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Identifying Differently Expressed Transcripts between a Novel Late-Ripening *Citrus sinensis* Mutant and its Wild Variety during Fruit Ripening using cDNA-AFLP

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ABSTRACT

'Fengjiewancheng' (FJWC) is a late-ripening bud sport of 'Fengjie72-1' navel orange (FJ72-1). To investigate transcript differences during fruit ripening between the two cultivars and to get clues as to the mechanisms monitoring the late-ripening traits, we compared their transcript profiles during fruit ripening using cDNA-AFLP. As a result, 144 transcript-derived fragments (TDFs) were found differently expressed in FJWC compared with FJ72-1 of which 129 TDFs were recovered and assembled into 51 uniTDFs. Blast annotation indicated that 39 uniTDFs were assigned with putative function in signal transduction, control of gene expression and carbohydrate biosynthesis. Some TDFs shared relatively high identities with known functional genes and showed differential expression patterns in fruit pulp or peel between FJWC and FJ72-1 during fruit ripening, implying a potential role in the formation of the late-ripening phenotype.

Keywords: blast2go, bud sport, navel orange, transcript analysis

Abbreviations: **AFLP**, Amplified fragment length polymorphism; **B2G**, Blast2GO; **cDNA-AFLP**, cDNA-amplified fragment length polymorphism; **DAF**, days after flowering; **FJ72-1**, 'Fengjie72-1' navel orange; **FJWC**, 'Fengjiewancheng'; **GO**, Gene Ontology; **RT-PCR**, reverse transcription polymerase chain reaction; **TDF**, transcript-derived fragment

INTRODUCTION

Fruit development and maturation are intricate and coordinated processes, which correlates with lots of attributes to fruit quality from the standpoint of agriculture and economy (Giovannoni 2001). Fruits can be separated into dehiscent and fleshy fruit, of which the later is classically divided into climacteric and nonclimacteric types. However, most information of fruit developmental and ripening process is from *Arabidopsis* (dehiscent fruit) and tomato (climacteric fruit) because of the availability of suitable fruit ripening-related mutants and other advantages such as available genomic information and a short inter-generation time (Giovannoni 2001; Giovannoni 2004). Citrus is one of the most important fruit crops in the world and belongs to nonclimacteric fruit type. It has received considerable attention for molecular analysis of fruit development and ripening. Until now, a number of novel ripening-related genes or genes associated with quality characters were identified (Cercós *et al.* 2006; Terol *et al.* 2007; Alós *et al.* 2008). Some fruit metabolism, for example citric acid utilization (Cercós *et al.* 2006) and carotenoids pathway (Rodrigo *et al.* 2003; Kato *et al.* 2004; Liu *et al.* 2007), were well illustrated. But an overall developmental regulatory pathway for citrus fruit is still poorly understood.

'Fengjiewancheng' (FJWC), is a late-ripening *Citrus sinensis* bud sport of 'Fengjie72-1' navel orange (FJ72-1). Experiments and field observation in different locations for several years confirmed that this mutant is genetically stable. Academically, FJWC might be a useful experiment system with its parental line for the investigation of complex regulation ripening process of a non-climacteric fruit like citrus. Though we had revealed the physiological differences in sugar, acid and peel color between FJWC and FJ72-1 during fruit ripening (Liu *et al.* 2006; Liu *et al.* 2007), the underlying molecular mechanism is still unknown. In our initial experiment, we tried to detect their DNA variation between the two cultivars using AFLP (Amplified Fragment Length Polymorphism) technique, and no reproducible DNA polymorphic bands between them were found after screening 150 pairs of AFLP-selective primers.

cDNA-amplified fragment length polymorphism (cDNA-AFLP) developed by Bahem *et al.* (1996), was regarded as both an effective alternative to cDNA microarrays with low cost and a valid transcript profiling technique for the organisms that lack a genomic platform (Alba *et al.* 2004). Currently, cDNA-AFLP is widely used for gene expression analysis (Breyne *et al.* 2003; Bove *et al.* 2005; Bae *et al.* 2006) and gene isolation (Eckey *et al.* 2004; Leymarie *et al.* 2007). As a sensitive and reliable technique, cDNA-AFLP was employed here to investigate transcript difference during fruit ripening between FJWC and its wild type to get clues about the mechanisms monitoring the late-ripening traits.

MATERIALS AND METHODS

Plant materials

'Fengjiewancheng' (FJWC) and 'Fengjie72-1' navel orange (FJ72-1, *Citrus sinensis* Osbeck) were grafted on trifoliate orange in 2000 and cultivated together in an orchard of Fengjie county, Chongqing, China. Three trees of each cultivar were selected for sampling. Fruits of both cultivars were collected at 20~30 days' interval from the pre-color break stage [165 days after flowering (DAF)] to the deep orange stage (263 DAF), which were decided according to the wild type. Each time for each cultivar, 12 fruits were randomly selected from the outer crown of trees. Then each fruit was longitudinally and symmetrically cut into 10 aliquot portions. One portion of each of the 12 fruits were mixed together.

