Plant Mol Biol (2015) 88:233–248
DOI 10.1007/s11103-015-0317-y

Comparative analysis of chrysanthemum transcriptome in response to three RNA viruses: *Cucumber mosaic virus*, *Tomato spotted wilt virus* and *Potato virus X*

Hoseong Choi[1] · Yeonhwa Jo[1] · Sen Lian[1] · Kyoung-Min Jo[1] · Hyosub Chu[1] · Ju-Yeon Yoon[2] · Seung-Kook Choi[2] · Kook-Hyung Kim[1] · Won Kyong Cho[1]

Received: 26 September 2014 / Accepted: 2 April 2015 / Published online: 23 April 2015
© Springer Science+Business Media Dordrecht 2015

Abstract The chrysanthemum is one of popular flowers in the world and a host for several viruses. So far, molecular interaction studies between the chrysanthemum and viruses are limited. In this study, we carried out a transcriptome analysis of chrysanthemum in response to three different viruses including *Cucumber mosaic virus* (CMV), *Tomato spotted wilt virus* (TSWV) and *Potato virus X* (PVX). A chrysanthemum 135K microarray derived from expressed sequence tags was successfully applied for the expression profiles of the chrysanthemum at early stage of virus infection. Finally, we identified a total of 125, 70 and 124 differentially expressed genes (DEGs) for CMV, TSWV and PVX, respectively. Many DEGs were virus specific; however, 33 DEGs were commonly regulated by three viruses. Gene ontology (GO) enrichment analysis identified a total of 132 GO terms, and of them, six GO terms related stress response and MCM complex were commonly identified for three viruses. Several genes functioning in stress response such as chitin response and ethylene mediated signaling pathway were up-regulated indicating their involvement in establishment of host immune system. In particular, TSWV infection significantly down-regulated genes related to DNA metabolic process including DNA replication, chromatin organization, histone modification and cytokinesis, and they are mostly targeted to nucleosome and MCM complex. Taken together, our comparative transcriptome analysis revealed several genes related to hormone mediated viral stress response and DNA modification. The identified chrysanthemums genes could be good candidates for further functional study associated with resistant to various plant viruses.

Keywords Chrysanthemum · Virus infection · Transcriptome · Microarray · *Cucumber mosaic virus* · *Tomato spotted wilt virus* · *Potato virus X*

Introduction

The chrysanthemum plants belonging to the family Asteraceae have been widely cultivated for horticulture, gardening, floriculture, herbal medicine and insecticide (Hitmi et al. 2000; Wu et al. 2010). Polyploidy and hybridization have been known as major factors for the speciation and genetic diversity of the genus Chrysanthemum (Liu et al. 2012; Yang et al. 2006). So far, numerous commercial chrysanthemum cultivars with polyploidy have been developed for cut flowers and pot plants using hybridization (Ding et al. 2008; Li et al. 2013).

Several pathogens including viruses, viroids, fungi and phytoplasma cause serious damages on the production of chrysanthemum cultivars. In particular, the chrysanthemum is the host for at least nine viruses including *Chrysanthemum*

Hoseong Choi and Yeonhwa Jo have contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11103-015-0317-y) contains supplementary material, which is available to authorized users.

✉ Kook-Hyung Kim
kookkim@snu.ac.kr

✉ Won Kyong Cho
wonkyong@gmail.com

[1] Department of Agricultural Biotechnology, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Republic of Korea

[2] Virology Unit, Department of Horticultural Environment, National Institute of Horticultural and Herbal Science, RDA, Wan-Ju 565-852, Republic of Korea

B virus (CVB), *Cucumber mosaic virus* (CMV), *Potato virus Y*, *Potato virus X* (PVX) *Tobacco mosaic virus*, *Tomato aspermy virus*, *Tomato spotted wilt virus* (TSWV) and *Turnip mosaic virus* (Verma et al. 2003) as well as two viroids like *Chrysanthemum stunt viroid* and *Chrysanthemum chlorotic mottle viroid* (Cho et al. 2013). To control viruses and viroids with chemical treatments and culturing approaches are still challenging in chrysanthemum plants. To make chrysanthemum cultivars resistant to viruses and viroids, it is important to identify chrysanthemum cultivars or to generate genetically modified (GM) chrysanthemums resistant to viral stresses.

Development of genetically modified (GM) chrysanthemums is one of effective ways to introduce new traits into the chrysanthemum. For instance, GM chrysanthemums with white flowering variety have been developed using chimeric *chalcone synthase* (*CHS*) gene (Courtney-Gutterson et al. 1994). Therefore, it is necessary to identify and characterize useful chrysanthemum genes for the GM chrysanthemum. Recently, several studies have identified chrysanthemum genes related to flowering and abiotic stresses (An et al. 2014; Fu et al. 2014; Liu et al. 2014; Song et al. 2014a, b; Tong et al. 2009; Yang et al. 2014). Of them, the overexpression of chrysanthemum *dehydration responsive element binding factor 1* (*DREB1*) showed delayed flowering and strong resistance against freezing and drought stresses (Tong et al. 2009). Another chrysanthemum *BBX24*, which is a zinc finger transcription factor gene, has been characterized to function flowering and abiotic stresses (Yang et al. 2014). Both *DREB1* and *BBX24* genes can be usefully applied for GM chrysanthemum plants with delayed flowering and tolerance to abiotic stresses.

Although the chrysanthemum plants are one of economically important flower plants, biochemical and molecular genetic studies for the chrysanthemum are limited. One of main obstacles for chrysanthemum genetic and genome study is polyploidy. Therefore, transcriptome based approach has been recently performed to provide expressed sequence tags (ESTs) from chrysanthemum inflorescence (Chen et al. 2009). Moreover, next generation sequencing (NGS) has provided a large number of chrysanthemums mRNAs (Jo et al. 2014; Wang et al. 2013, 2014). In addition, NGS can be used to examine gene expression profiles of chrysanthemum under dehydration stress (Xu et al. 2013) and *Alternaria tenuissima* inoculation (Li et al. 2014). Furthermore, the generated sequencing data has been used for development of simple repeat markers (Wang et al. 2013). Furthermore, cDNA-amplified fragment length polymorphism (AFLP) technology has been applied to find genes associated with floral development (Ren et al. 2013) and salt stress (He et al. 2012).

Several genome wide transcriptome analysis have been performed for model plants in response to diverse plant viruses. For instance, PVX infection in *Nicotiana benthamiana* (García-Marcos et al. 2009), for CMV infection in *Arabidopsis* (Marathe et al. 2004; Whitham et al. 2003), *N. tabacum* (Lu et al. 2012), and tomato (Lang et al. 2011), for TSWV infection in tomato (Catoni et al. 2009) have performed to establish expression profiles upon virus infection. However, study on gene expression profiles of the non-model plants such as chrysanthemum is limited.

In this study, we for the first time established a microarray system to examine gene expression profiles of chrysanthemum in response to different plant viruses. For that, we selected three viruses which infect a wide range of plants including many commercially important vegetables. Finally, two positive single strand (ss) RNA viruses such as PVX in the genus *Potexvirus* and CMV in the genus *Cucumovirus* as well as one negative ssRNA virus such as TSWV in the genus *Tospovirus* were chosen. Expression profiles using a chrysanthemum microarray revealed commonly and differentially regulated chrysanthemum genes in response to three different viruses. The identified chrysanthemum genes will be good candidates for functional characterization in association with virus infection and they can be applied to develop GM chrysanthemum plants resistant to various plant viruses in near future.

Materials and methods

Plant propagation and virus inoculation

Dendranthema grandiflorum Ramatuelle cultivar Shinma was obtained from the National Institute of Horticultural and Herbal Science in Korea and propagated in vitro in Murashige and Skoog (MS) solid medium containing 30 % sucrose and 0.1 % activated carbon. Five-week old chrysanthemum plants propagated by cuttage which were around 30 cm tall containing several leaves were used for virus inoculation. For virus inoculation, sap was prepared from approximately 0.5 μg of each virus infected tissues by homogenizing with the buffer containing 50 Mm potassium phosphate (pH 7.5). Mechanical inoculation was carried out on the leaves of chrysanthemum using carborundum. Mock samples were treated with inoculation buffer. After inoculation, plants were continually grown in growth chamber. Three different plants for each virus were inoculated. Three viruses were not co-inoculated. As a result, nine and three plants were used for virus and mock inoculation, respectively. After virus infection, no viral symptoms were observed for all virus inoculated chrysanthemum plants at 5 days after virus inoculation. We performed RT-PCR to confirm infection of each virus in inoculated leaves (Supplementary Fig. 1). In addition, we also inoculated same virus sap for PVX, CMV, and TSWV,

respectively, on the leaves of *Nicotiana benthamiana*. We could detect viral RNAs but did not observe any virus symptoms on the inoculated leaves. All chrysanthemum plants used in this study were grown in growth chamber at 25 °C for 16 h/8 h. We collected virus inoculated leaves which did not display any viral symptoms and used for total RNA extraction.

Samplings of seedlings and total RNA extraction

To examine expression profiles in local symptoms, inoculated leaves were used. Each virus-infected and mock-treated samples were harvested at 5 days post infection and immediately frozen in liquid nitrogen. Total RNA was extracted using Qiagen RNeasy plant mini kit (Qiagen GmbH, Hilden, Germany) following manufacturer's manual. To confirm virus infection in each sample, reverse-transcription polymerase chain reaction (RT-PCR) was performed. First strand cDNA was synthesized using GoScript™ Reverse Transcriptase (Promega Corp, Madison, USA) and PCR amplification was done using Takara Ex Taq™ Polymerase (Takara, Kusatsu, Japan) using virus specific primers (Supplementary Table S1). PCR products were analyzed by agarose gel electrophoresis (Supplementary Fig. S2).

