Production of mono- and polyclonal antibodies to Citrus leprosis virus C2 and their application in triple antibody sandwich ELISA and immunocapture RT-PCR diagnostic assays

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Abstract

The newly discovered *Citrus leprosis virus* cytoplasmic type 2 (CiLV-C2) is one of the causal virus of citrus leprosis disease complex; which leads to substantial loss of citrus production in the states of Meta and Casanare of Colombia. Specific and sensitive detection methods are needed to monitor the dissemination of CiLV-C2 in Colombia, and to prevent introduction of CiLV-C2 to other citrus growing countries. Toward this end, putative coat protein gene (CPG) of CiLV-C2 was amplified from CiLV-C2 infected citrus tissues. The CPG was cloned, expressed and purified a recombinant coat protein of ~31 kDa which used to generate monoclonal antibodies and polyclonal antisera. Four monoclonal antibodies and two polyclonal antisera were selected as being specific following Western blotting. The monoclonal antibody MAb E5 and polyclonal antiserum PAb UF715 were selected testing with an extract of CiLV-C2 infected leaves using triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA). In addition, an immunocapture RT-PCR was standardized using MAb E5 for specific and sensitive detection of CiLV-C2. The standardized TAS-ELISA and IC-RT-PCR were able to detect CiLV-C2 in the extracts of symptomatic citrus leprosis tissues up to the dilutions of 1:160 and 1:2,580, respectively. Result demonstrated that CiLV-C2 is present in citrus orchards in Meta and Casanare citrus growing areas of Colombia. TAS-ELISA could be used for routine detection of CiLV-C2, epidemiological studies, and for border inspections for quarantine purposes. IC-RT-PCR could be valuable for CiLV-C2 validation and viral genome analysis.

Keywords: Citrus leprosis disease; Citrus virus; CiLV-C2; Antibodies; TAS-ELISA

1. Introduction

Citrus leprosis virus (CiLV), the causal agents of Citrus leprosis disease, is an economically important and rapidly spreading virus affecting the citrus industry in South and Central America (Bastianel et al 2010). Citrus growers suffer severe economic losses every year due to damage caused by CiLV (Rodrigues et al. 2001, Locali et al., 2004). Viruses in the CiLV complex are transmitted by tenuipalpid false spider mite of genus *Brevipalpus* to citrus plants (Rodrigues et al. 2000, 2003, Roy et al., 2013). CiLV can infect the leaves, fruits and stems of citrus species and the symptoms of disease appear as localized lesions where viruliferous mites feed because CiLV movement is not systemic. The localized lesions appear circular and chlorotic with dark-brown centers in leaves, flat or depressed spots on fruits, and small, chlorotic, shallow or dark-brown lesions in stems (Rodrigues et al., 2001). Advanced stages of infection result in severe defoliation, fruit drops, dieback, and death of trees (Bitancourt, 1955). Almost all citrus species are susceptible to CiLV with sweet orange being the most sensitive, followed by tangerine and grapefruit while lime, lemon and tangelo are less susceptible (Rodrigues et al., 2001).

To date, two major types of CiLV have been described, cytoplasmic and nuclear type based on the presence of viral particles in symptomatic lesion tissues. CiLV particles are found only in the symptomatic lesion tissues and not in the surrounding asymptomatic tissues (Kitajima et al., 1972). Citrus leprosis virus cytoplasmic type (CiLV-C) is spreading rapidly in South and Central America and also reported from Mexico (Castillo et al, 2011). The citrus leprosis virus nuclear type (CiLV-N) was found in small areas of Sao Paulo, Rio Grande do Sul, and Minas Gerais in Brazil, the Boquete region in Panama and recently observed in the state of Queretaro, Mexico (Bitancourt, A. A. 1955, Roy et al., 2013). In 2009, citrus leprosis disease like symptoms were reported on leaves and bark of citrus *volkameriana* tree in Oahu, Hawaii and confirmed to

contain cytoplasmic type CiLV particles by TEM and later was characterized as Hibiscus green spot virus (HGSV), a new member of genus *Higrevirus* (Melzar et al., 2012).