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Table 1 Sequences of primers and adaptors used for cDNA-AFLP analysis.

ID	Sequence (5′ to 3′)
*Eco*RI top adaptor	CTCGTAGACTGCGTACC
*Eco*RI bottom adaptor	AATTGGTACGCAGTCTAC
*Eco*RI pre-amplified primer	GTAGACTGCGTACCAATTCA
*Eco*RI selective primer	AATTGGTACGCAGTCTACNN*
*Mse*I top adaptor	GACGATGAGTCCTGAG
*Mse*I bottom adaptor	TACTCAGGACTCAT
*Mse*I pre-amplified primer	GACGATGAGTCCTGAGTAAC
*Mse*I selective primer	GACGATGAGTCCTGAGTAACNN

*N – refers to nucleotide A, C, G or T.

Table 2 Primers used for semi-quantitative RT-PCR

ID	Sequence (5′ to 3′)		PCR cycles	
	Forward	Reverse	Pulp	Peel
fjfw1	TCCTGAGTAACGATTCCTTGA	CAGGATTCAGTCACCACTGC	28	26
fjfw9	TGATCTAGGCTTGGCAACAA	TCCAAGGTTGAGGCTAATGG	26	26
fjfw10	CCCTATGGTTATGCTTGGGA	CTTTGTCTCCTTCATGCTCC	26	26
fjfw14	AGTTGCTTGAAGGCTTTCAGG	ATTGAGTGTTCAGCAGCTGAG	28	26
fjfw17	GAGAGATTGCTGGGCTTCAA	CTGCGGAACTCATGGACACT	30	26
fjfw18	GGATTCAGATTCCACCCAAC	GCCAGGGGTCAAACTTGTAA	28	28
fjfw23	TGCAGTCATCTCCTGTTCCA	GACATGCTCCAACGGCTATT	28	26

Gene-specific primers were designed according to the sequence of differential TDFs with Primer 3 software.

The sample mixture was subsequentally divided into peel and pulp sections and were ground into fine powder in liquid nitrogen and then stored at -80°C until use.

RNA isolation and cDNA synthesis

Total RNAs of fruit peel and pulp of FJWC and FJ72-1 at five different ripening stages (165, 187, 207, 227 and 263 DAF) were isolated respectively according to a protocol described before (Liu *et al.* 2006). Ten RNA pools were made by an equal mixture of peel and pulp RNA (200 μg) of each cultivar at each ripening point for mRNA purification (PolyATtract® mRNA Isolation Systems III Kit, Promega, USA). Double strand cDNA was synthesized using the M-MLV RTase cDNA synthesis Kit [TaKaRa Biotechnology (Dalian) Co., Ltd., China] according to the manufacturer's instructions.

cDNA-AFLP analysis

cDNA-AFLP was performed according to Bachem *et al.* (1998) using the *Eco*RI/*Mse*I enzyme combination with the adaptors or primers listed in **Table 1**. The cDNA fragments were visualized by silver-staining according to the instruction of SILVER SEQUENCE™ DNA sequencing system (Promega, USA). cDNA-AFLP procedure of each pair of selective primer combination was reproduced independently at least twice for each cDNA sample. When results were identical, we could consider the result as effective.

Transcript-derived fragments sequencing, processing and annotation

Differential TDFs were excised from the gel and eluted cDNA was re-amplified under the same condition as for the cDNA-AFLP selective amplification. The re-amplified cDNAs were ligated to pMD18-T vector (TaKaRa, Dalian, China) and were then transformed into *E. coli* DH-5α competent cells. Recombinant clones of differential TDFs were sequenced at Shanghai United Gene holdings, Ltd.

The sequence of vector contamination was screened by VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Reading assembly was performed with the CAP3 program (Huang and Madan 1999), using read quality and defaults parameters. A Blastn (http://www.ncbi.nlm.nih.gov/BLAST/) search for each differential TDF against a citrus-specific EST database was carried out to extend the TDFs' length. Sequence description and Gene Ontology (GO) annotation of differential TDFs were generated by Blast2GO (B2G) analysis (Forment *et al.* 2005) with default parameters. Minimum values for BLAST e-value and number of sequence distribution cutoff for pie charts were set to e-03 and 3, respectively.

