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Identification of genes related to floral organ development in pak choi by expression profiling

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ABSTRACT. Pak choi is a highly nutritious vegetable that is widely grown in China, Southeast Asia, and other parts of the world. Because it reproduces by seed, it is very important to understand the mechanism of floral organ development. Therefore, using the Chinese cabbage genome as a reference, this study analyzed the expression profiles of shoot apex genes at flower bud differentiation stages 1 and 5, in order to identify genes related to floral organ development. The results showed that the proportion of mapped genes was high, with 84.25 and 83.80% of clean reads from the two sample saligned to the reference genome, respectively. A total of 525 differentially expressed genes (DEGs) were identified, 224 of which were upregulated and 301 were downregulated. The expression levels of genes homologous to Chinese cabbage flowering genes were also analyzed at stages 1 and 5; the expression

levels of Bra012997 (*ap1*), Bra000393 (*SOC1*), and Bra004928 (*SOC1*) were significantly upregulated at stage 5, suggesting that these three genes positively regulate floral development in pak choi. DEGs involved in floral organ development were analyzed with homologous genes from *Arabidopsis thaliana*; the homologous genes Bra029281 (*AGL42*), Bra026577 (*ARPN*), Bra022954 (*SPL3*), Bra029293 (*ARF2*), Bra007978 (*AtRLP12*), Bra033221 (*SPL8*), Bra008037 (*LOX4*), Bra001598 (*IAA19*), Bra003892 (*PATL1*), Bra038778 (*AT4G21323*), Bra025315 (*KLCR2*), and Bra013906 (*DTX35*) are directly related to floral organ development in *Arabidopsis*, suggesting that these genes have corresponding functions during flower organ development in pak choi, and could be candidates for further genetic research. These results provide a foundation for research on the molecular mechanism of flower organ development in pak choi and other *Brassica rapa* vegetables.

Key words: Pak choi; Floral organ; Expression profile; Gene

INTRODUCTION

Pak choi (*Brassica rapa* ssp *chinensis* 'Makino') is a crop in the botanical family Brassicaceae that originated from China. It has a wide distribution area, many varieties, and is highly nutritious. It contains glucosinolates, which have anticarcinogenic properties and are precursors of isothiocyanates. Pak choi plays an extremely important role in the production and supply of vegetable crops in China, particularly in the Yangtze River Valley and south China (Chen, 2010). Currently, the main methods used to produce F1 hybrids that take advantage of heterosis are artificial emasculation, chemical hybridization, and the use of self-incompatible and male-sterile lines (Yu, 2002). The production of hybrid seeds is difficult and expensive; therefore, it is important to understand the molecular mechanisms underlying flower organ development in pak choi.

Flowers are reproductive organs in angiosperms, and have undergone large structural changes over the course of evolution. Although flower development has been studied for over 200 years (Xu and Liu, 1998), research on floral organs in traditional biology has mainly focused on morphological descriptions and the physiological mechanism of flowering. Beginning in the 1980s through research on floral mutants of *Arabidopsis* and *Antirrhinum*, our understanding of the molecular mechanisms of floral organ development has advanced significantly with the development of molecular genetics, and has become an attractive "hotspot" in the field of plant developmental biology (Xu, 1999). Since the ABC model of flower development was first presented by Coen and Meyerowitz (1991), the key genes related to floral organ development have been successfully cloned from a variety of plants, and the regulation of flower development is now well understood, which has taken research on flower development from the ecological, physiological, and biochemical levels to the molecular genetic level. With the discovery of new mutants, the genetic model of flower organ development has been continuously updated and improved, progressing from the ABC model to the ABCDE and four factors models (Tang et al., 2013).

Recently, with the application of molecular biology in many areas of reproductive biology, the molecular mechanism of flower development in *Brassica rapa* has attracted more

