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Identification of differentially expressed genes in 72 h styles from self-incompatible *Citrus reticulata*

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ABSTRACT

Self-incompatibility (SI) is one important factor that can result in seedless fruits of *Citrus*. Our previous study showed that 'Wuzishatangju' mandarin was gametophytic self-incompatibility (GSI) and 72 h was the crucial stage for the shift from a self-compatible to a self-incompatible state. However, it is not clear what genes are involved in the process. In this study, two SSH libraries were constructed to screen differentially expressed genes using 72 h styles after self-pollination of 'Wuzishatangju' and cross-pollination of 'Wuzishatangju' × 'Shatangju'. 106 and 97 differentially expressed cDNA clones were sequenced and identified from forward and reverse SSH libraries, respectively. Differentially expressed ESTs are possibly involved in the SI reaction of 'Wuzishatangju' through S-phase kinase-associated protein1 (SKP1)-like activity, autoinhibited Ca^{2+}-ATPase 1, U-box domain protein, and serine/threonine kinase. Results from quantitative real-time PCR (qPCR) showed that *SKP1-like* and *U-box* were obviously up-regulated in ovaries before pollination of 'Wuzishatangju', and approximately 20- and 2-fold higher than that of 'Shatangju', respectively. The *SKP1-like* and *U-box* were obviously up-regulated in pistils at 4 d after self-pollination of 'Wuzishatangju', and approximately 20- and 800-fold higher than that the same stage after cross-pollination of 'Wuzishatangju' × 'Shatangju', respectively. The potential involvement of these genes in the SI reaction is discussed.

1. Introduction

Seedlessness is an important agronomic trait for citrus, and self-incompatibility (SI) is one main cause for seedless fruits in citrus (Soost, 1964; Yamashita, 1978; Yamamoto et al., 2006; Ye et al., 2009; Ngo et al., 2010). In angiosperms, SI can be classified into sporophytic self-incompatibility (SSI) and gametophytic self-incompatibility (GSI) according to the genetic control of pollen behavior (Takayama et al., 2001; Meng et al., 2011). *Citrus* belongs to GSI and several GSI species have been found in 'Hyuganatsu' (*Citrus tamurana* Hort. ex Tanaka) (Yamashita, 1978), 'Keraji' (*C. keraji* hort. ex Tanaka) (Yamamoto et al., 2006), 'Guanxi' and 'Duwei' (*C. grandis*) (Wang and Lü, 2009), 'Zigui shatian' (*C. grandis* Osbeck) (Chai et al., 2011a,b), 'Wuzishatangju' (*C. reticulata* Blanco) (Ye et al., 2009), and 'Clementine' (*C. clementina* Hort. Ex Tan) (Caruso et al., 2012). The growth of pollen tubes was arrested in the stigmas of Orlando tangelo (*Citrus paradise* Macf. × *C. reticulata* Blanco) (Kahn and DeMason, 1986), in the upper styles of 'Commune' clementine (Distefano et al., 2009), in the lower one-third of styles of 29 citrus cultivars (Ngo et al., 2010), in the ovaries of 'Guanxi' and 'Duwei' pomelo (*C. grandis*) (Wang and Lü, 2009) and 'Wuzishatangju' (*C. reticulata* Blanco) (Ye et al., 2009). Currently, RNase activity (Roiz et al., 1995; Chai et al., 2011a; Miao et al., 2011a), *F-box* gene (Chai et al., 2011b; Caruso et al., 2012), S-phase kinase-associated protein1 (SKP1)-like gene (Chai et al., 2010), aspartic-acid rich (Asp-rich) protein genes (Caruso et al., 2012), S1 SI locus-linked pollen 3.15 protein gene, *ubiquitin-activating enzyme E1* gene (Miao et al., 2011b), and Ca^{2+}-binding protein gene (Miao et al., 2013) have been isolated from *Citrus*. However, the molecular mechanism of SI in *Citrus* is not clear yet.

'Wuzishatangju' (*C. reticulata* Blanco) is an excellent mandarin cultivar (seedless, very tasty, and easy to peel) derived from a seedy 'Shatangju' cultivar (Self-compatibility, SC). Our previous studies showed that self-pollinated pollen tubes of 'Wuzishatangju' grew well in the stigmas and styles; however, they became twisted and could not enter the ovaries at 72 h after self-pollination. These results suggested that 72 h may be the curial stage from

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SC to SI (Ye et al., 2009). However, it is not clear what genes or factors are involved in the process. In this study, two SSH libraries were constructed to screen differentially expressed genes using 72 h styles after artificial self-pollination of 'Wuzishatangju' and cross-pollination of 'Wuzishatangju' × 'Shatangju'. Expression characteristics of all candidate genes were analyzed using semi-quantitative RT-PCR (SqRT-PCR) and quantitative real-time PCR (qPCR). The aim of this study was to identify differentially expressed genes in 72 h styles from 'Wuzishatangju' and to discuss the possible roles of the identified candidate genes in the SI response of 'Wuzishatangju'.

