The Correlation between Cellular Features and Gene Expression in ‘Korla’ Fragrant Pear

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Additional index words. cell wall, 4CL, HCL, CCR, POD, PsTTY, stone cell, coarse pear, normal pear

Abstract. ‘Korla’ fragrant pear (*Pyrus sinkiangensis* T.T. Yu) variety has shown severe coarse skin in recent years. The intrinsic quality of its coarse fruit shows an increase in the number of stone cells and poor taste. In this study, stone cells and the cell wall of coarse pear (CP) and normal pear (NP) during various development stages were compared using paraffin-sectioning and transmission electron microscopy (TEM), and the relationships between lignin-related genes and stone cell formation and cell wall thickening were also analyzed. Our results show that giant stone cells are formed and distributed in the core of pear, whereas many of these crack 60 days after flowering (DAF). The period of stone cell fragmentation occurs later in CP fruits than in NP fruits. Parenchyma cell wall development in CP and NP fruits varies from 120 DAF to maturity. The parenchyma cell wall of CP fruits thickens, whereas that of NP fruits is thinner during the same period. The expression pattern of five genes (*Pp4CL1-l*, *PpHCT-l*, *Pp4CL2-l*, *PpPOD4*, and *PpPOD25*) coincides with changes in stone cell content in the pulp. Correlation analysis demonstrates a significant correlation between stone cell content and the expression level of the five genes ($p < 0.05$). In addition, the expression of those five genes and *PpCCR1* genes in CP fruits significantly increases during maturation and is highly correlated with the thickness of the parenchyma cell wall. The aim of this work is to provide insights into the mechanism of stone cell and parenchyma cell wall development in pear fruits and identify important candidate genes to regulate the quality of fruit texture using bioengineering methods.

‘Korla’ fragrant pear is a famous pear species that has been cultivated in China for more than 1300 years. It is generally favored

by consumers because of its distinctive aroma, sweet taste, juiciness, unique shape, and bright colors (Gao et al., 2005). It has been nominated as a geographic indication product by the Ministry of Agriculture and Rural Affairs of the People’s Republic of China. However, in recent years, coarse ‘Korla’ fragrant pear has been frequently observed in the production area. The intrinsic qualities of coarse fruits include light taste, low sugar content, higher acidity, more stone cells, and poor taste (Ma, 2010). Coarse ‘Korla’ fragrant fruits began to appear rough-skinned or exhibit hard end symptoms 90 DAF and can be divided into three phenotypes: rough-skinned fruit, hard end fruit, and mixed type at maturity. Previous studies have shown that hard end is a pear fruit disorder in which the tissue of the calyx end of the ripe fruit is hard and dry (Ogawa and English, 1991). The disorder has been reported primarily in most of the European cultivars, such as Bartlett, Anjou, and Winter Nelis

(Pierson et al., 1971; Yamamoto and Watanabe, 1983). Possible causes of hard end disorder in pear fruits include water stress, lignin accumulation, and calcium deficiency (Lu et al., 2015; Welsh, 1979). The disorder significantly affects fruit flavor and consumer acceptance (Rose et al., 1951).

Previous studies have reported that the diameter and density of stone cell mass are larger at the calyx end of coarse pears than normal pears during the maturation stage, and the shape of parenchyma cells around the stone cells in coarse pear differs from normal pears (Ma, 2010). Stone cells are a type of sclerenchyma cells formed by the secondary thickening of cell walls, followed by the deposition of lignin on the primary walls of parenchyma cells. In pears, stone cells in the fruit flesh are an important determinant of fruit texture (Tao et al., 2009) and distributed in the pulp in either the isolated or aggregated forms. The development of stone cells mainly depends on the synthesis, transfer, and deposition of lignin (Cai et al., 2010). In hard end pear fruits, the number of stone cells and lignin content are higher, which results in greater firmness and lower market value, suggesting that the hard end disorder in pear fruits is caused by abnormal lignin accumulation (Cai et al., 2006; Passardi et al., 2005; Veitch, 2004). The size and shape of parenchyma cells around the stone cells are related to the texture of pear flesh (Gu et al., 1989).

