RESEARCH ARTICLE

Evidence for pre-climacteric activation of AOX transcription during cold-induced conditioning to ripen in European pear (*Pyrus communis* L.)

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Abstract

European pears (*Pyrus communis* L.) require a range of cold-temperature exposure to induce ethylene biosynthesis and fruit ripening. Physiological and hormonal responses to cold temperature storage in pear have been well characterized, but the molecular underpinnings of these phenomena remain unclear. An established low-temperature conditioning model was used to induce ripening of 'D'Anjou' and 'Bartlett' pear cultivars and quantify the expression of key genes representing ripening-related metabolic pathways in comparison to non-conditioned fruit. Physiological indicators of pear ripening were recorded, and fruit peel tissue sampled in parallel, during the cold-conditioning and ripening time-course experiment to correlate gene expression to ontogeny. Two complementary approaches, Nonparametric Multi-Dimensional Scaling and efficiency-corrected 2-(ΔΔCt), were used to identify genes exhibiting the most variability in expression. Interestingly, the enhanced alternative oxidase (AOX) transcript abundance at the pre-climacteric stage in 'Bartlett' and 'D'Anjou' at the peak of the conditioning treatments suggests that AOX may play a key and a novel role in the achievement of ripening competency. There were indications that cold-sensing and signaling elements from ABA and auxin pathways modulate the S1-S2 ethylene transition in European pears, and that the S1-S2 ethylene biosynthesis transition is more pronounced in 'Bartlett' as compared to 'D'Anjou' pear. This information has implications in preventing post-harvest losses of this important crop.

Introduction

The fruit is a specialized organ unique to angiosperms that provides a protective environment for the seeds to develop and mature. In order for the seeds to be disseminated, the fruits undergo a highly-orchestrated set of physiological and biochemical processes that result in

senescence or ripening [1, 2]. The process of ripening is characterized by the breakdown of chlorophyll and accumulation of anthocyanins or carotenoids and xanthophylls; the resulting vivid colors make the fruits visually appealing to potential seed dispersers [3]. The accompanying evolution of aromatic and volatile compounds, conversion of starches to sugars and softening of the mesocarp or cortical tissue make the fruits attractive to consumers [4]. The ripening process is categorized as 'climacteric' when there is a respiratory burst along with a peak in ethylene production [5]. All other modes of ripening that do not demonstrate this characteristic behavior are categorized as 'non-climacteric.' While the latter mode of ripening is represented by various fruits such as citrus, strawberry (*Fragaria* × *ananassa*), grapes (*Vitis sp.*), etc., the climacteric mode of ripening is exemplified by bananas (*Musa sp.*), tomato (*Solanum lycopersicum* L.), apple (*Malus* x *domestica* Borkh.) and pear, to name a few.

In climacteric fruit, the biochemistry of ethylene biosynthesis is well understood [6, 7]. As a result of enhanced auto-stimulatory production of ethylene during respiratory climacteric, referred to as System 2 ethylene synthesis, the fruit develops a complete profile of desirable sensory qualities for consumption [8–10]. This is accomplished by the activity of ethylene-precursor synthesizing and ethylene-synthesizing enzymes, ACC SYNTHASE (ACS) and ACC OXIDASE (ACO), respectively. In addition to their modulatory effects, these rate-limiting enzymes are themselves under extensive regulation at the transcriptional, post-transcriptional and post-translational levels as demonstrated in banana, tomato, etc. [11–14]. Through a combination of physiological and molecular analyses of climacteric fruit, a comprehensive model of S1 to S2 transition is emerging [5, 15–19] involving numerous phytohormones and molecular signals.

Differential abundance of the ABA catabolic gene transcripts ABSCISIC ACID 8'-HYDROXYLASE 1 and 2 (CYP707A1 and CYP707A2) was shown to correlate with the upregulation of ACS transcripts and specific developmental stage [20]. These genes inhibit expression of NCED-like genes in strawberry and tomato, thereby reducing ABA biosynthesis and promoting cell wall breakdown and ripening [19]. Similar work in peach showed a correlation between endogenous ABA levels with sensitivity to chilling injury and regulation of induction of fruit ripening [17, 21]. Auxin is involved in modulating acute and long-term cold exposure in plants [22, 23]. In climacteric Japanese plum (*Prunus salicina* L.) and melting-flesh peach (*Prunus persica* L.), development and ripening coincides with prolonged cold temperature exposure and changes in auxin metabolic processes, indicating that cold responses in fruit tissues may be influenced in part by intracellular auxin concentrations, though species and cultivars vary in sensitivity [24, 25]. This, in turn, may be controlled by transport, conjugation, biosynthetic, and catabolic mechanisms [23]. For example, the ethylene-signaling repressors EIN3 BINDING FACTOR 1 and 2 were down-regulated in tomato in response to exogenous auxin treatment, thereby propagating the ethylene signal [26]. Similarly, ACS expression was reported to increase in banana fruit upon exogenous auxin application [27].

In addition to phytohormonal regulation, The MADS-BOX TRANSCRIPTION FACTOR Rin (MADS-RIN) protein has long-been considered essential for ripening in climacteric fruits, and RIN binding motifs have been identified in the promoter regions of many genes involved in ethylene biosynthesis and response [28]. Recently, gene-editing based reevaluation of the role of RIN demonstrated that mutation of this gene results in the production of a protein that actively inhibits ripening induction [29]. During ripening, the RIN is known to form a transcriptional regulatory complex that recruits numerous other proteins including specific APE-TELA 1-like (AP1-like) and SQUAMOSA PROMOTER BINDING PROTEIN-like (SBP-like) in the activation of downstream ETHYLENE RESPONSE FACTOR (ERFs), including those leading to altered ACS or ACO gene expression and protein accumulation [11]. Studies in banana and tomato have further elucidated the components of this transcriptional activation

complex, thereby adding to the understanding of its function, and the functions of additional MADS-box proteins in maturing fruits [30, 31]. Recently, the MADS-RIN protein has been implicated in cold-induced ripening of 'Bartlett' pear [32].

Climacteric respiration primarily consists of the combined activity of cytocyrome c electron transport and electron transport via the alternative oxidase (AOX) pathway, both of which take place in the mitochondria. The alternative respiratory pathway provides a secondary shunt for electrons at times in which respiratory demands are high and the cytochrome pathway is at full capacity. As such, AOX activity prevents overreduction of the ubiquinone pool and serves to help maintain cellular redox homeostasis. Several studies in climacteric fruit have suggested that alternative oxidase activity affects ripening through the propagation of a mitochondria-derived signal [33–35]. This signal is believed to be initiated as a result of imbalance in the cellular redox state when presence of reactive oxygen species is increased, often under conditions of stress and in the presence of ethylene signaling [36]. Furthermore, several studies implicate crosstalk between ethylene, ROS signaling, and AOX activity in maintenance of metabolic homeostasis [37]. In mango, the climacteric stage is facilitated by the up-regulation of cytochrome chain components, and AOX transcript and protein abundance increase after the climacteric peak, reaching a maximum when the fruit is ripe [38]. Moreover, stimulation of AOX by exogenous pyruvate enhanced apple respiration via the alternative respiration pathway at climacteric under cold storage [39]. Similar observations have been recorded in banana, cucumber, and tomato where cold treatment enhanced AOX abundance [40–42]. Collectively, these studies demonstrate that AOX is a product of post-climacteric events and contributes to senescence after the ripening phase.

The transition from the autoinhibitory System 1 to autocatalytic System 2 ethylene production occurs naturally during the developmental course of many fruits such as tomato, apple, peach, and banana. The postharvest cold treatment has been shown to enhance and synchronize production of System 2 ethylene in some pome fruits such as 'Conference' pear and 'Golden Delicious' and 'Granny Smith' apple [43, 44]. Pre- or post-harvest exposure to cold has also been shown to induce ethylene production, and fruit softening in avocado (*Persea americana*) and kiwifruit (*Actinidia deliciosa*) [45, 46]. However, in some cultivars of European pear (*Pyrus communis* L.), a period of exposure to cold temperature after harvest, also called as pre-ripening period, is required for induction of System 2 ethylene production [47, 48]. Such post-harvest cold exposure to ripen fruit has been termed 'conditioning' [49–51]. Previous studies have characterized the conditioning needs for different pear cultivars in terms of storage temperatures, exogenous ethylene exposure and preharvest treatments [49, 50, 52, 53]. In the absence of exogenous ethylene, conditioning can be achieved by storing the fruit at -1˚ to 10˚C for 1–15 days for 'Bartlett' to 60 days for 'D'Anjou' to 90 days for 'Passe Crassane' [51]. At the other end of the spectrum are several Japanese pear (*Pyrus pyrifolia*) cultivars which have no conditioning requirements and are regarded as non-climacteric. In this context, *Pyrus* displays a spectrum of fruit phenotypes in terms of response to cold, induction of S2 ethylene, and the onset of ripening [20]. Interestingly, exogenous application of ethylene can either replace or reduce the need for conditioning and initiate the ripening climacteric, suggesting the existence of cold-induced regulatory processes that act independently of the S1-S2 transition [51].

While general biochemical pathways involved in ripening of model climacteric fruits are well studied, more targeted research is necessary to understand cultivar-specific kinetics and interactions of key ripening-related enzymes in European pears, especially in response to low-temperature conditioning. Previously documented biochemical and genomics data on pear ripening have revealed a complex regulatory crosstalk between numerous phytohormones, secondary messengers, signaling pathways, respiration and chromatin modification [5, 11, 32,

54–56]. Differential regulation of these pathways can generate a spectrum of ripening or post-harvest phenotypes, including delayed or accelerated senescent fruit, and fruit with altered sugar, volatile and nutritional content [15, 57]. Cold-induced physiological responses have been shown to involve various phytohormones such as abscisic acid (ABA), auxin, jasmonic acid, and respiration-related signaling [32].

Several facets of cold-induced ripening in *Pyrus communis* are becoming clearer. Extensive studies in fruit and non-fruit crops have identified the presence of C-REPEAT BINDING FACTOR (CBF)-dependent and independent signaling pathways. A CBF-independent pathway has been shown to modulate the propagation of the cold-signal in various plant tissues through the concerted roles of various phytohormone, messenger, and additional elements [58]. It is expected that phytohormone, respiration and environmental-signaling pathway-targeted analysis of gene expression across European pear cultivars that differ in their cold requirement may reveal insights that integrate and regulate the critical transition from S1 to S2 ethylene production [59, 60].

This study was conducted with a focus on expression changes of key genes involved in ripening-associated biochemical pathways as fruit cultivars representing extreme ends of the chilling requirement spectrum, 'Bartlett' and 'D'Anjou,' underwent physiological conditioning by exposure to predetermined amounts of cold. Nonmetric multidimensional scaling (NMDS) was used to assess relationships between multiple experimental factors of genotype and physiology and the associated expression of key genes. NMDS is a multivariate data reduction technique that identifies axes describing variability among sample units with many measured response variables [61]. The method condenses the many measured variables in a multivariate data set into a reduced number of axes that maximize explained variance. Unlike a number of other methods, however, the method does not require that the measured variables be linear or scaled similarly. This method was used to accommodate the disparity in a large number of data points represented by expression values of individual genes, and a relatively lower number of biological replicates [62]. The NMDS analysis of physiological ripening and expression of target genes revealed that 'Bartlett' and 'D'Anjou' fruits follow two dissimilar vectors in response to cold conditioning, which has implications in preventing post-harvest losses of this important crop.

