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Identification of differentially expressed genes in pistils from self-incompatible *Citrus reticulata* by suppression subtractive hybridization

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Abstract Self-incompatibility (SI) is one important factor that can result in *Citrus* seedlessness. However, the molecular mechanism of SI in *Citrus* is not clear yet. To isolate the pistil's SI-related genes, a suppression subtractive hybridization library was constructed using mature pistils of 'Wuzishatangju' mandarin (SI) as the tester and mature pistils of 'Shatangju' mandarin (self-compatibility, SC) as the driver. 229 differentially expressed cDNA clones from 967 positive clones were sequenced and identified. Differentially expressed ESTs are possibly involved in the SI reaction of 'Wuzishatangju' through a regulating signaling pathway, serine/threonine phosphatase activity, receptor kinase, embryonic development, gibberellin stimulus, or transcription. 11 out of 36 SI candidate genes displayed different expression patterns in various tissues and stages after self- and cross-pollination of 'Wuzishatangju'. The expression of CaBP (WY65), a senescence-protease (WY372), an unknown gene (WY-283), and a WRKY (WY17) were up-regulated in the styles of 'Wuzishatangju' while higher expression of WY190 was observed in styles of 'Shatangju'. Highest expression levels of WY65, WY372, an annexin (WY598), the zinc-finger protein (WY376), a C2-protein (WY291), and an unknown gene (WY318) were detected in styles at 3 days after self-pollination of 'Wuzishatangju' while lowest levels were observed in styles at 3 days after cross-pollination of 'Wuzishatangju' × 'Shatangju'. The potential involvement of these genes in the SI reaction is discussed.

Keywords *Citrus reticulata* Blanco · Self-incompatibility · SSH · Pistil · Expression analysis

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Introduction

Self-incompatibility (SI) is a barrier against inbreeding that allows the pistils of a flower to reject genetically related pollen and promotes outcrossing in angiosperms [1]. SI can be classified into homomorphic and heteromorphic types based on whether it is associated with floral polymorphism. Homomorphic SI is further classified into sporophytic self-incompatibility (SSI) and gametophytic self-incompatibility (GSI) according to the genetic control of pollen behavior. In the SSI system of *Brassicaceae*, *S*-locus cysteine-rich protein (*SCR*)/*S*-locus protein-11 (*SP11*) and *S*-locus receptor kinase (*SRK*) have been identified as being able to regulate a signal transduction cascade in the

stigmatic papillae [2]. In the GSI system of *Rosaceae*, *Solanaceae*, and *Scrophulariaceae*, SI was mainly determined by pistil-expressed *S* locus-encoded ribonuclease (*S-RNase*) genes [3, 4] and pollen-expressed *S*-haplotype-specific *F-box* (*SFB*)/*S*-locus *F-box* (*SLF*) genes [5–8]. In the GSI system of *Papaveraceae*, pistil *S*-determinant *PrsS* (*Papaver rhoeas* stigmas *S* determinant) interacted with incompatible pollen to inhibit pollen growth via a Ca^{2+}-dependent signaling network, resulting in programmed cell death (PCD) of 'self' pollen [9].

Self-incompatibility operates as a complex, multi-gene synergistic process involving multiple factors. HT-B is a pistil-expressed non-*S*-factor and was first identified from *Nicotiana* [10]. It is involved in destabilization of the pollen tube vacuole which can result in breakdown of the vacuole and release S-RNase into the cytoplasm of the self pollen [11]. Other non-*S*-factors, such as the stylar 120 kDa glycoprotein (120 K) [12] and a 4936-factor [13], are also required for pollen rejection. *S-RNase* remains sequestered and unable to cause rejection if there is no expression of HT-B or 4936-factor in pistils [10]. Five potential SI candidate genes i.e. β-expansin 2 precursor, serine/threonine-protein kinase NAK, MYB-like DNA-binding domain, calcium-dependent protein kinase, and SCP-like extracellular protein were isolated from perennial ryegrass (*Lolium perenne* L.). A rapid increase in expression of those five genes was detected within 2 min after pollen-stigma contact and reached a maximum between 2 and 10 min, implying their roles in the SI response [14]. Compared to SC clementine 'Monreal', stress-induced genes, transcription factors and genes related to calcium and hormone signaling were found in unpollinated and self-pollinated styles and stigmas of SI clementine (*Citrus clementina* Hort. ex Tan) 'Comune' [15]. Higher expression levels of a G-protein, a microtubule protein, and inositol phosphatase were verified in pistils after self-pollination of SI tea (*Camellia sinensis*) cv. 'Longjing Changye' [16].

Self-incompatibility is one important factor that can result in seedless fruit in *Citrus* [17–21]. Research of this aspect is essential to understand the relationship between the SI mechanism and *Citrus* seedlessness. *Citrus* belongs to the GSI system [17]. Cytological analysis showed that growth of pollen tubes was arrested in the upper styles of 'Commune' clementine [15], in the lower one-third of styles of 29 citrus cultivars [22], in the ovaries of 'Guanxi' and 'Duwei' pomelo (*Citrus grandis*) [23] and 'Wuzishatangju' mandarin (*Citrus reticulata* Blanco) [21]. Currently, RNase activity has been identified in stigmas from calamondin (*C. reticulata var. austera* × *Fortunella* sp.) [24], in ovaries from 'Zigui shatian' pummelo (*C. grandis* Osbeck) [25], and in pistils from 'Wuzishatangju' (*C. reticulata* Blanco) [26]. Recently, a novel *F-box* gene [27], the *Skp1-like* gene [28], and three different aspartic-acid rich (Asp-rich) protein genes potentially regulating Ca^{2+} homeostasis during self-pollen recognition [29] were isolated from an SI *C. clementina*. However, other factors involved in the SI response of *Citrus* pistils have not been reported yet.