Lignin synthesis involves biosynthesis of lignin precursors, their transport to the cell walls, and polymerization. Lignin is a complex phenolic polymer mainly derived from *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (known as monolignols) (Barros et al., 2015; Boerjan et al., 2003). In the lignin biosynthetic pathway, four-coumarate: CoA ligase gene (4CL) plays multiple roles in plant growth and development by catalyzing the formation of a CoA ester. As a key enzyme involved in the biosynthetic pathway, 4CL is a plant-derived phenylpropane derivative and is related to the synthesis of flavonoids and lignin. The gene family encoding these enzymes comprises multiple isoforms of 4CL, which are potentially associated with diverse and unknown functions (Costa et al., 2005; Gui et al., 2011). Shikimate hydroxycinnamoyl transferase (HCT) is an enzyme involved in the monolignol pathway, catalyzing the reactions both immediately preceding and following the insertion of the 3-hydroxyl group into monolignol precursors (Hoffmann et al., 2003, 2004). Cinnamoyl-CoA reductase (CCR) is the first enzyme specific to the monolignol pathway (Lacombe et al., 1997). Class III peroxidases (CIII Prxs) are primarily considered as secreted/apoplastic/CW proteins (Welinder, 1992), although vacuolar isoforms exist (Carter et al., 2004). The complex roles of CIII Prxs could be explained according to the diversity of their substrates and the spatiotemporal regulation of their expression. Thus, their functional analysis remains a challenge.

Consumers mainly eat the middle part of the fruit pulp. However, information on the cell structure and the molecular mechanism

Received for publication 1 Oct. 2019. Accepted for publication 29 Oct. 2019.

Published online 16 December 2019.

This study was supported by the Conservation and Utilization of Crop Germplasm Resources (111821301354052020), the Funds for Basic Scientific Research Business of Public Welfare Research Institutions in Autonomous Region (ky2018041), and the Natural Science Foundation of Xinjiang Uygur Autonomous Region (2017D01B22).

We thank LetPub (www.letpub.com) for providing linguistic assistance during the preparation of this article.

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in the middle pulp of coarse 'Korla' fragrant fruit during development is limited. In the present study, stone cell development and distribution and cell wall thickening in the middle pulp of CP (rough-skinned fruit and hard end fruit mixed-type) and NP fruits were monitored and compared during fruit development. The relationships between lignin-related genes and stone cell formation and cell wall thickening were investigated. Our results provide insight into the mechanism of stone cell and cell wall development in pear fruits and identify important candidate genes that regulate the quality of fruit texture using bioengineering methods.

Materials and Methods

Fruit materials. Pear fruits were harvested from trees grown in an experimental orchard located in the National Fruit Tree Germplasm Repository, Academy of Xinjiang Agricultural Sciences, Luntai, Xinjiang, China (lat. 45°19'N, long. 86°03'E). All of the experimental trees were planted in rows 4 × 5 m apart in a north-south orientation in 1994. Fertilization management and pest control were conducted according to standard practices, and drip irrigation was used to water the fruit trees. The NP tree was a singletree plot with a completely randomized design carrying replicates of 10 individual trees. The CP tree was a singletree plot with 10 individual trees as replicates, with no pruning. During the 2017 harvest season (from 10 May to 7 Sept.), fruits were picked from 10 trees at each stage (30, 60, 90, 120, and 150 DAF). Three replicates were used for each stage of the NP and CP fruits. The equatorial section of fruits was cut into small pieces without kernel. The samples were immediately frozen in liquid nitrogen and stored at -70 °C.

Determination of the stone cell content. The stone cell content was measured using the method of Cai et al. (2010). Pulp (100 g) was collected from 2.0 mm under the peel to 0.5 mm outside the core and stored at -20 °C for 24 h, followed by the addition of 100 mL H₂O and homogenized at 20,000 rpm for 3 min. The homogenized pulp was incubated with water, followed by the addition of 5 mL hydrochloric acid. After 1 hour, the upper

suspension was decanted. This procedure was repeated several times. The collected stone cells were dried and weighed. The stone cell contents were calculated as follows: $SCC(\%) = \frac{W_1}{W_2} \times 100$, in which SCC is the number of stone cells per 100 g of fresh pulp, W₁ is the dry weight (DW) of the stone cells, and W₂ is the fresh weight (FW) of the pulp.

