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RESEARCH ARTICLE

Microarray analysis of *Arabidopsis thaliana* exposed to single and mixed infections with *Cucumber mosaic virus* and turnip viruses

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Abstract *Cucumber mosaic virus* (CMV), *Turnip mosaic virus* (TuMV) and *Turnip crinkle virus* (TCV) are important plant infecting viruses. In the present study, whole transcriptome alteration of *Arabidopsis thaliana* in response to CMV, TuMV and TCV, individual as well as mixed infections of CMV and TuMV/CMV and TCV were investigated using microarray data. In response to CMV, TuMV and TCV infections, a total of 2517, 3985 and 277 specific differentially expressed genes (DEGs) were up-regulated, while 2615, 3620 and 243 specific DEGs were down-regulated, respectively. The number of 1222 and 30 common DEGs were up-regulated during CMV and TuMV as well as CMV and TCV infections, while 914 and 24 common DEGs were respectively down-regulated. Genes encoding immune response mediators, signal transducer activity, signaling and stress response functions were among the most significantly upregulated genes during CMV and TuMV or CMV and TCV mixed infections. The NAC, C3H, C2H2, WRKY and bZIP were the most commonly presented transcription factor (TF) families in CMV and TuMV infection, while AP2-EREBP and C3H were the TF families involved in CMV and TCV infections. Moreover, analysis of miRNAs during CMV and TuMV and CMV and TCV infections have demonstrated the role of miRNAs in the down regulation of host genes in response to viral infections. These results identified the commonly expressed virus-responsive genes and pathways during plant–virus interaction which might develop novel antiviral strategies for improving plant resistance to mixed viral infections.

Keywords *Arabidopsis thaliana* · Defense responses · Differentially expressed genes · Microarray · Virus infection

Introduction

Plants are subjected to a variety of biotic and abiotic stresses which adversely affect the crop growth and yield (Suzuki et al. 2014; Sharma et al. 2013). Among biotic stresses, viruses are highly devastating and causing major losses to economically important host plants (Nicaise 2014; Chen and Wei 2020). Plant viruses are infectious agents that can only replicate and survive within their host cells. Due to their obligate nature, they depend on host cellular machinery to complete their reproduction cycles (replication and movement processes) (Ahlquist et al. 2003; Whitham and Wang 2004). Among viruses, *Cucumber mosaic virus* (CMV) (genus *Cucumovirus*, family *Bromoviridae*) is one of the most important aphids transmitted plant pathogens which infects various plant species (Palukaitis and Garcia-Arenal 2003). In addition, *Turnip mosaic*

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virus (TuMV) (genus *Potyvirus*, family *Potyviridae*) is another aphid transmitted virus that is considered as one of the most damaging plant pathogens infecting vegetable crops around the world, and is likely the most common and economically important virus infecting various species of *Brassicaceae* (Nguyen et al. 2013; Yasaka et al. 2015; Edwardson and Christie 1991; Walsh and Jenner 2002). Moreover, *Turnip crinkle virus* (TCV) (genus *Carmovirus*, family *Tombusviridae*) is a positive single-strand RNA virus that is transmitted by chrysomelid beetles with a wide host range (Broadbent and Heathcote 1958; Hollings and Stone 1972). TuMV induces various symptoms including necrosis, stunting and plant death in the host plants (Guerret et al. 2017; Edwardson and Christie 1991), resulting in yield losses and poor product quality in crops (Jiang et al. 2010; Spence et al. 2007). TuMV infections negatively affect developmental traits like flower viability in *A. thaliana* (Walsh and Jenner 2002; Sánchez et al. 2015). It has also been reported that CMV and TCV are considered as highly virulent pathogens on *Arabidopsis* plants (Van Regenmortel et al. 2000; Cohen et al. 2000). Moreover, CMV and TCV can tackle plant defense mechanisms, thus resulting in severe losses in crops around the world (Manfre and Simon 2008). CMV and TCV induce markedly different symptoms in *A. thaliana*. CMV infections cause small leaves and stunting, while TCV infections express chlorotic symptom in *A. thaliana* (Yang et al. 2010). Therefore, CMV, TuMV and TCV are capable of infecting a wide number of plants. *Arabidopsis thaliana* is a genetically model plant in dicots that is a susceptible host for these viruses (Sommerville and Koornneef 2002) and therefore it is a suitable model plant to study plant-pathogen interactions (Pagan et al. 2010; Bethke and Glazebrook 2019). A successful infection requires counteracting host defense mechanisms and other cellular factors (Harries and Ding 2011). Therefore, viruses recruit various strategies to utilize cellular resources of host plants to facilitate their infections. Consequently, viral infections induce a variety of specific and general changes in plant gene expression of multiple regulatory and host defense responses at host physiological and developmental processes (Golem and Culver 2003; Lecellier and Voinnet 2004; Maule et al. 2002; Whitham et al. 2006; Pesti et al. 2019). Gene expression of plants is transiently changed to help viral gene expression and to create a favorable cellular environment for viral infections (Jelitto-Van Dooren et al. 1999). Therefore, studying the global transcriptome alteration in response to virus infection and host-virus interactions using gene expression profiling analysis including DNA microarray is widely used to understand the mechanism of virus-host interaction and the host response mechanism (Moustafa and Cross 2016; Shaik and Ramakrishna 2013; Pashaiasl et al. 2016). Among differentially expresses genes, transcription factors (TFs) play a critical role in responses against environmental stresses. The TFs are DNA-binding proteins that modulate the genes expression by binding to specific DNA sequences in the genes promoter and act as transcriptional activators/repressors to regulate other gene (Seo and Choi 2015; Li et al. 2014a; Seki et al. 2002). Plant micro RNAs (miRNAs) are endogenous, noncoding and small RNAs that regulate gene expression via interactions with their specific target mRNAs (Liu et al. 2008). MiRNAs play a vital role in many biological and metabolic processes including regulation of plant growth, development and in particular response to biotic and abiotic stresses (Shukla et al. 2008; Liu et al. 2008; Jones-Rhoades and Bartel 2004; Kulshrestha et al. 2020). It has also been observed that miRNAs play pivotal roles in plant–virus interactions (Huang et al. 2016), which mediate defense responses in plants against viruses (Pacheco et al. 2012). Since, different viruses show various responses in a common host, hence we cannot ignore mixed infections which frequently occurs in nature and resulted into antagonistic or synergistic viral interactions (Senthil et al. 2005; Whitham et al. 2003; Syller 2012; Moreno and López-Moya 2020). It has also been observed that in the mixed infection, CMV leads to synergistic interaction with TuMV (Martínez et al. 2013; Takeshita et al. 2012), while antagonistic interaction with TCV in *A. thaliana* (Chen et al. 2014; Yang et al. 2010). It was also observed that mixed infection of CMV and TuMV cause more severe symptoms than single viral infections in *Nicotiana benthamiana* (Takeshita et al. 2012). Mixed infection of CMV and TCV showed symptoms similar to those caused by single TCV infection (Yang et al. 2010). TCV infection results in resistance to CMV during mixed infection of these viruses in *Arabidopsis* showing suppression of CMV infection by TCV (Chen et al. 2014). However, their mechanisms in terms of common responses remained unknown. The present study has been designed to discover transcriptome response of *Arabidopsis* to CMV and TuMV and to CMV and TCV infections to better understand the virus-host interaction mechanisms through different approaches including GO, KEGG and network analysis which were used to explain the complexity of *Arabidopsis* reaction to different viral infections and their common responses. The function of TFs and miRNAs during viral infections has been studied. To our knowledge, this is the first comparative study of common and specific genes of *A. thaliana* in response to different virus infections compared with healthy plants.