and more attention. It has been recognized that flower development in *B. rapa* is a complex process that is regulated by multiple genes. Taking male sterility as a starting point, several studies of pollen development genes in pak choi have been conducted since 2001 (Han, 2011). A series of genes related to pollen development have been isolated, including *CYP86MF* (Yu et al., 2004), *BcMF1* (Wang, 2003), *BcMF2* (Wang et al., 2005), *BcMF3* and *BcMF4* (Liu et al., 2007), *BcMF5* and *BcMF6* (Zhang et al., 2008), *BcMF7* (Huang et al., 2007), *BcMF8* (Huang et al., 2008a), *BcMF9* (Huang et al., 2009), *BcMF10* (Huang et al., 2008b), *BcMF11* (Song et al., 2007), *BcMF12*, *BcMF13* (Li et al., 2008), *BcMF19* (Huang et al., 2011), *BcMF22* (Li, 2011), *BcMFJMJ30*, *BcBGAL11*, and *MIR158* (Jiang, 2014), *BcMF20* (Han, 2011), *BcAHA8* (Qiu, 2012) and *BcSKS11* (Zhang, 2011), *BcMYB*, and *BjMF6*. The functions of some of these genes have been described, including the cytochrome P450 gene *CYP86MF*, the pectinesterase gene *BcMF3*, the polygalacturonase genes *BcMF2*, *BcMF6*, and *BcMF9*, the arabinogalactan protein genes *BcMF8* and *BcMF18*, the beta-galactosidase gene *BcBGAL11*, and the pollen wall protein gene *BcMF5*. However, no research has been conducted on floral organ genes in pak choi. With the rapid development of bioinformatics, the genomes of the model plants *Arabidopsis* and Chinese cabbage have been sequenced, and genomic data are continuously accruing, which enable studies on gene expression and the regulation of flower organ development in pak choi (Qiu, 2012). Therefore, using the Chinese cabbage genome as a reference, this study compared gene expression profiles at flower bud differentiation stages 1 and 5 to identify genes associated with floral organ development, which will establish a foundation for further research into reproductive development in *B. rapa* ssp *chinensis*.

MATERIAL AND METHODS

Plant materials and RNA extraction

The pak choi inbred line '75#' that bolts easily was used in our experiments. This line was obtained from the Institute of Vegetable Research, Shanxi Academy of Agricultural Sciences. Germinating seeds were maintained at 4°C for 20 days for the low temperature treatment, after which, seedlings with cotyledons were transplanted to a tray containing soil. Traditional field management was practiced, and flower bud differentiation was observed. The shoot apices of flower buds at differentiation stages 1 and 5 were sampled on days 16 and 17 after transplanting (Song et al., 2015); these samples, each weighed 0.15 g, were named V16 and V17, respectively, and were used for the extraction of total RNA. Total RNA was extracted from each sample using an RNeasy Plant Mini Kit (Qiagen, Cat. No. 74903) following the manufacturer's instructions. The extracted RNA was stored at -80°C for subsequent analysis. cDNA library construction, sequencing, gene expression analysis, and functional annotation was conducted by Biomarker Technologies Co. Ltd. The methods of DNA sequencing, gene expression analysis, and functional annotation were previously described by Sun et al. (2015).

Screening of differentially expressed genes (DEGs) related to floral organ development

DEGs that were potentially related to floral organ development were screened by Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. In addition, the flowering genes from Chinese cabbage published in BRAD (http://brassicadb.org) were compared with the profiles to identify genes involved in flower

organ development in pak choi. These screened genes were compared to homologous genes from *Arabidopsis* to predict their function in pak choi flowering.

Quantitative real-time polymerase chain reaction (RT-qPCR)

To validate the RNA-Seq results, a RT-qPCR was performed using gene-specific primers for four randomly selected genes. For reverse transcription, the concentration of each RNA sample was adjusted to 500 ng/μL. Single-stranded cDNA was synthesized using a PrimeScript® RT reagent kit (Perfect Real Time; TaKaRa, Cat. No. DRR037A) following the manufacturer's instructions. Specific primers used for RT-qPCR were designed using Primer 3 software (http://primer3.ut.ee/) based on the gene sequence, and the pak choi *ACTIN* gene was chosen as an internal reference gene. These four primers were all adjusted to 10 μM. RT-qPCR was conducted using SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus; TaKaRa, Cat. No. RR820A) in a 25-μL reaction volume. The reaction mixture included 20 ng cDNA (2 μL), 8.5 μL ddH_2O, 12.5 μL SYBR® mix, and 2 μL primers. The thermocycler program was as follows: 95°C for 1 min, followed by 40 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Real-time PCR analysis was performed in an ABI 7500 apparatus and the relative expression level was calculated using the ΔΔCt method (Stanko et al., 2014).

Data access

The transcriptome sequencing data from this study have been deposited in the National Center for Biotechnology Information Sequence Read Archive database, and are accessible through accession number SRP075755 (http://www.ncbi.nlm.nih.gov/sra).

RESULTS

Quality assessment of the sequencing results

Gene expression profiles were analyzed using RNA-Seq technology. As shown in Table 1, 10,709,754 and 10,333,666 reads were obtained from the V16 and V17 libraries, respectively. Clean reads obtained by filtering out adaptor sequences, contaminating sequences, and low-quality reads accounted for 99.86% (V16) and 99.84% (V17) of the total reads, meaning that the quality of the sequencing was excellent.

Table 1. Illumina DNA sequencing reads from the two datasets and mapping results.