Microstructural observations. Transverse and longitudinal sections of pear fruits were manually prepared and treated with 5% phloroglucinol in a 95% ethanol solution for 5 min and mounted in 6 M HCl to examine whether the cinnamaldehyde groups of the lignin were present before the coverslips were applied (Pradhan and Loque, 2014).

Sample preparation was performed as described by Yang et al. (2017). Briefly, fruits were detached at 30, 60, 90, 120, and 150 DAF; fixed in FAA [1 formalin: 1 acetic acid: 18 ethanol (70%)], and dehydrated through an ethanol gradient (70%, 80%, 95%, 100%, and 100% for 1 h and 40, 30, 30, and 30 min, respectively). After 50% absolute ethanol and 100% dimethylbenzene, 100% dimethylbenzene for 20 min per stage, the samples were embedded in paraffin at 60 °C. The embedded samples were sectioned transversely with a rotary microtome at a thickness of 5 µm. Before staining, the sections were deparaffinized in dimethylbenzene for 10 min and rehydrated in an ethanol series for 5 to 10 min (100%, 95%, 85%, and 70%). The samples were dyed with safranin (0.5 g safranin in 100 mL 70% ethanol) for 6 h and then dehydrated in a graded series of ethanol dilutions (70%, 85%, and 95% for 10 s per stage). The samples were then stained with fast green (0.5 g in 100 mL 95% ethanol) for 10 s and observed under a microscope.

TEM observation. For sample preparation, fruits were detached at 120 and 150 DAF. The small slices (1 mm thickness) were sectioned from the fruit flesh with a razor blade using freehand and fixed with a freshly prepared mixture comprising 3% glutaraldehyde (v/v) and 0.1% osmium acid (w/v) in 0.1 M phosphate buffer (pH 7.4) and processed as described by Musetti et al. (2005) before embedding in epoxy resin.

Gene expression analysis by real-time reverse transcriptase polymerase chain reaction.

Total RNA was extracted from the pear flesh tissue using an RNA plant reagent (TianGen, Shanghai, China) according to the manufacturer's instructions. DNA contamination was removed using DNase (Fermentas, Vilnius, Lithuania). The first cDNA strand was reverse transcribed using a Revert Aid First Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's instructions. At each time point, three batches of RNA were isolated as three biological replicates for separate cDNA synthesis.

The primer sets used for real-time transcriptase polymerase chain reaction (PCR) analysis were designed by Primer3 (<http://primer3.ut.ee>). Pear actin was used as internal control to normalize small differences in template amounts. 'Korla' fragrant pear actin gene (forward: 5'-CCATCCAGGCTGTTCTCTC-3', reverse: 5'-GCAAGGTCCAGACGAAGG-3') was used as reference. The primer sequences of the target genes are shown in Table 1.

Real-time PCR was performed using a real-time PCR instrument (Roche 480, Switzerland). The total volume of 20 µL included 2 µL of the cDNA template, 2 µL of each primer, and 10 µL 2 × SYBR Green PCR Master Mix (Roche, Basel, Switzerland). The PCR protocol at 94 °C for 5 min, 94 °C for 15 s, and 40 cycles at 60 °C for 1 min. A negative control without a template for each primer pair was included in each run. Quantification was achieved by normalizing the number of target transcripts to the reference actin gene using the comparative 2^{-ΔΔCT} method (Livak and Schmittgen, 2001). Three RNA isolates and cDNAs were used as replicates for real-time PCR analysis. All of the relative intensities were calculated from the standard curves for each gene.

Statistical analysis. Three biological replicates were used for all experiments, and the results were expressed as the mean and standard error of triplicate experiments. The graphs were plotted using Origin Pro 7.5 G (Microcal Software, Inc., Northampton, MA). Statistically significant differences were analyzed by Fisher's protected least significant difference test at a *P* = 0.05 level or *P* = 0.01 level. SPSS 10.01 (SPSS Inc., Chicago, IL) was used for correlation analysis between

Table 1. Primers used in real-time polymerase chain reaction analysis.