Material and methods

Virus inoculations and microarray analysis

To identify common differential expression genes against CMV and TuMV or CMV and TCV infection in *Arabidopsis* leaf tissues, microarray data were obtained from four independent experiments deposited in ArrayExpress database (https://www.ebi.ac.uk/arrayexpress/). The *Arabidopsis* plants were inoculated with CMV (E-GEOD-37921), TuMV (E-MEXP-509 and E-GEOD-20278) and TCV (E-MEXP-1218). Detailed protocol information of each project is available at the ArrayExpress. In all experiments, the inoculated plants were maintained under controlled growth chamber with 16/8 h light/dark period, 60–80% humidity at 21–25 °C temperature. All the CEL data were analyzed using FlexArray software version 1.6.3, data normalization was conducted by Robust Multiarray Average (RMA) algorithm and RMA signal values were transformed into $\log_2$ (Khojasteh et al. 2018). Moreover, the quality control of CEL data was checked. The adjusted false discovery rate with P value less than 0.05 was selected for identification of DEGs (Khojasteh et al. 2018). Consequently, a list of genes was retrieved for further analysis and a Venn graph was depicted using http://bioinformatics.psb.ugent.be/webtools/Venn/. Moreover, The DEGs over the *Arabidopsis* genome were shown by using circus (Cheong et al. 2015).

Functional enrichment analysis

Gene Ontology (GO) analysis of differentially expressed genes in response to each individual virus infection was studied using AgriGO tools (http://bioinfo.cau.edu.cn/agriGO/). Moreover, GO and Singular Enrichment Analysis (SEA) analysis of common up-regulated and down-regulated genes in response to CMV and TuMV and to CMV and TCV infections were studied. GO enrichment analysis of DEGs in response to virus infections were identified (Du et al. 2010). To understand the biological significance of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, enrichment analysis of common up/down-regulated genes were performed using KOBAS 3.0 database (Xie et al. 2011). The corrected P value $<$ 0.05 was considered as significant.

Identification of TFs and miRNA targets

The TF families of up-regulated genes in response to virus infections were determined using AGRIS database (Yilmaz et al. 2011). To identify a gene association network in individual or between virus infections and the protein interaction relationships corresponding to the DEGs regulated by the TFs, the Search Tool for the Retrieval of Interacting Genes was used (STRING v.10) (Szklarczyk et al. 2014). A STRING was used to determine relationships among TFs and to identify significant protein pairs with combined score $>$ 0.7. Subsequently, Genevestigator (https://genevestigator.com/gv/) database was used to analyze individual and common up-regulated TF genes expression at different developmental stages and tissues of *A. thaliana* (Zimmermann et al. 2004). The miRNA targets were identified using default parameters in psRNATarget (http://plantgrn.noble.org/psRNATarget/) database (Dai and Zhao 2018).

Validation analysis

In order to validate the results of our analysis, a meta-analysis was initially conducted with "*metaSeq*" package in Rstudio (Tsuyuzaki and Nikaido 2020). In this step, we confirmed the presence of the hub genes in our database through Fisher and Stouffer methods in this package (Fig. S2A). Subsequently, a cross-validation analysis based on Support Vector Machine (SVM) was carried out through "*e1071*" package on expression values of selected genes (Dimitriadou et al. 2009; Tahmasebi et al. 2019).

Results and discussion

Identification and functional enrichment analysis of differentially expressed genes

The experiments were designed to dissect the mechanism of transcriptional regulation of *Arabidopsis* genes in response to viral infections. All virus infections in *A. thaliana* have substantially stimulated plant gene expression. Single virus infection by CMV, TuMV and TCV resulted in up/down-regulation of 2517/2615, 3985/3620 and 277/243 genes, respectively (Fig. 1). In CMV and TuMV or CMV and TCV treatments, a total of 1222/914 and 30/24 genes were up/down-regulated, respectively (Fig. 1). The quality of array was checked (Fig. S1) and full description of up and down-regulated genes has been mentioned in Tables S1 and S2. The viral infections significantly offer the expression of common genes which are potential candidates for the genetic manipulation of plants to improve resistance to mixed viral infections. The information of common genes during viral infections might be an effective approach for the identification of important pathways involved in plant-virus interaction as well as virus to virus interactions. This study has further provided insight into the common gene expression profiling in *Arabidopsis* during viral infections. GO distribution of the

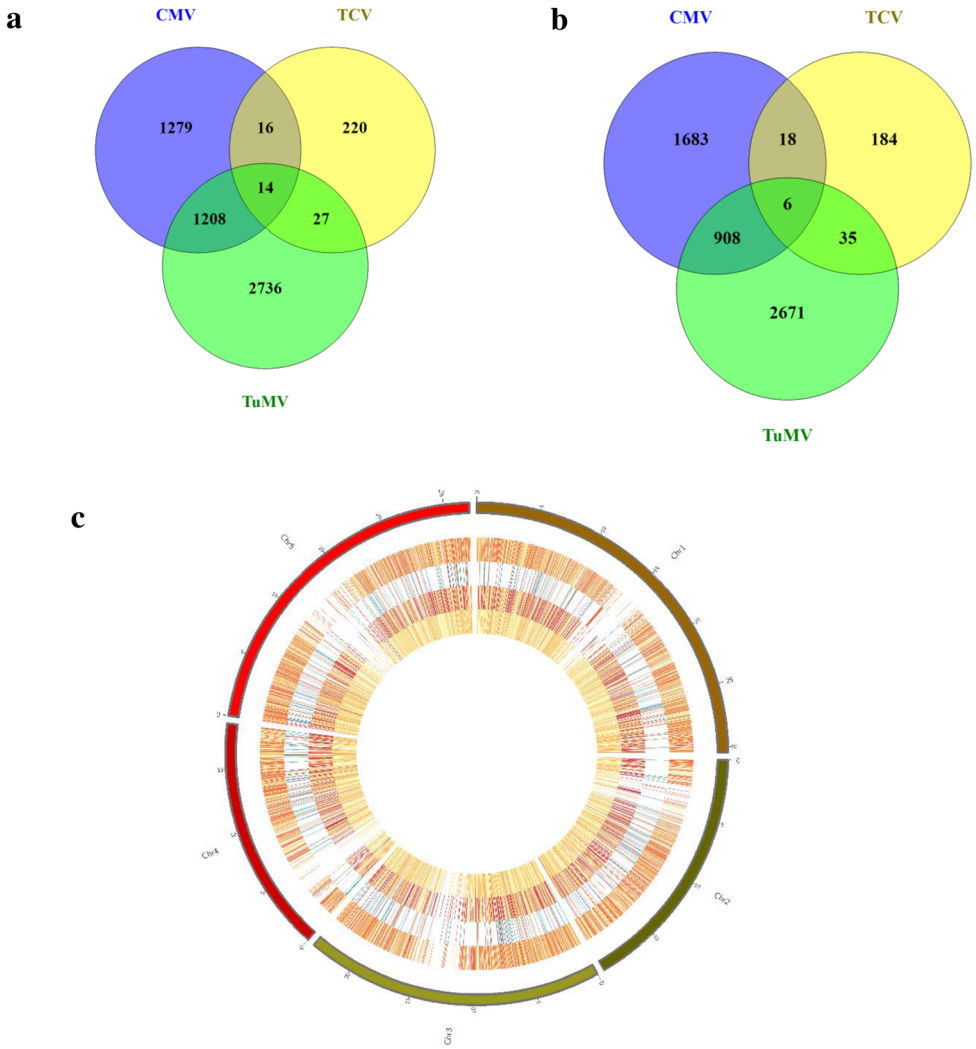

Fig. 1 Global transcription of differentially expressed genes in *Arabidopsis thaliana*. **a** Venn diagram of up-regulated genes in response to CMV, TCV and TuMV viruses and **b** down-regulated genes. **c** Transcriptional responses of genes shown in circos on 5 chromosomes

