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Full Length Article

Transcriptional and Characteristic Analysis of Expressed Sequence Tags from a Normalized Full-Length cDNA Library of Loquat Fruit at Different Developmental Stages

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Abstract

Loquat (*Eriobotrya japonica* Lindl.) fruit is popular for its delicious taste, rich nutrients and medical value. But the available genome resources on loquat fruit are very limited. In this study, an improved normalized full-length cDNA library construction was established mainly by the duplex-specific nuclease (DSN) normalization strategy and the SMART^{TM} Kit. Sequencing results from randomly 8972 expressed sequence tags (ESTs) in loquat fruit cDNA library revealed 7451 unigenes with 70.3% non-redundancy rate and an average length 1.1 kb. Further, 2896 unigenes with potential full-length cDNAs were functionally classified and annotated by Gene Ontology (GO). Besides, some significant functional genes related to loquat fruit development and ripening were characterized and analyzed their expression patterns including three ethylene receptors (*EjETR 1-3*) and one ethylene transcription factor (*EjETF*), six cell wall expansin genes (*EjEXPA 1-6*) and the three key enzyme genes of carbohydrate metabolism (SS-S, SS-C, SPS). The establishment of normalized cDNA library with high ratio full-Length unigenes will make great contribution in discovering novel genes resource and promoting fruit quality improvement genetic engineering and molecular breeding of loquat.

Keywords: Loquat fruit; Normalized cDNA library; Expressed sequence tags (ESTs); Gene expression

Introduction

Loquat (*Eriobotrya japonica* Lindl.) is an evergreen fruit tree that originates in China and plants currently in other many countries involving Japan, Brazil and Spain. As one of non-climacteric fruits, loquat fruit is popular for its delicious taste, rich nutrients and medical value. Most researches on loquat mainly focus on molecular marker identification, construction of genetic map and postharvest biology (Ding *et al.*, 2006; Martínez-Calvo *et al.*, 2008; Cao *et al.*, 2009; Gisbert *et al.*, 2009), but the available genome resources on loquat fruit development and ripening are very limited presently and no public genomics or Expressed Sequence Tags (ESTs) data are available.

cDNA library and ESTs sequencing are the efficiently common approaches for discovering new genes. Conventional method of cDNA library construction generally contains a large number of 5'-ESTs fragments due to the premature stop of reverse transcription and the tending formation of mRNA secondary structures (Wellenreuther *et al.*, 2004). The SMART^{TM} technology is prevailing for straight forward isolation of full-length cDNAs and requires only 0.025-1 µg of mRNA (Zhu *et al.*, 2001). This technology utilizes the property of some MMLV reverse transcriptases to add a few C residues at 3' end of the first strand cDNA, but not at prematurely terminated reverse transcripts (Bogdanova *et al.*, 2008). Moreover, the percentage of full-length clones with SMART^{TM} technique was much higher compared with libraries with other full-length enriching techniques (Wellenreuther *et al.*, 2004).

Due to the differences of high abundant genes among various plant species, it is significant for equivalent cDNA molecule quantities that cDNA normalization to increase existence quantity of rare or long cDNA genes by which conventional methods of cDNA library construction are not appropriate (Sugahara *et al.*, 2001). Duplex-specific nuclease (DSN) from the hepatopancreas of Kamchatka crab has a good preference in cleaving dsDNA and DNA-RNA hybrid duplexes (Sugahara *et al.*, 2001). DSN normalization involves the formation of a normalized single-strand (ss) fraction and a non-target double-strand (ds) fraction. Because DSN enzyme has a thermostable active at 70°C, the degradation of ds-cDNA can be performed at the

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same temperature, which avoids effectively a non-specific hybridization of cDNA during the renaturation and the loss of mRNAs due to the formation of secondary structures. DSN normalization produces about 65−70% high frequency of full-length cDNAs and a 250-fold decrease in the representation of high abundance cDNAs (Bogdanova *et al.*, 2008).

This study established a normalized full-length cDNA library method mainly by the DSN normalization strategy and the SMART^{TM} Kit, which provides an insight way in discovering rare functional genes during development and ripening of loquat fruit. A large number of unigenes were classified and annotated functionally. Some significant functional genes related to loquat fruit development and ripening were characterized and analyzed their expression patterns including ethylene receptors (*EjETR 1-3*) and ethylene transcription factor (*EjETF*), cell wall expansin genes (*EjEXPA 1-6*) and carbohydrate-metabolizing key enzyme genes (SS-S, SS-C, SPS). The establishment of normalized cDNA library with high ratio full-Length unigenes will make great contribution in discovering novel genes resource and promoting fruit quality improvement genetic engineering and molecular breeding of loquat.