Semi-quantitative RT-PCR analysis

Five micrograms of RNA of fruit peel and pulp tissues at each sampling points were reverse transcribed using RevertAid™ First Strand cDNA Synthesis Kit (MBI, USA) according to its instructtions. The reaction components, amplification program and electrophoresis conditions of RT-PCR analysis was performed as before (Liu *et al.* 2007). Primer sequences and amplification cycles are listed in **Table 2**. RT-PCR was conducted in parallel and the amplification result was considered effective if the agarose gel detection was uniform.

RESULTS

Differential transcript-derived fragments between the two varieties during fruit ripening

In this research, a total of 120 selective primer combinations corresponding to *Eco*RI/*Mse*I were analyzed. A representative example of a cDNA expression profile after polyacrylamide gel electrophoresis and silver-staining is shown in **Fig. 1**. In this research, five different ripening points according their flavedo color were sampled for cDNA-AFLP analysis (**Fig. 2**). Differential expressed genes specific to the two varieties were divided into two types: up- and down-expression in FJWC. Up-expression in FJWC meant that cDNA fragments were present or had stronger intensity in FJWC than that in FJ72-1 (**Fig. 1A, 1B**), and the reverse is down-expression in FJWC (**Fig. 1C, 1D**). As a result, around 60,000 clear transcript-derived fragments (TDFs) were generated in acrylamide gels with band sizes from 50 to 500 bp. However, most TDFs were nearly identical and only 144 TDFs could be detected as differentially expressed between the two cultivars at different ripening stages. Out of the overall differential TDFs, 127 were down-expressed (88.2%) and 17 were up-expressed (11.8%) in FJWC. Furthermore, numbers of differential TDFs varied in ripening points (**Fig. 2**). At 165 DAF when two cultivars were in the green stage, 20 genes were found to be down-expressed and none of these differential genes was shown to be up-expressed. At 187 and 227 DAF, numbers of differential TDFs were equal with little difference in the proportion of up- and down-expression. The fewest differential TDFs (13 with 6 down-expression and 7 up-expression) of the five ripening points occurred at 207 DAF when FJWC was in the yellow stage and FJ72-1 was in the light orange stage, while most

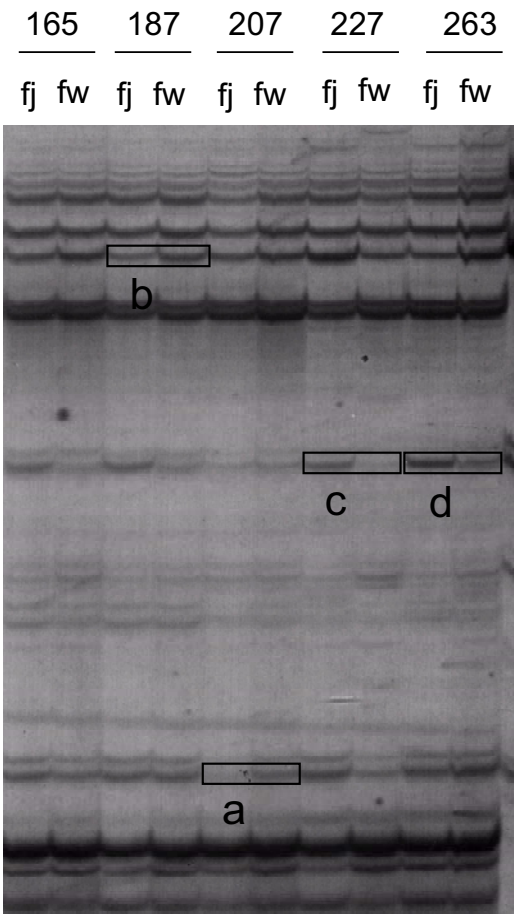

Fig. 1 A representative picture of a silver-stained cDNA gel showing the differential expression of the genes specific to FJWC (fw) and FJ72-1(fj). Differently expressed genes specific to the two varieties were detected by visual judgment: a, absence in fj and presence in fw; b, weak intensity in fj and strong intensity in fw; c, presence in fj and absence in fw; d, strong intensity in fj and weak intensity in fw.

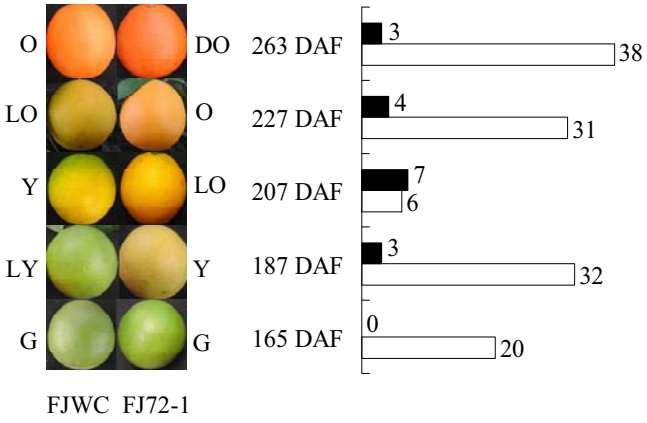

Fig. 2 Stages of flavedo coloration (DO-deep orange, O-orange, LO-light orange, Y-yellow, LY-light yellow, G-green) and TDFs distribution of down-expression (□) and up-expression (■) in FJWC compared with FJ72-1 at different ripening points. Value on column indicates the TDFs numbers of each sampling point.

