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Influence of an m-type thioredoxin in maize on potyviral infection

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Abstract Expression of many host genes can be altered during virus infection. In a previous study of sugarcane mosaic virus (SCMV) infection in maize (*Zea mays*), we observed that expression of *ZmTrm2*, a gene encoding thioredoxin *m*, was up-regulated at about 10 days post-inoculation (dpi). In this present study we determined that *ZmTrm2* silencing in maize by virus-induced gene silencing significantly enhanced systemic SCMV infection. In contrast transient over-expression of *ZmTrm2* in maize protoplasts inhibited accumulation of SCMV viral RNA. Furthermore, we found that in inoculated *Nicotiana tabacum* leaves transient expression of *ZmTrm2* inhibited accumulation of the RNA of tobacco vein-banding mosaic virus (TVBMV), a potyvirus infecting dicotyledonous plants. Interestingly in *ZmTrm2* transiently expressed *N. tabacum* leaves, we detected by semi-quantitative RT-PCR a reduced level of the mRNA of class I beta-1, 3-glucanase (*GluI*), a protein known to have a role in cell wall callose deposition and viral movement. Our data indicate that the maize *ZmTrm2* plays an inhibitory role during infection of plants by SCMV and TVBMV.

Keywords *Zea mays* · *ZmTrm2* · SCMV · TVBMV · Potyvirus infection

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Introduction

To establish infection in plants, viruses require host factors for their replication, cell-to-cell and long-distance movement. During various steps of virus infection, expression profiles of many host genes involved in metabolism, signal transduction and defence responses were found to be altered (Collazo et al. 2006; Escalettes et al. 2006; Alfenas-Zerbini et al. 2009). Since the molecular mechanisms by which plant viruses affect their hosts are largely unclear (Maule et al. 2002), studies on differentially expressed genes after virus infection can lead to elucidating the relationship between specific genes and virus infection.

Sugarcane mosaic virus (SCMV), a member of the genus *Potyvirus*, causes considerable losses in different field crops in many countries including China (Shi et al. 2006). Previous studies utilizing suppression subtractive hybridization (SSH) and microarrays identified multiple candidate genes and their potential involvement in SCMV resistance in maize (*Zea mays* L.)(Shi et al. 2006; Użarowska et al. 2009). However, the relationship between individual candidate genes and SCMV infection in maize has not been determined.

In the present study, we analyzed a maize gene encoding an m-type thioredoxin and its role in SCMV infection of maize and TVBMV infection of *Nicotiana tabacum*. Thioredoxins are a group of small proteins that contain a dithiol-disulphide active site with two redox-active cysteine residues (Holmgren 1985). Thioredoxins were known to be involved in numerous cellular processes including enzyme regulation, oxidative stress response, transcription and translation (Balmer and Buchanan 2002; Montrichard et al. 2009). The two cysteine residues in the conserved motif are critical for stability, conformation, and regulation of thioredoxin-targeted protein structure through redox state interchange (Aslund and Beckwith 1999; Montrichard et al. 2009). Different types of thioredoxins have been identified in plant chloroplasts and mitochondria. Twenty-two thioredoxin genes have been reported for *Arabidopsis* (Ishiwatari et al. 1995; Rivera-Madrid et al. 1995; Meyer et al. 2002, 2005; Gelhaye et al. 2004; Juárez-Diaz et al. 2006). Of them thioredoxin *f* and *m* were reported to be two distinct light-dependent chloroplast proteins showing low sequence identity (Eklund et al. 1991). Thioredoxin *f* activates enzymes involved in the Calvin cycle, including fructose 1,6-bisphosphatase (FBPase), phosphoribulokinase, sedoheptulose bisphosphatase, and H^+-ATPase (Nishizawa and Buchanan 1981; Schwarz et al. 1997), whereas thioredoxin *m* mainly plays roles in activating NADP-dependent enzyme malate dehydrogenase, which can also be activated *in vitro* by thioredoxin *f* (Geck et al. 1996; Hodges et al. 1994). It has also been reported that thioredoxin *m* was involved in regulating glucose 6-phosphate dehydrogenase and other metabolic processes (Wenderoth et al. 1997). Thioredoxin *m* in maize is unlike those in other plants because of its unique heat susceptibility (Lunn et al. 1995).