Read type	Stage 1	Stage 5
Total read	10,709,754	10,333,666
Clean read	10,695,085	10,317,519
Mapped reads	9,010,384	8,645,982
Unique-mapped reads	8,454,786	8,113,061
Multiple-mapped reads	555,598	532,921

In order to obtain gene expression information, high-quality reads from the two libraries were aligned to the Chinese cabbage genome sequence. A total of 84.25 and 83.80% of the clean reads from the V16 and V17 libraries, respectively, were mapped to the reference genome, including unique-mapped reads and multiple-mapped reads. The proportion of

mapped genes was high, indicating that the sequences and reference genome were suitable for further analysis. The unique-mapped reads accounted for 93.83 and 93.84% of the total mapped reads in the V16 and V17 libraries, respectively, so could be used for further analysis.

Screening of DEGs

The unique reads that aligned perfectly to Chinese cabbage genes from the two shoot apex libraries at flower bud differentiation stages 1 (V16) and 5 (V17) were used for DEG analysis (Table 2). A total of 525 DEGs were identified, 224 and 301 of which were upregulated and downregulated, respectively. DEGs in which the fold change was between 2 and 10 comprised the majority (474), and included 194 genes that were upregulated and 280 that were downregulated. The expression fold changes for 47 genes were between 10 and 50, with 26 upregulated and 21 downregulated. Only four genes exhibited a >50-fold change, and all of them were upregulated.

Table 2. Characteristics of the differentially expressed genes (DEGs).

DEG fold change		Number of DEGs between stages 1 and 5
>2	Total	525
	Up	224
	Down	301
2-10	Total	474
	Up	194
	Down	280
10-50	Total	47
	Up	26
	Down	21
>50	Total	4
	Up	4
	Down	0

GO functional analysis of DEGs

For the GO enrichment analysis, a GO term with a corrected P value of ≤0.05 was considered significantly enriched. Figure 1 shows that the numbers of significantly enriched terms in cell composition, molecular function, and biological process were 4, 9, and 24, respectively. The GO enrichment analysis revealed that the proteins encoded by these DEGs were mainly located in the apoplast, extracellular regions, and cell wall, and had the molecular functions of hydrolase activity, xyloglucosyl transferase activity, and acid phosphatase activity, which are involved in the biological processes of glucose metabolism, phenylalanine metabolism, the low temperature response, and the gibberellic acid (GA) response.

KEGG pathway analysis of DEGs

The DEGs were annotated and classified in the KEGG database. The main biochemical metabolism and signal transduction pathways in which the DEGs were involved were identified by enrichment analysis. The 525 DEGs were mapped to referable and canonical KEGG pathways. Overall, these genes were distributed in 64 metabolic pathways, but only the following five were significant: flavonoid biosynthesis, phenylpropanoid biosynthesis, linoleic acid metabolism, arginine and proline metabolism, and phenylalanine metabolism

(Table 3), suggesting that these pathways play an important role in the process of flower bud differentiation from stage 1 to stage 5 in pak choi.

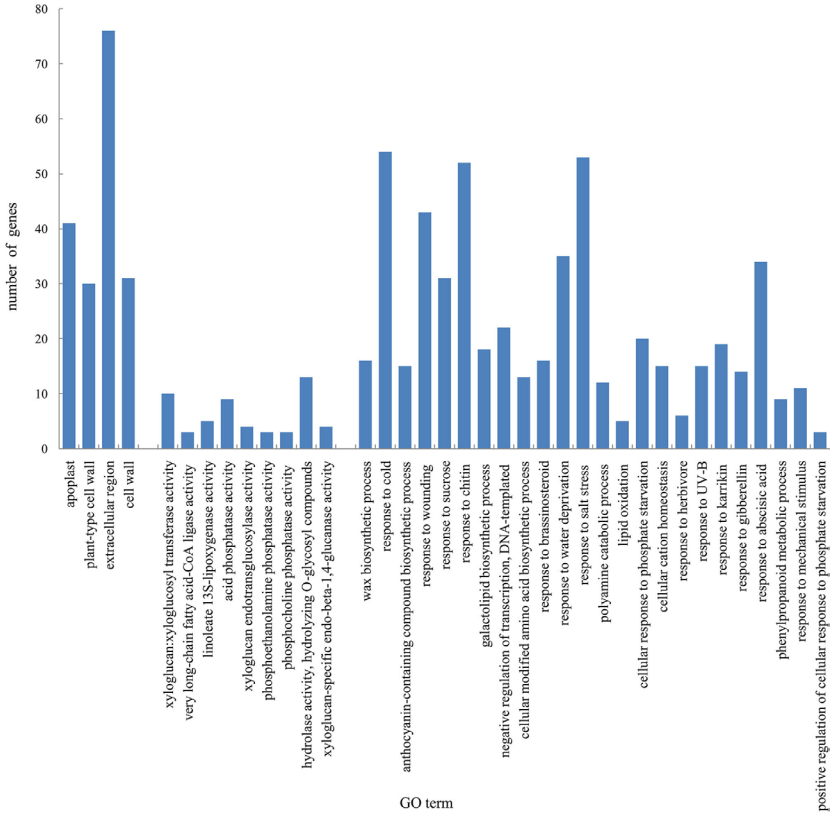

Figure 1. Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs). A corrected P value threshold of ≤0.05 was used to identify significantly enriched GO terms in the DEGs.

