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RESEARCH PAPER

Transcriptome analysis of a spontaneous mutant in sweet orange [*Citrus sinensis* (L.) Osbeck] during fruit development

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Abstract

Bud mutations often arise in citrus. The selection of mutants is one of the most important breeding channels in citrus. However, the molecular basis of bud mutation has rarely been studied. To identify differentially expressed genes in a spontaneous sweet orange [*C. sinensis* (L.) Osbeck] bud mutation which causes lycopene accumulation, low citric acid, and high sucrose in fruit, suppression subtractive hybridization and microarray analysis were performed to decipher this bud mutation during fruit development. After sequencing of the differentially expressed clones, a total of 267 non-redundant transcripts were obtained and 182 (68.2%) of them shared homology (E-value $\leqslant 1\times10^{-10}$) with known gene products. Few genes were constitutively up- or down-regulated (fold change $\geqslant 2$) in the bud mutation during fruit development. Self-organizing tree algorithm analysis results showed that 95.1% of the differentially expressed genes were extensively coordinated with the initiation of lycopene accumulation. Metabolic process, cellular process, establishment of localization, response to stimulus, and biological regulation-related transcripts were among the most regulated genes. These genes were involved in many biological processes such as organic acid metabolism, lipid metabolism, transport, and pyruvate metabolism, etc. Moreover, 13 genes which were differentially regulated at 170 d after flowering shared homology with previously described signal transduction or transcription factors. The information generated in this study provides new clues to aid in the understanding of bud mutation in citrus.

Key words: Bud mutation, candidate genes, cDNA microarray, *Citrus*, real-time RT-PCR, suppression subtractive hybridization (SSH).

Introduction

Mutations have proved to be a key resource for functional genomics studies in model plant species (Chatelet *et al.*, 2007). Besides the mutants artificially generated in model plants, naturally occurring bud mutants are extensively found for most species (Koornneef *et al.*, 2004). These can be of particular scientific value for citrus.

Bud mutations (bud sports), a consequence of genetic variation of somatic cells leading to the occurrence of phenotypic alteration in plants, arise often in citrus (Raghuvanshi, 1962). Mutations occurred spontaneously in buds and limbs, representing the main natural source of new cultivars (Spiegel-Roy and Goldschmidt, 1996). When these bud sports are vegetatively propagated by clonal techniques, the new phenotype is generally maintained, leading to a new variety (Marcotrigiano, 1997). Mutants are generally detected by the growers themselves in branches of trees showing altered horticultural traits, such as maturity and flowering time or fruit characteristics (Bernet and Asins, 2003). To date, many bud mutants with elite characteristics such as early ripening and red-flesh in citrus fruit have been discovered (Zhang and Deng, 2006).

Genetic improvement in some woody perennial plants such as citrus, apples, and grapes by hybridization has been inefficient, long lasting and time consuming due to their heterozygosity and long juvenility (Asins *et al.*, 1999; Aradhya *et al.*, 2003; Kenis and Keulemans, 2005).

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Improvement in citrus has been largely the result of selection of naturally occurring bud mutants. Thus, exploring mutants is one of the most important breeding methods to obtain new cultivars with superior traits in citrus. Most cultivars of clementine mandarin (*Citrus clementina* Hort. ex Tan.), satsuma mandarin (*C. unshiu* Marc), and sweet orange [*C. sinensis* (L.) Osbeck] have resulted from bud mutations (Cameron and Frost, 1968; Asins *et al.*, 1999).

Although bud mutations have been important to the citrus industry, the molecular basis behind the generation of sports in citrus is not well understood. Most molecular genetic approaches to study bud mutations in citrus are limited to the detection of genetic variations of bud mutants from their original cultivars by molecular markers, such as RFLPs, RAPDs, AFLPs, ISSR, and SCAR (Moore, 2001; Fanizza *et al.*, 2003; Tao *et al.*, 2006; Mase *et al.*, 2007). However, minor genetic variation existing between the bud mutant and its original cultivars could not be efficiently distinguished by these kinds of markers (Deng *et al.*, 1995; Fang and Roose, 1997; Breto *et al.*, 2001). Several mechanisms that might be the molecular basis of bud mutations have been hypothesized, such as transposon activity, gene mutation, and DNA methylation (Breto *et al.*, 2001). Transposable elements, first recognized by Barbara McClintock in maize (McClintock, 1951), have been identified in many species including citrus (Rico-Cabanas and Martinez-Izquierdo, 2007), grape (Kobayashi *et al.*, 2004), and apple (Yao *et al.*, 2001), and, in grape and apple, the mobility of the transposable elements can be responsible for changes in fruit colour. The deletion of two regulatory genes of the berry locus was responsible for the colour change of grape berries of two bud mutants (Walker *et al.*, 2006). A spontaneous epigenetic mutation in a gene encoding an SBP-box transcription factor resulted in the *Colorless non-ripening* (*Cnr*) mutant in tomato (Manning *et al.*, 2006).

Despite such understanding of the mechanism of bud mutations, little information is available on the whole genome level regarding the candidate genes linked to the altered phenotype in mutant fruits of citrus. Until recently, transcriptomic and proteomic profiling and metabolite analysis of a stay-green mutation in the *Navel Negra* citrus mutant was conducted, and elevated Chl levels and photooxidative stress were associated with the mutant (Alos *et al.*, 2008). A spontaneous bud mutation in sweet orange [*C. sinensis* (L.) Osbeck] 'Hong Anliu', which results in fruits with lycopene accumulation, low citric acid, and high sucrose was reported in a previous study (Liu *et al.*, 2007). To identify differentially expressed genes linked to this bud mutation, techniques combining suppression subtractive hybridization (SSH) and microarray were used. First, by means of SSH (Diatchenko *et al.*, 1996), two libraries of differentially expressed clones were obtained. Then, these clones were printed on a microarray and subsequently used for a global comparison between the mutant fruits and the wild type, and the results were validated with real-time reverse transcriptase polymerase chain reaction (RT-PCR).

Materials and methods

Accession numbers

All the EST sequences generated in this study were deposited in GenBank with accession numbers from FE659063 to FE659327, plus FE660221 and FE660222.

Microarray data and experimental information from this work were deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE10729.

Plant material and sample preparation

'Anliu' sweet orange [*Citrus sinensis* (L.) Osbeck] and its red-flesh mutant, 'Hong Anliu', cultivated at the Institute of Citrus Research located in Guilin, Guangxi Province, China, were used in the present investigation. Both of them were of the same age, grown in the same orchard and subjected to standard cultivation practices. Fruits of each genotype were collected from three different trees, 10 representative fruits from each tree, for a total of 30 fruits per genotype. These samples were collected at five time points from August to December: 120, 150, 170, 190, and 220 d after flowering (DAF) [fig. 1 in Liu *et al.*, 2007). Sampled fruits were frozen in liquid nitrogen immediately, and kept at –80 °C until analysed.