Materials and Methods

Experimental Material and Treatments

Loquat fruit ('Jiefangzhong' variety) samples were gathered from 10 year-old trees grown in the Loquat Germplasm preservation orchard of South China Agricultural University (Guangzhou, China). Twenty-five fruits were randomly collected at 10-day interval from 60 to 150 post-anthesis days (PAD) until ripening. The slices of loquat fruit from three independent samples were treated as three replications at each stage. All fruit tissues were stored at -80°C after liquid nitrogen treatment.

Experimental Methods

Determination of sugar contents: Determination of sugar contents ($g.kg^{-1}$) was performed according to the method of Song *et al.* (2016). 5 g of fruit sample was grinded into fine power in 20 mL of cold ethanol (95%, v/v) before filtering with a 0.2 μm membrane. Then, 6 mL extract was filtered with a Sep-Pak C18 cartridge (Waters, Milford, MA, USA). Take 20 μL filter liquor for detecting in liquid chromatograph (Waters, Mildford, MA) with a carbohydrate analysis column (Transgenomic, San Jose, CA). Sugar components were distinguished by the times and areas of peak in agreement with each sugar standard products.

Determination of total soluble solids (TSS) and titratable acidity (TA) contents: According the description of Cao *et al.* (2008), 5 g of fruit sample were grinded into fine power followed by centrifugation at 13,000 g for 20 min. TSS (%) was measured by a digital refractometer (PAL-a, ATAGO, Tokyo, Japan). TA (%) content (calculated with malic acid content) was detected by titration with 0.1 mol L^{-1} NaOH.

Total RNA Isolation and First-strand cDNA Synthesis

Total RNA was isolated by Trizol kit (Life Technologies Inc., United States). The synthesis of first-strand cDNA was performed by the SMART^{TM} PCR cDNA Synthesis Kit (Clontech, United States) according to the manufacture instruction. The 50 μL amplification system and sixteen cycles of PCR were adopted.

Normalization of First-strand cDNA and ssDNA Amplification

After purification with the Qiagen Kit (Tokyo, Japan), first-strand cDNA products were diluted to 100 mg L^{-1} concentration to normalize by DSN enzyme according to Zhulidov *et al.* (2004). The normalized product is single-strand DNA (ssDNA) and then was amplified in 50 μL reaction system with the advantage 2 PCR kit (Clontech, United States) according to the manufacture instruction. 25 cycles of PCR amplification were adopted to produce a final about 20 mg L^{-1} ssDNA.

Construction and Evaluation of cDNA Library

cDNA normalized products were digested by *Sfi* followed cloning by the vector of pDNR-LIB (Promega, Madison, WI). A number of positive colonies is normalized cDNA library. 278 random colonies were amplified in 30 cycles system by M13 primers for the size analysis of the cDNA insertion.

Sequence Processing and Annotation

Sequence assembling was carried out using a GS de novo Assembler (Newbler) v2.5.3 (Roche Applied Science, Indianapolis, IN, USA). Newbler explicitly accounts for splice variants in its cDNA mode operation and constructs isotigs. According to Müller-Herbst *et al.* (2014), the singleton sequences were blasted by NCBI protein databases (Blast X) and analyzed information for function, motif and 5'-end of full-length cDNAs using the InterPro member databases (http://www.ebi.ac.uk/InterProScan). Gene Ontology (GO) terms were assigned to UniESTs by using Blast2GO (Conesa *et al.*, 2005). The functional annotation includes the three aspects of molecular functions, biological processes and cellular components according to GO terms.

Gene Expression Analysis by Real-time qPCR

Real-time PCR was performed using The 10 ng of first-strand cDNA was amplified by Real-Time qPCR

cycler (Roche Diagnostics) with SYBR Green I Master (ROX). The program included 95°C denaturation for 5 min, 40 cycles amplification (95°C for 10 s, 60°C for 10 s, 72°C for 70 s). Melting curve condition was 60−95°C range with a rising rate of 0.1°C/sec and real-time fluorescence detection. *β*-action gene was applied in an internal control. The primers were listed in Table 1.