Virus-induced gene silencing (VIGS) has been utilized extensively for functional studies of genes in many plants, and VIGS based on an infectious clone of *Brome mosaic virus* (BMV) was established for monocot plants (Ding et al. 2006). It was used to identify maize genes that are functionally involved in the interaction with a pathogen (van der Linde et al. 2011). In a recent study of ours, a maize gene encoding thioredoxin *m* (designated as *ZmTrm2*) was found to be up-regulated upon virus infection (unpublished data). This result demonstrated the impact of *ZmTrm2* expression on SCMV replication and systemic accumulation in maize. We propose that expression of *ZmTrm2* negatively influences SCMV infection in maize and TVBMV in *N. tabacum*, therefore implicating a role in defence responses against these two and possibly other potyviruses. In the present research, the BMV-based VIGS was employed for transient silencing of a target gene in maize leaves in order to test this hypothesis.

Materials and methods

Plant growth and virus sources

Maize (*Zea mays*-inbred line Zong 31 and cv. Va35) plants were grown in a controlled growth chamber set at 23/21°C (day and night) and with 16 h light and 8 h dark. SCMV-BJ (accession number AY042184) was isolated from diseased maize leaves in the northern suburbs of Beijing (Fan et al. 2003) and maintained in maize inbred line Zong 31. TVBMV was kindly provided by Prof. Xiangdong Li from Shandong Agricultural University.

Construction of plasmids

The full-length coding sequence of *Z. mays* thioredoxin m-type (*ZmTrm2*) was amplified from total RNA of maize cv. Zong 31 by RT-PCR using primers P1 and P2 (Table 1) and inserted into the T-cloning vector pMD18-T (TaKaRa Bio Inc., Otsu, Shiga, Japan) to produce pZmTrm2. Primers P1 and P2 were designed according to the *ZmTrm2* sequence deposited in the GenBank (NM_001157280.1).

The *ZmTrm2* full-length coding sequence was then amplified again from pZmTrm2 by PCR using primers P3 and P4 (Table 1), digested with restriction enzymes *Sal*I and *Sac*I, and was cloned into the pGFP vector (a derivative of pUC19, TaKaRa Bio Inc.) to

Table 1 Primers used for polymerase chain reaction (PCR) amplification

Primer name	Sequence*	Restriction Site	Usage
P1	5'-ATGGCCTCCCGCCTCGCCGT-3'		Full-length coding sequence
P2	5'-TCACCTCCCACCAACGTACTTGTC-3'		
P3	5'- TA*GTCGAC*GGATGGCCTCCCGCCTCGCCG-3'	*Sal* I	pGFPZmTrm2 construction
P4	5'-CGC*GAGCTC*TCACCTCCCACCAACGTACTTG-3'	*Sac* I	
P5	5'- ACT*AAGCTT*TCGTGTGCCAGGCCCAG-3'	*Hind* III	Silencing vector construction
P6	5'- GTG*AAGCTT*TACACGGTCCACACCATG-3'	*Hind* III	
P7	5'- ATA*ATCGAT*ATGGCCTCCCGCCTCGCCG-3'	*Cla* I	PVXTrm2 construction
P8	5'- AGC*GTCGAC*TCACCTCCCACCAACGTACTTG-3'	*Sal* I	
P9	5'- GGAAAAACCATAACCCTGGA-3'		*Ubiquitin* gene analysis
P10	5'- ATATGGAGAGAGGGCACCAG-3'		
P11	5'- GCTAAACACCGACGAGAACC-3'		qRT-PCR analysis of *ZmTrm2*
P12	5'-GCCCATCCTTGAATCCCT-3'		
P13	5'- GGCGAGACTCAGGAGAATACA-3'		SCMV RNA analysis
P14	5'-ACACGCTACACCAGAAGACACT-3'		
P15	5'-GTGTTGGATTCTGGTGATGGTG-3'		Tobacco *actin* RNA analysis
P16	5'-TGTCAAGCTCCTGCTCGTAGT-3'		
P17	5' -CACTGCCACAGATGTCAATCG-3'		TVBMV RNA analysis
P18	5' -CCAAGCGTCACCTACAAGAATAG-3'		