'Wuzishatangju' (*C. reticulata* Blanco) is an excellent mandarin cultivar (seedless, very tasty, and easy to peel) derived from a bud sport variation of a seedy 'Shatangju' cultivar (SC). It has become one of the newly grown varieties during the last decade in China. Our previous studies showed that there was no significant difference in pollen viable and germination rates between 'Wuzishatangju' and 'Shatangju' which indicated that the male gamete of 'Wuzishatangju' was normal [21]. Pollen tubes of cross-pollinated 'Wuzishatangju' could grow normally in the stigmas, styles and ovaries, and they entered into the embryo sac resulting in successful fertilization. The pollen tubes of self-pollinated 'Wuzishatangju' grew well in the stigmas and styles; however, when they entered into the ovaries, they became twisted and could not enter the embryo sac. These results suggested that GSI caused seedlessness in 'Wuzishatangju' by blocking fertilization in the ovaries [21]. Therefore, the two cultivars are excellent materials to study the SI/SC mechanisms in *Citrus*. Suppression subtractive hybridization (SSH) is an effective tool to screen the expression of different genes and has been successfully used to identify genes associated with SI/SC genes from perennial ryegrass [14] and 'Shatangju' [30]. In this study, an SSH library was constructed to identify SI-related genes using mature pistils of 'Wuzishatangju' as the "tester" and mature pistils of 'Shatangju' as the "driver". The aim of this study was to identify differentially expressed genes from pistils of SI 'Wuzishatangju' mandarin based on our previous studies [21, 26]. These results may provide valuable information concerning the SI mechanism in *Citrus*.

Materials and methods

Plant materials

Flower buds (1.0 × 0.5 cm^2) were collected from six 6-year-old trees of 'Wuzishatangju' and four 'Shatangju' mandarin trees in an orchard of South China Agricultural University. Buds, leaves, petals, filaments, stigmas, styles, ovaries, pistils, and anthers were removed, separated using tweezers and immediately frozen in liquid nitrogen and stored at −80 °C until later analysis. Pistils were used to construct SSH libraries while buds, leaves, petals, filaments, stigmas, styles, ovaries, and anthers were used for expression analysis of SI-related genes. Stigmas, styles, and ovaries of 0 h, and 1–7 days after artificial self-pollination of 'Wuzishatangju' and cross-pollination of 'Wuzishatangju' × 'Shatangju' were collected,

immediately frozen in liquid nitrogen and stored at −80 °C for expression analysis.

Total RNA extraction and mRNA purification

Total RNA was extracted using a CTAB method [31] and pretreated with RNase-free DNase I (TaKaRa, Dalian, P. R. China). The quality and concentration of total RNA were examined by ethidium bromide (EB) staining, 1.2 % (w/v) agarose gel electrophoresis and spectrophotometric (Bio-RAD, USA) analysis. mRNA was purified using a Poly Attract mRNA Isolation Systems Kit III according to the manufacturer's instructions (Promega, USA).

Construction of the SSH library

The subtraction was carried out using cDNA from mature pistils of 'Wuzishatangju' as the tester and cDNA from mature pistils of 'Shatangju' as the driver with the PCR-Select^{TM} cDNA Subtractive Kit (TaKaRa, Dalian, P. R. China) following the procedure of Miao et al. [30]. 50 ng of the second PCR product were inserted into pMD19-T vector (TaKaRa, Dalian, P. R. China) and transformed into *Escherichia coli*-competent DH5α cells. All the recombinant clones were selected to form the SSH library.

Screening the SSH library using colony-PCR and reverse northern analysis

Inserted fragments were screened by colony-PCR using M13 primers (M13-F: GAGCGGATAACA ATTTCACACAGG; M13-R: CGCCAGGGTTTTCCCAGTCACGAC) (TaKaRa, Dalian, P.R. China). The PCR mixture (final volume of 25 μl) contained 1 × PCR buffer, 0.1 mM dNTP mixture, 0.4 μM M13 primers, and 1.0 unit r*Taq* DNA polymerase (TaKaRa, Dalian, P. R. China). The PCR parameters were: 94 °C for 4 min then 35 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 1.5 min, with a final 72 °C for 10 min. Reverse northern analysis was carried out to screen for up-regulated clones using the method of Miao et al. [30].

Bioinformatics analysis of expressed sequence tags (ESTs)

Clones (sequences) showing up-regulated expression were sequenced after reverse northern screening. All vector sequences were removed using VecScreen software (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Then sequences were subjected to the GenBank database at NCBI (http://www.ncbi.nlm.nih.gov) with basic local alignment search tool (BLAST) sequence comparison algorithms. All contigs and singlets were annotated according to the GO classification and the hierarchical structure using the Blast2GO suite. The Blast2Go program, which assigns the GO terms based on the BLAST definitions, was applied with an E-value of $<10^{-03}$. Thirty-six putative SI-related genes were obtained from the SSH library and registered (Table 1) in NCBI (gb-admin@ncbi.nlm.nih.gov).

Expression analysis of SI-related genes by SqPCR and qPCR

The first strand cDNA was synthesized from 1 μg of total RNA using a cDNA synthesis kit (TaKaRa) and Oligo $(dT)_{16}$ primers. Thirty-six putative SI-related genes were obtained and first screened by SqPCR (semi-quantitative reverse transcription polymerase chain reaction) using corresponding primers and PCR procedures (Additional File 1). Expression patterns of candidate genes were detected by SqPCR and qPCR using the *citrus actin* gene (accession No. GU911361) as a control. qPCR was performed in an iQ5 real-time PCR detection system (Bio-Rad, USA) using the SYBR *Ex*Script RT-PCR Kit (TaKaRa). Expression levels of these genes were verified in triplicate and calculated using the $2^{-\Delta\Delta CT}$ method [32]. All data were analyzed using iQ5 software in an iQ5 real-time PCR detection system (Bio-Rad, USA).

