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Construction and characterization of a cDNA library from floral organs and fruitlets of *Citrus reticulata*

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Abstract

To explore and isolate genes related to flowering and fruit development, we constructed a cDNA library from floral organs and fruitlets of Ponkan mandarin (*Citrus reticulata* Blanco). A total of 661 high-quality expressed sequence tags (ESTs) were generated and submitted to GenBank with the accession numbers from GO343532 to GO344192. All these ESTs were assembled into 43 contigs and 296 singletons (totally 339 unigenes). The *BLAST2GO* software was employed to annotate the unigenes, among which 77 ones had no significant homology with the sequences in NCBI non-redundant proteins database by *BLASTX* analysis. Additionally, gene ontology (GO) analysis revealed an overview of sequences distribution, which implied some specially expressed genes related to flower and fruit development. Furthermore, some abundantly expressed unigenes involved in several crucial metabolic pathways related to fruit quality were highlighted and three types of homologues of miraculin-like protein2 were analyzed by both semi-quantitative RT-PCR and real-time PCR. The results showed different expression profiles of these genes, which meant that they contribute distinctly to fruit development.

Additional key words: expressed sequence tags, Ponkan mandarin.

Introduction

Citruses are important fruits in subtropical areas and Ponkan mandarin is a commercial cultivar in Southern China. Traditional breeding approaches are constrained for the peculiarities of citrus reproductive biology such as long juvenile period, nucellar polyembryony, self- and cross-incompatibility and low genetic diversity. Thus modern biotechnologies including tissue culture, molecular markers and genetic transformation are widely used for citrus improvement (Deng 2005). Generation and characterization of gene libraries is the priority for citrus breeders because it is fundamental platform in diverse aspects as genetic and physical mapping, molecular marker, new genes isolation and identification, and comparative genomics research (Talon and Gmitter 2008).

In the past few years, a series of cDNA libraries have been constructed from a number of citrus species for a range of tissues with and without exposure to pests, pathogens, and abiotic stresses (Forment *et al*. 2005). Thousands of candidate genes associated with citrus fruit development were discovered and analyzed functionally. To elucidate the mechanism of citric acid utilization, Cercos *et al*. (2006) examined the expression patterns of 7000 genes during the ripening of fruit flesh of citrus Clementina using cDNA microarray. In order to identify useful genes associated with fruit quality, production and salinity tolerance, Terol *et al*. (2007) constructed a normalized full-length cDNA library and nine additional standard libraries, and more than 5 000 novel citrus genes were obtained. Meanwhile, Fujii *et al*. (2007) identified 1493 ethylene-responsive genes in mature mandarin fruit by 22 K oligoarray containing 21 495 independent ESTs

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Abbreviations: EST - expressed sequence tags; NCBI - National Center for Biotechnology Information; ORF - open reading frame; RT-PCR - reverse transcription-polymerase chain reaction.

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from citrus species. Martinez-Godoy *et al*. (2008) designed and constructed a genome-wide 20 K citrus microarray based on 85 965 high quality EST sequences for global gene expression analysis. Wang *et al*. (2009) cloned a *ptCBF* gene by RT-PCR and RACE methods from *Poncirus trifoliata* to elucidate its ability of cold resistance. However, fruit development is a complicated process involving in a series of components. Some important agronomic traits, such as color of juice sacs, seedlessness and easy peeling are different among citrus cultivars (Tao *et al*. 2006). Both BAC (bacterial artificial chromosome) (Baig *et al*. 2009) and cDNA libraries of Ponkan mandarin were constructed in our group recently. This study provided a primary exploration of functional genes involved in Ponkan mandarin fruit development.

Materials and methods

Flowers and fruits of Ponkan mandarin (*Citrus reticulata* Blanco) genotype E-gan No. 1 were collected from the Fruit and Tea Research Institute of Hubei, Wuhan, China. Both floral organs and fruitlets were collected from April to June at eight different developmental stages including squaring period, medium bud stage, in bud stage, full-bloom stage and fruitlets at 15, 25, 35 and 45 d after flowering (DAF).