Gene name	GenBank accession no.	Primer name	Primer sequence
<i>Pp4CL1-I</i>	XP_009370961.1	<i>Pp4CL1-I-F</i>	AGGTTGACATCAGACCAGACG
		<i>Pp4CL1-I-R</i>	TCCACAAAGCAATACCGAGTT
<i>Pp4CL2-I</i>	XP_009334566.1	<i>Pp4CL2-I-F</i>	CGGAAACTGAGGAAGACGGT
		<i>Pp4CL2-I-R</i>	TATGGAACAAGGGCAGCAGC
<i>Pp4CL7-I</i>	XP_009356915.1	<i>Pp4CL7-I-F</i>	TCACGCTAAACACGACACAA
		<i>Pp4CL7-I-R</i>	CGCCGTGAAGTACTGAATGA
<i>PpHCT-I</i>	NP_001289240.1	<i>PpHCT-I-F</i>	GGTAGAACGGCACAAGAGC
		<i>PpHCT-I-R</i>	GAGGAGACGCCAAAGAAGG
<i>PpCCR1</i>	AHM10331.1	<i>PpCCR1-F</i>	GGGTCACCTTCCAGTCCATA
		<i>PpCCR1-R</i>	TTTTACAATACCATCGCAGC
<i>PpPOD3</i>	XP_009342265.1	<i>PpPOD3-F</i>	CCCGAAAGCAGAGAAGATTGT
		<i>PpPOD3-R</i>	GGGTTAGATTTGGGGAAGCAT
<i>PpPOD4</i>	AFY97687.1	<i>PpPOD4-F</i>	TGCCATCTCACCCAAAGAG
		<i>PpPOD4-R</i>	CATGCATGTGGTCAGTCGTTT
<i>PpPOD25</i>	XP_009368847.1	<i>PpPOD25-F</i>	ATGCTTGGTTTATGGTCGGG
		<i>PpPOD25-R</i>	GGATGGCAGGGTCTCTTTGT

gene expression and stone cell content in the CP and NP fruits.

Results

Changes in stone cell content during fruit development. Compared with the stone cell content in NP, although the stone cell content in CP fruits was higher throughout the fruit development period, their trends were similar (Fig. 1). In addition, stone cell content continuously increased before 60 DAF and was significantly higher in CP than in NP fruits ($P < 0.01$). After 60 DAF, stone cell content decreased, reaching its lowest level at 150 DAF. At 120 DAF, stone cell content was also significantly higher in CP fruits than in NP fruits ($P < 0.05$).

Distribution of stone cells in fruits and changes in cell walls. After staining with phloroglucinol, the density and distribution range of stone cells in the cross section of CP fruit were higher than in NP fruit (Fig. 2).

Fig. 1. The stone cell content of coarse (CP) and normal (NP) pears at five development stages [30, 60, 90, 120, and 150 days after flowering (DAF)]. Each histogram represents the mean value, and the bar is the standard error of three biological replicates. The symbols a, b and A, B indicate significant differences between CP and NP at $P < 0.05$ and $P < 0.01$, respectively.

Fig. 2. Microstructure of coarse (CP) and normal (NP) pears during the five fruit development periods [30, 60, 90, 120, and 150 days after flowering (DAF)]. CP represents the stained sections of the stone cells of CP fruits during five developmental periods. NP represents the stained sections of the stone cells of NP fruits at five developmental periods. Arrow indicates the location of the stone cells.

From 30 DAF to 150 DAF, the distribution of stone cells was radial around the core, and the density of stone cell was high. From 90 to 150 DAF, the distribution range and density of stone cells gradually decreased.

Assessment of the microstructure of the whole pear fruit in both CP and NP fruits revealed that the area of stone cells increased initially and subsequently decreased during fruit development (Fig. 2). In the CP and NP fruits, the major stone cells were at the primitive stage at 30 DAF and appeared as loose aggregates near the fruit core, and the thick-walled cells were scattered near the peel. At 60 DAF, the area of the stone cells increased, the diameter of the stone cells in the fruits continuously increased until it was adjacent to the core, and the stone cells appeared near the peel. Compared with NP, no cracks in giant stone cells were observed. The stone cell area rapidly increased before 60 DAF. However, the stone cell areas decreased at 90 DAF, and apparently additional giant stone cells had cracked. Compared with NP, stone cell cracking was delayed and inhibited. At 120 DAF, the stone cell areas continued to decrease, and although the giant stone cells had fragmented into small pieces, these still existed in the core of fruit. At 150 DAF, the stone cell area decreased to its lowest level. Giant stone cells in NP were mainly near the core and were scattered in the whole pulp. The number of stone cells with diameters greater than 200 μm was higher in CP than in NP at maturity.