Results

Identification of inserted gene fragments from the SSH library

An SSH library was constructed to isolated differentially expressed genes using mature pistils of SI 'Wuzishatangju' as the "tester" and mature pistils of SC 'Shatangju' as the "driver". According to the results of colony-PCR, a total of 967 positive colonies were obtained and the size of inserted cDNA fragments ranged from 300 to 1000 bp, although most were 400–700 bp (Additional File 2). Based on reverse northern analysis, 229 clones in 'Wuzishatangju' showing different intensities in dot screening (Additional File 3A and 3B) were sequenced (BGI, Shenzhen, P. R. China).

General statistics of the SSH library

A total of 229 unique sequences were obtained and compared with an available database to find similarities with known sequences (Additional File 4). Dynamic translation (Blastx) was carried out and matched sequences with an E-value of $<10^{-03}$ were considered to be homologous

Table 1 All potential pistils SI-related genes obtained from the SSH library

Seq. name	Seq. description	GenBank accession No.	Length (bp)	E-value	Similarity (%)	Annotation
WY51	Protein-phosphatase1	JK724800	349	2.43E−28	68.60	Serine/threonine phosphatase activity
WY-65	Ca^{2+}-binding protein	JK724801	349	1.04E−45	86.95	Calcium ion binding
WY88	F-box kelch-repeat protein	JK724802	252	4.16E−12	60.25	Degradation of proteins
WY163	Acid phosphatase	JK724803	247	5.60E−13	89.20	Acid phosphatase activity
WY190	U4/U6 small nuclear ribonucleoprotein	JK724804	347	2.92E−29	90.60	Embryonic development
WY252	Senescence-associated protein	JK724805	342	1.52E−57	95.60	Ageing
WY291	C2-domain containing protein	JK724806	314	2.42E−51	94.15	Electron carrier activity
WY372	Senescence-associated cysteine protease	JK724807	309	9.00E−06	97.00	Cysteine-type endopeptidase activity
WY376	Zinc-finger protein	JK724781	289	4.52E−13	80.06	Transcription factor activity
WY377	Cu/Zn superoxide dismutase	JK724808	527	3.04E−47	71.15	Antioxidant activity
WY433	Cap-binding protein nCBP	JK724809	717	1.77E−102	94.75	Translation initiation factor
WY447	Chaperone protein dnaJ	JK724810	603	3.96E−49	72.45	Unfolding protein binding
WY598	Annexin p34	JK724811	809	1.39E−133	90.05	Calcium ion binding
WY800	Ca^{2+}-dependent membrane-binding protein annexin	JK724812	971	2.61E−75	82.00	Calcium ion binding
WY889	Polyubiquitin1	JK724813	266	1.00E−04	71.00	Ubiquitin
WY17	WRKY transcription factor 2	JK724814	749	1.64E−51	90.90	Transcription, DNA binding
WY22	PIL5 transcription factor	JK724815	294	8.59E−41	83.25	Transcription regulator activity
WY75	bZIP transcription factor	JK724816	621	2.00E−15	59.7	Receptor kinase
WY191	MYB transcription factor	JK724817	502	4.25E−40	65.75	Response to gibberellin stimulus
WY774	Translation initiation factor eIF1	JK724818	342	1.41E−40	96.35	Translation initiation
WY926	NAP-like transcription factor	JK724819	276	3.80E−41	92.25	Flower development
WY5	Unknown protein	JK724820	308			
WY19	Ring-h2 finger protein	JK724821	362	4.38E−12	60.38	Metal ion binding
WY89	DNA binding protein	JK724822	313	1.98E−06	59.25	Unknown
WY283	Unknown protein	JK724823	264	8.00E−33	83.20	Molecular function
WY318	Unknown protein	JK724824	289	2.26E−06	64.50	Unknown
WY460	Act domain–containing protein	JK724827	341	8.25E−40	83.50	Embryonic development
WY605	DNA binding protein	JK724828	289	2.21E−06	64.50	Unknown
WY630	Lactoylglutathione lyase-like protein	JK724829	649	1.56E−124	93.65	Metal ion binding
WY729	Cox190-like CHCH family protein	JK724830	404	2.54E−23	83.55	Protein binding
WY925	Desiccation protectant protein	JK724832	269	1.10E−11	85.80	Embryonic development
WY929	Unknown protein	JK724833	209	2.17E−18	75.80	Nucleus
WY361	Unknown protein	JK724825	309			
WY385	Unknown protein	JK724826	214			
WY844	Unknown protein	JK724831	279			
WY937	Unknown protein	JK724834	251			

sequences while sequences with an E-value of $>10^{-03}$ were labeled as undescribed. Homologous sequences accounted for 74.5 % of the sequences in the SSH library (Additional File 4). Among the homologous sequences, eight in the SSH library (8/188, 4.3 %) lacked annotation. Additionally, there were in total 40 no-mapping sequences in the SSH library. All data are summarized in Fig. 1.