Two mRNA pools were built for the construction of SSH libraries: R (the mutant 'Hong Anliu') and CK (the wild type 'Anliu'). Pool R and pool CK were enriched for equal

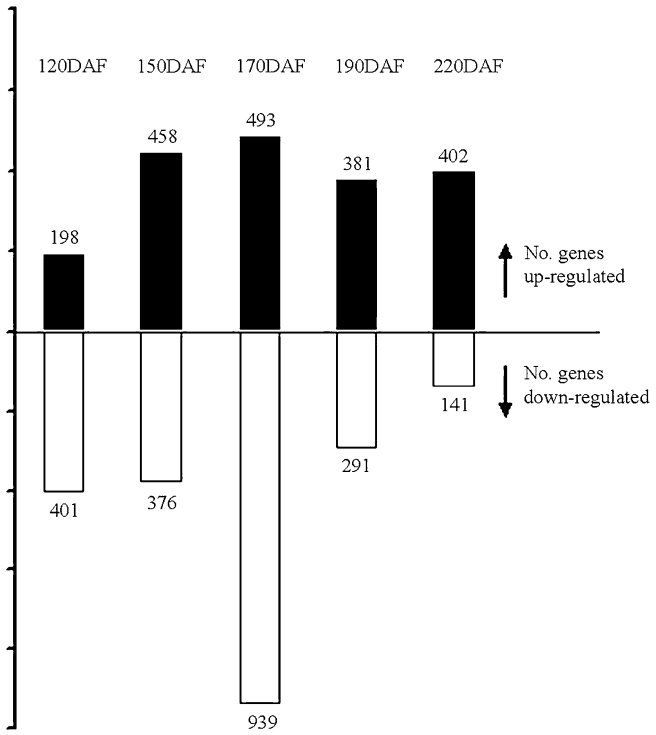

Fig. 1. Profile of gene expression during fruit development. Number of SSH cDNA clones significantly up- or down-regulated in the sweet orange mutant during fruit development. DAF, Days after flowering.

amount of mRNA at each time point from the mutant and wild-type fruits, respectively.

Total RNA and mRNA isolation

Total RNA was extracted from fruits following Liu *et al.* (2006). Isolated RNA was treated with DNase I at 37 °C for 1 h to remove genomic DNA contamination. For SSH, equal amounts of total RNA for each sample from 'Hong Anliu' and wild type were mixed and the mRNA was purified from the mixed total RNA using PolyATract® mRNA Isolation System I (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Construction of subtracted cDNA library

The cDNA reversely transcribed from 2 μg of the mixed mRNA mentioned above was used for SSH with the PCR-selected cDNA subtraction kit (BD Biosciences Clontech, San Jose, CA, USA). Both forward (mutant as tester and wild type as driver) and reverse (wild type as tester and mutant as driver) SSH libraries were constructed following the manufacturer's instructions.

Amplification of cDNA inserts

The three thousand cDNA clones, which were randomly picked from each subtracted SSH library, were cultured in 384-well plates overnight at 37 °C and used as templates. PCR amplification was conducted following Ouyang *et al.* (2007). PCR products were precipitated with anhydrous ethanol–sodium acetate (25:1), resuspended in 40 μl sterile water, and run on 1.2% agarose gel and examined by Bio-Rad UV spectroscopy (Bio-Rad Laboratories, Washington, DC, USA) to ensure both the quality and quantity.

cDNA microarray slides preparation

The PCR products were precipitated again by adding 100 μl of anhydrous ethanol and resuspended in 15 μl of 50% dimethylsulphoxide at a final concentration of 0.1–0.5 μg μl^{-1} and then spotted onto amino-silaned glass slides (CapitalBio Corp., Beijing, China) with a SmartArrayer™ microarrayer (CapitalBio Corp.). Each clone was printed in triplicate. After printing, the slides were baked for 1 h at 80 °C and stored dry at room temperature till use. Prior to hybridization, the slides were rehydrated over 65 °C water for 10 s, snap dried on a 100 °C heating block for 5 s, and UV cross-linked at 250 mJ cm^{-2}. The unimmobilized PCR products were washed off with 0.5% SDS for 15 min at room temperature, and SDS was removed by dipping the slides in anhydrous ethanol for 30 s. The slides were spun dry at 1000 rpm for 2 min. Eight sequences derived from intergenic regions in yeast genome, showing no significant homology to all existing citrus sequences, were spotted multiple times onto the microarray as exogenous controls. Total citrus RNA was spiked with a mixture of these exogenous control RNAs to validate the semi-quantitative microarray result.

Preparation of fluorescent dye-labelled cDNA and hybridization

The relative gene expression profiles of 'Hong Anliu' fruits at 120, 150, 170, 190, and 220 DAF compared with those of wild type at the corresponding stages were investigated by microarray analysis. An aliquot of 5 μg total RNA was used to produce Cy5/Cy3-labelled cDNA employing an RNA amplification combined with Klenow enzyme labelling strategy according to a previously published protocol (Guo *et al.*, 2005).

Cy5/Cy3-labelled cDNA was hybridized with the microarray at 42 °C overnight. Each hybridization was performed in duplicate by dye swap. After that, the arrays were washed with 0.2% SDS, 2× SSC at 42 °C for 5 min, and then with 0.2% SSC for 5 min at room temperature.

Microarray data analysis

Arrays were scanned with a confocal laser scanner, LuxScan™ 10K (CapitalBio Corp.), and the resulting images were analysed with SpotData Pro 2.0 software (CapitalBio Corp.). Spots with fewer than 50% of the signal pixels exceeding the local background value for both channels (Cy3 and Cy5) plus two standard deviations of the local background were removed. cDNA spots with less than four out of a total of six data points in each replicated hybridization were removed. A spatial and intensity-dependent (LOWESS) normalization method was employed (Yang *et al.*, 2002). Normalized ratio data were then log transformed. Differentially expressed genes were identified using a *t*-test, and multiple test corrections were performed using false discovery rate (FDR) (Benjamini and Hochberg, 1995). Genes with FDR <0.01 and a fold change $\geqslant 2$ were identified as differentially expressed genes.