Table 3. Kyoto Encyclopedia of Genes and Genomes enrichment analysis of differentially expressed genes.

Pathway	ID	Frequency	Corrected P value
Flavonoid biosynthesis	ko00941	8	2.13E-05
Phenylpropanoid biosynthesis	ko00940	13	0.000228
Linoleic acid metabolism	ko00591	5	0.000505
Arginine and proline metabolism	ko00330	9	0.014962
Phenylalanine metabolism	ko00360	9	0.041315

Correspondence analysis of Chinese cabbage flower genes with the expression profiles of pak choi

The expression of Chinese cabbage flowering genes in the BRAD database was analyzed at flower bud differentiation stages 1 and 5 in pak choi (Table 4). We found that the homologous gene expression of Bra012997 (*ap1*), Bra000393 (*SOC1*), and Bra004928

Table 4. Correspondence analysis of Chinese cabbage flower genes and pak choi expression profiles.

#	Gene name	Gene ID	RPKM		Up or Down
			Stage 1	Stage 5	
1	*AGL24*	Bra019221	122.9	115.7	↓
2	*AGL24*	Bra013891	0	0	→
3	*ap1*	Bra038326	1.7	2.3	↑
4	*ap1*	Bra004007	0.6	3.3	↑
5	*ap1*	Bra011021	8.1	4.0	↓
6	*ap1*	Bra029347	1.6	2.5	↑
7	*ap1*	Bra012997	3.7	14.4	↑
8	*ap1*	Bra035952	1.1	4.8	↑
9	*ap1*	Bra004361	4.2	12.0	↑
10	*AP2*	Bra011741	24.3	36.4	↑
11	*AP2*	Bra017809	24.4	32.7	↑
12	*ARP6*	-	-	-	
13	*CDF1*	Bra029261	6.9	5.5	↓
14	*CDF1*	Bra010082	3.2	2.4	↓
15	*CO*	Bra008669	0.3	0	↓
16	*CO*	Bra023541	0.5	1.3	↑
17	*COP1*	Bra005541	12.1	13.2	↑
18	*COP1*	Bra021818	0	0	→
19	*CRY1*	Bra030568	39.7	43.6	↑
20	*CRY1*	Bra015313	6.8	5.2	↓
21	*CRY2*	Bra037880	67.9	53.7	↓
22	*CSTF64*	Bra003913	8.3	10.4	↑
23	*CSTF64*	Bra007985	4.4	6.7	↑
24	*CSTF77*	Bra016588	6.8	10.4	↑
25	*DHF*	-	-	-	
26	*FCA*	Bra038446	7.8	10.2	↑
27	*FD*	Bra010504	5.7	7.7	↑
28	*FD*	Bra011648	13.7	24.2	↑
29	*FD*	Bra017735	7.1	4.5	↓
30	*FKF1*	Bra038831	7.1	9.9	↑
31	*FKF1*	Bra038830	9.9	10.1	↑
32	*FKF1*	Bra038832	0.1	0.1	→
33	*FLC*	Bra009055	31.6	23.9	↓
34	*FLC*	Bra028599	17.3	12.3	↓
35	*FLC*	Bra006051	14.1	16.2	↑
36	*FLC*	Bra022771	2.6	2.6	→
37	*FLD*	Bra001357	10.2	9.6	↓
38	*FLK*	Bra001111	52.7	44.8	↓
39	*FLM*	-	-	-	
40	*FPA*	Bra004761	9.9	10.7	↑
41	*FRI*	Bra035723	3.3	2.7	↓
42	*FT*	Bra022475	0	0	→
43	*FT*	Bra004117	0.4	0	↓
44	*FT*	Bra015710	0	0	→
45	*FT*	Bra010052	0	0	→
46	*FVE*	Bra031085	53.9	50.2	↓
47	*FVE*	Bra040678	43.7	42.3	↓
48	*FVE*	Bra036717	54.6	49.9	↓
49	*FVE*	Bra011133	4.1	4.1	→
50	*FVE*	Bra040681	1.2	0.8	↓
51	*FY*	Bra006202	13.2	16.1	↑
52	*FY*	Bra023416	10.5	11.5	↑
53	*GA1*	Bra036239	0.9	0.6	↓
54	*GA1*	Bra000864	0.3	0	↓
55	*GAI*	Bra024875	153.9	146.8	↓
56	*GAI*	Bra017443	173.6	136.7	↓
57	*GI*	Bra024536	11.2	13.0	↑
58	*GID1A*	Bra039460	27.3	26.9	↓
59	*GID1A*	Bra009970	17.0	19.4	↑
60	*GID1A*	Bra003520	0.5	1.1	↑

RPKM, reads per kb per million reads; ↑, upregulated; ↓, downregulated; →, unchanged.