common up/down-regulated genes and DEGs response to each virus signaled the biological process, cellular component and molecular function categories (Figs. 2, 3). Genes encoding immune response mediators, signal transducer activity, signaling and stress response functions were among the most significantly upregulated genes (Fig. 2a), which were also indicated in cellular components including cell (88.3 and 90%), cell part (88.22 and 90%) and organelles (67.59 and 73.33%), during CMV and TuMV or CMV and TCV infections, respectively in upregulated genes (Fig. 2a). Whereas, most of the down-regulated genes were related to cellular components including cell (90.1 and 87.5%), cell part (90.1 and 87.5%) and organelles (79.08 and 70.83%), respectively during CMV and TuMV or CMV and TCV infections (Fig. 2b). Viruses rely on host cell machinery and their infection can fine-tune gene expression in cells, which might cause upregulation and downregulation of the genes. It has also been observed that viruses disrupt a few processes in plant cells leading to changes in metabolic responses (Alazem and Lin 2015). Furthermore, the presence of organelles like mitochondria (Tables S1 and S2) in GO analysis of differentially expressed genes during viral infections might provide energy balance required for defense responses including programmed cell death (Rojas et al. 2014; Lam et al. 2001), which may play pivotal roles in *Arabidopsis's* response to the viruses. In molecular function process, the largest group of up-regulated genes has belonged to the binding with 52.63 and 46.66% followed by catalytic activity with 44.03 and 53.33% for CMV and TuMV or CMV and TCV infections, respectively (Fig. 2a). The GO results revealed up/down-regulated genes related to plant defense responses and plant-virus interaction during CMV, TuMV and TCV infections. As compared to common DEGs between CMV and TCV, some functional categories such as positive regulation of biological process, locomotion, nutrient reservoir activity, molecular function regulator, electron carrier activity, supramolecular fiber, cell junction, symplast, extracellular region parts were up-regulated between CMV and TuMV (Fig. 2a), while growth, negative

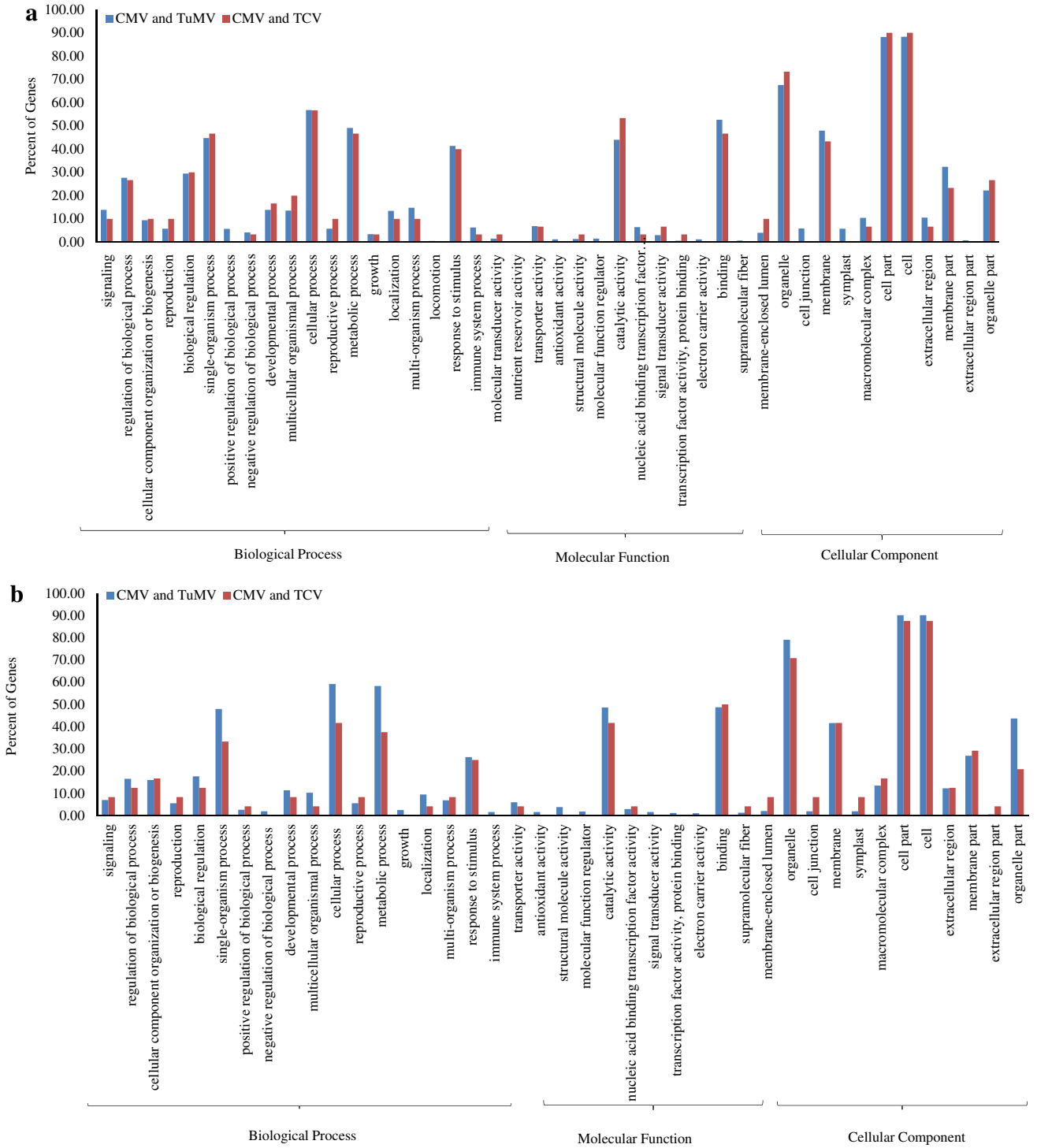

Fig. 2 Gene Ontology (GO) analysis of **a** the common up-regulated genes in response to CMV and TuMV and to CMV and TCV and **b** the common down-regulated genes in response to CMV and TuMV and to CMV and TCV in *Arabidopsis*. The *x*-axis represented GO terms classify based on three functional categories and the *y*-axis represented the percent of incorporated genes

regulation of biological process, immune system process, antioxidant activity, structural molecule activity, molecular function regulator, signal transducer activity, TF activity, protein binding related DEGs were down-regulated (Fig. 2b). Their down-regulation in the *Arabidopsis* in response to viral infections could suggest a functional part for these factors in biotic stresses. These transcriptome alterations were distinctly different between two viral