EST sequence analysis

All the clones differentially expressed in at least one of five stages were single-pass sequenced (AuGCT Biotechonology Co. Ltd, Beijing, China). The software SeqClean was used for performing vector removal, poly(A) removal, trimming of low quality segments at the 5′ and 3′ ends, and cleaning of low complexity regions. RepeatMasker was used to mask repeats (Smit, 2007). Reading assembly was performed with the CAP3 program (Huang and Madan, 1999), using the read quality and defaults parameters. Cluster analysis was performed by the self-organizing tree algorithm (SOTA) (Herrero *et al.*, 2001), using linear correlation coefficient as the distance between genes. The tree was allowed to grow up using a variability threshold of 40% as the training condition.

The Blast2Go (Conesa *et al.*, 2005) program was used for the gene ontology (GO) data mining.

Quantitative real-time PCR verification

Total RNA was extracted from 'Anliu' and 'Hong Anliu' fruits collected at five different development stages according to Liu *et al.* (2006). Primer pairs were designed with the Primer Express software (Applied Biosystems, Foster City, CA, USA). Primer sequences are provided in Table S3

Table 1. Selected list of relevant candidate genes for the formation of the phenotype of the red-flesh bud mutant grouped in functional categories

The complete list of genes is given in Table S1 in Supplementary data available at *JXB* online. For each gene, the EST GenBank accession numbers and the putative molecular function are given. The putative molecular functions were assigned according to the biological process categories of GO annotation. *n* is the number of sequenced clones in the libraries.

Biological process	GenBank Accession no.	Description	BLAST E-value	*n*
Cellular metabolic process				
Organic acid metabolic process	FE659316	12-Oxophytodienoate reductase	1E-84	1
Organic acid metabolic process	FE659229	Glutamate decarboxylase	1E-68	1
Organic acid metabolic process	FE659194	Glyoxysomal malate dehydrogenase	1E-14	1
Organic acid metabolic process	FE659103	Phosphoenolpyruvate carboxykinase	1E-36	2
Organic acid metabolic process	FE659289	Stearoyl-acyl carrier protein desaturase	1E-16	1
Organic acid metabolic process	FE659140	Malonyl-acyl carrier protein transacylase	1E-30	3
Aromatic compound metabolic process	FE659159	Flavonol synthase	1E-58	1
Primary metabolic process				
Lipid metabolic process	FE659078	Lipoxygenase	1E-38	2
Lipid metabolic process	FE659085	Myo-inositol-1-phosphate synthase	1E-58	2
Lipid metabolic process	FE659245	Beta-carotene hydroxylase	1E-27	1
Lipid metabolic process	FE659242	Aspartic proteinase	1E-79	1
Localization				
Transport	FE659068	Lipid transfer protein	1E-14	2
Transport	FE659304	Glycosyl hydrolase family 17 protein	1E-12	1
Transport	FE659183	Sugar transporter	1E-43	1
Transport	FE659246	Glucose-6-phosphate translocator	1E-20	1
Transport	FE659238	Iron inhibited ABC transporter 2	1E-29	1
Transport	FE659222	ABC transporter	1E-15	1
Transport	FE659240	ABC transporter	1E-20	1
Transport	FE659184	Cytochrome *c*	1E-25	1
Macromolecule metabolic process				
Biopolymer metabolic process	FE659260	Ubiquitin-conjugating enzyme e2	1E-66	1
Biopolymer metabolic process	FE659110	UBC36 ubiquitin-protein ligase	1E-19	2
Biopolymer metabolic process	FE659206	Aldose 1-epimerase family protein	1E-48	1
Biopolymer metabolic process	FE659105	2-Oxoglutarate dehydrogenase e2 subunit	1E-32	2
Biopolymer metabolic process	FE659179	Pyruvate kinase	1E-20	1
Biopolymer metabolic process	FE659122	UDP-glucose pyrophosphorylase	1E-58	2
Biopolymer metabolic process	FE659182	Soluble acid invertase	1E-121	1
Biopolymer metabolic process	FE659239	Nucleotide sugar epimerase	1E-55	1
Biopolymer metabolic process	FE659287	Glyoxalase i	1E-90	1
Transcription	FE659120	Abscisic stress ripening protein	5E-171	9
Transcription	FE659326	WRKY-type transcription factor	1E-29	1
Transcription	FE659307	NAC domain protein	1E-53	1
Transcription	FE659294	Zinc finger protein	1E-28	1
Transcription	FE659300	Zinc finger transcription factor-like protein	1E-30	1
Transcription	FE659156	*S*-adenosyl-l-homocysteine hydrolase	1E-49	9
Transcription	FE659308	C-repeat binding factor	1E-10	1
Transcription	FE659195	Agamous-like protein	1E-38	1
Transcription	FE659124	Homeobox protein expressed	1E-92	7
Translation	FE659323	Elongation factor 1-expressed	1E-25	1
Translation	FE659121	Ribosomal protein l19	1E-35	7
Nitrogen compound metabolic process				
Amine metabolic process	FE659309	*S*-adenosylmethionine decarboxylase	1E-28	1
Cell communication				
Signal transduction	FE659190	Aux1-like permease	1E-15	1
Signal transduction	FE659263	Calmodulin	1E-68	1
Signal transduction	FE659279	WD-40 repeat family protein	1E-15	1
Response to stimulus				
Response to hormone stimulus	FE659093	Stem-specific protein expressed	1E-30	3
Response to jasmonic acid stimulus	FE659089	Dehydroascorbate reductase	1E-39	3
Response to oxidative stress	FE659293	Monodehydroascorbate reductase	1E-35	1
Response to protein stimulus	FE659161	KDA class i heat shock protein	1E-38	1
Response to water	FE659261	Dehydrin	1E-28	1
Response to stress	FE659086	Late embryogenesis-abundant protein	1E-30	12
Response to cold	FE659301	BAP2 (bon association protein 2)	1E-12	1
Response to DNA damage stimulus	FE659119	Thiazole biosynthetic enzyme	1E-170	15

available in Supplementary data at *JXB* online. Real-time PCR verification was performed according to Liu *et al.* (2007).

Results

Construction of SSH libraries and overall features of the mutant-responsive expression profile

To isolate genes differentially expressed in the mutant 'Hong Anliu' sweet orange compared with its wild type 'Anliu' during fruit development, forward (mutant as tester and wild type as driver) and reverse (wild type as tester and mutant as driver) subtractions were conducted between fruits of the red-flesh mutant and its wild type. Two thousand nine hundred and eighty-nine clones were randomly picked from each SSH library. The average insert size of the SSH clones was around 0.4 kb. The clones from the two SSH libraries were amplified and used for microarray analysis. RNA samples of mutant and wild-type fruits collected at 120, 150, 170, 190, and 220 DAF were used for microarray hybridization.