Total RNA was extracted by modified *Trizol* method described by Liu *et al*. (2006), and poly (A) mRNA was purified using *PolyATtract*® mRNA isolation kit (*Promega*, Madison, WI, USA). First-strand cDNA was synthesized from the mRNA and then amplified by long distance (LD) PCR to generate the double-strand (ds) cDNA according to the manual of the *Creator*™ *SMART*™ cDNA library construction kit (*Clontech,,* Mountain View, CA, USA). About 5 mm^3 PCR products were analyzed on 1.8 % agarose/EtBr gels to assess the quality and quantity of cDNA. Then approximately 50 mm^3 double-stranded cDNAs were digested by proteinase *K* and *SfiI*. The digested ds cDNA were further fractionated by *Chroma Spin-400* column (*Clontech*). Fragments longer than 400 bp were collected to be ligated to the pDNR-LIB vector at 16 °C overnight. Then recombinant plasmids were transformed into ElectroMax™ DH5α-E™ cells (*Invitrogen*, Carlsbad, CA, USA) with electroporation method and the cellular suspension was transferred into the centrifuge tube containing 970 mm^3 Luria Bertani (LB) liquid medium (supplied with 30 mg dm^{-3} chloramphenicol) and incubated at 225 rpm and 37 °C for 1 h. Finally, the cultured bacterial liquid was plated onto LB media (with 30 mg dm^{-3} chloramphenicol) plates at 37 °C for 18 - 20 h to generate the original cDNA library.

The tittering and conservation of the cDNA library were according to *Creator*™ *SMART*™ cDNA library using construction kit user manual IX and X. 16 clones were randomly selected and PCR-amplified to determine the insertion fragments by using screening primers M13+ (5'-GTAAAACGACGGCCAGT-3') and M13– (5'-GGA AACAGCTATGACCATGTTCA-3'). Approximately 820 clones were randomly picked and incubated in LB broth containing 30 mg dm^{-3} chloramphenicol for 16 to 24 h. PCR reaction was conducted to eliminate the non-single clones, then all certified clones were subjected to 5' end single-pass sequencing by *BGI Life Tech* (Beijing, China).

Total RNA of tissues at six different development stages including young ovaries, flowers at in bud stage, medium bud stage, squaring stage, mature and young leaves were extracted with the method mentioned above. The primer pairs (Table 1) used in reverse transcriptase polymerase chain reaction (RT-PCR) and real-time PCR were designed with the *Primer 5.0* and *Primer express* software, respectively (*Applied Biosystems*, Foster City, CA, USA). In order to normalize the cDNA templates for PCR, the concentration of starting RNA was determined by the *Nanodrop 1000* spectrophotometer (*Thermo Scientific*, Wilmington, DE, USA), and then adjusted to an advisable volume for single strand cDNA synthesis with the prescription of the *RevertAid*™ first strand cDNA synthesis kit (*Fermentas China*, Shenzhen, China). Then each cDNA sample was pre-amplified using the specific primer pairs of citrus actin gene and adjusted to identical concentration for subsequent semi-quantitative RT-PCR and real-time PCR. The PCR program differed in terms of the annealing temperature of each primer pair and the length of the predicted PCR products. The real-time PCR was performed using the *ABI 7500* real time system (*PE Applied Biosystems*) with the method as described by Liu *et al*. (2007).

Results

The results showed that the original and amplified library contained 1.13×10^8 and 1.04×10^9 clones, respectively. Sequencing results of 360 randomly picked clones indicated that the recombinant rate was 97 %. Randomly selected 16 clones showed that the size of the insertion fragments ranged from 400 to 2 000 bp, with an average size of 900 bp (Fig. 1). A total of 661 high-quality ESTs were obtained after eliminating the leading vector, poor-

Table 1. Forward (F) and reverse (R) primers used in semi-quantitative RT-PCR and real-time PCR. The specific primers named qF and qR were used in real-time PCR.