Design of microarray probes

The 13,687 consensus sequences were obtained from 18,784 ESTs after removing redundant sequences. In addition, 85 sunflower chloroplast genes were also included (Timme et al. 2007). Seven probes with 60-nt long were designed for each gene started from 250 bp ahead to the end of stop codon by shifting 15 bp gradually. As a result, seven probes covered 150 bp in the 3′ region of each gene. Moreover, 50 mitochondrial genes and several selection markers encoding GFP, GUS, HYG, BAR, and KAN were included. In total, 92,448 probes were designed. The average size of probe was 60-nt long with adjusting its Tm value from 75 to 85 °C. The microarray chips were manufactured at NimbleGen Inc (http://www.nimblegen.com/). Random GC probes (40,000) to monitor the hybridization efficiency and four corner fiducial controls (225) were included to assist with overlaying the grid on the image.

cDNA library preparation and microarray hybridization

Twelve different total RNAs were subjected for cDNA library preparation. For the synthesis of double strand cDNAs, RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Ontario, Canada) was used. Briefly, 1 µl of oligo dT primer (100 mM) and 10 µl (10 µg) of total RNA were mixed and denatured at 70 °C for 5 min and renatured by cooling the mixture on ice. The 4 µl of 5X First Strand Buffer, 1 µl of RiboLock™ Ribonuclease Inhibitor, 2 µl of 10 mM dNTP mix and 1 µl of RevertAid™ H Minus M-MuLV Reverse Transcriptase enzyme were mixed together and used for First strand DNA synthesis by incubating at 42 °C for 1 h. To stop the reaction, mixture was heated at 70 °C for 10 min. To synthesize the second strand, 66.7 ml of nuclease free water, 5 µl of 10X reaction buffer for DNA Polymerase I (Fermentas), 5 µl of 10X T4 DNA ligase buffer (Takara, Kyoto, Japan), 3 µl of 10 U/µl DNA Polymersase I (Fermentas), 0.2 µl of 5U/µl Ribonuclease H (Fermentas) and 0.1 µl of 350 U/µl T4 DNA ligase (Takara) were added to the first strand reaction mixture and the reaction was proceeded at 15 °C for 2 h. Double stranded cDNA mixture was purified using MinElute Reaction Cleanup Kit (QIAGEN, Valencia, USA). For the synthesis of Cy3-labeled target DNA fragments, 1 mg of double strand cDNA was mixed with 30 µl (1 OD) of Cy3-9mer primers (Sigma-Aldrich, St. Louis, USA) and denatured by heating at 98 °C for 10 min. The reaction was further proceeded by adding 10 µl of 50X dNTP mix (10 mM each), 8 µl of deionized water, 2 µl of Klenow fragment (50 U/ml, Takara) and incubating at 37 °C for 2 h. DNA was precipitated by centrifugation at 12,000×*g* after adding 11.5 µl of 5 M NaCl and adding 110 µl of isopropanol. Precipitated samples were rehydrated with 13 µl of water. The concentration of each sample was determined by using spectrophotometer. 10 µg of DNA was used for microarray hybridization. The sample was mixed with 19.5 µl of 2X hybridization buffer (Nimblegen, Delaware, USA) and finalized to 39 µl with deionized water. Hybridization was performed with MAUI chamber (Biomicro, Salt Lake City, USA.) at 42 °C for 16–18 h. After the hybridization, the microarray was removed from MAUI Hybridization Station and immediatly immersed in the shallow 250 ml Wash I (Nimblegen) at 42 °C for 10–15 s with gentle agitation and then transferred to the second dish of Wash I and incubated for 2 min with gentle agitation. The microarray was transferred into dish of Wash II and further washed in Wash III for 1 min with agitation. The microarray was dried in a centrifuge for 1 min at 500 g and scanned using GenePix scanner 4000B (Axon, Union City, USA)

Microarray data analysis

The microarray was scanned with Genepix 4000 B (Axon) preset with a 5 µm resolution and for Cy3 signal. Signals were digitized and analyzed by Nimblescan (Nimblegen). The grid was aligned to the image with a chip design file, NDF file. The alignment was checked by ensuring that the

grid's corners are overlaid on the images corners. This was further checked by uniformity scores in the program. An analysis was performed in a two-part process. First pair reports (.pair) files were generated in which sequence, probe, and signal intensity information for Cy3 channel were collected. Data-based background subtraction using a local background estimator was performed to improve fold change estimates on arrays with high background signal. The data was normalized and processed with cubic spline normalization using quantiles to adjust signal variations between chips (Workman et al. 2002). Probe-level summarization by Robust Multi-Chip Analysis (RMA) using a median polish algorithm implemented in NimbleScan was used producing calls files. The method identifies probes that are outliers in the overall behavior of the expression measured for a given gene and the contribution by those outliers is reduced in the reported gene expression level. This improves the sensitivity and reproducibility of microarray results (Irizarry et al. 2003). Raw data were deposited in NCBI's GEO database with an Accession Number GSE55985.

Multiple analysis was performed with limma package in R computing environment (Smyth 2004). The package adopts the linear modeling approach implemented by lmFit and the empirical Bayes statistics implemented by eBayes. Genes of which adjusted p value or false discovery below 0.05 were collected and further selected for those gene expressions were higher than 1 or less than -1 at least at the virus infected sample compared to that of mock sample. Multivariate statistical tests such as clustering, principal component analysis, and multidimensional scaling were performed with Acuity 3.1 (Axon Instruments). Hierarchical clustering was performed with similarity metrics based on squared Euclidean correlation and average linkage clustering was used to calculate the distance of genes using genesis (Sturn et al. 2002).

Gene enrichment analysis

We divided differentially expressed gene (DEG) into down-regulated and up-regulated DEG at each virus infected condition. To identify enriched GO terms in each group containing lists of DEG, we used the Gossip package (Blüthgen et al. 2005) implemented in Blast2GO (Conesa et al. 2005) which uses the Fisher's Exact Test and corrects for multiple testing. For chrysanthemum annotation, all EST sequences used for microarray probes were subjected for Blast2GO analysis and the obtained gene ontology was used as the reference file. To identify enriched GO terms, GO terms with FDRs less than 0.05 were collected.

Quantitative real-time RT-PCR

The primers used for real-time PCR were designed using PrimerSelect (DNASTAR, Madison, USA) following the manual and were listed in Supplementary Table 1. Two fragments for a actin gene (Accession Number JN638568.1) was used as a reference gene based on the previous study which demonstrated that the actin gene among eight tested reference genes was generally stable in various stress conditions for quantitative real-time RT-PCR in chrysanthemums (Gu et al. 2011). cDNAs of each sample were synthesized by SuperScript® III (Invitrogen, Carlsbad, USA) reverse transcriptase mixture using oligo (dT 20) (Invitrogen) as reverse primers. Real-time PCR was performed with a Bio-Rad CFX384 Real-time PCR system (Bio-Rad, Hercules, USA) in Bio-Rad iQTM SYBR® Green Supermix (Bio-Rad) reagents according to manufacturer protocols. Briefly, real-time PCR amplification reaction for the each genes was performed in 10 μL volume including 5 μL of iQTM SYBR® Green Supermix, 10 ng cDNA and 10 pm forward and reverse primers, respectively. The PCR cycling condition consisted of an initial 3 min 95 °C cycle followed by 40 cycles of 30 s at 95 °C, and 30 s at 55 °C. Data analysis was carried out by Bio-Rad CFX Manager software (Version 3.1).

Results

Generation of a chrysanthemum microarray and transcriptome analysis in response to three different viruses

To examine changes of the chrysanthemum transcriptome in response to diverse viruses, we generated a microarray based on previously published ESTs data derived from flower and leave tissues (Chen et al. 2009; Jo et al. 2014). The microarray probes were consisted of chrysanthemum 13,251 nuclear genes as well as 85 chloroplast-encoded genes of the sun flower belonging to the family Asteraceae (Timme et al. 2007). One month-old chrysanthemum plants were prepared by cuttage. Three different viruses were independently infected by the direct rub-inoculation with sap and mock treated plants were used as controls. A total of 12 samples as well as 12 DNA chips were used for microarray analysis (Supplementary Table 2). Microarray and data analyses were performed as described in materials and methods.

Identification of differentially expressed genes in response to three different viruses

We compared expression profiles of each virus infected condition to those of mock-treated condition to get expression ratios (fold changes) and respective p values by averaging data from three different biological replicates (Supplementary Table 3). To identify DEG in response to

each virus infection, we applied a fold-change cutoff of 2 and *p* value threshold of 0.05 (Supplementary Table 4). To examine global gene expression patterns, volcano plots were generated by using fold changes and *p* values (Fig. 1a). CMV and PVX caused dramatic transcriptional changes of the chrysanthemum transcriptome than TSWV. Only less than 1 % of genes were identified as DEGs. In final, we identified 127 DEGs (CMV), 72 DEGs (TSWV), and 125 DEGs (PVX) (Supplementary Table 4). Interestingly, the number of up-regulated genes was more than two times as compared to that of down-regulated genes by CMV and PVX, respectively (Fig. 1b).

Next, we compared the number of DEG among three conditions. Many genes displayed virus specific expression. For example, 46, 39 and 26 genes were specifically expressed by CMV, TSWV and PVX, respectively (Fig. 2a). Thirty-three genes were commonly identified in three conditions (Table 1; Fig. 2a). Of them, 22 genes showed up-regulation while 11 genes displayed down-regulation (Fig. 2b, c). In addition, 35 genes (CMV), 6 genes (TSWV), and 25 genes (PVX) exhibited virus specific up-regulation (Fig. 2b). Moreover, 11 genes (CMV), 20 genes (TSWV), and 13 genes (PVX) showed virus specific down-regulation (Fig. 2c).