Materials and methods
Physiological conditioning
For this study, fruit was harvested at physiological maturity from two commercial lots in central Washington state. The fruit was obtained within five days of harvest after 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 the time of the initiation of the experiment. Pears of each cultivar were divided into two replicate groups of 1920 fruits each, which were then maintained at 10˚C (Fig 1) for conditioning. Control fruit that did not undergo conditioning were maintained at 20˚C for the entirety of the experiment [49]. After the conditioning period, 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. The fruit was evaluated for firmness at three-time points: at harvest (i.e., 0% conditioning), 100% conditioning, and 100% ripened, which comprised 7 d after completion of conditioning. Tissue samples containing both peel and flesh peeled from the fruit were also collected at the stages mentioned above for subsequent comparative gene expression analysis.
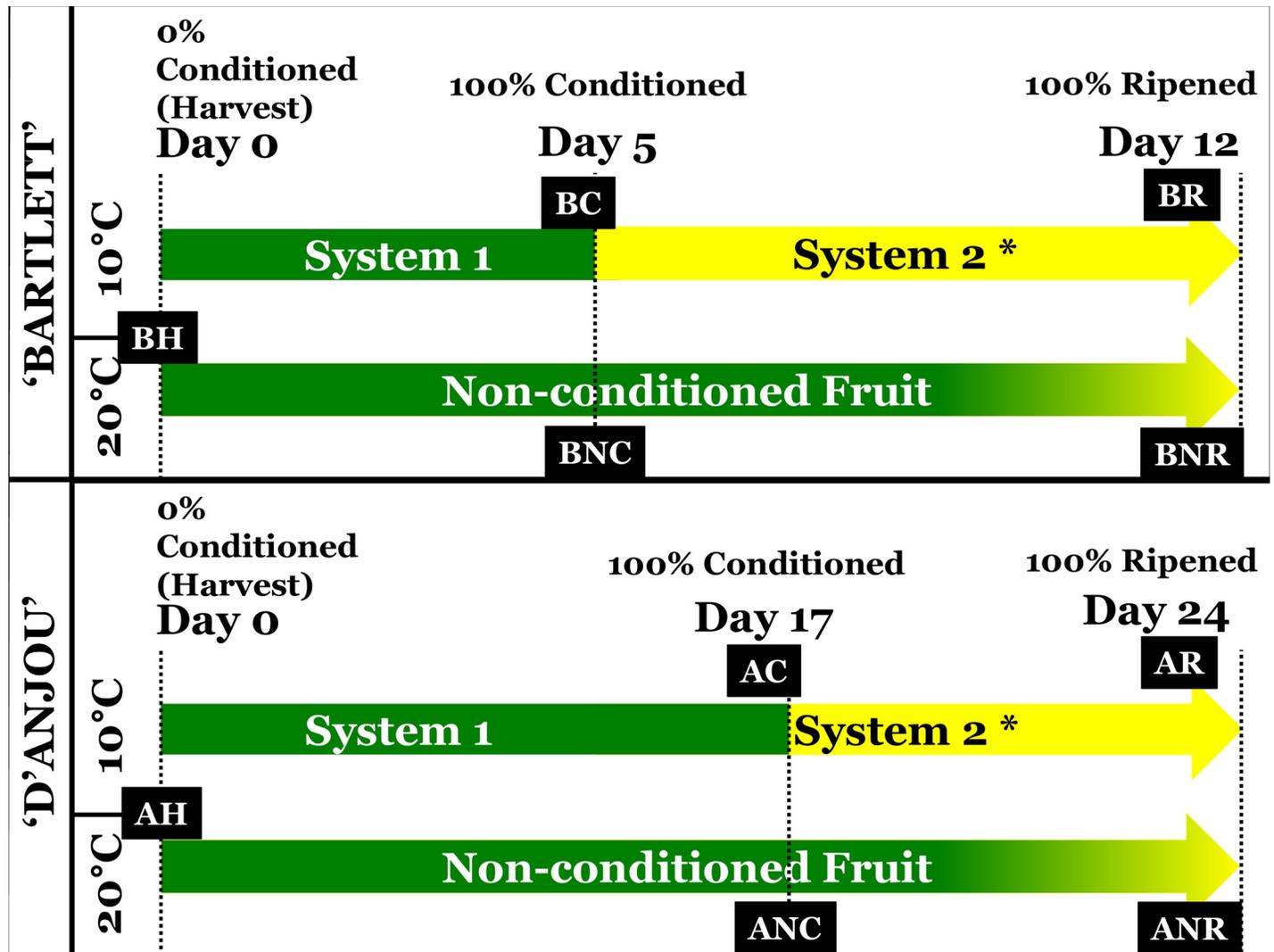
Fig 1. Treatment and sampling scheme for 'Bartlett' and 'D'Anjou' fruit. 1,920 fruit of each cultivar were equally distributed. Tissue samples were obtained from Non-Conditioned control fruit maintained at 20°C in parallel to a sampling of fruit that received conditioning treatment. Conditioned fruit was moved to isolated flow-through respiration chambers at 20°C for one week and samples harvested at that time. BH and AH–'Bartlett' and 'D'Anjou' fruit two days after harvest; BC, AC—'Bartlett' and 'D'Anjou fruit at 100% conditioning timepoint; 'BNC, ANC—'Bartlett' and 'D'Anjou' Non- Conditioned control fruit corresponding to 100% conditioning timepoint for fruit that received conditioning; BR, AR—'Bartlett' and 'D'Anjou' fruit at 100% Ripened stage; BNR, ANR—'Bartlett' and 'D'Anjou' Non-Conditioned control fruit corresponding to 100% ripening timepoint for fruit that received conditioning.

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Fruit firmness

Fruit firmness was measured at each sampling time point, and peel tissue samples were collected from 10 replicate fruit. Firmness was obtained from two equidistant points around the equatorial region of each fruit after removal of the peel with a GS-14 Fruit Texture Analyzer (GÜSS Instruments, South Africa) equipped with an 8.0 mm probe set at 5.0 mm flesh penetration. To determine significant factors impacting changes in fruit firmness, data were assessed using ANOVA, following the statistical approaches described previously [53, 63].

RNA isolation and cDNA preparation

Peel tissue was obtained from 1 cm wide equatorial region of 3 randomly selected fruit for each treatment and flash-frozen in liquid nitrogen. The tissues were then ground using a SPEX

Freezer/Mill 6870 (Metuchen, NJ USA). Total RNA was extracted from 3 representative time points–harvest, fully conditioned fruit maintained at 10˚C, and fully ripened fruit derived from fruit conditioned at 10˚C following the method described previously [64]. For these time points, corresponding control tissues were also sampled from fruit maintained at 20˚C. For cDNA preparation, RNA samples were treated with DNaseI to eliminate any DNA contamination according to the manufacturer's methods (NEB, Ipswich, MA USA). The RNA concentration was determined for each sample using a Nanodrop ND-8000 (ThermoFisher, MA, USA). The 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 random primers provided in the Invitrogen VILO kit (Life Technologies, Carlsbad, CA USA). Product integrity was checked using agarose gel electrophoresis. Concentration for each cDNA preparation was evaluated using a Qubit fluorimeter (Life Technologies–Carlsbad, CA, USA). 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). The genes were selected based on a comprehensive literature review to represent phytohormone, secondary messenger and environmental signaling pathways (S3 File summarizes the source of literature used to develop the list of genes involved in S1-S2 ethylene transition and regulation). Reactions were prepared according to the manufacturer's protocols with 100 ng template cDNA. For the amplification phase, samples were denatured at 95˚C for 2:30 min, followed by 50 cycles of 30 s at 95˚C, 30 s at 60˚C annealing temperature and 30 s at 72˚C. For the dissociation phase, samples were denatured for one cycle at 95˚C for 30 s, annealed at 60˚C for 30 s and denatured gradually to 95˚C in increments of 0.5˚C to obtain the dissociation curve.

Primer design for qRT-PCR

Primers were designed using Primer3 software (http://frodo.wi.mit.edu/) using either the doubled haploid 'Comice' genome [65], or from *Pyrus* ESTs and *Malus × domestica* genome [66] as a template, and were procured from Sigma–Aldrich (St. Louis, MO). The primers were evaluated to ensure single amplicon amplification. Amplicons were gel-extracted, sequenced, then annotated and validated using BLASTX against the NCBI nr database. Primer name, sequences, target gene, and amplicon sequence, are summarized in S3 File.

Quantitative analysis of targeted gene expression, NMDS analysis

To account for PCR efficiency in the data, Cq values and efficiencies were calculated for each reaction using the LinRegPCR tool [67, 68] (S4 File). Confidence in Cq values resulting from efficiencies below 1.80 or above 2.20 was marked where appropriate by the gene target in further analyses. The Cq values whose efficiency were within these bounds, but exceeded (or equaled) 40.00, were deemed unacceptable and identified in downstream analysis. Similarly, Cq values between (or equal to 35.00–39.99) were marked as '*Low confidence'*, where appropriate, by the gene target in further analyses.

Following this, fold-change expression was determined from Cq values of all gene targets (across 4 replicates of all samples) between 'D'Anjou' and 'Bartlett' cultivars using the Pfaffl method [69]. Expression of individual genes was normalized in reference to the geometric mean of *Pyrus communis* β-tubulin and RELATED TO UBIQUITIN1 (RUB1) Cq values, identified as ideal reference genes with NormFinder [70–72] (S6 File). Sequences of these amplicons were determined using Sanger sequencing, then checked for target amplification using BLASTX against the NCBI nr database (S3 File) [73, 74]. This allowed identification of variable expression of individual genes between samples, following methods reviewed previously [75].

NMDS. To determine genes contributing the most to variability in the experiment, Cq values for all remaining gene targets in all biological replicates of all samples were converted into a community matrix (*n* samples by *p* genes) for nonmetric multidimensional scaling (NMDS) using the R package '*Vegan*' [76, 77]. The NMDS process assigns rank-order of each gene expression measurement (Cq in this case) across all samples, then depicts variability in a reduced dimensional space [78]. Graphical or statistical assessment of the grouping of individuals within treatment groups may then be performed to examine dissimilarity in overall gene expression within and among treatment groups. In the present study, these group membership factors included pear cultivar ('Bartlett' and 'D'Anjou'), phenology (harvest, fully conditioned, fully ripened) and conditioning treatment (conditioned, non-conditioned control). To the assess goodness of fit of the final ordination, a stress coefficient was calculated from the data matrix (S5 File).

Within the resulting ordination space (NMDS axis 1 × NMDS axis2), the radial distance of individual gene-associated ordination scores from the origin was calculated and represented as an assessment of contribution of that gene to variability. Pear cultivar membership appeared to be strongly related to NMDS axis 1, while phenology (*harvest*, *conditioned*, *or ripened*) appeared to be strongly related to NMDS axis 2 (S10 File). Sorting radial distance of the plotted points from the vertex produced a list of genes in order of descending contribution to variability. Some additional targets were added to the final list of targets for which additional technical replicates were sought based on *ab initio* and prior unpublished data. From the original set of 90 selected candidates, genes that had the top 25% of joint biplot lengths (radial distance) (S7 File), along with a few additional genes known to be involved in the regulation of ripening were selected. A total of four replicate reactions were performed for 36 gene targets in all biological replicates of all samples. A second two-axis NMDS ordination was performed for 36 targets to visualize variability as a function of each treatment and gene target. A centroid hull plot was generated from expression data among the unique variety-conditioning-phenology treatment combinations in RStudio (raw output in S11 File). Finally, a ray biplot was generated from 12 ABA, auxin, ethylene, cold-signaling and respiration-related genes among this final set to indicate relative contributions of pear cultivar and ripeness to the expression of these highly variable transcripts (raw output in S12 File).

2- ΔΔCt, Comparative analyses and visualization. Cq values were calculated following methods described previously by Pfaffl [79]. In addition to NMDS ordination, fold-change values for individual genes were analyzed to identify highly variable expression at equivalent stages of fruit phenology between conditioned and nonconditioned samples. Gene-by-gene comparisons were conducted after the fold-change data were rendered into a heatmap using a web-accessible tool, Morpheus (raw output as S13 File) [80]. In this study, due to the specific experimental design and limitations of assumptions associated with ANOVA or pairwise t-tests, differences in expression were visualized using a heatmap, and then ranked in a decreasing order before being compared with the results of NMDS ordinations. Genes that showed low-confidence qRT-PCR reaction efficiency or exceeded the parameters described above were marked on the resulting heatmap with an *.

Results and discussion

In the United States, 97% of the pear orchards exist as low-density plantings with large three-dimensional trees [81–83]. The tree architecture and orchard organization have a significant impact on the physiological quality of the fruit [84, 85]. In order to reduce the extent of variability in fruit quality, fruit used in this study were procured from a commercial warehouse that had been pre-sorted for size. However, it should be noted, that sorting for size does not

necessarily control for variability in the physiological maturity of the fruit, which is affected by canopy position [85]. The conditioning treatment provided to fruit resulted in uniform ripening as evident from changes in fruit firmness, as demonstrated previously [51].
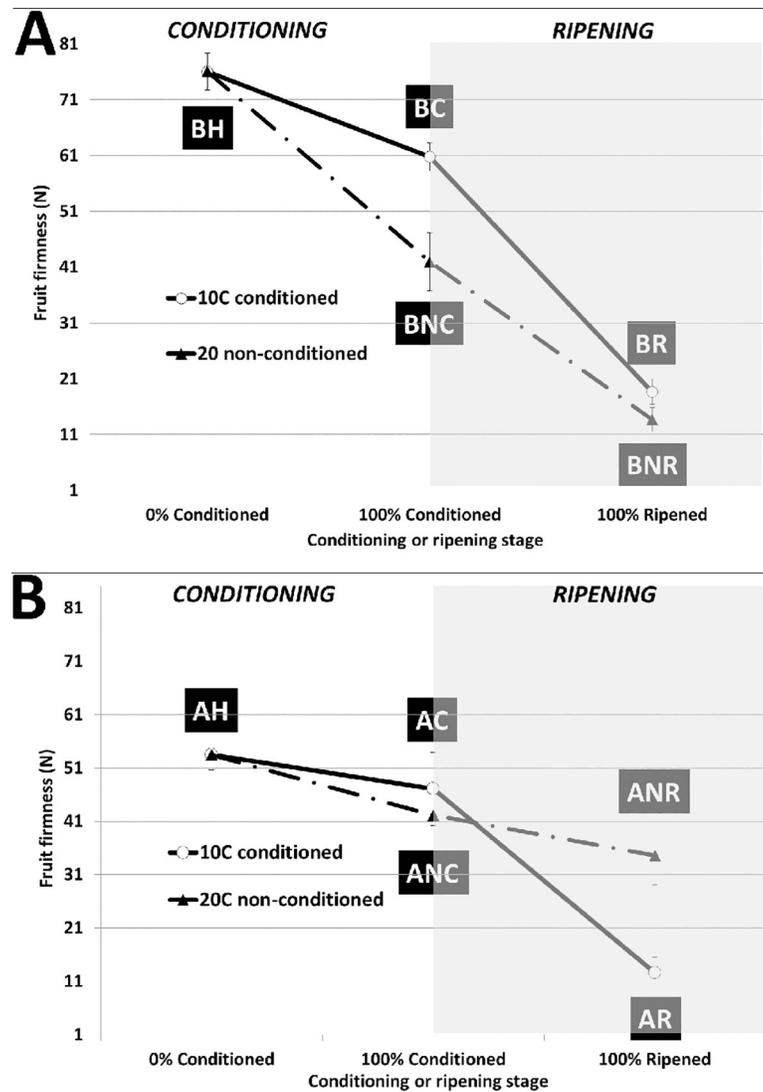
Fruit firmness

Cold conditioning of the fruit at 10°C resulted in a reduction of fruit firmness in both 'Bartlett' and 'D'Anjou' cultivars as was demonstrated previously [49, 53, 86]. 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' (Fig 2A). Ripening of 'Bartlett' requires 15 d of cold conditioning, while 'D'Anjou' typically requires 60 d of -1°C to attain ripening competency [87, 88]. The duration of cold conditioning, however, was reduced when conditioning temperatures were increased to 10°C [49]. 'D'Anjou' pears at advanced physiological maturity stages, achieved through delayed harvest, also ripened with markedly shorter conditioning periods [49]. The rate of softening showed high variability throughout ripening, particularly during the post-conditioning week while the fruit was maintained at 20°C (Fig 2, S1 File). Conversely, fruit that did not receive cold conditioning, particularly D'Anjou' fruit, failed to soften appreciably (Fig 2B).

