Transcriptomic changes in *Nicotiana tabacum* leaves during mosaic virus infection

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Abstract

To provide a detailed insight into the early biological process of tobacco mosaic disease, transcriptomic changes in tobacco leaves were surveyed at 1, 3 and 5 days after mono-infected by *Tobacco mosaic virus* (TMV) and co-infected by *Cucumber mosaic virus* (CMV) and TMV. At the three different stages, there were 2372, 3168 and 2045 differentially expressed genes (DEGs) in mono-infected leaves, and 2388, 3281 and 3417 DEGs were identified in co-infected leaves. There were 836, 1538 and 1185 common DEGs between the mono-infection and co-infection at the three time points, respectively. These common DEGs were enriched in the pathways, such as photosynthesis, biosynthesis of secondary metabolites, plant–pathogen interaction, porphyrin and chlorophyll metabolism, phenylalanine metabolism and phenylpropanoid biosynthesis. Photosynthesis pathway was observably down-regulated, and defense response pathways were markedly up-regulated. These pathways have been found to be related to tobacco mosaic disease. Of these common DEGs, the changes in expression of argonaute proteins, thioredoxins and peroxidases showed that the activation of RNA silencing and the destruction of redox balance can be induced by tobacco mosaic virus infection, resulting in the reset of biology process and damage in tobacco plants. Additionally, the occurrence of symptoms in co-infected tobacco plants was more early and serious than mono-infection, indicating that there is synergy between TMV and CMV in co-infected tobacco plants. The timely usage of antiviral agents and plant resistance inducers can decrease the incidence of tobacco mosaic disease through changing the expression of some DEGs, indicating that these genes can be used to screen novel plant resistance inducers and antiviral agents. Overall, our results were helpful in clarifying the mechanism of tobacco mosaic disease and provided novel strategies for the prevention of tobacco mosaic disease.

Keywords Transcriptomic changes · Multiple infections · Tobacco mosaic disease · RNA silencing · Redox balance

Introduction

In the field of agriculture, the virus diseases can cause qualitative and quantitative losses of yield. It is still a challenging problem to improve the control methods of eliminating the viral diseases. Several viruses including *Tobacco mosaic*

virus (TMV), *Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY), *Tobacco etch virus* (TEV) and *Tobacco vein banding mosaic virus* (TVBMV) are the major viruses infecting tobacco crops in China (Zhao et al. 2017). Tobacco mosaic disease is often caused by multiple infections of TMV and CMV, and it can cause the drastic decrease in yield, the appearance characters and intrinsic quality of flue-cured tobacco (Dai et al. 2012). Therefore, tobacco mosaic disease often brings out huge economic losses, and has become an important constraint for production of high-quality tobacco leaves. At present, since it is difficult to control the spread of tobacco mosaic disease, prevention and surveillance is very important for tobacco production.

Tobacco crop diseases result from the interaction of tobacco plant, pathogen and environment. The infections of plant RNA viruses can obviously induce reprogramming of host gene expression and the transcriptional changes

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(Whitham et al. 2003, 2006; Havelda et al. 2008). After infection of mosaic virus, tobacco plants produced some physiological responses through altering genes expression and enzymes activities, and then resulted in the occurrence of disease symptoms. Deep-sequencing technology has recently become a powerful tool that improves the efficiency and speed of gene discovery. RNA sequencing has the ability to easily perform quantitative gene expression comparisons without potential bias, allowing for a more sensitive and accurate transcriptional profile that more closely resembles the biology of the cell. A previous study has reported global changes in gene expression in tobacco plants infected by M-CMV, which is helpful in understanding the molecular mechanisms of the symptom development process at 6–20 days after infection (Lu et al. 2012). The important KEGG pathways influenced by M-CMV infection included ribosome, biosynthesis of secondary metabolites, photosynthesis, carbon fixation in photosynthetic organisms, porphyrin and chlorophyll metabolism, anthocyanin biosynthesis, carotenoid biosynthesis and plant–pathogen interaction (Lu et al. 2012). In the initial pathogenesis process, energy metabolism (including the ‘carbon fixation in photosynthetic organisms’, ‘photosynthesis’ and ‘sulfur metabolism’ pathways) and pigment metabolism (including ‘porphyrin and chlorophyll metabolism’, ‘anthocyanin biosynthesis’ and ‘carotenoid biosynthesis’) were down-regulated by CMV infection. The up-regulated pathways, such as ‘diterpenoid biosynthesis’ and ‘zeatin biosynthesis’, were related to plant defense (Lu et al. 2012). Quantitative transcriptional changes showed that coat protein (CP) mutation of CMV governed the expression level of chloroplast- and photosynthesis-related genes, which are closely associated with the induction of chlorosis (Mochizuki et al. 2014). The alterations in the gene expression profile induced by TMV were analyzed in *Arabidopsis thaliana* through cDNA microarrays (Golem and Culver 2003). There were 68 genes that displayed significant and consistent changes in expression levels after TMV infection, including transcription factors, antioxidants, metabolic enzymes and transporters (Golem and Culver 2003). The metabolic components and miRNA profile were analyzed after *Nicotiana benthamiana* and *Nicotiana tabacum* were infected by TMV, and the involvement of a systemic signaling on early miRNAs alteration was proposed (Bazzini et al. 2011; Yin et al. 2015).