Of identified DEGs, genes encoding dehydrin (ESTC0 08904), nuclease HARBI1-like protein (ESTC000886), and 9-*cis*-epoxycarotenoid dioxygenase chloroplastic-like protein (ESTC000686) were strongly up-regulated by CMV (Supplementary Table 4). TSWV infection strongly up-regulated genes encoding nematode resistance HSPRO2-like protein (ESTC000887), ethylene responsive factor (ERF)/AP2 transcription factor (TF) (ESTC004608) and ERF109-like protein (ESTC001650). By PVX infection, genes encoding nuclease HARBI1-like protein (ESTC000886), alcohol dehydrogenase-like 3-like protein (ESTC001297) and nematode resistance HSPRO2-like protein (ESTC000887) were strongly up-regulated. Twenty-two genes such as genes encoding calcium-binding protein, glycosyltransferase

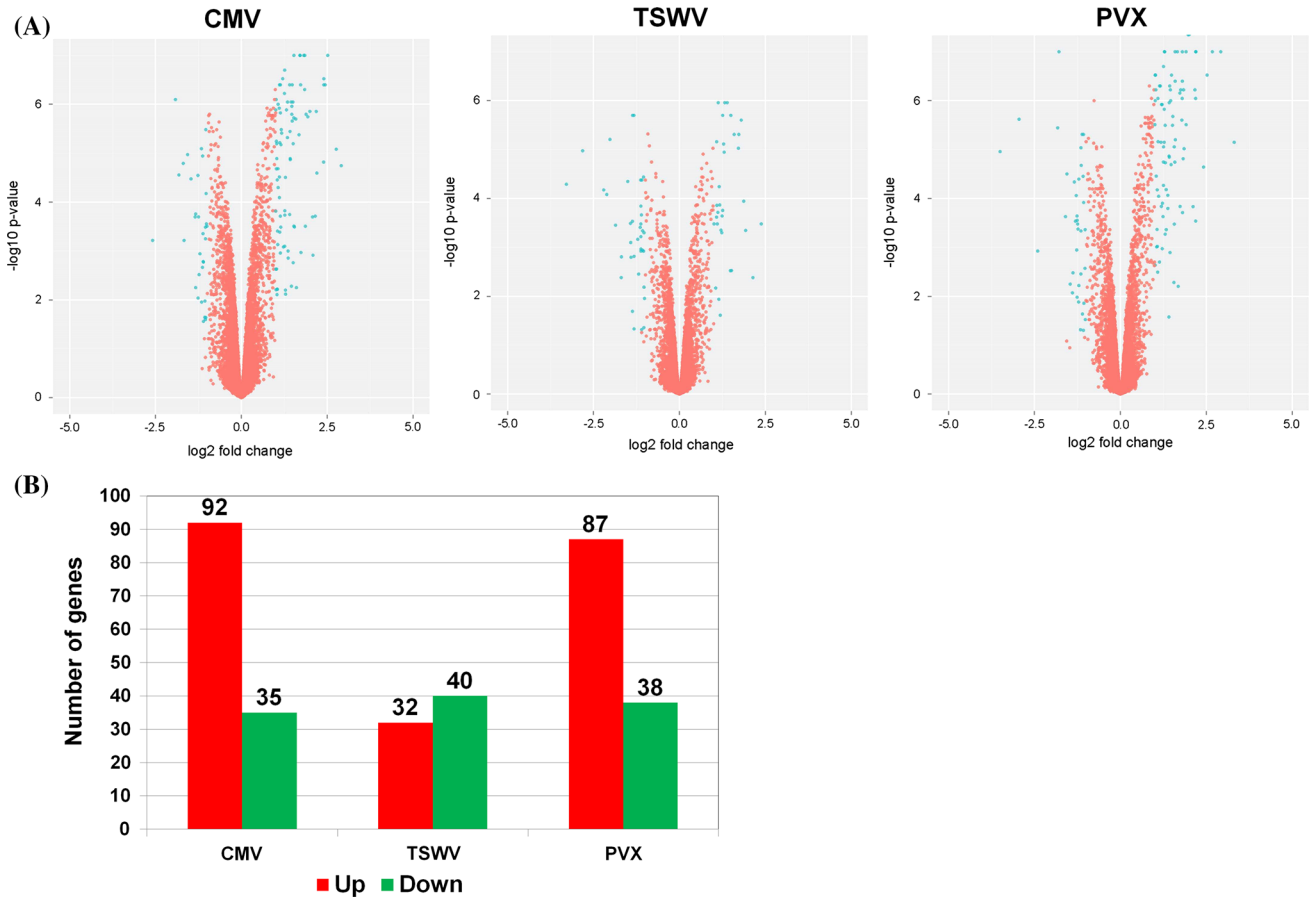


Fig. 1 Identification of differentially expressed chrysanthemum genes in response to infection of three different viruses. **a** Volcano plots display log2 converted fold changes and *p* values. To identify differentially expressed genes, each virus infected sample was compared to mock treated sample. Obtained fold changes and *p* values were converted to log2 to generate volcano plots using ggplot2 program implemented in R program. More than twofold changes and *p* values less than 0.05 were applied to identify DEG indicated by blue-colored dots. **b** The number of DEG in response to individual virus infection. *Blue* and *red colored bars* indicate down- and up-regulated DEGs, respectively, in each virus infected sample

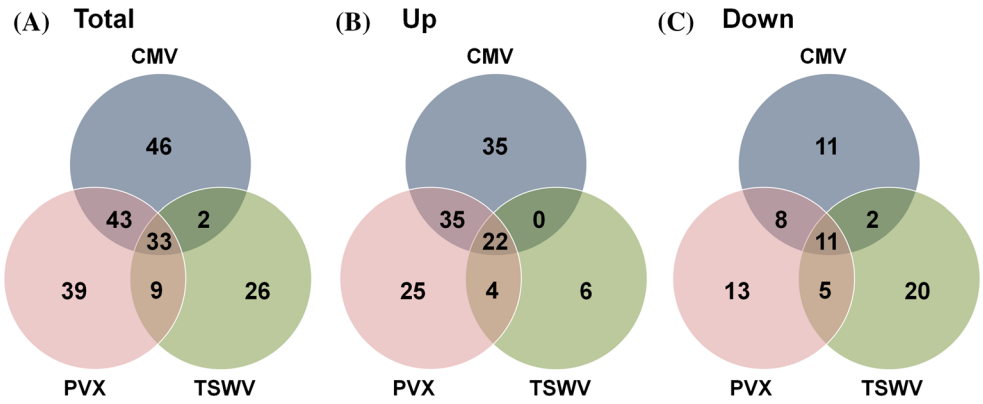


Fig. 2 Comparison of the number of DEGs among three different gene sets. *Venn diagrams* display the number of DEGs from three different gene sets. A total of 127, 72, 125 DEGs from CMV, TSWV, and PVX, respectively, were compared. *Total*, *up* and *down* indicate identified total number of DEGs, only up-regulated DEGs and only down-regulated DEGs, respectively

family protein and an ERF TF were up-regulated by all three viruses while 11 genes including genes encoding xyloglucan-specific fungal endoglucanase inhibitor protein precursor and ribonucleoside-diphosphate reductase small chain-like protein were down-regulated by three viruses.

Go enrichment analysis to identify important functions associated with virus infection

To reveal function of DEGs in response to each virus, we conducted gene ontology (GO) enrichment analysis implemented in Blast2GO program. A total of 132 GO terms were significantly enriched in three conditions (Supplementary Table 5, Fig. 3a). Only 8 and 39 terms were identified from CMV and PVX, respectively while 85 GO terms were obtained from TSWV. Six GO terms were commonly identified in three conditions (Fig. 3b). They are related to endogenous stimulus, chitin, organonitrogen compound response, and DNA unwinding function according to biological process (Fig. 3c) and MCM complex was solely identified according to cellular component (Fig. 3d).

CMV infection up-regulated expression of genes involving in stresses as well as beta-carotene 15-monooxygenase and serine/threonine phosphatase activities (Supplementary Table 5). TSWV infection resulted in up-regulation of genes related to external or internal stimulus (TSWV) (Supplementary Fig. 2A). Of them, genes responding to chitin including as CCR4-associated factor, nematode resistance HSPRO2 and protein phosphatase 2C (PP2C) were identified as major DEGs (Table 2). In particular, up-regulation of three ERF TF genes indicated association of ethylene mediated signaling pathway for plant defense response against TSWV infection. Down-regulated genes in response to TSWV were related to DNA metabolic process (Supplementary Table 5 and Supplementary Fig. 2B). For example, these genes were involved in DNA replication, chromatin organization and cytokinesis by cell plate formation. The genes including mitotic spindle checkpoint protein, ribonucleoside-diphosphate reductase, prolifera, kinesin, cyclin-dependent kinase and DNA replication licensing factor are required for DNA-dependent DNA replication (Table 2). Four genes encoding histone required for chromatin organization were down-regulated by TSWV. These genes including kinesin-1, cyclin-dependent kinase b2-2, DNA replication licensing factor MCM3 homolog 1 are required for assembly of chromatin, nucleosome, and MCM (eukaryotic genome DNA replication) complex. In addition, genes required for methylation like histone methylation and phosphorylation were also up-regulated.

Up-regulated genes by PVX were also involved in response to various stresses such as chitin, nitrogen compound and oxygen-containing compound (Supplementary Table 5 and Supplementary Fig. 2C). Those genes were also up-regulated by PVX and were related to defense response to establish host immune system (Table 3). In addition, genes required for nucleic acid processing function such as mRNA 3′-end processing, nuclear-transcribed mRNA poly(A) tailing shortening, nuclear-transcribed mRNA catabolic process and 3′-5′ exonuclease activity were up-regulated by PVX infection (Supplementary Table 5). The genes involving in mRNA 3′-end processing are identified as four chrysanthemum CCR-associated factor 1 gene (Table 3). Furthermore, PVX infection up-regulated ethylene response related genes encoding CCR-associated factor 1 and ERF TFs (Table 3).

Gene expression of TFs and chloroplast genes

Using known *Arabidopsis* TF database, at least 610 chrysanthemum TFs were present on microarray chips. To