*The restriction sites are indicated in italic type

generate pGFPZmTrm2. The BMV-based vector pC-$BMV_{A/G}$ was used in this study to silence the *ZmTrm2* gene in maize (cv. Va35). This pC-$BMV_{A/G}$ vector consists of pF1-11 for RNA1, pF2-2 for RNA2 and pF3-5/13'$_{A/G}$ for RNA3. A 139-bp PCR fragment of *ZmTrm2* was amplified using primers P5 and P6 (Table 1). The PCR fragment was digested with *Hin*dIII restriction enzyme and cloned into the pF3-5/13'$_{A/G}$ to produce pF3-5/13'$_{A/G}$-ZmTrm2. The combination of pF3-5/13'$_{A/G}$-ZmTrm2 and two other plasmids (pF1-11 and pF2-2) of pC-$BMV_{A/G}$ was named pBMVZmTrm2.

A vector constructed from *Potato virus X* (PVX) (van Wezel et al. 2002) was used to express *ZmTrm2* transiently in *N. tabacum* leaves. The full-length coding sequence of *ZmTrm2* was amplified using primers P7 and P8 (Table 1) and the fragment was cloned into the *Cla*I and *Sal*I sites to produce PVXTrm2.

Maize protoplast preparation and transfection

Maize protoplasts were prepared and transfected according to Sheen's protocol (Sheen 1991) with minor modifications. A total of 2×10^5 maize protoplasts were transfected with 20 μg of individual plasmids or co-transfected with one plasmid and 10 μg of purified SCMV viral RNA via electroporation using the Gene Pulser Xcell as instructed (Bio-Rad, Hercules, CA, USA), and the electroporated protoplasts were incubated at 25°C in the dark for 12 h before RNA analysis. SCMV viral RNA used for transfection was extracted from viral particles according to a previously described protocol (Dijkstra and de Jager 1998).

In vitro transcription and inoculation

RNA transcripts were produced individually from pBMVZmTrm2 and pC-$BMV_{A/G}$ through *in vitro* transcription using T3 RNA polymerase (Promega). The RNA transcripts were then mixed and mechanically inoculated onto the leaves of 10-day-old Va35 seedlings as previously described (Ding et al. 2006). The first systemic leaf showing chlorotic streaks at about 10 days post inoculation (dpi) was re-inoculated with crude sap from SCMV-infected maize leaves. The first and second systemic leaves above the SCMV-inoculated leaf were collected at 5 dpi by SCMV and used for analyzing *ZmTrm2* and virus accumulation. In later experiments using PVX, RNA transcripts were produced from plasmids PVX

and PVXTrm2 through *in vitro* transcription using T7 RNA polymerase (Promega). The RNA transcripts were mechanically inoculated onto leaves of *N. tabacum* plants. At 5 dpi, the inoculated leaves were then re-inoculated with crude sap from TVBMV-infected *N. tabacum* plants. The inoculated leaves were collected for analysis of TVBMV RNA accumulation at 3 dpi by TVBMV.

Semi-quantitative and quantitative RT-PCR (qRT-PCR) analysis

Total RNA was extracted from maize or *N. tabacum* leaves using TRIzol (Invitrogen, Carlsbad, CA, USA) and treated with 5 U of RNase-free DNase (TaKaRa) at 37°C for 30 min. The DNase-treated total RNAs were recovered by ethanol precipitation. First-strand cDNAs were synthesized from total RNA (0.5 μg total RNA per 20 μl reaction) using M-MLV reverse transcriptase (Promega) and random 6-mer primers (TaKaRa). The RT products were individually diluted 10-fold in the Easy Dilution buffer (TaKaRa) to be used as templates for the subsequent quantitative RT-PCR (qRT-PCR) analysis. The maize *ubiquitin* gene was amplified with primers P9 and P10 and used as an internal control for both semi-quantitative and qRT-PCR analyses. Primers P11 and P12 were used for qRT-PCR analysis of *ZmTrm2* with P12 being specific for the non-translated region of the *ZmTrm2* gene (NM_001157280.1). Primers P13 and P14 were used for both semi-quantitative and qRT-PCR detection of SCMV infection. For SCMV detection, PCR amplification was conducted with 1 μl of RT product in a 25-μl reaction at the following conditions: 94°C for 2 min followed by 30 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 20 s, and then 72°C for 10 min. The PCR products were visualized in a 1.5% agarose gel after staining with ethidium bromide and analyzed by an AlphaImager 2200 (Alpha Innotech.). For TVBMV accumulation analysis, primer pairs P15 and P16, and P17 and P18 were used to detect accumulation of tobacco (*N. tabacum*) *actin* mRNA (served as an internal control) and TVBMV genomic RNA, respectively. PCR amplification conditions were 94°C for 2 min followed by 25 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, and a final 72°C for 10 min.