Tobacco mosaic disease is the most prevalent viral disease in tobacco plants and responsible for large crop losses every year, so the rapid diagnosis is of great importance to set measurements for constraining the spread of TMV in the field. The study on molecular mechanism in the early stage of tobacco mosaic disease is the basis for its rapid diagnosis. In the tobacco field production, tobacco mosaic disease is often caused by multiple infections of TMV and CMV. However, the gene expression profile of multiply

infected tobacco plants by mosaic viruses has been rarely studied. To provide a detailed insight into the early stage of tobacco mosaic disease, the transcript profiles of tobacco leaves at 1, 3 and 5 days after the two kinds of infection were investigated in present study. In line with results from previous studies (Lu et al. 2012; Zhu et al. 2018), we found that photosynthesis, porphyrin and chlorophyll metabolism, plant–pathogen interaction, and biosynthesis of secondary metabolites were influenced by mosaic virus infection. Except for these pathways in previous reports, it was found that mosaic virus infection can induce the changes in expression of argonaute proteins and then activate RNA silencing in the early stage of tobacco mosaic disease. In addition, the destruction of redox balance can also contribute to the occurrence of tobacco mosaic disease. The analysis of DEGs contributes to not only clarifying the physiological responses of tobacco plants to mosaic viruses but also the prevention of tobacco mosaic disease.

Materials and methods

Plant materials

Tobacco plants (*Nicotiana tabacum* cv. K326) were grown in a greenhouse on a cycle of 16 h light at 30 °C and 8 h dark at 25 °C. TMV (common strain) and CMV (common strain) were, respectively, multiplied in tobacco plants. The infected leaves were harvested for purifying mosaic viruses with the Gooding’s method (Gooding and Hebert 1967), and then the concentrations of TMV and CMV were determined with an ultraviolet spectrophotometer. The purified viruses were diluted to 50 µg/ml with 0.01 mol/L phosphate-buffered saline (PBS) before used. Tobacco plants were cultivated in pots and divided into 36 groups. There were ten tobacco plants in every group. The experiments were conducted when the plants grew to 4–5 leaf stage. Tobacco leaves were mono-infected with TMV solution or co-infected with the mixed solution of TMV and CMV according to the proportion of 1:1. The mock-inoculated tobacco plants groups were used as the control, which were only treated with 0.01 mol/L PBS. The incidence of tobacco mosaic disease was surveyed at 1 day, 3 days and 5 days after infection. Each treatment was replicated three times. All leaf samples were obtained and then pooled group-wise at the three time points after infection. These samples were immediately frozen in liquid nitrogen, and then stored at – 80 °C until used.

To detect the effects of antiviral agents and plant resistance inducers on the expression of the genes related to tobacco mosaic disease, the following experiments were executed. (1) Tobacco plants were sprayed with amino-oligosaccharin (0.167 g per plant) or guanidine-copper acetate (0.25 g per plant) at 1d after infection of TMV.

The leaves were sampled at 1d after spraying. (2) Tobacco plants were sprayed with moroxydine hydrochloride (0.05 g per plant) or N-glycoside morpholine guanine (0.05 g per plant) at 7d before infection of TMV. The leaves were sampled at 1d after infection. All samples were immediately frozen in liquid nitrogen, and then stored at -80°C until used.

RNA isolation and library preparation for DGE

Five biological replicates were performed for each treatment. The leaves from five individual tobacco plants were sampled and then mixed for RNA extraction of each treatment. Total RNA was isolated from each treatment of tobacco plants using the CTAB method. All RNA samples were checked by capillary electrophoresis and spectrophotometer. The extracted RNA samples were selected based on 28S/18S rRNA band intensity (2:1) and spectroscopic A260/A280 readings between 1.8 and 2.0, A260/A230 readings greater than 1.5. The mRNA was purified with oligo (dT) magnetic beads and then produced short fragments in fragmentation buffer. These short fragments were used as a template for first and second-strand cDNA synthesis with random hexamers primer. After purified with QiaQuick PCR kit, the sequencing adaptor was ligated. The cDNA fragments were amplified to generate the library for RNA sequencing with Illumina HiSeq™ 2500.