Statistical Analysis

The mean values of the data were obtained from three replicate of loquat fruit samples and subjected to statistical analysis with the SPSS (Version 15.0). One-way ANOVA was adopted for variance analysis on sugar, TA, TSS and gene expressions based on the means of the standard error (±SE). Significance of differences ($p < 0.05$) between means was carried out according to Tukey comparison tests.

Results

Changes of Sugars, TSS and TA Contents during Loquat Fruit Development

During the initial stage, a small amount of sugar accumulation was detected. After 90 PAD, sucrose accumulation was faster than any other sugar until 120 PAD, then declined until fruit ripening (Fig. 1a). Fructose significantly climbed up after 110 PAD and played dominant role in the period of loquat fruit ripening. Although glucose increased along with fruit development and ripening, its percentage in total sugars was decreasing.

TA was gradually raised during the development and ripening stages of loquat fruit (Fig. 1b). The highest TA content was recorded up to 150 PAD. A similar rise of TSS was also shown from 60 to 150 PAD (Fig. 1c). A continuous increase in the ratio of TSS/TA was also determined in maturation period of the fruit from 90 PAD to 150 PAD (Fig. 1d).

The Construction and Characterization of Normalized cDNA Library from Loquat Fruit

Generation of normalized library and the full-length cDNAs enrichment: To understand fruit development and sugar accumulation of loquat, construction of a high-quality cDNA library is necessary during fruit development. An improved DSN (Duplex-specific Nuclease) enzyme digestion method was used to remove the dsESTs in the mixed sample at the different development stages of loquat fruit. The first-strand and the amplified cDNAs were used for nondirectional and directional cloning of cDNA libraries. From Table 1, it can be known that the rate of non-redundant unigenes was high about 70.3% and the redundancy rate was rapidly declined in the dendrogram analysis of the EST sequences from the normalized cDNA library by improved DSN method. It greatly helps to enrich rare genes of cDNA library in loquat fruit.

Table 1: Summary of the normalized cDNA library

Description	Number
Total number of successful sequences	8972
Unique sequences	7451
5’ end unigenes	4021
3’ end unigenes	3503
Full-length genes	2896
Number of known genes	2210
Unique unknown genes	5241

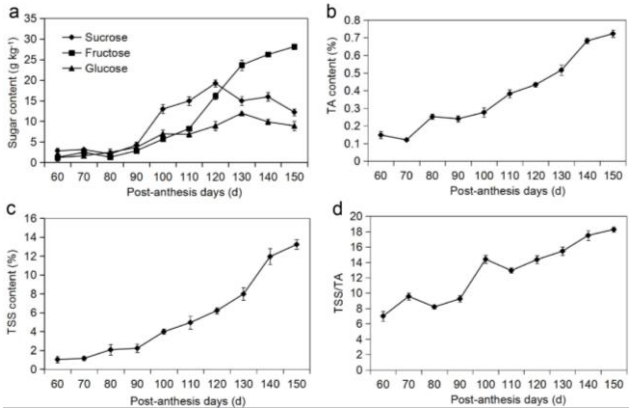

Fig. 1: Changes in sugar components (a), TA (b), TSS (c) and TSS/TA (d) during loquat fruit development. The presented data are the means of three replicates (25 fruit samples each) from three experiments. Vertical bars represent the standard errors of the means

Characterization of cDNA Library and the Full-length Unigenes

The full-length ratios of cDNA library was subjected to X-Gal/IPTG screening and PCR amplification. 278 positive clones were picked randomly to test the enrichment of full-length genes. The primary concentration of cDNA library was 1×10^6 cfu.mL^{-1} covered 99% recombinants. The size of inserted cDNA fragments ranged from 0.6 kb to 3.5 kb of which an average length is 1.1 kb (Fig. 2).

More than 9000 clones were randomly selected and sequenced from cDNA library. After removing vector and other contamination sequences, 8972 clone sequences were obtained successfully and compared with the nonredundant (nr) protein database using BlastX (Table 1). Of 7451 unigenes, 2210 ones (29.6%) matched with the known genes and 53.9% of unigenes were predicted to comprise the ATG initiation codon. 38.9% (2896 of 7451) of unigenes had the potential for encoding full-length genes. There was 5241 putative novel unigenes composed of 86.1% singletons and 13.9% contigs in the nonredundant protein databases in NCBI.