qPCR analysis was conducted using an ABI 7500 thermocycler (Applied Biosystems, Foster City, U.S.A.). The qPCR reaction consisted of 10 μl of 2× SYBR Premix Ex *Taq* DNA polymerase, 200 nM each of the two gene-specific primers (P11/P12) (Table 1), 1 μl of diluted reverse-transcribed cDNA, 0.4 μl ROX Dye II, in a total volume of 20 μl reaction as instructed by the manufacturers (TaKaRa). The qPCR amplification conditions were as follows: 95°C for 30 s, 40 cycles at 95°C 5 s, 58°C 20 s and 72°C 35 s.

qRT-PCR quantification was based on the relative abundance, as determined by ZmTrm2 Ct values compared with the ubiquitin Ct values according to the formula $\Delta Ct = 2^{-(CtZmTrm2 - CtUbiquitin)}$.

Northern blot analysis

Total RNAs were extracted from *N. tabacum* leaves using TRIzol as described above, and loaded (35 μg per sample) onto a 1% agarose–formaldehyde gel, blotted to Hybond N-membrane, UV cross-linked, and then detected by hybridization with DIG-labeled TVBMV-specific probe which was synthesized by using PCR DIG Probe Synthesis Kit (Roche) with TVBMV specific primers P17/P18 (Table 1).

Western blotting

Total proteins were extracted from maize leaves using a protein extraction buffer (20 mM MES, pH6.1, 0.25 M sucrose, 0.1 M EGTA, 1 mM DTT, 0.2 mg/ml BSA) and protein concentration was determined via a Coomassie blue assay (Bradford 1976). The total protein (40 μg) of each sample was separated on a 10% sodium dodecyl-sulphate-polyacrylamide gel by electrophoresis and then transferred onto a nitrocellulose membrane. The viral protein was detected by an antiserum against a SCMV coat protein which was prepared and kept in our laboratory, followed by the alkaline phosphatase-conjugated protein A (Sigma-Aldrich) and visualized after staining with NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) (Thompson and Larson 1992).

Results

Up-regulation of *ZmTrm2* in SCMV systemically infected maize leaves

To dissect the response of maize to SCMV infection, a suppression subtractive hybridization

(SSH) library was constructed and screened for maize genes whose expression altered after SCMV infection (unpublished data). A gene encoding a maize m-type thioredoxin was found to be highly up-regulated after SCMV infection (unpublished data) and was selected for further analysis. This gene is designated as *ZmTrm2* (HM582205) due to its sequence identity to other m-type thioredoxins. GenBank searching revealed two more m-type isoforms and are designated as *ZmTrm1* (NM_001111860.1) and *ZmTrm3* (NM_001159098), respectively. Alignment and homological analysis showed that sequence identity among the three isoforms was low. Our data shows that the *ZmTrm2* shares 59% nucleotide (nt) sequence identity and 45% amino acid (aa) identity with the *ZmTrm1*, and 51% nt identity and 45% aa identity with the *ZmTrm3*, respectively.

To determine *ZmTrm2* mRNA transcription alterations after SCMV infection, total RNA was isolated from the first systemic leaves of either mock or SCMV-inoculated plants at 2, 6, 10 and 14 dpi, respectively. Relative expression levels of *ZmTrm2* mRNA in different leaf samples were determined through qRT-PCR, using the expression levels of the *ubiquitin* gene as the internal control. Our results showed that at 10 dpi the expression levels of *ZmTrm2* mRNA were approximately 2.5-fold higher than that observed in the tissues from the mock-inoculated plants (Fig. 1). The expression levels of *ZmTrm2* mRNA in SCMV-infected leaf tissues was, however, similar to that in tissues from the mock-inoculated plants at 2, 6 and 14 dpi. This indicates that the up-regulation of *ZmTrm2* mRNA in SCMV-infected tissues was transient.