(41 with 38 down-regulation and 3 up-regulation) appeared at 263 DAF when FJWC was in the mature stage and FJ72-1 was in the over-mature stage.

Differently expressed transcript-derived fragments in function categories

Of a total of 144 differential TDFs, only 129 were successfully recovered and sequenced. The average length of the TDFs was 230 bp with a range of 77-392 bp. To reduce the TDF redundancy, CAP3 soft (Huang and Madan 1999) was employed and produced 51 uniTDFs (21 contigs and 30 singletons). Sequence data were submitted to the GenBank data library with accession numbers listed in **Table 3**.

The GO annotation of the differential TDFs was performed using the B2G program (Forment *et al.* 2005). A previous publication showed that a large number of ESTs shorter than 500 bp were prone to fail to produce significant hits by BLASTX, which was possibly due to these sequences containing no coding sequence (Terol *et al.* 2007). Moreover, sequences with at least 98% nucleotide identity over a minimum of 100 bp could be assumed to derive from the same transcript. To avoid the risk of annotation by shot matches with a low e-value and uncover additional information about potential gene function for these differential uniTDFs, we carried out a nucleotide blast search in NCBI for each differential TDFs against citrus-specific EST database to extend their length. Only hits with max identity over 98% and query coverage over 80% or 100 bp were included for uniTDFs assemble using the CPA3 program. Results showed that 35 out of a total of 51 uniTDFs had significant hits in the citrus EST database, and assembled into superTDFs. Annotation of the assembled superTDFs and uniTDFs by a BLASTX against the GenBank non-redundant protein database in NCBI with an e-value cutoff of e-03 indicated that a large proportion of sequences (82.4%) had significant hits with clear description. The average of similarity mean value for the blast results was 76% with a range of 43-96% (**Table 3**).

GO annotation of these 51 differently expressed sequences indicated that a total of 39 sequences were annotated with 192 annotations among the three GO categories: molecular function (69), biological process (62) and cellular component (61). Almost half of them (19 of 51 sequences) had functional annotation among the three principle categories, and 13 sequences were annotated with two categories (**Fig. 3A**). Under the molecular function category, seven functional subsets were produced, of which hydrolase activity contributed 23%, followed by ATP binding (15%), protein binding (13%), zinc ion binding (13%) and nucleic acid binding (13%). The oxidoreductase activity and protein serine/threonine kinase activity contributed equally for 11% (**Fig. 3B**). In the biological process, these ESTs are mainly involved in response to stimulus (12%), transport (12%), cellular metabolic process (12%), post-translational protein modification (12%), micromolecule biosynthesis (12%), transcription (10%), cellular localization (10%), phosphate metabolic process (10%) and developmental process (10%) (**Fig. 3C**). As for the cellular component category (**Fig. 3D**), many of the sequences were related with the mitochondria (26%), and membrane system, including cytoplasmic membrane-bound vesicle (14%), integral membrane (12%), organelle membrane (9%) and endomembrane system (9%), followed by intracellular organelle (12%), chloroplast (9%) and protein complex (9%).

Expression analysis of some differently expressed transcript-derived fragments between fruit peel and pulp of the two cultivars

Differential TDFs above were generated from RNA pools combined RNAs of fruit peel and pulp tissues. We here selected seven possible important TDFs to investigate their expression profiles in the fruit peel and pulp tissues respectively between FJ72-1 and FJWC during fruit ripening process. Results showed that some expression differences were existed between the fruit pulp or peel or both tissues of the two cultivars except for TDF *fjfw18* of which the expression patterns in fruit pulp and peel between the two cultivars were similar (**Fig. 4**). Among them, differences in transcripts of TDF *fjfw9* could be found in both fruit pulp and peel between FJ72-1 and FJWC. In FJ72-1, the expression of TDF *fjfw*9 could be detected in both fruit pulp and peel during ripening, while in FJWC's fruit peel or pulp tissue, it

Table 2 Basic characteristic of cDNA-AFLP fragments differently expressed in FJWC and FJ72-1 during fruit ripening.