In total, 2394 differentially expressed cDNA clones (fold change $\geqslant 2$ and FDR <0.01) were identified. However, only one clone (GenBank accession no. FE659117) was constitutively down-regulated, and no clone was constitutively up-regulated in the mutant versus its wild type during fruit development. The number of up- or down- regulated clones was largest at 170 DAF when lycopene began to accumulate in the juice sacs of the mutant 'Hong Anliu' (Fig. 1). At this time point, there are more clones down-regulated than up-regulated in mutant compared with the wild type, and the number of down-regulated clones was twice that up-regulated. The gene expression profiles of up- and down-regulated seemed to be symmetrically opposite each other except for the time point of 170 DAF. The number of up-regulated genes increased from 120 DAF to 170 DAF, and then it remained almost the same from 190 DAF to 220 DAF. On the contrary, the number of down-regulated genes remained almost the same from 120 DAF to 150 DAF, and then it decreased from 170 DAF to 220 DAF.

EST analysis

All the clones which showed differential expression for at least one time point out of five between the mutant 'Hong Anliu' and wild type were selected. Single-run sequencing of the selected clones yielded 698 readable sequences longer than 100 bp. Of these, 526 were grouped into 96 contiguous sequences (contigs) and 171 were single sequences (singletons) with the CAP3 program (Huang and Madan, 1999). Thus, in total, 267 independent sequences were obtained. Sequence redundancy was 61.6%. BLASTX analysis showed that 68.3% of the independent sequences (116 singletons and 66 contigs) exhibited high sequence homology with known proteins in the NCBI non-redundant protein sequences database (E-value $\leqslant 1\times10^{-10}$) and that 85 (55 singletons and 30 contigs) were classified as no hits (E-value $>e^{-10}$). Of the 267 genes, 255 (95.1%) showed differential expression at 170 DAF.

The relative expression profiles of the 267 genes were subjected to cluster analysis using the SOTA algorithm (Herrero *et al.*, 2001). To group relative gene expression profiles on the basis of similar trends and not of similar expression levels, the Pearson correlation coefficient was used as the distance function. Figure 3 shows hierarchical clustering of transcript accumulation and eight relative expression patterns observed in the mutant versus its wild type at five time points, and demonstrates that gene expression changes are highly coordinated during fruit development. As expected, 95.1% of the prominent expression patterns observed in this study correlated well with the initiation of lycopene accumulation.

GO categories were assigned to 267 non-redundant genes with BLASTX hit using Blast2GO (Conesa *et al.*, 2005). Table 1 showed a selected list of genes with putative functions that could be important for this mutant. The complete list is given in Table S1 available in Supplementary data at *JXB* online. Interestingly, 31% of the ESTs were found to be potentially new genes having no similarity in the public databases, while hypothetical proteins having

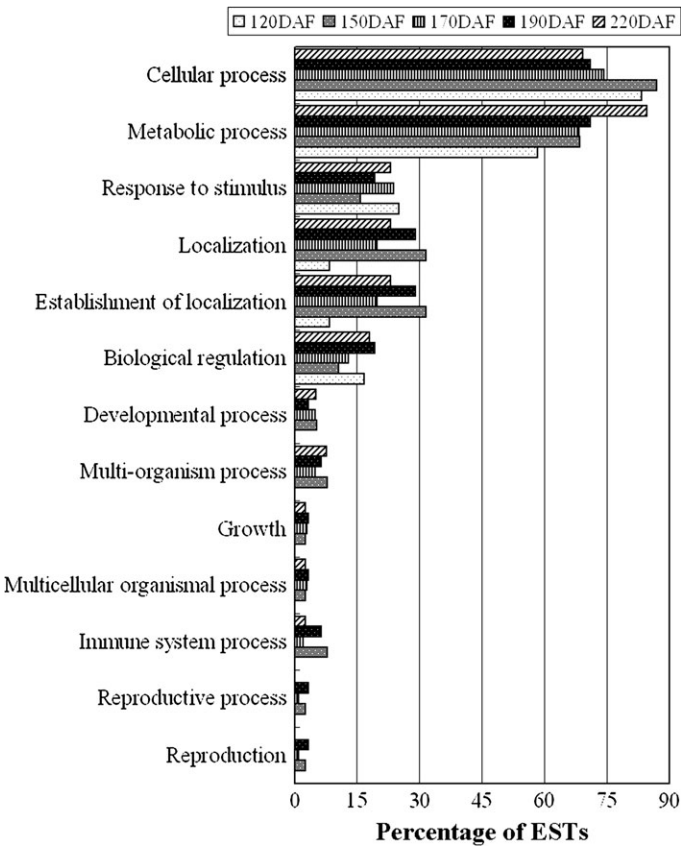

Fig. 2. Distributions of differentially expressed ESTs according to the biological process part of GO (2nd level GO terms) consortium during fruit development. The total numbers of unique ESTs annotated for the biological process are 124. Since a gene product could be assigned to more than one GO term, the percentages in each main category will add up to >100%.

no defined biological process annotation constituted 22% of the EST set. Figure 2 shows the percentage distributions of GO terms (2nd level GO terms) according to the biological process part of GO consortium during fruit development. Metabolic process and cellular process were the major 2nd level terms annotated to the biological process GO category. The percentage distributions of metabolic process increase, while the percentage distributions of cellular process decrease during fruit development.

Metabolic pathways involved in the formation of the phenotype of the mutant fruit

Thirty-nine different metabolic pathways were altered by the bud mutation, most of the genes in these pathways were differentially expressed at 170 DAF, and the identities of these genes are listed in Table S2 available in Supplementary data at *JXB* online. Pyruvate metabolism, glycolysis/gluconeogenesis, pentose and glucuronate interconversions, and carbon fixation were among the most altered metabolic pathways. Figure 4 showed relative expressions of selected genes which took part in pyruvate metabolism by real-time PCR. Cysteine protein precursor, the only gene which was consecutively down-regulated in the bud mutation during fruit development, showed a low expression in 'Hong Anliu' at the green stage (120–150 DAF). After the green stage, the expression of cysteine protein precursor in 'Hong Anliu' was barely detectable (170–220 DAF).

