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Comparative transcript profiling of gene expression between seedless Ponkan mandarin and its seedy wild type during floral organ development by suppression subtractive hybridization and cDNA microarray

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Abstract

Background: Seedlessness is an important agronomic trait for citrus, and male sterility (MS) is one main cause of seedless citrus fruit. However, the molecular mechanism of citrus seedlessness remained not well explored.

Results: An integrative strategy combining suppression subtractive hybridization (SSH) library with cDNA microarray was employed to study the underlying mechanism of seedlessness of a Ponkan mandarin seedless mutant (*Citrus reticulata* Blanco). Screening with custom microarray, a total of 279 differentially expressed clones were identified, and 133 unigenes (43 contigs and 90 singletons) were obtained after sequencing. Gene Ontology (GO) distribution based on biological process suggested that the majority of differential genes are involved in metabolic process and respond to stimulus and regulation of biology process; based on molecular function they function as DNA/RNA binding or have catalytic activity and oxidoreductase activity. A gene encoding male sterility-like protein was highly up-regulated in the seedless mutant compared with the wild type, while several transcription factors (TFs) such as AP2/EREBP, MYB, WRKY, NAC and C2C2-GATA zinc-finger domain TFs were down-regulated.

Conclusion: Our research highlighted some candidate pathways that participated in the citrus male gametophyte development and could be beneficial for seedless citrus breeding in the future.

Keywords: Citrus, cDNA microarray, Differential transcript, Male sterility-like protein, Seedlessness

Background

Seedlessness is a desired fruit trait for consumers, and a fruit is considered to be seedless if it produces no seeds, traces of abortion seeds, or significant reduced-number of seeds [1]. Some plants can set seeds asexually through apomixis. However, in most flowering plants, seed initiation requires signals activated by the double fertilization event that occurs in the embryo sac, and seed is produced sexually from the fertilized ovule [2,3]. Various phytohormones such as gibberellins (GAs), auxins and cytokinins are involved in this signaling process [4-6]. GAs and

jasmonic acid/jasmonate derivatives (JAs) were found to play crucial roles in plant reproductive development [7,8].

Citrus is one of the most important fruit crops with great economic and health value around the world [9]. However, some citrus varieties are seedy, and seedy fruits have constrained the development of fresh citrus market. Therefore, breeding seedless citrus varieties is a long-term pursuit for citrus breeders worldwide [10,11]. Nowadays, Satsuma mandarin and navel orange are two of the most famous and widely grown citrus varieties, mainly due to their seedless trait. For decades, great progress on seedless citrus breeding was made by traditional approaches such as sexual hybridization, seedling and bud sport mutation. However, due to the peculiarities of citrus reproductive biology such as long juvenile period and nucellar polyembryony,

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traditional breeding is inefficient and costly [12]. Modern biotechnological approaches (e.g. somatic hybridization) have potential to effectively expedite breeding process of citrus [13-15]. As most citrus varieties can produce fruits parthenocarpically [16], male or female sterility, embryo sac abortion, self-incompatibility, polyploidy and even environmental stress can result in seedless citrus fruits [17,18]. Actually there were some successful reports about seedless fruit production by genetic transformation. Ectopic expression of *iaaH* gene with *DefH9* as promoter to elevate auxin levels in placenta or ovules resulted in seedless fruits [19,20]. Another effective strategy was by specific expression of toxin proteins during early development of plant reproductive organs. Typical cases were the ectopic transformation of the *Barnase* gene from *Bacillus amyloliquefaciens* [21,22]. Potential cases were by specific expression of enzymes such as chloroplast Chaperonin 21 and ubiquitin extension protein S27a to induce cell disruption of seed tissues for parthenocarpic plants [11,23,24]. And in our laboratory, the *Arabidopsis thaliana* *MAC12.2* gene had been introduced into precocious trifoliolate orange (*Poncirus trifoliata* [L.] Raf) for production of potential seedless fruits [25].

Male sterility (MS) is one of the main causes for seedless fruit production in citrus. In recent years, great progress on MS was made with annual plants especially rice [26,27], *Arabidopsis* [28] and oil-rape [29], and a serial of genes regulated tapetum, anther and pollen development were identified. However, there remained very limited information on MS of perennial woody plants such as citrus. Ponkan mandarin (*Citrus reticulata* Blanco) is a widely grown citrus variety in China. Within this variety, many variants were derived through sexual hybridization and mutation such as bud sport mutation. 'Qianyang seedless' Ponkan mandarin (QS) is an elite seedless variant selected from bud sport mutation of a common seedy Ponkan mandarin, and it can set fruits with no seeds (even no seed rudiments) in open orchard [30,31]. In this article, QS and a common seedy Ponkan mandarin 'Egan NO.1' (EG) were used for comparative study. These two mandarins shared highly close genetic relationship based on molecular marker analysis and showed no distinctly morphological differences except that QS was completely male sterile while Egan No 1 has normal flower. In order to gain general understanding on genes involved in this MS mutation, suppression subtractive hybridization (SSH) [32] combining with cDNA microarray was performed to detect differentially expressed genes. Several candidate genes and related pathways were focused in particular. Our research identified some useful genes which could be beneficial to citrus seedless breeding. The results could help to reveal the molecular mechanism of male sterility of Ponkan mandarin and shed light on

seedless trait formation of other perennial woody plant at the gene expression level.

Results

Phenotype analysis of the floral organs of QS

Previous studies suggested that the floral organs (actually the whole plant) of QS had no morphological difference from the wild type. To further validate the phenotype of this seedless Ponkan mandarin, we measured the length of filament and pistil, and the average ratio of filament to pistil (filament length/pistil length) was 0.83 ± 0.01 for EG and 0.79 ± 0.01 for QS. And for EG, the pistil was 0.155 ± 0.01 cm longer than filament while for QS, the pistil was 0.166 ± 0.009 cm longer than filament. Above data further confirmed that the floral organs of both EG and QS had no morphological difference, and the seedless trait was not caused by malformation of reproductive organs. However, the number of pollen grains per anther of QS was 9.5% less than that of EG. The pollen dying viability of QS was $6.0\% \pm 1.0\%$ (or $6.5\% \pm 1.0\%$ for I_2 -KI₂ staining) in striking contrast to the high viability of $93.8\% \pm 0.9\%$ (or $89.6\% \pm 2.5\%$ for I_2 -KI₂ staining) for EG. Pollen germination test found that no pollen of QS could germinate. Furthermore, SEM assays showed abnormal structures of the pollen grains of QS (Figure 1), confirming that QS is male sterile.

Construction of SSH-cDNA libraries and overall feature of the differential transcript profiling

To identify genes associated with the MS of QS, SSH cDNA libraries (both forward and reverse) were constructed from floral organs of QS and EG. A total of 6,048 cDNA clones derived from the SSH-cDNA libraries including 4,195 from the forward library and 1,853 from the reverse one were successfully amplified, and then used for a custom cDNA microarray. Each cDNA clone has triplicate spots on the array. The RNA samples of the four developmental stages (SE, ME, BF and OV) were used for array-hybridization. The fluorescent dye-labelled cDNA and hybridization strategy was employed for the microarray assay.

