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# Effect of ATP treatment on enzymes involved in energy and lipid metabolisms accompany peel browning of 'Nanguo' pears during shelf life after low temperature storage

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ABSTRACT

The postharvest ripening and senescence of 'Nanguo' pears (*Pyrus ussuriensis Maxim.*) can be effectively delayed by low temperature storage. However, peel browning (PB) often occurs in pear fruit during shelf life at room temperature. In this study, ATP treatment on 'Nanguo' pears has efficiently inhibited the occurrence of PB. Higher flesh firmness, ATP concentration and energy charge (EC) were detected in ATP-treated fruit during shelf life. Malondialdehyde (MDA) concentration and electrolyte leakage were lowered in ATP-treated fruit. Gene expression levels of ATP synthase (*ATPase*), NADH dehydrogenase (*NDA*) and vacuolar proton-inorganic pyrophosphatase (*VPP*) were promoted by ATP treatment, and the activities of *ATPase*, *NDA* and *VPP* were higher in ATP-treated fruit. The elevated activity and gene expression of phospholipase D (*PLD*) were restrained under ATP treatment. ATP treatment effectively alleviated the PB of 'Nanguo' pears and the possible mechanisms were discussed.

## 1. Introduction

Low temperature storage is widely used to retard the ripening and senescence in postharvest fruit (Wang et al., 2015b). However, some respiration climacteric fruit, such as pear fruit, are highly vulnerable to chilling injury (CI). 'Nanguo' pear (*Pyrus ussuriensis* Maxim.) is one of the most famous and economically important varieties in Liaoning province, China, but the postharvest storage life of the fruit at ambient temperatures is less than 20 d (Wang et al., 2017a). The traditional method for prolonging the postharvest storage period of the fruit is low temperature storage, but peel browning (PB) often occurs in pears during shelf life at room temperature after removal from long term refrigeration (Cheng et al., 2015; Wang et al., 2017a), which may cause a loss to quality and commodity value of the fruit.

The occurrence of PB in pear fruit is mainly caused by damage of cellular membrane and the consequent enzymatic oxidation of phenols by polyphenol oxidases (PPOs) which are originally separated in different cell compartments (Lin et al., 2016; Sheng et al., 2016; Wang et al., 2017a). Energy levels in fruit cells play an important role in maintaining the integrity of cellular membrane (Wang et al., 2013). Changes in energy status, including concentration of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and energy charge (EC), have often been reported in fruit underwent low temperature storage (Pan et al., 2017; He et al., 2017). In terms of postharvest fruit, cellular energy status plays key roles not only in controlling ripening and senescence, but also in affecting chilling tolerance and quality of the fruit during low temperature storage (Wang et al., 2013; Lin et al., 2018; Wang et al., 2017a). In 'litchi' fruit, ATP content increased significantly in developing preharvest litchi fruit and declined significantly during postharvest fruit senescence (Wang et al., 2013). The development of pericarp browning in harvested longans during storage was companied by the decreases of ATP content and EC (Lin et al., 2018).

Changing in cellular energy levels is mainly depended on energy metabolism in which several key enzymes are involved. Among the energy metabolism pathways, oxidative phosphorylation, which is the third stage of respiration metabolism conducted in the inner mitochondrial membrane, generates almost 95% of the energy in cells (Qin et al., 2009). ATP synthase (*ATPase*), NADH dehydrogenase (*NDA*) and vacuolar proton-inorganic pyrophosphatase (*VPP*) are key enzymes involved in oxidative phosphorylation (Wang et al., 2017b). Furthermore, integrity of cell membrane is closely related to the process of membrane lipid degradation. Phospholipase D (*PLD*), a key enzyme involved in glycerophospholipid metabolism pathway, is considered to

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play important role in membrane lipid degradation of cells (Sheng et al., 2016; Jincy et al., 2017). The activity and gene expression of these enzymes have been reported to change during low temperature storage (Sheng et al., 2016) or under postharvest approaches (Wang et al., 2017a; Pan et al., 2017), and the changes in activity and gene expression are associated with the variation of cellular energy and occurrence of CI. However, the feedback effect of exogenous energy treatment on the enzymes accompanying the occurrence of PB in 'Nanguo' pear fruit remains unknown.

The objective of this study was to investigate the effect of exogenous ATP treatment on energy and lipid related genes, as well as occurrence of PB in 'Nanguo' pear fruit during shelf life after removal from low temperature storage. To achieve this goal, changes in activities and gene expression levels of *NDA*, *VPP*, *ATPase* and *PLD* in fruit after cold storage for 120 d were investigated. Moreover, we examined the peel browning index, firmness, electrolyte leakage, MDA concentration, ATP concentration and EC.

## 2. Materials and methods

### 2.1. Fruit materials and sampling

Samples of pear (*Pyrus ussuriensis* Maxim. cv 'Nanguo') were harvested on September 12, 2016 at an orchard in Anshan, Liaoning Province, China. Fruit were immediately transported to the laboratory on the day of harvest. Fruit without mechanical injury or decay and with uniform size were selected for testing.

Pear samples were firstly kept at room temperature (20 ± 1 °C) for 5 d of pre-ripeness. Then the pears were randomly divided into two groups of 450 fruit each. The control group was dipped in distilled water for 3 min, then air dried and kept in low temperature storage at (0 ± 0.5) °C, while the ATP-treated group was immerged in 0.001 mol/L ATP solution for 3 min, then air dried and stored at (0 ± 0.5) °C. The fruit were stored for 120 d, and relative humidity (RH) was maintained at 80–85 % during the whole storage period. Both of the groups were transferred to 15 day's shelf life at 20 °C after removal from low temperature storage.