Gene	Primer	Sequence (5' to 3')	Ta [°C]	Amplicon size [bp]
Miralp33	F	CTATTGGGCATCTCATCAAACT	56	460
	R	AAGAAAGACAGACACTCGGACA	58	
	qF	TCAAATCCACCCTCGAGTATAAACT	58	65
	qR	GCCTAGGTGTGTGCTGAATCC	58	
Miralp65	F	AGAAACGAACTCTGCCCACTTGA	60	323
	R	CAAACGGAAGGACAGTGAACAAT	58	
	qF	GCGCGCTTGCTGTTGTT	59	67
	qR	GGGCATTCAAGACGCACAT	58	
Miralp81	F	TTAGCCTACACGGTGGCAGAA	56	350
	R	AACGGATGGACAATGAACGA	60	
	qF	ACGTTGGCCGTTCGTTTG	59	67
	qR	AATGCCGGCTCATCATCAC	58	
Actin	F	CCAAGCAGCATGAAGATGAA	60	100
	R	ATCTGCTGGAAGGTGCTGAG	60	

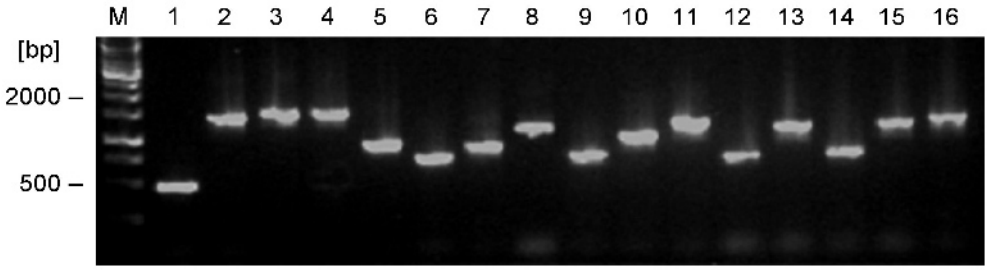

Fig. 1. Agarose gel electrophoresis of insertion fragments from randomly selected 16 clones. M - 1 kb ladder; *lanes* 1 to 16 - stand for the clones.

Table 2. Unigenes concerning metabolic pathways related to Ponkan fruit quality.

Pathway	Enzyme	EC	Number of unigenes
Glycolysis/ gluconeogenesis	6-phosphofructokinase	2.7.1.11	195
	aldehyde dehydrogenase	1.2.1.3	275
	pyruvate decarboxylase	4.1.1.1	318
Citrate cycle/TCA cycle	succinate-CoA ligase	6.2.1.5	299
	citrate synthase	2.3.3.8	299
Oxidative phosphorylation/ pentose phosphate cycle	ubiquinol-cytochrome-*c* reductase	1.10.2.2	277
	cytochrome-*c* oxidase	1.9.3.1	124
	H^+-transporting two-sector ATPase	3.6.3.14	298
	6-phosphofructokinase	2.7.1.11	195
Flavonoid biosynthsis	caffeoyl-CoA O-methyltransferase	2.1.1.104	188, 82
	naringenin-chalcone synthase	2.3.1.74	271
Phenylpropanoid biosynthesis	caffeoyl-CoA O-methyltransferase	2.1.1.104	188, 82
	cinnamoyl-CoA reductase	1.2.1.44	190
	peroxidase	1.11.1.7	193
Ascorbate and aldarate metabolism	inositol oxygenase	1.13.99.1	308
	aldehyde dehydrogenase	1.2.1.3	275
	L-ascorbate oxidase	1.10.3.3	219
	UDP-glucose-6-dehydrogenase	1.1.1.22	199
Fructose and mannose metabolism	6-phosphfructokinase	2.7.1.11	195
	mannose-1-phosphate guanylyltransferase (GDP)	2.7.7.22	215
	diphosphate-fructose-6-phosphate 1-phosphotransferase	2.7.1.90	195

quality and shorter than 100-nucleotide sequences. Then all these ESTs were assembled by *SEQMANII* software (*DNASTAR* package) and most of them ranged from 400 to 800 bp. *BLAST2GO* analysis was performed for the functional characterization of the unigene sequences (http://blast2go.bioinfo.cipf.es/start_blast2go). According to homology search with *BLASTX* against the NCBI non-redundant protein database, the *BLAST* results were parsed from the best hit name and description, the percentage of similarity and E-value. Based on the sequence conservation all the unigenes were classified as follows: 139 unigenes had very high similarities (80 - 100 %), 104 ones showed high similarities (60 - 80 %), while 13 unigenes had moderate similarities (40 - 60 %) and 83 unigenes displayed low similarities (< 40 %). In addition, 96 sequences had no definite function description including 77 sequences, which had neither significant hits nor similarities with cut-off default expect-values of 10^3, demonstrating that these 77 unigenes were putative new genes. In order to identify genes with relevant roles in fruit quality, homologues of structural enzymes involved in some crucial metabolic pathways were highlighted (Table 2). Then all unigenes were compared to annotations through the gene ontology, among which 197 annotated unigenes were created for gene ontology graphs under the categories of biological

Table 3. Redundantly expressed unigenes.