Data analysis

Raw sequence reads were filtered through the Illumina pipeline. The low quality sequences were excluded, and the remaining high-quality sequences (clean tags) were mapped to tobacco Genome Survey Sequences (GSS, www.ncbi.nlm.nih.gov) by SOAP allowing for only 2 bp mismatch. Clean tags that mapped to reference sequences from multiple genes were filtered. The remaining clean tags were designed as unambiguous clean tags. The number of unambiguous clean tags for each gene was calculated and normalized to the number of transcripts per million clean tags (TPM).

A rigorous algorithm was used to identify DEGs in comparisons of two samples. “false discovery rate (FDR) ≤ 0.001 and the absolute value of $\log_2\text{Ratio} \geq 1$ ” was used as the threshold to judge the significance of differential gene expression. More stringent criteria with smaller FDR and larger fold change values were used to identify differentially expressed genes. For pathway and GO enrichment analysis, all DEGs were mapped to terms in Kyoto Encyclopedia of Genes and Genomes (KEGG) and GO databases, and the

significantly enriched terms were identified in comparison with the genome background.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from leaves of tobacco plants using TRizol solution and treated with RNase-free DNase I to remove potential DNA contamination. First-strand cDNA was synthesized using 1 μg of total RNA according to One Step PrimeScript RT-PCR Kit (perfect Real Time) protocol. TaKaRa SYBR Premix Ex Taq™ II (Perfect Real Time) was used for qRT-PCR on a Bio-Rad IQ5 Real-Time PCR Detection System. The volume of qRT-PCR reaction was 25 μL , and actin was used as the endogenous reference gene. qRT-PCR was performed as described (Li et al. 2016). The three tobacco plants were selected as biological replications. Three replicates were performed for each sample. The primer pairs used for qRT-PCR are shown in Table S1.

The interactions network analysis of proteins

The proteins related to RNA silencing and redox balance from RNA-Seq data were entered into the search tool of STRING Version 11.0 (<https://string-db.org/>), and the interactions network of proteins was constructed. Functional enrichments in network were performed by Gene ontology (GO) analysis.

The determination of ascorbate peroxidase (APX) activity

The fresh tobacco leaves (0.15 g) were ground and extracted in 1.5 ml of 50 mmol/L PBS (pH 7.8) including 2 mmol/L ascorbate (AsA), 0.1 mmol/L EDTA and 1% PVP. The extract was centrifuged at 12,000g for 15 min, and the supernatant was used to determine the activity of APX. All extractions were performed at 4°C . The protein content was measured by Bradford method (Bradford 1976), using BSA as a standard.

APX was measured spectrophotometrically with a modified method of Nakano and Asada (1987). The reaction mixture (2.95 mL) contained 50 mmol/L $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 7.0), 250 $\mu\text{mol/L}$ AsA, 2 μL of 30% H_2O_2 (w/w) and 50 μL of extract. The decrease in A290 was measured as AsA was oxidized. One unit of APX enzyme activity corresponded to the oxidation of 1 μmol AsA per 1 min by 1 g of protein. All manipulations were made at 25°C and were performed three times for each sample.

The determination of H_2O_2 content

The content of H_2O_2 was detected with the modified titanium sulfate method (Eisenberg 1943; Zdenko et al. 2013).

Tobacco leaves (0.1 g) were ground in 2.5 ml precooled acetone, and then centrifuged at 3000g for 10 min. The supernatant was used to determine the content of H_2O_2 . Peroxide-titanium complex was formed in the mixture solution including 1 ml the extract, 0.1 ml of 20% titanium tetrachloride and 0.2 ml of ammonium hydroxide. After centrifuged at 3000g for 10 min, the precipitate was rinsed with acetone for three times, and it was dissolved in 5 ml of 2 mol/L sulfuric acid. The reaction solution was diluted to 10 ml with distilled water, and then the absorbance at 415 nm was measured. The content of H_2O_2 was calculated through comparing with the calibration curve determined from known concentrations of H_2O_2 .