Classification and Functional Analyses of Unigenes

The known unigenes were blasted with available genes in

the non-redundant database. Annotation included sequence homology comparisons, search for domains (12.5% of sequences), signal peptides (22.3% of sequences) and numbers of the enzyme commission (8.5% of sequences). The high-abundant genes were related to cell senescence, carbohydrate metabolism and protein transport (Table 2). Other genes could be involved in different metabolic pathway relevant for fruit development and ripening (Table 3). There was still 70.3% of that remained unknown for any putative functions.

The annotation of unigenes was carried out by the Blast2GO tool on Ontology. The 8972 unigenes were functionally classified into 'biological process', 'molecular function' and 'cellular component' category according to GO slim terms. The predicted function from 8972 loquat unigenes mainly included signal transduction, anabolism, catabolism and reproduction. The majority of unigenes (70.9%) were divided into 'other intra-cellular components', 'unknown cellular components' and 'other cytoplasmic components' (Fig. 3a). Besides, 3.6% unigenes were included in cellular component of chloroplast. Seen from metabolism processes, 8972 unigenes were diivded into nine different ways involving developmental processes (8.3%), protein metabolism (9.3%), transcription (3.6%), response to stress (3.2%) and unknown path. But the other most of unigenes were grouped into unknown biological processes (25.2%), other biological process (16.4%), other cellular process (12.1%) and other metabolic process (11.4%) (Fig. 3b). From molecular functions, 41.9% unigenes related to binding activities (24.9%), transporter activity (10.2%) and transferase activity (6.8%) (Fig. 3c), however, most of unigenes (55.8%) was attributed to unknown molecular function.

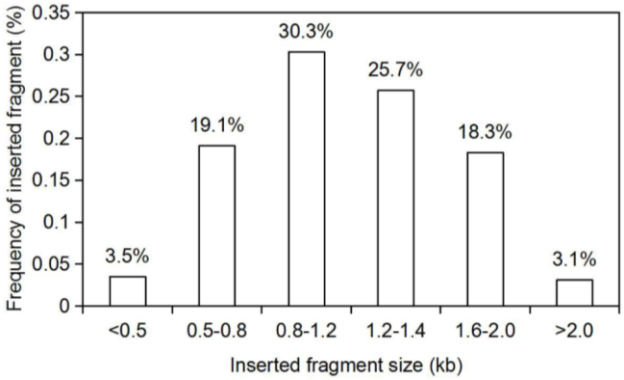

Fig. 2: Distribution of insert size in the normalized cDNA library of loquat fruit

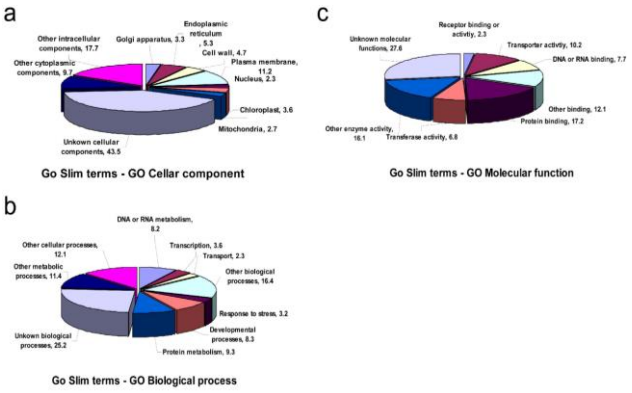

Fig. 3: Functional categorization of unigenes with GO terms by the Blast2GO program. Top 8-10 GO terms containing the large number of unigenes were calculated for three GO categories: cellular component (a), molecular function (b), and biological process (c)

Expression Pattern Analyses of some Significant Functional Genes Involved in Loquat Fruit Development and Ripening

Expression analyses of ethylene signal transduction genes: From normalized cDNA library of loquat fruit, four genes related to ethylene signal transduction including three of ethylene (C_2H_2) receptors (*EjETRs*) and one of ethylene transcription factor (*EjETF*) were isolated and aligned with other plants homologous genes. The transcription characteristics of *EjETR-1, -2, -3* and *EjETF* were examined using real-time PCR during fruit development. High level expression of *EjETRs* was displayed in young loquat fruits, subsequently declined acutely from 110 PAD to 150 PAD (ripening period). The three of *EjETR-1, -2, -3* genes had a similar expression pattern (Fig. 4a). These results indicated that ethylene production in young loquat fruit was an autocatalytic process. The kinetic traits were accompanied by *EjETF* expression (Fig. 4b).