(*SOC1*) was significantly upregulated at stage 5. In *A. thaliana*, *ap1* encodes a MADS-box protein involved in flowering that regulates the expression of *SOC1*, and is also upregulated by *SOC1*. In addition to Bra011021, the expression levels of four other *ap1* genes (Bra038326, Bra004007, Bra029347, and Bra004361) all increased, although they were not as high as that of Bra012997. Therefore, *ap1* genes may also be involved in the regulation of flowering in pak choi. *SOC1* controls flowering, and is required for *CO* to promote flowering. *SOC1* acts downstream of *FT*. The overexpression of *SOC1* suppresses not only late flowering in plants that have functional *FRI* and *FLC* alleles, but also delays phase transitions during the vegetative stages of development in *A. thaliana*. In addition to the expression of Bra004928 and Bra000393 being significantly upregulated, the other *SOC1* genes (Bra004927 and Bra000392) were also upregulated, whereas Bra039324 was slightly downregulated. Therefore, *SOC1* is also predicted to promote flowering in pak choi.

When other Chinese cabbage flowering genes were analyzed, five genes related to floral organ development were found; these included *AP2*, *GID1A*, *GID1B*, *GID1C*, and *PRC2*. *AP2* is involved in the specification of floral organ identity in *A. thaliana*. The expression of two *AP2* genes, Bra011741 and Bra017809, increased at stage 5. *GID1A*, *GID1B*, and *GID1C* encode a GA receptor ortholog of the rice GA receptor gene, which is involved in floral organ morphogenesis. In addition to the slightly decreased expression of Bra039460 and unchanged expression of Bra040420, the expression levels of Bra009970, Bra003520, and Bra007722 were upregulated (Table 4). *PRC2* encodes a polycomb group protein with a zinc-finger domain that is involved in the regulation of reproductive development. *PRC2* forms a complex with *FIE*, *CLF*, and *MSI1* that modulates the expression of target genes including *AG*, *PI*, and *AP3*. The expression of the homologous genes Bra032169, Bra015200, and Bra022541 all increased during flower bud differentiation in pak choi. Therefore, the pak choi homologs of Bra011741, Bra017809, Bra009970, Bra003520, Bra007722, Bra032169, Bra015200, and Bra022541 may also be involved in floral organ development.

In addition to the above Chinese cabbage flowering genes, there were genes related to light (light cycle, quality, signal transduction, and photomorphogenesis), such as *CDF1*, *CO*, *COP1*, *TEM1*, *CRY1*, *CRY2*, *LHP1*, *PHYA*, *PHYB*, and *SPA*, the floral transition-related genes *FKF1*, *FCA*, *FLC*, *FLD*, *CSTF64*, and *CSTF77*, the flowering-related genes *FD*, *FLK*, *FPA*, *Fri*, *FT*, *FVE*, and *FY*, the GA-related genes *GA1*, *GAI* and *GI*, *RGA*, *SPY*, and the vernalization-related genes *VIN3* and *VRN2*. The expression patterns of these genes were mostly consistent with those seen in Chinese cabbage during flower bud differentiation, indicating that the genetic mechanisms underlying floral development are similar in Chinese cabbage and pak choi.

Comparison of DEGs related to floral organ development with their homologs in *A. thaliana*

By analyzing the gene expression profiles, it was found that there were 35 DEGs related to floral organ development between flower bud differentiation stages 1 and 5 (Table 5). Of these, 22 were upregulated and 13 were downregulated, and three genes coincided with flowering genes in Chinese cabbage: Bra000393 (*SOC1*), Bra004928 (*SOC1*), and Bra012997 (*ap1*). The homologs of these genes from Chinese cabbage have been published, and are described in the previous section.

Table 5. Correspondence analysis of differentially expressed genes related to floral organ development in pak choi and their homologs in *Arabidopsis thaliana*.