TEM analysis showed that cell wall development between CP and NP differed from 120 d to maturity. Compared with cell walls of the NP pulp at 120 DAF (Fig. 3C), the cell walls of NP were thinner at 150 DAF (Fig. 3D) and thicker (Fig. 3C) than the cell walls of CP at 120 DAF (Fig. 3A). Compared with the cell walls of NP pulp at 150 DAF (Fig. 3D), the CP cell walls thicker (Fig. 3B). Conversely, the cell walls of CP at 150 DAF (Fig. 3B) were thicker than those at 120 DAF (Fig. 3A). The results showed a gradual

thinning of the cell walls of the NP pulp with fruit ripening, whereas the cell walls of CP thickened.

Expression of eight genes during fruit development. As shown in Fig. 4 and Table 1, the expression of eight homologous genes in the model plants was observed at 30, 60, 90, 120, and 150 DAF in the CP and NP fruits.

The expression of *Pp4CL1-l* was most similar to that of *Pp4CL2-l* and *PpHCT-l* genes, which peaked at 60 DAF and then decreased with fruit development. Compared with the NP fruits, the CP fruits showed significantly higher *PpHCT-l* expression before 60 DAF, whereas significantly lower *Pp4CL2-l* expression at 60 DAF ($P < 0.05$). The expression of *Pp4CL1-l*, *PpHCT-l*, and *Pp4CL2-l* genes in the CP fruits was significantly lower at 120 DAF and higher at 150 DAF ($P < 0.01$). These results suggested that the three genes were related to stone cell formation. *Pp4CL7-l* showed a different expression pattern in experimental fruits. The expression in the CP fruits peaked at 150 DAF, whereas the NP fruits peaked at 120 DAF. The expression of *Pp4CL7-l* in the CP fruits was significantly higher from 60 to 90 DAF and 150 DAF but lower at 30 DAF ($P < 0.01$). The expression of *PpCCR1* peaked at 30 DAF and decreased with fruit development. The expression of the *PpCCR1* gene in the CP fruits significantly decreased from 30 and 90 DAF to 120 DAF ($P < 0.01$). During the maturation period, the expression of the *PpCCR1* gene in CP fruits gradually increased, whereas that in NP fruits decreased. The expression of the *PpPOD3* gene in CP fruits gradually increased from 30 to 150 DAF and peaked at 150 DAF, while that in the NP fruits peaked at 90 DAF, and the lowest expression was observed at 60 DAF. The expression of *PpPOD3* in the CP fruits significantly decreased at 30 DAF and from 90 to 120 DAF and increased at 60 and 150 DAF ($P < 0.01$). Compared with *PpPOD4* in NP during the total fruit development period, the *PpPOD4* expression levels in CP fruits were lower but shared similar trends with the NP fruits. The expression of *PpPOD4* between the CP and NP fruits gradually increased during early and later fruit development periods. The expression of *PpPOD4* in CP fruits significantly decreased from 30 to 60 DAF and 120 DAF ($P < 0.05$). The expression of *PpPOD25* varied between the CP and NP pears, peaking in CP at 90 DAF, and the lowest level was observed at 120 DAF, compared with peak levels at 60 DAF and the lowest at 150 DAF in NP fruits. The expression of *PpPOD25* in CP was significantly lower at 60 and 120 DAF ($P < 0.01$).

Correlation between gene expression and stone cell content. As shown in Table 2, the correlation coefficients of CP SCC vs. *Pp4CL1-l*, *Pp4CL2-l*, *PpHCT-l*, and *PpPOD4* were $r = 0.909$ to 0.954 ($P < 0.01$), respectively. The correlation coefficients of NP SCC vs. *Pp4CL1-l*, *Pp4CL2-l*, *PpHCT-l*, *PpPOD4*, and *PpPOD25* were $r = 0.838$ – 0.947 ($P < 0.01$ and $P < 0.05$), respectively. The correlation

coefficients between CP SCC and NP SCC vs. *Pp4CL7-1* were $r = 0.131$ and 0.227 , respectively. The correlation coefficients between CP SCC and NP SCC vs. *PpCCR1* were $r = 0.485$ and 0.122 , respectively. Negative correlation coefficients between CP SCC and NP SCC vs. *PpPOD3* were $r = -0.472$ and -0.297 ($P < 0.05$), respectively. The correlation coefficients (r) of SCC vs. *PpPOD25* in CP fruits were significantly different from the NP fruits.

