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# Transcriptome analyses provide new possible mechanisms of aroma ester weakening of 'Nanguo' pear after cold storage

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ABSTRACT

'Nanguo' pear are popular for their unique aroma ester. However, we found that the fragrance faded undergoing long term low temperature (LT) storage, and the aroma-weakening mechanism has not been well understood. To this end, the transcriptome of the fruit on 0 d and optimum tasting period (OTP) during shelf life at room temperature (RT) and after cold storage were analyzed, respectively. On the OTP, the kind of volatile esters decreased to 18 and the total content of aroma ester decreased significantly after cold storage. A total of 2441 and 7513 genes were differentially expressed between 0 d and the OTP during shelf life and after cold storage, respectively. These genes were categorized into various functional groups and pathways according to the bio-information analysis. Notably, genes demonstrated significant differential expression only in the fruit after LT storage included several from the plant hormone signal transduction category such as *DELLA* and *JAR*, as well as some related to fatty acid metabolism such as *fadD*, *fabG*, *SCD*, *FAD*, *LOX2S* and *HPL* and transcription factors (TFs) MYBP, NFYA, and ERF1. These results revealed that the signal transduction of abscisic acid, salicylic acid, fatty acid metabolism, as well as TFs plays important roles in the aroma weakening after cold storage.

## 1. Introduction

Aroma constitutes a major factor affecting fruit quality and commodity value (El Hadi Muna et al., 2013; Li et al., 2014). The 'Nanguo' pear, a kind of *Pyrus Ussuriensis Maxim*, is usually harvested in mid to late September, the commercial harvest time, at which it is very hard and with little aroma, but after an approximately 8–10 d post-harvest ripening process at room temperature (RT, 20 ± 1 °C), the fruit reaches the optimum taste period (OTP) with rich aroma, soft flesh, and sweet taste (Ji et al., 2012; Zhou et al., 2014). The pears ripen and deteriorate quickly at RT after harvest and have a short shelf life. In production, cold storage is an effective technology for prolonging storage life and market supply by slowing fruit ripening and reducing decay development; however, in practice, it was found that the aroma of the fruit was obviously weakened, especially following long term cold storage that seriously affected the quality of the fruit (Ji et al., 2012; Zhang et al., 2013; Zhou et al., 2014; Shi et al., 2017). The plant responses to LT have typically been studied in vegetative tissues or in chilling sensitive fruit. The molecular mechanism of the aroma weakened caused by LT is not well understood.

Preliminary studies found that expression of the key genes lipoxygenase (*PuLOX*), alcohol dehydrogenase (*PuADH*), and alcohol acyltransferase (*PuAAT)* changed during the period of aroma formation, whereas key genes in the ethylene signaling pathway changed along with aroma weakening in the fruit following cold storage (Li et al., 2014; Zhou et al., 2015). In addition, previous studies have identified numerous cold-responsive transcription factors (TFs), such as ethylene response factor (ERF), WRKY, MYB, NAC, and b-ZIP in plants (Bensmihen et al., 2005; Liu et al., 2012; Qin et al., 2012). Specifically, the CBF/DREB1 family members comprise the most well-known cold tolerance pathway in plants, with over-expression of CBF genes increasing the cold tolerance (Fowler and Thomashow, 2002). However, the metabolism of aroma weakening still requires further study, and the genes and TFs that control or regulate the metabolism of aroma formation in the fruit following cold storage are not clear.

In recent years, the use of high-throughput sequencing technology has became common, characterized by high data output and low cost, and is widely used in plant transcriptome research (Marguerat and Bähler, 2010; Glenn, 2011). RNA sequencing (RNA-Seq) also comprises a currently common method to unravel a diversity of stress responses at the transcriptome level, especially in cases where the complete genome sequence and annotation are not yet available; in particular, researchers have used this method to identify and annotate gene expression at the whole mRNA level even without prior sequence of any transcript from

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any related organism (Li et al., 2017; Qi et al. 2013; Zhang et al., 2014). Using RNA-seq to analyze the transcriptome of 'Nanguo' pear, this study was conducted to investigate the effect of cold storage on the aroma forming during the fruit ripening at the whole mRNA level and provide a comprehensive sequence resource for further molecular genetic research in the cold stored fruit. We hope to find the potential controlling genes, and LT-induced pathways controlling the aroma forming of the fruit. Additionally, the potential role of selected TFs in regulating LT-associated aroma forming is discussed. We examined the effect of LT alone on expression of selected genes to clarify if they changed during the aroma forming. And we identified genes and pathways likely leading to loss of aroma in LT-treated fruit.

## 2. Materials and methods

### 2.1. Plant materials and treatment

'Nanguo' pears with homogeneous size and maturity, and without visible signs of defects or decay were picked from trees grown in a commercial orchard (Anshan, Liaoning Province, China) on September 17, 2016, and transported to the laboratory on the day of harvest. A total of 1200 pears were selected and randomly divided into two groups with three replicates, and placed in 0.04 mm thick polyethylene (PE) bags to maintain a relative humidity of about 80%–85% for a 5 d pre-ripening period. For the control group, 600 pears were stored at RT (20 ± 1 °C), whereas the other 600 pears were stored at 0 °C ± 0.5 °C, 80%–85% relative humidity for 150 d, followed by transfer to RT for up to 12 d.