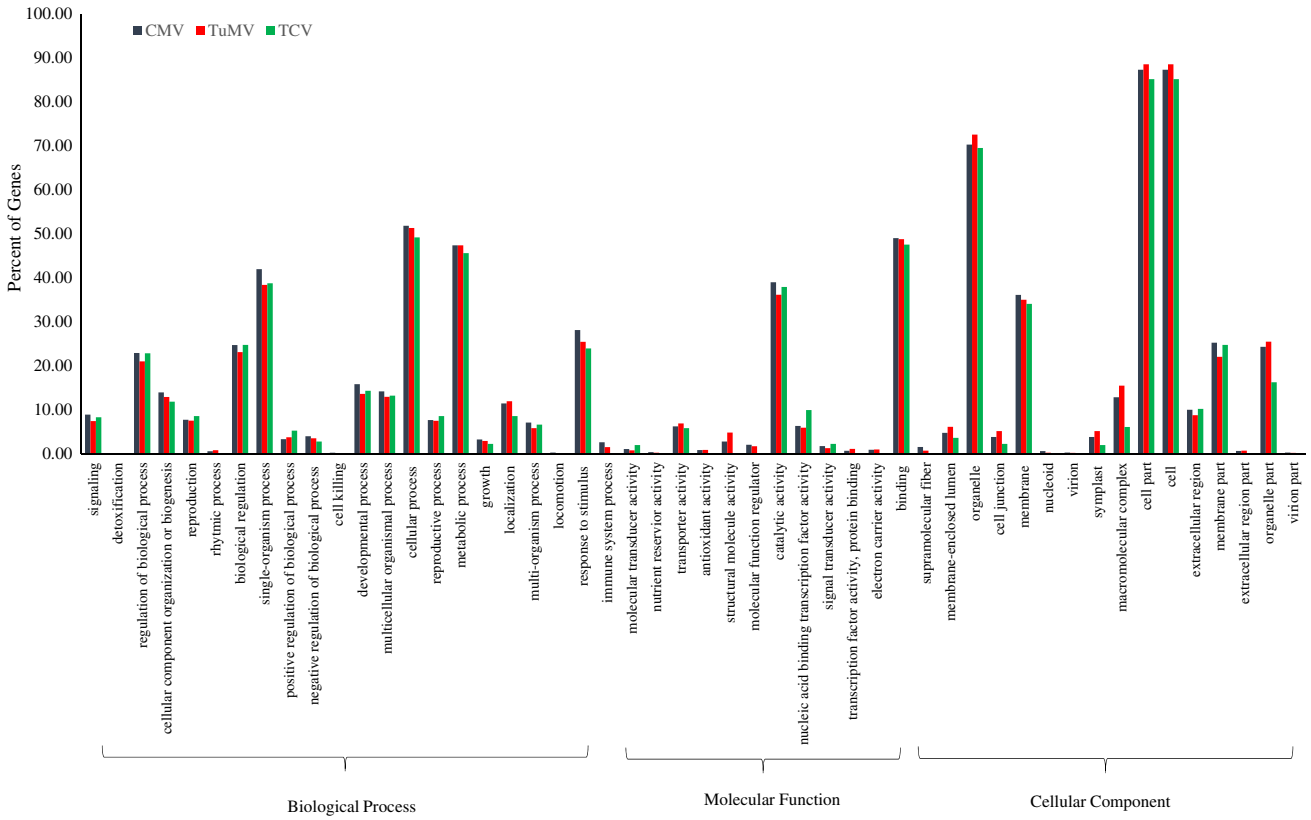

Fig. 3 Gene Ontology (GO) analysis of differentially expressed genes (DEGs) in response to CMV, TuMV and TCV in Arabidopsis. The *x*-axis represented GO terms classification based on three functional categories and the *y*-axis represented the percent of incorporated genes

interactions, and it might activate different host pathways resulting in various viral interactions. In this study, the responsive genes to biotic and abiotic stresses, immune and defense responses, response to hormones including abscisic acid, salicylic acid (SA) and jasmonic acid (JA) and leaf senescence were upregulated in *Arabidopsis* against the CMV and TuMV infections (Table S3). Plant hormones play a major role in various defense signaling pathways, and some viruses interact with these signaling pathways to enhance their infection process (Culver and Padmanabhan 2007; Bari and Jones 2009). Chemical signal compounds, such as SA and methyl jasmonate (MeJA), which have defensive and senescence-promoting functions, regulate the expression of certain senescence and pathogen-associated genes (Schenk et al. 2005). Abscisic acid is a key signaling molecule that mediates plant stress response by activating many stress-related genes (Cheng et al. 2016). Genes encoding photosynthesis and metabolism processes were downregulated in response to the viral infections (Table S4). Previous studies showed that plant photosynthetic activity is negatively regulated by virus infection (Rahoutei et al. 2000). The viral restrictions on photosynthesis, chlorophyll and metabolism processes might be due to nutrient deprivation as a common outcome of both viral infections (Bazzini et al. 2011). In addition, both viral infections can perturb metabolic process of *Arabidopsis* and activate plant-pathogen interaction, resulting in alterations of gene expression and cellular metabolism. As compared to individual infections of CMV or TuMV, positive regulation of biological process, reproductive process, molecular and signal transducer activity and nucleic acid binding transcription factor (TF) activity showed the highest percentage in TCV infection (Fig. 3). While, activities like cell killing, growth, immune system process, nutrient reservoir activity, antioxidant activity, molecular function regulator, TF activity and protein binding indicated lower percentage in TCV than those in individual infections of CMV and TuMV (Fig. 3). Some processes including developmental process, response to stimulus, immune system process, signaling, cellular component organization or biogenesis and catalytic activity showed the highest value in CMV infection compared to others infections in this study. The highest percentage of some processes like structural molecule activity, organelle, cell junction, symplast, cell and macromolecular complex was also observed in TuMV infection (Fig. 3). These findings revealed that immune system process contained the lowest and highest extent in response to TCV and CMV infections, respectively. Eight significant pathways were enriched with up-regulated DEGs in KEGG analysis,

including glutathione metabolism, regulation of autophagy, protein export, protein processing in endoplasmic reticulum, proteasome, SNARE interactions in vesicular transport and plant-virus interaction during CMV and TuMV (Fig. 4). On the other hand, down-regulated DEGs exhibited the enrichment of some pathways including metabolic processes, photosynthesis and biosynthesis and metabolism of amino acids and fatty acids during CMV and TuMV infections (Fig. 4). The DEGs of pathways including glutathione metabolism and autophagy were significantly induced in response to CMV and TuMV infections. Glutathione as an antioxidant and important regulator of redox signaling plays key role in modulating plant defense by the activation of defense genes following virus infections (Foyer and Noctor 2009). Autophagy as a conserved intracellular degradation pathway is also induced against various stresses like viral infections (Batoko et al. 2017; Hafren et al. 2017). In addition, autophagy has also been related to regulation of defense hormone signaling and host cell death (Kabbage et al. 2013; Dagdas et al. 2016). Protein export-related DEGs were also up-regulated during CMV and TuMV infections. Under stress conditions, nuclear import of certain proteins can be critical for the plant to reprogram cellular processes to combat the stress (Zhao et al. 2007), which can show protein export activity in response to viral infections. In this study, the DEGs of endoplasmic reticulum (ER) were up-regulated which is a membrane-bound compartment that plays important roles in many cellular processes such as calcium homeostasis, protein processing, synthesis of protein and membrane

