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Study of the structure and biosynthetic pathway of lignin in stone cells of pear

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ABSTRACT

Pear stone cell content is one of key determinants of fruit quality. Stone cells form by the deposition of lignin on primary cell walls, following secondary thickening of cell walls. Studies of the structure and metabolic pathway of pear lignin are rare. Stone cell and lignin content in the pulp of *Pyrus bretschneideri* cv. Dangshan Su were determined during fruit development. Lignin was extracted, purified and analyzed by ultraviolet (UV) light, Fourier-transform infrared (FTIR) spectroscopy and ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy. Intermediates of lignin biosynthesis were detected by high-performance liquid chromatography (HPLC). The results show that lignin content increases initially and decreases afterwards during fruit development, peaking twice at day 47 and day 63 after flowering. The lignin peaks precede both peaks of stone cell content (day 51 after flowering) and at the point at which sclereids reach their maximum diameter (day 67 after flowering). The milled wood lignin in pulp was identified as guaiacyl-syringyl-lignin by spectroscopic analyses. In the FTIR spectra, the peak intensity ratio of A_{1269}/A_{1227} was 1.25. HPLC analyses detected cinnamic acid and p-coumaric acid, but not caffeic acid or ferulic acid. In conclusion, during pear fruit development, lignin is first biosynthesized, and subsequently deposited on cell walls to form stone cells and sclereids. The biosynthesis of guaiacyl-syringyl- lignin in pear pulp may involve a phenylpropane-type metabolic pathway from phenylalanine to cinnamic acid and acyl-CoA ester, as pear pulp lignin contains more guaiacyl units than syringyl units.

1. Introduction

Stone cell content in pear pulp is one of the key determinants of pear quality. Pear stone cells, a type of brachysclereid, contain abundant lignin and cellulose. They are present in pulp in either isolated or aggregated forms known as sclereids (Schroeder, 1982; Qiao et al., 2005). Stone cells are a type of sclerenchyma cell formed by the secondary deposition of lignin on the primary walls of parenchyma cells. The development of stone cells is closely related to the synthesis, transfer, and deposition of lignin (Li et al., 2007; Tao et al., 2009). To date, there have been limited studies on the structure and biosynthetic pathway of lignin in pear stone cells. The influence of lignin on the formation of stone cells therefore remains unclear.

The metabolic pathway of lignin is complex. Lignin biosynthesis follows a phenylpropane metabolism (Meyermans et al., 2000; Humphreys and Chapple, 2002). This starts with the deamination of phenylalanine to form cinnamic acid, which then undergoes a series of hydroxylation, methylation and reduction reactions. Finally, the five major monomers in lignin biosynthesis are formed: p-coumasic acid, caffeic acid, ferulic acid, 5-hydroxy-ferulic acid, and sinapinic acid. These monomers then polymerize to form lignin and result in different metabolism pathways of lignin formation. Lignins are divided into three types according to their monomers (Meyermans et al., 2000). Syringyl lignin (S-lignin) is made of syringyl structure units, guaiacyl lignin (G-lignin) is made of guaiacyl units, and hydroxyphenyl lignin (H-lignin) consists of p-hydroxyphenyl propane units.

Pyrus bretschneideri cv. Dangshan Su, a native species of China, is grown widely across Asia and has the largest growing area of pear species in China. In recent years, due to factors such as deterioration of variety and poor field management, pear pulp stone cell content has increased, and the texture of the pulp has become rougher with more pomace. These changes have adversely affected pear flavor and quality (Nie et al., 2009; Tao et al., 2009).

The objectives of this study were to determine the relationship between stone cell formation and lignin metabolism, and to clarify the structure and metabolic pathway of lignin in pear stone cells. We analyzed and studied the correlation of stone cell and lignin content during the development of *P. bretschneideri* cv. Dangshan Su fruits. We extracted and purified lignin from pear fruits and studied

Abbreviations: DAD, diode array detector; FTIR, Fourier-transform infrared spectroscopy; G-lignin, guaiacyl lignin; G-S-lignin, guaiacyl-syringyl-lignin; H-lignin, hydroxyphenyl lignin; ^{1}H NMR, ^{1}H nuclear magnetic resonance spectroscopy; HPLC, high-performance liquid chromatography; MWL, milled wood lignin; S-lignin, syringyl lignin; UV, ultraviolet light.

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its spectral characteristics. We also determined the concentration of four lignin metabolic intermediates (cinnamic acid, p-coumaric acid, caffeic acid and ferulic acid). Our results provide the foundation for further research into pear stone cell development.

2. Materials and methods

2.1. Materials

Fruits of *P. bretschneideri* cv. Dangshan Su were obtained from 40-year-old pear trees grown on a farm (Dangshan, Anhui, China). Ten healthy and robust trees were selected from the same field in 2008 and 2009, labeled, and managed in a consistent manner. In April of each year (i.e. the bud stage), short branches bearing buds of similar developmental stage and size were selected from the medium layer of the south side of the crown and labeled. Two fruits were kept on each branch. Fruits were collected once every 4 days from 15 to 67 days after bloom. From 67 days after bloom, fruits were collected once every 7 days. Forty fruits of relatively uniform size were collected at each time point, refrigerated, transferred to the laboratory and tested.

2.2. Methods

2.2.1. Measurement of stone cell content

Stone cell content was measured using the method of Nie et al. (2009). Pulp (5.0 g), from 2.0 mm under the peel to 0.5 mm outside the core, was collected, stored at −20 °C for 24 h, and then homogenated at 20,000 rpm for 3 min. The homogenated pulp was added to water, allowed to rest, and the upper suspension was decanted. This procedure was repeated several times. The collected stone cells were oven-dried and then weighed three times. Stone cell content was calculated as: (stone cell content = dry weight of stone cells/5 × 100%).

