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RESEARCH ARTICLE

# Changes of chlorogenic acid content and its synthesis-associated genes expression in Xuehua pear fruit during development

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**Abstract**

According to synthetic pathway of plant chlorogenic acid (CGA), the expression patterns of genes encoding enzymes that are associated with CGA synthesis were studied in normally developed Xuehua pear fruit. The study demonstrated that CGA content in peel and flesh of Xuehua pear decreased as fruit development progressed, with a higher level in peel. The expression levels of *PbPAL1*, *PbPAL2*, *PbC3H*, *PbC4H*, *Pb4CL1*, *Pb4CL2*, *Pb4CL6*, *PbHCT1* and *PbHCT3* genes decreased in fruit, which was consistent with the pattern of variation in CGA content. That indicated that these genes might be key genes for influencing fruit CGA synthesis in Xuehua pear. However, *Pb4CL7* gene expression profile is not consistent with variation of CGA content, hence, it may not be a key gene involved in CGA synthesis.

**Keywords:** cinnamate 4-hydroxylase gene, hydroxy cinnamoyl CoA, shikimate/quinic acid hydroxycinnamoyl transferase gene, ρ-coumarate 3´-hydroxylase gene, 4-hydroxycinnamoyl-CoA ligase gene, phenylalanine ammonia lyase gene, Xuehua pear

## 1. Introduction

Pear fruit is rich in phenolic compounds, and chlorogenic acid (CGA) is the main one (Cui *et al.* 2005; Salta *et al.* 2010; Yuan *et al.* 2011; Li *et al.* 2014), which exerting crucial physiological function, such as anti-oxidation process. CGA exhibits a higher antioxidant capacity than caffeic acid, ferulic acid, syringic acid and tocopherol and has a wide range of biological activities (Bravo 1998; Donovan *et al.* 1998; Santos *et al.* 2006). CGA serves as an oxidative substrate catalyzed by polyphenol oxidase (PPO) for enzymatic browning of fruits (Wu *et al.* 1992; Tomás-Barberán and Espín 2001; Yuan *et al.* 2011). In addition, CGA is also implicated in stone cell development and biosynthesis of lignin in fruits (Tomás-Barberán and Espín 2001; Cai *et al.* 2010). Therefore, CGA content is closely associated with fruit quality. However, the researches on CGA biosynthesis in fruits are very limited.

Previous works on tobacco, tomato and *Coffea canephora* show that CGA is synthesized *via* phenylpropanoid pathway (Niggeweg *et al*. 2004; Mahesh *et al*. 2007). The pathway is initiated from the deamination of phenylalanine to cinnamic acid by phenylalanine ammonia ligase (PAL), followed by hydroxylation and methylation of cinnamic acid by cinnamate 4-hydroxylase (C4H) and 4-hydroxycinnamoyl-CoA ligase (4CL), respectively. Thus ρ-coumaric acid is produced, then hydroxycinnamoyl-CoA shikimate/quinate hydroycinnamoyltransferase (HCT/HQT) pathway

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begins, and CGA is synthesized finally (Niggeweg *et al.* 2004; Mahesh *et al.* 2007; Zhao *et al.* 2013). Hence, PAL, C4H, 4CL and HCT/HQT are important regulators related to CGA biosynthesis (Lepelley *et al.* 2007; Lallemand *et al.* 2012; Kim *et al.* 2013; Escamilla-Treviño *et al.* 2014; Yuan *et al.* 2014). Previous studies had shown that CGA levels were higher at the early development stages of apple and pear fruits and then decreased gradually to a steady level as fruits mature (Ju *et al.* 1995; Awad *et al.* 2001; Cui *et al.* 2005). However, the mechanism of molecular regulation of variation in CGA content of fruit is unclear.

Given the fact that CGA is a main phenolic compound in pear fruit with a variety of biological activities and functions, and Xuehua pear (*Pyrus bretschneideri* Rehd), as a typical white pear cultivar, is cultivated in large areas and widely consumed in China, it is paramount to examine the variation in CGA content and corresponding molecular regulation during fruit development.

*PbPAL* and *PbHCT* genes were cloned and their organ-specific expression patterns had been studied in our laboratory (Ge *et al.* 2012; Yan *et al.* 2014). Based on data of these studies, the variation in CGA content and the expression levels of CGA synthesis-associated genes during the development of Xuehua pear fruit were further studied, in an effort to discover key genes closely associated with CGA biosynthesis during pear fruit development, and thereby providing a novel evidence for further study on regulation of CGA synthesis.

## 2. Materials and methods

### 2.1. Materials

Xuehua pear fruit was sampled every 30 days from 55 days after full bloom (DAFB) at a normal orchard in Zhao County, Hebei Province, China. Single-layer brown paper bags were bagged to the fruit from 70 DAFB until harvest at maturity. All samples were collected at the height of 1.5–1.7 m above ground from the periphery of canopy, and 15 fruits were got at least for each tree at every sampling time. The sampled fruits were weighed, then peels and fleshes were frozen in liquid nitrogen immediately and stored in freezer at –80°C on the same day. Procedures were carried out in triplicates.

### 2.2. CGA content determination

2 g of ground frozen fruit sample were suspended in 10 mL 80% methanol and incubated with shaking in ultrasonic oscillator for 20 min. After centrifugation at 10 000×g for 10 min at 4°C, 1 mL supernatant was collected and subjected to solid phase extraction *via* $C_{18}$ column, followed by washing with 1 mL methanol and being filtered through a 0.45-μm organic membrane. The resulting filtrate was stored for further analysis. The content of CGA was measured *via* high-performance liquid chromatography (HPLC) according to the method of Awad *et al.* (2000). With a HITACHI L2000 HPLC System equipped with a Lachrom $C_{18}$ column (250 mm×4.6 mm, 5 μm), HPLC was performed in a mobile phase of 5% acetic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 1 mL $min^{-1}$ and a temperature of 30°C, and monitored at a wave length of 280 nm with injection of 5 μL samples. Samples were tested using external standard method, which used retention time to determine the quality and peak area for quantification. The analysis was performed in triplicates for each sample to obtain the mean value and standard deviation.

### 2.3. RNA extraction

Total RNAs were isolated from fruit peel and flesh, respectively, using improved hexadecyltrimethylammonium bromide (CTAB) method (Malnoy *et al.* 2001) and purified with recombinant DNase I. After electrophoresis analysis, genomic DNAs were eliminated with PrimeScript RT Reagent Kit With gDNA Eraser (Perfect Real Time)(TaKaRa Bio Inc., Japan) and first strand of cDNA was generated by reverse transcription.

### 2.4. Real-time PCR (RT-PCR) analysis

Primers for quantitative PCR were designed according to pear (*P. bretschneideri*) nucleotide sequences registered in GenBank of NCBI. The sequence of primers was shown in Table 1.

RT-PCR analysis was performed on ABI 7500 Real-Time System using SYBR Premix *ExTaq*TM Kit (TaKaRa Bio Inc., Japan) and SYBR Green I fluorescent dye. The pear *PbActin2* gene was used as internal reference (Yan *et al.* 2012) and the amount of gene expression in peel of Xuehua pear at 55 DAFB was defined as 1.0.

### 2.5. Statistical analysis

Data were analyzed and Figs. 1–6 were generated using GraphPad Prism 5. Statistical analysis was performed using SPSS software ver. 18 to test statistical significance. The different letters in Figs. 1–6 represented significant difference at 5% level.

## 3. Results

### 3.1. Variation in CGA content during fruit development

The CGA content in peel and flesh of Xuehua pear fruit exhibited a decreasing trend during development. In addi-