Expression analyses of several key-enzyme genes mediating sugar accumulation: Currently, the researches on sugar metabolism mainly concentrated on carbohydrate metabolism enzymes embodying in sucrose phosphate synthase (SPS) enzyme and sucrose synthase (SS) enzyme which includes SS-synthesis (SS-S) and SS-cleavage (SS-C) enzymes. SPS and SS-synthesis (SS-S) catalyze sucrose biosynthesis, whereas SS-cleavage (SS-C) cleaves sucrose into fructose and glucose (Ruan, 2014). In our results (Fig. 5), the coding carbohydrate-metabolizing enzyme homology genes (*SPS1-2, SS-C and SS-S*) were found and aligned. In contrast to low mRNA levels at the initial stage, *SPS-1* and *SPS-2* transcript levels gradually increased between 110 PAD and 150 PAD (Fig. 5a). Similarly, *SS-S* expression levels were also markedly up-regulated and exhibited obvious bio-function profiles (Fig. 5b). Changes of *SS-C* transcription levels might be relevant to enhance of sucrose hydrolysis and increase of fructose and glucose in loquat fruit.

Expression analyses of expansin genes related to fruit cell wall extension: Six distinct expansin genes (*EjEXPA1, -2, -3, -4, -5* and *-6*) which belong to a class of nonenzymatic cell wall proteins in α-expansin family

Table 2: List of abundant unigenes from the normalized cDNA library of loquat fruit

Unigene name	Putative function	Accession No.	Source organism	Identity (%)
Contig 5600	Polyphenol oxidase	FJ603649	*Eriobotrya japonica*	95
Contig 5601	Pyrophosphate--fructose 6-phosphate	KJ736836	*Eriobotrya japonica*	89
Contig 5602	Ethylene cinnamyl alcohol dehydrogenase	KF767459	*Eriobotrya japonica*	76
Contig 5603	ADP-glucose pyrophosphorylase subunit	AB710172	*Eriobotrya japonica*	85
Contig 5604	Sucrose phosphate synthase	AB710171	*Eriobotrya japonica*	94
Contig 5605	Heat shock protein 70	JF815560	*Eriobotrya japonica*	100
Contig 5606	Ethylene response factor	JF815559	*Eriobotrya japonica*	82
Contig 5607	Expansin	EU123922	*Eriobotrya japonica*	93
Contig 5608	TFL1-like protein	GU320722	*Eriobotrya japonica*	100
Contig 5609	Ethylene-responsive transcription factor	JF815559	*Eriobotrya japonica*	96
Contig 5610	Ethylene receptor	FJ624870	*Eriobotrya japonica*	97
Contig 5611	Phenylalanine ammonia lyase	EF685344	*Eriobotrya japonica*	94
Contig 5612	ATP synthase subunit	KF766121	*Eriobotrya japonica*	98
Contig 5613	Fructokinase	JF414124	*Eriobotrya japonica*	89
Contig 5614	Hexokinase	JF414121	*Eriobotrya japonica*	97
Contig 5615	Catalase 1	JX307086	*Eriobotrya japonica*	94
Contig 5616	Ribulose-1,5-bisphosphate carboxylase	XM003616402	*Medicago truncatula*	95
Contig 5617	Sucrose synthase	XM002266984	*Vitis vinifera*	65
Contig 5618	Phosphoglycerate kinase	M008384936	*Malus domestica*	71
Contig 5619	Lipid transfer protein 4 precursor	AY793558	*Lens culinaris*	68
Contig 5620	Myb transcription factor	XM008366832	*Malus domestica*	64
Contig 5621	UDP-glucosyltransferase	DR993941	*Malus domestica*	74
Contig 5622	Ethylene response factor (ERF)	JF412350	*Malus domestica*	83
Contig 5623	Senescence-related protein	XM002300415	*Populus trichocarpa*	65
Contig 5624	Poly(A)-binding protein	NM001198255	*Arabidopsis thaliana*	70
Contig 5625	Metallothionein-like protein	AF009959	*Malus domestica*	82
Contig 5626	Translation elongation factor	XM008340889	*Malus domestica*	87
Contig 5627	DELLA protein	KC434135	*Triticum aestivum*	79
Contig 5628	Vacuolar Ca^{2+}/H^{+} exchanger	AB012932	*Vigna radiata*	86
Contig 5629	ATP binding protein	XM002521829	*Ricinus communis*	76
Contig 5630	Phosphoric diester hydrolase	NM101237	*Arabidopsis thaliana*	72
Contig 5631	Serine/threonine-protein kinase	XM008391178	*Malus domestica*	82
Contig 5632	Cytochrome C reductase	X79275	*Solanum tuberosum*	85
Contig 5633	1-aminocyclopropane-1-carboxylic acid oxidase	AB003514	*Actinidia deliciosa*	75
Contig 5634	Vacuolar ATP synthase subunit G1	NM180158	*Arabidopsis thaliana*	68
Contig 5635	*β*-1,3- glucanase	AY548364	*Malus domestica*	62
Contig 5636	Alcohol dehydrogenase	L23548	*Zea mays*	78
Contig 5637	14-3-3 family protein	NM001247178	*Solanum lycopersicum*	85
Contig 5638	MADS box genes	AJ000759	*Malus domestica*	89