Quantitative analysis of ripening-related genes and NMDS analysis

A comprehensive literature review was used to shortlist 90 key ripening-related genes. The genes represented phytohormone, secondary messenger, and environmental signaling pathways. The expression of these genes was analyzed for both cultivars at different temporal stages during conditioning and ripening. A large number of gene targets in comparison with a limited number of biological replicates, as in most gene expression studies, presents a key challenge to the dimensionality of the experiment [62]. One methodological solution to this challenge is the use of data reduction methods such as ordination, in which the information encoded in many independent variables is distilled into a few dimensions that maximize explained variation [89]. The NMDS procedure assigns rank-order of the measurement associated with each unique treatment combination, then depicts variability in a dimensional space that displays dissimilarity between samples [76, 78, 90]. This approach allows for visualization and pattern recognition among data from numerous samples, including the potential influence of experimental treatment variables. To assess goodness of fit of the final ordination, a stress coefficient is calculated from the data matrix, representing the variability (dissimilarity and dispersion) captured by the set n dimensions (S8 and S9 Files).

The premise of this study, based on non-uniform fruit, warranted a reconsideration of the statistical tests to be applied to the results and their subsequent interpretation. Recent studies have suggested for a need to revisit the interpretation of ANOVA, and pairwise t-tests applied to biological data, including fruit texture analysis, gene expression and others [75, 91–93]. To provide physiological context and following protocols applied in prior studies, traditional ANOVA was utilized to identify significant treatment factors in flesh firmness data [63]. Some of these reports focus on the identification of differential expression from microarray data, which features much higher replication than the analytical qRT-PCR data utilized in this study. Indeed, analysis of qRT-PCR data and identification of significant variance in transcript abundance between samples remains challenging due to assumptions inherent to traditional ANOVA and t-tests. Specifically, these tests assume equal heterogeneity of variance across biological replicates. Prior reports have demonstrated variable maturation and phenology between fruit depending on location in the canopy, harvest time, and other factors which would introduce large amounts of variance in the fruit, and cDNA preparations derived from

Fig 2. Mean fruit firmness (N) through conditioning (white background) and ripening (grey background) in A. 'Bartlett', and B. 'D'Anjou' pear. Fruit was placed into conditioning two days after harvest. Error bars represent standard deviation from the mean of measurements recorded from 10 replicate fruit. Black boxes correspond to fruit treatment sampling stage as follows: BH and AH–'Bartlett' and 'D'Anjou' fruit two days after harvest; BC, AC—'Bartlett' and 'D'Anjou' fruit at 100% conditioning timepoint; 'BNC, ANC—'Bartlett' and 'D'Anjou' Non- Conditioned control fruit corresponding to 100% conditioning timepoint for fruit that received conditioning; BR, AR—'Bartlett' and 'D'Anjou' fruit at 100% Ripened stage; BNR, ANR—'Bartlett' and 'D'Anjou' Non-Conditioned control fruit corresponding to 100% ripening timepoint for fruit that received conditioning.

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them [85, 94, 95]. In order to account for the variability, several methods have been proposed including the TREAT-method (*t*-tests relative to a threshold), which frames p-values derived from *t*-tests against an *ab initio*-derived, biologically meaningful point of significance [91]. More recent responses to these challenges propose that inclusion of graphical-estimate approaches may complement, or supplement traditional null-hypothesis based statistical testing, [93]. In this study, nonmetric multidimensional scaling (NMDS) was used for the visualization of broad trends in fruit phenology, as well as the contribution of individual genes in the context of cultivar and treatment.

Nonlinear approaches such as those used in the analysis of qRT-PCR expression data have been used in prior work. Olsvik et al., visualized gene expression with Principal Component Analysis (PCA), also a nonparametric approach, to identify optimal reference genes from a list of targets [96]. This enabled rapid, intuitive selection of those targets exhibiting minimum global variability in the examination of responses in oceanic fish, a heterogeneous environment imparting high experimental dimensionality. Calculation of this global variability is also used in the NormFinder tool described above in the initial analysis of qRT-PCR data. Similarly, a 'progress curve' fitting approach has been proposed in which all fluorescence data points of all reactions are utilized in fitting to the sigmoidal or logistic-growth curve model [97]. NMDS was selected to assess patterns in gene expression with no assumptions of linearity in the data, similar scaling of expression values, or other constraints associated with several other ordination approaches. The reduction of many dimensions associated with a large number of measured genes to a much-reduced number of ordination axes allows the efficient exploration of variability within and among groups.

The initial NMDS ordinations achieved stability after 20 iterations, resulting in stress values ranging from 0.18–0.20 and capturing over 95% of the variability in gene expression data by the pear cultivar and phenology axes [*NMDS axis 1* (graphically inferred to be associated with cultivar) and *NMDS axis 2* (graphically inferred to be associated with phenology)] (S5 File). This indicated that substantial variability among the expression dataset was represented by cultivar and conditioning phenology (Fig 3).

Prior studies have reported a correlation between phenology and the expression of ripening related genes [56, 98, 99]. However, NMDS analysis provides an approach that can capture major axes of variance within a multivariate data set, regardless of the scaling of the variables, and allow for interpretation of the sources of variability. Sorting radial distance of variability in the expression of genes (according to NMDS axes 1 and 2) revealed numerous phytohormone and cold-signaling and additional genes in the approximate top third (S10 File). Further, expression data sorting revealed a tendency to form clusters by cultivar and treatment factors. A rightward-shift is seen in 'Bartlett'-derived expression data in initial and final ordination spaces, relative to 'D'Anjou'-derived expression values. Expectedly, unconditioned controls occupied different regions in the ordination space relative to conditioned fruit of the same cultivar. While unconditioned 'Bartlett' samples remained stationary along the NMDS axis 1 (cultivar), they grouped to the right of (higher axis 1 score) conditioned fruit. Alternatively, unconditioned 'D'Anjou' samples reverted to the left of (lower axis 1 score) conditioned samples. Together, this pattern in the ordination space reveals that there is a pre-existing genotypic variation between 'Bartlett' and 'D'Anjou' fruit regardless of conditioning treatments. This information could be used to devise more efficient, cultivar-specific, conditioning strategies to optimize fruit ripening and quality.

Cq values from ripened fruit samples were generally higher on axis 2 (ripeness) of the ordination space, possibly indicative of the shared physiological responses in the fruit of each cultivar once they acquired ripening capacity post-conditioning. However, 'D'Anjou' fruit exhibit different overall transcriptional responses compared to 'Bartlett' tissues after full conditioning. Of all the transcripts probed in this work, those from 'Bartlett' tissues exhibited a decline in their values on axis 2 of the ordination space initially, followed by a large increase when the fruit begins to ripen during the S1-S2 transition. However, 'D'Anjou' tissues remain comparatively stable for axis 2 in the ordination space, suggesting underlying differences in conditioning response of this cultivar as shown in Fig 3. 'BH' and 'AH'–'Bartlett' and 'D'Anjou' fruit at harvest represents the fruit when the experiments were initiated; BC, AC—'Bartlett' and 'D'Anjou fruit at 100% conditioning timepoint; 'BNC, ANC—'Bartlett' and 'D'Anjou' Non-Conditioned Fruit corresponding to 100% conditioning timepoint (see Fig 1);
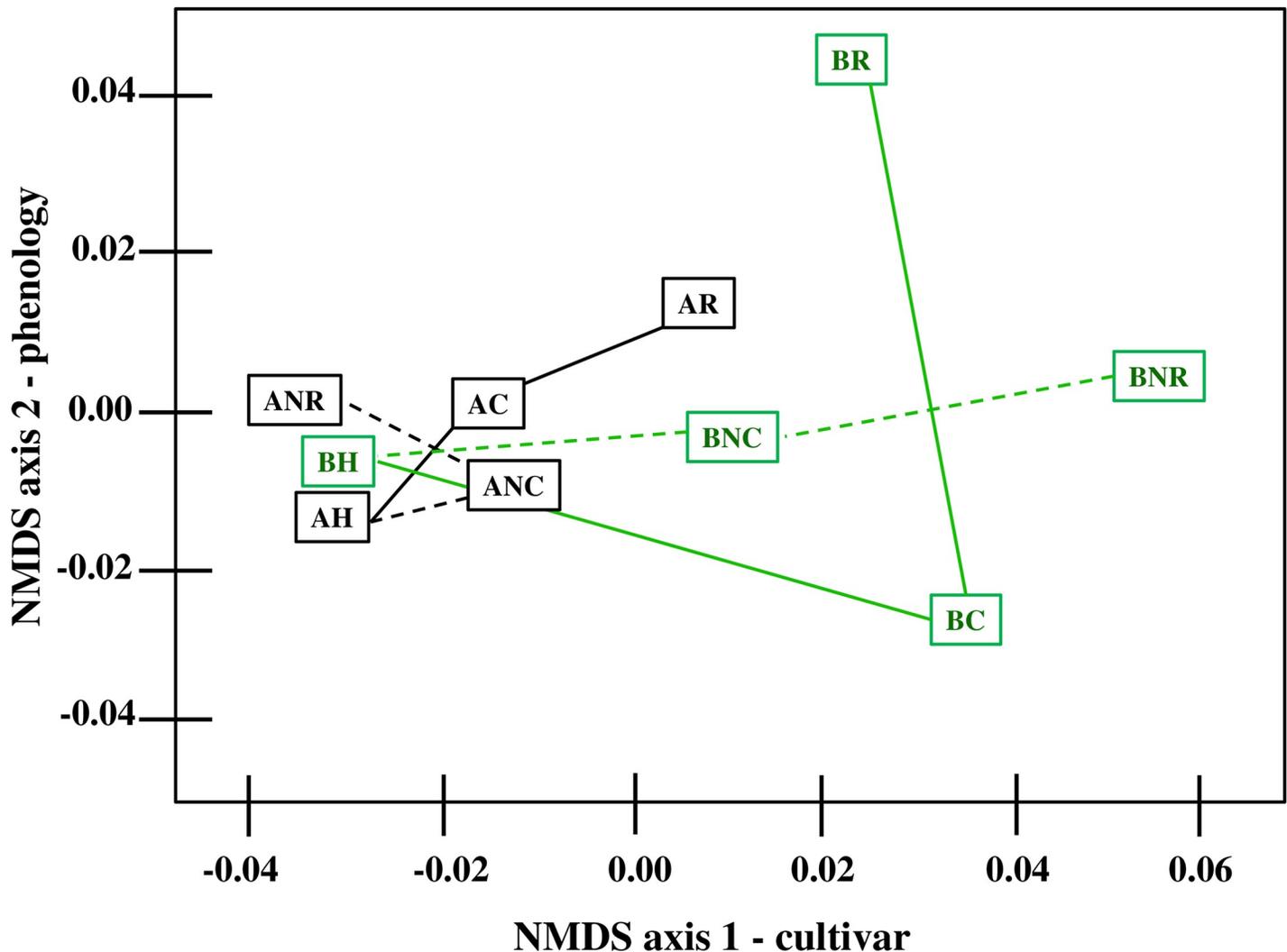
Fig 3. Geometric treatment group hull centroid plot from final NMDS ordination representing a grouping of expression data according to pear cultivar, ripeness and conditioning treatment in the two-dimensional NMDS ordination space of *x*-axis and *y*-axis (correlating to cultivar and phenology, respectively). Dashed lines indicate unconditioned control fruit held at 20°C while solid indicate samples given conditioning treatment. Black lines indicate 'D'Anjou' while green lines indicate 'Bartlett'. BH and AH–'Bartlett' and 'D'Anjou' fruit two days after harvest; BC, AC—'Bartlett' and 'D'Anjou' fruit at 100% conditioning timepoint; 'BNC, ANC —'Bartlett' and 'D'Anjou' Non- Conditioned control fruit corresponding to 100% conditioning timepoint for fruit that received conditioning; BR, AR—'Bartlett' and 'D'Anjou' fruit at 100% Ripened stage; BNR, ANR—'Bartlett' and 'D'Anjou' Non-Conditioned control fruit corresponding to 100% ripening timepoint for fruit that received conditioning. Graph recreated from raw NMDS output in R with Microsoft PowerPoint. Original R output available in S11 File.

BR, AR—'Bartlett' and 'D'Anjou' fruit at 100% Ripened stage; BNR, ANR—'Bartlett' and 'D'Anjou' Non-Conditioned Fruit corresponding to 100% ripening timepoint (see Fig 1). These findings suggest that axis 1 may be discriminating relative 'conditioning need' or 'propensity for S2-ethylene induction and ripening', while axis 2 may discriminate ontogeny of the fruit or relative stage of ethylene production, or ripening.

Data from non-conditioned control samples occupied different regions in the ordination space and followed a different vector relative to conditioned fruit of the same variety. While non-conditioned 'Bartlett' samples (see BH, BNC, BNR, Fig 3) remained unchanged along the NMDS axis 1, they grouped to the right of (higher axis 1 score) conditioned and ripened fruit (see BH, BC, BR, Fig 3). This is consistent with their ripening behavior, where prolonged

storage at 20°C can soften the fruit, but not necessarily ripen it completely. Alternatively, non-conditioned 'D'Anjou' samples reverted to the left of (lower axis 1 score) conditioned samples (see AH, ANC, ANR, Fig 3). These patterns in the ordination space illustrate inherent cultivar-specific differences between 'Bartlett' and 'D'Anjou' fruit before conditioning treatments. Overall, this plot helps visualize differential transcript abundance and provides a basis for understanding how ripening responses manifest in genetically different pear cultivars subjected to cold conditioning. 'Bartlett' pears transitioned from green to yellow as they ripened, while 'D'Anjou' pears generally retained the green peel color.