Unigenes	Sequence homology	Length [bp]	E-value	Amino acid homology [%]	Best match in GenBank
Unigene-23	lipid transfer protein precursor	666	1.0e-31	78.55	AAM21292.1
Unigene-25	acidic class IIchitinase	1267	1.0e-133	72.95	BAC20285.1
Unigene-33	miraculin-like protein 2	968	2.5e-114	97	AAG38518.1
Unigene-36	DNA binding protein	501	2.3e-5	60	ABO93453.1
Unigene-55	putative protein	461			
Unigene-65	miraculin-like protein 2	1073	7.8E-107	98	ABL67650.1
Unigene-81	miraculin-like protein 2	960	4.1E-112	97	BAE79511.1
Unigene-96	class II heat shock protein	411	1.0E-33	92.5	AAR25848.1

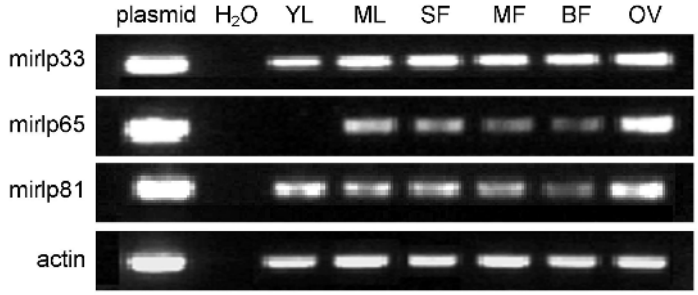

Fig. 2. Semi-quantitative RT-PCR expression analysis of 3 types of miraculin-like protein2. *Lanes* 1 to 6 represent cDNA templates of six different developmental stages including young (YL) and mature leaves (ML), squaring stage (SF), medium bud stage (MF), flowers at in bud stage (BF) and young ovaries (OV) respectively, H_2O as negative control and plasmid isolated from corresponding single-clone bacterial suspension as positive control.

processes, molecular functions and cellular components. The results showed that the unigenes involved in translation (16 %), abiotic (7 %) or other stress processes (11 %) and regulation (10 %) accounted for the majority of the biological function. The large proportion of unigenes was interfered in structure (20 %), protein binding (16 %), hydrolase activity (15 %) and nucleotide binding (10 %), while the cellular components consisted mainly of ribosome (21 %), mitochondrion (19 %), plastid (16 %) and cytosol (14 %) as well as cytoplasmic member-bounded vesicle (13 %).

As the EST sequences were obtained randomly, each unigene that comprised more than 10 ESTs was considered to be abundantly expressed gene (Boo *et al.*

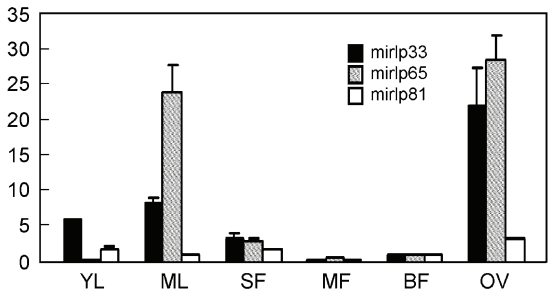

Fig. 3 Relative expression of three types of miraculin-like protein 2 in Ponkan mandarin during six stages of flower and leaf development (for abbreviations see Fig. 2). Means ± SE, $n = 4$.