Several genes in pathways closely related to the altered phenotypes were also found to be differentially expressed (Table 2). Beta-carotene hydroxylase (EC 1.14.13.–), a gene encoding a key enzyme in the carotenoid

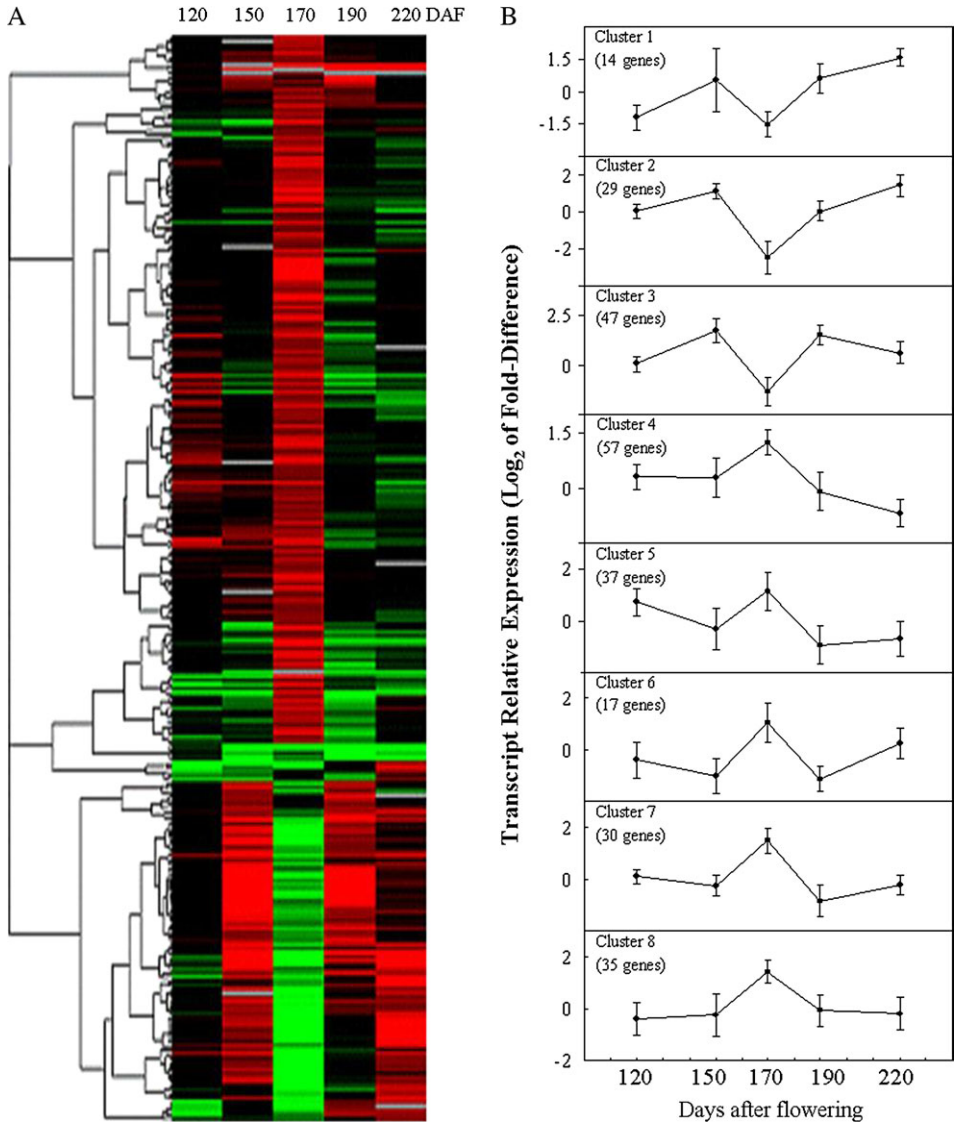

Fig. 3. Cluster analysis of expression profiles of differentially expressed gene in the mutant versus its wild type during fruit development. (A) Hierarchical clustering of transcript accumulation between five time points (120, 150, 170, 190, and 220 DAF) during fruit maturation. For each stage, the $\log_2$ value of the ratio between 'Hong Anliu' and its wild type was represented. (B) SOTA algorithm was used for cluster analysis. There are eight clusters according to SOTA analysis. Data are average relative expression values ±standard deviation. The number of differentially expressed genes in each cluster is also shown.

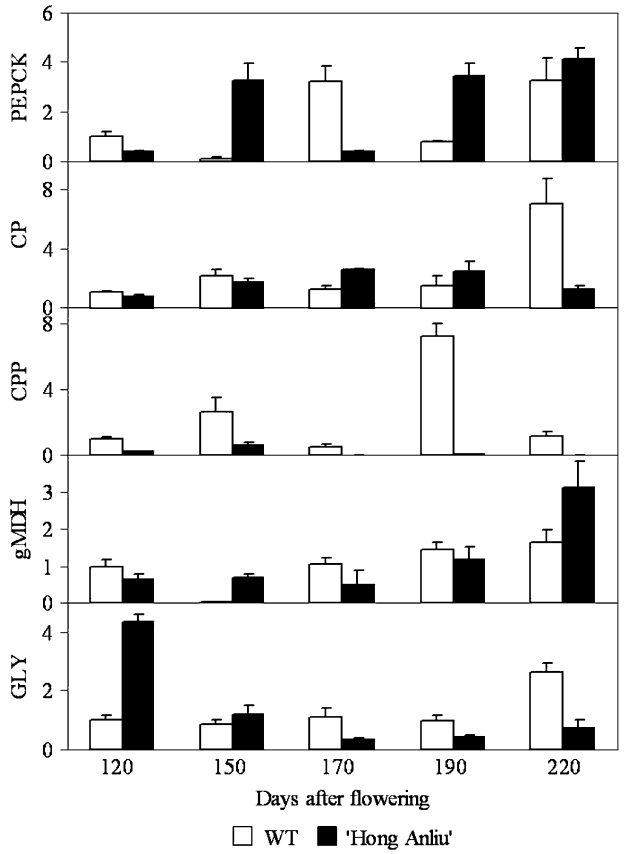

Fig. 4. The relative expression of pyruvate metabolism in the mutant 'Hong Anliu' versus its wild type (WT) by real-time PCR. Five differentially expressed genes in the SSH libraries were thought to be involved in pyruvate metabolism. All EST clones shown have been sequence verified. PEPCK, Phosphoenolpyruvate carboxykinase; CP, cysteine protease; CPP, cysteine protease precursor; gMDH, glyoxysomal malate dehydrogenase; GLY1, glyoxalase1.

biosynthesis pathway, was affected by the mutation. The expression of this gene was up-regulated at 170 DAF in mutant 'Hong Anliu' compared with its wild type, and was not significantly affected at the other four time points. Three genes involved in the citrate cycle were regulated by the mutation (Table 2). Cysteine protease (EC 3.4.22.–) was up-regulated at 170 DAF, and then down-regulated at 190 and 220 DAF, although it did not reach 2-fold. Phosphoenolpyruvate carboxylase (EC 4.1.1.49) and lipoic transsuccinylase (EC 2.3.1.61) were both up-regulated at 150 DAF and 190 DAF, and were not significantly altered at the other three time points. Several genes encoding key enzymes in the starch and sucrose metabolism and glycolysis were significantly altered; they included UDP-glucose pyrophosphorylase (EC 2.7.7.9), soluble acid invertase (EC 3.2.1.26), pyruvate kinase (EC 2.7.1.40), aldose 1-epimerase (EC 5.1.3.3), and fructose-bisphosphate aldolase (EC 4.1.2.13). Among these, UDP-glucose pyrophosphorylase was significantly down-regulated throughout all the five time points; and the other four were up-regulated at 170 DAF.

