treated samples (Tukey's test, p -value ≤ 0.05).

Fig. 8: Relative expression of genes encoding transcription factors during ripening at 20 °C of the control fruit in 2010 (no-low temperature conditioning treatment). Bars represent mean values of three biological replicates containing six pooled fruit each; error bars indicate standard errors. Different letters indicate significant differences in ANOVA (Tukey's test, p -value ≤ 0.05).

Fig. 9: Proposed molecular model in the regulation of low-temperature-mediated enhancement of pear ripening. Genes in blue italic font were down-regulated and genes in red font were up-regulated by low temperatures. For the color version of this figure, readers are referred to the web version of this article.

636 **Tables**637 **Table 1: Selected genes encoding transcription factors with log2FC (fold change) by RNA-seq and relative expression by real-time PCR validation.**

Gene ID	Accession No.	Putative function	Functional group	Log2FC RNA-seq		RE Realtime-PCR	
				0C/Ctrl	10C/Ctrl	0C/Ctrl	10C/Ctrl
PcM_59277	GBXL01000612	ACO (1-aminoacylcyclopropane-1-carboxylic acid oxidase)	Ethylene	2.29	3.29	7.02	8.09
PcM_61151	GBXL01003065	ETR2 (ethylene receptor)		2.32	3.28	1.77	2.50
PcM_57563	GBXL01007268	ERS1a (ethylene response sensor)		1.22	2.26	1.49	1.90
PcM_60321	GBXL01004555	EBF1 (EIN3-binding f-box protein)		1.26	2.44	1.18	2.60
PcM_40167	GBXL01025516	AOC1 (allene oxide cyclase)	Jasmonic acid	-1.97	-1.92	-1.95	-1.84
PcM_36557	GBXL01029695	AOC2 (allene oxide cyclase)		-4.30	-3.50	-4.31	-3.44
PcM_44588	GBXL01011629	AOS (allene oxide synthase)		-2.64	-2.36	-2.98	-2.94
PcM_51546	GBXL01024322	CaLB1 (Calcium-dependent lipid-binding)	Low temperature	-1.47	-0.98	-1.51	-0.89
PcM_40485	GBXL01046157	CaLB2 (Calcium-dependent lipid-binding)		-1.68	-1.07	-1.36	-0.76
PcM_27854	GBXL01032569	COR413 (cold-regulated 413)		-0.77	0.39	-0.63	0.73
PcM_58422	GBXL01005541	CAP160 (cold-acclimation protein)		0.02	-4.89	0.84	-4.97
PcM_67226	GBXL01055407	CBF1 (C-repeat binding factor)		-6.78	NA	-6.96	-3.75
PcM_49115	GBXL01024454	CBF4 (C-repeat binding factor)		-7.64	-2.74	-6.98	-4.59
PcM_53561	GBXL01010386	DREB2 (dehydration responsive element-binding)		-5.21	-2.06	-5.03	-2.16
PcM_35299	GBXL01015905	AGL24 (agamous - like 24)	Transcription factor	2.25	2.26	2.10	2.28
PcM_58018	GBXL01010173	CDF2 (cycling DOF factor)		-4.21	-5.23	-4.14	-5.44
PcM_16536	GBXL01026855	bHLH135 (basic helix-loop-helix)		0.89	1.70	0.26	1.18
PcM_55822	GBXL01017779	MYB1R1		-1.16	-2.17	-0.57	-1.86
PcM_59620	GBXL01012423	TCP4		-2.08	-2.11	-1.71	-2.07
PcM_46371	GBXL01014622	TCP9a		1.84	2.48	1.63	2.63
PcM_37893	GBXL01012046	TCP9b		-3.71	-2.94	-3.75	-2.92
PcM_56708	GBXL01013177	TCP14		-0.96	-2.28	-1.41	-2.79
PcM_53793	GBXL01007033	TCP20		-1.06	-1.04	-1.88	-1.20
PcM_58482	GBXL01021462	A6B (heat shock)		3.62	2.44	3.54	2.87
PcM_53369	GBXL01036079	HsfB2b (heat shock, winged helix)		3.35	2.38	3.02	2.46

638 **Additional files**

639 Sup. File 1: Experimental procedure. LT: Low temperature, Ctrl: Control, 1-MCP: 1-methylcyclopropene

640

641 Sup. File 2: Primers for Real-time PCR validation

642

643 Sup. File 3: Mapman functional annotation of differentially expressed genes between 0 °C and the

644 control fruit, and between 10 °C and the control fruit. 2nd: secondary, met.: metabolism, Other: amino

645 acid met., photosynthesis, redox, nucleotide met., minor CHO met., major CHO met., mitochondrial

646 electron transport/ATP and glycolysis, co-factor and vitamin met., TCA/org transformation, metal

647 handling, tetrapyrrole syn., C1-met., OPP, N-met., biodegradation of xenobiotics, fermentation,

648 polyamine met., gluconeogenesis/glyoxylate cycle, S-assimilation, micro RNA and natural antisense.

649

650 Sup. File 4: Number of differentially expressed transcripts according to Mapman functional classification

2010

2013

Day at 20°C