In Colombia, the symptoms of citrus leprosis disease were first observed in the eastern plains of Casanare in 2003, and in Meta in 2004 on leaves and fruits of valencia orange which initially identified as CiLV-C by TEM and later confirmed by reverse transcription-polymerase chain reaction (RT-PCR) (Leon et al., 2006). However, in 2011-2012 the symptomatic citrus leprosis tissues from Colombia were tested negative for CiLV-C by enzyme-linked immunosorbent assay (ELISA) and RT-PCR assays but TEM observations revealed the presence of CiLV-C like virus particles within the endoplasmic reticulum (Roy et al., 2013). Subsequent genome sequencing of this virus revealed the presence of a new cytoplasmic citrus leprosis virus cytoplasmic type 2 (CiLV-C2) which produced similar symptoms in citrus as elicited by CiLV-C. Genome organization of CiLV-C2 was found identical to CiLV-C except an additional hypothetical p7 protein in RNA2. CiLV-C2 was assigned to the genus *Cilevirus* (Roy et al., 2013). The genomic RNA of CiLV-C2 was 13,706 nucleotides long composed of RNA1 of 8,717 and RNA2 of 4,989 nucleotides and was found 52% and 40% identical compared to RNA1 and RNA2 of CiLV-C, respectively (Roy et al., 2013). To date, CiLV-C2 has been reported only from Colombia (Roy et al., 2013, 2015).

CiLV-C2 in extracts of symptomatic citrus leprosis tissue could not be detected with antibodies and conventional RT-PCR primers of CiLV-C (Locali et al., 2003, Choudhary et al., 2013, 2014, 2015) because CiLV-C2 and CiLV-C share only 33% amino acid and 47% nucleotide identity in CPG region (Roy et al., 2013). The currently available methods for detection of CiLV-C2 are TEM and conventional RT-PCR (Roy et al., 2013). The antibody-based diagnostic methods are sensitive, specific and economical for plant viruses. Highly specific antibody would be needed to

monitor the spread of CiLV-C2 in Columbia and to prevent introduction in other citrus growing areas.

In this study, CPG of CiLV-C2 was amplified by RT-PCR, cloned and expressed in *Escherichia coli*. The recombinant coat protein of CiLV-C2 was purified and used for production of monoclonal antibodies and polyclonal antiserum in mice and rabbits, respectively. Triple antibody sandwich- (TAS) ELISA, and immunocapture reverse transcriptase polymerase chain reaction (IC-RT-PCR) assays were successfully standardized for detection of CiLV-C2 in extracts of symptomatic citrus leprosis tissues.

2. Material and Methods

2.1 Expression and purification of coat protein

The p29 CPG sequence of CiLV-C2 genome (Accession number JX000024, JX000025) was used to synthesized forward; 5'-CGATTACATATGAGTAACATTGTGTCGTTTTTC-3' and reverse primer; 5'-CGATTAGGATCCTCACTCTTCCTGTTCATC-3' for amplification of CPG. Total RNA was extracted from CiLV-C2 infected citrus leaves collected from Colombia using RNeasy Plant Mini kit (Qiagen, Valencia, CA) and RT-PCR was carried out as previously described (Roy et al., 2013). The amplified CPG was cloned between *Nde*I and *Bam*HI restriction enzyme sites in pET28a bacterial expression vector. The positive recombinant plasmid clones containing CPG gene were sequenced and analyzed for its accuracy. The recombinant CP of CiLV-C2 was expressed through BL21(DE3) cells using the *E. coli* expression system (Life Technologies, Grand Island, NY) following the manufacturer's recommendations. The recombinant CP was purified from the insoluble fractions of induced *E. coli* cells using the Ni-

NTA purification system (Life Technologies, Grand Island, NY) following the manufacturer's recommendations.

2.2 Production of Monoclonal antibodies and Polyclonal antisera to recombinant coat protein of CiLV-C2

The purified recombinant CP of CiLV-C2 was used for production of four monoclonal (MAbs, E2, E5, 6E5 and E7) antibodies and two polyclonal antisera (UF715 and UF 716) commercially by GenScript (Piscataway, NJ, USA) and Cocalico Biological (Reamstown, PA, USA). Immunoglobulin G (IgGs) from each monoclonal antibodies and polyclonal antisera were purified from crude preparations using Protein G and Protein A agarose resin, respectively, following manufacturer's protocol (Bio-Rad, Hercules, CA). The standard working concentration of purified IgG was adjusted to 1.0 mg/ml using the NanoDropTM 1000 spectrophotometer (Fisher Scientific, Pittsburgh, PA) for further assays.