Observed changes in expression of the selected genes in the ordination space, which represent independent variables of phenology, cultivar and treatment, align well with the physiological and molecular models of ripening [15, 24, 47, 56, 98, 100]. Recently, Nham et al., identified multiple ABA, auxin, and jasmonic acid-related signaling transcripts as potential putative regulators of cold-induced ripening in European pear, supporting the outcomes of this study [32]. Biplot representation of selected gene vectors and relationship to NMDS ordination axes showed clear grouping according to pear cultivar, correlating calculated radial distance of genes from the vertex of the ordination plot (Fig 4).

Following the second NMDS ordination of expression data from the final set of selected gene targets, distinct associations between expression patterns of genes with NMDS axes 1 (cultivar) and 2 (phenology) were observed. This indicates that the NMDS approach is an additional avenue to visualize multiple independent variables in a statistically relevant space to identify the most important elements that contribute to variability. This may be especially relevant for RNAseq studies where the number of analyzed genes will always be overwhelmingly higher than the number of biological replicates.

The emerging pattern after two rounds of NMDS ordination was that the final third of gene targets focused on in subsequent 2-($\Delta\Delta$Ct)-parametric analysis comprised putative regulatory control points in the following signaling pathways: cold-perception, abscisic acid signaling, ethylene signaling, auxin signaling, mitochondrial or peroxisomal metabolite transport, and respiration-related genes. The expression behavior of selected genes is discussed in the context of the metabolic pathways they participate in during conditioning and ripening.

Cold-perception and ABA signaling. Conditioning temperatures for pear typically range between 0–10°C; this is well within the range of temperatures at which cold-stress is experienced in any other fruit tissues that do not require prolonged cold-conditioning to acquire ripening competency [16]. The range of conditioning requirements of European pear cultivars indicates variation in cold-stress responses, which is manifest via differential phytohormone and secondary messenger transmission of cold-induced signals. The data were analyzed to explore how the propagation of cold signals varied between 'Bartlett' and 'D'Anjou' tissues. In both Arabidopsis and climacteric fruits, cold acclimation requires activation of the CBF cold response pathway. ICE1 and HOS1 (INDUCER OF CBF EXPRESSION 1 and HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1, respectively) comprise initial signaling-elements in cold-perception and initiate the downstream CBF-pathway [21, 101, 102]. Relatively stable expression of the cold signal inducers across pear cultivars and in response to conditioning treatments suggests the presence of an alternative cold-responsive pathway compared to other fruit. Potential candidates could be ABA-signaling or a prolonged cold-responsive RARE COLD INDUCIBLE (RCI)-pathway specific to pear [103]. Differential expression of the cold-signaling transcription factor HOS1 was not observed, though it did appear among the genes contributing the most to total variability in expression among the data. Similarly, analysis of expression from initial technical qRT-PCR replicates did not reveal much variability in ICE1 or CBF-like expression. This highlights the important insights gained
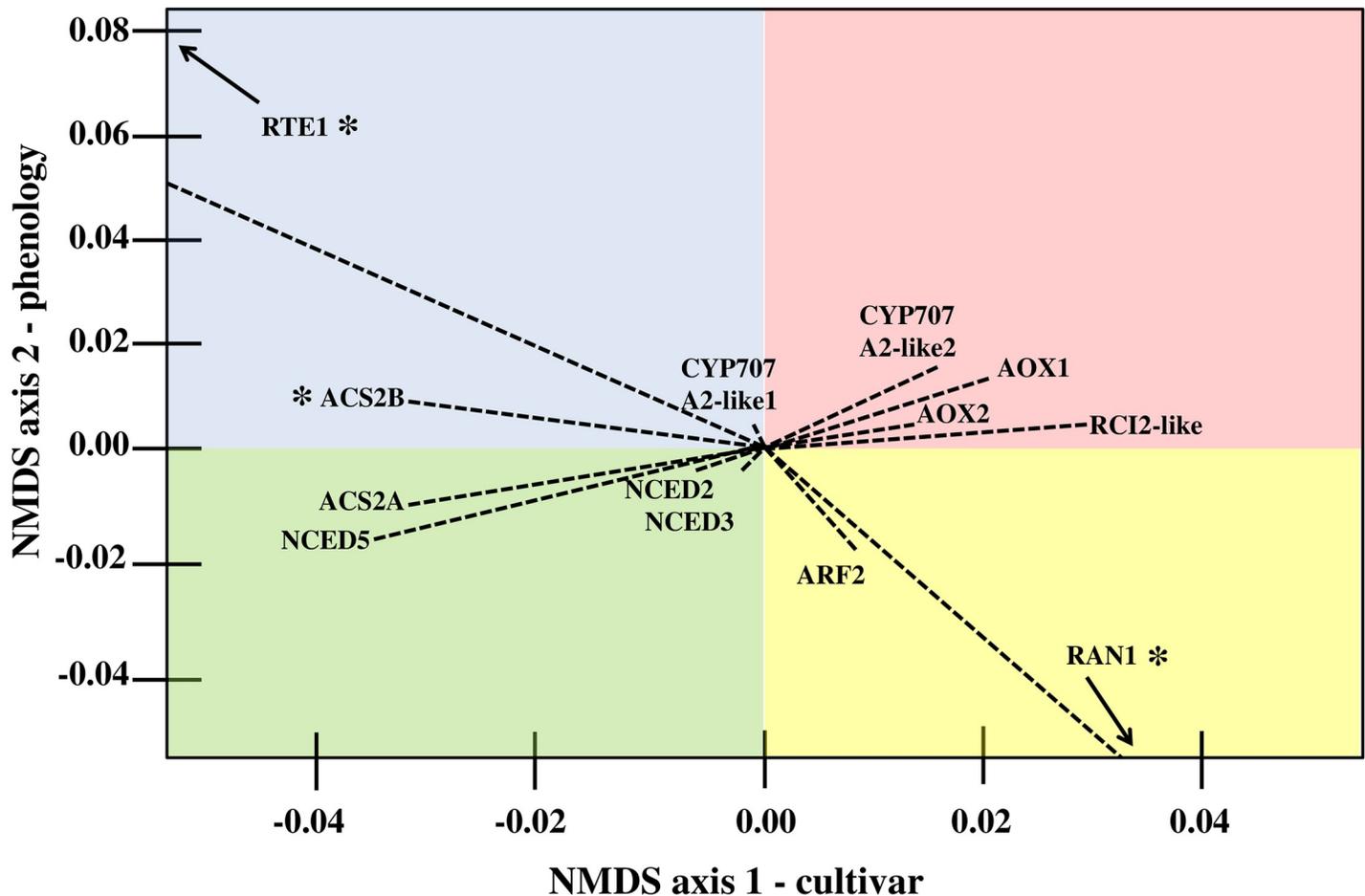
Fig 4. Ray biplot representation of NMDS axis 1 and axis 2 (correlating to cultivar and phenology on the *x*-axis and *y*-axis, respectively) contributions to expression variability between select gene targets from final NMDS ordination. Dashed lines represent vector associated with genes in ordination space. Image recreated from raw NMDS output in R. Quadrant shading added to highlight vector separation and is not correlated to, or suggestive of, any physiological state. *-indicates 'low confidence' values, defined as those from which mean Ct equaled or exceeded 35.00, or whose efficiency exceeded 1.80–2.20 in at least one replicate reactions. Raw R output available in S9 File.

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into gene expression patterns using a spatial reduction approach, compared to an ANOVA-based comparison of gene expression.

ABA and auxin-pathways have been implicated in cold-induced signaling [104, 105]. Variation of transcript abundance from ABA-biosynthetic and signaling genes was observed in this study. Two pear homologs of ABA-biosynthesis related 9-CIS-EPOXYCAROTENOID DIOOXGENASE (NCED)-like genes were found to associate with the left-side of the NMDS-ordination *x*-axis, corresponding to 'D'Anjou' fruit. Pear CYTOCHROME P450, FAMILY 707, SUBFAMILY A POLYPEPTIDE (CYP707A2)-like expression exhibited slightly divergent orientation in the ordination space. The CYP707A2-vector is extending toward the right of the NMDS axis 1, indicating an enhanced expression in 'Bartlett' throughout the experiment. ABA accumulation may positively influence the transition towards System 2 ethylene biosynthesis and ripening, with steady-state levels significantly impacted by NCED-like, and CYP707A2--like transcript abundance following harvest [20]. A similar impact of ABA accumulation has been reported in peach, where nearly 900 ABA-related differentially expressed genes were correlated with variable cold responsive-phenotypes [21]. Analysis of the data in this study

suggests that ABA biosynthetic activity may vary in pear and depend on the conditioning needs of individual cultivars.

The NMDS ordination suggests that the ABA-biosynthetic NCED-like gene showed a correlation with the increased cold requirement in 'D'Anjou,' while ABA-catabolic CYP707A2--like gene was associated with reduced need for cold in 'Bartlett.' Increased expression of ABA-biosynthetic NCED-like genes and reduced expression of ABA-catabolic genes was shown to correlate with the accumulation of ABA in cold-independent ripening of Asian pear. However, the expression of these genes remains unclear in cold-dependent ripening of Chinese White pear (*Pyrus bretschneideri*) [20], and in 'Braeburn' apple, which also requires cold-exposure to ripen [106]. The results in this study show variable transcript abundance of rate-limiting ABA-biosynthetic genes, providing a critical signature of cultivar-specific cold-response in European pear exposed to established conditioning protocols (Fig 4). Peak abundance of NCED-like and ABA-signaling transcripts showed a correlation to the completion of the conditioning treatment, particularly in 'D'Anjou.' At this phenological stage, 'D'Anjou' fruit had completed the conditioning requirement and had attained full ripening competency, which also marked the induction of S2-associated ACS ethylene biosynthesis [100]. The correlation between ABA-pathway transcript vectors in the ordination space share the trends and position with traditional ethylene-signaling elements, suggesting a collaborative role of these two pathways in regulating cold-induced S2-induction and ripening in European pear.

Ethylene perception and signaling. Cold conditioning initiates the S2 transition in European pear, which activates ethylene biosynthesis and downstream signaling processes. In this study, the negative and positive regulators of ethylene-signaling REVERSION-TO-ETHYLE-NE-SENSITIVITY1 (RTE1) and RESISTANT-TO-ANTAGONIST1 (RAN1), respectively, exhibited the largest changes in the biplot vector (Fig 4). The regulatory role of the two genes is evident in the leftward vector associated with RTE1 expression in the NMDS ordination space (Fig 4). Similarly, a RAN1-like transcript exhibited a rightward orientation, supporting the proposed role of this gene in promotion of ethylene signaling, and fruit ripening [107–109]. In Arabidopsis, RAN1 encodes a copper-transporting protein which physically interacts with the ETHYLENE RECEPTOR 1 (ETR1) to deliver the requisite Cu^{+1} ion required for ethylene sensitivity [110]. RTE1 and RAN1 exhibited notable directionality only in unconditioned 'Bartlett,' unconditioned 'D'Anjou,' and fully conditioned 'D'Anjou' samples.

Further, a large increase in fold-change values was observed only in the unconditioned 'D'Anjou' samples. Upregulation of an RTE1-like gene in unconditioned pear would fit with observed physiological responses in pear whose conditioning needs are not met; such fruit would fail to develop ethylene-sensitivity, engage System 2 ethylene biosynthesis or ripen. As a negative regulator of ethylene-signaling, upregulation of an RTE1 like transcript in unconditioned 'D'Anjou' may indicate a repression mechanism in these fruits. There is some evidence for a repressive role for this protein in Arabidopsis where *rte*1 mutants were able to restore ethylene sensitivity in the etr1-2 mutant [111]. In pear, expression of an RTE1-like transcript appears strongly influenced by the stage of conditioning/ripening and cultivar. The magnitude change in RAN1 vector indicates its abundance is strongly impacted by cultivar and phenology, providing the possibility of control of ethylene receptor biogenesis or sensitivity via access to the required copper ion cofactor. The autocatalytic feedback associated with S2 ethylene may be triggered as a result of this altered receptor activity. The pear qRT-PCR product showing homology to RAN1 CDS was sequenced, and its identity confirmed using BLAST (S3 File). A pear RAN homolog was induced at high levels in early ripening, possibly indicating its role in conferring enhanced ethylene sensitivity to the fruit, which is a requirement for S2 ethylene production [112]. Characterization of RAN and RTE1-like homologs in pear may help

determine if these genes indeed exhibit regulatory control over the ripening competency, ethylene signaling or biosynthesis, or cold-signaling.

Pear ACS2-like transcripts exhibited vectors near the vertex of the ripeness axis in the biplot but were located to the left, supporting the role of ACS2 in S1 to S2 transition and ethylene biosynthesis during conditioning [99]. This narrow transitionary period also appears to coincide with increased RARE COLD-INDUCIBLE (RCI)-like expression. The role of RCI-like genes is poorly understood outside of a few model systems. In Arabidopsis, RCI-proteins attenuated ethylene biosynthesis and cold-acclimation by destabilizing ACS proteins. The 14-3-3 protein RCI1 destabilizes all three ACS types in Arabidopsis [113] via a yet to be characterized CBF- and ABA-independent cold signaling pathway. This would agree with the general functional role of 14-3-3 proteins in modulating target protein activity through physical interaction [114]. Similarly, expression of RCI-like transcripts may be regulated through a CBF- and ABA-independent pathway of cold-responsive signal transduction [115]. An RCI2-like transcript oriented toward the right of the NMDS axis 1, in alignment with the recent reports of its role in the promotion of ripening in tomato [103].