Candidate regulatory genes for the formation of the phenotype of mutant fruit

Among the 267 genes described above, 13 (4.9%) were assigned to the categories of transcription factor (10 loci, E-value $\leqslant 1\times10^{-10}$) and signal transduction (3 loci, E-value $\leqslant 1\times10^{-10}$). Figure 5 depicts expression for six candidate regulatory genes, including three putative transcription factors: zinc-finger protein, NAC domain transcription factor, and C-repeat binding factor. All of the genes shown in Fig. 5 were in accordance with the initiation of lycopene accumulation. Candidate regulatory genes likely to encode signal transduction factors have also been identified. Three examples of genes belonging to this functional category are shown in Fig. 5, including aux1-like permease, wd-40 repeat family protein, and calmodulin.

Verification of microarray data

To validate expression profiles obtained using microarray analysis, quantitative RT-PCR was performed on 10 genes using gene-specific primer pairs (Table S3 available in Supplementary data at *JXB* online). Transcript abundance patterns were compared at five time points between mutant 'Hong Anliu' and its wild type 'Anliu' (Fig. 6). Linear regression [(microarray value) = a(RT-PCR value)+b] analysis showed coefficients of variation of 0.87. This confirmed the differential expression of all six selected genes.

Discussion

Reported here is a collection of differentially expressed genes and metabolic pathways caused by the bud mutation of sweet orange based on SSH, microarray comparison, and sequence homology, since this set of approaches has proved to be an efficient way to enrich and identify differentially expressed genes (Diatchenko *et al.*, 1996; Yang *et al.*, 1999; Derory *et al.*, 2006; Ouyang *et al.*, 2007; Terol *et al.*, 2007; Xu *et al.*, 2007). The results identified a list of genes probably associated with the novel traits of this mutant fruit.

In the mutant 'Hong Anliu', the expression levels of hundreds of genes have been altered (fold change $\geqslant 2$) compared with its progenitor 'Anliu' during fruit development (Fig. 1). Similar results have been found in several researches on mutant–progenitor pairs. Through microarray analysis, 564 and 268 genes were found to be differentially expressed in *Navel Negra* [*Citrus sinensis* (L.) Osbeck], an abnormal brown-coloured falvedo mutant, versus Washington navel orange (wild type) at mature green stage and ripe stage, respectively (Alos *et al.*, 2008). Two hundred and sixty genes were differentially expressed in blood orange 'Moro' compared with common orange 'Cadenera' through SSH and reverse northern analysis (Licciardello *et al.*, 2008). In tomato (*Solanum lycopersicum*=*Lycopersicon esculentum*), mutation of an ethylene receptor [*Never-ripe* (*Nr*)], which reduces ethylene sensitivity and inhibits ripening, altered the expression of 322 genes that were differentially

Table 2. List of differentially expressed genes involved in carotenoid, organic acid, and sugar metabolic pathway according to the KEGG pathway database (KEGG = Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/pathway.html)

Significant differences (FDR <0.01 and fold change >=2) in relative level are shown in bold.

KEGG pathways	EC*	Putative function	'Hong Anliu'/wild type				
			120 DAF†	150 DAF	170 DAF	190 DAF	220 DAF
Citrate cycle (TCA cycle)							
	3.4.22.–	Cysteine protease	–1.04‡	1.30	**2.29**	–1.69	–1.46
	4.1.1.49	Phosphoenolpyruvate carboxylase	–1.01	**2.91**	–1.97	**2.41**	–1.25
	2.3.1.61	Lipoic transsuccinylase	1.41	**2.38**	1.57	**3.37**	–1.03
Starch and sucrose metabolism							
	2.7.7.9	UDP-glucose pyrophosphorylase	–1.53	**–5.48**	**–3.04**	**–13.83**	**–9.64**
	3.2.1.26	Soluble acid invertase	1.40	–1.46	**2.57**	–1.61	1.08
Glycolysis							
	2.7.1.40	Pyruvate kinase	1.17	**–2.67**	**3.30**	–1.74	–1.21
	5.1.3.3	Aldose 1-epimerase	1.15	–1.14	**4.10**	**–2.10**	–1.32
	4.1.2.13	Fructose-bisphosphate aldolase	1.47	–1.05	**2.08**	1.21	–1.44
Carotenoid biosynthesis							
	1.14.13.–	Beta-carotene hydroxylase	–1.32	1.02	**2.03**	1.13	–1.33

* EC, Enzyme code.
† DAF, Days after flowering.
‡ '–' means the value of wild type/'Hong Anliu'.

expressed in developing tomato pericarp (Alba *et al.*, 2005); 477 unique genes were differentially expressed between a *Colorless non-ripening* (*Cnr*) and wild-type fruits through microarray and differential screen analysis (Eriksson *et al.*, 2004). According to current research, it is common that expression of hundreds of genes is altered by natural mutations.

As for all the differentially expressed genes identified in our SSH libraries during fruit development, most (95.1%) of them showed differential expression at the time point of 170 DAF when a large amount of lycopene started to accumulate, indicating that 170 DAF might be a key developmental stage for the formation of novel traits of the mutant 'Hong Anliu' fruits. However, few genes were constitutively up- or down-regulated (fold change $\geqslant 2$) in the bud mutation during fruit development. In our SSH libraries, only one gene (cysteine protease precursor, EST accession no. FE659117) was constitutively down-regulated by the bud mutation. This result was similar to that of a stay-green mutation in the *Navel Negra* citrus mutant. Although 11 distinct genes differentially expressed between the *Navel Negra* citrus mutant and its wild type during all three developmental stages, only one gene (SGR gene homology) showed constitutive down-regulation (Alos *et al.*, 2008).

All these differentially expressed genes were involved in many biological processes such as organic acid metabolism, lipid metabolism, transport, and pyruvate metabolism, etc. Moreover, 13 genes shared homology with previously described signal transduction or transcription factors which might be of particular interest.