The specificity of purified IgGs were determined in Western blot assays against the recombinant CP of CiLV-C2 as describes previously (Choudhary et al., 2013, 2014). Extracts from CiLV-C2 infected leaves were used to determine the sensitivity and specificity of the developed monoclonal antibodies and polyclonal antisera, and extracts of healthy citrus leaves were used as negative controls.

2.3 Detection of CiLV-C2 in ELISAs

Sixty-three symptomatic citrus leprosis samples of CiLV-C2 were collected from citrus growing areas of Yopal and Aguazul in Casanare state, Cumaral, Guamal and Villavicencio in Meta state, Colombia. The infected citrus samples were obtained under USDA APHIS permit for diagnosis

of CiLV-C2 to the biosecurity level 3 quarantine facilities at Foreign Disease Weed Science Research Unit (FDWSRU), Fort Detrick, MD, USA. The extracts of symptomatic citrus leprosis samples were used for detection of CiLV-C2 using developed antibodies by TAS-ELISA and IC-RT-PCR as described previously (Wu et al., 2009, Choudhary et al., 2014). CiLV-C and CiLV-N infected and healthy citrus leaves were also tested with the developed antibodies of CiLV-C2 to determine the specificity.

3. Results

3.1 Expression and purification of recombinant coat protein of CiLV-C2

The p29 CPG (795 bp) was successfully amplified from total RNA extracted from CiLV-C2 infected tissues by RT-PCR. The amplified product was inserted into pET28a expression vector. The insert was sequenced which showed no mismatch nucleotide and gene is also in correct open reading frame (ORF). The recombinant pET28a vector transformed in *E. coli* BL21(DE3) competent cells and overexpressed the recombinant coat protein by induction with IPTG. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis confirmed the presence of recombinant CP of ~31 kDa in the insoluble fractions of induced cells (Fig. 1a, lane 5) and absent in un-induced cells (Fig. 1a lane 4). The recombinant CP was successfully purified from insoluble fraction of induced BL21(DE3) cells lysate (Fig. 1a lane 6).

3.2 Selection of specific monoclonal antibodies and polyclonal antisera

The hybridoma cell lines 6D4E2, 4D4E5, 6H6E5 and 2B10E7 producing specific MAbs, E2, E5, 6E5 and E7, respectively and two polyclonal antisera, UF715 and UF716 were determined highly specific to recombinant CP of CiLV-C2 in Western blot analysis (Fig. 1b, lane 1). No

reactive signal was seen with extracts of healthy citrus tissues or CiLV-C2 infected citrus tissues (Fig. 1b, lane 2, 3).

In TAS-ELISA, positive reaction was measured in extracts of CiLV-C2 infected leprosis citrus tissues when using MAbs E2, E5, 6E5 and E7 as primary or capturing antibodies and PAb-UF715 and UF716 as secondary antibodies, which detected with goat anti-rabbit antibody conjugated with alkaline phosphatase enzyme. The absorbance values obtained with symptomatic CiLV-C2 infected tissues and healthy citrus tissue were compared to select best antibody pair. The maximum absorbance values were observed with MAb-E7/ PAb-UF716 pair while minimum absorbance observed with MAb-E2/ PAb-UF715 pair. A 21-fold higher absorbance value was calculated with MAb-E7/ PAb-UF715 pair compared to only 6-fold higher value with MAb-E7/ PAb-UF716 pair (Fig. 2). Based on these results MAb-E7/ PAb-UF715 pair (at dilutions of 1:1,000 w/v, mg mL⁻¹) along with goat anti-rabbit antibody conjugated with alkaline phosphatase (at a dilution of 1:5,000 w/v, mg mL⁻¹) were selected for diagnosis of CiLV-C2 in TAS-ELISA.

For IC-RT-PCR, MAb-E7 at a dilution of 1:50 (w/v, mg mL⁻¹) found the best CiLV-C2 capturing potential which amplified an expected single product of 795 bp from the CiLV-C2 infected citrus tissues and as expected no any amplification observed from healthy citrus tissues (Fig. 3). The amplified product was sequenced which showed 99-100% nucleotide sequence identity with CP gene of CiLV-C2.