Results

Symptoms of tobacco after infection of TMV and CMV

Rugosity, chlorosis and the “mosaic” or mottled pattern of light and dark green areas in the leaves are the important symptoms of tobacco mosaic disease. As shown in Fig. 1, the slight rugosity can be observed in the infected leaves at 1d after inoculated with mosaic virus. The “mosaic” or mottled pattern appeared at 3 days after infection, and it was more serious at 5 days after infection. The occurrence of symptoms in tobacco plants co-infected by TMV and

CMV was more early and serious than that mono-infected by TMV, indicating synergy between TMV and CMV infection. It was also observed that the expression of CP was obviously higher in co-infection than that in mono-infection (Fig.S1). Therefore, the co-infection can accelerate the replication and accumulation of TMV in tobacco plants, resulting in rapid and serious symptoms occurrence of tobacco mosaic disease.

Differentially expressed genes at the three time points after infection

The gene expression profiles of healthy tobacco plant were used as controls. If the gene expression in the infected tobacco plant recorded a twofold (or more) difference relative to the control ($FDR \leq 0.001$), this gene was regarded as the DEG. For infection only with TMV, there were 2372, 3168 and 2045 DEGs at 1 day, 3 days and 5 days after infection, respectively. The numbers of up-regulated DEGs were 802, 1197 and 1444, and the numbers of down-regulated DEGs were 1570, 1971 and 601 at the three time points (Fig. 2a). For co-infection with TMV and CMV, there were 1003, 1829 and 2750 up-regulated DEGs, which were more than mono-infection. The numbers of down-regulated DEGs were 1385, 1452 and 667 at the three time points. Apart from the third time point, the numbers of down-regulated DEGs were less than mono-infection. For the two kinds of infection, the number of up-regulated DEGs sharply

Fig. 1 Symptoms of tobacco plants after mono-infection and co-infection. *TMV* tobacco plants infected only by TMV; *TMV + CMV* tobacco plants co-infected by TMV and CMV; *Control* tobacco plants

without infection. *1d* 1 day after infection, *3d* 3 days after infection, *5d* 5 days after infection

Fig. 2 The differential expressed genes in tobacco leaves after infected with mosaic virus. **a** Changes of differential expressed genes after infected with mosaic virus. **b** Differential genes between mono-infected and co-infected tobacco. HV1, HV2 and HV3 denoted the DGEs at 1 day, 3 days and 5 days after co-infected with TMV and CMV. SV1, SV2 and SV3 represented the DGEs at 1 day, 3 days and 5 days after mono-infected with TMV

increased at the three time points, and the number of down-regulated DEGs increased at first two time points and then drastically decreased at the third time point. Since having the similar trend in changes of DEGs number, the biology processes of tobacco mosaic disease induced by the two kinds of infection should be alike. However, the cluster analysis of change trend of DEGs expressions showed that there was some difference between co-infection and mono-infection (Fig.S2). The most significant change trend in mono-infected tobacco plants was that DEGs expressions decreased at first and then increased. However, in co-infected tobacco plants, the most significant change trend was that DEGs expressions increased sharply at first and then kept unchanged. Virus synergism is widespread phenomenon when plant is co-infected by two or more virus (Takeshita et al. 2012). Therefore, it was easy to understand that tobacco mosaic disease induced by co-infection can occur more quickly and seriously than mono-infection.

The shared DEGs between the mono-infection and co-infection

The DEGs were further analyzed for commonalities and differences between the mono-infection and co-infection. Since

tobacco mosaic disease can be induced by both mono-infection and co-infection, we were especially interested in these shared DEGs to investigate the pathogenesis of tobacco mosaic disease. As shown in Fig. 2b, there were 836, 1538 and 1185 common DEGs between the mono-infection and co-infection at the three time points, respectively. Of these common DEGs, there were 226, 618 and 868 up-regulated genes at the three time points, and correspondingly the number of down-regulated genes were 610, 920 and 317. It was interesting that the number of up-regulated genes gradually increased, and that the number of down-regulated genes first increased and then decreased. These data were helpful for understanding the process of tobacco-virus interaction. At 1 day and 3 days after infection, the common DEGs were enriched in photosynthesis-antenna proteins, photosynthesis, phenylpropanoid biosynthesis, metabolic pathways, phenylalanine metabolism, biosynthesis of secondary metabolites and carbon fixation in photosynthetic organisms (Table S2 and S3). At 5d after infection, the main enriched pathways of the common DEGs were plant hormone signal transduction, plant-pathogen interaction, linolenic acid metabolism, phenylalanine metabolism, metabolic pathways and phenylpropanoid biosynthesis (Table S4). The pathway enrichment analysis of the common DEGs showed that