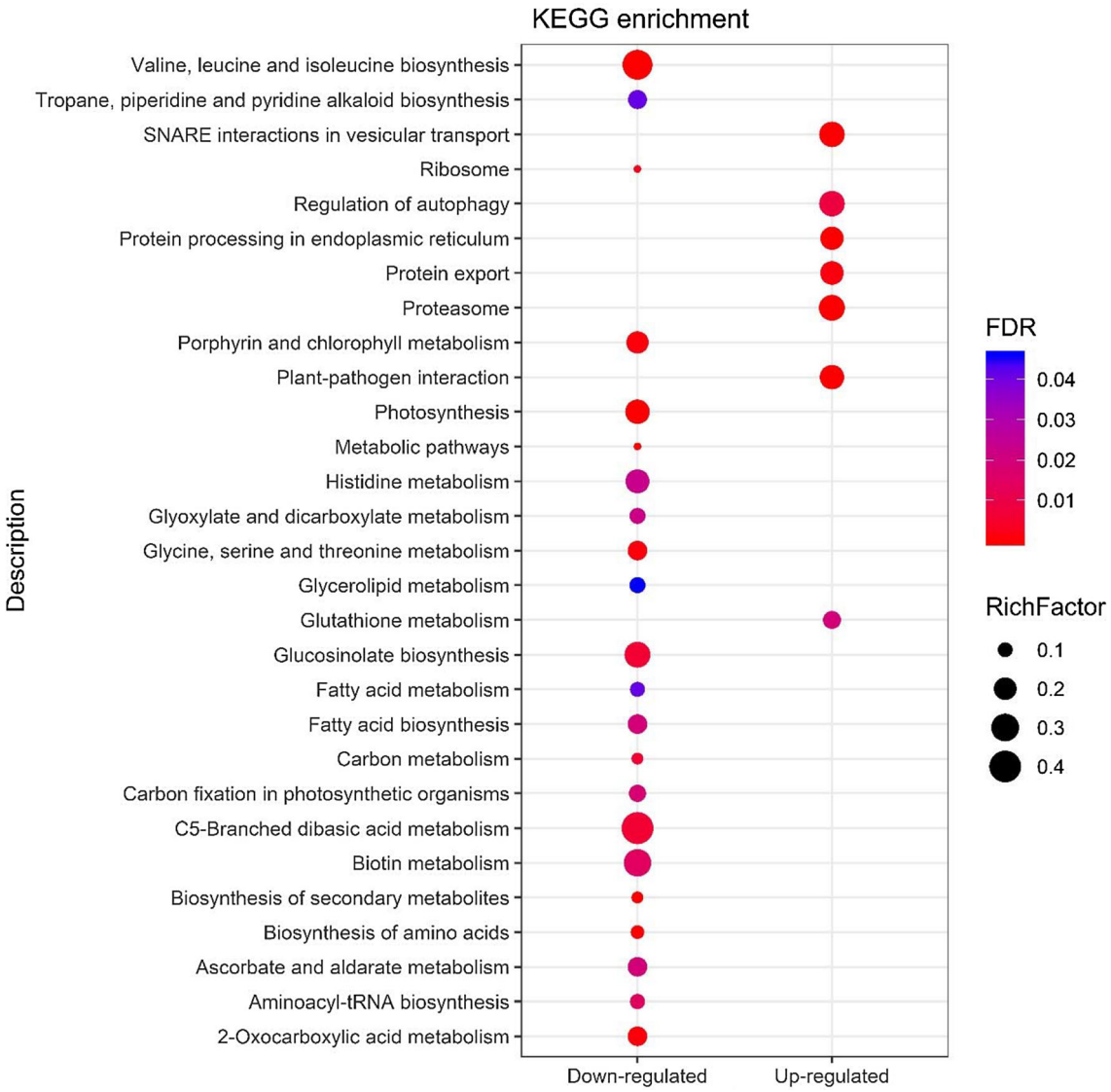

Fig. 4 KEGG pathway enrichment analysis of DEGs in response to CMV and TuMV in *Arabidopsis* (FDR rate < 0.05). The *y*-axis shows the enriched KEGG pathways. The color and the size of pathways descriptions represent the FDR and the Rich Factor, respectively. Rich factor is the ratio of the gene number to the total gene number in that specific pathway

lipid (Verchot 2014; Hetz 2012). Many viruses commandeer ER resident chaperones to contribute to virus replication and intercellular movement (Verchot 2014). Viruses make these changes on ER to create a cellular rich environment which is an essential step for viral infection. We have identified up-regulated proteasome-related DEGs that were responsive to the viral infections. Ubiquitin-26S proteasome system (UPS) is an important mechanism for protein removal (Vierstra 2009). UPS is also involved in the plant defense and in pathogen virulence program and plants-pathogens interactions (Citovsky et al. 2009; Dielen et al. 2010; Trujillo and Shirasu 2010), and the regulation of viral infection and plant-virus interactions (Alcaide-Loridan and Jupin 2012). Contrarily, UPS degradation of viral or cellular proteins is a major mechanism regulating viral infection (Citovsky et al. 2009; Alcaide-Loridan and Jupin 2012) which promotes viral replication and movement (Verchot 2016). The responsive up-regulated DEGs related to soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) proteins were identified. SNARE proteins are involved in facilitating vesicle traffic to ensure efficient targeting and delivery of specific membrane proteins (Rand and Parsegian 1989; Bock et al. 2001). Plant viruses utilize host endomembrane system and some viral proteins interact with SNARE-interacting proteins to promote virus systemic infection, replication and movement (Schoelz et al. 2011; Lewis and Lazarowitz 2010).