Gene ID	log_2FC	nr_annotation	Chinese cabbage floral gene	*Arabidopsis* gene or annotation
Bra000393	1.764137	MADS-box protein *AGL20*	*SOC1*	*SOC1*
Bra000568	-1.43187	Predicted protein	-	*TPS10*
Bra001598	-2.07617	Auxin-responsive protein *IAA19*	-	*IAA19*
Bra003892	-1.37326	Hypothetical protein	-	*PATL1*
Bra004836	1.62323	Beta glucosidase 15	-	*BGLU13*
Bra004928	1.381429	MADS-box protein	*SOC1*	*SOC1*
Bra007978	1.289333	Hypothetical protein	-	*RLP12*
Bra008037	-1.27487	Lipoxygenase 4	-	*LOX4*
Bra011184	1.38791	Diacylglycerol kinase 7	-	*DGK7*
Bra012997	1.917994	Agamous-like MADS-box protein *AGL8* homolog	*ap1*	*AGL8*
Bra013906	1.57119	Hypothetical protein	-	*DTX35*
Bra017839	-1.16143	BTB and TAZ domain protein 5	-	*BT5*
Bra018412	5.713405	Hypothetical protein	-	*WSD1*
Bra020349	-4.69827	Subtilase family protein	-	*AT5G58830*
Bra020816	1.63828	Uncharacterized protein	-	*AT4G23530*
Bra022161	1.165676	Hypothetical protein	-	*JR1*
Bra022954	2.29602	Hypothetical protein	-	*SPL3*
Bra024749	1.373295	Hypothetical protein	-	*CUT1*
Bra024848	-1.58058	Endoxyloglucan transferase	-	*ATXTH27*
Bra025315	-1.14993	Hypothetical protein	-	*KLCR2*
Bra025683	-1.29451	Uncharacterized protein	-	*AT1G18740*
Bra025914	-1.87012	*Epr1*	-	*EPR1*
Bra026577	1.401154	Predicted protein	-	*ARPN*
Bra028286	2.932274	Hypothetical protein	-	*AT5G51950*
Bra029224	-2.94055	Cytochrome P450	-	*CYP94B1*
Bra029281	1.491943	Protein agamous-like 42	-	*AGL42*
Bra029293	-1.22053	Auxin response factor 2-1	-	*AGE1*
Bra030284	1.835634	Glycine-rich RNA-binding protein GRP2A	-	*GRP7*
Bra031210	1.183685	Glycine-rich RNA-binding protein *GRP1A*	-	*GRP7*
Bra031249	1.437032	Hypothetical protein	-	*ABCG15*
Bra032670	2.095754	Protein ECERIFERUM 1	-	*CER1*
Bra033221	1.857669	SQUAMOSA-promoter binding protein-like 8	-	*SPL8*
Bra038778	1.043807	Hypothetical protein	-	*AT4G21323*
Bra039702	1.676074	Epithiospecifier protein	-	*TASTY*
Bra040837	-2.07617	Hypothetical protein	-	*AT4G27890*

FC, fold change.

Another 32 genes were analyzed by comparing them with their homologs in *A. thaliana*. The Bra001598 homolog is a primary auxin-responsive gene that is involved in the regulation of stamen filament development in *Arabidopsis*, which is similar to its function in pak choi. Bra003892 is probably involved in pollen tube growth and glycolysis in pak choi; the homologous gene *PATL1* encodes a novel cell-plate-associated protein that is involved in pollen tube growth in *Arabidopsis*. Bra007978 is presumed to play a role in stamen development and the jasmonic acid-mediated signaling pathway in pak choi, while the homologous gene *RLP12* in *Arabidopsis* encodes a *CLAVATA2* (*CLV2*)-related protein that regulates both meristem and organ development. It is predicted that Bra008037 is involved in stamen filament development, pollen development, and anther dehiscence in pak choi, because its homolog *LOX4* encodes a PLAT/LH2 domain-containing lipoxygenase family protein in *Arabidopsis* that has a similar function in pak choi. It is predicted that Bra011184 is involved in pollen tube growth in pak choi; the homologous gene in *Arabidopsis* encodes a diacylglycerol kinase, and applying a specific diacylglycerol kinase inhibitor to growth media results in reduced root elongation and plant growth. It is expressed throughout the plant, but

expression is strongest in flowers and young seedlings; although it does not have a function that is directly related to floral organ development, decreases in root and plant growth would probably impair the development of floral organs. Bra013906 is predicted to be involved in pollen development, anther dehiscence, pollen tube growth, and flavonoid metabolism in pak choi. Its homolog *DTX35* has a similar function in flavonoid metabolism, pollen development, and pollen release and viability in *Arabidopsis*. Bra020349 is predicted to play a role in the development of stamens and petals, because the homologous gene in *Arabidopsis* is expressed in the embryo sac central cell, the plant embryo, and plant sperm cells. *SPL3*, the homolog of Bra022954, encodes a member of the *SPL* (SQUAMOSA-promoter binding protein-like) family, may directly regulate *AP1*, and is involved in the regulation of flowering and the vegetative phase change. Bra025315 and the homologous gene in *Arabidopsis* have similar functions in pollen tube growth. Bra026577 is predicted to regulate flower development, and is involved in anther development and pollination. Its homolog in *A. thaliana* encodes plastacyanin, a blue copper protein, which is involved in anther development and pollination, and is expressed in the transmitting tract of the pistil. Bra029281 is predicted to regulate flower development, because its *Arabidopsis* homolog, *AGL24*, also regulates floral development, and is expressed during the floral transition phase. The Bra029293 homolog encodes an auxin response factor and positively regulates flower development, which is consistent with its function in pak choi. Bra033221 is predicted to be involved in micro- and megasporogenesis and anther development in pak choi; micro-and megasporogenesis, trichome formation on sepals, and stamen filament elongation are affected in mutants of its homolog in *Arabidopsis*, *SPL8*. *SPL8* is a member of the *SPL* gene family in *Arabidopsis*, and encodes an SBP-box protein. Bra038778 is thought to participate in cell wall composition and pollen tube growth in pak choi. Its homolog in *Arabidopsis* is expressed in pollen tube cells and is involved in pollen tube growth, which is consistent with its function in pak choi.