uniTDF ID	ESTs Accession Number	uniTDF Length bp	superTDFs length[a] bp	Hit sequence description	max eValue[b]	sim mean[c]
fjfw1	EH117766	189	719	transcription factor IIA large subunit	1.00E-52	74%
fjfw41	EH117767	367	990	ring zinc finger ankyrin protein	1.00E-67	70%
fjfw10	EH117768	133	1080	cinnamyl alcohol dehydrogenase	1.00E-165	91%
fjfw11	EH117769	148	-	cyclin c-like protein	1.00E-05	73%
fjfw33	EH117770	220	-	capsule polysaccharide biosynthesis	1.00E-21	73%
fjfw6	EH117772	391	956	leucine-rich repeat transmembrane protein	1.00E-91	65%
fjfw12	EH117773	214	-	Leucine-rich	1.00E-06	63%
fjfw3	EH117774	139	-	BGGP beta-1-3-galactosyl-*o*-glycosyl-glycoprotein	1.00E-10	80%
fjfw7	EH117775	208	1325	cathepsin b-like cysteine protease	1.00E-149	84%
fjfw8	EH117776	131	445	zinc dependent protease	1.00E-33	92%
fjfw9	EH117777	328	965	*trans*-cinnamate 4-hydroxylase	1.00E-123	96%
fifw5	EH117779	276	906	60s ribosomal protein bbc1 protein	1.00E-87	89%
fjfw2	EH117780	292	742	gamma glutamyl hydrolase	1.00E-76	73%
fjfw37	EH117781	290	-	receptor-like protein kinase	1.00E-04	67%
fjfw13	EH117782	290	-	-	-	-
fjfw14	EH117783	231	626	EREBP-4 like protein	1.00E-101	74%
fjfw36	EH117784	215	956	transport protein sec61	1.00E-08	90%
fjfw15	EH117785	119	724	OTU domain containing 6b	1.00E-53	62%
fjfw16	EH117786	237	-	40s ribosomal protein s6	1.00E-29	91%
fjfw40	EH117787	294	1251	nucleolar GTPase	1.00E-30	43%
fjfw17	EH117788	212	990	mitogen-activated protein kinase 3	1.00E-170	92%
fjfw18	EH117789	217	941	NAC domain transcription expressed	1.00E-141	75%
fjfw20	EH117790	340	924	dolichyl-diphosphooligosaccharide-protein glycosyltransferase	1.00E-110	73%
fjfw19	EH117791	287	-	nucleic acid binding	1.00E-16	64%
fjfw32	EH117792	289	1352	26s proteasome regulatory subunit	1.00E-117	69%
fjfw21	EH117793	156	676	glutathione peroxidase	1.00E-59	88%
fjfw23	EH117794	299	-	polygalacturonase	1.00E-43	80%
fjfw22	EH117795	251	751	SAH7 protein	1.00E-54	72%
fjfw24	EH117796	119	750	CCR4-associated factor	1.00E-124	82%
fjfw31	EH117797	185	1139	purple acid phosphatase-like protein	1.00E-127	78%
fjfw25	EH117798	358	1293	OMEGA-3 fatty acid desaturase	1.00E-76	90%
fjfw30	EH117799	93	999	proline-rich cell wall	1.00E-54	63%
fjfw34	EH117804	116	744	ATP binding helicase nucleic acid binding	1.00E-42	69%
fjfw42	EH117806	292	753	zinc-binding family protein	1.00E-81	83%
fjfw39	EH117807	125	1041	zinc finger (c3hc4-type ring finger) family protein	1.00E-57	72%
fjfw43	EH210584	119	421	FLU (fluorescent in blue light) binding	1.00E-24	77%
fjfw45	EH210587	102	-	-	-	-
fjfw46	EH210588	126	787	actin depolymerizing factor	1.00E-63	88%
fjfw54	EH210589	277	-	-	-	-
fjfw55	EH210590	274	-	-	-	-
fjfw47	EH210591	138	986	cell wall-associated hydrolase	1.00E-43	71%
fjfw50	EH210593	126	759	zinc finger (c3hc4-type ring finger) family protein	1.00E-55	74%
fjfw49	EH210595	77	-	-	-	-
fjfw56	EH210596	117	-	-	-	-
fjfw57	EH210598	260	-	-	-	-
fjfw58	EH210599	392	429	thioesterase superfamily member 2	1.00E-14	80%
fjfw60	EH210600	154	1297	pap13_arath probable plastid-lipid-associated protein chloroplast precursor	1.00E-52	76%
fjfw59	EH210601	254	1328	ATP synthase	1.00E-60	74%
fjfw61	EH210602	85	-	-	-	-
fjfw64	EH210604	380	1119	-	1.00E-60	49%
fjfw63	EH210605	89	960	NAM protein	1.00E-116	80%