Auxin perception and signaling.   Changes in free auxin concentrations have been positively correlated to ripening induction in many other climacteric fruits [5, 15, 26, 59, 116]. In the climacteric Japanese plum, the seasonal harvest time of fruit was correlated to TIR1-like auxin receptor haplotype [117]. However, expression of a TRANSPORT INHIBITOR RESPONSE 1 (TIR1)-like auxin receptor did not vary significantly in this study. While knowledge from other climacteric fruits suggests cold-adaptive capacity, auxin-sensitivity may be related to specific pear cultivars.

Cold-responses in plant tissues may be attenuated from intracellular auxin concentrations, which in turn may be controlled by transport, conjugation, biosynthetic, and catabolic mechanisms [22]. Recent work in Japanese plum also correlates cold-adaptive capacity to auxin-sensitivity, System 2 ethylene biosynthesis and fruit ripening through a mechanism not yet characterized [117]. The abundance of some, but not all, auxin-signaling related transcripts displayed variation in this study. The vector for a transcript bearing homology to an AUXIN-RESPONSE FACTOR 5 (ARF5) oriented toward the right of the cultivar axis (toward 'Bartlett'), but towards reduced ripeness. This agrees with prior studies where a correlation between ARF-like expression to the regulation of abscisic acid and ethylene-signaling has been demonstrated [12, 118–120]. In Arabidopsis, studies have linked induction of the ARF2 transcriptional activator to ABA, demonstrating the critical relationship between these two phytohormones in mediating the response to environmental cues. In tomato, an ARF2-like homolog functions at the intersection between activities of other phytohormones impacting ethylene, abscisic acid, cytokinins, and salicylic acid signaling [121].

Transcription and respiration activators.   Climacteric fruit is characterized by a concomitant increase in respiration and ethylene production [48], processes which are expected to be coordinated by various transcriptional activators. Two MADS-box like transcripts were found to be variably expressed between 'Bartlett' and 'D'Anjou' fruit that received conditioning treatment and were held at 20˚C. Transcripts bearing homology to the *Malus × domestica* MADS-RIN like transcription factor 8 and 9-like exhibited significant differential expression in response to conditioning and phenology, but not to the cultivar. This suggests that there may be a shared mechanism between 'Bartlett' and 'D'Anjou' during the acquisition of ripening competency that may be mediated by MADS-box transcriptional regulators. The intricate roles of these important regulatory players have been detailed extensively in tomato, and research has described the complex network of interactions and subsequent regulatory influence of the MADS-RIN protein on fruit ripening induction [28, 122]. MADS-RIN/AP1-like genes play a significant role in tomato ripening and are thought to recruit the redundant

FRUITFUL1 and 2 (FUL1 and 2) MADS-box proteins to regulate fruit ripening under ethylene-dependent and independent pathways [123, 124]. Conditioned (and not ripened) 'Bartlett' and 'D'Anjou' samples exhibited far more variability in expression of both transcriptional activators, suggesting that temporal variation in MADS-box gene expression results in differential regulation of the S1-S2 transition among pear cultivars. It seems likely that the expression of this transcription factor may serve as an indicator of pear ripening competency through conditioning treatments, lending support to the growing understanding of the MADS-box/AP1/SBP-transcriptional regulatory complex that acts during the respiratory climacteric.

Interestingly, accumulation of ALTERNATIVE OXIDASE (AOX) 1-like transcripts varied during phenology of 'Bartlett' and 'D'Anjou' samples. Transcript abundance peaked in the "100% Conditioned" stage of both cultivars, which is a pre-climacteric stage, as the fruit is yet to transition to S2 stage [35]. This is intriguing since the expression of AOX has been shown to coincide with the climacteric peak, a characteristic of climacteric fruit [39, 125]. However, AOX-1 expression may have peaked between sampling time points, particularly in initial responses to conditioning environments. These results were not observed for AOX2-like transcripts, differing from trends previously reported in mango and tomato fruit [34, 126, 127].

AOX1 and AOX2-like expression has been reported in many fruit systems, with AOX isoforms displaying responses to a broad range of stresses, including cold-stress. Knock-down *AOX* in tomato delayed ripening, indicating a regulatory role of AOX [128]. Notably, AOX overexpression tomato lines were shown to be far less responsive to the ethylene signaling inhibitor 1-methylcyclopropene (1-MCP), while knock-out lines were highly responsive. Thus, 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 [34, 129]. A mechanism for the observed variation in AOX transcripts between the tested pear cultivars and other model climacteric systems is unclear, though such variation in AOX expression and activity has been reported in many plants for some time [130, 131].

Comparative gene expression analyses using LinReg PCR-corrected 2-(ΔΔCt)

The LinRegPCR workflow was applied to the 2-(ΔΔCt) expression data for 27 genes. A heatmap of the relative expression was produced using Morpheus [80], which illustrates variable patterns of selected genes between 'Bartlett' and 'D'Anjou' (Fig 5). Nearly half of the genes included for final analysis exhibited down-regulation throughout fruit phenology in 'Bartlett' and 'D'Anjou' samples relative to harvest samples. The second half of genes exhibited upregulation, exhibiting cultivar or conditioning treatment-dependent deviations.

Variable transcript abundance at the fully conditioned stage was evident for NCED-, ABI3- and ARF-like 'D'Anjou' transcripts (columns 2 and 3 of Fig 5). Similar results were apparent for 'Bartlett' (columns 7 and 8), with variability in response to conditioning evident for FUL1, ACS2, RAN1, ARF5, and CYP707A2-like transcripts. 'Bartlett' tissues exhibited generally higher AOX1 and AOX2 transcript abundance, suggesting that AOX induction in 'D'Anjou' may be muted. Enhanced AOX transcript abundance and activity has been associated with accelerated ripening in other climacteric systems in which S2 ethylene-production is impaired [35, 125]. These results highlight shared and unique transcriptional responses in these two cultivars that have a very different conditioning requirement for the transition to S2.

In this analysis approach, increased AOX-like transcript abundance coincided with that of ACS, ARF, and ABA-related genes for 'Bartlett' and 'D'Anjou' samples, suggesting that these pathways comprise at least part of a coordinated transcriptional cascade that results in S2
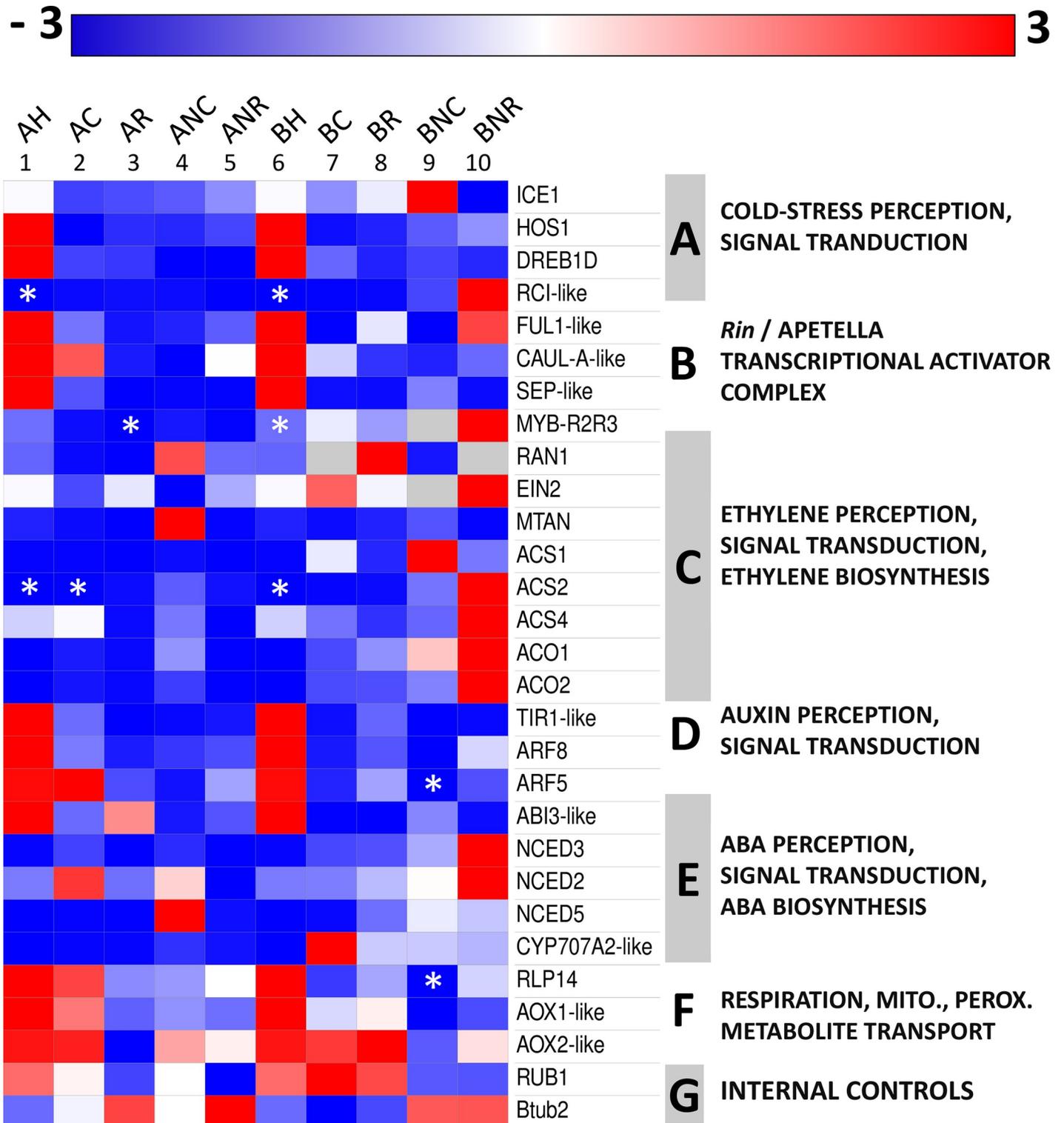
Fig 5. Fold-change gene expression values (from -3 to +3) of topmost variably expressed genes from the qRT-PCR analysis in D'Anjou (columns 1–5), and Bartlett (columns 6–10), following sorting from final NMDS ordination. Grey cells indicate that a gene was not detected in the sample. *-indicates 'low confidence' values, defined as those from which mean Cq equaled or exceeded 35.00, or whose efficiency exceeded 1.80–2.20 in at least one replicate reactions. Gene annotations on right side of the heatmap, labeled as letters A-G. A- cold signaling, B- transcriptional regulators, C- ethylene signaling, D- auxin signaling, E- abscisic acid signaling, F- peroxisomal or mitochondrial metabolite transport and respiration-related, G- internal controls. BH and AH–'Bartlett' and 'D'Anjou' fruit two days after harvest; BC, AC—'Bartlett' and 'D'Anjou fruit at 100% conditioning timepoint; 'BNC, ANC—'Bartlett' and 'D'Anjou' Non- Conditioned control fruit corresponding to 100%

conditioning timepoint for fruit that received conditioning; BR, AR—'Bartlett' and 'D'Anjou' fruit at 100% Ripened stage; BNR, ANR—'Bartlett' and 'D'Anjou' Non-Conditioned control fruit corresponding to 100% ripening timepoint for fruit that received conditioning. Heatmap generated with Morpheus with additional sample and pathways annotated using Microsoft Powerpoint. Raw Morpheus output is available in S13 File.

ethylene biosynthesis and acquisition of ripening competency (Fig 5). In this role, induction of the AOX pathway in pear may provide an additional hub of regulatory control beyond the MADS-RIN complex, which integrates signals from cold-signaling pathways, while relieving limited energy production and metabolic flux from the mitochondria. Some initial work in climacteric systems suggests this control point could affect increased ethylene biosynthesis through a retrograde signal derived from respiratory activity, metabolic flux or energy limitation [131]. The cold and phytohormone-responsive transcriptional activator MYB29 was shown in Arabidopsis to be a putative regulator of AOX activity via such a retrograde signaling mechanism, integrating numerous hormonal and signaling pathways with respiration [132]. MYB29-like and other R2R3-MYB genes have only recently been the subject of broad comparative analyses in other climacteric crops [133] and can be found in *P. communis* and *P. bretschneideri* genomes. Such genes were also found to be a target of miRNAs influencing post-cold storage physiological responses in Litchi [134].

Adding to this, sulfur signaling in plants may closely impact respiratory activity, oxidative signaling, ethylene signaling and may interact with nitric oxide pathways [135–138]. Among the most variably abundant transcripts in this work was the plastid-derived Rhodanese-like domain-containing protein 14, a sulfurtransferase localized to the thylakoid. The full complement of the functional relevance of this protein in plants is unclear, though, after full conditioning treatment, more of this transcript was found relative to 'Bartlett' tissue, which generally loses green pigmentation more rapidly upon ripening onset than 'D'Anjou'. Overall, these data, along with results of other recent studies, presents the possibility that in European pear and other climacteric fruit, variation in respiratory and phytohormone-pathway activity is not just a consequence of environmental factors, but also mediates physiological responses to them. Generally, the results from LinReg PCR-corrected 2-($\Delta\Delta$Ct) correspond to what was observed with the NMDS approach.

Utilizing a targeted gene approach, this study allowed for focused analysis of genes documented to play important roles in cold-induced conditioning and subsequent ripening in pear. It does not, however, capture transcript abundance of the breadth of genes including those regulating S2-ethylene and ripening related chromatin modifications, epigenetic regulation or small RNAs, all of which have been reported to impact these processes in model fruit systems.