photosynthesis, defense metabolism, plant–pathogen interaction and plant hormone signal transduction were involved in the response of tobacco to mosaic virus. Some proteins in photosynthesis pathway, such as chlorophyll *a/b* binding protein 1, photosystem II interacting protein, photosystem II subunit and photosystem I subunit, were down-regulated after infection, showing that photosynthesis was seriously influenced by mosaic virus. On the contrary, the defense response pathways, including plant hormone signal transduction, plant–pathogen interaction, biosynthesis of secondary metabolites and phenylpropanoid biosynthesis, were up-regulated by virus infection, which contributed to improving the resistance of tobacco plants to virus. Calcium-dependent protein kinase, RPM1-interacting protein 4-like isoform 2, calmodulin-related protein isoform 2, MAP kinase, SERK3A, caltractin-like and calmodulin were enriched in plant–pathogen interaction pathway.

Quantitative RT-PCR validation of DEGs

A subset of 12 genes, which responded to mosaic virus infection, was selected for qPCR analyses. The qPCR results

showed that the expression trends of DEGs were consistent with those found by RNA-Seq (Fig. 3). These selected DEGs were pathogenesis-related protein 1a (PR1a), pathogenesis-related protein 4 (PR4), heat shock protein 90 (HSP90), peroxidase (POD), the coat protein of virus (CP), Ntdin (a tobacco senescence-associated gene), hybrid-proline-rich protein (HyPRP), JOKA2 (a tobacco member of selective autophagy cargo receptors family), osmotin, phyloplanin, snakin and thionin-like protein. The increase of CP expression showed that mosaic virus had been largely replicated in the infected tobacco plants. The two pathogenesis-related proteins, PR1a and PR4, were increasingly expressed, indicating that the defense system of tobacco plants had been activated. Thionin-like protein and phyloplanin are two proteins related to resistance (Taveira et al. 2016; Shepherd et al. 2005). HSP90 is a kind of defensive heat shock protein. The increase in expression of HSP90, phyloplanin and thionin-like protein also showed that the defense capability can be induced by virus infection. HyPRP is involved in cell apoptosis. JOKA2 is related to the process of selective autophagy in response to environmental stresses. Snakin and osmotin are stress responsive proteins. The changes in these

Fig. 3 The qPCR validation of some differentially expressed genes. (□) Control, (▨) 1d, (▤) 3d, (▥) 5d. FPKM represents RNA sequencing data, and PCR denotes qPCR data. For qPCR analysis, the fold change was determined using the $2^{-\Delta\Delta C_t}$ method. The error bars represent the standard deviation of the mean (SD). Statistical significance: * $p < 0.05$ and ** $p < 0.01$

proteins expression suggested that the defense system of tobacco might be broken in some respects by virus infection.

The expression of the DEGs related to RNA silencing and redox balance

RNA silencing is an important mechanism to regulate gene expression and antiviral defense in plants. RNA decay is a very well conserved pathway to control endogenous gene expression by eliminating dysfunctional transcripts (Belostotsky and Sieburth 2009; Schoenberg and Maquat 2012). It has been shown that argonaute proteins (AGOs) are involved in RNA silencing, and that CCR4 (carbon catabolite repressor 4), RDR (RNA-dependent RNA polymerase), DCL (Dicer-like protein), RRP (3'-5'-exoribonuclease family protein), XRN (5'-3' exoribonuclease) and UPF (RNA helicase) are the important proteins in RNA decay pathway (Carbonell and Carrington 2015; Conti et al. 2017). Therefore, the expressions of these proteins can be used as the indication of RNA silencing and RNA decay pathway. The antiviral RNA silencing pathway has the same components with the endogenous miRNA and trans-acting siRNA pathways that regulate gene expression. When viral RNAs are degraded through RNA silencing, the decay of endogenous RNAs will also be activated, resulting in the symptom development of virus-induced disease. Of these common DEGs, there were some genes related to RNA silencing and RNA decay, including CCR4, DCL, exosome complex exonuclease and AGO family (Table S5). As shown in Fig. 4, the expression of AGO1, AGO2, AGO3, AGO4, AGO4B, AGO5, AGO18, CCR4a, RDR1, DCL1, RRP41, XRN3 and UPF1 markedly increased after infection. The interaction network of these proteins was shown in Fig. 5, and their biological processes were enriched in gene silencing by RNA. These data indicated that RNA silencing and RNA decay might be activated by the infection of TMV, which can be one of the reasons for symptom development of tobacco mosaic disease. In addition, it was interesting that the expression of AGO16 and AGO9 markedly decreased after infection, the further work is needed to clarify their role in the process of virus-induced disease.

