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Chemical composition, crystal morphology and key gene expression of cuticular waxes of Asian pears at harvest and after storage

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ABSTRACT

Cuticular wax of three Asian pear cultivars, 'Kuerle', 'Xuehua' and 'Yuluxiang' at harvest and after 7 months of storage, was analyzed to determine its chemical composition, crystal morphology, and expression levels of associated genes. The highest cuticular wax concentration was observed in 'Kuerle' and the lowest in 'Xuehua'. The surface wax was mainly composed of alkanes, primary alcohols, terpenoids, fatty acids, and aldehydes. After storage, the total wax concentration at all cultivars decreased, and the wax crystal structures became glossier. 'Yuluxiang' fruit showed the strongest resistance to *Alternaria* rot. Gene expression analysis indicated that four structural genes (*CER6*, *KCS9*, *KCS20* and *FDH1*) expressed at higher levels and three genes (*CER60*, *DGAT1* and *MAH1*) expressed at lower levels in stored fruit were involved in wax synthesis, and the expression levels of two wax transportation genes (*LTPG1* and *LTP4*) and a transcriptional activator (*MYB96*) were also consistent with the wax concentration of the cultivars. Overall, understanding the differences in the cuticular wax in fruit at harvest and after storage among the cultivars may lead to a better understanding of their contributions to disease resistance and postharvest storage properties.

1. Introduction

Wax is a protective barrier on the primary aerial surface of plants that plays significant roles in non-stomatal water loss reduction (Weng et al., 2010), abiotic and biotic stress resistance (McDonald et al., 1993; Sun et al., 2015; Yin et al., 2011), and helps to maintain plant surface cleanliness and prevent organ fusion (Barthlott and Neinhuis, 1997; Sieber et al., 2000). The components of cuticular waxes have been determined to include homologue series of very-long-chain (VLC) aliphatic compounds, terpenoids, some sterols and flavonoids obtained by extraction with organic solvents (Bernard and Joubès, 2013). How is cuticular wax formed? So far, the answer to this question is based mainly on the biosynthesis of cuticular wax derived from *Arabidopsis*. The whole process can be divided into three stages. First, C_{16} and C_{18} fatty acids, which are common precursors in the biosynthesis of all lipids that exist widely in plants, are formed from de novo synthesis in plastids. Next, in the endoplasmic reticulum, the saturated C_{16} and C_{18} fatty acids are extended to very-long-chain fatty acids (VLCFAs) of 20 or more carbons in length. Last, VLCFAs are further modified into alkanes, alcohols, aldehydes, ketones and other components. Notably, as wax precursors, VLCFAs are split into two biosynthesis pathways. One is the decarbonylation (alkane-forming) pathway, which mainly forms aldehydes, alkanes, secondary alcohols and ketones. The other is the acyl reduction (alcohol-forming) pathway, which forms primary alcohols and esters (Li et al., 2008; Samuels et al., 2008). The wax compositions of several fruit crops have been analyzed. For example, triterpenes and *n*-alkanes were the most prominent components of the epicuticular waxes of apple fruit (Belding et al., 2000), whereas triterpenes and alkanes are the main compounds in at harvest fruit of sweet cherries (Peschel et al., 2007). Alkanes were the predominant class of compounds in the cuticular wax from tomato fruit (Leide et al., 2007), whereas triterpenes were the main composition in cuticular wax from grapefruit (McDonald et al., 1993) and blueberry (Chu et al., 2017). Moreover, it has also been reported that the wax composition varies among citrus species (Wang et al., 2014, 2016).

It has been reported that cuticular waxes can inhibit the conidial germination of plant pathogens. For example, cuticle wax concentration was positively correlated with resistance to gray mold (*Botrytis cinerea*) in roses (Hammer and Evensen, 1994), tomato fruit (Rijkenberg et al., 1980) and grape berries (Gabler et al., 2003). Furthermore, a disease

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resistance analysis of compounds isolated from avocado cuticular wax showed that longer-chain alcohols ($\geq C_{24}$) could induce appressorium formation of *Colletotrichum gloeosporioides*, making it easier for this pathogen to invade fruit (Podila et al., 1993). *Alternaria* rot is a common disease in pear-growing areas, and it can cause severe losses during the post-harvest period. Yin et al. (2011) reported that fatty acids, *n*-alkanes and triterpenoids might have contributed to the antifungal properties of 'Pingguoli' pear fruit cuticular waxes, but the correlation of *Alternaria* rot resistance with the concentration of C_{24} to C_{30} longer-chain alcohols in pears still needs to be elucidated.

Pear is one of the most economically important fruit crops and is cultivated in more than 50 countries. 'Kuerle' pear (*Pyrus sinkiangensis* Yü.) is the most famous cultivar in China, not only for its pleasant appearance, aroma and taste but also for its long postharvest storage period. The 'Xuehua' pear (*Pyrus bretschneideri* Rehd.) is an old cultivar, mainly produced in North China, and has a shorter storage life than that of 'Kuerle' pear. 'Yuluxiang' is a relatively new cultivar, derived from the hybridization of 'Kuerle' pear and 'Xuehua' pear, which has inherited the pleasant appearance, aroma, taste and the longer storage period from the 'Kuerle' pear, and the larger fruit size from the 'Xuehua' pear. Generally, it is believed that among pear cultivars, the fruit cuticular wax concentration and its properties are the main factors determining postharvest performance, which includes storage life and disease resistance; however, no direct evidence in pear cultivars has yet been available. In the present study, three cultivars from different but genetically related pear species were selected for determination of the concentration and properties of cuticular wax through GC–MS, crystal structure and qRT-PCR analysis to provide information about cuticular wax functions and synthesis in pear fruit.