Responsive TFs

TF activity is one of the essential parts of molecular function among up-regulated genes in response to virus infections. It has been observed that TFs play a vital role in plant innate immunity (Seo and Choi 2015; Ashrafi-Dehkordi et al. 2018). TFs play pivotal roles in plant innate immunity by regulating genes related to pathogen-associated molecular pattern-triggered immunity and effector-triggered immunity (Seo and Choi 2015). Therefore, TFs role was investigated in response to individual virus or CMV and TuMV or CMV and TCV infections. The results indicated specific TFs including ARID, GeBP, C2C2-CO-like and CCAAT-HAP5 in TuMV, ARR-B and VOZ-9 in CMV, and BBR/BPC in TCV infection. TFs including WRKY, MYB and C3H in CMV, and C2H2, HSF, Homeobox and MADS in TuMV infection showed the highest TF numbers compared to those in the individual and common viral infections (Table 1). The highly expressed TFs might play a vital and distinct role in response to viral infections. In this study, twenty-six and two TF families were significantly up-regulated in response to CMV and TuMV or CMV and TCV infections, respectively (Table 1). The NAC, C3H, C2H2, WRKY and bZIP were the most commonly presented TF families in CMV and TuMV infection, while AP2-EREBP and C3H were the TF families involved in CMV and TCV infections (Table 1). The functional analysis of interactions between TFs and other proteins is very important for elucidating the role of these transcriptional regulators in different signaling cascades (Alves et al. 2014). Seven up-regulated basic leucine zipper (bZIP) TFs were identified during CMV and TuMV infections. bZIPs regulate disease resistance through interaction with other proteins in defense responses (Kaminaka et al. 2006). Furthermore, the number of twenty-three and eight Cys2/His2 (C2H2)-type zinc finger TF have been identified during TuMV and CMV and TuMV infections, respectively. C2H2 TFs are important components in the regulation of plant growth, development, hormone responses, defense responses to biotic and abiotic stresses (Jiang and Pan 2012; Ciftci-Yilmaz and Mittler 2008; Kiełbowicz-Matuk 2012). Moreover, C3H-type zinc finger TFs were identified among the most up-regulated TF families in response to CMV, CMV and TuMV and CMV and TCV infections. C3H TFs have been involved in multiple processes, including leaf senescence (Asad et al. 2013), and abiotic and biotic responses (Wang et al. 2008; Sui et al. 2012; Zhang et al. 2011). In addition, some C3H proteins are involved in multiple stress responses and hormonal pathways (Jan et al. 2013; Guo et al. 2009). The results revealed twelve and eight up-regulated WRKY TFs during CMV and CMV and TuMV infections. WRKY TFs are a large family of regulatory proteins forming such a network and are involved in diverse biological processes including biotic/abiotic stress-induced defense responses and senescence (Eulgem and Somssich 2007; Rushton et al. 2010; Jiang et al. 2014). WRKY transcription factor genes are also responsive to viral infections (Chen et al. 2013). The number of fourteen up-regulated NAC TFs were detected in response to CMV and TuMV infections. NAC TFs are one of the largest families of transcriptional regulators in plants, and have been suggested to play important roles in the regulation of plant stress responses (McLellan et al. 2013; Nuruzzaman et al. 2013; Jensen et al. 2010). Some NAC TFs can be targeted by various pathogens to enhance disease susceptibility and as negative regulators of the plant defense responses (McLellan et al. 2013). For instance, in rice seedlings, *NAC* genes were up regulated after virus infections (Nuruzzaman et al. 2010), and increases in the expression level of *NAC* genes have been monitored in response to virus infections (Ren et al. 2000; Selth et al. 2005).

Table 1 The up-regulated TF families in response to CMV, TCV, TuMV, and to both CMV and TuMV and to CMV and TCV infection in *Arabidopsis*

TF families	Genes number				
	CMV	TCV	TuMV	CMV and TuMV	CMV and TCV
ABI3VP1	ND	ND	1	ND	ND
Alfin-like	ND	ND	ND	3	ND
AP2-EREBP	7	ND	9	5	1
ARF	2	1	4	1	ND
ARID	ND	ND	1	ND	ND
ARR-B	1	ND	ND	ND	ND
BBR/BPC	ND	1	ND	ND	ND
bHLH	7	3	9	1	ND
bZIP	8	1	9	7	ND
BZR	1	ND	ND	1	ND
C2C2-CO-like	ND	ND	2	ND	ND
C2C2-Dof	1	ND	2	ND	ND
C2C2-Gata	1	ND	3	ND	ND
C2H2	8	2	23	8	ND
C3H	15	2	6	9	1
CAMTA	1	ND	ND	ND	ND
CCAAT-DR1	ND	ND	1	1	ND
CCAAT-HAP2	4	ND	2	2	ND
CCAAT-HAP3	1	ND	2	ND	ND
CCAAT-HAP5	ND	ND	4	ND	ND
CPP	1	ND	ND	ND	ND
E2F-DP	ND	ND	1	ND	ND
EIL	ND	ND	ND	1	ND
G2-like	1	1	1	2	ND
GeBP	ND	ND	4	ND	ND
GRAS	3	ND	7	1	ND
GRF	1	ND	3	ND	ND
Homeobox	3	1	10	1	ND
HSF	1	1	5	3	ND
JUMONJI	ND	ND	1	1	ND
MADS	ND	1	3	1	ND
MYB	8	ND	4	3	ND
NAC	4	2	6	14	ND
NLP	ND	ND	1	ND	ND
PHD	1	ND	ND	ND	ND
RAV	ND	ND	ND	2	ND
REM	ND	1	3	1	ND
SBP	2	ND	1	1	ND
TCP	1	ND	2	1	ND
Trihelix	3	1	3	1	ND
TUB	1	ND	1	1	ND
VOZ-9	1	ND	ND	ND	ND
Whirly	ND	ND	1	ND	ND
WRKY	12	ND	4	8	ND
ZF-HD	ND	ND	1	ND	ND

ND not detected

TF's network

TFs including WRKY and NF-YA in CMV, and NF-YC, NF-YA and NF-YB in TuMV showed the nodes with the score higher than 0.7 (Fig. 5b, c). Recently, it has been shown that plant NF-Y TFs play important roles in plant–microbe interactions and adaptation to stresses (Zanetti et al. 2017). To uncover transcriptional regulatory mechanisms by TFs in host innate immune responses of *Arabidopsis*, a TFs network was constructed by common differentially expressed TFs between CMV and TuMV interaction including about 40 nodes in *A. thaliana* (Fig. 5) *MYB15* (*AT3G23250*), *MYB51* (*AT1G18570*), *STZ* (*AT1G27730*), *WRKY40* (*AT1G80840*), *RHL41* (*AT5G59820*), *HSFA4A* (*AT4G18880*) and *ERF11* (*AT1G28370*) have revealed most interactions between differentially expressed genes during CMV and TuMV infections (Fig. 5a). The *RHL41* and *WRKY40* were primarily affected by other TFs such as, *STZ*, *MYB15*, *ERF11*, *WRKY46* (*AT2G46400*) and *HSFA4A* (Fig. 5a). We aimed to construct a gene network among differentially expressed TFs. Thus, it is suggested that *MYB15*, *MYB51*, *STZ*, *WRKY40*, *RHL41*, *HSFA4A* and *ERF11* play main role in compatible interaction during CMV and TuMV infection in *Arabidopsis*.

These TFs might play important roles in response to these viral infections. Additionally, several other signaling components, such as MYB, ERF and WRKY TFs,