From the 6,048 clones printed on the glass slide, 279 cDNA clones (278 non-redundant) were differentially expressed (false discovery rate (FDR) < 0.05 and a fold change ≥ 2) between QS and EG. Among these cDNA clones, 218 (78%) were down-regulated while only 61 (22%) showed up-regulated expression across the four developmental stages; and the differentially expressed clones peaked at full bloom stage (BF) (Figure 2). At this stage, many more clones showed down-regulated than up-regulated expression. During the four developmental stages, one clone (GenBank accession no. JU497336) encoding a putative cysteine protease (tr[B9RRA4]) showed

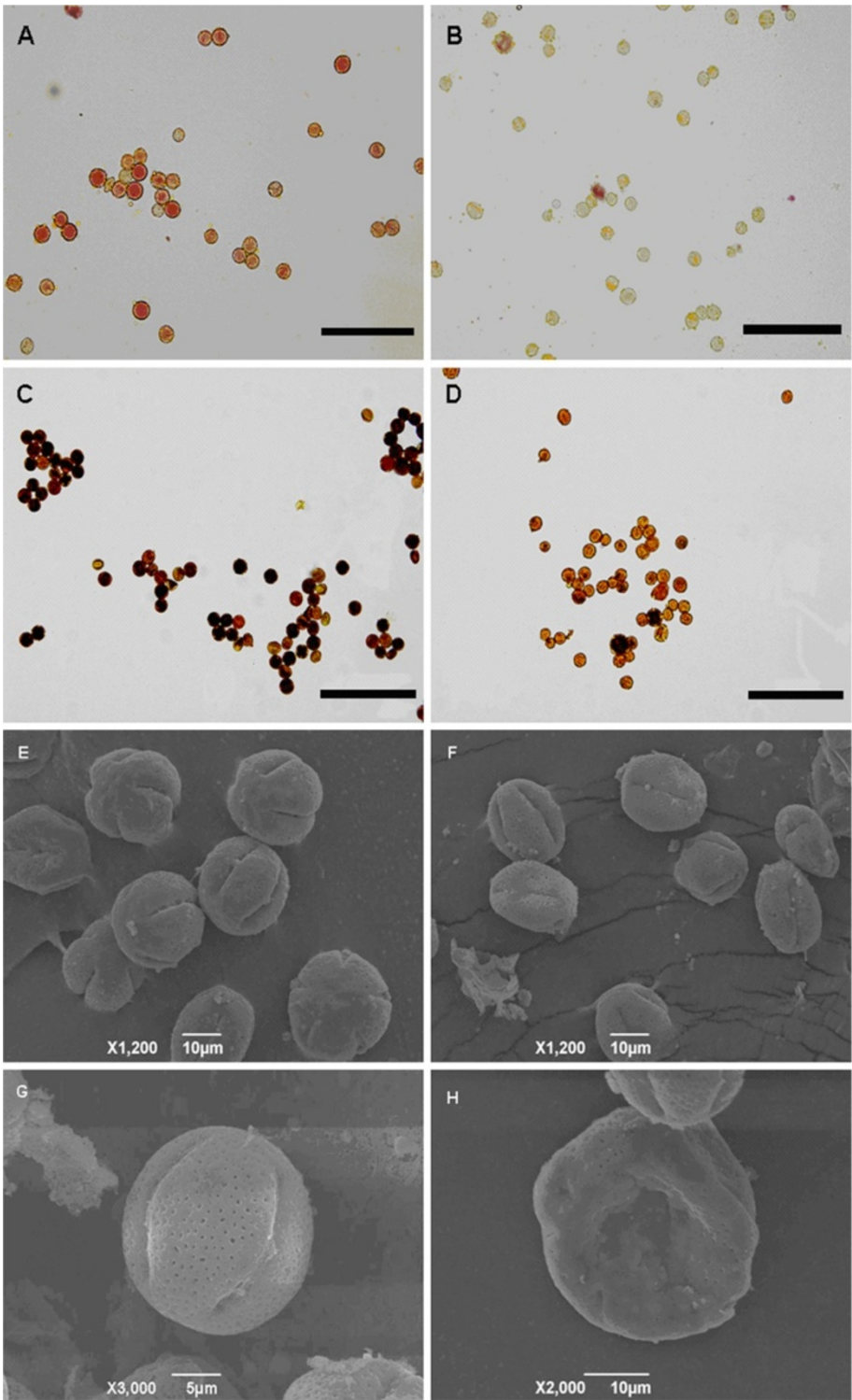


Figure 1 Viability assay and SEM analysis of pollen grains. A, C, E, G showed the pollen grains of EG; B, D, F, H showed the pollen grains of QS. Bar (A, B, C, D) = 100 μm.

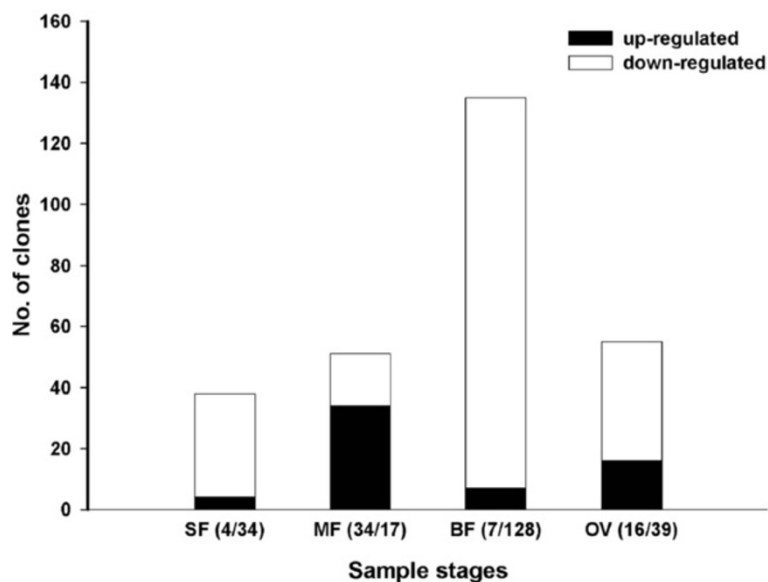


Figure 2 Number of clones significantly up- and down-regulated in QS during four developmental stages. Each two numbers in parentheses of x-axis indicated the numbers of clone up- and down-regulated respectively.

down-regulated expression at BF stage but up-regulated at OV stage (young ovaries of 2–3 days after flowering).

Sequencing of the differentially expressed clones and EST analysis

Among the 279 differentially expressed clones, 255 non-redundant clones were subjected to one single-pass sequencing. In all, 237 high-quality ESTs (average length was 496 bp) were yielded after eliminating vectors and unreliable sequences. These ESTs were assembled using CAP3 program, and 133 unigenes (43 contigs and 90 singletons) were obtained with sequence redundancy of 43.9%. The majority of the contigs (38) contained 2–5 ESTs, whereas only 5 contigs contained 6–11 ESTs, indicating an ideal normalization and subtraction. Of the 133 unigenes, 80 (60.1%) showed differential expression at BF stage. Subsequently, BLASTX search of the UniProt database showed that 20 unigenes (15.0%) did not have significant hits ($E\text{-value} \leq 1.0 \times 10^{-5}$). However, when the 20 unigenes were used in BLASTN ($E\text{-value} \leq 1.0 \times 10^{-10}$) search of the *Citrus clementina* transcript database [33,34] with local Blast software (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/release/LATEST/>), 17 genes had significant hits and high scoring pairs (HSP) showed high nucleotide identity. It suggested that these 20 unigenes were unique for citrus, and three of them were novel citrus genes.

Based on the microarray analysis, the relative expression profiles of all 255 ESTs were performed hierarchical clustering with cluster software (version 3.0). Four typical relative expression patterns were observed in QS versus EG at four developmental stages. Figure 3A and 3B showed a group of clones down-regulated mainly at

squaring stage (SF) and full bloom stage (BF), respectively, while the other two groups of clones were down/up-regulated constitutively during the developmental stages (Figure 3C and 3D). In addition, candidate genes with putative function that could be important for the MS of QS were specifically collected (Table 1). It is noteworthy that 27.7% of the unigenes (not listed in the table) were only annotated as putative proteins or with no defined biological process besides 15% unigenes with no hits in the database.

GO annotations were conducted and three categories representing molecular functions, biological processes, and cellular components were assigned. Figure 4 showed the percentage distributions of GO terms (2nd level GO terms) based on biological process. It indicated that during the floral organ development, the majority of differentially expressed genes were involved in metabolic process (46%) or responded to stimulus (27%) and regulation of biological process (18%). In addition, the other two GO categories (molecular functions and cellular components) were also generated (data not shown). In the molecular function category, large proportion of unigenes may have binding activity (59%), catalytic activity (19%), or oxidoreductase activity (11%), while the cellular components consisted mainly of intracellular (57%) and membrane (23%).