Table 1 The 33 chrysanthemum DEGs commonly regulated by three different viruses

EST ID	Arabidopsis	Function	CMV_logFC	TSWV_logFC	PVX_logFC
ESTC000200	AT5G22250.1	Probable ccr4-associated factor 1 homolog 11	1.256034	1.259653	1.5913322
ESTC000379	AT3G15210.1	Ethylene-responsive transcription factor 4	1.531765	1.3909958	1.6590381
ESTC000791	AT2G30020.1	Probable protein phosphatase 2c 25	1.405054	1.0822603	1.4890028
ESTC000886	AT5G12010.1	Nuclease harbi1	2.40309	1.7283498	2.6759604
ESTC000887	AT3G55840.1	Nematode resistance hspro2	1.736346	2.3807041	2.1903035
ESTC001052	AT2G41640.2	Glycosyltransferase family 61 protein	1.469965	1.3003534	1.8024769
ESTC001150	AT3G04290.1	GDSL esterase lipase at5g33370	−1.10589	−2.8175691	−2.9480555
ESTC001463	AT4G34150.1	Protein binding	2.442582	1.1181634	2.5234291
ESTC001529	AT4G33920.1	Probable protein phosphatase 2c 63	1.864904	1.1466094	2.1146498
ESTC001599	AT1G10560.1	u-box domain-containing protein 19	2.181185	1.1028794	1.913781
ESTC001953	AT5G42050.1	Uncharacterized loc101208173	1.583855	1.1578814	1.7975921
ESTC002279	AT3G27060.1	Ribonucleoside-diphosphate reductase small chain	−1.06478	−1.6942268	−1.3953897
ESTC002497	AT1G03220.1	Basic 7s globulin	−2.58393	−1.3201636	−2.4064899
ESTC002782	AT4G02060.2	Protein prolifera	−1.34655	−1.4244452	−1.2324075
ESTC002839	AT3G10520.1	Non-symbiotic hemoglobin 2	−1.56541	−1.5038269	−1.8249699
ESTC003432	AT1G03220.1	Basic 7s globulin	−1.03521	−1.034402	−1.4604319
ESTC004433	AT5G33370.1	GDSL esterase lipase at5g33370	−1.33041	−3.2903619	−3.5007152
ESTC004467	AT5G12010.1	Nuclease harbi1	1.283779	1.0807177	1.5997668
ESTC004645	AT4G34050.1	Caffeoyl-*o*-methyltransferase	1.262329	1.1283672	1.9689032
ESTC004760	AT1G76650.3	Probable calcium-binding protein cml31	1.528401	1.7985957	1.8379139
ESTC005732	AT5G46280.1	DNA replication licensing factor mcm3 homolog 1	−1.1621	−1.0931597	−1.14768
ESTC007280	No homology	Unknown function	−1.00369	−1.1226339	−1.3400621
ESTC007606	No homology	Ethylene-responsive transcription factor 3	1.423385	1.0057652	1.4166209
ESTC009326	No homology	Xyloglucan-specific fungal endoglucanase inhibitor protein precursor	−1.6924	−1.1213372	−1.5556363
ESTC009590	No homology	Protein ndr1	1.314914	1.4957855	1.454499
ESTC009824	No homology	Organ-specific protein s2	−1.3309	−1.2124214	−1.257847
ESTC010579	No homology	Yeast pheromone receptor	1.509424	1.7169262	2.1696602
ESTC010769	No homology	Probable calcium-binding protein cml45	1.818084	1.3183837	2.0020141
ESTC011407	No homology	Unknown function	2.909496	1.5155375	3.3121574
ESTC011821	No homology	Unknown function	1.546061	1.05514	1.5681043
ESTC012643	No homology	MYB transcription factor	1.066975	1.593313	1.7307589
ESTC013192	No homology	Unknown function	1.72668	1.2863566	2.1976883
ESTC013292	No homology	Unknown function	1.997028	1.1723039	2.920275

The homologous *Arabidopsis* genes were obtained by blast search. Function of each EST was obtained by blast search against NCBI non-redundant protein database implemented in Blast2GO program. LogFC indicates $\log_2$ converted fold changes

identify differentially expressed TFs, hierarchical clustering was performed to identify three groups of TFs (Fig. 4a). Forty-three TFs in the group A were down-regulated while 24 genes in group B and 88 genes in group C were up-regulated (Fig. 4b). In particular, eight genes in group B were members of the ERF TF family. In case of chloroplast encoded genes, most chloroplast genes were down-regulated by three viruses (Fig. 4c). However, some chloroplast genes encoding photosystem II and cytochrome b6/f complexes were slightly up-regulated by CMV.

Validation of microarray data by real time RT-PCR

To validate results of microarray analysis, we performed quantitative real time RT-PCR with six selected genes encoding ethylene-responsive transcription factor 4, phosphatase 2c 25, ribonucleoside-diphosphate reductase small chain, basic 7s globulin, GDSL esterase lipase and a unknown function protein (Supplementary Table 1). Completely independent biological samples were prepared for real time RT-PCR. Two fragments for an *actin* gene were

Table 2 Significantly enriched GO terms in up- and down-regulated gene sets by TSWV infection

EST ID	Arabidopsis	Function	CMV	TSWV	PVX
GO: response to chitin (up regulated by TSWV)					
ESTC000200	AT5G22250.1	Probable ccr4-associated factor 1 homolog 11	1.256034	1.259653	1.591332
ESTC000379	AT3G15210.1	Ethylene-responsive transcription factor 4	1.531765	1.390996	1.659038
ESTC000887	AT3G55840.1	Nematode resistance hspro2	1.736346	2.380704	2.190304
ESTC001052	AT2G41640.2	Glycosyltransferase family 61 protein	1.469965	1.300353	1.802477
ESTC001463	AT4G34150.1	Protein binding	2.442582	1.118163	2.523429
ESTC001485	AT5G47230.1	Ethylene-responsive transcription factor 5	0.9296	1.232555	1.827593
ESTC001529	AT4G33920.1	Probable protein phosphatase 2c 63	1.864904	1.146609	2.11465
ESTC001650	AT4G34410.1	Ethylene-responsive transcription factor erf109	−0.24512	1.927128	−0.90946
GO: DNA-dependent DNA replication (down regulated by TSWV)					
ESTC001056	AT3G25980.1	Mitotic spindle checkpoint protein mad2	−0.56604	−1.01625	−0.69255
ESTC002279	AT3G27060.1	Ribonucleoside-diphosphate reductase small chain	−1.06478	−1.69423	−1.39539
ESTC002782	AT4G02060.2	Protein prolifera	−1.34655	−1.42445	−1.23241
ESTC004117	AT4G05190.1	Kinesin-1	−0.87373	−1.32037	−0.96524
ESTC004140	AT1G20930.1	Cyclin-dependent kinase b2-2	−0.80614	−1.40868	−1.14895
ESTC005732	AT5G46280.1	DNA replication licensing factor mcm3 homolog 1	−1.1621	−1.09316	−1.14768
GO: chromatin organization (down regulated by TSWV)					
ESTC000196	AT5G65360.1	Histone	−0.77735	−1.04321	−0.69455
ESTC001056	AT3G25980.1	Mitotic spindle checkpoint protein mad2	−0.56604	−1.01625	−0.69255
ESTC002062	AT5G02560.1	Histone h2a	−0.70054	−1.12197	−0.758
ESTC002782	AT4G02060.2	Protein prolifera	−1.34655	−1.42445	−1.23241
ESTC003209	AT5G02560.1	Histone h2a	−0.77225	−1.36372	−0.92959
ESTC004117	AT4G05190.1	Kinesin-1	−0.87373	−1.32037	−0.96524
ESTC004140	AT1G20930.1	Cyclin-dependent kinase b2-2	−0.80614	−1.40868	−1.14895
ESTC005732	AT5G46280.1	DNA replication licensing factor mcm3 homolog 1	−1.1621	−1.09316	−1.14768
ESTC006025	AT5G65360.1	Histone	−0.6633	−1.12811	−0.87299
GO: cytokinesis by cell plate formation (down regulated by TSWV)					
ESTC001511	AT4G15830.1	Protein fam179b	−0.70367	−1.04558	−0.81109
ESTC003685	AT1G08560.1	Syntaxin-related protein knolle	−0.76062	−1.36695	−1.14713
ESTC004117	AT4G05190.1	Kinesin-1	−0.87373	−1.32037	−0.96524
ESTC004140	AT1G20930.1	Cyclin-dependent kinase b2-2	−0.80614	−1.40868	−1.14895
ESTC005732	AT5G46280.1	DNA replication licensing factor mcm3 homolog 1	−1.1621	−1.09316	−1.14768

Chrysanthemum genes with respective homologous Arabidopsis gene were assigned to each identified GO term. The number indicates $\log_2$ converted fold changes

used as a reference gene. Two repeated real time RT-PCR results displayed reliable standard deviations (Fig. 5a). Except one gene encoding basic 7s globulin, all five genes showed strong correlation between microarray results and RT-PCR results (Fig. 5b). For instance, fold changes of the PP2C 25 gene (ESTC000379) as compared to mock by RT-PCR were higher than microarray data.

Comparison of chrysanthemum microarray data with the previous *Arabidopsis* microarray data in response to RNA viruses

Previously, several studies performed transcriptome analyses in response to various plant viruses (Ascencio-Ibáñez et al. 2008; Marathe et al. 2004; Whitham et al. 2003). It might be of interest to compare DEGs between two different hosts upon same virus infection. Of them, we selected a study which performed a comprehensive *Arabidopsis* transcriptome analysis in response to five viruses including CMV and PVX using *Arabidopsis* GeneChip microarrays (Affymetrix) (Whitham et al. 2003). To compare two studies, all chrysanthemum DEGs were converted to corresponding *Arabidopsis* gene loci based on sequence similarity. However, many chrysanthemum DEGs were chrysanthemum specific genes. Unexpectedly, only a gene out of 94 non-redundant *Arabidopsis* DEGs in response to five RNA viruses was overlapped with the list of DEGs upon PVX infection (Supplementary Fig. 3). The