Conclusions

This study adds cultivar specific information regarding the response of European pear to cold conditioning and lends insight into the genetic changes that occur as fruit transitions to S2 ethylene production. Results of this work indicate that cold-sensing and signaling elements from ABA and auxin pathways modulate S1-S2 ethylene transition in European pears, and suggest that, while 'D'Anjou' pear is able to mitigate and cope with the effects of cold exposure, 'Bartlett' is comparatively less-equipped, resulting in a more pronounced S1-S2 ethylene biosynthesis transition. Interestingly, enhanced alternative oxidase transcript abundance in 'Bartlett' and 'D'Anjou' tissues at the peak of the conditioning treatments suggests that AOX plays a novel role in the achievement of ripening competency in European pear.

Supporting information

S1 File. 'Bartlett' and 'D'Anjou' pear flesh firmness raw data and ANOVA analysis. (XLSX)

S2 File. Quantitative RT-PCR reaction conditions, thermal profile.
(DOCX)

S3 File. Quantitative RT-PCR primer names, primer sequences, (Sanger) sequenced amplicons used and reference providing ab initio annotation to candidate regulatory transcript (s).
(XLSX)

S4 File. LinRegPCR input as raw fluorescence readings from the qRT-PCR instrument and PCR cycle, with resulting efficiency and Cq output with regression statistics (Ramakers et al., 2003). LinRegPCR run in plate-wide mean calculation mode. Data separated by tabs as input and output for each plate run in the qRT-PCR analysis.
(XLSX)

S5 File. Initial NMDS ordination of fold-change values from initial qRT-PCR reaction replicates from all gene targets.
(PPTX)

S6 File. NormFinder candidate reference gene input and output.
(XLS)

S7 File. Community master data matrix for initial and final NMDS ordination.
(XLSX)

S8 File. Stress plots from initial and final NMDS ordination plot. Stress plots of the initial (circles) and second (triangles) NMDS ordination procedures. Both instances produced a final stress coefficient of nearly 0.20 after 20 iterations.
(DOCX)

S9 File. Raw R code, NMDS modeling.
(TXT)

S10 File. The radial distance of 90 gene targets from initial NMDS ordination plot vertex, representing total variability as a function of ripeness and pear cultivar. The radial distance of final 36 gene targets from final NMDS ordination plot vertex, representing total variability as a function of pear cultivar and ripeness (NMDS axes 1 and 2, respectively). For NMDS-2, table of the radial distance of final 36 gene targets from initial NMDS ordination plot vertex, representing total variability as a function of pear cultivar and ripeness (NMDS axes 1 and 2, respectively).
(DOCX)

S11 File. Raw R output, centroid hull plot from 36 genes following initial NMDS ordination plot representing total Cq variability by treatment group as a function of pear cultivar and ripeness (NMDS axes 1 and 2, respectively).
(PPTX)

S12 File. Raw R output, final 12 gene-set vector plot following final NMDS ordination plot from the vertex, representing total Cq variability as a function of pear cultivar and ripeness (NMDS axes 1 and 2, respectively).
(PPTX)

S13 File. Raw Morpheus heatmap tool output in PDF format.
(PDF)

Acknowledgments

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References

1. White PJ. Recent advances in fruit development and ripening: an overview. Journal of Experimental Botany. 2002; 53(377):1995–2000. https://doi.org/10.1093/jxb/erf105 PMID: 12324524

2. Anwar R, Mattoo AK, Handa AK. Ripening and Senescence of Fleshy Fruits. Paliyath G, Subramanian J., Lim L., Subramanian K., Handa AK, Mattoo AK, editors2018. 15–51 p.

3. Karlova R, Chapman N, David K, Angenent GC, Seymour GB, de Maagd RA. Transcriptional control of fleshy fruit development and ripening. Journal of Experimental Botany. 2014; 65(16):4527–41. https://doi.org/10.1093/jxb/eru316 PMID: 25080453

4. Granell A, Rambla José L. Biosynthesis of Volatile Compounds. The Molecular Biology and Biochemistry of Fruit Ripening. 2013. https://doi.org/10.1002/9781118593714.ch6

5. Cherian S, Figueroa CR, Nair H. 'Movers and shakers' in the regulation of fruit ripening: a cross-dissection of climacteric versus non-climacteric fruit. Journal of Experimental Botany. 2014; 65 (17):4705–22. https://doi.org/10.1093/jxb/eru280 PMID: 24994760

6. S F Yang a, Hoffman NE. Ethylene Biosynthesis and its Regulation in Higher Plants. Annual Review of Plant Physiology. 1984; 35(1):155–89. https://doi.org/10.1146/annurev.pp.35.060184.001103

7. Tatsuki M. Ethylene Biosynthesis and Perception in Fruit. Journal of the Japanese Society for Horticultural Science. 2010; 79(4):315–26. WOS:000283277200001.

8. Barry CS, Giovannoni JJ. Ethylene and fruit ripening. Journal of Plant Growth Regulation. 2007; 26 (2):143–59. https://doi.org/10.1007/s00344-007-9002-y WOS:000248582800006.

9. Barry CS, Llop-Tous MI, Grierson D. 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. 2000; 123(3):979. https://doi.org/10.1104/pp.123.3.979 PMID: 10889246

10. Tucker G, Yin XR, Zhang AD, Wang MM, Zhu QG, Liu XF, et al. Ethylene and fruit softening. Food Quality & Safety. 2017; 1(4):253–67. https://doi.org/10.1093/fqsafe/fyx024 WOS:000424579100002.

11. Han Y, Kuang J, Chen J, Liu X, Xiao Y, Fu C, et al. Banana Transcription Factor MaERF11 Recruits Histone Deacetylase MaHDA1 and Represses the Expression of MaACO1 and Expansins during Fruit Ripening. Plant Physiology. 2016; 168(1):357–76. http://dx.doi.org/10.1104/pp.16.00301.

12. Liu M, Pirrello J, Kesari R, Mila I, Roustan JP, Li Z, et al. A dominant repressor version of the tomato Sl-ERF. B3 gene confers ethylene hypersensitivity via feedback regulation of ethylene signaling and response components. The Plant Journal, 76(3), 406–419. The Plant Journal. 2013;73(6):406–19. https://doi.org/10.1111/tpj.12305 PMID: 23931552

13. Xiao YY, Chen JY, Kuang JF, Shan W, Xie H, Jiang YM, et al. Banana ethylene response factors are involved in fruit ripening through their interactions with ethylene biosynthesis genes. Journal of experimental botany, 64(8), 2499–2510. Journal of Experimental Botany. 2013;64(8). https://doi.org/10.1093/jxb/ert108 PMID: 23599278

14. Zhou M, Guo S, Zhang J, Zhang H, Li C, Tang X, et al. Comparative dynamics of ethylene production and expression of the ACS and ACO genes in normal-ripening and non-ripening watermelon fruits. Acta Physiologiae Plantarum. 2016; 38(9). https://doi.org/10.1007/s11738-016-2248-x

15. El-Sharkawy I, Sherif S, Mahboob A, Abubaker K, Bouzayen M, Jayasankar S. Expression of auxin-binding protein1 during plum fruit ontogeny supports the potential role of auxin in initiating and enhancing climacteric ripening. Plant Cell Reports. 2012; 31(10):1911–21. https://doi.org/10.1007/s00299-012-1304-2 PMID: 22739723

16. Leng P, Yuan B, Guo YD. The role of abscisic acid in fruit ripening and responses to abiotic stress. Journal of Experimental Botany. 2014; 65(16):4577–88. https://doi.org/10.1093/jxb/eru204 WOS:000342928000008. PMID: 24821949

17. Soto A, Ruiz K. B., Ravaglia D., Costa G., & Torrigiani P. ABA may promote or delay peach fruit ripening through modulation of ripening-and hormone-related gene expression depending on the developmental stage. Plant Physiology and Biochemistry. 2013; 64:11–24. Plant Physiology and Biochemistry. https://doi.org/10.1016/j.plaphy.2012.12.011 PMID: 23337357

18. Weng L, Zhao F, Li R, Xu C, Chen K, Xiao H. The Zinc Finger Transcription Factor SlZFP2 Negatively Regulates Abscisic Acid Biosynthesis and Fruit Ripening in Tomato. Plant Physiology. 2015; 167 (3):931–49. https://doi.org/10.1104/pp.114.255174 PMID: 25637453

19. Jia H, Jiu S, Zhang C, Wang C, Tariq P, Liu Z, et al. Abscisic acid and sucrose regulate tomato and strawberry fruit ripening through the abscisic acid-stress-ripening transcription factor. Plant Biotechnology Journal. 2016; 14(10):2045–65. https://doi.org/10.1111/pbi.12563 PMID: 27005823

20. Dai S, Li P, Chen P, Li Q, Pei Y, He S, et al. Transcriptional regulation of genes encoding ABA metabolism enzymes during the fruit development and dehydration stress of pear 'Gold Nijisseiki'. Plant Physiology and Biochemistry. 2014; 82:299–308. https://doi.org/10.1016/j.plaphy.2014.06.013 PMID: 25038474

21. Pons C, Martí C, Forment J, Crisosto CH, Dandekar AM, Granell A. A bulk segregant gene expression analysis of a peach population reveals components of the underlying mechanism of the fruit cold response. PLoS One. 2014; 9(3). https://doi.org/10.1371/journal.pone.0090706.

22. Rahman A. Auxin: a regulator of cold stress response. Physiol Plantarum. 2013; 147(1):28–35. https://doi.org/10.1111/j.1399-3054.2012.01617.x PMID: 22435366

23. Ashraf MA, Rahman A. Hormonal Regulation of Cold Stress Response. In: Wani SH, Herath V, editors. Cold Tolerance in Plants: Physiological, Molecular and Genetic Perspectives. Cham: Springer International Publishing; 2018. p. 65–88.

24. El-Sharkawy I, Sherif S, Mila I, Bouzayen M, Jayasankar S. Molecular characterization of seven genes encoding ethylene-responsive transcriptional factors during plum fruit development and ripening. Journal of Experimental Botany. 2009; 60(3):907–22. https://doi.org/10.1093/jxb/ern354 PMID: 19213809

25. Tatsuki M, Nakajima N, Fujii H, Shimada T, Nakano M, Hayashi K-I, et al. Increased levels of IAA are required for system 2 ethylene synthesis causing fruit softening in peach (Prunus persica L. Batsch). Journal of Experimental Botany. 2013; 64(6):1049–59. https://doi.org/10.1093/jxb/ers381.

26. Yang Y, Wu Y, Pirrello J, Regad F, Bouzayen M, Deng W, et al. Silencing Sl-EBF1 and Sl-EBF2 expression causes constitutive ethylene response phenotype, accelerated plant senescence, and fruit ripening in tomato. Journal of Experimental Botany. 2009; 61(3):697–708. https://doi.org/10.1093/jxb/erp332 PMID: 19903730

27. Choudhury SR, Roy S, Sengupta DN. Characterization of transcriptional profiles of MA-ACS1 and MA-ACO1 genes in response to ethylene, auxin, wounding, cold and different photoperiods during ripening in banana fruit. J Plant Physiol. 2008; 165(18):1865–78. https://doi.org/10.1016/j.jplph.2008.04.012 PMID: 18554749

28. Fujisawa M, Nakano T, Shima Y, Ito Y. 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. 2013; 25(3):371–86. http://dx.doi.org/10.1105/tpc.112.108118.

29. Ito Y, Nishizawa-Yokoi A, Endo M, Mikami M, Shima Y, Nakamura N, et al. Re-evaluation of the rin mutation and the role of RIN in the induction of tomato ripening. Nature plants. 2017; 3(11):866. https://doi.org/10.1038/s41477-017-0041-5 PMID: 29085071

30. Elitzur T, Yakir E, Quansah L, Zhangjun F, Vrebalob JT, Khayat E, et al. Banana MaMADS transcription factors are necessary for fruit ripening and molecular tools to promote shelf-life and food security. Plant Physiology. 2016. http://dx.doi.org/10.1104/pp.15.01866.

31. Osorio S, Scossa F, Fernie AR. Molecular regulation of fruit ripening. Front Plant Sci. 2013; 4. https://doi.org/10.3389/fpls.2013.00198.

32. Nham NT, Macnish AJ, Zakharov F, Mitcham EJ. 'Bartlett' pear fruit (Pyrus communis L.) ripening regulation by low temperatures involves genes associated with jasmonic acid, cold response, and transcription factors. Plant Science. 2017; 260:8–18. https://doi.org/10.1016/j.plantsci.2017.03.008 PMID: 28554478

33. Berkowitz O, De Clercq I, Van Breusegem F, Whelan J. Interaction between hormonal and mitochondrial signalling during growth, development and in plant defence responses. Plant, Cell and Environment. 2016; 39(5):1127–39. https://doi.org/10.1111/pce.12712 PMID: 26763171

34. Perotti VE, Moreno AS, Podestá FE. Physiological aspects of fruit ripening: the mitochondrial connection. Mitochondrion. 2014; 17:1–6. https://doi.org/10.1016/j.mito.2014.04.010 PMID: 24769052

35. Dhingra A, Hendrickson C, inventorsControl of ripening and senescence in pre-harvest and post-harvest plants and plant materials by manipulating alternative oxidase activity. USA patent 9,591,847. 2017.

36. Szal B, Rychter A. Alternative oxidase-never ending story. Postepy biochemii. 2016; 62(2):138–48. PMID: 28132465

37. Sewelam N, Kazan K, Schenk PM. Global plant stress signaling: reactive oxygen species at the crossroad. Frontiers in plant science. 2016; 7:187. https://doi.org/10.3389/fpls.2016.00187 PMID: 26941757

38. Considine MJ, Daley DO, Whelan J. The expression of alternative oxidase and uncoupling protein during fruit ripening in mango. Plant Physiol. 2001; 126(4):1619–29. https://doi.org/10.1104/pp.126.4.1619 PMID: 11500560

39. Duque P, Arrabaca JD. Respiratory metabolism during cold storage of apple fruit. II. Alternative oxidase is induced at the climacteric. Physiol Plantarum. 1999; 107(1):24–31. https://doi.org/10.1034/j.1399-3054.1999.100104.x ISI:000083523200004.