Fruit from the two groups were sampled at a 3-day interval during the shelf life. At each sampling point, measurement of browning index, firmness, electrolyte leakage, MDA concentration, ATP concentration and EC value, enzyme activities of *NDA*, *VPP*, *ATPase* and *PLD* was conducted on 3 replicates of 18 fruit. Peel of 9 fruit was frozen in liquid nitrogen for analysis of genes expression of *NDA*, *VPP*, *ATPase* and *PLD*. Brown peel tissue was taken for measurement when PB was appeared.

### 2.2. Analysis of firmness

Firmness was measured using a texture analyzer (TA-XT2iPlus; StableMicro System, Guildford, UK) equipped with a 2-mm plunger tip, and the penetration rate was 3 mm $s^{-1}$ with a final penetration depth of 5 mm. Four measurements were carried out on opposite sides of fruit.

### 2.3. Analysis of browning index

Measurement of browning index (BI) was conducted by using the method of Yang et al. (2010). Fruit PB degrees were graded by the area of browning as follows: 0 = no browning, 1 = less than 1/3 browning, 2 = 1/3 - 2/3 browning, 3 = more than 2/3 browning. Finally, BI was calculated by the equation: BI (%) = (Σ (browning degree × fruit number of this degree)) / (the highest browning degree × total fruit number) × 100%.

### 2.4. Analysis of malondialdehyde (MDA) and electrolyte leakage

MDA concentration was determined using the method described by Sun et al. (2011). Sample of peel (1 *g*) was homogenized in 5 mL of 1 *g* $L^{-1}$ trichloroacetic acid (TCA), and centrifuged for 20 min at 10,000 *g*. Then 2 mL of the supernatant was mixed with 2 mL of 6.7 *g* $L^{-1}$ thiobarbituric acid (TBA), and the mixture was heated for 20 min at 100 °C. The mixture was cooled immediately on ice and centrifuged for 10 min at 5000 *g*. The absorbance of supernatant was detected at 450 nm, 532 nm, and 600 nm using a spectrophotometer (TU-1810 DSPC, Beijing Puxi Instrument Co., Beijing, China). The MDA concentration was calculated by using the equation: [6.45 × (A532 - A600) - 0.56 × A450] × 5.

Measurement of electrolyte leakage was conducted using the method described by Zhu et al. (2009). A total of 20 peel pieces of 1 cm diameter were immerged in 40 mL of double-distilled water. Electrolyte leakage was detected using a conductivity meter (DDS-307, Shanghai Precise Science Instrument Co., Shanghai, China). P0 was determined as initial electrolyte leakage, and P1 was measured 10 min later. P2 was measured after the samples boiling for 10 min and cooling to 20 °C. The electrolyte leakage of peel sample was calculated by using the equation: Electrolyte leakage (%) = (P1 - P0) / (P2 - P0).

### 2.5. Analysis of ATP, ADP and AMP concentrations and energy charge

ATP, ADP and AMP concentrations were measured according to the method of Wang et al. (2013). Peel samples (2 *g*) were ground in liquid nitrogen with 5 mL of 0.6 mol $L^{-1}$ perchloric acid. The mixture was centrifuged at 20,000 *g* for 10 min at 4 °C, then 3 mL supernatant was collected and the pH was adjusted immediately to 6.5–6.8 using 1 mol $L^{-1}$ KOH. The solution was diluted to 4 mL and passed through a 0.45 mm filter (Millipore Corp., Bedford, MA, USA). The concentrations of ATP, ADP and AMP were measured using a HPLC instrument (Agilent 1100, Agilent Corp., Santa Clara, CA, USA) with a reversed-phase Nova-Pak C18 column (5 μm, 5 × 250 mm; Agilent Corp., Santa Clara, CA, USA). Mobile phase A consisted of 0.06 mol $L^{-1}$ $K_2HPO_4$ and 0.04 mol $L^{-1}$ $KH_2PO_4$ in deionized water and adjusted to pH 7.0 using 0.1 mol $L^{-1}$ KOH. Mobile phase B was pure acetonitrile. A linear gradient program was performed with 75–100 % A and 0–25 % B for 7 min, and the flow rate was 1.2 mL $min^{-1}$. The wavelength of UV detection was 254 nm and injection volume was 10 μL. The concentrations of ATP, ADP, and AMP were measured using the external standard program, and EC was calculated using the equation: [ATP + (1/2) ADP] / [ATP + ADP + AMP].

### 2.6. Analysis of enzyme activity

Enzyme activities of *NDA*, *VPP*, and *ATPase* were measured using the method described by Mushtaq et al. (2013) and Yin et al. (2015) respectively. The measurement of enzyme activity of *PLD* was carried out by using the method of Sheng et al. (2016).

### 2.7. RNA isolation and cDNA synthesis

RNA was extracted from 0.05 - 0.1 g frozen peel samples using RNAprep Pure Plant (Polysaccharide & Polyphenolics-rich) kit (Tiangen, Beijing, China). A spectrophotometer (NanoDropND-2000, Thermo, Germany) was used to determine the purity and concentration of total RNA at 260 nm. The integrity of RNA was investigated using 1.0% agarose gels electrophoresis. Synthesis of first strand cDNA was carried out using TIANScript RT kit (Tiangen, Beijing, China).

### 2.8. Analysis of gene expression

Analysis of gene expression levels was performed using an iQ5 real-time PCR system (Bio-Rad, USA). The operation parameter was set up following the recommendation of RealMasterMix (SYBR Green) (Tiangen). The primers for analysis (Table 1) were designed according to the related genes sequences using Primer Premier 5.0 software. Triplicate independent replicates were carried out for each sample.