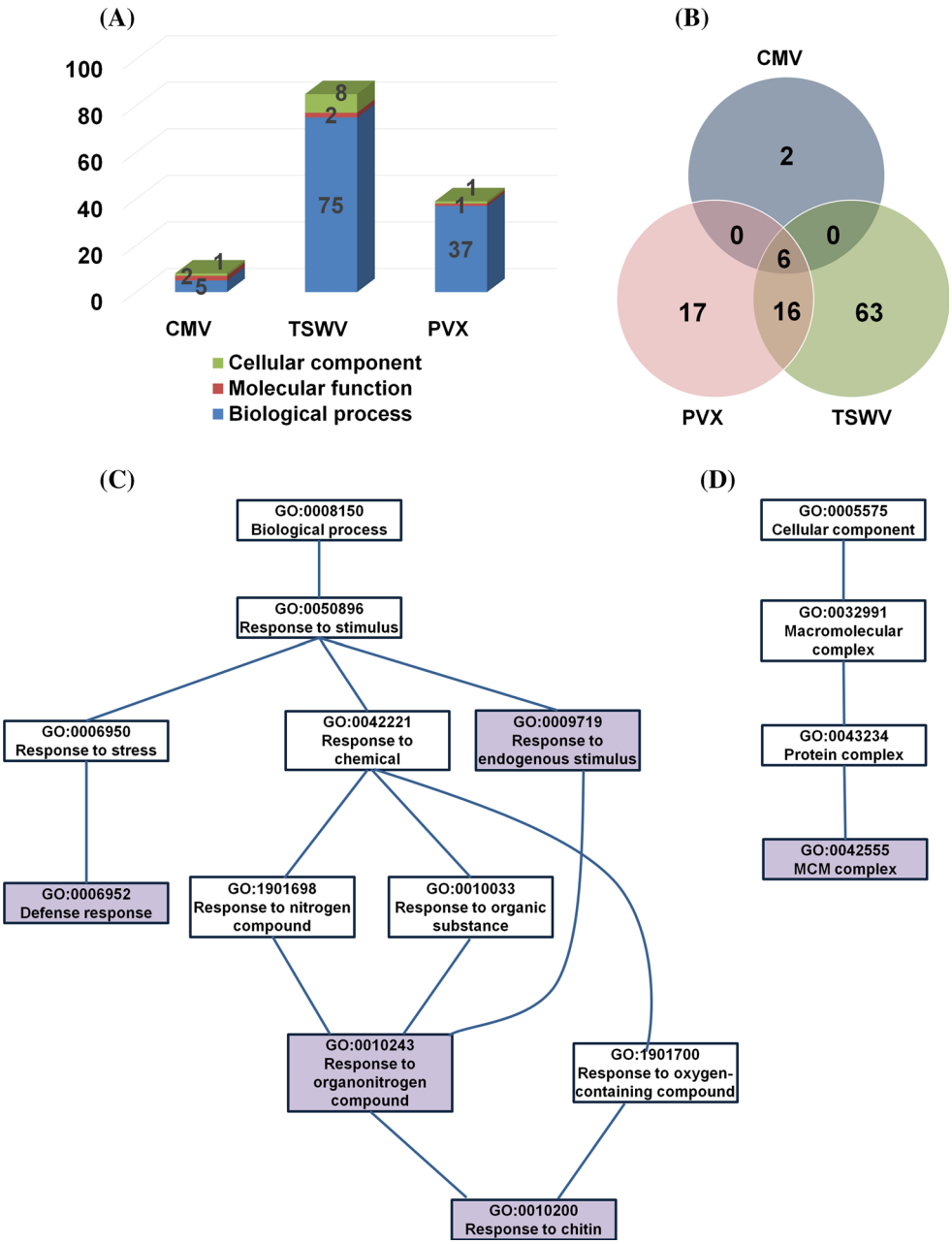


Fig. 3 Comparison of identified enriched GO terms from three different gene sets. **a** The number of identified GO terms by gene enrichment analysis according to three categories. **b** Comparison of identified enriched GO terms among three different gene sets. *Venn diagram* illustrating the number of overlapped and specific GO terms for each condition. Commonly identified GO terms according to biological process (**c**) and cellular component (**d**). A detailed information for enriched GO terms is available in Supplementary Table 3. The directed acyclic graph (DAG) illustrates the relationships of the GO terms, which are highly over-represented in the chrysanthemum transcriptome in response to three virus infection. *Violet colored box* indicates identified enriched GO terms

gene (AT2G22500) is one of the mitochondrial dicarboxylate carriers (Palmieri et al. 2008). However, members of some gene families such as protein kinase, phosphatase, ubiquitin, heat shock protein, ethylene responsive factor and cytochrome P450-like protein were commonly identified in both studies.

Discussion

In this study, for the first time, we generated a chrysanthemum microarray based on EST data and demonstrated its application to examine expression profiles of chrysanthemum transcriptome in response to three different viruses. In addition, infection of three different viruses in a same host led to identify virus specific genes and genes commonly regulated by three viruses. In general, the early stage of virus infection does not display any disease symptoms in the infected host plants, but the host initiates to establish the host immune system by manipulating transcriptional machinery via several signaling pathways. Thus, it is interesting to monitor transcriptional changes of the host regulated by different viruses at early stage. Based on microarray data, CMV and PVX led to regulate more genes than TSWV at transcriptional level. However, gene enrichment analysis revealed the number of enriched GO terms for TSWV infection was much higher than

Table 3 Significantly enriched GO terms in up-regulated gene sets by PVX infection

EST ID	Arabidopsis	Function	CMV	TSWV	PVX
GO: response to chitin (up regulated by PVX)					
ESTC000200	AT5G22250.1	Probable ccr4-associated factor 1 homolog 11	1.256034	1.259653	1.591332
ESTC000379	AT3G15210.1	Ethylene-responsive transcription factor 4	1.531765	1.390996	1.659038
ESTC000887	AT3G55840.1	Nematode resistance hspro2	1.736346	2.380704	2.190304
ESTC001052	AT2G41640.2	Glycosyltransferase family 61 protein	1.469965	1.300353	1.802477
ESTC001463	AT4G34150.1	Protein binding	2.442582	1.118163	2.523429
ESTC001485	AT5G47230.1	Ethylene-responsive transcription factor 5	0.9296	1.232555	1.827593
ESTC001496	AT5G22250.1	Probable ccr4-associated factor 1 homolog 11	0.891229	0.919774	1.195916
ESTC001529	AT4G33920.1	Probable protein phosphatase 2c 63	1.864904	1.146609	2.11465
ESTC002310	AT3G25250.1	Serine threonine-protein kinase oxi1	0.887997	0.914517	1.30367
ESTC002795	AT1G49780.1	u-box domain-containing protein 26	0.802413	0.574014	1.098318
ESTC004366	AT3G16720.1	Ring-h2 finger protein atl2	1.708853	0.974331	1.911415
ESTC004883	AT2G24570.1	Probable wrky transcription factor 11	0.797976	0.730538	1.331684
ESTC008822	No homology	Probable ccr4-associated factor 1 homolog 11	0.773999	0.688032	1.009727
GO: mRNA 3′-end processing (up regulated by PVX)					
AT5G22250.1	AT5G22250.1	Probable ccr4-associated factor 1 homolog 11	1.256034	1.259653	1.591332
ESTC001496	AT5G22250.1	Probable ccr4-associated factor 1 homolog 11	0.891229	0.919774	1.195916
ESTC008822	No homology	Probable ccr4-associated factor 1 homolog 11	0.773999	0.688032	1.009727
GO: immune system process (up regulated by PVX)					
ESTC000200	AT5G22250.1	Probable ccr4-associated factor 1 homolog 11	1.256034	1.259653	1.591332
ESTC000379	AT3G15210.1	Ethylene-responsive transcription factor 4	1.531765	1.390996	1.659038
ESTC000887	AT3G55840.1	Nematode resistance hspro2	1.736346	2.380704	2.190304
ESTC001463	AT4G34150.1	Protein binding	2.442582	1.118163	2.523429
ESTC001485	AT5G47230.1	Ethylene-responsive transcription factor 5	0.9296	1.232555	1.827593
ESTC001496	AT5G22250.1	Probable ccr4-associated factor 1 homolog 11	0.891229	0.919774	1.195916
ESTC001529	AT4G33920.1	Probable protein phosphatase 2c 63	1.864904	1.146609	2.11465
ESTC001953	AT5G42050.1	Uncharacterized loc101208173	1.583855	1.157881	1.797592
ESTC004366	AT3G16720.1	Ring-h2 finger protein atl2	1.708853	0.974331	1.911415
ESTC004760	AT1G76650.3	Probable calcium-binding protein cml31	1.528401	1.798596	1.837914
ESTC004883	AT2G24570.1	Probable wrky transcription factor 11	0.797976	0.730538	1.331684
ESTC006406	AT3G07040.1	Disease resistance protein rpm1	1.263495	0.742705	1.136991
ESTC009590	No homology	Protein ndr1	1.314914	1.495786	1.454499
ESTC009952	No homology	Serine threonine-protein kinase protein ccr3	1.458572	0.275332	1.557205
GO: ethylene mediated signaling pathway (up regulated by PVX)					
ESTC000200	AT5G22250.1	Probable ccr4-associated factor 1 homolog 11	1.256034	1.259653	1.591332
ESTC000379	AT3G15210.1	Ethylene-responsive transcription factor 4	1.531765	1.390996	1.659038
ESTC001485	AT5G47230.1	Ethylene-responsive transcription factor 5	0.9296	1.232555	1.827593
ESTC001496	AT5G22250.1	Probable ccr4-associated factor 1 homolog 11	0.891229	0.919774	1.195916
ESTC007606	No homology	Ethylene-responsive transcription factor 3	1.423385	1.005765	1.416621
ESTC008822	No homology	Probable ccr4-associated factor 1 homolog 11	0.773999	0.688032	1.009727

Chrysanthemum genes with respective homologous *Arabidopsis* gene were assigned to each identified GO term. The number indicates $\log_2$ converted fold changes

those of CMV and PVX. This result indicates that the number of DEGs is not correlated with the functional diverse of identified DEGs. Although several functions associated with viral infection were identified in this study, we discuss more on genes associated with defense/stress related gene, hormone signaling pathway and DNA modification.

Up-regulation of defense/stress genes in response to three viruses

It is natural most commonly identified DEGs were related to defense/stress response and those genes were preferentially up-regulated. Of identified defense/stress related GO terms, endogenous stimulus and organonitrogen compound were significantly enriched. More specifically, genes involving in response to chitin were significantly up-regulated. Chitin is a polymer of *N*-acetyl-D-glucosamine which an important structural component in the fungal cell walls as well as a well-known elicitor of plant immune system associated with pathogenic fungi (Sánchez-Vallet et al. 2014; Zipfel 2008). Plants do not possess chitin but produce chitin degrading enzymes named as chitinases which disrupt the integrity of fungal cell walls releasing chitin (Sánchez-Vallet et al. 2014). Several studies strongly demonstrated that plants recognize chitin by various pattern recognition receptors such as LysM receptor like kinases to induce pathogen-triggered immunity (PTI) (Miya et al. 2007; Wan et al. 2008). Thus, it seems that virus infection induces a series of host genes which are involved in chitin signaling and plant disease resistance. In addition, a previous study treated rice seedling with chitosan as a fungal elicitor, which is produced by deacetylation of chitin, and found the accumulation of pathogenesis-related proteins. These results showed that chitosan or chitin affect defense response of the rice plants (Agrawal et al. 2002). However, it seems that the genes involving in response to chitin are generally homolog to known many stress responsive genes. For example, several ERF TFs and a WRKY11 TF functioning in chitin response in our study have been frequently identified. Many previous studies demonstrated roles of those TF as diverse viral stress responsive genes (Catoni et al. 2009; García-Marcos et al. 2009; Lu et al. 2012). Of known TF families, many WRKY TFs function in a complex defense signaling acting as both positive and negative regulators in coping with various biotic and abiotic stresses (Pandey and Somssich 2009).

Among identified defense/stress response genes, many genes are involved in resistance against *Pseudomonas syringae*. Especially, the chrysanthemum *WRKY11* like gene (ESTC004883), which was up-regulated by three viruses, was homolog to the loss of *Arabidopsis WRKY11* gene led to susceptible to virulent *P. syringae* (Journot-Catalino et al. 2006). Another example is a CCR4-associated factor 1 (CAF1) homolog 11 gene. This gene was first characterized in the yeast to function in mRNA deadenylation (Tucker et al. 2001). In *Arabidopsis thaliana*, expression of two genes, *AtCAF1a* and *AtCAF1b*, was up-regulated by various stresses, and overexpression of *AtCAF1a* showed increased resistance against *P. syringae* infection (Liang et al. 2008). Similarly, the nematode resistance *HSPRO2* has showed increased resistance against *P. syringae*, and expression of *HSPRO2* was also up-regulated by *P. syringae* infection (Murray et al. 2007). Therefore, it seems the chrysanthemum *WRKY11*, *CAF1* and *HSPRO2* genes might play a role as regulators against various biotic stresses including viruses and bacteria.

