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LETTER TO THE EDITOR

P22 of tomato chlorosis virus, an RNA silencing suppressor, is naturally expressed in the infected plant

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Since its first discovery nearly two decades ago (*1*), tomato chlorosis virus (ToCV) (the genus *Crinivirus* of the family *Closteroviridae*) has emerged as a serious disease problem in field and greenhouse tomato production worldwide (*2–9*). The infected tomato plants exhibit symptoms including interveinal chlorosis, leaf brittleness, and limited necrotic flecking or leaf bronzing (*10*). ToCV has a bipartite genome of single-stranded positive-sense RNA (*1*). RNA 1 encodes four open reading frames (ORFs) and RNA 2 encodes up to nine ORFs. The proteins are not well studied and their putative functions are associated with virus replication, encapsidation, movement, and whitefly transmission (*11–13*). The p22 protein encoded in the RNA1, as well as the major (CP) and minor (CPm) coat proteins encoded in the RNA2 of ToCV are implicated as RNA silencing suppressors (RSS) (*14*). Until now, the CP is the only viral protein being detected in ToCV-infected tomato plants (*15*). *In vitro* assays demonstrated that the p22 protein is bound preferentially to the long dsRNAs, thus protecting these dsRNAs from dicer cleavage (*16*). Since an antibody is not yet available for p22, until now, there is no direct evidence showing p22 is expressed naturally in the ToCV-infected plants. The objective of the present study was to provide a direct physical evidence for the presence of p22 protein in a ToCV-infected plant. By developing an infectious clone of ToCV with a triple-FLAG tag fused to the C-terminus of p22, using an anti-FLAG monoclonal antibody, we present here, for the first time, a physical evidence for the natural expression of p22 in ToCV-infected *Nicotiana benthamiana* plant.

To develop an infectious cDNA clone of ToCV Beijing isolate (ToCV-BJ)(*17*), reverse transcription and polymerase chain reactions (RT-PCR) were used to synthesize and amplify full-length cDNA of ToCV-BJ RNA 1 and 2. Viral genomic RNAs were prepared from purified virions. Specific primers were designed based on the complete genome sequence of ToCV-BJ (accession number KC887998 and KC887999) (*17*). First strand cDNA was synthesized with moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, USA) according to the manufacturer's protocol. PCR amplifications were performed using Phusion® High-fidelity DNA Polymerase (New England Biolabs, Kallang, Singapore). The full-length cDNAs of ToCV RNA1 and 2 were cloned under a double 35S promoter in the binary vector pCass-RZ (*18*) to produce the two constructs pCa-ToCR1 and pCa-ToCR2 for ToCV RNA1 and RNA2, respectively. Biological activity of the constructs pCa-ToCR1 and pCa-ToCR2 was demonstrated through agroinfection of *N. benthamiana* plants according to reported protocols (*15*).

To detect the natural expression of p22 *in vivo*, triple FLAG-tag (DYKDDDDK) sequences were fused in frame to the C-terminus of ORF(p22) in pCa-ToCR1 to obtain

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Abbreviations: ToCV = tomato chlorosis virus; ORFs = open reading frame(s); RSS = RNA silencing suppressor

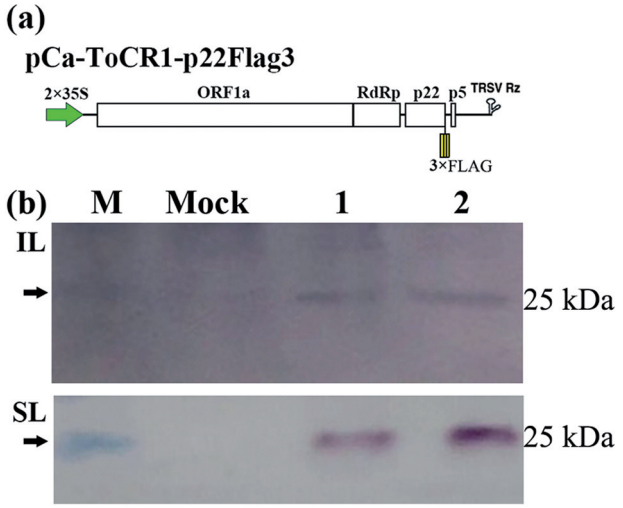

Fig. 1

Detection of p22 in ToCV-BJ-infected plants

(a) A triple FLAG-tag coding sequence was fused to the C-terminus of ORF (p22) in RNA1. (b) Western blots analyses with anti-FLAG monoclonal antibody detected the accumulation of p22 in the inoculated (IL, upper panel) and upper systemic leaves (SL, lower panel). The arrows indicate the bands with molecular weight 25 kDa.

construct pCa-ToCR1-p22FLAG$_3$ (the figure panel A). An overlap-PCR with primers C1c-F (5'-CCGGGATGAATCT AGAAACT-3') and V31-R (5'-CGTCGTCGTCTTT GTAGTCTTTGTCGTCGTCGTCTTTGTAGTCTATA TCACTCCCAAAG-3') (for upstream fragment) and V32-F (5'-ACAAAGACGACGACGACAAAGACTACAAAGA CGACGACGACAAATAATCTGGTTAATATTC-3') and C1c-R (5'-ACAGGGTATCGGATCCGACCTATTTAT TTATATACTAG-3') (for downstream fragment) was used to construct pCa-ToCR1-p22FLAG$_3$. The triple FLAG-tag insertion was verified by DNA sequencing. *N. benthamiana* plants were agroinoculated with *Agrobacterium tumefaciens* containing pCa-ToCR1-p22FLAG$_3$ and pCa-ToCR2. The test plants were maintained in a growth chamber under a 16 h light at 24 °C /8 h dark at 22 °C with 60% humidity.

To test for the presence of fusion p22 in Western blot, the agroinoculated and upper uninoculated *N. benthamiana* leaves, respectively, were collected at 5 and 25 days post inoculation (dpi). Total proteins were extracted from leaf tissue using buffer containing 220 mmol/l Tris-HCl (pH7.4), 250 mmol/l sucrose, 1 mmol/l $MgCl_2$, 50 mmol/l KCl and 10 mmol/l β-mercaptoethanol. The extracted proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5 % gels, and then subjected to Western blotting with anti-FLAG M2 monoclonal antibody to detect the p22FLAG$_3$ fusion protein accumulation in *N. benthamiana* plants as described previously (*19*). The result showed that the p22FLAG$_3$ protein (with molecular weight 25 kDa) had a relatively lower accumulation in the inoculated leaves (Fig. 1, panel b IL), whereas a relatively higher level of p22FLAG$_3$ fusion protein was detected in the upper uninoculated leaves (Fig. 1, panel b SL). These results offered direct physical evidence for the presence of p22 in ToCV-infected plants.

As an RNA silencing suppressor (RSS), p22 is important for ToCV infection and it might also be involved in viral pathogenicity (*14*). The RSS property of p22 has been further supported by its preferential binding to long dsRNAs *in vitro* and protecting these long RNAs from cleavage by dicer (*16*). In the present study, using an antibody against the fusion triple FLAG-tag, we provided direct evidence for the natural expression of p22 in ToCV-infected plants.

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