[a] a nucleotide blast search was carried out in NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) against citrus-specific EST database. Only hits with max identity over 98% and query coverage over 80% or 100bp were included for assembling superTDFs using CPA3 program.
[b] E-value of the best hit of against the GenBank non-redundant protein database in NCBI with an e-value cutoff of e-03.
[c] mean similarity value for the blast results. This value was computed as the average hsp-similarity value for all the hits of a given sequence.

was relatively low or negligible at 165 and 187 DAF and then increased or remained relatively steady at 207, 227 and 263 DAF. The expression profiles of TDF *fjfw*10, 14 and 17 differed in the fruit pulp though they were similar during fruit ripening between the fruit peel of the two cultivars. The expression of TDF *fjfw*10 in the pulp of FJ72-1 could be detected all the time, although highest expression was found at 165 and 187 DAF. In FJWC, it was negligible at 165 and 263 DAF and remained steady at a low level from 187 to 227 DAF. For TDF *fjfw*14, the expression was strong at 207 and 227 DAF in the pulp of FJ72-1, while in that of FJWC, it was low or negligible at 163, 187 and 207 DAF, and highest expression was found at 227 DAF. As for the expression of TDF *fjfw*17, in FJ72-1 it was weak at 165 and 187 DAF, and then remain relatively low and steady at the following three sampling points, while in the pulp of FJWC, its expression level was found to be relatively low and steady all the time. The difference in the expression of TDF *fjfw*1 and 23 between the two cultivars mainly existed in the fruit peel tissue. The expression of TDF *fjfw*1 in FJ72-1 was strong at 165 DAF and then decreased to a low level and remained relatively steady, while in FJWC, its expression was strong and remained steady during the ripening process. The expression of TDF *fjfw*23 in FJWC accumulated and amounted to a relatively high level at the last two sampling points, while in FJ72-1 its expression was relatively high and remained relatively steady all the time.

