

Expression Analysis of the ANR and LAR Gene in *Fragaria × ananassa* cv. Toyonaka

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Abstract

Strawberry is an economically valuable crop all over the world. Proanthocyanidin is one polyphenol compound rich in strawberry fruits owned significant antioxidant capacity and claimed beneficial health effects. Anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR) are both key enzymes of the branch of proanthocyanidins biosynthesis pathway, which are responsible for the production of (-)-epicatechin and (+)-catechin, respectively. In this study, the expression levels of the *Fa-ANR* and *Fa-LAR* gene and the total PAs concentration at seven developmental stages of the strawberry fruit were investigated. The results showed that the PAs contents gradually reduced along with the fruit maturation; while the expression patterns of *Fa-ANR* and *Fa-LAR* were consistent with the PAs. These results indicated that LAR and ANR were both key enzymes in proanthocyanidin biosynthesis.

Keywords: proanthocyanidins, LAR, ANR, strawberry fruit, developmental stages

1. Introduction

Proanthocyanidins (PAs), also known as condensed tannins, is oligomers or polymers of flavan-3-ol units synthesized via the flavonoid biosynthetic pathway (Ferreira et al., 2002; Marles et al., 2003). As one of the most ubiquitous groups of plant phenolics, they are widespread throughout the plant kingdom with diverse biological and biochemical activities including protection against predation and pathogen attack (Lamb et al., 1989). In recent years, more and more attention has been drawn to PAs and their monomers because of their beneficial effects on human health, such as the activities of immunomodulatory and anticancer (Zhao et al., 2007), antioxidant (Rao et al., 2004),

Anti-inflammatory (Subarnas et al., 2000) and antithrombotic effects (Sano et al., 2005). Besides, PAs also provide the flavors of bitterness and astringency to beverages, especially the wines, where they have a significant influence on the mouth feel and the color alternation of the products (Lee et al., 2008; Peleg et al., 1999; Fei et al., 2009).

Anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR) are both key enzymes of the branch of PAs biosynthesis pathway (Tanner et al., 2003; Xie et al., 2003). They are responsible for the production of (-)-epicatechin and (+)-catechin, respectively (Xie et al., 2003; Pfeiffer et al., 2006). To date, these two enzymes are the last known steps in proanthocyanidin synthesis, whereas the condensation processes remain unclear. ANR and LAR have been studied at both the genetic and the biochemical level in various plants, including grapes and strawberries (Bogs et al., 2005; Bogs et al., 2007; Fabrizio et al., 2009), leading to significant progress in our understanding of the proanthocyanidin metabolism (Soizic et al., 2010). However, the mostly wide used cultivar 'Toyonaka' was not covered therein.

Strawberry is an economically valuable crop, widely grown in all temperate regions of the world. Its fruits contain large amounts of polyphenol compounds, proanthocyanidin for instance, with significant antioxidant capacity and claimed beneficial health effects (Fabrizio et al., 2009). In this study, we used the most extensive cultivar (*Fragaria × ananassa* cv. Toyonaka) applied in forcing culture of strawberry to investigate the total PAs concentration and expression levels of the *Fa-ANR* and *Fa-LAR* gene at seven developmental stages, and analyze

the correlation between them. This study will provide further molecular evidences to understand the affection of genetic background and developmental clues on the ripening physiology of strawberry fruits.

2. Materials and Methods

2.1 Plant Material

Strawberry fruits from the cultivar ‘Toyonaka’ were collected at seven time points during fruit development indicated by Hou et al. (2009) as follows: small green (SG, 7 days after fruit set), large green (LG, 15 days after fruit set), green ripe (GR, white fruits), turning red (TR, 1/4 red), half red (HR, 1/2 red), red ripe (RR, >1/2 red), full red (FR) (Figure 1). They were sampled from the Shuangliu country of Sichuan Province in 2011. All fruits were frozen in liquid nitrogen upon harvesting in the field and stored at -80°C until use.

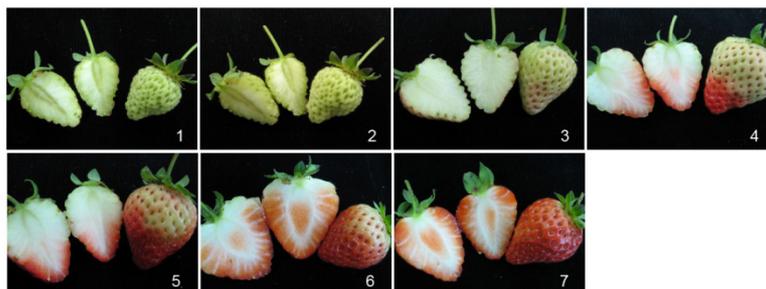


Figure 1. Seven development stages of strawberry fruit

Description: 1, SG, small green; 2, LG, large green; 3, GR, green ripe; 4, TR, turning red; 5, HR, half red; 6, RR, red ripe; 7, FR, full red.