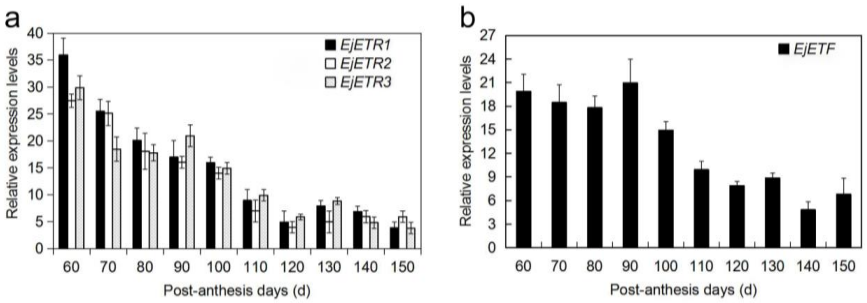

Fig. 4: Real-time PCR analyses of *EjETR* and *EjETF* transcript profiles during loquat fruit development. (a) *EjETR1-3* expression profiles. (b) *EjETF* expression profiles. Each point is the mean of three determinations. Vertical bars represent the standard errors of the means (n = 3)

were detected during loquat fruit development. The results showed that *EjEXPA-2,-4* were expressed fiercely in young fruit, then dispeared rapidly at 110 PAD (Fig. 6a). Therefore, *EjEXPA-2,-4* presumably contributed to change cell wall properties during fruit cell division. *EjEXPA-5* was expressed in the later stages of fruit development between 110 PAD and 150 PAD (Fig. 6b), which might be related to cell expansion and fruit maturation. In contrast, *EjEXPA-3,-6* displayed an expression level with decreasing-rising-dropping changes at the peak in 120 PAD (Fig. 6c). However, *EjEXPA-1* expression pattern was very different, it presented in high levels at all stages of fruit development, but with a small reduction in 150 PAD (Fig. 6d).

Table 3: Genes involved in different metabolic pathway during fruit development and ripening

Metabolic Pathway	Novel Genes	Genes described previously
Flavonol biosynthesis	1	2
Biosynthesis of unsaturated fatty acids	2	3
Ethylene Biosynthesis	1	4
Jasmonic Acid	0	5
Sucrose Metabolism	0	7
Terpenoid Biosynthesis	3	4
Photosynthesis	0	3
Citrate cycle (TCA cycle)	0	4
Glycolysis/Gluconeogenesis	3	2
Fatty acid metabolism	3	2
Steroid biosynthesis	1	1
Pentose phosphate pathway	1	0
Abscisic Acid	0	2
Gibberellin Biosynthesis	0	2
Lignin Biosynthesis	2	1
Carotenoid Biosynthesis	1	1
Phenylalanine metabolism	1	2
Nitrogen metabolism	0	2

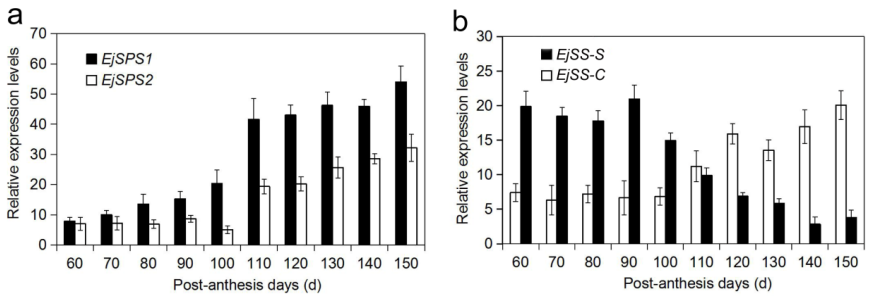

Fig. 5: Real-time PCR analyses of *EjSPS* and *EjSS* transcript profiles during loquat fruit development. (a) *EjSPS1-2* expression profiles. (b) *EjSS-C* and *EjSS-S* expression profiles. Each point is the mean of three determinations. Vertical bars represent the standard errors of the means (n = 3)