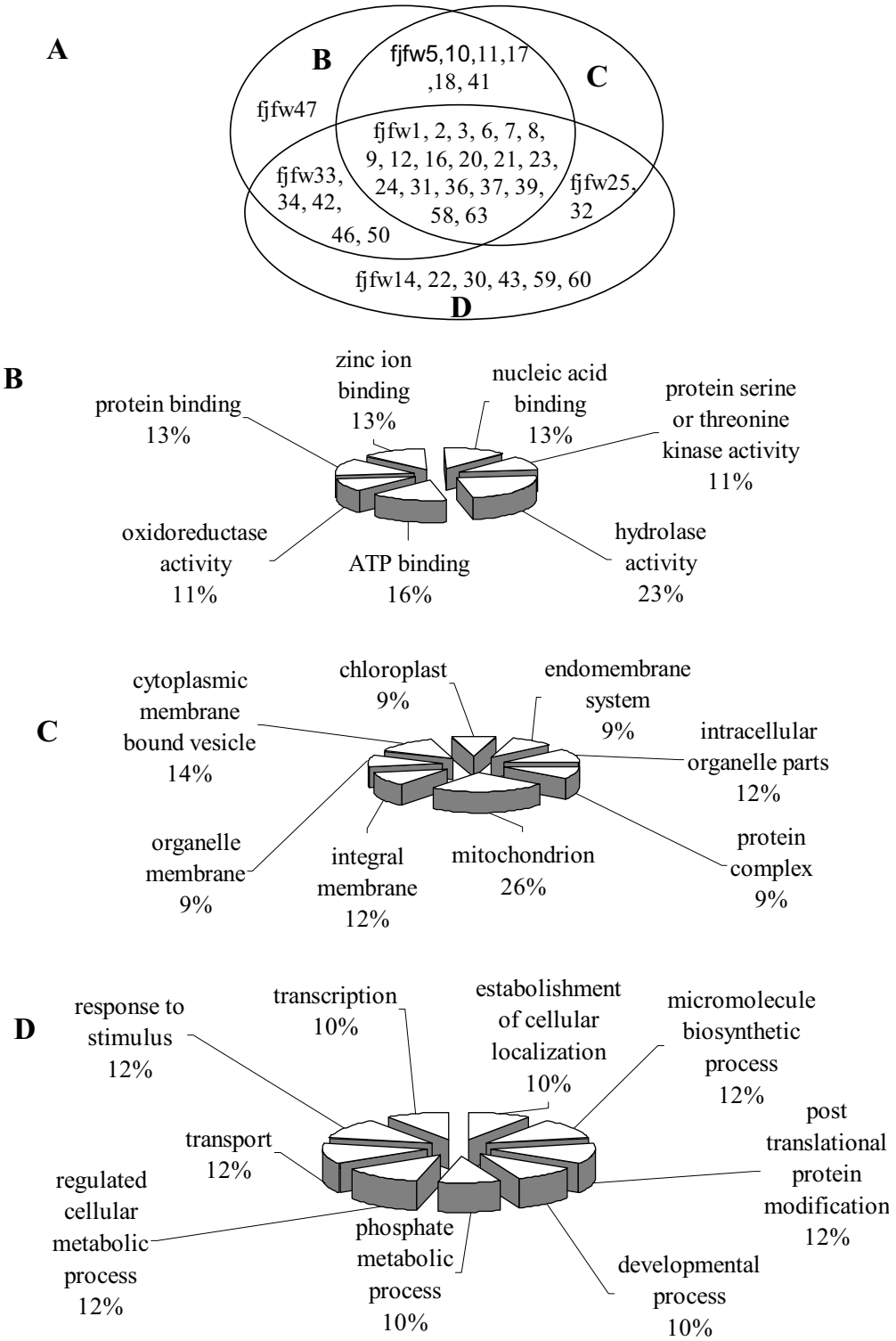

Fig. 3 Sequence distribution among GO slim categories of differential TDFs (Filtered by #Seq cutoff = 3.0). Functional annotation was carried out using Blast2GO (Forment *et al.* 2005) with default parameters. The pie diagrams show GO distribution by multilevel. A, Transcript-derived fragments distribution among the three GO principles; B, molecular function; C, biological process; D, cellular component.

DISCUSSION

In plants, mutants have played a great role in the elucidation of a wide variety of complex pathways or developmental process, including the biosynthesis of carotenoids (Rodrigo *et al.* 2003; Liu *et al.* 2007), chlorophyll metabolism (Alós *et al.* 2008) and fruit ripening of climacteric fruit (Giovannoni 2004, 2007). The majority of new navel oranges originate from a mutation or genetic change in a vegetative bud, commonly called as a "bud sport". To date, there are many late-ripening citrus bud sport released in the world, such as 'Lane Late' and 'Summer Gold'. Unfortunately, little information was reported in this area with respect to transcriptional difference between a late-ripening bud sports with its original variety.

FJWC is a late ripening mutant of FJ72-1. Previous evaluation showed that the ripening process of FJWC, such as color transition and sugar accumulation, were modified in a comprehensive scale (Liu *et al*. 2006, 2007). In this study, we compared their fruit expression profiles during the ripening process, and 144 TDFs were found as differential fragments, most of which were down-expressed in FJWC (**Fig. 1**). Growth of citrus fruit is a process characterized by very active metabolism, and a comparison between a mutant and its wild type at a certain stage of ripening should give many differently-expressed transcripts (Cercós *et al.* 2006; Alós *et*

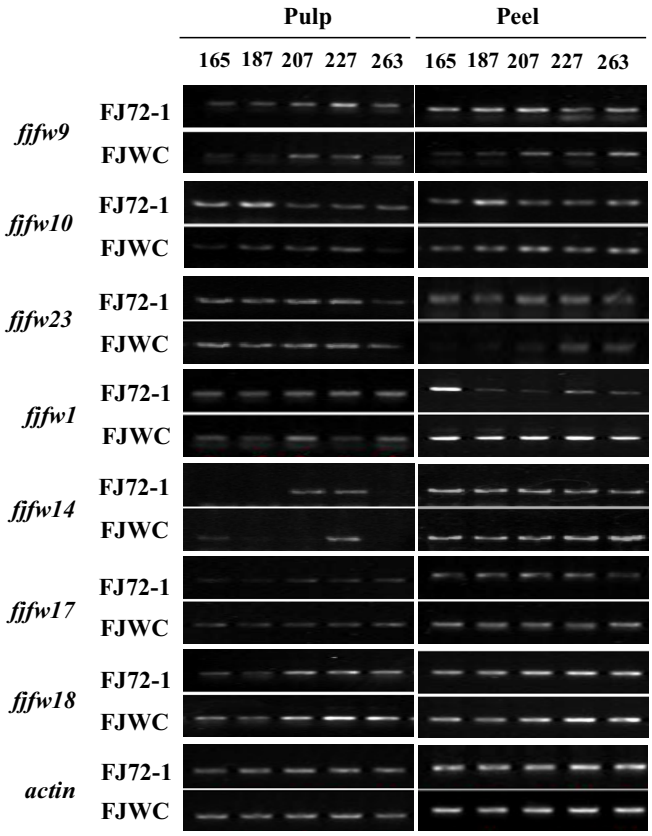

Fig. 4 Semi-quantitative RT-PCR analysis of TDF *fjfw1*, *fjfw9*, *fjfw10*, *fjfw14*, *fjfw17*, *fjfw18* and *fjfw23* between fruit pulp and peel of FJWC and FJ72-1. The result showed that differently expressed TDFs possibly had differential expression patterns between fruit peel or pulp or both of the two cultivars, which might be involved in the formation of late-ripening phenotype.