3.3 Sensitivity analysis and detection of CiLV-C2

Serial dilutions of extracts of CiLV-C2 infected citrus leprosis tissues were used to determine the sensitivity of CiLV-C2 detection in TAS-ELISA and IC-RT-PCR. CiLV-C2 can be detected by TAS-ELISA and IC-RT-PCR up to the dilution of 1:320 (w/v, g mL⁻¹) and 1: 2,560 (w/v, g mL⁻¹),

respectively. This suggests, IC-RT-PCR assay was 8-fold more sensitive compared to TAS-ELISA for detection of CiLV-C2 (Fig. 3 and 4).

All sixty-three symptomatic citrus leprosis samples collected from the Casanare and Meta areas of Colombia were found positive for CiLV-C2 by TAS-ELISA and IC-RT-PCR assays. Healthy citrus tissues and the citrus samples infected with CiLV-C, CiLV-N, citrus tatter leaf virus, *Citrus tristeza virus* (genotypes T30, T36 and T68), and citrus leaf rugose virus tissues were tested negative with antibodies of CiLV-C2 (data not shown). These results confirm that selected monoclonal antibody MAb E7 and polyclonal antisera PAb UF715 are highly specific for detection of CiLV-C2.

4. Discussion

CiLV-C2 is a definitive member of genus *Cilevirus*, transmitted by flat mite species *Brevipalpus yothersi* (previously called: *B. phoenicis*) to citrus plants (Roy et al., 2013, 2015). CiLV-C2 is the second cytoplasmic type virus that causes leprosis disease in citrus and affect citrus production in Colombia. The current diagnostic methods for CiLV-C2 are based on visible observation of symptoms, RT-PCR and transmission electron microscopy (TEM). The visual detection based on symptomatology is inadequate because both CiLV-C and CiLV-N types of virus produces the similar lesion symptoms in citrus. TEM requires expensive equipment and specialized technicians, and cannot conclusively identify CiLV-C2 since all CiLV virions have a similar morphology and size. The economical detection methods are crucial to develop effective management plans for CiLV-C2. Serological assays being specific, sensitive, inexpensive and suitable for high throughput applications are widely acceptable in developing or underdeveloped countries for diagnosis of plant viruses (Shang et al., 2011; Wu et al., 2009, 2011). The amount

of CiLV-C2 needed for antibody production is difficult to purify from localized lesions because virions accumulate at very low titer. Therefore, we resolved the problem by cloning the CiLV-C2 CPG amplicon and expressing the coat protein in-vitro for monoclonal antibodies and polyclonal antisera production. The recombinant coat protein expresses in the bacterial cells due to abundant protein expression, stability and simplicity of purification (Wu et al., 2009).

The amino acid sequence of coat protein of CiLV-C and CiLV-C2 were only 33% identical that's why the antibodies produced against expressed coat protein of CiLV-C failed to detect the CiLV-C2 in ELISA (Choudhary et al., 2013, 2014, Roy et al., 2013). This suggests, probably coat protein of CiLV-C and CiLV-C2 do not share any common epitopes.

The RT-PCR amplified p29 gene of CiLV-C2 was cloned into the pET28a vector which can be preserved indefinitely and protein can be expressed and purified quickly whenever required in the future. The recombinant coat protein of CiLV-C2 was purified by affinity chromatography and injected into mice and rabbits to produce the monoclonal antibodies and polyclonal antisera, respectively. The antibodies thus produced a positive result against the expressed CP in Western blots and confirmed specificity of the antibodies. However, the developed antibodies failed to react with extracts of CiLV-C2 infected citrus tissues from Colombia. The reason could be that after SDS treatment the conformation of epitopes of coat protein were changed in a way that they were not able to be detected with the produced antibodies.