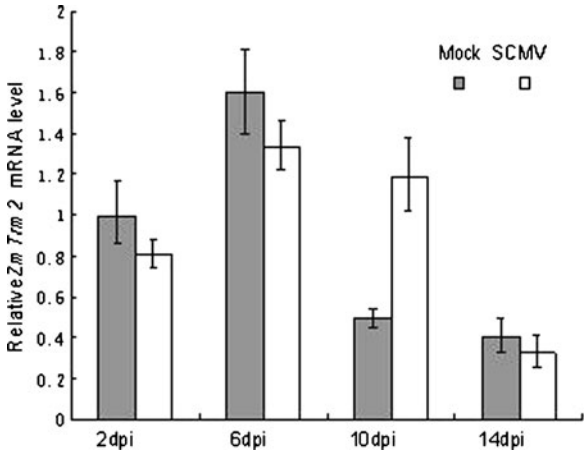

Fig. 1 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *ZmTrm2* mRNA transcripts in the first systemic leaf of mock-inoculated (Mock) and SCMV-inoculated (SCMV) maize at different periods. The qRT-PCR quantification of *ZmTm2* was based on the calculation of amplification efficiency against that of the ubiquitin gene used as an internal control. Three individual plants were pooled for each sample and the resulting mean values of three independent experiments were presented as relative *ZmTrm2* mRNA level. All data are the mean value ± standard deviation (SD) normalized to that of 2 dpi mock (negative control)

Silencing of *ZmTrm2* in maize leaves enhanced SCMV accumulation

To investigate the potential role of ZmTrm2 on SCMV infection in maize, a *ZmTrm2* silencing vector, pBMVZmTrm2, was constructed using the C-BMV$_{A/G}$-based silencing vector (Ding et al. 2006). Our data showed that the mRNA levels of *ZmTrm2* in the first systemic leaves above the SCMV inoculated leaves were about 40% of that in leaves from the mock - or only C-BMV$_{A/G}$-inoculated control plants (Fig. 2a). At the same time, SCMV RNA accumulated to a much higher level in *ZmTrm2*-silenced leaves than that in control leaves by semi-quantitative RT-PCR (Fig. 2b). Western blot analysis of SCMV CP accumulation further confirmed the results (Fig. 2c). Similar results were obtained when the second systemic leaves above the SCMV-inoculated leaves were analyzed (data not shown). Hence, SCMV accumulation was significantly increased in the *ZmTrm2* silenced leaves.

Transient over-expression of ZmTrm2 in maize protoplasts impaired virus replication

Since ZmTrm2 showed a negative impact on SCMV accumulation in maize leaves, we decided to test whether this negative impact is correlated with SCMV replication. The pGFPZmTrm2 and pGFP vectors were inoculated individually into maize protoplasts and the *ZmTrm2* mRNA over-expression was determined by qRT-PCR using specific primers. Results of experiments indicated that *ZmTrm2* mRNA level was about 1000-fold higher in protoplasts transfected with pGFPZmTrm2 than that in protoplasts transfected with the pGFP vector (Fig. 3a). We also determined that SCMV