Fig. 3. Ultrastructure of parenchyma cell wall of coarse (CP) and normal (NP) pear fruit flesh. (A) Cell wall of CP at 120 days after flowering (DAF), (B) cell wall of CP at 150 DAF, (C) cell wall of NP at 120 DAF, (D) cell wall of NP at 150 DAF. CW = represents cell wall. Bars = 2 μ m.

Discussion

Stone cell content is a key determinant of fruit quality in pears. Several studies have revealed that stone cells in pears originate from sclerenchyma cells (Cai et al., 2010; Jin et al., 2013; Nii et al., 2008; Zhao et al., 2013). Recent studies have investigated stone cell content, lignin, and lignin-related genes in young fruits and at postharvest (Lu et al., 2015; Zhang et al., 2017). However, studies on stone cell development and cell wall alterations in coarse ‘Korla’ fragrant pears during fruit maturation are limited. In addition, these studies used partial pulp in their investigations. A few studies investigated the microstructure of the whole pear fruit. In the present study, we investigated the accumulation and distribution of stone cells and changes in cell wall thickness during fruit development and screened the potential key genes related to lignin formation.

In this study, we determined the stone cell content, distribution, and area in normal and coarse fruits. On the basis of microscopic evaluation of the total landscape of pear pulp, the diameter of the stone cells in CP and NP fruits continued to increase near the core before 60 DAF and eventually cracked. The present study supports the conclusion that stone cells appear in the pear flesh at about 15 DAF and persist until 60 DAF (Liu et al., 2005; Qiao et al., 2005; Tao et al., 2009). However, we also observed that giant stone cells obviously cracked in CP and NP fruits 60 DAF, which was contrary to previous conclusions suggesting that the stone cells remained at a certain level 2 months before maturity

(Liu, 2005; Qiao, 2005). Jiang (2013) reported that stone cells in ‘Gold-Nijisseiki’ (Japanese pear) gradually disintegrated and decreased in size after flowering for 2 months. The present study provides further evidence supporting that conclusion, and we also found that stone cell cracking in CP fruits occurred later compared with NP fruits. During the mature period, stone cells measuring more than 200 μ m in NP fruits mainly accumulated near the core, while those in CP fruits were dispersed within the whole pulp. The number of stone cells measuring greater than 200 μ m in diameter was higher in CP than in NP fruits. These results were concordant with the findings of a previous study suggesting that the diameter of stone cells exceeded 300 μ m, and the fruits had coarser flesh (Tian et al., 2011).

Hiwasa et al. (2004) suggested that cell wall degradation was correlated with a decrease in firmness during ripening, and the parenchyma cell walls became extremely thin and fragile (Ben-Arie et al., 1979). Our results showed that the parenchyma cell walls of NP fruits became thin during maturation, whereas those of CP increased in thickness.

Previous studies have shown that lignin synthesis is catalyzed by multiple enzymes. In the present study, the eight genes showed high homology with their counterparts in *Arabidopsis thaliana* and *Nicotiana tabacum*. The expression levels of structural genes (*Pp4CL1-1*, *Pp4CL2-1*, *PpHCT-1*, *PpPOD4*, and *PpPOD25*) related to lignin metabolism in fruits peaked before 60 DAF (Fig. 4), which was consistent and significantly correlated with stone cell content in pear flesh (Fig. 1; Table 2), suggesting that these five

Fig. 4. Expression patterns of eight genes (*Pp4CL1-1*, *Pp4CL2-1*, *Pp4CL7-1*, *PpHCT-1*, *PpCCR1*, *PpPOD3*, *PpPOD4*, and *PpPOD25*) in coarse (CP) and normal (NP) pear during five developmental stages: 30, 60, 90, 120, and 150 days after flowering (DAF) based on quantitative real-time polymerase chain reaction analysis. Data represent the mean $\pm$ SE of three biological replicates. In this and the following figures, the symbols a and b represent significant differences between CP and NP at $P < 0.05$ and A and B represent significant differences between CP and NP at $P < 0.01$.

Table 2. Analysis of the correlation between eight genes and stone cells in two fruit phenotypes at different developmental stages.