Up-regulation of genes involved in hormone mediated stress signaling pathways in response to three viruses

TFs play an important role in gene expression in higher plants. Of known TFs, for instance, up-regulation of several ERF TFs by three viruses in our study suggests their involvement in virus infection and ethylene mediated signal pathway. It has known that members of AP2/ERF TF family are highly conversed in plant kingdom and many studies demonstrated their functional roles in mediating stress responses and plant developments (Licausi et al. 2013). Our study, at least three ERFs TFs such as *ERF3-5* as well as three *CAF1 homolog 11* genes are involved in ethylene mediated signaling pathway. Those genes are highly up-regulated in response to all three viruses indicating that this could be a common signaling pathway to establish a basal stress resistance against viral infection. In addition, the nematode resistance *HSPRO2* was also involved in basal resistance to *P. syringae* by negatively regulating jasmonic and ethylene signaling (Murray et al. 2007).

The members of the PP2C family have known to function as regulators in various signal pathways including abscisic acid (ABA) signal transduction (Meyer et al. 1994; Rodriguez 1998). Our study as well as a previous study suggests the function of identified chrysanthemum PP2C homology gene might function in viral stress signaling pathway (Schweighofer et al. 2004). Many members of threonine-protein kinase families are involved in signal pathways in response to various stresses (Afzal et al. 2008). In our study, one of threonine-protein kinases name as *OXI1* was up-regulated by three viruses. The expression of *Arabidopsis OXI1* gene was induced by H_2O_2 generating stimuli and is required for active oxygen species processes by activating mitogen activated protein kinases (Rentel et al. 2004). Taken together, it is likely that expression of genes associated with diverse hormone mediated signaling pathways was up-regulated to establish basal immune system in response to diverse viruses. However, the functional role of such genes acting as positive or negative

regulator against virus infection should be elucidated in further study.

Down regulation of DNA modification related genes in response to three viruses

It is of interest that TSWV infection led to down-regulate expression of genes involved in DNA metabolic process including DNA replication, chromatin organization, histone modification, cytokinesis, and chromatin and spindle assembly. They are genes encoding mitotic spindle checkpoint protein MAD2, syntaxin-related protein knolle, kinesin-1, cyclin-dependent kinase b2-2, histone, and DNA replication licensing factor MCM3 homolog 1 and they are preferentially targeted to nucleosome and MCM complex. Expression of those genes were strongly down-regulated by not only TSWV but also CMV and PVX. This result suggests their expression is commonly regulated by all three viruses.

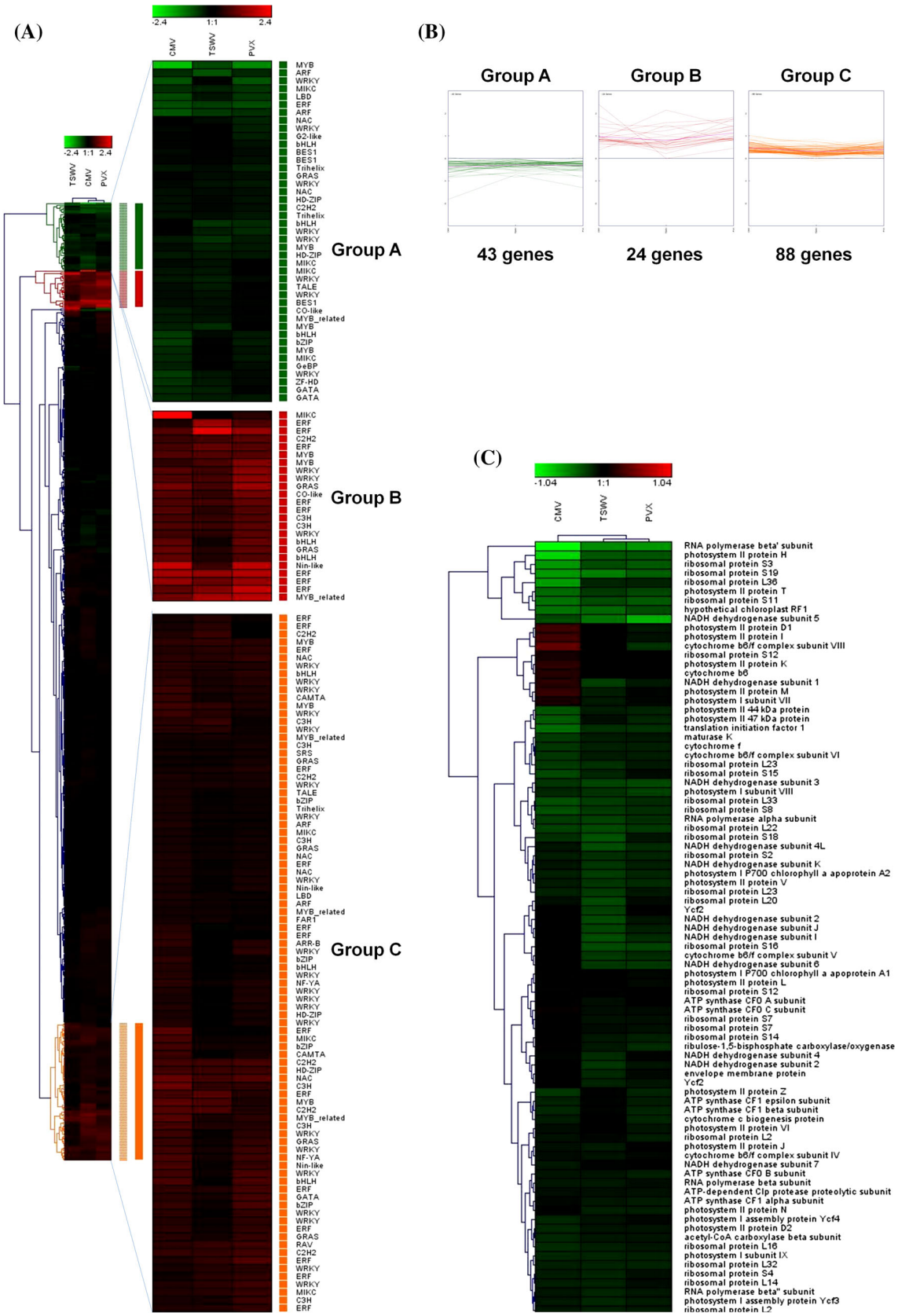
A hexameric minichromosome maintenance (MCM) complex composed of six proteins (MCM2-7) are highly conserved in the eukaryotic organisms and play essential roles at the initiation step of DNA synthesis (Tye 1999). MCM proteins are also important for DNA unwinding which might be affected by external stimuli. In higher plants, MCM like protein referred as PROLIFERA (*PRL*) has been identified in *Arabidopsis* by gene trap tagging (Springer et al. 1995). The *Arabidopsis PRL* gene encoding MCM7 protein localizing to the nucleus is required for DNA replication and cytokinesis during early *Arabidopsis* development (Holding and Springer 2002; Springer et al. 2000). Another study has shown that the expression of pea *MCM6* was up-regulated by salinity and cold stresses, and overexpression of *MCM6* gene in tobacco plants displayed salinity tolerance (Dang et al. 2011). It is likely that two genes *MCM3* (ESTC005732) and *MCM7* (ESTC002782), which were strongly down-regulated by three viruses, might involve in viral infection, but detailed functional studies should be carried out.

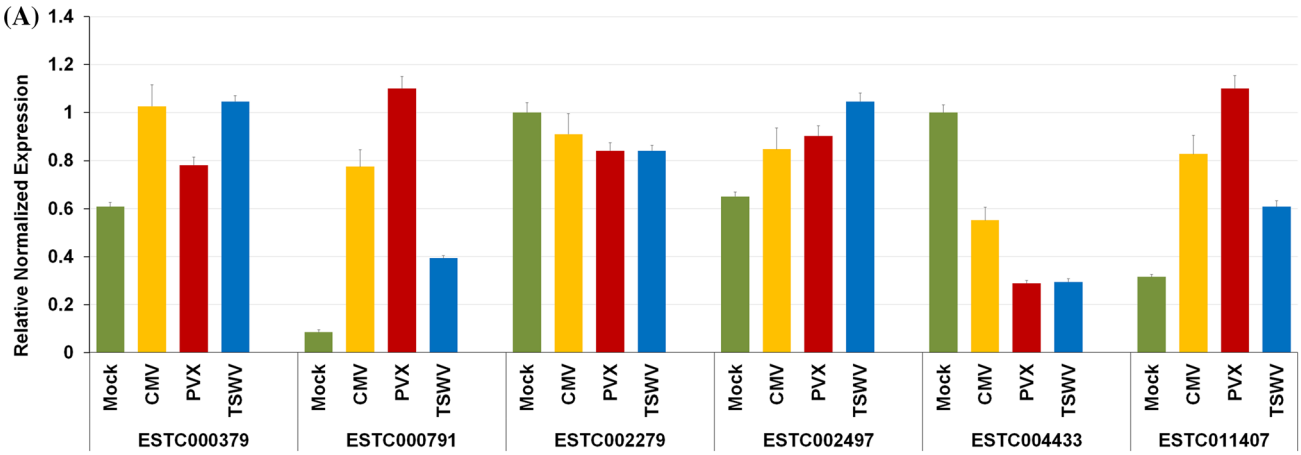
The *MAD2* gene (mitotic arrest deficient 2) encoding spindle assembly protein has been firstly identified in yeast (Li and Murray 1991) and highly conserved in eukaryotic organisms functioning in cell division (Caillaud et al. 2009). The chrysanthemum *kinesin-1* gene (ESTC004117) is homologous to *Arabidopsis ATK5* gene which is required for early spindle assembly during cell division (Ambrose and Cyr 2007). Strong down-regulation of DNA replication related genes such as *MAD2* and *kinesin-1* by ssRNA viruses indicates that viruses might inhibit transcription of genes involving in host DNA replication. By contrast, DNA viruses such as geminiviruses with ssDNA genomes replicate through double-stranded intermediates and induce transcripts associated with DNA replication machinery for their viral DNA synthesis (Hanley-Bowdoin et al. 1999). A previous *Arabidopsis* microarray analysis in response to *Cabbage leaf curl virus* (CaLCuV), which is a member of geminiviruses, revealed that expression of cell cycle-associated genes was changed by CaLCuV infection (Ascencio-Ibáñez et al. 2008). For instance, genes expressed during S and G2 stages were up-regulated while genes expressed in G1 and M were down-regulated by CaLCuV. This result provided strong evidence that transcriptional reprogramming plant cell cycle by a small ssDNA virus (Hanley-Bowdoin et al. 2013). Thus, the host transcriptional machinery associated with plant cell cycle is affected by both ssRNA and ssDNA viruses, however, transcriptional modulation by ssRNA and ssDNA viruses might be different from each other.