Redox signaling has an important role in the response of plants to multiple stresses. As shown in Table S5, the expression of some genes related to redox balance, such as thioredoxin, peroxiredoxin and protein disulfide isomerase, changed markedly at the three time points after infection. TRXX (thioredoxin X) and TRXM (thioredoxin M3) belong to thioredoxin family. TPX (thioredoxin peroxidase) and APX (ascorbate peroxidase) are two kind of peroxidase. As shown in Fig. 6, the expression of these genes changed obviously. The interaction network of these proteins was shown in Fig. 7, and their biological process were enriched in cellular oxidant detoxification, suggesting that the redox

balance in tobacco plants had been possibly broken by the infection of mosaic virus. APX is the key enzyme eliminating H_2O_2 in chloroplast. After infected by TMV and CMV, the activity of APX decreased and the accumulation of H_2O_2 increased (Fig. 8), showing that the chloroplasts might have been damaged. Therefore, it is easy to understand the chlorosis symptom and weakened photosynthesis in tobacco plants infected by TMV and CMV.

The effects of antiviral agents and resistance inducers on the expression of several genes related to tobacco mosaic disease

The usage of antiviral agents is an effective measure to control the incidence of tobacco mosaic disease. As shown in Table 1, the incidences of tobacco mosaic disease in the treatments with antiviral agents at 1 day after infection were obviously lower than that at 3 days after infection, indicating that antiviral agents should be timely used for effective control of tobacco mosaic disease. As shown in Fig. 9, the antiviral agents could drastically decrease the expression of CP in tobacco plants, showing that the amplification speed of TMV had been slowed. As previously mentioned, the expressions of PR1a, PR4, POD, HSP90 and AGO1 could be increased by the infection of TMV. When tobacco plants were sprayed with the antiviral agents, the inductive effects of TMV infection on PR1a, PR4 and POD were obviously subdued, and the inductive effects on HSP90 and AGO1 were observably enhanced.

Plant resistance inducers (PRIs) have been successfully used in the *Solanaceae* plant family to protect against pathogens by activating the plant's own defence (Alexandersson et al. 2016). Here, two kinds of PRIs, moroxydine hydrochloride and N-glycoside morpholine guanidine were used as to improve the resistance of tobacco plants. As shown in Table 1, the incidences of tobacco mosaic disease were 36.70% or 30.00% after spraying moroxydine hydrochloride or N-glycoside morpholine guanidine, respectively, which were markedly lower than the control treatment. Like antiviral agents, the qPCR results showed that PRIs could also subdue the inductive effects of TMV infection on PR1a, PR4 and POD, and enhance the inductive effects on HSP90 and AGO1 (Fig. 10). Therefore, these genes can be used to screen novel PRIs and antiviral agents for controlling tobacco mosaic disease.

Discussion

Some plant viruses can interact with other viruses to produce more serious disease than that caused by either virus alone. Mosaic viruses, including TMV and CMV, are plant viruses inducing the leaves to have a speckled appearance. Both

Fig. 4 The expression analysis of genes related to RNA silence. CK: control; 6 h: 6 h after infection; 18 h: 18 h after infection. Fold change was determined using the $2^{-\Delta\Delta C_t}$ method and error bars represent the standard deviation of the mean (SD). Statistical significance: * $p < 0.05$ and ** $p < 0.01$

TMV and CMV can cause tobacco mosaic disease, so there are considerable opportunities for synergistic interaction leading to the enhanced symptoms. It was also reported that CMV replicated in rice protoplasts by itself, whereas TMV

did so only with the aid of CMV (Okada et al. 1988). In the present study, the mosaic disease symptoms of tobacco plants co-infected by TMV and CMV appeared more early and seriously than that mono-infected by TMV. In addition,

Fig. 5 The interaction network of proteins related to RNA silence. Line thickness indicates the strength of data support. *FDR* false discovery rate; *CCR4* CRINKLY4 related 4; *XRN3* 5'-3' exoribonuclease 3; *RRP41* exosome complex component RRP41; *AGO1* argonaute family protein 1; *AGO2* argonaute family protein 2; *AGO3* argonaute

family protein 3; *AGO4* argonaute family protein 4; *AGO5* argonaute family protein 5; *UPF* RNA helicase; *AGO9* argonaute family protein 9; *AGO10* argonaute family protein 10; *RDR1* RNA-dependent RNA polymerase 1; *DCL1* endoribonuclease dicer homolog 1

the replication and accumulation of TMV in tobacco plants can be enhanced by co-infection. Therefore, there is synergy between TMV and CMV during the infection of mosaic viruses. In the field of tobacco production, tobacco mosaic

disease was often caused by the co-infection of TMV and CMV (Yang et al. 2010).