Gene ontology analysis

Gene ontology analysis was carried out using the Blast2GO program and three ontology categories i.e. biological process, molecular function and cellular components were obtained (Fig. 2; Additional File 5). In the category of "biological process", the most frequent process was the

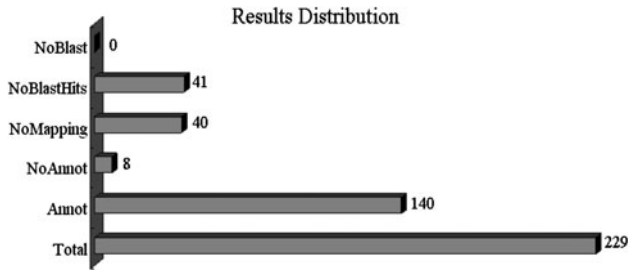

Fig. 1 Gene distribution from the SSH library. The *Arabic numerals* represent the number of unique sequence at each step of the annotation process. *NoBlast* no blast result; *NoAnnot* no annotation; *Annot* annotation. Data analysis and visualization of results were performed by the Blast2GO software

metabolic process (83/311, 26.7 % in the SSH), followed by the cellular process (70/311, 22.5 % in the SSH). In addition, stimulus response (40/311, 12.9 % in the SSH) which might be related to pollen germination and tube growth accounted for the more proportion than that of other functional classes. For the "molecular function", catalytic activity was the most frequent activity (63/107, 58.9 % in the SSH), followed by transporter activity (19/107, 17.8 % in the SSH) and enzyme regulator activity (8/107, 7.5 % in the SSH). For the "cellular component", the most frequent activity was cell (81/155, 52.3 % in the SSH), followed by organelle (53/155, 34.2 % in the SSH) then macromolecular complex (13/155, 8.4 % in the SSH).

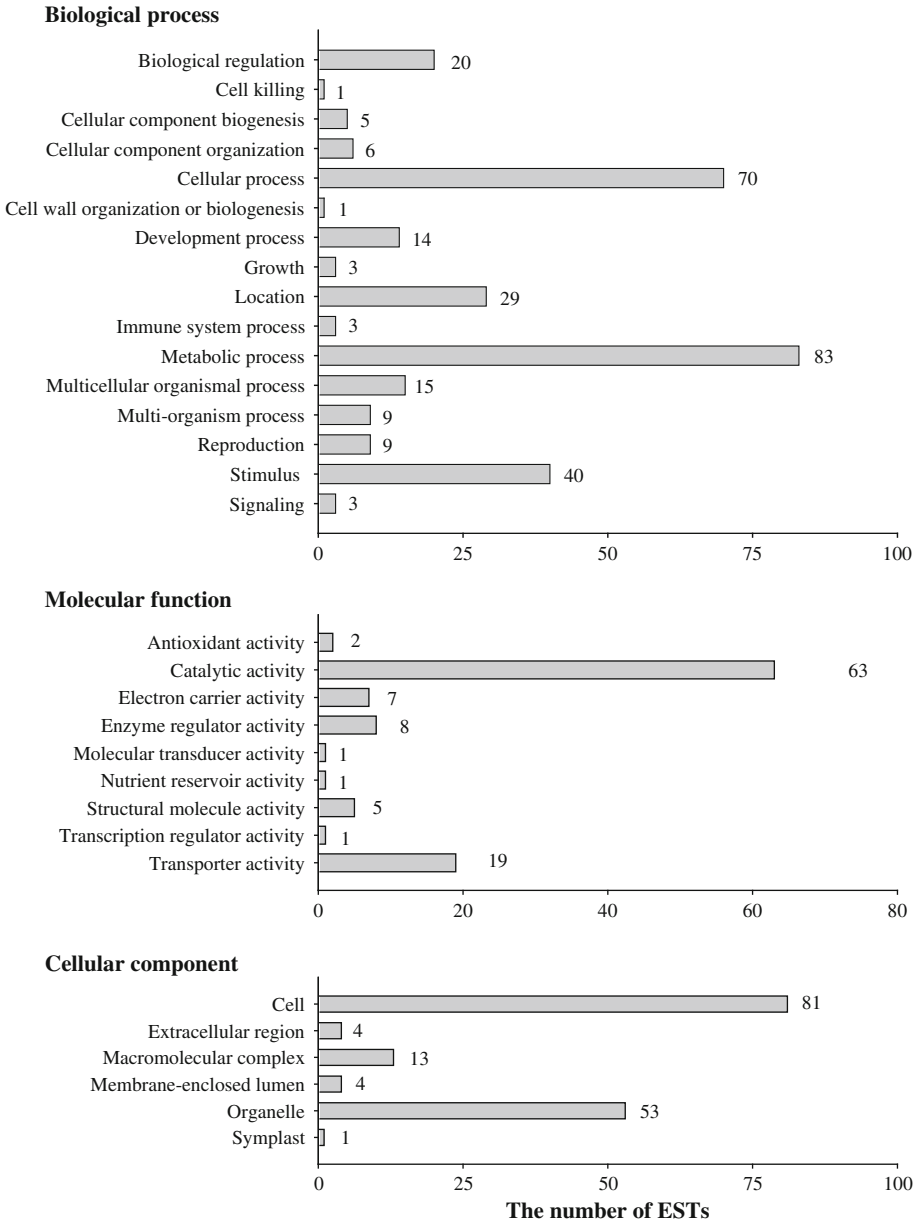

Fig. 2 Classification of the unique sequence from the SSH according to Gene Ontology criteria. The *Arabic numerals* represent the number of unique sequence at each step of the classification process. The biological process combined graph was made based on ontology level 2

Expression analysis of 36 candidate genes by SqPCR

According to the bioinformatics analysis (Table 1), 36 SI candidate genes were selected and preliminarily analyzed using SqPCR (Additional File 6). Among those 36 genes, 11 showed different expression patterns in various tissues of 'Wuzishatangju' and 'Shatangju' and different stages after self- and cross-pollination of 'Wuzishatangju' (Fig. 3). Compared to 3 days after cross-pollination, eight genes (WY65, WY372, WY598, WY376, WY291, WY447, WY283, and WY17) were up-regulated in styles at 3 days after self-pollination of 'Wuzishatangju' (shown by arrows) (Fig. 3). WY318 was up-regulated in 'Wuzishatangju' stigmas. The expression levels of the WY65, WY372, WY283, and WY17 genes in styles of 'Wuzishatangju' were higher than that in 'Shatangju'. Compared to 'Shatangju', WY447 was preferentially expressed in the ovaries of 'Wuzishatangju' (Fig. 3).