2007) (Table 3). Based on this principle, we specifically analyzed 3 types of miraculin-like protein2 genes named *mirlp33*, *mirlp65* and *mirlp81*. These three unigenes contained complete open reading frames (ORFs) and showed high amino acid homology (up to 98 %) according to *BLASTx* analysis. However, *BLASTn* analysis showed relatively low similarity identities (64 - 71 %) by aligning every two of these sequences. These results revealed that they were likely to be members of one gene family. To further validate that these genes were expressed abundantly, we employed both semi-quantitative RT-PCR and quantitative real-time PCR methods. Six different developmental stages including young (YL) and mature leaves (ML), squaring stage (SF), medium bud stage (MF), flowers at in bud stage (BF) and young ovaries (OV) were chosen to analyze the relative expression levels of these genes (Figs. 2,3). The results showed that these genes shared a similar variation of expression during the six developmental stages. The expression of *mialp33* and *mirlp65* increased during the maturation of leaves, but with the development of flowers, expression of all the three genes decreased to a low level especially at medium bud stage, then increased and stabilized at relatively high level at young ovary stage. However, the expression levels of the three genes were somehow different, especially at stages of YL, ML and OV. These consequences might involve some different roles and functions of the three members during the development of Ponkan mandarin.

Discussion

It was reported that fruit trees could respond differently to normal or adverse conditions, and this was probably associated with differential regulation of gene expression rather than the sequences divergence (Terol *et al*. 2007). Many pivotal enzymes or transcription factors related to the metabolism of fruit quality formation were highlighted. We also targeted some redundantly and extensively expressed genes/unigenes such as gene family encoding miraculin-like protein2 in the library. Miraculin-like protein is not sweet itself but elicits sweetness when an acid is tasted and in Japan, miraculin is produced commercially. It is a 50 kDa homodimeric glycoprotein existing in the miracle fruit (*Synsepalum dulcificum*, *Sapotaceae*) and similar to Kunitz-type trypsin inhibitor (Masuda *et al*. 1995). Some putative transcripts of these proteins had been isolated in immature fruit flavedo of grapefruit (Shatters *et al*. 2004). These genes have also been confirmed expressing abundantly in young fruit of Satsuma mandarin (Boo *et al*. 2007). However, little is known about these genes, especially the three members of the miraculin-like protein gene family, in Ponkan mandarin. Based on the cDNA library, we obtained the full-length cDNA sequences and used both semi-quantitative RT-PCR and real-time RT-PCR to detect their expression profiles. The expression pattern of miraculin-like protein in Ponkan mandarin was somewhat different with the results reported in *Citrus jambhir*i, where it was found that the transcripts of miraculin-like proteins specifically accumulated in fruits but not in leaves or stems (Tsukuda *et al*. 2006). However, in Ponkan mandarin, these genes seemed to be expressed constitutively, and the transcripts were detected obviously in leaves and floral organs, even at low expression level in some tissues. The different results might imply the diverse expression pattern of the miraculin-like protein2 genes interspecies. Nevertheless, little information was available about the exact function of these genes except previous evidences of involving in defense against pathogens (Tsukuda *et al*. 2006).

Additionally, we chose floral organs and fruitlets of eight different developmental stages as materials, and expected that genes involved in fruit quality formation, embryogenesis and some other metabolic processes would be included in this library. In fact, some unigenes involved in phytohormone metabolism/regulation such as auxin-independent growth promoter (unigene No. 52), auxin response factor (unigene No. 10) and gibberellin-regulated family protein (unigene No. 15) widely exist in this library, and other unigenes related to embryo and seed development also exist extensively including gene encoding a type of ubiquitin extension protein (unigene No. 2), This protein was involved in cell growth and organ differentiation, and minor expression level variations during carpel and ovule development may lead to anatomical abnormalities and seeds abortion (Hanania *et al*. 2009). Ankyrin (ANK) repeat family proteins (unigene No. 263) as well as ankyrin-like proteins (unigene No. 54) were also obtained in this library. In plants, ANK proteins were found to be involved in diverse important processes such as embryogenesis (Albert *et al*. 1999), pollen germination and pollen tube growth (Huang *et al*. 2006), resistance to biotic and abiotic stresses (Yan *et al*. 2002, Seong *et al*. 2007), and maintenance of nitrogen-fixing symbiosis in root nodules (Kumagai *et al*. 2007). In conclusion, our research provided an initial exploration of fruit development about Ponkan mandarin and further endeavors should be focused on functional analysis of more candidate genes.

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