40. Holtzapffell RC, M Finnegan P, Millar A, R Badger M, Day D. Mitochondrial protein expression in tomato fruit during on-vine ripening and cold storage2002. 827–34 p.

41. Lei T, Feng H, Sun X, Dai Q-L, Zhang F, Liang H-G, et al. The alternative pathway in cucumber seedlings under low temperature stress was enhanced by salicylic acid. Plant Growth Regul. 2009; 60(1):35. https://doi.org/10.1007/s10725-009-9416-6

42. Yang Q-S, Wu J-H, Li C-Y, Wei Y-R, Sheng O, Hu C-H, et al. Quantitative proteomic analysis reveals that antioxidation mechanisms contribute to cold tolerance in plantain (Musa paradisiaca L.; ABB Group) seedlings. Molecular & cellular proteomics: MCP. 2012; 11(12):1853–69. https://doi.org/10.1074/mcp.M112.022079 PMID: 22982374

43. Jobling JJ, McGlasson WB. A comparison of ethylene production, maturity and controlled atmosphere storage life of Gala, Fuji and Lady Williams apples (Malus domestica, Borkh.). Postharvest Biol Tec. 1995; 6(3):209–18. https://doi.org/10.1016/0925-5214(94)00002-A.

44. Knee M, Looney NE, Hatfield SGS, Smith SM. Initiation of Rapid Ethylene Synthesis by Apple and Pear Fruits in Relation to Storage Temperature. Journal of Experimental Botany. 1983; 34(146):1207–12.

45. Hershkovitz V, Friedman H, Goldschmidt EE, Feygenberg O, Pesis E. Induction of ethylene in avocado fruit in response to chilling stress on tree. J Plant Physiol. 2009; 166(17):1855–62. Epub 2009/ 07/14. https://doi.org/10.1016/j.jplph.2009.05.012 PMID: 19592132.

46. Mworia EG, Yoshikawa T, Salikon N, Oda C, Asiche WO, Yokotani N, et al. Low-temperature-modulated fruit ripening is independent of ethylene in 'Sanuki Gold' kiwifruit. Journal of Experimental Botany. 2012; 63(2):963–71. https://doi.org/10.1093/jxb/err324 PMC3254691. PMID: 22058408

47. Lelievre JM, Tichit L, Dao P, Fillion L, Nam YW, Pech JC, et al. Effects of chilling on the expression of ethylene biosynthetic genes in Passe-Crassane pear (Pyrus communis L.) fruits. Plant Mol Biol. 1997; 33(5):847–55. Epub 1997/03/01. https://doi.org/10.1023/a:1005750324531 PMID: 9106508.

48. Hartmann C, Drouet A, Morin F. Ethylene and ripenig of apple, pear and cherry fruit. Plant Physiology and Biochemistry. 1987; 25(4):505–12.

49. Sugar D, Einhorn TC. Conditioning temperature and harvest maturity influence induction of ripening capacity in 'd'Anjou'pear fruit. Postharvest Biol Tec. 2011; 60(2):121–4. http://doi.org/10.1016/j.postharvbio.2010.12.005.

50. Sugar D, Basile SR. Integrated ethylene and temperature conditioning for induction of ripening capacity in 'Anjou'and 'Comice'pears. Postharvest Biol Tec. 2013; 83:9–16. http://doi.org/10.1016/j.postharvbio.2013.03.010.

51. Sugar D, Basile SR. Integrated ethylene and temperature conditioning for inducing pear ripening capacity. 2015:567–72. https://doi.org/10.17660/ActaHortic.2015.1094.76

52. Chiriboga MA, Schotsmans WC, Larrigaudière C, Dupille E, Recasens I. Responsiveness of 'Conference'pears to 1-methylcyclopropene: the role of harvest date, orchard location and year. Journal of Science of Food and Agriculture. 2013; 93(3):619–25. https://doi.org/10.1002/jsfa.5853 PMID: 22936181

53. Zucoloto M, Antoniolli LR, Squeira DL, Czermainski ABC, Salomao LCC. Conditioning temperature for inducing uniform ripening of 'Abate Fetel' pears. Revista Ciência Agronômica. 2016; 47(2):344–50. https://doi.org/10.5935/1806-6690.20160040

54. Smékalová V, Doskočilová A, Komis G, Šamaj J. (2014). Crosstalk between secondary messengers, hormones and MAPK modules during abiotic stress signalling in plants., 32(1), 2–11. Biotechnology Advances. 2014; 32(1):2–11. https://doi.org/10.1016/j.biotechadv.2013.07.009 PMID: 23911976

55. Nham NT, Willits N, Zakharov F, Mitcham EJ. A model to predict ripening capacity of 'Bartlett' pears (Pyrus communis L.) based on relative expression of genes associated with the ethylene pathway. Postharvest Biol Tec. 2017; 128:138–43. https://doi.org/10.1016/j.postharvbio.2017.02.006.

56. Nham NT, de Fretas ST, Macnish AJ, Carr KM, Kietikul T, Guilatco AJ, et al. A transcriptome approach towards understanding the development of ripening capacity in 'Bartlett' pears (Pyrus communis L.). Bmc Genomics. 2015; 16(1). https://doi.org/10.1186/s12864-015-1939-9 PMID: 26452470

57. Paul V, Pandey R, Srivastava GC. The fading distinctions between classical patterns of ripening in climacteric and non-climacteric fruit and the ubiquity of ethylene—an overview. Journal of Food Science and Technology. 2012; 49(1):1–21. https://doi.org/10.1007/s13197-011-0293-4 PMID: 23572821

58. Catala R, Medina J, Salinas J. Integration of low temperature and light signaling during cold acclimation response in Arabidopsis. Proceeding of National Academy of Sciences, USA. 2011; 108 (39):16475–80. https://doi.org/10.1073/pnas.1107161108 PMID: 21930922

59. Kondo S, Meemak S, Ban Y, Moriguchi T, Harada T. Effects of auxin and jasmonates on 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase gene expression during ripening of apple fruit. Postharvest Biol Tec. 2009; 51(2):281–4. http://doi.org/10.1016/j.postharvbio.2008.07.012.

60. Tacken EJ, Ireland HS, Wang YY, Putterill J, Schaffer RJ. Apple EIN3 BINDING F-box 1 inhibits the activity of three apple EIN3-like transcription factors. AoB Plants. 2012. https://doi.org/10.1093/aobpla/pls034.

61. McCune B, Grace J. Analysis of ecological communities. MJM Software Design, Gleneden Beach, OR2002.

62. Donoho D. High-Dimensional Data Analysis: The Curses and Blessings of Dimensionality. AMS Math Challenges Lecture2000.

63. Val J, Monge E, Risco D, Blanco A. Effect of Pre-Harvest Calcium Sprays on Calcium Concentrations in the Skin and Flesh of Apples. Journal of Plant Nutrition. 2008; 31(11):1889–905. 10.1080/01904160802402757

64. Gasic K, Hernandez A, Korban SS. RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. Plant Molecular Biology Reporter. 2004; 22 (4):437–8. https://doi.org/10.1007/BF02772687

65. Dhingra A. Pre-publication Release of Rosaceae Genome Information https://genomics.wsu.edu/research/ Washington State University; 2013 [cited 2016 December 10]. Available from: https://genomics.wsu.edu/research/

66. Velasco R, Zharkikh A, Affourtit J, Dhingra A, Cestaro A, Kalyanaraman A, et al. The genome of the domesticated apple (Malus [times] domestica Borkh.). Nature Genetics. 2010; 42(10):833–9. https://doi.org/10.1038/ng.654 PMID: 20802477

67. Ramakers C, Ruijter JM, Deprez RHL, Moorman AF. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neuroscience Letters. 2003; 339(1):62–6. https://doi.org/10.1016/s0304-3940(02)01423-4 PMID: 12618301

68. Ruijter JM, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ, et al. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res. 2009; 37 (6):e45. https://doi.org/10.1093/nar/gkp045 PMID: 19237396

69. Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 2001; 29(9):e45–e. https://doi.org/10.1093/nar/29.9.e45 PMID: 11328886

70. Andersen CL, Ledet-Jensen J., T. Ø. Normalization of real-time quantitative RT-PCR data: a model based variance estimation approach to identify genes suited for normalization—applied to bladder- and colon-cancer data-sets. Cancer Research. 2004; 64:5245–50. https://doi.org/10.1158/0008-5472.CAN-04-0496 PMID: 15289330

71. Imai T, Ubi BE, Saito T, Moriguchi T. Evaluation of reference genes for accurate normalization of gene expression for real time-quantitative PCR in Pyrus pyrifolia using different tissue samples and seasonal conditions. PLoS One. 2014; 9(1). https://doi.org/10.1371/journal.pone.0086492.

72. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology. 2002; 3(7). https://doi.org/10.1186/gb-2002-3-7-research0034 PMID: 12184808

73. Altschul S, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990; 215. https://doi.org/10.1016/s0022-2836(05)80360-2

74. Gish W, States DJ. Identification of protein coding regions by database similarity search. Nat Genet. 1993; 3(3):266–72. Epub 1993/03/01. https://doi.org/10.1038/ng0393-266 PMID: 8485583.

75. Rieu I, Powers SJ. Real-time quantitative RT-PCR: design, calculations, and statistics. Plant Cell. 2009; 21(4):1031–3. Epub 2009/04/28. https://doi.org/10.1105/tpc.109.066001 PMID: 19395682; PubMed Central PMCID: PMC2685626.

76. Kruskal JB. Nonmetric multidimensional scaling: A numerical method. Psychometrika. 1964; 29 (2):115–29. https://doi.org/10.1007/bf02289694

77. Oksanen J. Multivariate analysis of ecological communities in R: vegan tutorial. R Package version. 2011; 1(7):11–2.

78. Krzywinski M, Altman N. Points of significance: Nonparametric tests. Nature Methods. 2014; 11 (5):467–8. https://doi.org/10.1038/nmeth.2937 PMID: 24820360

79. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acid Research. 2002; 30(9). https://doi.org/10.1093/nar/30.9.e36.

80. Morpheus. Broad Institute https://software.broadinstitute.org/morpheus2019.

81. USDA. Agricultural Statistics 2016. USDA NASS; 2016.

82. Elkins R, Bell R, Einhorn TC. Needs Assessment for Future US Pear Rootstock Research Directions Based on the Current State of Pear Production and Rootstock Research. J Am Pom Soc. 2012; 66 (3):153–63.

83. Guzman D, Dhingra A. Challenges and Opportunities in Pear Breeding. In: Lang G, editor. Achieving sustainable cultivation of temperate zone tree fruits and berries. 2. Cambridge, UK: Burleigh Dodd Science Publishing Limited; 2019.

84. Minas IS, Tanou G, Molassiotis A. Environmental and orchard bases of peach fruit quality. Scientia Horticulturae. 2018; 235:307–22. https://doi.org/10.1016/j.scienta.2018.01.028.

85. Serra S, Sullivan N, Mattheis JP, Musacchi S, Rudell DR. Canopy attachment position influences metabolism and peel constituency of European pear fruit. BMC Plant Biology. 2018; 18(1):364. https://doi.org/10.1186/s12870-018-1544-6 PMID: 30563450

86. Agar IT, Biasi WV, Mitcham EJ. Temperature and exposure time during ethylene conditioning affect ripening of Bartlett pears. J Agric Food Chem. 2000; 48(2):165–70. Epub 2000/02/26. https://doi.org/10.1021/jf990458o PMID: 10691611.

87. Sugar D, Mitcham E, Kupferman G. Rethinking the chill requirement for pear ripening. Good Fruit Grower. 2009.

88. Villalobos-Acuna M, Mitcham EJ. Ripening of European pears: the chilling dilemma. Postharvest biology and technology. 2008; 49(2):187–200.

89. Wayland M. A Practical Approach to Microarray Data Analysis. Briefings in Functional Genomics & Proteomics. 2003; 2(1):82–4. https://doi.org/10.1093/bfgp/2.1.82.

90. Young FW. Nonmetric multidimensional scaling: Recovery of metric information. Psychometrika. 1970; 35(4):455–73. https://doi.org/10.1007/BF02291820

91. McCarthy DJ, Smyth GK. Testing significance relative to a fold-change threshold is a TREAT. Bioinformatics. 2009; 25(6):765–71. https://doi.org/10.1093/bioinformatics/btp053 PMID: 19176553

92. Dalman MR, Deeter A, Nimishakavi G, Duan Z-H. Fold change and p-value cutoffs significantly alter microarray interpretations. BMC Bioinformatics. 2012; 13(2):S11. https://doi.org/10.1186/1471-2105-13-s2-s11 PMID: 22536862

93. Ho J, Tumkaya T, Aryal S, Choi H, Claridge-Chang A. Moving beyond P values: Everyday data analysis with estimation plots. bioRxiv. 2019:377978. https://doi.org/10.1101/377978

94. Jajo A, Rahim MA, Serra S, Gagliardi F, Jajo NK, Musacchi S, et al. Impact of tree training system, branch type and position in the canopy on the ripening homogeneity of 'Abbé Fétel' pear fruit. Tree Genetics & Genomes. 2014; 10(5):1477–88. https://doi.org/10.1007/s11295-014-0777-2

95. Rudell DR, Serra S, Sullivan N, Mattheis JP, Musacchi S. Survey of 'd'Anjou' Pear Metabolic Profile Following Harvest from Different Canopy Positions and Fruit Tissues. 2017; 52(11):1501. https://doi.org/10.21273/hortsci12375-17

96. Olsvik PA, Søfteland L, Lie KK. Selection of reference genes for qRT-PCR examination of wild populations of Atlantic cod Gadus morhua. BMC Research Notes. 2008; 1(1):47. https://doi.org/10.1186/1756-0500-1-47 PMID: 18710500

97. Liu M, Udhe-Stone C, Goudar CT. Progress curve analysis of qRT-PCR reactions using the logistic growth equation. Biotechnology progress. 2011; 27(5):1407–14. Epub 2011/07/19. https://doi.org/10.1002/btpr.666 PMID: 21766473.