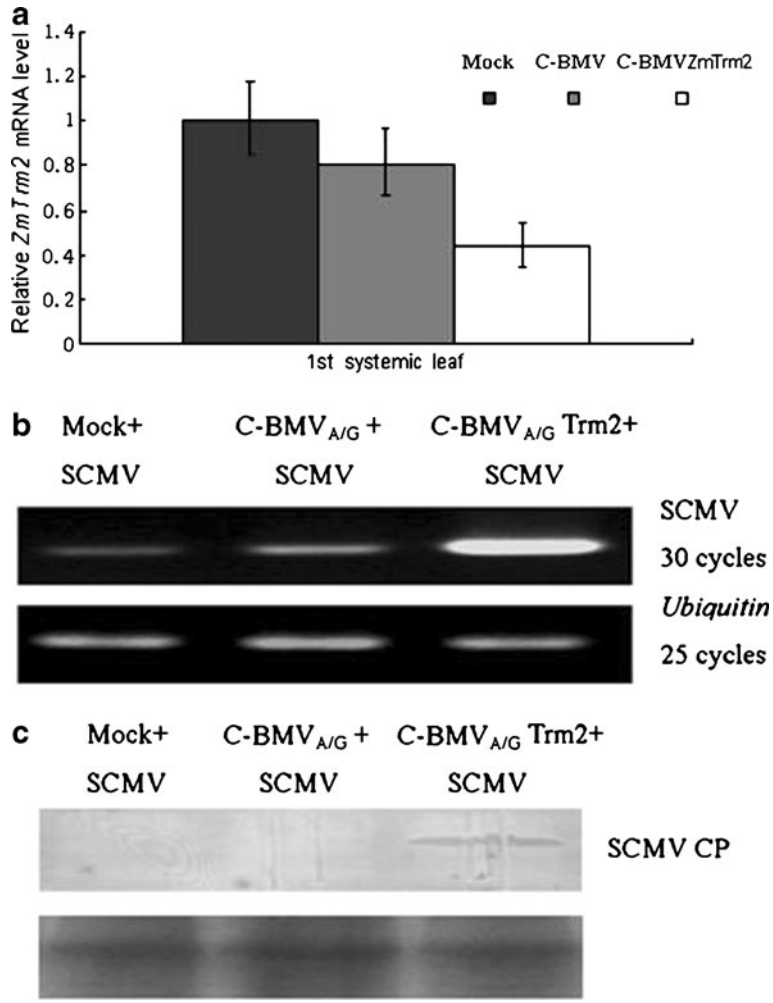

Fig. 2 Transient silencing of ZmTrm2 facilitated SCMV infection in systemic leaves above SCMV inoculated maize seedling leaves. **a** Real time reverse transcription-polymerase chain reaction (RT-PCR) analysis of ZmTrm2 transient silencing in first systemic leaf upper SCMV inoculated leaves at 5 dpi, which were challenge-inoculated with SCMV ten days after BMV and BMVZmTrm2 inoculation. The amplification efficiency of *ubiquitin* gene was calculated as an internal control. The resulting mean values of three independent experiments were presented as relative *ZmTrm2* mRNA level. All data are the mean value ± standard deviation (SD) normalized to that of mock-inoculated. **b** Semi-quantitative RT-PCR analysis of SCMV accumulation in first systemic leaf at 5 dpi. *Ubiquitin* gene was amplified as internal control. RT-PCR products of SCMV mRNA were separated by agarose gel as indicated. **c** Western blot detection of SCMV accumulation in the first systemic leaf at 5 dpi using antiserum against SCMV CP. The Coomassie brilliant blue-stained 12% sodium dodecylsulphate-polyacrylamide gel (bottom) for different samples was used as loading control

RNA accumulation in protoplasts co-transfected with pGFPZmTrm2 and SCMV RNA was significantly reduced compared with that in protoplasts co- transfected with pGFP and SCMV RNA (Fig. 3b). These results support the finding described above and suggest that ZmTrm2 plays an inhibitory role in SCMV replication.

Overexpression of ZmTrm2 also reduced accumulation of a different potyvirus in tobacco leaves

To reveal further the role of ZmTrm2 in potyvirus accumulation, a PVX-based expression vector was used to transiently express *ZmTrm2* in *N. tabacum*