Metabolic pathways involved in formation of seedless fruit

As large proportion of altered expressed genes were involved in varieties of metabolic processes. Based on the KEGG (Kyoto Encyclopedia of Genes and Genomes)

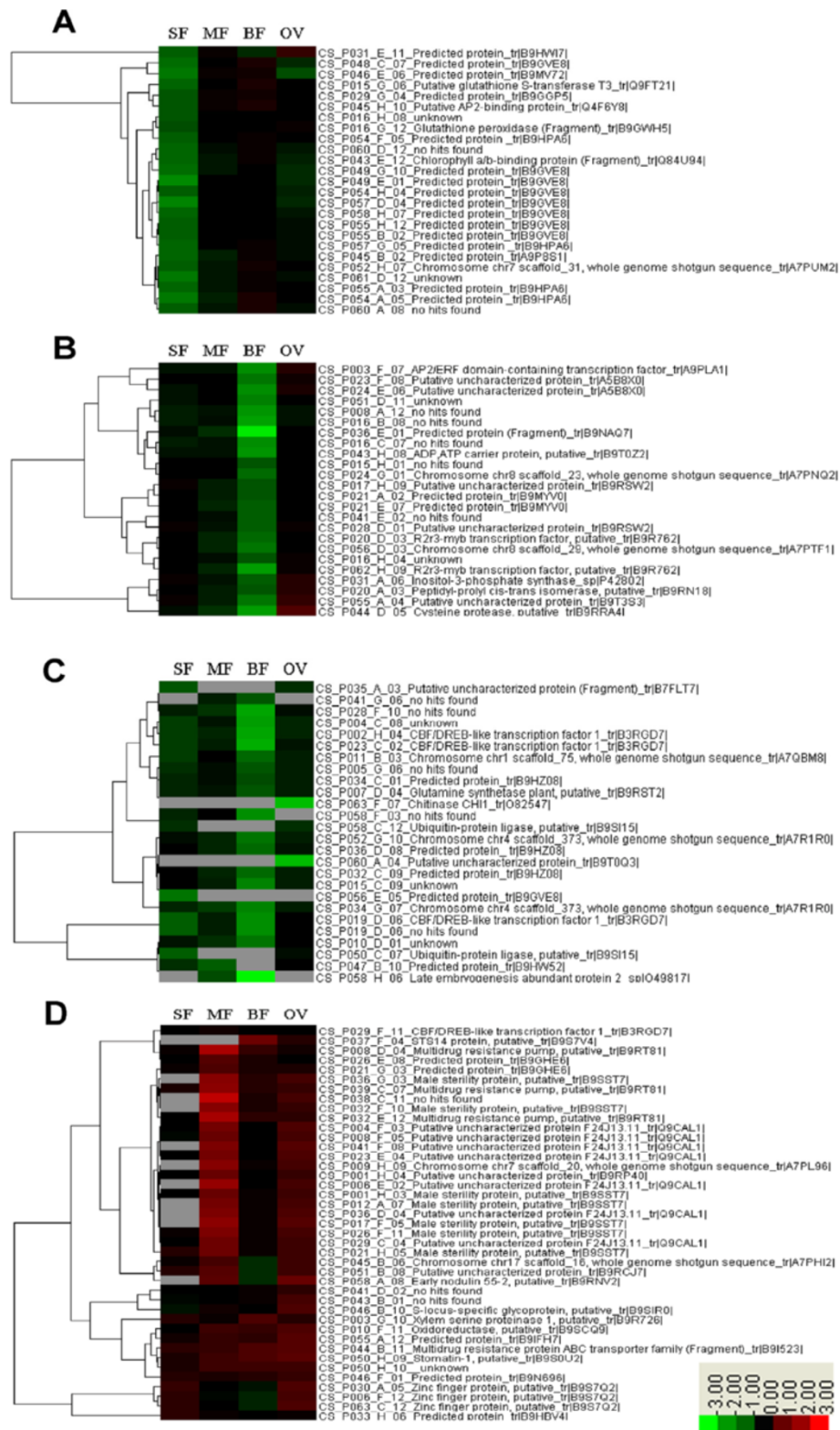


Figure 3 (See legend on next page.)

(See figure on previous page.)

Figure 3 Cluster analysis of expression profiles of altered expressed genes in the QS versus EG. **A** showed a cluster of ESTs that were down-regulated mainly at squaring stage (SF) when the tetrads were produced and the microsporocyte underwent meiosis. **B** showed ESTs that were down-regulated especially at full bloom stage (BF). **C** and **D** suggested a cluster of ESTs that were down-regulated and up-regulated constitutively during the four developmental stages respectively. The ratio value was log₂ transformed for each gene and used for the hierarchical clustering analysis.

analysis, 36 different metabolic pathways were altered during the four developmental stages. Among these pathways, nine (25%) were related to amino acid metabolic pathway (Table 2), and genes involved in carbohydrate and energy metabolism showed down-regulated expression during subsequent developmental stages of floral organs. Besides, genes related to specific secondary metabolism such as terpenoids and polyketides metabolism were also found to be altered. Interestingly, a gene (JU497309) encoding fatty acyl-CoA reductase, which may be involved in lipid metabolic process, was identified (Table 1). This gene was found highly homologous with putative male sterile protein (GI: 255576327) in castor bean, fatty acyl-CoA reductase 3 (GI: 359500474) in poplar and male sterile 2-like protein (*MS2*) (GI: 3549681) in *Arabidopsis*. Herein, this gene was named as male sterile-like protein. And qRT-PCR analysis showed its expression level increased from SF to BF stages and then declined at OV stage. The expression pattern was similar in both QS and EG; however, it showed obviously higher expression level in QS than in EG during the developmental process (Figure 5).

Differential expression of transcription factor genes

It is noteworthy that among the 133 unigenes, 12 were assigned to the category of transcription factor (TF) based on plant TF database (<http://planttfdb.cbi.edu.cn/>). Figure 6 showed the specific expression pattern of six AP2-ERF family TFs, two zinc-finger TFs, one MYB TF and one NAC TF using qRT-PCR assay. These TFs (except of NAC TF) had similar expression profile during the four developmental stages between EG and QS. For instance, among six AP2-ERF TFs, four (AP2-EREBP TF1, AP2-EREBP TF3, AP2/ERF domain containing TF2 and CBF/DREB-like TF) showed co-expression pattern like "V" type. It showed that the gene expression level in QS was higher than that in EG from SF stage to MF stage; however, these genes were subsequently repressed more obviously in QS from MF stage to BF stage, and the gene expression level was down-regulated mostly at BF stage. Two zinc-finger TFs (GATA TF8 and *Cys2-His2* type) and one R2R3-MYB TF likewise showed similar "V" type-variation tendency. The other two AP2-ERF TFs (AP2-EREBP TF2 and AP2/ERF domain containing TF1) showed "V"-like type expression pattern in QS. However, the expression pattern of AP2/ERF domain containing

TF1 was somehow different from others, as it showed relatively stabilized expression level during the four stages in EG. As for NAC TF, its expression level was down-regulated obviously at BF and OV stages in QS compare with EG. It was notable that no expression was observed at OV stage in QS. The results suggested that these TFs could play important roles in the seedless phenotype formation, and the relative expression level in QS versus EG seemed to be key factor in this process.

Verification of microarray data

Two approaches were used to examine the quality of the microarray data. First, as one contig was assembled by several ESTs that were arrayed at random location in the microarray, so these ESTs sharing similar sequence or encoding the same gene would share similar expression pattern. Additional file 1: Figure S1 showed that four ESTs (F2-13 G, F6-15I, F7-18O, F8-12A) were assembled into one unigene (JU497321) which encoded methionine synthase, and these four ESTs truly shared similar expression pattern. For the other approach, qRT-PCR was performed on 11 unigenes using gene-specific primer pairs. Expression patterns were compared at the four developmental stages between QS and EG. Additional file 2: Figure S2 showed the correlation analysis of the ratio values of differential expression level from microarray to that from qRT-PCR. Linear regression [(average microarray ratio value) = a (qRT-PCR value) + b] analysis showed a good coefficient of variation ($R^2 = 0.847$). These results confirmed the reliability of the microarray data.