Expression patterns of 11 candidate SI genes in different tissues from 'Wuzishatangju' and 'Shatangju' mandarin by qPCR

The 11 candidate genes were further investigated using qPCR. Compared to 'Shatangju', WY376 was up-regulated in the stigmas of 'Wuzishatangju' (Fig. 4). The expression levels of WY65, WY372, WY318, and WY17 in the styles of 'Wuzishatangju' were approximately ten-, three-, two-, three-, and fivefold higher, respectively than that in the styles of 'Shatangju' (shown by arrows) (Fig. 4). Moreover, WY447 showed up-regulated expression in the ovaries of 'Wuzishatangju' and was sixfold higher than that in the ovaries of 'Shatangju' (Fig. 4). The highest expression levels of WY190, WY291, and WY447 were detected in styles of 'Shatangju' while lowest levels were observed in the styles of 'Wuzishatangju' (Fig. 4).

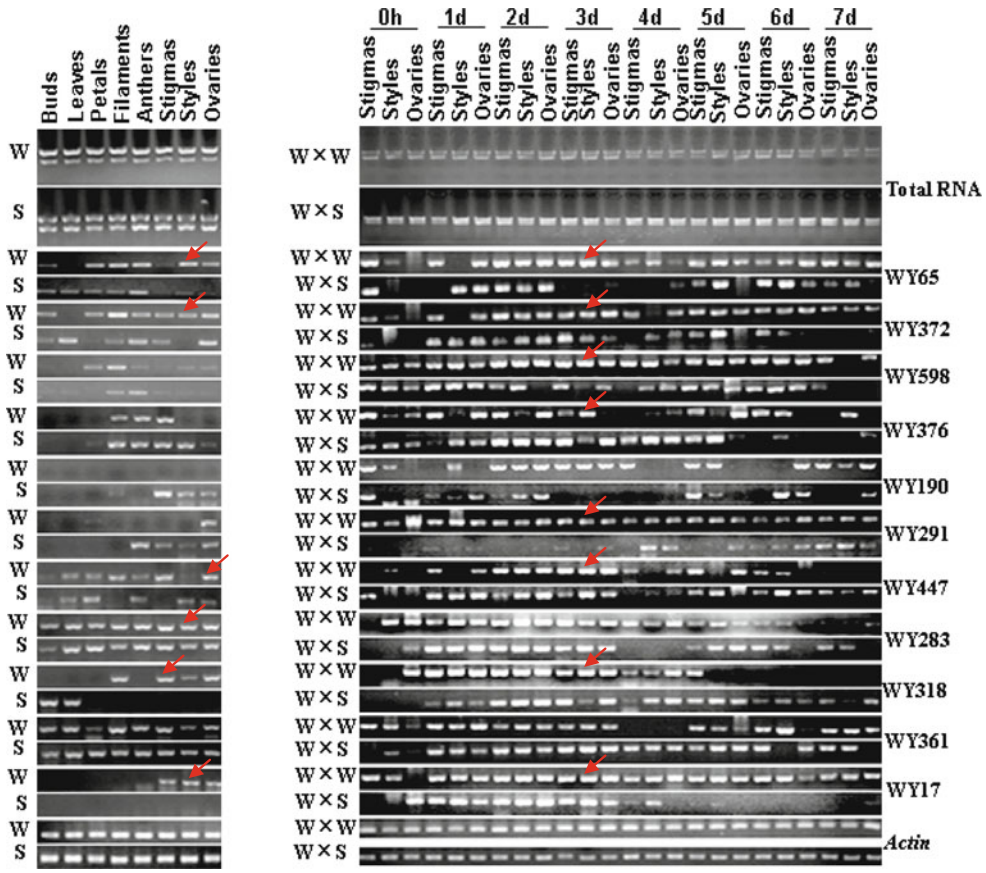

Fig. 3 Expression analyses of 11 pistils SI-related genes in different tissues of 'Wuzishatangju' (W) and 'Shatangju' (S) and different stages after self-pollination of 'Wuzishatangju' × 'Wuzishatangju' (W × W) and cross-pollination of 'Wuzishatangju' × 'Shatangju' (W × S) using SqRT-PCR. *WY65* Ca^{2+}-binding protein; *WY372* Senescence-associated cysteine protease; *WY598* Annexin p34; *WY376* Zinc-finger protein; *WY190* U4/U6 small nuclear ribonucleoprotein; *WY291* C2-domain containing protein; *WY447* Chaperone protein; *WY283* Unknown protein; *WY318* Unknown protein; *WY361* Unknown protein; *WY17* WRKY transcription factor 2