2.2 RNA Isolation and cDNA Synthesis

Fruits at different developmental stages were used for total RNA isolation by the protocol adopted by Djami-Tchatchoua et al. (2012). First strand cDNA was synthesized from 2 µg of the RNA by M-MuLV reverse transcriptase with Oligo-(dT)₁₈ primer according to the instructions of the Easy-Go™ RT Premix kit (SBS Genetech, China) after treated with RNase-free DNase I (Sangon, Shanghai, China).

2.3 Quantitative Real Time PCR (qRT-PCR) Primer Design

The qRT-PCR primers (Table 1) for *Fa-ANR*, *Fa-LAR* and *Fa-Actin* gene were designed to span the intron boundaries in Beacon Designer 7 (Primer, USA). The gene sequences used were those deposited in Genbank database of NCBI including: *Fa-ANR* (Genbank ID: DQ664192), *Fa-LAR* (Genbank ID: DQ087253) and *Fa-Actin* (Genbank ID: JN616288).

Table 1. Primers used for quantitative real time PCR analysis

Gene	Description	Sequences (5'-3')	Amplicon (bp)
<i>Fa-ANR</i>	Forward	CCATCATCTCAACCAAGTCT	95
	Reverse	GACAGCATAGCCCTTCTC	
<i>Fa-LAR</i>	Forward	TGTCACCTTCTATTGCCTCTG	81
	Reverse	CGACGAACTTGCCGATGA	
<i>Fa-Actin</i>	Forward	ACCTTCAATGTGCCTGCTAT	101
	Reverse	ACACCATCACCAGAGTCAAG	

2.4 qRT-PCR for Expression Analysis

The expression levels of *Fa-ANR* and *Fa-LAR* gene at seven stages of strawberry fruits were determined by qRT-PCR, using SYBR green method on a CFX96 real-time cycler (BIO-RAD, USA). Each PCR reaction (20 µl) contained 0.6 µl primer F, R (10 µM), 1 µl cDNA (10 ng), and 10 µl 1×Takara SYBR Premix (Takara, Dalian,

China). The qRT-PCR conditions were: 1 cycle at 95°C for 3 min; 40 cycles at 95°C for 10 s and 59°C (*LAR*) or 55.7°C (*Actin* and *ANR*) for 30 s, followed by a melt cycle from 65.0°C to 95.0°C. The *Fa-Actin* gene was served as an internal control. Three replicates of all qRT-PCR reactions were carried out on each sample. Amplification efficiency of all primers used was primarily determined prior to sample investigation. Relative expression values were firstly calculated as $2^{-\Delta CT}$, normalizing against the internal control *Fa-Actin* gene. The maximal expression level of each gene observed was served as a calibrator (1.0) respectively, and the rest was expressed as ratios in relation to the calibrator (relative expression ratio).

2.5 Total PAs Determination

Extractions of PAs from strawberry fruits at seven stages followed procedures described by Prior et al. (2010). Frozen powders of strawberry fruit were weighed (1.5~3 g) into a 50 mL conical tube. The PAs extraction solution (20 mL) (150 mL acetone was transferred to a glass bottle containing 49 mL of deionized water and 1 mL of acetic acid was added) was added to the samples. Samples were then placed on an orbital shaker for 1 h and subsequently centrifuged at 10000×g for 20 min at 12°C (Eppendorf 5804R, Germany). The supernatant was collected and transferred into a new tube and ready for analyzing instantly by UV spectrophotometry at 640 nm. Each sample was extracted three times. Then data was analyzed in SPSS18.0, and plotted by Sigmaplot 12.0.

3. Results

3.1 Total PAs Contents

The concentration of PAs was assayed by UV spectrophotometry at 640 nm. As shown in Figure 2, our results indicated that the PAs content was highest in the fruit at the small green stage (SG), and then gradually declined till the full red stage (FR).

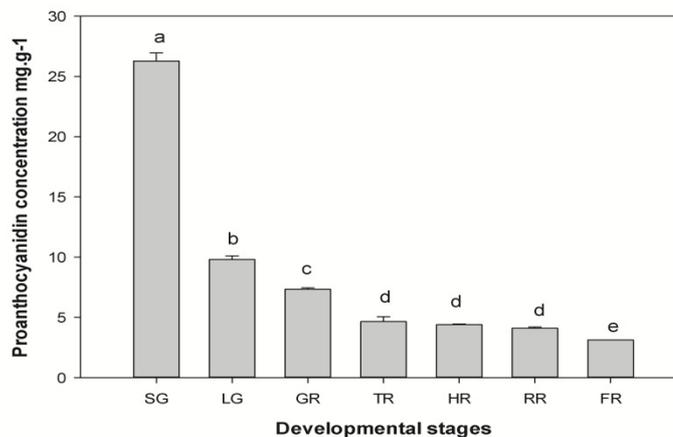


Figure 2. Change patterns of proanthocyanidins in ripening strawberries. Each value represents the mean \pm standard deviation of seven berries with triplicated replication