Discussion

Here, we combined SSH and microarray techniques to investigate potential mechanism underlying seedlessness in Ponkan mandarin. SSH was proved to be an efficient and popular approach to enrich and identify differentially expressed genes between wild-type and its mutant or treatment [35,36]. However, because of high sensitivity of SSH, usually a large number of clones could be obtained but inevitably included some false-positive ones. Screening the SSH libraries to identify some candidate genes using microarray and to validate using qRT-PCR has proved to be a high-throughput and efficient way [37-39]. However, relatively few clones were isolated in this study. Of the 6,000 clones, only 279 cDNA clones

Table 1 List of selected candidate functional genes related to the formation of the phenotype of QS

GenBank accession no.	EMI ^a	Description ^b	e-Value ^c	Clones ^d
Up-regulated				
JU497309	B9SST7	Male sterility protein	2E-77	7
JU497311	B9S7V4	STS14 protein	6E-23	3
JU497315	Q5CD81	(E)-beta-ocimene synthase	1E-120	2
JU497324	B9RXQ0	Tryptophan synthase beta chain	6E-91	2
JU497327	B9RT81	Multidrug resistance pump	5E-50	3
JU497333	B9S7Q2	Zinc finger protein	1E-67	3
JU497348	A9ZN18	Geranyl-diphosphate synthase	3E-11	5
JU497417	B9R726	Xylem serine proteinase 1	7E-24	1
JU497359	B9SCQ9	Oxidoreductase	2E-36	1
JU497418	A1ECJ7	Putative miraculin-like protein 2	7E-24	1
JU497422	A9XCN2	Putative DNA binding protein	2E-24	1
JU497389	B9I523	Multidrug resistance protein ABC transporter family	1E-121	2
JU497397	B9SIR0	S-locus-specific glycoprotein	2E-45	1
JU497403	B9S0U2	Stomatin-1, putative	1E-07	1
Down-regulated				
JU497308	B9S7Q1	Zinc finger protein	1E-140	2
JU497318	B9S1E9	Transcription factor AtMYC2	4E-20	2
JU497321	B2VQE0	Methionine synthase	1E-107	4
JU497323	P42802	Inositol-3-phosphate synthase	1E-112	4
JU497331	B9SR02	Multicopper oxidase	6E-78	2
JU497332	B3RGD7	CBF/DREB-like transcription factor	6E-44	4
JU497336	B9RRA4	Cysteine protease	2E-25	2
JU497338	Q8VWL8	Beta-mannosidase	6E-64	2
JU497342	O82547	Chitinase CHI1	8E-28	7
JU497343	O49817	Late embryogenesis abundant protein	2E-22	2
JU497344	B9SI15	Ubiquitin-protein ligase	2E-54	2
JU497351	A9PLA1	AP2/ERF domain-containing transcription factor	2E-46	1
JU497352	B9T724	GATA transcription factor	4E-62	1
JU497353	B9RZK6	Protein COBRA	4E-82	1
JU497354	A2IB54	Mitogen-activated protein kinase	4E-85	1
JU497356	B9RST2	Glutamine synthetase plant	3E-71	1
JU497357	Q3KN68	Isoflavone reductase-like protein 5	2E-56	1
JU497361	B9SQM6	Transcription factor	3E-34	1
JU497362	Q9FT21	Putative glutathione S-transferase T3	2E-07	1
JU497364	B9GWH5	Glutathione peroxidase (Fragment)	2E-25	1
JU497367	B9RN18	Peptidyl-prolyl cis-trans isomerase	3E-54	1
JU497368	B9R762	R2R3-myb transcription factor	1E-69	1
JU497369	O82547	Chitinase CHI1	8E-28	1
JU497419	P83948	Pectinesterase-3	1E-46	1
JU497372	B9NBQ9	AP2/ERF domain-containing transcription factor	1E-108	1
JU497373	Q9ZRC9	ACC oxidase	6E-36	1
JU497421	A5YWA9	NAC domain protein	1E-51	1
JU497378	Q7Y066	Plasma membrane H ⁺ -ATPase	1E-114	1

Table 1 List of selected candidate functional genes related to the formation of the phenotype of QS (Continued)

JU497423	B9RIP3	Hevamine-A	1E-22	1
JU497424	B9T0Z2	ADP/ATP carrier protein	1E-06	1
JU497427	Q84U94	Chlorophyll a/b-binding protein (Fragment)	2E-14	1
JU497388	A7XUL4	dehydration-responsive element binding protein	1E-14	1
JU497391	Q4F6Y8	Putative AP2-binding protein	6E-15	1
JU497398	Q6EV47	Non-specific lipid-transfer protein (Fragment)	1E-45	1
JU497401	B9S JL5	Amine oxidase	1E-57	1
JU497405	B9S925	Zinc finger protein	3E-88	1
JU497406	B9T868	Putative peroxidase C3 (Fragment)	8E-26	1
JU497434	Q8H2A1	Caffeoyl CoA O-methyltransferase (Fragment)	3E-11	1
JU497412	B9HGW6	Glutaredoxin	6E-20	1
JU497413	B9R762	R2R3-myb transcription factor	3E-21	1

^a The EMI codes of the most similar genes to the EST sequences.

^b The description of sequences based on Uniprot database.

^c The best e-value from a BLASTx search for corresponding EST sequences.

^d The number of sequenced clones in the libraries.

were identified as differentially expressed. Such results may suggest that there were little variations between QS and EG mandarins in gene expression. It was hypothesized that bud sport mutant was likely caused by single

gene mutation, DNA methylation or retroelement activity [40,41]. In this research, various types of DNA markers including SCAR [42], and SSR (172 pairs of primers), MSAP (96 pairs of primers) and AFLP (13