Organic acid metabolic process

This bud mutation had a profound effect on the organic acid content of mutant ('Hong Anliu' sweet orange) fruits (Liu *et al.*, 2007). Seven genes involved in organic acid metabolic process were differentially expressed in the mutant 'Hong Anliu' versus its wild type in our library. Glutamate decarboxylase (GenBank accession no. FE659229) is an enzyme catalysing the conversion of L-glutamate to γ-aminobutyric acid. In plants, glutamate decarboxylase is activated by acidic pH (Snedden *et al.*, 1995, 1996; Shelp *et al.*, 1999) and γ-aminobutyric acid accumulates in response to cytosolic acidification (Shelp *et al.*, 1999). Thus, it is possible that glutamate decarboxylase could participate in regulating the cytosolic pH of the mutant fruit. Glyoxysomal malate dehydrogenase (FE659194), another organic acid-related gene, belongs to the glyoxylate cycle, which bypasses the two decarboxylative steps of the citric-acid cycle and redirects the carbon flow toward gluconeogenesis (Guex *et al.*, 1995). A 12-oxophytodienoate reductase (FE659316) was involved in jasmonate biosynthesis (Schaller *et al.*, 2000), which implied that jasmonic acid metabolism has been affected by the mutation. Phosphoenolpyruvate carboxykinase (EC 4.1.1.49, FE659103) in plants is a cytosolic enzyme that catalyses a reversible reaction which lies at an important crossroads involved in the metabolism of lipids, organic acids, and amino acids (Chen *et al.*, 2004).

Lipid metabolic process

In citrus fruits, carotenoid accumulation was considered to be the result of coordination of the genes encoding key enzymes in the carotenoid biosynthesis pathway (Kato *et al.*, 2004; Liu *et al.*, 2007). A differentially expressed gene encoding beta-carotene hydroxylase (EC 1.14.13.–, FE659245) was present in our library. This gene showed 97 % similarity to ABB49053, which has been identified as a key member of carotenoid biosynthesis in higher plants

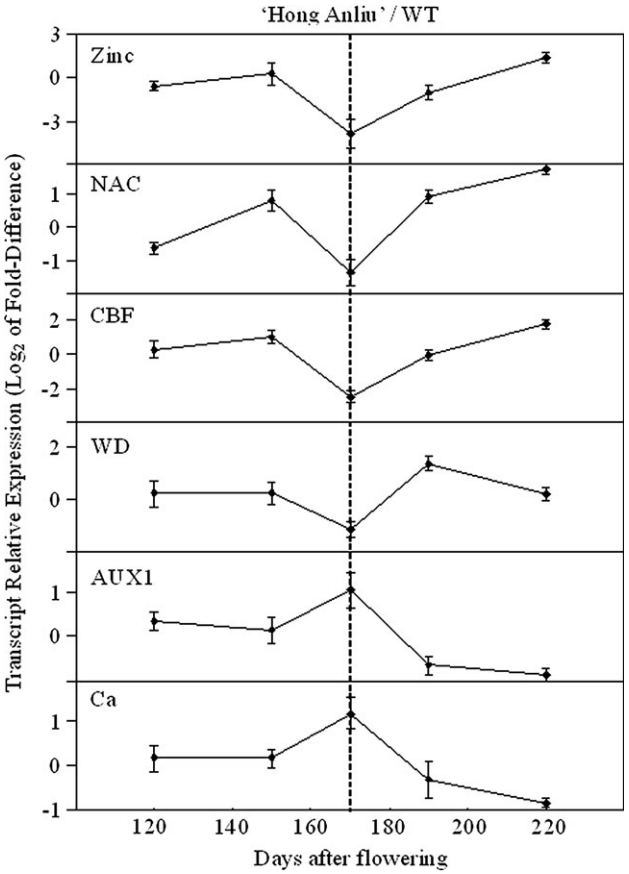

Fig. 5. Candidate regulatory genes for the bud mutation. Since the expression profile of each gene in the same gene family was similar, one representative gene of each family was chosen. Six candidate regulatory genes identified in this study were shown. Columns show the relative expression of 'Hong Anliu' versus its wild type by real-time PCR. Only ESTs with substantial sequence homology were considered (E-value $<1\times10^{-10}$), and all EST clones shown have been sequence verified. The vertical dashed line denotes the stage when dramatic lycopene accumulation was initiated in the fruit. Zinc, Zinc-finger protein; NAC, NAC-domain protein; CBF, C-repeat binding factor; WD, WD-40 repeat family protein; AUX1, aux1-like permease; Ca, Calmodulin.

(Sun *et al.*, 1996). The lipoxygenase activity was inhibited by beta-carotene (Serpen and Gokmen, 2006). Plant lipoxygenases (EC 1.13.11.12, FE659078) are thought to be involved in the biosynthesis of lipid-derived signalling molecules and jasmonic acid (Bell *et al.*, 1995), and play roles in conferring resistance against pathogens and early potato tuber development (Feussner and Wasternack, 2002). The organic acid and sugar content of the mutant 'Hong Anliu' were altered by the mutation. Aspartic proteinases (EC 3.4.23, FE659242) were most active at acidic pH (Milisavljevic *et al.*, 2007). Myo-inositol 1-phosphate synthase (EC 5.5.1.4, FE659085) catalyses the formation of myo-inositol 1-phosphate from glucose-6-P. A synergistic effect of sugar and abscisic acid was found on myo-inositol-1-phosphate synthase expression (Yoshida *et al.*, 2002).

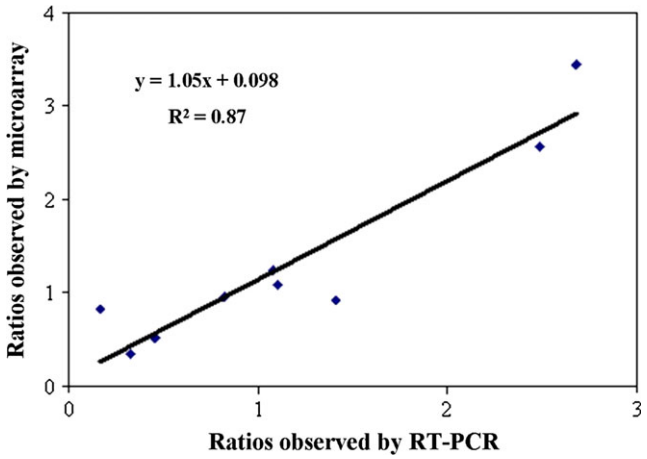

Fig. 6. Comparison of gene expression ratios observed by the microarray and by quantitative real-time RT-PCR. Data were from 10 probe sets at five time points between mutant 'Hong Anliu' and its wild type 'Anliu'. The microarray $\log_2$ (expression ratio) values (*y*-axis) are plotted against the $\log_2$ (expression ratio) obtained by quantitative real-time RT-PCR (*x*-axis).