3.2 Expression of *FaANR* and *FaLAR* in Strawberry Fruit Tissues

ANR and *LAR* are both key enzymes of the branch of PAs biosynthesis pathway, which catalyze the formation of epicatechine or catechin respectively (Tanner et al., 2003; Xie et al., 2003). The results in our work showed that their expression levels decreased continuously during fruit developments (Figure 3 and 4). The expression of *FaANR* and *FaLAR* was significantly different during fruit development. As shown in Figure 3. The expression level of the *FaANR* was highest at the small green stage (SG), gradually decreased to the minimum at the full red stage (FR), with relative transcript abundance 1.00, 0.62, 0.50, 0.89, 0.41, 0.46, 0.22, respectively. However, different from *FaANR*, the amount of transcripts of the *FaLAR* was highest at the large green stage (LG), then gradually decreased to the minimum at the full red stage (FR), with the relative transcript abundance 0.91, 1.00, 0.75, 0.12, 0.80, 0.04, 0.02, respectively.

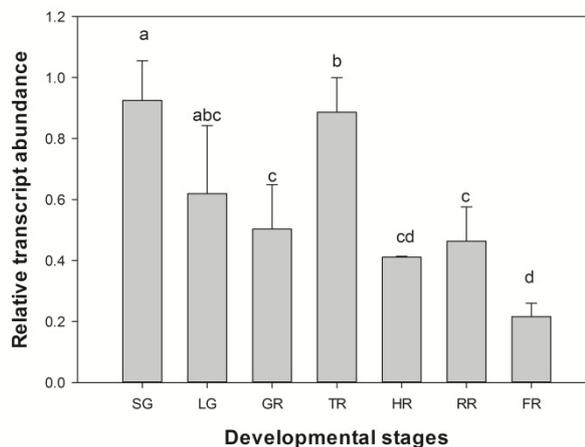


Figure 3. Expression of ANR gene in strawberry fruit. The figure was generated by a log transformation of the real-time PCR data presented as ΔC_T ($C_{T \text{ target}} - C_{T \text{ actin}}$)

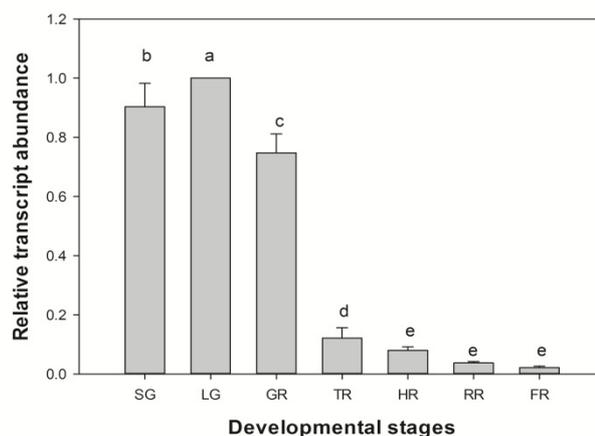


Figure 4. Expression of LAR gene in strawberry fruit. The figure was generated by a log transformation of the real-time PCR data presented as ΔC_T ($C_{T \text{ target}} - C_{T \text{ actin}}$)

The Pearson correlation coefficients were calculated between gene expression and final products accumulation. The correlation coefficient of the PAs concentration and expression was 0.717 and 0.701 for *FaANR* and *FaLAR* respectively. Correlation of the PAs concentration and expression of *FaANR* was higher than that of the PAs and *FaLAR*.

4. Discussion

The concentration of PAs was gradually reduced as the strawberry fruits maturation. The result was consisting with that was found in blackberry and strawberry fruit (Chen et al., 2012; Fabrizio et al., 2009). However, PAs accumulation in grape occurred immediately after fruit-set and the maximum levels of accumulation were reached around *Véraison* (Kennedy et al., 2001). Consequently, the differences might be due to various species under control of different mechanisms.

The expression level of the *FaANR* and *FaLAR* gradually decreased to the minimum as fruits matured. The lowest level was observed at the full red stage (FR). This was differed from the findings reported by Fabrizio et al at 2009, which showed that transcript level of the *FaANR* diminished throughout fruit ripening, but transcript level of the *FaLAR* was lowest at the green ripe stage (GR). It gradually increased until the full red stage (FR). We speculated that the difference might be due to various species used or environmental difference. To sum up, our

results demonstrated the PAs metabolism during the developmental stages in strawberry fruits, and confirmed the roles of LAR and ANR in proanthocyanidins biosynthesis.

The concentration of PAs was highest at the small green stage (SG), accordingly, expression of *FaANR* and *FaLAR* were also higher. Nevertheless, through the stage of large green (LG) to the turning red stage (TR), when the concentration of PAs gradually decreased, the expression of *FaANR* increased but the level of *FaLAR* did not decrease until the turning red stage (TR) (Figures 3 and 4). In planta, final content of plant components is determined by the biosynthesis and degradation metabolism. Thus, this might be because that the concentration of PAs began to degrade at the large green stage (LG), and an unknown degradation occurred at this stage, although *FaANR* and *FaLAR* were still synthesized at high transcript level.

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