2. Materials and methods

2.1. Plant materials

Flower buds (1.0 cm × 0.5 cm) were collected from 6 six-year-old trees of 'Wuzishatangju' and 4 'Shatangju' (*C. reticulata* Blanco) 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. 72 h styles after artificial self-pollination of 'Wuzishatangju' and cross-pollination of 'Wuzishatangju' × 'Shatangju' were used to construct SSH libraries while pistils of 0, 1, 2, 3, 4, 5, 6, and 7 d after artificial self-pollination of 'Wuzishatangju' and cross-pollination of 'Wuzishatangju' × 'Shatangju' were collected for gene expression analysis.

2.2. Total RNA extraction and mRNA purification

Total RNA was extracted using the CTAB method (Luo et al., 2003) and pretreated with RNase-free DNase I (TaKaRa, Dalian, P.R. China). The quality and concentration of the total RNA were measured by ethidium bromide (EB) stained–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).

2.3. Construction of the SSH library

Two SSH libraries were constructed to isolate differentially expressed genes using 72 h styles after self-pollination of 'Wuzishatangju' and cross-pollination of 'Wuzishatangju' × 'Shatangju' with the PCR-Select™ cDNA Subtractive Kit (TaKaRa) following the procedure of Miao et al. (2011b). 50 ng of the second PCR product were inserted into pMD19-T vector (TaKaRa) and transformed into *Escherichia coli*-competent DH5α cells. All the recombinant clones were selected to form the SSH library.

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

Inserted fragments were screened by colony-PCR using M13 primers (M13-F: GAGCGGATAACAATTTCACACAGG; M13-R: CGCCAGGGTTTTCCCAGTCACGAC) (TaKaRa). The PCR mixture (final volume of 25.0 μl) contained 1× PCR buffer, 0.1 mM dNTP mixture, 0.4 μM M13 primers, and 1.0 unit r*Taq* DNA polymerase (TaKaRa). 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 according to the method of Miao et al. (2011b).

2.5. 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.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 $<10^{-03}$.

2.6. Expression analysis of differentially expressed genes by SqPCR and qPCR

1.0 μg of total RNA was used as a template for cDNA synthesis by using a first-strand cDNA synthesis kit (TaKaRa) according to the instructions of the manufacturer. Putative SI candidate genes were obtained and first screened by SqPCR using corresponding primers and procedures (Table S1). Expression characteristics of the candidate genes were further 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 (Livak and Schmittgen, 2001). All data were analyzed using Optical System Software (Version 2.0) in an iQ5 real-time PCR detection system (Bio-Rad, USA).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scienta.2013.07.013.

3. Results

3.1. Identification of gene fragments from two SSH libraries

Two SSH libraries were constructed to isolate differentially expressed genes from 72 h styles after self-pollination

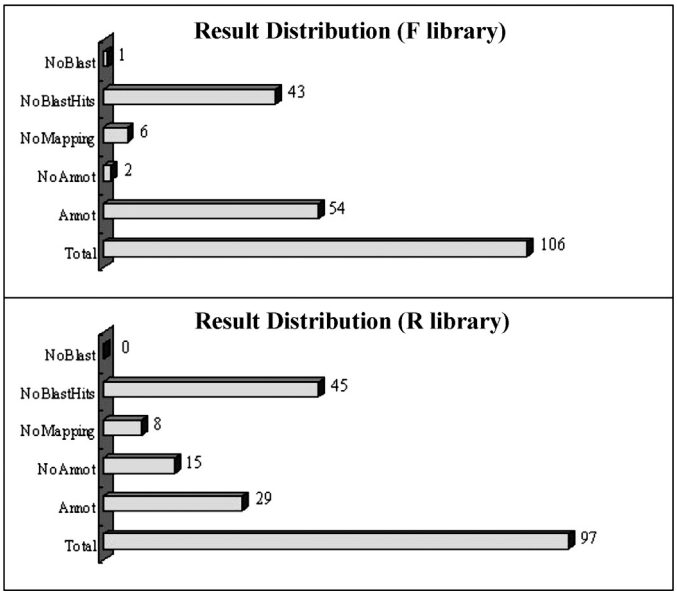

Fig. 1. Gene distribution from two SSH libraries. The Arabic numerals represent the number of unique sequence at each step of the annotation process. NoBlast, no blast result; NoAnnot, no annotation; Annot, annotation. Data analysis and visualization of results were performed by the Blast2GO software.