However monoclonal antibodies and polyclonal antisera of CiLV-C2 were able to detect CiLV-C2 in extracts of symptomatic leprosis citrus samples by TAS-ELISA and IC-RT-PCR assays. Monoclonal antibody was identified as successful captured antibody for CiLV-C2 in both assays which are necessary for uniform detection of CiLV-C2 in symptomatic citrus leprosis samples from Colombia. In TAS-ELISA, the captured CiLV-C2 virions are likely to provide multiple

native epitopes of coat protein of CiLV-C2 and to allow binding of multiple copies of secondary polyclonal antiserum of CiLV-C2 that enhances the sensitivity of detection by the alkaline phosphatase conjugated goat-anti rabbit antibody in TAS-ELISA. The coat protein of CiLV-C2 was not detected in ELISA when the CiLV-C2 polyclonal antiserum used as capturing antibody and the CiLV-C2 MAb was used as secondary antibody followed by detection with the alkaline phosphatase conjugated goat-anti mouse antibody (data not shown). The reason could be that, these four MAbs were not able to detect CiLV-C2 when used as a secondary antibody, is unknown. Similar findings has been reported previously for detection of CiLV-C, cucumber green mottle virus, and tomato spotted wilt virus (Choudhary et al., 2014, Wu et al., 2009, 2011). In the present study, accurate, specific and sensitive detection of CiLV-C2 in the symptomatic citrus leprosis samples using IC-RT-PCR was shown to be more sensitive than TAS-ELISA assays.

TAS-ELISA and IC-RT-PCR assays were performed on sixty-three field citrus leprosis samples collected from the Casanare and Meta states of Colombia and it was determined that the CiLV-C2 is prevalent in these areas (data not shown). Negative test results for CiLV-C2 in symptomatic leprosis citrus samples collected from Panama and Mexico suggests that the developed antibodies are specific to CiLV-C2.

In conclusion, the monoclonal antibodies and polyclonal antisera developed to the expressed CP of CiLV-C2 for the accurate diagnosis of CiLV-C2 in the symptomatic citrus leprosis tissues were successfully used in TAS-ELISA and IC-RT-PCR assays. IC-RT-PCR was shown to be more sensitive assays however TAS-ELISA is the preferred method for the detection of large numbers of symptomatic leprosis sample. Since CiLV-C is already present in the citrus growing regions of many countries of South and Central America it is important to determine the

emergence and spread of CiLV-C2 into new citrus areas. The *Brevipalpus* mite that vectors CiLV-C2 is present in all citrus orchards and once acquired by the mite, CiLV-C2 may spread rapidly. An effective management strategy to control spread should include early detection of CiLV-C2 using the sensitive and specific TAS ELISA to screen field samples followed by confirmation of select samples using the IC-RT-PCR. Newly developed tools will aid to develop strategies to decrease the spread of CiLV-C2 in Colombia and also prevent the spread of CiLV-C2 to other citrus growing countries.

5 Conflict of interests

The authors declare no conflict of interests.

6. Acknowledgements

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7. References

- Bastianel, M., Noveli, V.M., Kubo, K. S., Kitajima, E. W., Bassanezi, R., Machado, M. A., Freitas-Astua, J., 2010. Citrus leprosis: Centennial of an unusual mite–virus pathosystem Plant Dis. 94, 284–292.
- Bitancourt, A.A., 1955. Estudos sobre a leprose dos citros. I-Distribuição geográfica e sintomatologia. Arq. Inst. Biol. 22, 161–184.
- Castillo, I.I., Diaz, L.F.Z., Mendez, W., Colina, G.O., Freitas-Astua, J., Locali-Fabris, E.C., Moraes, G.J., Calegario, R.F., Tassi, A.D., Kitajima, E.W., 2011. Confirmation of the