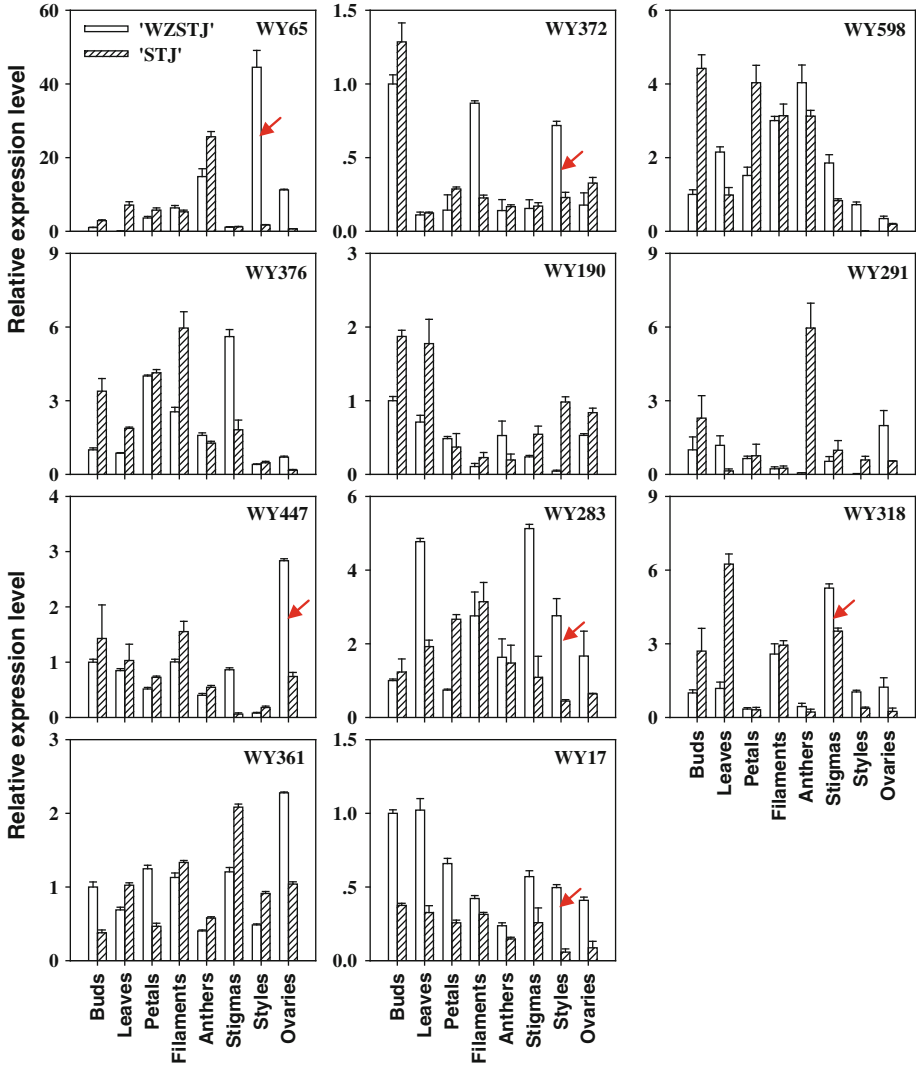

Fig. 4 Expression analyses of 11 pistils SI-related genes in different tissues of 'Wuzishatangju' and 'Shatangju' mandarin using qPCR. *WY65* Ca^{2+}-binding protein; *WY372* Senescence-associated cysteine protease; *WY598* Annexin p34; *WY376* Zinc-finger protein; *WY190* U4/U6 small nuclear ribonucleoprotein; *WY291* C2-domain containing protein; *WY447* Chaperone protein; *WY283* Unknown protein; *WY318* Unknown protein; *WY361* Unknown protein; *WY17* WRKY transcription factor 2

Expression patterns of 11 candidate SI genes at different stages after self-pollination of 'Wuzishatangju' and cross-pollination of 'Wuzishatangju' × 'Shatangju' by qPCR

Expression levels of WY65, WY372, WY598, WY376, WY291, WY447, WY318, and WY17 in styles at 3 d after self-pollination of 'Wuzishatangju' were approximately 65-, 20-, 10-, 10-, 2-, 2-, 10-, and 400-fold higher, respectively than at 3 days after cross-pollination of 'Wuzishatangju' × 'Shatangju' (shown by arrows) (Fig. 5). The highest expression level of WY190 was in styles at 4 days of self-pollinated 'Wuzishatangju', which was approximately 12-fold higher than at 0 h. For cross-pollinated 'Wuzishatangju' × 'Shatangju', the expression of WY190 was very weak in styles at 4 days (Fig. 5). The expression of WY65, WY372, WY598, WY376, and WY17 was up-regulated in ovaries at 3 days after self-pollination of 'Wuzishatangju' but was lowest in ovaries at 3 days after cross-pollination of 'Wuzishatangju' × 'Shatangju' (Fig. 5). Lower expression levels of WY361 were observed in stigmas, styles, and ovaries at 3 days after self-pollination of 'Wuzishatangju' and after cross-pollination of 'Wuzishatangju' × 'Shatangju' (Fig. 5). For self-pollinated styles of 'Wuzishatangju', expression levels of WY318 increased gradually at 0 h and 1 days, and reached a maximum at 2 days, followed by a decrease thereafter (Fig. 5).

Discussion

In the SI response of perennial ryegrass, a complex pathway is triggered by the recognition process between pollen and pistils, leading to the inhibition of pollen growth in the pistils [14]. Therefore, genes involved in the SI response can be identified from pistils (including stigmas, styles, and ovaries) of 'Wuzishatangju' (SI) and 'Shatangju' (SC) through