98. Nham NT, Macnish AJ, Zakharov F, Mitcham EJ. 'Bartlett'pear fruit (Pyrus communis L.) ripening regulation by low temperatures involves genes associated with jasmonic acid, cold response, and transcription factors. Plant Science. 2017; 260:8–18. https://doi.org/10.1016/j.plantsci.2017.03.008 PMID: 28554478

99. Sharkawy IE-S, Jones B, Gentzbittel L, Lelièvre J-M. Differential regulation of ACC synthase genes in cold-dependent and -independent ripening in pear fruit. Plant, Cell & Environ. 2004; 27(10):1197–210. https://doi.org/10.1111/j.1365-3040.2004.01218.x

100. El-Sharkawy E-I, Jones B, Gentzbittel L, Lelivre JM, Pech JC, Latché A. Differential regulation of ACC synthase genes in cold-dependent and-independent ripening in pear fruit. Plant, Cell & Environ. 2004; 27(10):1197–210. https://doi.org/10.1111/j.1365-3040.2004.01218.x

101. Liu M, Pirrello J, Chervin C, Roustan J-P, Bouzayen M. Ethylene Control of Fruit Ripening: Revisiting the Complex Network of Transcriptional Regulation. Plant Physiology. 2015; 169(4):2380–90. https://doi.org/10.1104/pp.15.01361 PMID: 26511917

102. Shan W, Kuang JF, Lu WJ, Chen JY. Banana fruit NAC transcription factor MaNAC1 is a direct target of MaICE1 and involved in cold stress through interacting with MaCBF1. Plant, Cell and Environment. 2014; 37(9):2116–27. https://doi.org/10.1111/pce.12303 PMID: 24548087

103. Sivankalyani V, Geetha M, Subramanyam K, Girija S. Ectopic expression of Arabidopsis RCI2A gene contributes to cold tolerance in tomato. Transgenic Research. 2015; 24(2):237–51. https://doi.org/10.1007/s11248-014-9840-x PMID: 25260337

104. Shi Y, Yang S. ABA Regulation of the Cold Stress Response in Plants. In: Zhang D-P, editor. Abscisic Acid: Metabolism, Transport and Signaling. Dordrecht: Springer Netherlands; 2014. p. 337–63.

105. Eremina M, Rozhon W, Poppenberger B. Hormonal control of cold stress responses in plants. Cellular and Molecular Life Sciences. 2016; 73(4):797–810. https://doi.org/10.1007/s00018-015-2089-6 PMID: 26598281

106. Tian MS, Prakash S, Zhang N, Ross GS. Chilling-induced ethylene biosynthesis in Braeburn apples. Plant Growth Regul. 2002; 38(3):249–57. https://doi.org/10.1023/a:1021552002676

107. Chang J, Clay JM, Chang C. Association of cytochrome b5 with ETR1 ethylene receptor signaling through RTE1 in Arabidopsis. The Plant journal: for cell and molecular biology. 2014; 77(4):558–67. https://doi.org/10.1111/tpj.12401 PMID: 24635651.

108. Ma Q, Du W, Brandizzi F, Giovannoni JJ, Barry CS. Differential control of ethylene responses by GREEN-RIPE and GREEN-RIPE LIKE1 provides evidence for distinct ethylene signaling modules in tomato. Plant Physiology. 2012; 160(4):1968–84. https://doi.org/10.1104/pp.112.205476 PMID: 23043080

109. Qiu L, Xie F, Yu J, Wen CK. Arabidopsis RTE1 is essential to ethylene receptor ETR1 amino-terminal signaling independent of CTR1. Plant Physiol. 2012; 159(3):1263–76. Epub 2012/05/09. https://doi.org/10.1104/pp.112.193979 PMID: 22566492; PubMed Central PMCID: PMC3387708.

110. Binder BM, Rodríguez FI, Bleecker AB. The copper transporter RAN1 is essential for biogenesis of ethylene receptors in Arabidopsis. Journal of Biological Chemistry. 2010; 285(48):37263–70. https://doi.org/10.1074/jbc.M110.170027 PMID: 20876528

111. Resnick JS, Wen C-K, Shockey JA, Chang C. REVERSION-TO-ETHYLENE SENSITIVITY 1, a conserved gene that regulates ethylene receptor function in Arabidopsis. Proceedings of the National Academy of Sciences. 2006; 103(20):7917–22. https://doi.org/10.1073/pnas.0602239103 PMID: 16682642

112. Zhang M-Y, Xue C, Xu L, Sun H, Qin M-F, Zhang S, et al. Distinct transcriptome profiles reveal gene expression patterns during fruit development and maturation in five main cultivated species of pear (Pyrus L.). Scientific Reports. 2016; 6:28130. https://doi.org/10.1038/srep28130 https://www.nature.com/articles/srep28130#supplementary-information. PMID: 27305967

113. Catalá R, López-Cobollo R, Castellano MM, Angosto T, Alonso JM, Ecker JR, et al. The Arabidopsis 14-3-3 protein RARE COLD INDUCIBLE 1A links low-temperature response and ethylene biosynthesis to regulate freezing tolerance and cold acclimation. The Plant Cell. 2014; 26(8):3326–42. https://doi.org/10.1105/tpc.114.127605 PMID: 25122152

114. Obsilova V, Kopecka M, Kosek D, Kacirova M, Kylarova S, Rezabkova L, et al. Mechanisms of the 14-3-3 protein function: regulation of protein function through conformational modulation. Physiol Res. 2014; 63 Suppl 1:S155–64. Epub 2014/02/26. 24564655.

115. Medina J, Rodríguez-Franco M, Peñalosa A, Carrascosa MJ, Neuhaus G, Salinas J. Arabidopsis mutants deregulated in RCI2A expression reveal new signaling pathways in abiotic stress responses. The Plant Journal. 2005; 42(4):586–97. https://doi.org/10.1111/j.1365-313X.2005.02400.x PMID: 15860016

116. Pattison RJ, Csukasi F., & Catalá C. (2014). Mechanisms regulating auxin action during fruit development. Physiol Plantarum. 2014; 151(1):62–72. https://doi.org/10.1111/ppl.12142 PMID: 24329770

117. El-Sharkawy I, Sherif SM, Jones B, Mila I, Kumar PP, Bouzayen M, et al. TIR1-like auxin-receptors are involved in the regulation of plum fruit development. Journal of Experimental Botany. 2014; 65 (18):5205–15. https://doi.org/10.1093/jxb/eru279 PMID: 24996652

118. Robles LM, Deslauriers SD, Alvarez AA, Larsen PB. A loss-of-function mutation in the nucleoporin AtNUP160 indicates that normal auxin signalling is required for a proper ethylene response in Arabidopsis. Journal of experimental botany. 2012; 63(5):2231–41. Epub 2012/01/11. https://doi.org/10.1093/jxb/err424 PMID: 22238449.

119. Schaffer RJ, Ireland HS, Ross JJ, Ling TJ, David KM. SEPALLATA1/2-suppressed mature apples have low ethylene, high auxin and reduced transcription of ripening-related genes. AoB Plants. 2013; 5:pls047. https://doi.org/10.1093/aobpla/pls047 PMID: 23346344; PubMed Central PMCID: PMC3551604.

120. Tacken EJ, Ireland HS, Wang YY, Putterill J, Schaffer RJ. Apple EIN3 BINDING F-box 1 inhibits the activity of three apple EIN3-like transcription factors. AoB Plants. 2012; 2012:pls034. https://doi.org/10.1093/aobpla/pls034 PMID: 23585922; PubMed Central PMCID: PMC3624930.

121. Breitel DA, Chappell-Maor L, Meir S, Panizel I, Puig CP, Hao Y, et al. AUXIN RESPONSE FACTOR 2 Intersects Hormonal Signals in the Regulation of Tomato Fruit Ripening. PLoS genetics. 2016; 12(3). https://doi.org/10.1371/journal.pgen.1005903.

122. Dong T, Hu Z, Deng L, Wang Y, Zhu M, Zhang J, et al. A tomato MADS-box transcription factor, SlMADS1, acts as a negative regulator of fruit ripening. Plant Physiology. 2013; 163(2):1026–36. https://doi.org/10.1104/pp.113.224436 PMID: 24006286

123. Bemer M, Karlova R, Ballester AR, Tikunov YM, Bovy AG, Wolters-Arts M, et al. The Tomato FRUIT-FULL Homologs TDR4/FUL1 and MBP7/FUL2 Regulate Ethylene-Independent Aspects of Fruit Ripening. The Plant Cell. 2012; 24(11):4437–51. https://doi.org/10.1105/tpc.112.103283 PMID: 23136376

124. Shima Y, Fujisawa M, Kitagawa M, Nakano T, Kimbara J, Nakamura N, et al. Tomato FRUITFULL homologs regulate fruit ripening via ethylene biosynthesis. Bioscience, Biotechnology and Biochemistry. 2014; 78(2):231–7. http://dx.doi.org/10.1080/09168451.2014.878221.

125. Xu F, Yuan S, Zhang DW, Lv X, Lin HH. The role of alternative oxidase in tomato fruit ripening and its regulatory interaction with ethylene. J Exp Bot. 2012; 63(15):5705–16. Epub 2012/08/24. ers226 [pii] https://doi.org/10.1093/jxb/ers226 PMID: 22915749.

126. Borecký J, Nogueira F. T., De Oliveira K. A., Maia I. G., Vercesi A. E., & Arruda P. (2006). The plant energy-dissipating mitochondrial systems: depicting the genomic structure and the expression profiles

of the gene families of uncoupling protein and alternative oxidase in monocots and dicots. Journal of Experimental Botany. 2006; 57(4):849–64. https://doi.org/10.1093/jxb/erj070 PMID: 16473895

127. Feng H, Guan D, Sun K, Wang Y, Zhang T, Wang R. Expression and signal regulation of the alternative oxidase genes under abiotic stresses. Acta Biochimica et Biophysica Sinica. 2013; 45(12):985–94. https://doi.org/10.1093/abbs/gmt094 PMID: 24004533

128. Xu F, Yuan S, Zhang D-W, Lv X, Lin H-H. The role of alternative oxidase in tomato fruit ripening and its regulatory interaction with ethylene. J Exp Bot. 2012; 63(15):5707–16. https://doi.org/10.1093/jxb/ers226.

129. Xu F, Yuan S, Zhang DW, Lv X, Lin HH. The role of alternative oxidase in tomato fruit ripening and its regulatory interaction with ethylene. Journal of Experimental Botany. 2012; 63(15):5705–16. https://doi.org/10.1093/jxb/ers226 PMID: 22915749

130. Wagner AM. A role for active oxygen species as second messengers in the induction of alternative oxidase gene expression in Petunia hybrida cells. FEBS Lett. 1995; 368(2):339–42. Epub 1995/07/17. https://doi.org/10.1016/0014-5793(95)00688-6 PMID: 7628633.

131. Vanlerberghe GC. Alternative Oxidase: A Mitochondrial Respiratory Pathway to Maintain Metabolic and Signaling Homeostasis during Abiotic and Biotic Stress in Plants. International Journal of Molecular Sciences. 2013; 14(4):6805–47. https://doi.org/10.3390/ijms14046805 PMC3645666. PMID: 23531539

132. Zhang X, Ivanova A, Vandepoele K, Radomiljac JD, Van de Velde J, Berkowitz O, et al. The transcription factor MYB29 is a regulator of ALTERNATIVE OXIDASE 1. Plant Physiology. 2017. http://dx.doi.org/10.1104/pp.16.01494.

133. Fan ZQ, Ba LJ, Shan W, Xiao YY, Lu WJ, Kuang JF, et al. A banana R2R3-MYB transcription factor MaMYB3 is involved in fruit ripening through modulation of starch degradation by repressing starch degradation-related genes and MabHLH6. Plant J. 2018; 96(6):1191–205. Epub 2018/09/23. https://doi.org/10.1111/tpj.14099 PMID: 30242914.

134. Yao F, Zhu H, Yi C, Qu H, Jiang Y. MicroRNAs and targets in senescent litchi fruit during ambient storage and post-cold storage shelf life. BMC plant biology. 2015; 15:181–. https://doi.org/10.1186/s12870-015-0509-2 PMID: 26179282.

135. Antoniou C, Savvides A, Christou A, Fotopoulos V. Unravelling chemical priming machinery in plants: the role of reactive oxygen–nitrogen–sulfur species in abiotic stress tolerance enhancement. Current Opinion in Plant Biology. 2016; 33:101–7. https://doi.org/10.1016/j.pbi.2016.06.020 PMID: 27419886

136. Wawrzynska A, Moniuszko G, Sirko A. Links Between Ethylene and Sulfur Nutrition—A Regulatory Interplay or Just Metabolite Association? Front Plant Sci. 2015; 6. https://doi.org/10.3389/fpls.2015.01053 PMID: 26648954

137. Ziogas V, Molassiotis A, Fotopoulos V, Tanou G. Hydrogen Sulfide: A Potent Tool in Postharvest Fruit Biology and Possible Mechanism of Action. Front Plant Sci. 2018; 9(1375). https://doi.org/10.3389/fpls.2018.01375 PMID: 30283483

138. Moniuszko G. Ethylene signaling pathway is not linear, however its lateral part is responsible for sensing and signaling of sulfur status in plants. Plant Signal Behav. 2015; 10(11):e1067742. Epub 2015/09/05. https://doi.org/10.1080/15592324.2015.1067742 PMID: 26340594; PubMed Central PMCID: PMC4883965.