RNA-Seq can provide sensitive and accurate transcriptional profile. Here, the sequencing results showed the

Fig. 6 The expression analysis of several genes related to redox balance. CK: control; 6 h: 6 h after infection; 18 h: 18 h after infection; 24 h: 24 h after infection; 72 h: 72 h after infection. Fold change was determined using the $2^{-\Delta\Delta C_t}$ method and error bars represent the standard deviation of the mean (SD). Statistical significance: * $p < 0.05$ and ** $p < 0.01$

Fig. 7 The interaction network of proteins related to redox balance. Line thickness indicates the strength of data support. FDR, false discovery rate. *MDHAR* monodehydroascorbate reductase; *TY1* thioredoxin Y1; *DHAR1* dehydroascorbate (DHA) reductase 1; *PDIL2-2* protein disulfide isomerase-like 2-2; *APX1* ascorbate peroxidase 1; *CAT* catalase; *CcdA* m-type thioredoxin (Trx-m); *PRXIIF* peroxire-

Biological Process (GO)		
GO-term	description	FDR
GO:0098869	cellular oxidant detoxification	3.96e-24
GO:0045454	cell redox homeostasis	3.96e-24
GO:0019725	cellular homeostasis	7.07e-22
GO:0055114	oxidation-reduction process	1.61e-20
GO:0006979	response to oxidative stress	1.76e-14

Molecular Function (GO)		
GO-term	description	FDR
GO:0016209	antioxidant activity	1.33e-24
GO:0016491	oxidoreductase activity	3.88e-19
GO:0015036	disulfide oxidoreductase activity	2.10e-14
GO:0016668	oxidoreductase, acting on NAD(P)	1.34e-11
GO:0016651	oxidoreductase, acting on NAD(P)H	3.03e-11

doxin-2F; *CXIP2* glutaredoxin; *GR* glutathione reductase; *PAP17* purple acid phosphatase 17; *PRXQ* thioredoxin Q; *NTRC* NADPH-dependent thioredoxin reductase; *TRX-M4* thioredoxin M4; *TRX5* thioredoxin H-type 5; *PER1* peroxiredoxin 1; *THX* thioredoxin X; *PRX-2E* peroxiredoxin-2E

Fig. 8 Changes of APX activity and H₂O₂ content in tobacco leaves after infection. 0 days: non-infection; 1 day: 1 day after infection; 3 days: 3 days after infection; 5 days: 5 days after infection. Fold

change was determined using the $2^{-\Delta\Delta C_t}$ method and error bars represent the standard deviation of the mean (SD). Statistical significance: * $p < 0.05$ and ** $p < 0.01$

Table 1 The effects of antiviral agents and resistance inducers on the incidence of tobacco mosaic disease

Treatment	Antiviral agents at 1 day after infection			Antiviral agents at 3 days after infection			Resistance inducers		
	CK	AO	GC	CK	AO	GC	CK	MH	MG
Incidence (%)	93.50 ± 4.03	23.30 ± 1.15	33.30 ± 1.56	96.70 ± 4.26	83.45 ± 4.03	76.67 ± 4.07	93.50 ± 4.03	36.70 ± 1.52	30.00 ± 1.09

The incidences of tobacco mosaic disease were surveyed at 7 days after infection. The resistance inducers were used at 7 days before infection *CK* (control) infection of TMV, *AO* spraying with amino-oligosaccharin after infection, *GC* spraying with guanidine-copper acetate after infection, *MH* spraying with moroxydine hydrochloride, *MG* spraying with N-glycoside morpholine guanidine

infection of mosaic viruses could produce drastic changes in transcriptional profile. After tobacco plants were mono-infected or co-infected, the number of up-regulated DEGs sharply increased at the three time points, and the number

of down-regulated DEGs increased at the first two time points and then drastically decreased at the third time point. Although having the similar trend in changes of DEGs number, tobacco plants had some difference in the cluster

Fig. 9 The effects of antiviral agents on the expression of several genes related to tobacco mosaic disease. CK (control), without infection. (1) Infection with TMV. (2) Spraying with amino-oligosaccharin at 1d after infection. (3) Spraying with guanidine-copper acetate at 1 day after infection. Fold change was determined using the $2^{-\Delta\Delta C_t}$ method and error bars represent the standard deviation of the mean (SD). Statistical significance: * $p < 0.05$ and ** $p < 0.01$