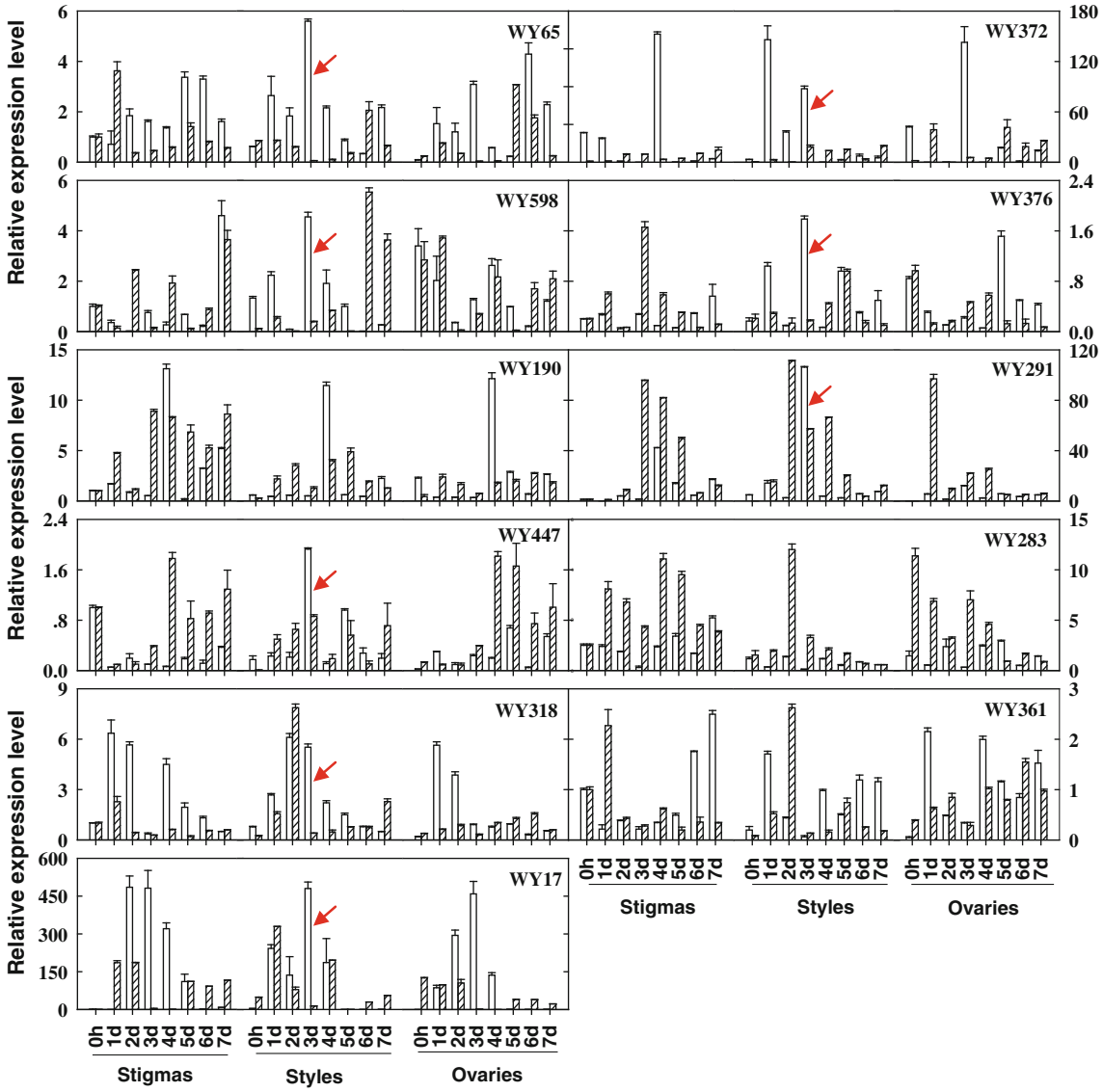

Fig. 5 Expression analyses of 11 pistils SI-related genes in different stages after self-pollination of 'Wuzishatangju' × 'Wuzishatangju' (W × W) and cross-pollination of 'Wuzishatangju' × 'Shatangju' (W × S) using qPCR. *WY65* Ca^{2+}-binding protein; *WY372* Senescence-associated cysteine protease; *WY598* Annexin p34; *WY376* Zinc-finger protein; *WY190* U4/U6 small nuclear ribonucleoprotein; *WY291* C2-domain containing protein; *WY447* Chaperone protein; *WY283* Unknown protein; *WY318* Unknown protein; *WY361* Unknown protein; *WY17* WRKY transcription factor 2

differential gene expression analysis. In this study, an SSH library was constructed to identify SI-related genes using mature pistils from SI 'Wuzishatangju' as the "tester" and SC 'Shatangju' as the "driver". Several SI-related genes such as the signaling pathway (WY65, WY372), serine/threonine phosphatase activity (WY51), receptor kinase (WY75), embryonic development (WY190, WY460, WY925), gibberellin stimulus (WY191), and transcription (WY17) (Table 1) were detected.

Ca^{2+} plays an important role in SI signal transduction. In the GSI system of *Papaveraceae*, increasing free Ca^{2+} in pollen tubes is a specific SI reaction that could transfer extracellular signals into the intracellular signaling of SI [33]. Extracellular Ca^{2+} currents, as part of the complex signaling cascade, contribute to the inhibition of pollen tube tip growth [34] while excess or inadequate Ca^{2+} concentrations inhibit the growth of the pollen tube [35]. Ca^{2+}-binding proteins also play a key role in pollen-pistil recognition, pollen tube growth, and fertilization in the SSI system of *Brassica* [36, 37]. C2-protein is required for pollen fertility by regulating Ca^{2+} and phospholipid signaling pathways [38]. The localization of C2-protein is altered in pollen tubes rejected by the SI mechanism [39, 40]. Moreover, C2-protein is preferentially expressed in pistils and pollen but down-regulated by pollination while knockdown of C2-protein expression could lead to partial