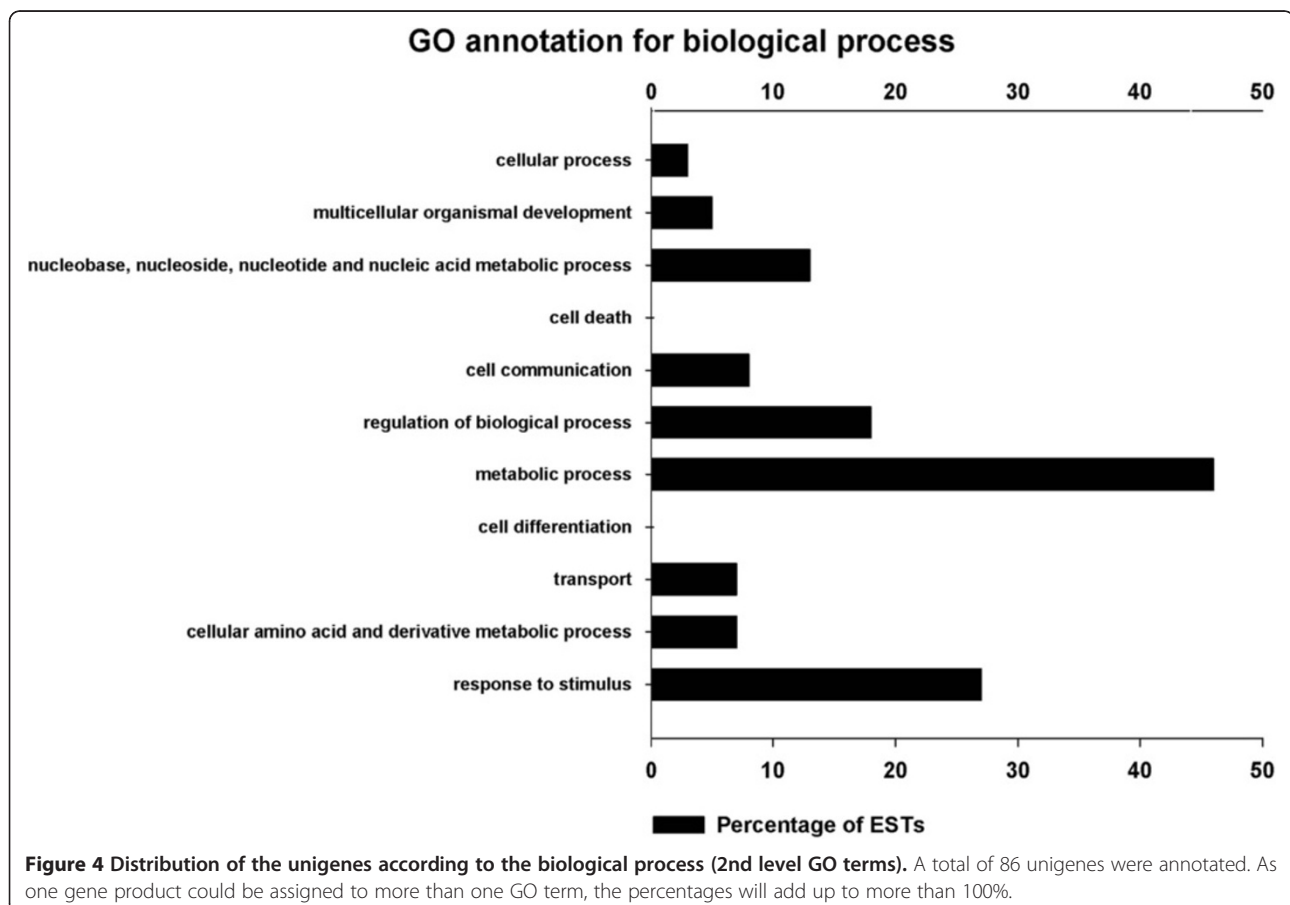


Table 2 List of differentially expressed genes involved in amino acid, carbohydrate, energy, terpenoid and polyketides metabolism based on KEGG pathway database

KEGG pathways	EC number	Putative function	QS/EG			
			SF	MF	BF	OV
Amino acid metabolism						
JU497356	6.3.1.2	Glutamate-ammonialigase	0.71±0.03	0.84±0.05	0.50±0.01	0.79±0.03
JU497374	6.3.5.4	Asparagine synthase	0.50±0.03	1.15±0.08	1.20±0.09	1.02±0.06
JU497373	1.14.17.4	Aminocyclopropane carboxylate oxidase	0.90±0.02	1.52±0.05	0.52±0.03	1.15±0.09
JU497321	2.1.1.14	5-methyltetrahydropteroyltriglutamate -homocysteine-methyltransferase	1.62±0.11	0.97±0.08	0.50±0.02	0.90±0.08
JU497330	4.2.1.78	(S)-norcochlorine synthase	1.04±0.11	1.94±0.28	1.29±0.04	1.17±0.13
JU497338	3.2.1.21	Beta-glucosidase	0.94±0.08	0.47±0.04	0.18±0.01	0.88±0.17
JU497364	1.11.1.1	Phospholipid-hydroperoxide -glutathione peroxidase	0.53±0.04	0.97±0.10	0.97±0.05	1.08±0.01
JU497324	4.2.1.20	Tryptophan synthase	1.86±0.13	2.64±0.16	1.03±0.05	0.70±0.05
JU497377	2.4.1.12	Indole-3-acetate-beta-glucosyl transferase	0.51±0.01	1.51±0.04	1.25±0.02	0.48±0.04
Carbohydrate metabolism						
JU497385	3.2.1.14	Chitinase	1.15±0.15	1.28±0.05	1.00±0.17	0.45±0.02
JU497313	1.13.99.1	Inositol oxygenase	1.04±0.09	1.08±0.04	0.79±0.07	0.49±0.03
JU497323	5.5.1.4	Inositol-3-phosphate synthase	1.11±0.08	1.17±0.20	0.48±0.03	1.42±0.13
JU497357	1.3.1.45	2{prime}-hydroxy isoflavone reductase	1.10±0.05	1.28±0.07	0.44±0.01	1.17±0.04
Energy metabolism						
JU497406	1.11.1.7	Peroxidase	0.99±0.05	1.02±0.01	1.12±0.09	0.54±0.06
JU497378	3.6.3.6	Proton-exporting ATPase	0.99±0.05	0.90±0.02	0.51±0.02	0.94±0.03
Terpenoids and polyketides metabolism						
JU497376	1.1.1.295	Momilactone-Asynthase	1.42±0.54	1.09±0.20	1.05±0.03	1.10±0.10
JU497315	4.2.3.15	Myrcene synthase	1.24±0.04	2.28±0.16	0.52±0.01	1.10±0.11
JU497325	4.2.3.20	(R)-limonene synthase	1.27±0.18	0.91±0.03	0.50±0.02	1.10±0.17

pairs of primers) were employed to analyze the polymorphism between these two mandarins, and no repeatable polymorphic bands were detected (data not shown). These results suggested that very few nuclear genes were altered during the developmental stages.

For the four developmental stages we chose, immense efforts were taken to determine which time-point was pivotal for stamen development, but there has no criteria for citrus gametophyte development. Though criteria for gametophyte development was available in model plant *Arabidopsis* [43], it can not be directly applied herein. Semi-thin and paraffin sections were performed in this study to survey the microsporogenesis of QS, and it was found that abnormal tetrads produced at the tetrad stage and subsequently the microsporocyte underwent abnormal meiosis. This process mainly occurred at SF stage (the diameter of floral organs is about 3 mm) (unpublished data). Additionally, large proportion (about 59.7%) of differentially expressed genes was found in BF when the

anthers and pollen grains were almost mature, indicating that this time-point might be also important.

Amino acid metabolic process

Of the metabolic pathways with altered expressed genes, 25% were involved in amino acid metabolism. Amino acids were not only primary metabolic products for normal growth and development but also cell signaling molecules and regulators of gene expression and protein phosphorylation cascade [44]. Interestingly, among these amino acid metabolism pathways, two genes were down-regulated across the developmental stages in QS versus EG, one (JU497356) encoding glutamate-ammonialigase (EC 6.3.1.2), the other (JU497338) encoding beta-glucosidase (EC 3.2.1.21). In higher plants, glutamate-ammonialigase catalyzes ATP-dependent conversion of glutamate and ammonia into glutamine which occupies a central position of amino acid metabolic pathway [45], and this metabolic process is critical for coordinating

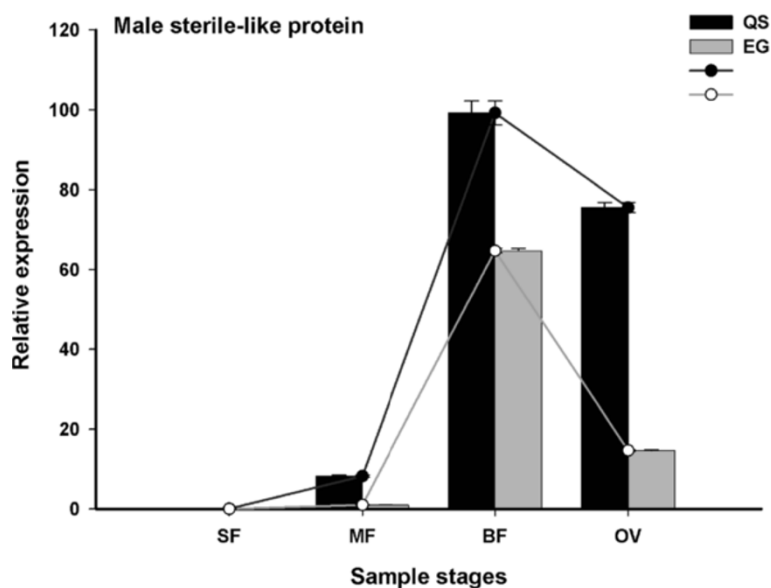


Figure 5 Relative expression (y-axis) of male sterile-like protein in QS versus EG during four developmental stages (x-axis) by qRT-PCR. Columns and bars represent the means and standard errors ($n = 3$) respectively.