For the fruit subjected to two groups, measurements of fruit firmness and total soluble solids (TSS) were performed on the same sample of fruit at 0, 3, 6, 9, 12 d, respectively, whereas the aroma assessment was performed on the day that the fruit was refrigerated (C0), on the day it was removed from LT (LT0), and on the OTP (COTP and LTOP for non- and cold stored fruit, respectively), with three replicates of eighteen fruits used each time. We also used the eighteen fruits for the analyses described below. The samples for RNA-Seq were also sampled on 0 d and the OTP for each group, frozen immediately in liquid nitrogen, then stored at −80 °C.

### 2.2. Measurement of fruit firmness, ethylene production and TSS

Fruit firmness was measured using a texture analyzer with a 2 mm plunger tip (TA-XT2i Plus, Stable Micro System, Godalming, UK). The test rate was 1 mm·s$^{-1}$, to 5 mm depth. The test was performed on opposite sides on the equator of eighteen fruits after the removal of a slice of skin, every fruit for four measurements and averaged the results.

The production of ethylene was measured by a gas chromatograph (CP-3800, Varian, USA) equipped with a flame ionization detector (Injector, detector, and oven temperatures were 110 °C, 140 °C, and 90 °C, respectively.). Four fruits were weighed and placed in 1.2 L fresh-keeping boxes, sealed, and placed for 5 h at 20 ± 1 °C, and ethylene was collected in the boxes headspace.

TSS was measured using a digital hand-held refractometer (PAL-13810, Atago, Tokyo, Japan) and expressed as percentages (%). The test was performed by the flesh of eighteen fruits, every fruit for two measurements, and averaged the results.

### 2.3. Measurement of fruit aroma

The methods described by Zhou et al. (2014) were used for the extraction and determination of the fruit aroma. Three Nanguo pear were randomly selected at every sampling point, cut 12 chunks from the fruits and pulped, then filtered by 4 layer cheesecloth. 8 m L juice were transferred to a 20 mL headspace vial, added 2.5 g NaCl and 10 u L 3-octanone (1.644 mg·mL$^{-1}$) were added as an internal standard, then the vial was sealed with a teflon septum aluminum cap and mixed. The condition of extraction was as Zhou et al. (2014). Aroma were analyzed by GC–MS (7890 A-5975C, Agilent Technologies, USA) apparatus equipped with HP-INNOWAX (30 m length × 0.25 mm i.d. × 0.25 u m film thickness) fused silica capillary column. The heating program and MS conditions were as Zhou et al. (2014). Compounds were quantified using the internal standard method, where the concentrations of various aroma components were normalized to that of 3-octanone.

### 2.4. RNA extraction and RNA-seq

'Nanguo' pears in the two groups were sampled on 0 d and the OTP. Total RNA was extracted from the fruit peel, which contained both skin and a small amount of flesh tissue peeled from two opposite sides of eighteen fruits (six fruits of every replicate, three biological replicates for each point), then frozen in liquid $N_2$ (Sheng et al., 2016). These samples were divided into six control samples (C01-C03 and COTP1-3) and six LT samples (LT01-03 and LTOTP1-3) for RNA isolation and sequencing. The extraction step was performed according to the manufacturer's instructions of the OminiPlant RNA kit (KangWeiShiJi, Beijing, China). The quality of RNA was monitored by gel electrophoresis and $OD_{260}/OD_{280}$ ratio. High quality RNA samples ($> 5 \times 10^{-2}$ g/L) were used for cDNA library preparation and massive sequencing with a HiSeq 4000 SBS Kit (300 cycles) System (Illumina, San Diego, CA, USA). Illumina library preparation and sequencing of 12 samples (two shelf-life times and three biological replicates) were completed following standard protocols (Erlich et al., 2008). The integrity and quantity of total RNA were examined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Carlsbad, CA, USA), and mRNA was isolated from total RNA using oligo-d(T) (Invitrogen, Life Technologies, Carlsbad, CA, USA). The mRNA libraries were constructed by following the TruSeq protocol (Illumina). Individual libraries were prepared with barcodes and pooled for sequencing on a lane of the Illumina HiSeq 4000 platform.

### 2.5. Analysis of RNA-Seq data

Reads were assembled separately from the whole library using the Trinity method (http://trinityrnaseq.sourceforge.net/) (Broad Institute, USA). There are three programs in Trinity including Inchworm, Chrysalis, and Butterfly, which were utilized sequentially to process RNA-Seq reads. Firstly, the Inchworm program was used to assemble reads into contigs. Secondly, the Chrysalis program was used to cluster the minimal overlapping contigs. Thirdly, the Butterfly program was used to construct the transcripts. Finally, the multiple sequence alignment tool BLAST was used to cluster the transcripts by the similarity of correct match length. The coding sequences (CDS) of the unigenes were predicted using EMBOSS (http://emboss.sourceforge.net/). Expectation-Maximization (RSEM) (http://www.biomedsearch.com/nih/RSEM-accurate-transcript-quantification-from/21816040.html) was used to predict all assembled transcribed sequences and count the read number mapped to each unigene. The reads per kilobase of million mapped reads (RPKM) of each gene was then calculated based on the length of the gene and read counts mapped thereto.

### 2.6. DEGs analysis and functional annotation

For RNA-Seq with three biological replicates prior to differential gene expression analysis, the read counts were adjusted using the edgeR program package (http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html) through one scaling normalized factor for each sequenced library. Differential expression analysis of the four libraries was then performed using edgeR. A corrected *P*-value of 0.05 and log2 (fold change) of 1 were set as the threshold for significant differential expression.

GO enrichment analysis of DEGs was implemented using Goatools (https://github.com/tanghaibao/GOatools) to categorize the total