Recent studies suggest that plant gene expression regulated by various stresses sometimes relies on nucleosome histone post-translational modification including DNA methylation (Bruce et al. 2007; Chinnusamy and Zhu 2009). As shown in many previous studies, down-regulation of four chrysanthemum genes related to histone indicates suppression of chromatin organization and DNA methylation. A previous tomato transcriptome analysis upon TSWV infection also identified DEGs involved in chromatin organization and DNA packaging indicating involvement of viral stress in epigenetic changes (Catoni et al. 2009). It seems that TSWV infection causes similar impact of the host transcriptome regardless of host range.

Taken together, we present the first study of transcriptional changes of chrysanthemum plants by a microarray analysis. In addition, the changes of chrysanthemum transcriptome response to three different viruses including CMV, TSWV and PVX showed many novel interesting findings including virus specific gene expression and up-regulation of hormone mediated stress responsive genes and down-regulation of genes associated with DNA replication and histone modification.

Fig. 4 Expression profiles of chrysanthemum TFs and chloroplast genes. **a** Three group of chrysanthemum TFs which were differentially expressed. To identify chrysanthemum TFs differentially expressed by infection of three different viruses, Log_2 converted fold changes of a total of 610 chrysanthemum TFs were subjected for hierarchical clustering using the genesis program. Detailed information for TFs can be found in Supplementary Table 4. **b** Average expression views of TFs in each group showing three distinct expression patterns. **c** Expression of 85 chloroplast encoded genes in response to infection of three different viruses


(A)
-2.4 1:1 2.4
CMV TSWV PVX
Group A
Group B
Group C
(B)
Group A
Group B
Group C
43 genes
24 genes
88 genes
(C)
-1.04 1:1 1.04
CMV TSWV PVX



(B)

		CMV		PVX		TSWV	
EST number	Function	Microarray	RT-PCR	Microarray	RT-PCR	Microarray	RT-PCR
ESTC000379	Ethylene-responsive transcription factor 4	1.53	0.76	1.66	0.36	1.39	0.78
ESTC000791	Protein phosphatase 2c 25	1.41	3.19	1.49	3.70	1.08	2.21
ESTC002279	Ribonucleoside-diphosphate reductase small chain	-1.06	-0.13	-1.40	-0.25	-1.69	-0.25
ESTC002497	Basic 7s globulin	-2.58	0.38	-2.41	0.47	-1.32	0.69
ESTC004433	GDSL esterase lipase	-1.33	-0.86	-3.50	-1.79	-3.29	-1.77
ESTC011407	Unknown function	2.91	1.39	3.31	1.80	1.52	0.95

Fig. 5 Quantitative real time RT-PCR to validate microarray data. **a** Normalized expression of six selected genes with standard errors. To normalize expression, two actin genes were used. **b** The detailed fold changes of six genes by microarray and real time RT-PCR. *Red* and *green color* indicate up- and down-regulation as compared to mock sample. The numbers are $\log_2$ converted fold changes

Acknowledgments This work was carried out with the support of "Next-Generation BioGreen21 Program (Project No. PJ01130902)" Rural Development Administration, Republic of Korea.

References

Afzal AJ, Wood AJ, Lightfoot DA (2008) Plant receptor-like serine threonine kinases: roles in signaling and plant defense. Mol Plant Microbe Interact 21:507–517. doi:10.1094/MPMI-21-5-0507

Agrawal GK, Rakwal R, Tamogami S, Yonekura M, Kubo A, Saji H (2002) Chitosan activates defense/stress response (s) in the leaves of *Oryza sativa* seedlings. Plant Physiol Biochem 40:1061–1069. doi:10.1016/S0981-9428(02)01471-7

Ambrose JC, Cyr R (2007) The kinesin ATK5 functions in early spindle assembly in Arabidopsis. Plant Cell 19:226–236. doi:10.1105/tpc.106.047613

An J, Song A, Guan Z, Jiang J, Chen F, Lou W, Fang W, Liu Z, Chen S (2014) The over-expression of *Chrysanthemum crassum* CcSOS1 improves the salinity tolerance of chrysanthemum. Mol Biol Rep 41:4155–4162. doi:10.1007/s11033-014-3287-2

Ascencio-Ibáñez JT, Sozzani R, Lee T-J, Chu T-M, Wolfinger RD, Cella R, Hanley-Bowdoin L (2008) Global analysis of Arabidopsis gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. Plant Physiol 148:436–454. doi:10.1104/pp.108.121038

Blüthgen N, Brand K, Čajavec B, Swat M, Herzel H, Beule D (2005) Biological profiling of gene groups utilizing gene ontology. Genome Inform 16:106–115

Bruce TJ, Matthes MC, Napier JA, Pickett JA (2007) Stressful "memories" of plants: evidence and possible mechanisms. Plant Sci 173:603–608. doi:10.1016/j.plantsci.2007.09.002

Caillaud M-C, Paganelli L, Lecomte P, Deslandes L, Quentin M, Pecrix Y, Le Bris M, Marfaing N, Abad P, Favery B (2009) Spindle assembly checkpoint protein dynamics reveal conserved and unsuspected roles in plant cell division. PLoS ONE 4:e6757. doi:10.1371/journal.pone.0006757

Catoni M, Miozzi L, Fiorilli V, Lanfranco L, Accotto GP (2009) Comparative analysis of expression profiles in shoots and roots of tomato systemically infected by *Tomato spotted wilt virus* reveals organ-specific transcriptional responses. Mol Plant Microbe In 22:1504–1513. doi:10.1094/MPMI-22-12-1504

Chen S, Miao H, Chen F, Jiang B, Lu J, Fang W (2009) Analysis of expressed sequence tags (ESTs) collected from the inflorescence of Chrysanthemum. Plant Mol Biol Report 27:503–510. doi:10.1007/s11105-0009-0103-6

Chinnusamy V, Zhu J-K (2009) Epigenetic regulation of stress responses in plants. Curr Opin Plant Biol 12:133–139. doi:10.1016/j.pbi.2008.12.006

Cho WK, Jo Y, Jo K-M, Kim K-H (2013) A current overview of two viroids that infect chrysanthemums: *Chrysanthemum stunt viroid* and *Chrysanthemum chlorotic mottle viroid*. Viruses 5:1099–1113. doi:10.3390/v5041099

Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21:3674–3676. doi:10.1093/bioinformatics/bti610

Courtney-Gutterson N, Napoli C, Lemieux C, Morgan A, Firoozabady E, Robinson KE (1994) Modification of flower color in florist's Chrysanthemum: production of a white-flowering

variety through molecular genetics. Nat Biotech 12:268–271. doi:10.1038/nbt0394-268

Dang HQ, Tran NQ, Gill SS, Tuteja R, Tuteja N (2011) A single subunit MCM6 from pea promotes salinity stress tolerance without affecting yield. Plant Mol Biol 76:19–34. doi:10.1007/s11103-011-9758-0

Ding L, Chen FD, Teng NJ, Fang WM (2008) Analysis of genetic diversity of cultivars in Dendranthema × grandiflorum based on POD and SOD isozyme. Scientia Agricultura Sinica 4:027

Fu J, Wang L, Wang Y, Yang L, Yang Y, Dai S (2014) Photoperiodic control of *FT*-like gene *ClFT* initiates flowering in *Chrysanthemum lavandulifolium*. Plant Physiol Biochem 74:230–238. doi:10.1016/j.plaphy.2013.11.004

García-Marcos A, Pacheco R, Martiáñez J, González-Jara P, Díaz-Ruíz JR, Tenllado F (2009) Transcriptional changes and oxidative stress associated with the synergistic interaction between *Potato virus X* and *Potato virus Y* and their relationship with symptom expression. Mol Plant Microbe Interact 22:1431–1444. doi:10.1094/MPMI-22-11-1431

Gu C, Chen S, Liu Z, Shan H, Luo H, Guan Z, Chen F (2011) Reference gene selection for quantitative real-time PCR in Chrysanthemum subjected to biotic and abiotic stress. Mol Biotechnol 49:192–197

Hanley-Bowdoin L, Settlage SB, Orozco BM, Nagar S, Robertson D (1999) Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. Crit Rev Plant Sci 18:71–106. doi:10.1080/07352689991309162

Hanley-Bowdoin L, Bejarano ER, Robertson D, Mansoor S (2013) Geminiviruses: masters at redirecting and reprogramming plant processes. Nat Rev Microbiol 11:777–788. doi:10.1038/nrmicro3117

He H, Yajing N, Huawen C, Xingjiao T, Xinli X, Weilun Y, Silan D (2012) cDNA-AFLP analysis of salt-inducible genes expression in *Chrysanthemum lavandulifolium* under salt treatment. J Plant Physiol 169:410–420. doi:10.1016/j.jplph.2011.09.013

Hitmi A, Coudret A, Barthomeuf C (2000) The production of pyrethrins by plant cell and tissue cultures of *Chrysanthemum cinerariaefolium* and Tagetes species. Crit Rev Biochem Mol Biol 35:317–337. doi:10.1080/07352680091139187

Holding DR, Springer PS (2002) The Arabidopsis gene PROLIFERA is required for proper cytokinesis during seed development. Planta 214:373–382. doi:10.1007/s00425-0001-0686-0

Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP (2003) Summaries of Affymetrix GeneChip probe level data. Nucl Acids Res 31:e15–e15

Jo Y, Jo K-M, Park S-H, Kim K-H, Cho WK (2014) Transcriptomic landscape of chrysanthemums infected by *Chrysanthemum stunt viroid*. Plant Omics 7:1–11

Journot-Catalino N, Somssich IE, Roby D, Kroj T (2006) The transcription factors WRKY11 and WRKY17 act as negative regulators of basal resistance in *Arabidopsis thaliana*. Plant Cell 18:3289–3302. doi:10.1105/tpc.106.044149