	<i>Pp4CL1-l</i>	<i>Pp4CL2-l</i>	<i>Pp4CL7-l</i>	<i>PpHCT-l</i>	<i>PpCCR1</i>	<i>PpPOD3</i>	<i>PpPOD4</i>	<i>PpPOD25</i>
CP SCC	0.954**	0.936**	0.131	0.914**	0.437	-0.472	0.909**	0.473
NP SCC	0.838**	0.943**	0.227	0.901**	0.122	-0.297	0.947**	0.918**

Significant correlation at ** $P < 0.01$ or * $P < 0.05$.

genes play a potential role in stone cell formation.

CCR catalyzes the first specific step in lignin monomer synthesis. *PpCCR1* shows a high degree of homology with *A. thaliana* CCR1 (*AtCCR1*). In CP and NP fruits, the expression levels of *PpCCR1* were correlated with the thickness of parenchyma cell wall (Figs. 3 and 4). The *AtCCR1* gene improved lignin content and increased the number of noncondensed lignin units and was more specifically involved in the assembly of S2 in the cell wall (Ruel et al., 2009). These results suggest that the *PpCCR1* gene may mediate lignin and cell wall formation. Our results show that the expression of *PpCCR1* was higher in NP than in CP from 30 to 120 DAF, indicating higher levels of noncondensed lignin in NP than in CP fruits. Noncondensed lignin more easily dissolved compared with condensed structures (Jin et al., 2013).

The *PpPOD25* gene shows a high degree of homology with *AtPrx25*, which encodes cationic cell-wall-bound peroxidase belonging to CIII Prxs and is considered as an extracellular protein (Francoz et al., 2014). *AtPrx25* is classified as an all-round peroxidase that can oxidize guaiacyl and syringyl moieties that are located in the cell wall. *AtPrx25* plays a role in cell wall stiffening and altered cell wall thickness in the stems by increasing the number of β -O-4 linked syringyl units (Shigeto et al., 2013, 2014, 2015). Previous studies have suggested that the *PpPOD25* gene may alter cell wall thickness and the number of β -O-4 linked syringyl units. Our results show that the expression of *PpPOD25* was higher in NP than in CP both in young fruits and at 120 DAF, which suggested the presence of additional β -O-4-linked syringyl units in NP compared with CP. In CP, the expression of *PpPOD25* coincided with the thickness of parenchyma cell wall ultrastructure (Figs. 3 and 4), and similar results were observed in NP fruits. These results suggest that the *PpPOD25* gene may play an important role in parenchyma cell wall thickening.

Despite the presence of lignin up to only 20% to 30% in mature stone cells, its formation is considered to involve lignification. Several studies have suggested that lignin plays an important role not only in cell wall thickening but also in stone cell formation (Crist and Batjer, 1931; Ranadive and Haard, 1973; Tao et al., 2009). The expression levels of five genes (*Pp4CL1-l*, *PpHCT-l*, *Pp4CL2-l*, *PpPOD4*, and *PpPOD25*) and the *PpCCR1* genes of CP fruits significantly increased during the maturation period and were highly correlated with the thickness of the parenchyma cell wall. The expression of the *PpPOD25* and *PpCCR1* genes in NP was

significantly higher before maturation (Fig. 4), indicating the presence of additional β -O-4-linked syringyl units in NP than in CP. The β -O-4 linkage plays an important role in the dissolution of lignin (Jiang, 2008; Wang, 2012a, 2012b), indicating the possibility of further noncondensed lignin in NP than in CP. Therefore, *PpCCR1* and *PpPOD25* may play important roles in parenchyma cell wall alterations.

Conclusion

Stone cell content is significantly higher in CP than in NP at three developmental stages ($P < 0.05$). The giant stone cells are formed and distributed in the core of pear, while many of these crack after 60 DAF. Compared with NP, the stone cells in the CP fruits subsequently crack, and stone cell content and diameter are higher at the maturation stage. The parenchyma cell wall of CP fruits is thicker, whereas the parenchyma cell wall of NP is thinner during the same period. The expression of structural genes (4CL, HCT, CCA, and POD) in CP fruits increased compared with NP fruits at the young fruiting stage and maturation. The expression pattern of genes is significantly correlated with changes in stone cell content and parenchyma cell wall thickness.

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