2. Materials and methods

2.1. Plant material

Commercial maturity fruit of three pear cultivars, without disease infection or physical injuries, were selected for experiments. 'Kuerle' fruit (*Pyrus sinkiangensis* Yü.) were harvested on September 9, 2015, at Xiangli Fruit Institute in Xinjiang Province, China. The 'Xuehua' fruit (*Pyrus bretschneideri* Rehd.) were harvested on September 4, 2015, at Wandi Farm in Laiyang County, Hubei Province, China. 'Yuluxiang' fruit ('Kuerle' × 'Xuehua') were picked on September 7, 2015, at Shanxi Fruit Institute in Jinzhong, Shanxi Province, China. All fruit were packed and delivered immediately to the laboratory at Nanjing Agricultural University. 100 fruit of each cultivar at harvest were examined immediately; another 100 fruit of each cultivar were stored at 3 °C (60–80% relative humidity) for seven months and tested at room temperature (warm fruit).

2.2. Extraction of the cuticular wax

The cuticular wax was extracted according to the method of Li et al. (2014). After being washed with tap water and air dried, three groups of five were fully immersed and agitated twice for 1 min in 600 mL chloroform under a fume hood. The solution containing the waxes was then transferred into pre-weighed vials and evaporated under a stream of nitrogen (JHD-001S, Shanhai Jiheng Industries Company Limited) at 40 °C, and the wax weights were recorded.

2.3. Determination of the cuticular wax concentration

The surface area of the pear fruit was calculated according to the method of Yin et al. (2011). Cuticular wax was extracted from five pears per cultivar and weighed as above. The wax concentration (g m^{-2}) was calculated as: $(W_1 - W_0)/Sa$; W_1 = final weight of the vials (g); W_0 = initial weight of the vials (g); Sa = total surface area of 5 pear fruit (m^{-2}).

2.4. Chemical analysis by GC–MS

Samples of 1 mg wax extractions dissolved with 1.2 mL chloroform were analyzed according to the method of Li et al. (2014). A sample analysis was completed using a Bruker 450-GC, coupled with a Bruker 320-MS and a BR–5 ms capillary column (FS 30 m, 0.25 μm ID, 0.25 μm df). Helium was used as a carrier gas at a flow rate of 1.2 mL min^{-1}. The following parameters were employed: inlet temperature, 280 °C; MS transfer line temperature, 280 °C; ion source temperature, 250 °C; quadrupole temperature, 150 °C; electron impact (EI), 70 eV; and m z^{-1} range, 50–650.

GC was carried out at the following temperature settings. First, the temperature was set to 50 °C for 2 min. Next, it was increased to 200 °C at a rate of 40 °C min^{-1} and held at this temperature for 2 min. Finally, it was increased to 320 °C at a rate of 3 °C min^{-1} and held at this temperature for 30 min. The mass spectra of the individual wax components were compared to the mass spectra of the standards.

2.5. Electron microscopy

Pericarp pieces (3 × 3 × 1 mm) from the equatorial sections of three fruit for each cultivar were excised using a blade and fixed in 2.5% glutaraldehyde for 1 h, followed by a phosphate buffer solution (PBS, 0.1 mol L^{-1}, pH 7.4) wash that was completed three times for 10 min each time. Next, the pieces were treated with 2% osmic acid for 1 h and were washed with PBS again in the same fashion as in the previous step. The samples were desiccated with ethanol at various concentrations 30%, 50%, 70%, 80%, 90% for 10 min each time; then, 100% ethanol was used for dehydrating twice (each for 10 min). Next, the samples were subjected to an ethanol and *tert*-butyl alcohol mixture (1:1) for 10 min. Finally, the products were placed in *tert*-butyl alcohol, and lyophilizate for 6 h by a Hitachi ES-2030 freezing drier. The dehydrated samples were attached to a sample stage with conductive tape and coated with gold particles using a Hitachi E-1045. The coated samples were examined using a Hitachi 4800 field emission scanning electron microscope.

2.6. Gene expression analysis by quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using the Column Plant RNA Out kit (Fuji, China), and cDNA was synthesized using RevertAid 1ST cDNA Synth Kit (Thermo, USA). Gene-specific primers were designed using Primer Premier 5 software (Table S1). Quantitative real-time PCR amplification reactions were carried out using a Light Cycler 480 (Roche, USA). The 20 μL reaction mixtures contained 10 μL SYBR Green I Mix, 5 ng cDNA, ddH_2O, and a final primer concentration of 0.4 μM. The reaction mixtures were incubated for 10 min at 95 °C for pre-incubation, followed by 45 amplification cycles of 15 s at 95 °C, 15 s at 60 °C and 20 s at 72 °C. Relative transcript abundance was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Non-wounding inoculation

2.7.1. Preparation of spore suspension

The causal agent of *Alternaria* rot (*Alternaria alternata*) was supplied by the College of Plant Protection at Nanjing Agricultural University. The spores were removed from 7-day-old potato dextrose agar (PDA) cultures and suspended in sterile distilled water. The suspension was filtered through three layers of sterile lens-cleaning paper to remove any adhering mycelia. To make the spore dispersion uniform, the sample was placed in an oscillator, which performed oscillations for 15 s. The spore concentration of *A. alternata* was determined with the aid of a hemocytometer and adjusted to 1×10^6 spores mL^{-1} with sterile distilled water.