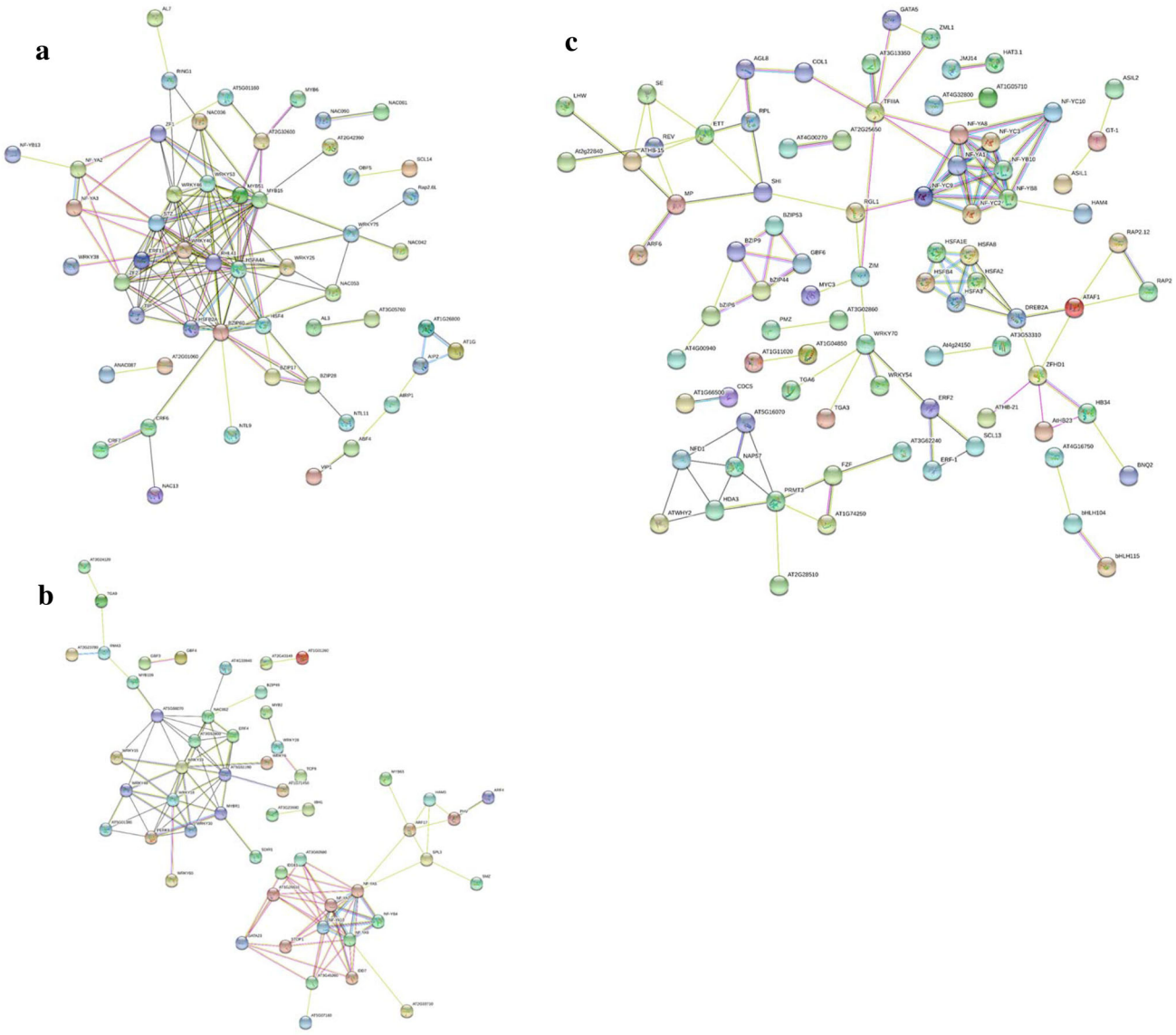

Fig. 5 Gene network of up-regulated TFs in response to **a** both CMV and TuMV, **b** CMV and **c** TuMV in *Arabidopsis*. The lines represent interaction between differentially expressed genes

contribute in regulation of defense response against virus infection. Previous studies have shown the importance of ERF in biotic and abiotic stress tolerance mechanisms (Sharma et al. 2010). Nevertheless, MYBs essentially contribute in plant growth, development, primary and secondary metabolism, and response to biotic and abiotic stresses (Oh et al. 2003; Van der Ent et al. 2008). WRKY TF positively regulates defense response upon *Tobacco mosaic virus* infection in *Capsicum annuum* and is involved in resistance via transcriptional reprogramming of pathogenesis-related (PR) gene expression (Huh et al. 2015). In conclusion, TFs and their networks are important regulators of biological processes that provide insight into the regulation of defense mechanisms which are useful in plant breeding to enhance virus resistance.

Expression pattern of TFs in different developmental stages and tissues

Genevestigator database analysis showed maximum expression of the majority of TFs genes occurred at the senescence stage in leaf protoplast (Fig. 6a–e and Fig. S3). It indicates that senescence rates are influenced by different mechanisms upon viral infections that might be due to different viral infections by inducing nutrient competition. Leaf senescence is a fine-tuned and natural developmental process that involves cell death for recycling and reuse of valuable resources (Gan and Amasino 1997), and which has also been induced by abiotic and biotic stresses (Haffner et al. 2015; Navabpour et al. 2003; Xiong et al. 2005). Pathogen infection influence leaf senescence via modulation of the plant metabolite status directly affecting primary metabolism or by regulating levels of plant hormones (Masclaux-Daubresse et al. 2010; Fagard et al. 2014; Diaz-Mendoza et al. 2016). The expression of senescence-associated genes was also increased during compatible interactions between plants and viruses (Espinoza et al. 2007). Senescence-related genes may play key roles in the modulation of plant susceptibility to virus infection by supporting virus infections in systemically infected host plants (Fernandez-Calvino et al. 2016). Where, NAC and WRKY showed the common presented TF families in *A. thaliana* during CMV and TuMV infection. NAC and WRKY families are among the largest groups of senescence-related TFs (Balazadeh et al. 2008; Breeze et al. 2011), which play important roles in leaf senescence. Thus, our results support the notion that senescence may be closely related to NAC-mediated stress responses (Nuruzzaman et al. 2013). Moreover, further studies are needed to study the convergence between senescence and pathogen defense in viral infections.

Identification of miRNA targeting down-regulated genes

Earlier findings revealed that plant miRNAs are involved in response to stresses (Gao et al. 2011; Sunkar and Jagadeeswaran 2012). Analysis of miRNAs during CMV and TuMV and CMV and TCV infections demonstrated eleven and five miRNA families, respectively indicating the role of these miRNAs in the down regulation of host genes in response to viral infections (Table 2 and Table S5). We also detected the miRNA's functional roles in growth, metabolism and biotic and abiotic stress responses. The ath-miR5021, ath-miR854, ath-miR5658, ath-miR172 and ath-miR414 were predicted to have the highest number of gene targets with 43, 35, 23, 19 and 17 targets among down-regulated genes, respectively, in CMV and TuMV infection, whereas the ath-miR5021 and ath-miR393 were identified with the highest targets (four and two targets) during CMV and TCV infection that were significantly higher than the other miRNAs (Table 2 and S3). There was ath-miR5021 miRNA with 20 nt in length, while the other miRNAs including ath-miR838, ath-miR414, ath-miR4239 with 21 nt were identified in CMV and TCV infections. The ath-miR5021 and ath-miR156 miRNAs represented 20 nt in length, while other identified miRNAs with frequencies more than five showed 21 nt in length during CMV and TuMV infections. The ath-miR5021 was recently found to have role in biotic stress responses and targets a diverse set of genes including global transcription factors and disease resistance proteins (Das et al. 2015; Kharonov 2013). The role of miR854 in the plant's response against arsenic (As) stress was already shown (Srivastava et al. 2012). The miR5658 targets various transcription factors, the nucleotide binding site leucine-rich repeat (NBS-LRR) resistance protein, proteins involved in signal transduction, molecular chaperone regulators, growth regulators, protein ligases, WRKY transcription factor, argonaute protein etc. (Han et al. 2014; Li et al. 2014b; Joshi 2018). Some miRNAs such as miRNA172 and miR393 are involved in pathogen defense (Sunkar and Jagadeeswaran 2012). MiR393 affects various processes including plant response to biotic stress and can fine-tune plant defense responses (Etemadi et al. 2014; Wong et al. 2014; Robert-Seilaniantz et al. 2011). Responsive abscisic acid genes were up-regulated during both viral infections. Accordingly, miRNAs such as miR172a and miR5658 may imply a complex crosstalk between the global regulation of miRNA metabolism and abscisic acid signaling functions which enable the fine-tuning of stress response in plants (Matsui et al. 2013). MiR414 targets transcriptional factors including the bZIP, WRKY, MYB, B3, scarecrow, heat shock proteins and TCP that play roles in plant growth, development, physiological and morphological changes, metabolism