2.2.2. Section observation of stone cells and measurement of sclereid size

Stone cells were sectioned using a procedure reported by Nie et al. (2009). Pulp, from 2.0 mm under the peel to 0.5 mm outside the core, was collected, cut into appropriate sizes, and fixed with FAA fixative solution (50 ml 50% ethanol, 5 ml glacial acetic acid, 5 ml formalin). Pulp sections were cut manually, stained with 0.1% Safranin, and studied under an optical microscope. Sclereid size was measured under a 40× objective and a 15× ocular micrometer. Each measurement was repeated 10 times. The actual size of sclereid = digital reading × 1000/15 × 40 (μm).

2.2.3. Measurement of lignin content

Lignin content was measured using the Klason method (Raiskila et al., 2007). Pulp (5.0 g), from 2.0 mm under the peel to 0.5 mm outside the core, was collected, oven-dried, ground into a uniform powder and passed through a 200-mesh sieve. The powder was extracted with methanol and oven-dried. A small amount (0.2 g) of this powder was extracted with 15 ml of 72% H_2SO_4 at 30 °C for 1 h, added to 115 ml of distilled water and boiled for 1 h. The volume was kept constant during boiling. The liquid mixture was filtered and the residue was rinsed with 500 ml of hot water, air-dried and weighed.

2.2.4. Extraction and purification of milled wood lignin

Milled wood lignin (MWL) was extracted and purified by a method from Kevin et al. (2006) and Chang et al. (2006). Pulp was cut into small pieces, oven-dried and ground into powder. The powder was passed through a 40-mesh sieve, extracted with a benzene-ethanol mixture, and oven-dried at 40 °C. Using a powder/extracting liquid ratio of 1:10, the powder was extracted with reflux mixture (dioxane/water 8:2) for 30 h at 20 °C. Raw lignin was obtained after solvent in the extract was removed by rotary evaporation under vacuum at 40 °C. The raw lignin was completely dissolved in a mixture of pyridine, glacial acetic acid and water (volumetric ratio 9:1:4), added to trichloromethane and separated using a separating funnel. The trichloromethane layer (bottom layer) was collected and added to ether to form a precipitate. The precipitate was separated by centrifugation, rinsed repeatedly with ether until any odor of pyridine was absent, and dried at 40 ± 2 °C under vacuum over P_2O_5. The material obtained after drying was purified MWL.

2.2.5. UV spectra of milled wood lignin

MWL (2.0 mg) was dissolved in a mixture of dioxane and water (volumetric ratio 8:2), transferred into a quartz cuvette, and scanned with a UV–vis spectrophotometer in the range of 190–400 nm (Sun et al., 2005; Chang et al., 2006).

2.2.6. Infrared spectra of milled wood lignin

MWL (2.0 mg) was mixed with 100 g of KBr in a dry atmosphere, ground into powder and pressed into pellets. The pellets were scanned with a Nexus-870 FTIR spectrophotometer in the range of 500–5000 cm^{-1} (Chang et al., 2006; Raiskila et al., 2007).

2.2.7. ^1H NMR of acetylated lignin

The ^1H NMR method was adapted from Kevin et al. (2006) and Li et al. (2007). Lignin (10.0 mg) was dissolved in a 2.0 ml mixture of pyridine and acetic anhydride (1:1). The flask was filled with nitrogen gas and stored at room temperature in the dark for 72 h. The reactant was precipitated by dropwise addition of ether, separated by centrifugation at 5000 rpm, and rinsed six to eight times with ether until the odor of any pyridine was absent. The resulting material was fully acetylated lignin. It was dissolved in 0.5 ml of $CDCl_3$, and studied with a Bruker-400 superconducting NMR spectrometer at 400 Hz using tetramethylsilicone as the internal standard (Pu et al., 2002; Qin et al., 2004).

2.2.8. Determination of cinnamic acid, p-coumaric acid, caffeic acid and ferulic acid

The acids were determined by HPLC (Agilent 1100) following the method of Wen et al. (2005). Quantifications were performed by external standardization with each acid, using the established regression equations (peak area) (Robbins and Bean, 2004).

Preparation of sample solutions: Pulp (2.0 g), from 2.0 mm under the peel to 0.5 mm outside the core, was accurately collected, added to 80% ethanol (pulp/liquid ratio 1:5), extracted over a 60 °C water bath for 2 h, and centrifuged at 12,000 rpm for 5 min. The supernatant was taken, condensed to half of the original volume, and filtered through a 0.45 μm microfiltration membrane. The filtrate was kept for analysis.

Preparation of standards: A 10 mg sample of each acid was dissolved in 10 ml HPLC-grade methanol under sonication. The solutions were stepwise diluted and combined to make a series of mixed standard solutions.

HPLC conditions: An Atlantis dC18 column (5 μm, 3.9 mm × 150 mm) and a diode array detector (DAD) were used. The mobile phase was made from water (phase A) and methanol (phase B), both added to 0.1% formic acid. Gradient elution parameters were as follows: 10% methanol from 0 to 5 min, increasing to 30% methanol from 5 to 6 min, 52% from 6 to 27 min, and 100% from 27 to 28 min; and a final elution with 100% methanol for 3 min. The flow rate was 1.0 ml/min, the injection volume was 10 μl, and the column temperature was 25 °C.

Selection of detection wavelength: Using full spectrum scans with DAD, the maximum absorption wavelength was determined to be 227 nm for cinnamic acid, 310 nm for p-coumaric acid, and