In addition, our data demonstrate that Bra000568, Bra017839, and Bra039702 probably participate in the specification of floral organ identity; Bra004836, Bra020816, Bra024749, and Bra025683 probably participate in pollen tube development; and Bra018412 and Bra031249 may participate in petal morphogenesis in pak choi. Bra022161, Bra028286, and Bra040837 are probably involved in stamen development; Bra024848 may regulate stamen filament development; Bra025914 may play a role in the regulation of flower development; and Bra029224, Bra030284, Bra031210, and Bra032670 are probably involved in anther development, although their homologous genes in *Arabidopsis* have no similar functions with respect to floral organ development.

Based on the results of the above analysis, most of the DEGs had functions related to floral development. Some genes act in stamen filament and pollen development, some in flower transition, and some in gamete formation. The *Arabidopsis* homologs of Bra001598, Bra003892, Bra007978, Bra008037, Bra011184, Bra013906, Bra020349, Bra022954, Bra025315, Bra026577, Bra029281, Bra029293, Bra033221, and Bra038778 have functions directly related to floral organ development; therefore, we predict that these genes have corresponding functions during the process of flower organ development in pak choi, and need to be studied further.

Validation of DEGs by RT-qPCR

To validate the RNA-Seq data, Bra022954, Bra000393, Bra004928, and Bra033221

were selected for RT-qPCR analysis at flower bud differentiation stages 1 and 5 (Figure 2). The expression patterns of these genes obtained by RT-qPCR and RNA-Seq were similar, indicating that the results obtained from the RNA-Seq were reliable.

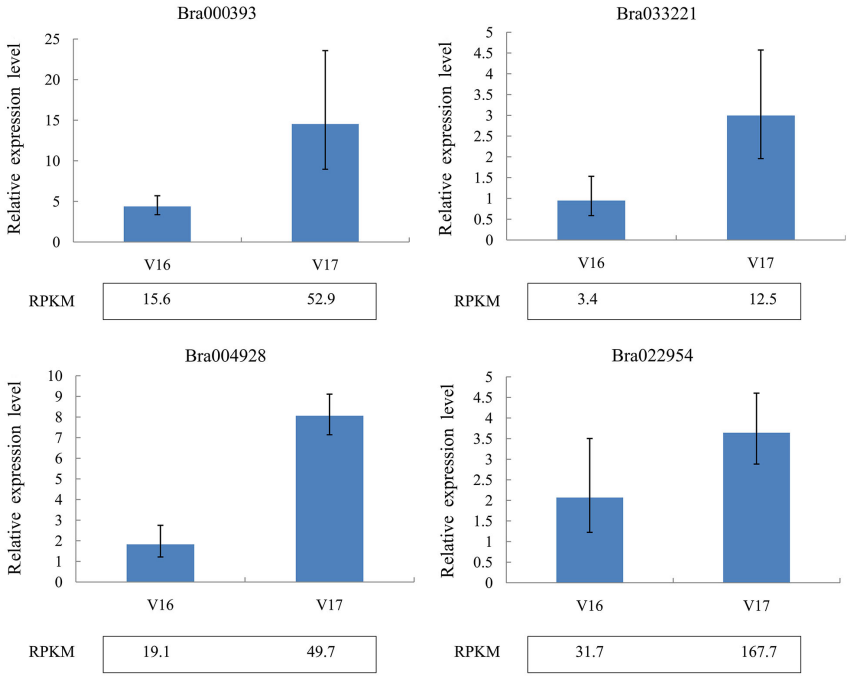

Figure 2. Quantitative real-time polymerase chain reaction validation of RNA-Seq results based on gene expression level. RPKM, reads per kb per million reads. Columns with error bars indicate relative quantification (RQ); RQmax and RQmin were calculated using an ABI 7500.