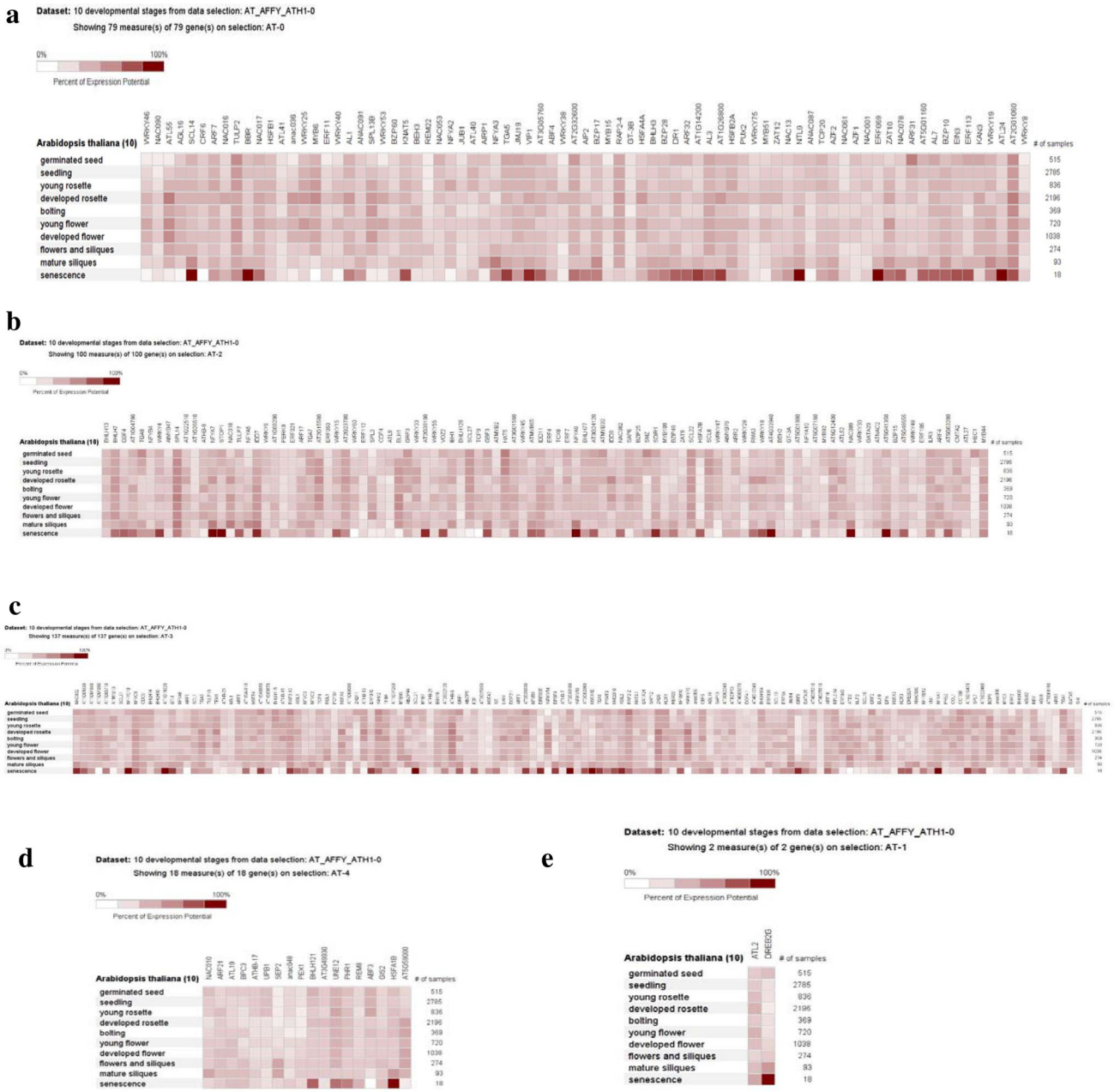

Fig. 6 Heat map representation for expression pattern of up-regulated TFs in 10 different developmental stages in **a** CMV and TuMV, **b** CMV, **c** TuMV, **d** TCV and **e** CMV and TCV

defense responses and abiotic stress regulation (Li et al. 2014b; Palatnik et al. 2003; Guo et al. 2007; Romanel et al. 2009). In addition, genes involved in proteasome degrading pathway such as ubiquitin conjugating enzyme are regulated by miRNAs including miR414 (Das and Mondal 2010). Therefore, the results of this study showed that these miRNAs could affect some common genes and pathways in the viral infections. In addition, the identification of miRNAs and target genes have a great capacity to progress plant genetic improvement that can result in transgenic plants with improved productivity.

Cross-validation analysis

The validation analysis of our data was implemented through two approaches, a *metaSeq* package in Rstudio was conducted to confirm the accuracy of our data. All of the common genes in our database were confirmed through this package in Fisher and Stouffer methods (Fig. S2A). Then, SVM method in Cross-validation analysis was used to determine the accuracy of our data between samples under normal and stressful circumstances. Therefore, this analysis was correctly categorized as the expression level of

Table 2 A list of miRNA families potentially targeting down-regulated genes in response to both (CMV and TuMV) viruses (where frequency > 5 and all miRNA families are related to CMV and TCV viruses)

Response to viruses	MiRNA family	Number of down-regulated target genes with the miRNA motif	Mean UPE	Mean expectation	Inhibition mechanism
CMV and TuMV	ath-miR5021	43	10.24	2.51	Cleavage/translation
	ath-miR854	35	7.65	3	Cleavage/translation
	ath-miR5658	23	9.34	2.15	Cleavage/translation
	ath-miR172	19	16.77	2.31	Cleavage/translation
	ath-miR414	17	11.79	2.55	Cleavage/translation
	ath-miR157	9	16.99	3	Cleavage/translation
	ath-miR395	9	19.81	2.16	Cleavage
	ath-miR838	9	15.21	2.88	Cleavage/translation
	ath-miR156	8	13.83	2.75	Cleavage/translation
	ath-miR1886	7	9.52	2.78	Cleavage/translation
	ath-miR159	6	12.94	2.75	Cleavage/translation
CMV and TCV	ath-miR5021	4	9.42	2.25	Cleavage/translation
	ath-miR393	2	10.68	2.5	Cleavage
	ath-miR4239	1	19.93	2.5	Cleavage
	ath-miR414	1	10.44	3	Cleavage
	ath-miR838	1	9.92	3	Translation

UPE: maximum energy to unpair the target site

selected genes with the accuracy of 77.5% with area under curve (AUC) value of 0.96 (Fig. S2B and C).

Conclusion

The dissection of the molecular network of virus-host interactions will help us to better understand the molecular mechanisms and regulatory cellular pathways of defense in plants, identify essential host gene pathways required for resistance to viruses, clarify the interactions among different TFs, and determine responsive miRNAs and identify candidate genes during viral infections. These observations will be important to identify differentially and commonly expressed virus-responsive genes and unique and conserved pathways in plant-virus interaction. The study of important conserved genes between CMV and turnip viruses might be an effective approach for the understanding of host responses during synergistic and antagonistic interactions of viruses which might provide common targets and develop novel plant antiviral strategies for improving plant resistance to mixed viral infections using genetic engineering approaches.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Consent for publication The manuscript has been read and approved by all named authors.

Ethical approval This study did not include any experiments with human participants or animals performed by all the authors.

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