metabolic balance in rice [46]. And beta-glucosidase could be used for the cellulosic ethanol industry [47] and has diversity of functions in plants. In maize, *Zm-p60.1* encoding a beta-glucosidase could release active cytokinin, and might function *in vivo* to supply the developing maize embryo [48]. Additionally, some beta-glucosidases affect the properties of cell wall [49] and are associated with freezing tolerance, such as the *SFR2* in *Arabidopsis* [50]. Some beta-glucosidases are related to the efficiency of microspore embryogenesis [51]. It is noteworthy that a gene (JU497374) encoding asparagine synthase (EC 6.3.5.4) was down-regulated exclusively at SF (early stage of stamen development). And asparagine is one central intermediate in nitrogen assimilation and transportation in plant [52,53]. Recent studies showed that this gene played important role in defense against pathogens and salt stress [54,55]. Additionally, genes related to carbohydrate metabolism and energy metabolism also showed down-regulated expression in QS mainly at BF and OV (late stage of stamen development). These results suggested that the vital activities of QS weakened during early development stages of stamen, and the metabolic process of nutrition and energy was also impaired at subsequent stages of stamen development especially when the stamen was mature.

Two genes involved in cysteine/methionine metabolism and participated in the biosynthesis of ethylene were also identified in this study. One (JU497321) encodes 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase (EC 2.1.1.14) is likely involved in the biosynthesis of L-methionine. And the methionine can be transformed into S-adenosylmethionine (SAM) (the

precursor of ethylene) [56]. The other one (JU497373) encodes aminocyclopropane carboxylate oxidase (EC 1.14.17.4) and is a pivotal enzyme during the biosynthesis of ethylene. In addition, genes involved in the synthesis of IAA (indole-3-acetic acid) were also identified such as a gene (JU497377) encoding Indole-3-acetate-beta-glucosyltransferase (EC 2.4.1.121). These results implied that the endogenous phytohormones might be involved in the male gametophyte development of citrus.

Transcription factors

It was known that floral organ formation and function were influenced by TFs regulation. In our research, twelve unigenes were assigned to the category of transcription factor, and six of them were identified as AP2-ERF family members. AP2-ERF TF containing highly conserved AP2/ERF DNA-binding domain, is a large family unique in plant. In our research, four AP2-ERF members showed similar expression pattern. AP2-EREBP TF1 was closely homologous with atERF107 (AT1G19210). This gene was likely involved in the regulation of gene expression by stress factors and by components of stress signal transduction pathways. However, until now, no experimental evidence was available. AP2-EREBP TF3 showed high similarity with ERF5 (AT5G47230.1). ERF5 might play an important role in plant innate immunity likely through coordinating chitin and other defense pathways [57]. Other research suggested that ERF5 and ERF6 might potentially overlap in their function and acted as positive regulators of JA/ethylene-mediated defense [58]. In tomato, this gene was mainly involved in responses to drought and salt stresses [59]. As for AP2/ERF

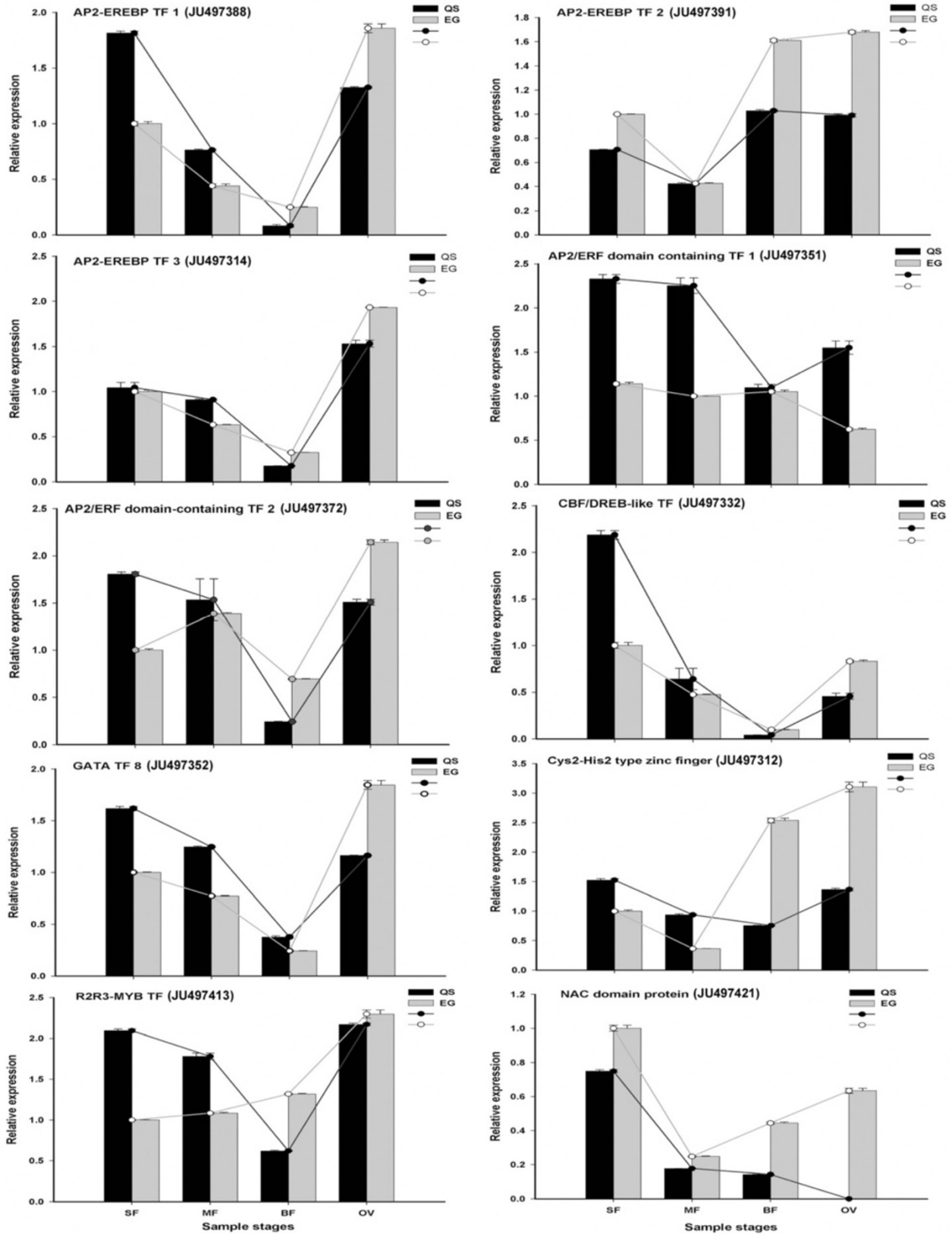


Figure 6 (See legend on next page.)

(See figure on previous page.)

Figure 6 Relative expression pattern of six AP2-ERF family TFs, two zinc-finger TFs, one MYB family TF and one NAC domain TF. The accession number of each TF was given inside the parenthesis. Relative expression was defined as the expression level in QS versus EG. Columns and bars represent the means and standard errors ($n = 3$) respectively.

domain containing TF2, its closest relative was ERF104 (AT5G61600.1). Recent studies showed that ERF104 was *in vivo* substrate of MPK6, and ethylene could release ERF104 and allow liberated ERF104 to access target genes related to plant defense [60]. CBF/DREB-like TF was of high similarity with CBF4 (AT5G51990.1) which was critical regulator involved in cold acclimation and drought adaptation [61,62].