Discussion

The changes of sugar types and accumulation levels were closely related to fruit development and quality. Sugars are not only energy resource, but also proved to be signal molecules to regulate gene expression (Ruan, 2014). Our research results on the changes of sugar components and levels in "Jiefangzhong" variety of loquat is similar with those previously found in other loquat cultivars (Amorós *et al.*, 2002), in which fruit fast growing period was harmonious with sugar increasing phase. The increases of glucose and fructose are closely related to the enzymatic activities of sucrose hydrolysis, a dominant translocated sugar in loquat phloem (Bantog *et al.*, 1999). However, the transposition of the sugars is mainly relevant to different cultivars and fruit development stages. Our previous research (Song *et al.*, 2016) showed that fructose and glucose were main types of sugars in the flesh of loquat 'cv. Baiyu'. In 'cv. Mogi' loquat fruit, major sugars were sucrose, glucose and fructose during fruit development (Ding *et al.*, 1998), while fructose was the most dominant sugar at maturity (Hamauzu *et al.*, 1997).

The content of TA was gradually enhanced during the development and ripening stages of loquat fruit, which is in accord with the report of Amorós *et al.* (2003). A similar rise of TSS was also shown from 60 to 150 PAD (Fig. 1c), this might be due to depolymerisation of polysaccharides and switch starch to sugars (Adams-Phillips *et al.*, 2005). Similarly, TSS/TA was increased markedly towards the last growth stage of pineapple fruit, (Saradhuldhat and Paull, 2007).

Conventional cDNA libraries were inefficient in discovering rare genes because of the repeatable existence of abundant EST fragments. The normalization of ESTs/unigenes for reducing the redundancy of abundant ESTs was essential for rare gene discovery before sequencing, this mainly owns to DSN strategy was adopted to remove the double-stranded cDNAs formed by abundant transcripts in the mixed sample of loquat fruits at different fruit development stages in our improved method. In addition, the selective PCR amplification following SMART^{TM} cDNA synthesis also improves the abundance of rare genes. Moreover, sixteen cycles in PCR amplification were helpful to produce an equal amount of PCR cloning on large ESTs compared to smaller ones, and also avoid the redundant raise and error

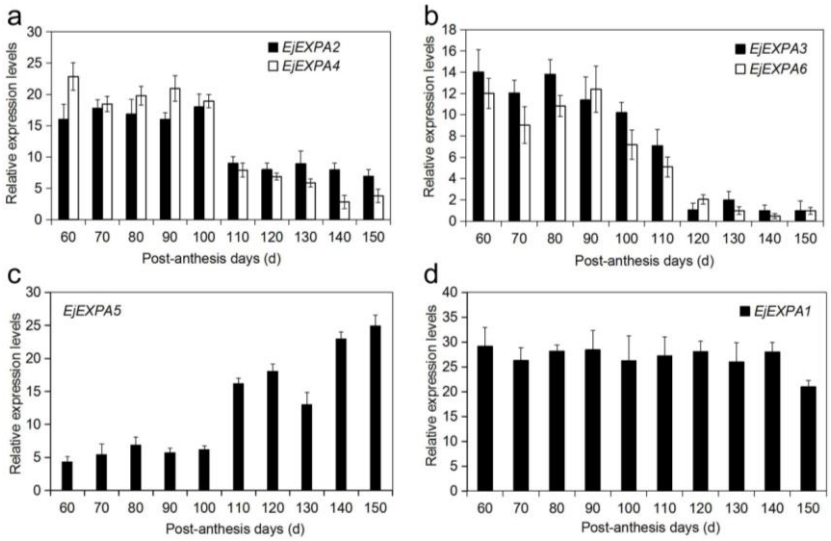

Fig. 6: Real-time PCR analyses of *EjEXPA* transcript profiles during loquat fruit development. (a) *EjEXPA2* and *EjEXPA4* gene expression profiles. (b) *EjEXPA5* gene expression profiles. (c) *EjEXPA3* and *EjEXPA6* gene expression profiles. (d) *EjEXPA1* gene expression profiles. Each point is the mean of three determinations. Vertical bars represent the standard errors of the means (n = 3)

reducing caused by PCR polymerase.