Transport

Genes encoding transport-related enzymes are also represented in the library. ATP binding cassette (ABC) transporters are membrane proteins known for their function of translocating a broad range of substances across biological membranes, including lipids, sterols, and drugs (Terol *et al.*, 2007). The library contains two up-regulated clones (FE659222 and FE659240) which show high similarity to ABC transporters (BAD93879, 85% similarity and 72% similarity, respectively). A differentially expressed lipid transfer protein (FE659068) which was found to catalyse transfer of carotenoids between lipohphorins of *Bombyx mori* (Tsuchida *et al.*, 1998) was found in the library. This supports the hypothesis that carotenoid transfer leads to lycopene accumulation in the albedo and segment membrane of mutant fruits. A glucose-6-phosphate translocator (FE659246) was represented in the library, and the acute inhibition of this gene leads to increased *de novo* lipogenesis in rats (Bandsma *et al.*, 2001). The sugar content of the mutant fruits was altered by the mutation, and a gene-encoding sugar transporter (FE659183) was identified in this study, the function of which was found to mediate carbon distribution within cells and between organs (Chiou and Bush, 1996).

Pyruvate metabolic pathway

It is worthy to note that five differentially expressed genes belonging to the pyruvate pathway, the metabolic pathway most altered by the bud mutation, were identified and their expressions were verified by real-time PCR (Fig. 4). The pyruvate pathway is closely related to all three major altered traits of the mutant 'Hong Anliu': carotenoid accumulation, low citric acid, and high sugar content. The key precursor of carotenoid biosynthetic pathway,

isopentenyl diphosphate, is provided by the pyruvate pathway (Lichtenthaler *et al.*, 1997). Pyruvate, an important intermediate compound of pyruvate pathway, is the output of the anaerobic metabolism of glucose known as glycolysis. Moreover, pyruvate could be converted into acetyl-coenzyme A, which is a substrate for citric acid metabolism (Ke *et al.*, 2000). Since no gene sequence difference of all the related pathways was observed between the mutant 'Hong Anliu' and its wild type, it is likely that the pyruvate pathway might play an important role in the alteration of the traits of the mutant 'Hong Anliu' fruit.

Transcription regulation

Nine genes encoding transcription factors were identified by microarray analysis. Among the group of transcription factors, four genes belonging to the zinc-finger protein family of transcription factors (FE659294, FE659300, FE659303, and FE659177) were identified in our library. Some zinc-finger proteins are known to play a regulatory role by interacting with the *cis* elements of specific target genes. A type of Cys_2/His_2-type zinc finger (CX_4–$CX_{22-23}HX_1H$) unique to plants was found within the conserved regions of the WRKY family of proteins (Takatsuji, 1998). Zinc-finger proteins, especially those members mediating stress responses, are uniquely expanded in plants (Ross *et al.*, 2007). WRKY proteins (FE659326) were involved in the regulation of abscisic acid signalling in aleurone cells (Xie *et al.*, 2005). Thus, it is possible that the abscisic acid metabolism of the mutant ('Hong Anliu' sweet orange) has been altered by the bud mutation.

Two genes belong to the NAC (petunia NAM, *Arabidopsis* ATAF1,2 and CUC2 genes) proteins (FE659187 and FE659307) which constitute one of the largest families of plant-specific transcription factors (Olsen *et al.*, 2005). Genes from this family participate in various biological processes including development, defence, and biotic and abiotic stress (Hegedus *et al.*, 2003; Olsen *et al.*, 2005). An NAC gene, *OsNAC2*, was greatly induced in mutant plants of rice and overexpression of *OsNAC2* contributes to tiller bud outgrowth (Mao *et al.*, 2007). However, little is known about the functions of NAC genes in citrus. Recently, an NAC-line gene in 'Navel' orange fruit response to post-harvest stresses was cloned and characterized (Fan *et al.*, 2007).

A putative transcription factor, ASR (FE659120), was found in our library. The expression of the ASR gene could be induced by abscisic acid, stress, and ripening (Iusem *et al.*, 1993; Amitai-Zeigerson *et al.*, 1994). A grape ASR gene was found to be involved in sugar and abscisic acid signalling (Cakir *et al.*, 2003). Moreover, an ASR protein was identified in a low acid pummelo mutant fruit (Canel *et al.*, 1995). It is notable that the ASR gene (similar to the *Lycopersicon esculentum* gene *asr4*; K Yu, unpublished data) in our library showed a different expression in the mutant 'Hong Anliu' compared with the wild type (Fig. 7). And the sugar and acid content were altered by the mutation. Thus it is hypothesized that the ASR might be involved in the alteration of the traits of the mutant 'Hong Anliu' fruit.

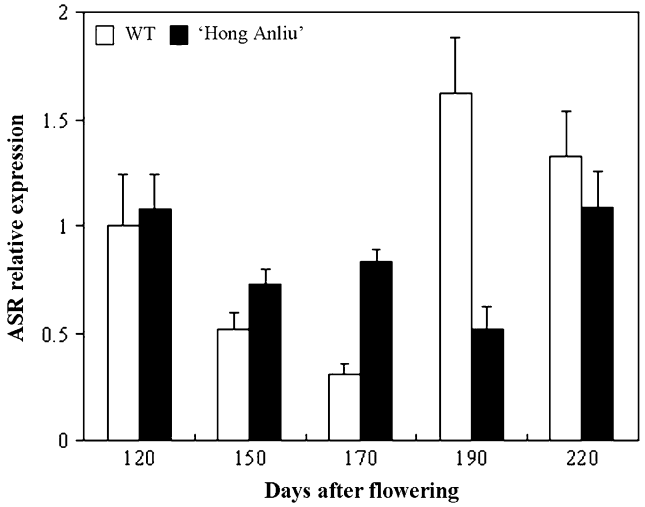

Fig. 7. Real-time PCR determination of ASR (EST accession no. FE659120) expression during development of wild-type (WT, open columns) and mutant ('Hong Anliu' filled columns) fruits. Columns and bars represent the means and standard error (n=3), respectively.

Other putative transcription factors related to this bud mutation, such as Agamous-like protein (FE659195), C-repeat binding factor (FE659308), and homeobox 2 protein (FE659124) were also identified in this study for the first time.

In conclusion, our SSH library contains a set of structural enzymes that are probably good candidates linked to the phenotype of the mutant fruit. Moreover, a number of interesting regulatory candidate genes was identified. However, much more work is needed to elucidate these genes' functions, and further experiments aiming at understanding which gene(s) have played the key role for the mutant can be designed on the basis of this study.

Supplementary data

Table S1. List of unique ESTs from the subtractive library and their relative expression patterns in the mutant 'Hong Anliu' to wild type comparison at five time points.

Table S2. Metabolic pathways involved in the bud mutation 'Hong Anliu' sweet orange according to the KEGG pathway database.

Table S3. Primers used for amplifying candidate and control genes for real-time PCR.

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