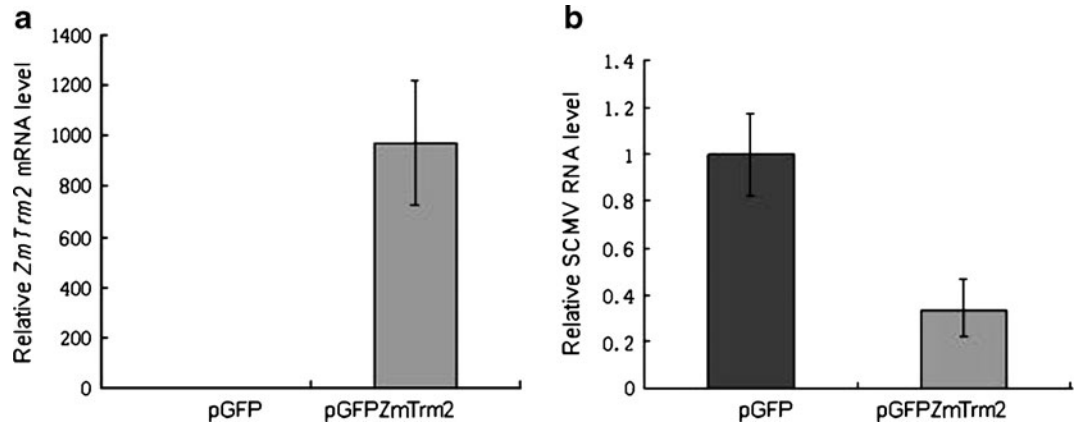

Fig. 3 *ZmTrm2* expression in maize chloroplasts inhibited SCMV replication. **a** Real time reverse transcription-polymerase chain reaction (RT-PCR) analysis of the expression of *ZmTrm2* mRNA in maize protoplasts 12 h after transformation. The amplification efficiency of *ubiquitin* gene was calculated as an internal control. The resulting mean values of three independent experiments were presented as relative *ZmTrm2* mRNA level. All data are the mean value ± standard deviation (SD) normalized to that of pGFP-transformed protoplasts. **b** Real-time RT-PCR analysis of the expression of SCMV mRNA in maize protoplasts 12 h after transformation. The amplification efficiency of *ubiquitin* gene was calculated as an internal control. All data are the mean value ± standard deviation (SD) normalized to that of pGFP-transformed protoplasts

leaves. At 5 dpi with either PVX or PVXTrm2 RNA, the inoculated leaves were challenge-inoculated with a different potyvirus *Tobacco vein banding mosaic virus* (TVBMV). The re-inoculated leaves were harvested at 3 dpi and analyzed for TVBMV RNA accumulation by both semi-quantitative RT-PCR and northern blotting. As shown in Fig. 4, *ZmTrm2* transient expression in tobacco leaves clearly inhibited TVBMV RNA accumulation in the inoculated leaves. Interestingly, the mRNA level of class I beta-1, 3-glucanase (*GluI*), which is known to be associated with callose deposition and plant virus movement (Iglesias and Meins 2000), was also decreased in *ZmTrm2*-expressing and TVBMV-inoculated tobacco leaves (Fig. 4a) suggesting that ZmTrm2 may also involve in regulating potyvirus intercellular movement, in addition to virus replication.

Discussion

Here we provide evidence that the expression of *ZmTrm2* can be up-regulated upon SCMV infection in maize. We also show in this paper that ZmTrm2 has an inhibitory role in SCMV infection in maize and

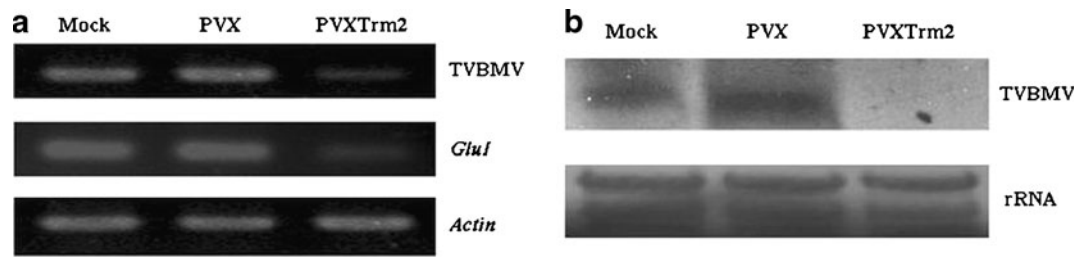

Fig. 4 ZmTrm2 expression in *N. tabacum* via a PVX infectious clone reduced TVBMV accumulation in inoculated leaves as well as *GluI* mRNA transcripts. **a** Semi-quantitative RT-PCR analysis of TVBMV accumulation and class I ß-1,3-glucanase (Glu I) level in inoculated leaves at 3 dpi, which were challenge-inoculated with TVBMV five days post inoculation with PVX and PVXTrm2. PCR reaction was conducted for 25 cycles for each sample. Maize *actin* gene was used as an internal control. RT-PCR products were separated by agarose gels as indicated. **b** Northern blot detection of TVBMV accumulation in PVX, PVXTrm2 and mock-inoculated leaves at 3 dpi. Total RNA blots were hybridized with a *ZmTrm2*-specific probe

TVBMV infection in tobacco. Because this negative role was observed in both monocotyledonous and dicotyledonous hosts and with two different potyviruses, it is possible that this gene can affect multiple potyviruses in many different hosts.