In addition, AP2-EREBP TF2 was highly homologous with RAP2.4 (AT1G78080.1). RAP2.4 acted at or downstream of a converging point of light and ethylene signaling pathways, and it coordinately regulated multiple developmental processes and stress responses [63]. As for AP2-ERF domain containing TF1, its expression pattern was different from other five members. It showed high similarity with DREB26 (AT1G21910.1). In plant, RAP2.6, RAP2.6 L, DREB26 and DREB19 exhibited tissue specific expression and participated developmental processes as well as biotic and/or abiotic stress signaling [64]. Though previous researches emphasized the functions of these AP2-ERF TFs on resistance against biotic and abiotic stresses, AP2-ERF TFs were also participated in plant development such as embryo patterning [65], and stamen emergence [66].

Additionally, two MYB (R2R3-MYB) transcription factors also showed differential expression between QS and EG. In plant, MYB TF family was categorized into 3 sub-families according to the number of adjacent repeats of MYB-domain. Of them, R2R3-MYB subfamily contains the largest number of members. Like the AP2-ERF TF family proteins, MYB family proteins also function in various plant-specific processes. In *Arabidopsis*, MYB TFs were found as key regulators involved in development, metabolism and biotic and abiotic stress responses. Among these MYB TFs of *Arabidopsis*, AtMYB26 is involved in determining endothelial cell development within the anther and is essential for anther dehiscence [67]. AtMYB33 and AtMYB65 redundantly facilitate anther and pollen development [68]. AtMYB80 regulates exine formation and acts downstream of AtMYB35; and AtMYB103 is required for tapetal development and microsporogenesis, especially for callose dissolution and exine formation [69,70]. AtMYB125 positively control male germ cell division and commit progenitor germ cells to sperm cell differentiation [71,72]. In rice, CSA gene encoding MYB TF functions as a key transcriptional regulator for sugar partitioning during male reproductive

development, and the CSA mutant showed reduced levels of sugars and starch in floral organs which lead to MS.

Interestingly, in our results, one MYB TF showed similar expression pattern with AP2-ERF TFs that down-regulated at BF stage when the anther and pollen grains are mature. This MYB TF termed as R2R3-MYB TF was closely related to ATMYBR1/ATMYB44 (AT5G67300.1), and AtMYB44 was likely to enhance drought and salt stress tolerance by suppressing the expression of genes encoding PP2Cs, which was described as negative regulators of ABA signaling [73]. Previous report showed that AtMYB44 was with changed expression during late embryogenesis and seed maturation [74]. And notably there was a NAC domain protein (JU497421) highly homologous with ANAC102 (AT5G63790.1). ANAC102 was an important regulator of seed germination and activated a seed-specific subset of genes under low-oxygen stress; it was also necessary for the viability of *Arabidopsis* seeds following low-oxygen treatment [75].

In summary, these results suggested that these AP2-ERF TFs and the MYB TF functioned redundantly and coordinated with other TFs which involved in the complex network regulating floral organ development. Further research should emphasize on the isolation of proteins interacted with these TFs.

Conclusion

An integrative approach combining SSH and microarray was employed to explore the transcriptional changes of a seedless bud sport mutant of Ponkan mandarin. A number of differentially expressed genes were identified. And the majority of genes were down-regulated in the mutant, especially those related to basic metabolic process. Metabolism of nutrition and energy might be impaired during male gametophyte development of the mutant, and TFs and phytohormones might play important regulatory roles during this process. Our research gained general information of citrus MS at transcription level and could provide some clues for further exploration of MS in citrus species.

Methods

Accession numbers of sequences and microarray data

All the sequences generated in the study were deposited in GenBank with accession numbers from JU497308 to JU497435. Five sequences which are shorter than 200 bp longer than 100 bp are attached in Additional file 3.

Microarray data and experimental information from this study were deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE38094.

Plant materials and phenotype analyses

Two Ponkan mandarin (*Citrus reticulata* Blanco) cultivars, Qianyang seedless (QS, mutant type) and Egan NO.1 (EG, a common seedy Ponkan mandarin, wild type) were grown in the same orchard of 'Fenghuangshan' citrus production area in the city of Dangyang, Hubei province, China. These two scion cultivars were seven years old when sampling in 2010, with trifoliate orange (*Poncirus trifoliata* L. Raf.) as the rootstock. Flower samples were collected from both cultivars in parallel including 4 continuous phenologically developmental stages (Figure 7C): squaring stage (SF, about 20 DBF), medium bud stage (MF, about 10 DBF), flowers at full bloom stage (BF) and young ovaries of 2–3 days after flowering (OV). All the flowers were bagged to prevent cross-pollination, and when sampled in the field, all the samples were frozen in liquid nitrogen as quickly as possible and then stored at -80°C until needed.

The morphology of mature anthers were investigated with fluorescence stereo-microscope (Figure 7A; 7B) (Leica MZ FLIII, German) and image was captured with a digital camera (Nikon Coolpix, Japan). The pollen grain number per anther was counted. In brief, anthers from mature flowers were collected and mixed randomly, each time 40 anthers were dissected and pollen

grains were suspended in 25 mL sterile water with 4–5 drops of surfactant (Tween-20, Amresco solon, OH). The viability of mature pollen grains were evaluated by dying with 1% acetic acid magenta as well as 1% iodine potassium iodide ($\text{I}_2\text{-KI}_2$) solution. After staining for 5 min, pollen grains were observed using BX-61 fluorescence microscope (Olympus, Japan) and Images were captured with DP70 CCD digital camera system. At least 1,000 pollen grains were counted. These experiments were repeated three times. The morphology of pollen grains was examined by scanning electron microscope (SEM) (NTC JSM-6390LV, Japan). For SEM, anthers at various developmental stages were pre-fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h, dehydrated twice using a gradient ethanol serial (30%-50%-70%-85%-95%-100%), then replaced ethanol with isopentyl acetate for 20 min. After that, samples were dried with critical-point drying method then sputtered coating with gold. Representative images were captured.

RNA extraction and mRNA isolation

The materials (floral organs) for RNA extraction were sampled from at least six independent plants, and mixed randomly. Total RNA from flower samples at four stages (SF, MF, BF and OV) were extracted with modified Trizol method according to [76]. The RNA pellets were washed with 75% (V/V) ethanol twice, dissolved in RNase-free water and stored at -80°C until use. By mixing equal amount of RNA of the four stages, RNA pools from both

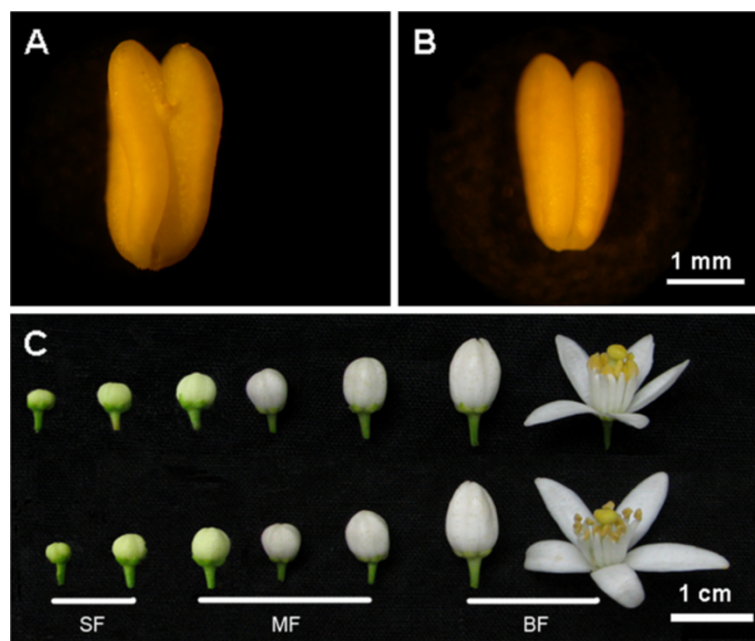


Figure 7 Flower organs at different developmental stages and mature anthers. A, B showed the anthers of EG and QS respectively; C showed the flower organs including SF, MF, BF stages, the upper row was EG.