pollen fertility and poor seed set [38]. Annexins are a homologous family of proteins, characterized by a Ca^{2+}-dependent affinity for phospholipids [41]. Annexins initially inhibit the growth of pollen tube tips in maize (*Zea mays*) [42]. In tomato (*Lycopersicon esculentum* cv Money Maker), annexins p34 and p35 were found to bind to F-actin in a calcium- and pH-dependent interaction [43]. The enzyme activity of annexins p34 and p35 could be abolished by their specific Ca^{2+}-dependent interaction or binding with phospholipids, suggesting their roles in mediating Ca^{2+}-dependent events involving interactions of the cytoskeleton and cellular membranes [43]. In this study, three genes putatively linked to Ca^{2+} signaling i.e. Ca^{2+}-binding protein (WY65), C2-protein (WY291), and annexin p34 (WY598), were obtained from the SSH library (Table 1). WY65 was up-regulated in the styles, ovaries, and anthers of 'Wuzishatangju' mandarin (Fig. 3), a pattern which mimics the expression of Ca^{2+}-binding protein in *Brassica* [37]. WY291 has putative C2-domain phospholipid-binding activity, sharing 83 % amino acid homology with *Arabidopsis lyrata* (XP002864397). Higher expression levels of C2-protein were detected in the unpollinated ovaries of 'Wuzishatangju' and unpollinated anthers of 'Shatangju' (Fig. 4). After self-pollination of 'Wuzishatangju', no expression of C2-protein was detected in ovaries (Fig. 5). Annexin p34 (WY598) was obtained and shared 74 % amino acid sequence homology with annexin p34 from *Solanum lycopersicum* (NP001234104). The expression pattern of WY598 was similar to that of WY65 and WY291 (Fig. 4), suggesting that the three genes are likely to act as a Ca^{2+} and phospholipid signaling factor to inhibit the development of pollen and seed of 'Wuzishatangju' mandarin.

Programmed cell death is a mechanism used by many organisms to destroy unwanted cells in a precisely regulated manner [44]. PCD provides a strategy to specifically target and destroy incompatible pollen in the SI system [45]. Senescence-associated cysteine protease plays a critical role in PCD and was involved in PCD of the pollen tube in *Papaver* SI [46] and in germinating seeds of *Petunia* [47]. Up-regulating the expression of senescence-associated cysteine proteases or cDNA clones encoding cysteine proteases can regulate the senescence of various plants [48, 49]. Zinc-finger protein is involved in flowering senescence and PCD [50]. During the opening and senescence of *Mirabilis jalapa* flowers, a remarkable abundance of transcripts of a gene encoding a RING zinc finger ankyrin protein increased 40,000-fold as the flowers senesced [50]. In our study, zinc-finger protein (WY376), a senescence-associated protein (WY252), and a senescence-associated cysteine protease (WY372) were isolated from the SSH library (Table 1). WY376, belonging to the ring finger and CHY zinc finger domain-containing protein 1, was obtained and shared 47 % amino acid homology with the zinc-finger protein of *Bruguiera gymnorhiza* (BAG15864). The expression of WY376 and WY372 was up-regulated within 3 days after self-pollination of 'Wuzishatangju' (Fig. 5), which was similar to the expression pattern of the *S-RNase-like* gene of 'Wuzishatangju' [26]. These results suggest that WY372 may be involved in PCD of the 'Wuzishatangju' mandarin pollen tube.

U4/U6-proteins are required for precursor mRNA splicing [51] and are involved in embryonic development (Table 1). RNA biogenesis is essential and vital for accurate expression of genes and cells cannot continue normal metabolism when RNA splicing is interfered with [51]. However, the role of U4/U6-proteins in SI has not been reported yet. In this study, U4/U6-proteins (WY190), belonging to the U4/U6 small nuclear ribonucleoproteins, were obtained and shared 88 % amino acid homology with the U4/U6 small nuclear ribonucleoprotein of *Ricinus communis* (XP002525342). Higher expression of WY190 was detected within 4 days after self-pollination of SI 'Wuzishatangju' (Fig. 5), which was consistent with our previous studies [21].

Besides SI-related genes, other transcriptional factors such as WRKY2 (WY17), PIL5 (WY22), bZIP (WY75), and MYB52 (WY191) were also obtained from the SSH library (Table 1). WRKY genes can regulate embryogenesis, seed size, seed coat, trichome development, and senescence [52]. PIL5, a phytochrome-interacting bHLH protein, regulates both gibberellic acid (GA) metabolism and responsiveness. In SI 'Clemenules' mandarin, GA_3 inhibited pollen tube elongation and seed fertilization [53]. Over-expression of bZIP transcription factor triggered PCD in *Podospora anserina* [54]. MYB transcription factors may be involved in the down-stream signaling transduction of the SI response to regulate expression of other candidate genes [14]. Higher expression levels of WRKY2 were detected within 3 days after self-pollination of SI 'Wuzishatangju' (Fig. 5), implying that WRKY2 may be involved in the signaling transduction of the SI response as a transcription factor by regulating the expression of other candidate genes.

Moreover, about 25.5 % of the isolated unigenes had no homology with recorded plant genes (Additional File 4), which may be due to the insufficient length of some sequences. Among these unknown sequences, 8 out of 37 significantly matched plant EST or *Citrus* genomic clones deposited in GenBank (Additional File 4) and two genes (WY283 and WY318) showed up-regulated expression in the stigmas of 'Wuzishatangju' (Fig. 3). The expressional characteristics suggested that WY283 and WY318 may be new factors involved in the SI response of 'Wuzishatangju'.

These results suggest that the up-regulated expression of WY65, WY291, WY598, WY447, WY372, WY376, WY17,

WY190, WY283, and WY318 in pistils (stigmas, styles, and ovaries) and 3 days after self-pollination may be involved in the SI response of 'Wuzishatangju' mandarin through regulation of Ca^{2+} signaling pathways, PCD, embryonic development, gibberellin stimulus, and unknown function genes. Further studies are required to elucidate why different expression levels of these genes in different tissues and stages after self-pollination of 'Wuzishatangju' and cross-pollination of 'Wuzishatangju' × 'Shatangju' could result in the occurrence of SI in 'Wuzishatangju'.

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