Previous studies on chloroplast thioredoxins provided insights into the diverse cellular reactions that may involve thioredoxin. For example, thioredoxin was reported to have roles in vitamin biosynthesis, protein assembly/folding, protein degradation, protein transport, starch degradation, etc. (Balmer et al. 2003; Bartsch et al. 2008; Motohashi et al. 2001). Results presented in this paper showed the first evidence that a maize m-type thioredoxin can also function in potyvirus infection in both monocotyledonous and dicotyledonous hosts. To our knowledge, no study has been reported on m-type maize thioredoxins for their functions in virus infection.

Viruses rely on various host factors to establish infection in the plant. To counterattack virus infection, the plant has evolved different defence mechanisms including up-regulating and down-regulating specific genes with different functions (Chen et al. 2008; Huang et al. 2010). Dynamic changes in the *ZmTrm2* mRNA level at various days post SCMV inoculation were determined via qRT-PCR. The qRT-PCR was performed using samples from three independent experiments and the results indicated that ZmTrm2 expression level was significantly increased at 10 dpi. In the same 10 dpi samples, however, the ZmTrm1 and ZmTrm3 showed no significant differences when compared with the controls (data not shown). Thus, ZmTrm2 is an m-type thioredoxin in maize which responds actively to potyviral infection. Transient silencing of *ZmTrm2* in maize (cv. Va35) was found to promote SCMV infection in upper systemic leaves. This result suggests that ZmTrm2 functions as a host defence factor during potyviral infection. In order to silence *ZmTrm2* specifically but not the other two isoforms, the *ZmTrm2* PCR fragment to be amplified was chosen carefully. A fragment of 306 bp including both the ORF and non-translated sequence was amplified with primers P11/P12 (Table 1) and inserted into the C-BMV silencing vector. Sequence analysis showed that the PCR fragment did not have any continuous identical 21 nucleotides with the other two isoforms. Our western blot data showed that SCMV coat protein can be detected in *ZmTrm2*-silenced maize leaves by 5 dpi but not in non-silenced (e.g. mock or C-$BMV_{A/G}$-inoculated) leaves after SCMV inoculation. The absence of SCMV CP in non-silenced maize leaves may be due to the sensitivity of detection method or low accumulation of virus at this time point.

Because *ZmTrm2* negatively influenced SCMV replication in maize protoplasts, we utilized a yeast two-hybrid assay to test whether SCMV proteins could interact with ZmTrm2 directly. Our assays with yeast two-hybrid were unable to detect the interaction between ZmTrm2 and any of the ten SCMV proteins including P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, NIb, and CP (excluding PIPO, Chung et al. 2008) (data not shown), although we cannot exclude the possibility that ZmTrm2 might interact with one or more of the viral proteins in maize cells. This might imply that ZmTrm2 may influence SCMV replication and/or intercellular movement by regulating other host responses.

A different potyvirus, TVBMV, was also tested in this study for its infection in *N. tabacum* to test the putative antiviral function of ZmTrm2 in dicots. Our data show clearly that TVBMV accumulation was inhibited in *ZmTrm2* over-expressed leaves, implying the negative impact of ZmTrm2 can also happen on dicot-infecting potyviruses. TRX-m3 from *Arabidopsis* shares 45.6% full-length coding sequence and 28.8% amino acid sequence identity with ZmTrm2 and was reported to regulate intercellular transport (Benitez-Alfonso et al. 2009). Considering the role of thioredoxin m in enzyme regulation, we investigated the possible effects of a thioredoxin on class I beta-1, 3-glucanase (*GluI*) mRNA expression. Class I beta-1, 3-glucanase was previously reported to have roles in callose deposition in cell walls and viral movement (Beffa et al. 1996; Iglesias and Meins 2000). In our study, the *GluI* expression level was found to be inhibited in *ZmTrm2*-expressing tobacco leaves. Therefore, we propose that *ZmTrm2* may also function in potyvirus intercellular movement by regulating *GluI* expression in cells, in addition to its role in virus replication or accumulation, although this needs to be confirmed by other approaches.

In conclusion, our findings suggest that ZmTrm2 is involved in plant defence against potyviral infection. Regulation of *GluI* expression and SCMV replication by ZmTrm2 shed light on the mechanisms by which ZmTrm2 influences potyviral infection. Further studies are needed to confirm this broad-range antiviral activity.

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