Fig. 10 The effects of resistance inducers on the expression of several genes related to tobacco mosaic disease. CK (control), without infection. (1) Infection with TMV. (2) Spraying with moroxydine hydrochloride at 7 days before infection. (3) Spraying with N-glycoside morpholine guanidine at 7 days before infection. Fold change was determined using the $2^{-\Delta\Delta C_t}$ method and error bars represent the standard deviation of the mean (SD). Statistical significance: * $p < 0.05$ and ** $p < 0.01$

analysis of change trend of DEGs expressions between co-infection and mono-infection (Fig.S2). The most significant change trend of DEGs expressions in mono-infected tobacco plants showed down first and then up. However, in co-infected tobacco plants, the most remarkable change trend was that DEGs expressions increased sharply first and then kept unchanged. These results are helpful to understand that the co-infection can induce more early and serious symptoms of tobacco mosaic disease than mono-infection. The common DEGs between co-infection and mono-infection were enriched in photosynthesis, defense metabolism, plant-pathogen interaction and plant hormone signal transduction, which will contribute to clarifying the symptoms of tobacco mosaic disease.

RNA silencing is a widespread and important defense mechanism to control viral infections. In plants, viral infections can induce Dicer-like ribonuclease to process virus RNA into siRNAs or miRNAs. AGOs associate with small RNAs and then are assembled into RNA-induced silencing

complex (RISC) which targets and represses complementary virus RNA. AGOs can also associate with endogenous small RNAs to regulate host gene expression and promote antiviral defense. The antiviral defense mechanism of plant has driven most viruses to evolve viral suppressors of RNA silencing (VSRs) which can attenuate or inhibit this defense process (Ren et al. 2010; Qu and Morris 2005). However, it has been found that the endogenous gene silencing mechanisms are also altered. Therefore, the viral disease symptoms result from changes in host gene expression regulation which are caused by the immune mechanism of host plant and the interference of VSRs (Bazzini et al. 2007, 2009, 2011; Wiczorek and Obrepalska-Stepelowska 2015; Zavallo et al. 2015). It had been demonstrated that TMV induces RNA decay pathways to modulate gene silencing and disease symptoms (Conti et al. 2017). Here, it was found that the DEGs included some genes related to RNA silencing and RNA decay, such as CCR4, DCL, exosome complex exonuclease

and AGO family, showing that mosaic virus can activate the RNA silence and RNA decay pathway for antiviral defense.

Recent studies have demonstrated that oxidative stress via RNA virus infection can contribute to several aspects of viral disease pathogenesis (Camini et al. 2017). The imbalance of the antioxidative systems at the subcellular level is an important reason for oxidative stress (Hakmaoui et al. 2012). Here, the changes in the expression of some genes related to redox balance, such as thioredoxin and peroxiredoxin, were induced by mosaic virus. Thioredoxin is one of the two major thiol antioxidants, playing essential roles in redox homeostasis and signaling (Fan et al. 2017). It had been found that thioredoxin can impart early resistance to sugarcane mosaic virus in maize (Liu et al. 2017). In the present study, the expression of several thioredoxin proteins, such as TRXX and TRXM, obviously changed after infection. Peroxiredoxins (Prx) are a family of antioxidant proteins and perform important functions in intracellular signal transduction. APXs are a kind of crucial enzymes for removing reactive oxygen species (ROS) in plant cell. The expression and activity of APX markedly decreased after infection, which caused the accumulation of H₂O₂ in tobacco plant. These results showed that mosaic virus infection can induce oxidative stress in tobacco plants, and then result in the symptoms of tobacco mosaic disease.

Molecular biomarkers can inform about the current phenotypic state and, more importantly, may also be predictive of future phenotypic trait endpoints. Here, we found that antiviral agents and PRIs can decrease the incidence of tobacco mosaic disease through altering the expression of some DEGs, such as HSP90, PR1a, PR4 and AGO1. Therefore, these genes can be used to screen novel PRIs and antiviral agents for controlling tobacco mosaic disease.

Conclusion

Tobacco mosaic disease is often caused by multiple infections of TMV and CMV in China, and the synergism of the two mosaic viruses occur in infected tobacco plant. After the infection of TMV, the oxidative stress and RNA silencing contribute to the symptoms development of tobacco mosaic disease. For controlling tobacco mosaic disease, some DEGs can be used to screen novel PRIs and antiviral agents.

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Compliance with ethical standards

Conflict of interest The authors declare no competing or financial interests.

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