QS and EG were established in parallel. Then mRNA was isolated from each of the RNA pools using the Oligotex mRNA mini kit (Qiagen, Germany). The quality of RNA was determined by Nanodrop 1000 spectrophotometer (*Thermo Scientific*, Wilmington, DE, USA) and 1.2% agarose gel electrophoresis.

Suppression subtractive hybridization (SSH) cDNA libraries construction and cDNA inserts amplification

Two micrograms of mRNA was used to synthesize cDNA for suppression subtractive hybridization (SSH). The SSH was performed with the PCR-selectTM cDNA subtraction kit (Clontech, Palo Alto, CA, USA) according to the user manual. And both forward (the seedless cultivar QS as tester and the seedy cultivar EG as driver) and reverse (EG as tester while QS as driver) SSH were conducted. For cDNA libraries construction, two hybridizations were performed followed by two rounds of PCR amplifications to enrich the desired differentially expressed sequences. Then the second PCR-amplified cDNAs were purified and ligated into the T/A cloning vector pMD18-T (Takara, Japan) overnight at 4°C. Then the ligated products were transformed into Electro MAXTM DH5 α -ETM cells (Invitrogen, USA) and incubated at 37°C, 160 r/m for 1 h, then cultured on SOB-MgCl₂ solid media with ampicillin (60 μ g ml⁻¹) to generate the primary cDNA libraries. The transformed white bacteria were randomly picked and grown on 384-well plates containing Luria Broth (LB) liquid media with ampicillin (100 μ g ml⁻¹) at 37°C overnight (about 16 h). Glycerol (Amresco, USA) (4.4% final) was added for storage at -80°C.

A total of 8,000 cDNA clones were randomly picked from forward and reverse SSH libraries and used as for subsequent PCR templates. Each PCR was performed in a 100 μ l reaction mixture using nested primers of SSH according to [77]. The PCR products were precipitated with equal amount of isopropyl alcohol and washed with 75% (V/V) ethanol, then re-suspended in 40 μ l sterile water. The yield and quality of the PCR products were determined by Nanodrop 1000 spectrophotometer (*Thermo Scientific*, Wilmington, DE, USA), and then run on 1.2% agarose gel and examined by Bio-Rad UV spectroscopy (Bio-Rad Laboratories, Washington, DC, USA) to confirm single clone (Additional file 4: Figure S3). Finally the validated PCR products were stored at -80°C for custom microarray.

Microarray slides fabrication and preparation of fluorescent dye-labelled cDNA

About 40 microlitre of PCR products were re-precipitated by adding 100 μ l of anhydrous ethanol and were dissolved in EasyArrayTM spotting solution (CapitalBio Corp, China) at a final concentration of 0.1-0.5 μ g μ l⁻¹ and then printed on amino-silaned glass slides with a SmartArrayerTM

microarrayer (CapitalBio Corp). Each clone was printed triplicate. The particular procedures for microarray fabrication were conducted according to [37].

The relative gene expression profiles of QS at four developmental stages (SE, ME, BF and OV) compared with the corresponding four stages of EG were investigated by microarray analysis. For each stage, three sets of total RNA samples were extracted independently, and then RNA pool was constructed by mixing aliquot of RNA from the three sets of RNA samples. An aliquot of 5 μ g total RNA from the RNA pool was used to produce Cy5/Cy3-labelled cDNA employing an RNA amplification combined with Klenow enzyme labeling strategy according to the protocol by [78]. Cy5/Cy3-labelled cDNA was hybridized with the microarray at 42°C overnight. Hybridization was performed in duplicate by dye swap (Cy5-labelled cDNA of QS versus Cy3-labelled cDNA of EG, and Cy5-labelled cDNA of EG versus Cy3-labelled cDNA of QS). And then the arrays were washed with 0.2% SDS, 2 \times SSC at 42°C for 5 min, and 0.2% SSC for 5 min at room temperature.

Microarray data analysis and EST sequence analysis

Arrays were scanned with a confocal laser scanner, LuxScanTM-scanner (CapitalBio Corp.) and the resulting images were analyzed with LuxScanTM 3.0 software (CapitalBio Corp.). cDNA spots were screened and identified with the methods described by [77]. A spatial and intensity-dependent (LOWESS) normalization method was employed and normalized ratio data were then log transformed [79]. Differentially expressed genes were identified using a t-test, and multiple test corrections were performed using FDR. Genes with FDR <0.05 and a fold change \geq 2 were identified as differentially expressed genes.

All the clones differentially expressed in at least one of the four stages were subjected to single-pass sequence using standard high throughput sequencing by BGI-Wuhan, China. All sequences were edited to omit vectors and low quality segments at 5' and 3' ends, then removal of sequences shorter than 100 bp with SeqClean software. Sequence reads were assembled by CAP3 program [80] with default parameters. Then all the unigenes were annotated using BLASTx with a cut-off value of 1.0×10^{-5} by searching the UniProt database (<http://www.ebi.ac.uk/uniprot/>). GO-KEGG-EC annotation was performed based on Annot8r platform [81]. Hierarchical clustering of transcript accumulation was performed with Cluster software (version 3.0) [82].

Quantitative real-time PCR verification and candidate TFs analysis

Total RNA was extracted from QS and EG collected at four different developmental stages with the Trizol

methods mentioned above. Primer pairs were designed with the Primer Express software (Applied Biosystems, Foster City, CA, USA). Primer sequences of 11 candidate genes for verification were provided in Additional file 5: Table S1, and primer sequences of 10 TFs were provided in Additional file 6: Table S2. Single strand cDNA was synthesized with the prescription of the Revert Aid™ first strand cDNA synthesis Kit (Fermentas, Life Science, EU). Then each cDNA sample was pre-amplified using the citrus house-keeping gene β -actin and normalized for subsequent real-time quantitative PCR (qRT-PCR). The PCR program differed in terms of the annealing temperature of each primer pair and the length of the predicted PCR products. The qRT-PCR was performed using the ABI 7500 Real Time System (PE Applied Biosystems, Foster City, CA, USA) with the method as described by [83]. And relative transcript change was analyzed by $2^{-\Delta\Delta Ct}$.

Additional files

Additional file 1: Figure S1. Comparison of expression patterns of 4 ESTs assembled the some contig encoding Methionine synthase. Y-axis represents the average Cy5 (Cy3) to Cy3 (Cy5) ratio in array hybridization. X-axis represents the four developmental stages.

Additional file 2: Figure S2. The correlation of gene expression ratios between cDNA microarray and qRT-PCR. Data were from 11 probe sets at four developmental stages. The gene expression ratios based on cDNA microarray were in log2 transformed.

Additional file 3: Sequences shorter than 200 bp but longer than 100 bp.

Additional file 4: Figure S3. The purified PCR products for microarray probe. 100 bp molecular ladders were used.

Additional file 5: Table S1. qRT-PCR primers for 11 candidate genes and citrus actin gene.

Additional file 6: Table S2. qRT-PCR primers for 10 transcription factors (TFs).

Abbreviations

AP2: APETALA2; AFLP: Amplified fragment length polymorphism; C2H2 TF: Cys2His2 transcription factor; CBF: C-repeat DNA replication-related element binding factor; DREB: DNA replication-related element binding; ERF/EREBPs: Ethylene-responsive element binding proteins; MSAP: Methylation-sensitive amplification polymorphism; NAC TFs: (NAM, ATAF and CUC) transcription factors; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; SCAR: Sequence characterized amplified region; SSR: Simple sequence repeat.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

WMQ performed the experiments and interpreted the results. ADZ carried out the ESTs annotation. YW helped with phenotype analysis. WMQ and WWG drafted the manuscript. LJC and XXG participated in the SSH-cDNA construction. XXD participated in research design. WWG proposed and supervised the overall project. All authors read and approved the final manuscript.

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