In our normalized cDNA library, the rate of non-redundant unigenes was ca. 70.3% (Table 1), which is great larger than 30−40% from conventional cDNA libraries (Lu and Wallis, 2007; Sakurai *et al.*, 2007). Therefore, the normalization of cDNA library greatly helps rare genes enrichment of cDNA library and reduces the cost of clone sequencing.

The Relationship Analyses between some Significant Functional Genes and Loquat Fruit Development and Ripening

Ethylene signal plays important roles during climacteric fruit development (Liu *et al.*, 2015). Currently, however, very little is known about the role of ethylene in non-climacteric fruits during their ripening, especially the downstream components. This may be because that non-climacteric fruits have no respiration and ethylene peak at fruit ripening stage. In our experiment, high transcription levels of *EjETR-1, -2, -3*, three ethylene receptor genes, were detected during loquat young fruit development, then sharply decreased from 110 PAD until ripening. This confirms that ethylene mass production mainly promotes non-climacteric fruit development but not ripening. The expression patterns of ethylene and its biosynthesis elements were also reported in some other non-climacteric fruits (Wang *et al.*, 2010). Inexplicably, three ethylene receptors in strawberry fruit presented high expression levels in the ripe fruit (Trainotti *et al.*, 2005). This maybe has relevant to ethylene other functions of promoting coloring, sugar conversion, synthesis of aroma volatiles, etc (Cara and Giovannoni, 2008). Exogenous ethylene enhanced anthocyanin accumulation in grape berries (El-Kereamy *et al.*, 2003). These results braced the argument that the genes of dependent and independent ethylene were modulated by the conserved and primary regulating factors through ethylene release in both types of climacteric and non-climacteric fruits (Adams-Phillips *et al.*, 2005; Liu *et al.*, 2015).

A comprehensive understanding on the sugar-signaling mechanism is helpful to fruit quality improvement. Currently, published reports on sugar metabolism mainly concentrated on carbohydrate metabolism enzymes including sucrose phosphate synthase (SPS) and sucrose synthase (SS) enzymes. SPS and SS-synthesis (SS-S) catalyze sucrose biosynthesis, whereas SS-cleavage (SS-C) cleaves sucrose into fructose and glucose (Ruan, 2014). In our results, *SPS-1, -2* and *SS-S* transcript levels gradually up-regulated between 110 PAD and 150 PAD (Fig. 5). These gave a good explanation on that sucrose sharply increased during fruit growth and maturation of loquat and high fructose contents were kept during the whole development (Fig. 1a). Changes of *SS-C* transcription levels might be related to enhance of sucrose hydrolysis and the increase of fructose and glucose in loquat fruit. Fructose and glucose were immediately phosphorylated by fructokinase and hexokinase when them produced by invertase or SS-C (Li *et al.*, 2012). SPS enzyme reversibly catalyzed the switch from 6-phosphate-fructose and UDP-glucose into 6-phosphate-sucrose. In fact, 14-3-3 proteins (shown in Table 2) can affect SPS activity to modulate the synthesis of sucrose (Zuk *et al.*, 2005; Yu *et al.*, 2012) and avoided SPS from hydrolysis of proteinase (Börnke, 2005).

Cell wall enlargement and extension plays a

significant role in modulating fruit development and maturity. In our results, *EjEXPA-2* and *EjEXPA-4* contributed to change cell wall properties during loquat fruit cell division. Our results indicate different expansins (*EjEXPA* 1-6) were potentially correlated with mediating fruit size or maturity (Fig. 6). Transgenic endogenous *EXPAs* was proved to promote plant vegetative growth (Cho and Cosgrove, 2000; Choi *et al.*, 2003). Similar expression patterns of expansins were shown in other fruit development (Harrison *et al.*, 2001; Kitagawa *et al.*, 2005). The accumulation level of *EjEXPA-1* mRNA was consistent with the raise of fruit firmness, which might be associated with lignifications (Yang *et al.*, 2008). *EjEXPA-1* might encode a protein of redundant functions (Li *et al.*, 2002; Yang *et al.*, 2008). Besides, cell wall breakdown may require other components. In strawberry ripening, cellulose and pectatelyase interacted synergistically with expansins to affect cell wall disassembly (Jiménez-Bermúdez *et al.*, 2002).

Conclusion

The accessibility of depolymerases to cell wall was regulated indirectly by expansin genes. However, it needs further study how the spatial distributions of expansions coordinated with other factors to regulate development and maturity of loquat fruit.

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