- presence of the Citrus leprosis virus C (CiLV-C) in Southern Mexico. *Tropical Plant Pathology* 36, 400–403.
- Choudhary Nandlal, Wei G., Govindarajulu A., Roy Avijit, Li Wenbin, Picton Deric D., Nakhla M. K., Levy L., Brlansky R. H. (2015). Detection of *Citrus leprosis virus C* using specific primers and TaqMan probe in one-step real-time reverse-transcription polymerase chain reaction assays. *J. Virol. Methods* (224) 105-109.
- Choudhary, N., Roy, A., Govindrajulu, A., Nakhla, M.K., Levy, L., Brlansky, R.H., 2014. Production of monoclonal antibodies for detection of Citrus leprosis virus C in enzyme-linked immuno- assays and immunocapture reverse transcription-polymerase chain reaction. *J. Virol. Methods* 206, 144–149.
- Choudhary, N., Roy, A., Leon, M.G., Picton, D.D., Wei, G., Nakhla, M.K., Levy, L., Brlansky, R.H., 2013. Immunodiagnosis of Citrus leprosis virus C using a polyclonal antibody to an expressed putative coat protein. *J. Virol. Methods* 193, 548–553.
- Kitajima, E.W., Müller, G.W., Costa, A.S., Yuki, W., 1972. Short, rodlike particles associated with citrus leprosis. *Virology* 50, 254–258.
- Leon, G.A., Realpe, C.E., Garzon, P.A., Rodriguez, J.A., Moreno, M.G., Childers, C.C., Achor, D., Freitas-Astúa, J., Antonioli-Luizon, R., Salaroli, S.B., Cobo, N.C.M., Kitajima, E.W., 2006. Occurrence of *Citrus leprosis virus* in Llanos Orientales, Colombia. *Plant Dis.* 90, 682.
- Locali, E.C., Freitas-Astua, J., Machado, M.A., 2004. Leprose dos citros: biologia e diagnóstico. *Laranja* 25, 53–68.
- Locali, E.C., Freitas-Astua, J., Souza de, A.A., Takita, M.A., Astua-Monge, G., Antonioli, R., Kitajima, E.W., Machado, M.A., 2003. Development of a molecular tool for the diagnosis of leprosis, a major threat to citrus production in the Americas. *Plant Dis.* 87, 1317–1321.
- Melzer, M. J., Sether, D. M., Borth, W. B., and Hu, J. S. 2012. Characterization of a virus infecting *Citrus volkameriana* with citrus leprosis-like symptoms. *Phytopathology* 102, 122–127.
- Rodrigues, J.C.V., Childers, C.C., Kitajima, E.W., Machado, M.A., Nogueira, N., 2001. Uma estratégia para o controle da leprose dos citros. *Laranja* 22, 412–423.

- Rodrigues, J.C.V., Kitajima, E.W., Childers, C.C., Chagas, C.M., 2003. *Citrus leprosis virus* vectored by *Brevipalpus phoenicis* (Acari:Tenuipalpidae) on citrus in Brazil. *Exp. Appl. Acarol.* 30, 161–179.
- Rodrigues, J.C.V., Machado, M.A., Kitajima, E.W., Muller, G.W., 2000. Transmission of *Citrus leprosis virus* by *Brevipalpus phoenicis* (Acari: Tenuipalpidae). Pages 174-178 in: *Proc. 14th Conf. Int. Org. Citrus Virol.* J. V. da Graca, R. F. Lee, and R. K. Yokomi, eds. IOCV, Riverside, CA.
- Roy, A., Choudhary, N., Guillermo, L.M., Shao, J., Govindarajulu, A., Achor, D., Wei, G., Picton, D.D., Levy, L., Nakhla, M.K., Hartung, J.S., Brlansky, R.H., 2013. A novel virus of the genus *Cilevirus* causing symptoms similar to citrus leprosis. *Phytopathology* 103, 488–500.
- Roy, A., Hartung, J.S., Schneider, W.L., Shao, J., Guillermo, L.M., Melzar, M.J., Beard, J.J., Otero-Colina, G., Bauchan, G.R., Ochoa, R., Brlansky, R.H., 2015. Role Bending: Complex Relationships Between Viruses, Hosts, and Vectors Related to Citrus Leprosis, an Emerging Disease. *Phytopathology* 105, 1013-1025
- Shang, H.L., Xie, Y., Zhou, X.P., Qian, Y.J., Wu, J.X., 2011. Monoclonal antibody-based serological methods for detection of Cucumber green mottle mosaic virus. *Virol. J.* 8, 228.
- Wu, J.X., Meng, C.M., Shang, H.L., Rong, S., Zhang, C., Hong, J., Zhou, X.P., 2011. Monoclonal antibody-based triple antibody sandwich-enzyme-linked immunosorbent assay and immunocapture reverse transcription-polymerase chain reaction for Odontoglossum ringspot virus detection. *J. Virol. Methods.* 171, 40–45.
- Wu, J.X., Yu, C., Yang, C.Y., Deng, F.L., Zhou, X.P., 2009. Monoclonal antibodies against the recombinant nucleocapsid protein of tomato spotted wilt virus and its application in the virus detection. *J. Phytopathol.* 157, 344–349.