Lang Q, Jin C, Gao X, Feng J, Chen S, Lai L, Chen J (2011) Profiling of *Cucumber Mosaic Virus* responsive mRNAs in tomato using Paraflo microfluidics microarrays. J Nanosci Nanotech 11:3115–3125. doi:10.1166/jnn.2011.3860

Li R, Murray AW (1991) Feedback control of mitosis in budding yeast. Cell 66:519–531

Li T, Je Guo, Li Y, Ning H, Sun X, Zheng C (2013) Genetic diversity assessment of chrysanthemum germplasm using conserved DNA-derived polymorphism markers. Sci Hort 162:271–277. doi:10.1016/j.scienta.2013.08.027

Li H, Chen S, Song A, Wang H, Fang W, Guan Z, Jiang J, Chen F (2014) RNA-Seq derived identification of differential transcription in the chrysanthemum leaf following inoculation with *Alternaria tenuissima*. BMC Genomic 15:9. doi:10.1186/1471-2164-15-9

Liang W, Li C, Liu F, Jiang H, Li S, Sun J, Wu X, Li C (2008) The Arabidopsis homologs of CCR4-associated factor 1 show mRNA deadenylation activity and play a role in plant defence responses. Cell Res 19:307–316. doi:10.1038/cr.2008.317

Licausi F, Ohme-Takagi M, Perata P (2013) APETALA2/ethylene responsive factor (AP2/ERF) transcription factors: mediators of stress responses and developmental programs. New Phytol 199:639–649. doi:10.1111/nph.12291

Liu P-L, Wan Q, Guo Y-P, Yang J, Rao G-Y (2012) Phylogeny of the genus *Chrysanthemum* L.: evidence from single-copy nuclear gene and chloroplast DNA sequences. PLoS ONE 7:e48970. doi:10.1371/journal.pone.0048970

Liu P, Chen S, Song A, Zhao S, Fang W, Guan Z, Liao Y, Jiang J, Chen F (2014) A putative high affinity phosphate transporter, CmPT1, enhances tolerance to Pi deficiency of chrysanthemum. BMC Plant Biol 14:18. doi:10.1186/1471-2229-14-18

Lu J, Du Z-X, Kong J, Chen L-N, Qiu Y-H, Li G-F, Meng X-H, Zhu S-F (2012) Transcriptome analysis of *Nicotiana tabacum* infected by *Cucumber mosaic virus* during systemic symptom development. PLoS ONE 7:e43447. doi:10.1371/journal.pone.0043447

Marathe R, Guan Z, Anandalakshmi R, Zhao H, Dinesh-Kumar S (2004) Study of *Arabidopsis thaliana* resistome in response to *Cucumber mosaic virus* infection using whole genome microarray. Plant Mol Biol 55:501–520. doi:10.1007/s11103-004-0439-0

Meyer K, Leube MP, Grill E (1994) A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. Science 264:1452–1455. doi:10.1105/tpc.9.5.759

Miya A, Albert P, Shinya T, Desaki Y, Ichimura K, Shirasu K, Narusaka Y, Kawakami N, Kaku H, Shibuya N (2007) CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. Proc Natl Acad Sci 104:19613–19618. doi:10.1073/pnas.0705147104

Murray SL, Ingle RA, Petersen LN, Denby KJ (2007) Basal resistance against *Pseudomonas syringae* in Arabidopsis involves WRKY53 and a protein with homology to a nematode resistance protein. Mol Plant Microbe Interact 20:1431–1438. doi:10.1094/MPMI-20-11-1431

Palmieri L, Picault N, Arrigoni R, Besin E, Palmieri F, Hodges M (2008) Molecular identification of three *Arabidopsis thaliana* mitochondrial dicarboxylate carrier isoforms: organ distribution, bacterial expression, reconstitution into liposomes and functional characterization. Biochem J 410:621–629. doi:10.1042/BJ20070867

Pandey SP, Somssich IE (2009) The role of WRKY transcription factors in plant immunity. Plant Physiol 150:1648–1655. doi:10.1104/pp.109.138990

Ren H, Zhu F, Zheng C, Sun X, Wang W, Shu H (2013) Transcriptome analysis reveals genes related to floral development in chrysanthemum responsive to photoperiods. Biochem Genet 51:20–32. doi:10.1007/s10528-012-9541-1

Rentel MC, Lecourieux D, Ouaked F, Usher SL, Petersen L, Okamoto H, Knight H, Peck SC, Grierson CS, Hirt H (2004) OXI1 kinase is necessary for oxidative burst-mediated signalling in Arabidopsis. Nature 427:858–861. doi:10.1038/nature02353

Rodriguez PL (1998) Protein phosphatase 2C (PP2C) function in higher plants. Plant Mol Biol 38:919–927. doi:10.1023/A:1006054607850

Sánchez-Vallet A, Mesters JR, Thomma BP (2014) The battle for chitin recognition in plant–microbe interactions. FEMS Microbiol Rev. doi:10.1093/femsre/fuu003

Schweighofer A, Hirt H, Meskiene I (2004) Plant PP2C phosphatases: emerging functions in stress signaling. Trends Plant Sci 9:236–243. doi:10.1016/j.tplants.2004.03.007

Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol. doi:10.2202/1544-6115.1027

Song A, An J, Guan Z, Jiang J, Chen F, Lou W, Fang W, Liu Z, Chen S (2014a) The constitutive expression of a two transgene construct enhances the abiotic stress tolerance of chrysanthemum. Plant Physiol Biochem 80:114–120. doi:10.1016/j.plaphy.2014.03.030

Song A, Zhu X, Chen F, Gao H, Jiang J, Chen S (2014b) A chrysanthemum heat shock protein confers tolerance to abiotic stress. Int J Mol Sci 15:5063–5078. doi:10.3390/ijms15035063

Springer PS, McCombie WR, Sundaresan V, Martienssen RA (1995) Gene trap tagging of PROLIFERA, an essential MCM2-3-5-like gene in Arabidopsis. Science 268:877–880. doi:10.1126/science.7754372

Springer PS, Holding DR, Groover A, Yordan C, Martienssen RA (2000) The essential Mcm7 protein PROLIFERA is localized to the nucleus of dividing cells during the G (1) phase and is required maternally for early Arabidopsis development. Development 127:1815–1822

Sturn A, Quackenbush J, Trajanoski Z (2002) Genesis: cluster analysis of microarray data. Bioinformatics 18:207–208. doi:10.1093/bioinformatics/18.1.207

Timme RE, Kuehl JV, Boore JL, Jansen RK (2007) A comparative analysis of the *Lactuca* and *Helianthus* (Asteraceae) plastid genomes: identification of divergent regions and categorization of shared repeats. Am J Bot 94:302–312. doi:10.3732/ajb.94.3.302

Tong Z, Hong B, Yang Y, Li Q, Ma N, Ma C, Gao J (2009) Overexpression of two chrysanthemum DgDREB1 group genes causing delayed flowering or dwarfism in Arabidopsis. Plant Mol Biol 71:115–129. doi:10.1007/s11103-009-9513-y

Tucker M, Valencia-Sanchez MA, Staples RR, Chen J, Denis CL, Parker R (2001) The transcription factor associated Ccr4 and Caf1 proteins are components of the major cytoplasmic mRNA deadenylase in *Saccharomyces cerevisiae*. Cell 104:377–386

Tye BK (1999) MCM proteins in DNA replication. Annu Rev Biochem 68:649–686. doi:10.1146/annurev.biochem.68.1.649

Verma N, Sharma A, Ram R, Hallan V, Zaidi A, Garg I (2003) Detection, identification and incidence of *Chrysanthemum B carlavirus* in chrysanthemum in India. Crop Protection 22:425–429. doi:10.1016/S0261-2194(02)00184-9

Wan J, Zhang X-C, Neece D, Ramonell KM, Clough S, S-y Kim, Stacey MG, Stacey G (2008) A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in Arabidopsis. Plant Cell Online 20:471–481. doi:10.1105/tpc.107.056754

Wang H, Jiang J, Chen S, Qi X, Peng H, Li P, Song A, Guan Z, Fang W, Liao Y (2013) Next-generation sequencing of the *Chrysanthemum nankingense* (Asteraceae) transcriptome permits large-scale unigene assembly and SSR marker discovery. PLoS ONE 8:e62293. doi:10.1371/journal.pone.0062293

Wang Y, Huang H, Ma Y, Fu J, Wang L, Dai S (2014) Construction and de novo characterization of a transcriptome of *Chrysanthemum lavandulifolium*: analysis of gene expression patterns in floral bud emergence. Plant Cell Tissue Organ Cult (PCTOC) 116:297–309. doi:10.1007/s11240-013-0404-1

Whitham SA, Quan S, Chang HS, Cooper B, Estes B, Zhu T, Wang X, Hou YM (2003) Diverse RNA viruses elicit the expression of common sets of genes in susceptible *Arabidopsis thaliana* plants. Plant J 33:271–283. doi:10.1046/j.1365-313X.2003.01625.x

Workman C, Jensen LJ, Jarmer H, Berka R, Gautier L, Nielser HB, Saxild H-H, Nielsen C, Brunak S, Knudsen S (2002) A new non-linear normalization method for reducing variability in DNA microarray experiments. Genome Biol 3:1–16. doi:10.1186/gb-2002-3-9-research0048

Wu L-Y, Gao H-Z, Wang X-L, Ye J-H, Lu J-L, Liang Y-R (2010) Analysis of chemical composition of *Chrysanthemum indicum* flowers by GC/MS and HPLC. J Med Plants Res 4:421–426

Xu Y, Gao S, Yang Y, Huang M, Cheng L, Wei Q, Fei Z, Gao J, Hong B (2013) Transcriptome sequencing and whole genome expression profiling of chrysanthemum under dehydration stress. BMC Genom 14:662. doi:10.1186/1471-2164-14-662

Yang W, Glover BJ, Rao GY, Yang J (2006) Molecular evidence for multiple polyploidization and lineage recombination in the *Chrysanthemum indicum* polyploid complex (Asteraceae). New Phytol 171:875–886. doi:10.1111/j.1469-8137.2006.01779.x

Yang Y, Ma C, Xu Y, Wei Q, Imtiaz M, Lan H, Gao S, Cheng L, Wang M, Fei Z (2014) A zinc finger protein regulates flowering time and abiotic stress tolerance in chrysanthemum by modulating gibberellin biosynthesis. Plant Cell 23:2038–2054. doi:10.1105/tpc.114.124867

Zipfel C (2008) Pattern-recognition receptors in plant innate immunity. Curr Opin Immunol 20:10–16. doi:10.1016/j.coi.2007.11.003