DISCUSSION

Previous research has shown that cells divide and differentiate into a layer of four sporophytic cells in the early stage of anther development, and that the mutation of early genes will cause a male-sterile phenotype (Scott et al., 2004). *SPL/NZZ* is a MADS-box transcription factor that is involved in the isolation and differentiation control of early cell differentiation in *Arabidopsis*. In *SPL/NZZ* mutant anthers, the formation of the archesporial cells is normal, but the primary sporogenous cells fail to differentiate into microsporocytes and the anther wall. The same problem also appears in the pistil mutant; therefore, either male or female *SPL* mutants are sterile (Schiefthaler et al., 1999; Yang et al., 1999). Therefore, *SPL/NZZ* plays an important role in the process of flower organ development. SPL8 is a member of the SBP-box protein family. Studies in *Arabidopsis* have shown that *SPL8* mutants exhibit a strong reduction in fertility. This reduced fertility is primarily attributable to abnormally developed microsporangia, which exhibit premeiotic abortion of the sporocytes (Unte et al., 2003). Another study showed that the constitutive overexpression of *SPL8* affects fertility due to non-dehiscent anthers, which probably result from a constitutive GA response, suggesting a positive role for *SPL8* in GA-mediated anther development (Zhang et al., 2007b). The results

of the present study suggest that the *SPL8* homolog Bra033221 is involved in micro- and megasporogenesis and anther development in pak choi; its expression was more upregulated in flower bud differentiation stage 5 than in stage 1, which is consistent with previous results in *A. thaliana*. This suggests that Bra033221 has similar functions as the *A. thaliana SPL8* gene in pak choi. However, the ways in which Bra033221 affects microspore development need further clarification. *SPL3* is also a member of the SBP-box protein family. Yamaguchi et al. (2009) suggested that *SPL3* acts together with other microRNA-regulated *SPL* transcription factors to control the timing of flower formation. Cardon et al. (1997) suggested that *SPL3* recognizes a conserved sequence motif in the promoter region of the *A. thaliana* floral meristem identity gene *AP1*. Similar to *AP1*, the constitutive expression of *SPL3* results in early flowering. In our study, the expression of the *SPL3* homolog Bra022954 was more upregulated in flower bud differentiation stage 5 than in stage 1, which is consistent with previous results in *A. thaliana.*

Flavonoids are a large class of important secondary metabolites in plants. Besides providing pigmentation to flowers, fruits, seeds, and leaves, flavonoids also play key roles in signaling between plants and microbes, in male fertility in some species, in defense as antimicrobial agents and feeding deterrents, and in ultraviolet protection (Winkel-Shirley, 2001). *DTX35* is a gene related to flavonoid metabolism in *A. thaliana.* Another study showed that *FFT* (*AtDTX35*) is highly transcribed in floral tissues. Mutant analysis has demonstrated that the absence of *FFT* transcripts affects flavonoid levels in the plant, and that altered flavonoid metabolism has wide-ranging consequences; root growth, seed development and germination, and pollen development, release, and viability are all affected (Thompson et al., 2010). In our experiment, flavonoid metabolism was significantly enriched in flower bud differentiation stages 1 to 5, indicating that flavonoid metabolism is closely related to the development of floral organs in pak choi. Bra013906, which is homologous to *AtDTX35*, is predicted to function in pollen development, anther dehiscence, pollen tube growth, and flavonoid metabolic processes in pak choi, which is consistent with previous results in *A. thaliana.*

Phenylpropanoids are a diverse class of plant natural compounds that are composed of a benzene ring connected to a three-carbon propene tail, and are members of a larger class of phenolic compounds. In this study, phenylpropanoid biosynthesis was significantly enriched in the process of floral organ development. Although no studies have found a direct relationship between phenylpropanoids and floral organ development, some have shown that the biosynthesis of flavonoid pigments in flowers requires the coordinated expression of genes that encode enzymes in the phenylpropanoid biosynthetic pathway (Sablowski et al., 1994). Moreover, flavonoid pigments have a direct relationship with floral organ development. Therefore, it is reasonable to assume that phenylpropanoid biosynthesis is significantly enriched during the development of floral organs in pak choi.

Linoleic acid is a natural antioxidant (Zhang et al., 2007a). The oleic acid dehydrogenase gene is an important functional gene that catalyzes the conversion of oleic acid to linoleic acid in peanuts. Research has shown that the relative expression level of the oleic acid dehydrogenase gene is low in roots, stems, and leaves of the peanut, while its expression is significantly higher in flowers than in other organs, which indirectly indicates that the linoleic acid content is highest in peanut flowers (Yin et al., 2013). We also found that linoleic acid metabolism was significantly enriched during flower organ development, but the specific relationship between linoleic acid and flower organ development requires further study.

We found no significant differences between flower bud differentiation stages 1 and 5 in the expression of most of the Chinese cabbage flowering genes, and their expression

patterns were mostly consistent with those observed in Chinese cabbage during flower bud differentiation. Moreover, the fold changes in the DEGs that were related to floral organ development were not large, meaning that the relative difference in floral organ developmental gene expression is not large between flower bud differentiation stages 1 and 5 in pak choi.

Conflicts of interest

The authors declare no conflict of interest.

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