al. 2008). Here, we did not discover differential TDFs as much as we expected during fruit ripening mainly due to that we screened differential TDFs by visual judgment. cDNA-AFLP was established by Bachem *et al.* (1996), and its reliability was verified consecutively (Bachem *et al.* 1998; Bove *et al.* 2005). Its rationality and accuracy was improved when combined with AFLP-QuantarPro image analysis software (Breyne *et al.* 2003). Though, we also assessed its validity in our study by DIG northern blot and semi-quantitative RT-PCR before further analysis (data not shown), it should be pointed out that we did not employ AFLP-QuantarPro software to quantify the transcript fingerprints for the selection of differential TDFs. Although differential TDFs were selected by visual judgment, which undoubtedly lost some information, some probably important TDFs differently expressed between the two varieties during fruit ripening were still revealed (**Table 3**). For example, TDF *fjfw9*, *fjfw10* and *fjfw23* showed putative relation with cinnamate-4-hydroxylase, cinnamyl alcohol dehydrogenase and polygalacturonase respectively, which involved in cell wall metabolism and softening (lignin biosynthetic and carbohydrate metabolic process), one of important processes of fleshy fruit ripening (Giovannoni 2001). Further analysis indicated that their transcript levels displayed differently in pulp or peel or both between the two cultivars (**Fig. 4**).

Generally, ripening of citrus fruit is always accompanied by obvious soluble sugar accumulation, acid decrease, and color transition from green to yellow or orange. We had discovered that the higher expression level of carotenoids-metabolism-related genes in the peel of the mutant were delayed(Liu *et al.* 2006), and the transcript levels of some sucrose- and citric-metabolic enzymes displayed difference between the two cultivars (Liu *et al.* 2007). However, we did not discover differential TDFs here directly related to them. It is well known that fruit ripening requires a complex coordination of pathways and processes, and mainly subjects to the regulation of gene expression in a spatio-temporal way which was controlled by signal transduction and a vast of transcription factors (Brivanlou and Darnell 2002; Adams-Phillips *et al.* 2004; Giovannoni 2007). Resent research indicated that a Stay-Green citrus mutant may be related with an regulatory step modulating *SGR* expression (Alós *et al.* 2008). As for molecular function of GO categories in this research, though differential TDFs could be divided into seven subsets, half of them including protein binding, zinc ion binding, nucleic acid binding and protein serine/threonine kinase activity (**Fig. 3B**) seemed to be related with signal transduction and gene expression control according to Brivanlou' classification (2002). For example, TDFs *fjfw14* had relationship with EREBP-4 like protein which is a member of a large family of transcription factors involving control of gene expression(Durrant *et al.* 2000). TDFs *fjfw17* showed high similarity (mean sim=92%) or low e-value (max eValue=1e-170) with mitogen-activated protein kinase genes which have emerged as a universal signal transduction mechanism that connects diverse receptors/sensors to cellular and nuclear responses in eukaryotes (Tena *et al.* 2001; Eckey *et al.* 2004). NAC proteins constitute one of the largest families of plant-specific transcription factors and participate in regulation of different biological function including senescence of plant tissues(Olsen *et al.* 2005). In this study, two TDFs (*fjfw18* and *fjfw63*) seemed to be related with NAC protein-like genes (**Table 3**) and TDFs *fjfw18* might involve in citrus fruit ripening process based on its transcript patterns analysis (**Fig. 4**). Taken together, the lack of differential TDFs directly related to carotenoids-, sucrose- or acid metabolism suggests that the mutation of FJWC is possibly associated with regulatory elements that control fruit ripening process, rather than exerting a direct effect on a specific aspect of catabolism.

In conclusion, to our knowledge, this is the first time to detect transcriptional difference between late-ripening citrus bud sport and its original line using cDNA-AFLP and B2G analysis. We presented transcriptional difference between the two varieties during fruit ripening and clearly showed various genes involving in signal transduction, control of gene expression and cell wall metabolism and softening, which should be related with citrus fruit ripening and are worthy of in depth research. Furthermore, they were mainly located in the mitochondria, and membrane system (**Fig. 3**). We could provide such hypothesis that the formation of late-ripening traits was due to some genes, which are mainly located in mitochondria and membrane system with the function of signal transduction and gene-expression control, were altered. However, what caused the change of transcriptional profiles of FJWC during fruit ripening was still elusive. The onset of fruit ripening process might be subject to the control of fruit earlier developmental stages (Giovannoni 2001; Cercós *et al.* 2006). Hence, a differential expression profile between the two cultivars during the earlier stages of fruit development should be investigated in the future, which will conduce to clarify possible mechanisms responsible for FJWC late-ripening phenotype.

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