Transgenic Plants Expressing a Single-chain Fv Antibody to Tomato spotted wilt virus (TSWV) are Resistant to TSWV Systemic Infection

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Abstract

Hybridoma cell lines secreting monoclonal antibodies to TSWV were characterized, and one cell line was selected for cloning of the antibody genes. Universal degenerate oligonucleotides were designed to prime PCR amplification of the variable regions of the heavy and light chains of a monoclonal antibody. The PCR products were ligated to produce one single-chain variable fragment (scFv) antibody gene construct that was used to transform Nicotiana tabacum ‘Turk’. PCR analyses showed 48% of the 120 regenerated R0 plants contained TSWV scFv genes. The integration and transcription of the scFv gene in the transgenic tobacco plants were confirmed by Southern and northern blot hybridizations, respectively. The expression of TSWV scFv genes in transformed plants was examined by ELISA. TSWV scFv antibodies accumulated to only low levels in transformed plants. Antigen-binding analysis showed that plant-produced TSWV scFv antibodies had the same antigen-binding specificity as the murine TSWV monoclonal antibody. Two TSWV scFv-transformed plants were resistant to TSWV systemic infection as determined by the absence of systemic infection symptoms following TSWV inoculation. The level of virus resistance was correlated with the level of the transgene RNA transcript and scFv antibody production. This resistance was inherited into the R1 generation, and was specific to a lettuce isolate of TSWV (TSWV-L). No resistance was found to Tobacco mosaic virus (TMV) or Cucumber mosaic virus (CMV).

INTRODUCTION

Tomato spotted wilt tospovirus (TSWV) is the type member of the genus Tospovirus, in the family Bunyaviridae (Murphy et al., 1995). TSWV causes devastating diseases of floriculture and vegetable crops in Hawaii and worldwide (Cho et al., 1989; German et al., 1992), and is the limiting factor for lettuce, tomato, celery, and pepper production in Hawaii (Cho et al., 1989). The virus has a very wide host range, infecting more than 500 species in 50 families (German et al., 1992), and is transmitted in a persistent manner by several species of thrips (Ullman et al., 1995). These characteristics make this virus very difficult to control in field situations (Bautista et al., 1995; Cho et al., 1989). Transgenic plants resistant to TSWV infections have been developed using such pathogen-derived resistance approaches (de Haan et al., 1992; Kim et al., 1994; Pang et al., 1993; Sherman et al., 1998).

Single-chain Fv antibody (scFv) molecules, antibody derivatives consisting of only the V_L and V_H domains linked by a short polypeptide, have been shown to retain the same antigen specificity as monoclonal antibodies whose V_L and V_H sequences were used to construct the truncated molecules (Raag and Whitlow, 1995). Transgenic plants that express scFv antibodies against the coat protein of a Tombusvirus have also been produced. These plants showed delayed symptom development and reduced virus titers after virus challenge, suggesting a possible role for the antibodies in plant protection (Tavladoraki et al., 1993). In addition, transgenic tobacco plants expressing full-size antibodies to TMV were found to be resistant to TMV infection, and the resistance levels in these plants were positively correlated with antibody production (Voss et al., 1995). Functional expression in plants of an scFv antibody fragment against an epitope of the
TSWV-G1 protein (Franconi et al., 1999), through a viral vector, gave promising results. We report here the production of transgenic tobacco plants that express functional scFv antibodies to TSWV and show that they are resistant to systemic infection by TSWV.

MATERIALS AND METHODS

Cloning of TSWV-MAb scFv Gene

Hybridoma cell lines secreting monoclonal antibodies directed against TSWV were produced and their specificities determined by ELISA and Western blot analyses (Hsu et al., 1990). One cell line (TSWV-MAb8C4D6), which secretes broad-spectrum TSWV antibodies that react to the nucleoprotein of TSWV, was selected for cloning of antibody genes. Preparations enriched for polyadenylated mRNAs were isolated from this cell line using oligo-dT affinity chromatography (Chomczynski and Sacchi, 1987). Oligonucleotide probes specific to the conserved regions of the V\textsubscript{L} gene were prepared and used in Northern blot hybridization analysis to examine these RNAs. RNAs that hybridized to these probes were isolated and used as templates for the synthesis of complementary DNAs (cDNAs) using oligo-dT as the primer for reverse transcription. Specific cDNAs made to TSWV-MAb mRNA were used as templates in PCR using universal degenerate primers designed for PCR amplification of the V\textsubscript{L} and V\textsubscript{H} DNA fragments of IgG (Coloma et al., 1991). Examination of PCR products by blot hybridizations showed that they were specific to the IgG genes. These PCR products were ligated to form a single-chain Fv antibody (scFv) gene construct and cloned into the Nco I site of the plasmid vector pBI525.

Production and Screening of TSWV-resistant Transgenic Plants

The scFv gene under control of the double 35S promoter from CaMV and the NOS terminator in pBI525 were subcloned between the Hind III and EcoRI sites of the plant transformation vector pBI121, thus replacing the GUS gene construct in this vector. The resulting plasmid (pTSWVscFv) was transformed in Escherichia coli DH5\textalpha and then transferred into A. tumefaciens strain EHA105 by triparental mating. Tobacco (Nicotiana tabacum ‘Turk’) leaf discs were transformed with A. tumefaciens strain EHA105 containing pTSWVscFv as described previously (Kim et al., 1994). Kanamycin-resistant callus tissues were induced to regenerate shoots and roots, and the putative transgenic plants were tested for expression of neomycin phosphotransferase II (NPT II) protein by ELISA and for integration of the target gene by PCR using the degenerate V\textsubscript{L} and V\textsubscript{H} chain primers as described (Coloma et al., 1991). ELISA for NPT II activity in transformed plants was performed on total proteins extracted from transformed and non-transformed plants using the NPT II ELISA Kit according to the manufacturer's instructions (5Prime-3Prime). The absorbance at 405nm of each well was measured using a Bio-Rad® 450 multiwell plate reader. Total nucleic acids were isolated from transgenic plants (Doyle and Doyle, 1990) and used as templates for detection of the scFV gene. The TSWV scFv gene was amplified using the TSWV MAb light-chain gene primers V\textsubscript{L}-5’ (5’-GGTGTCGTCGACGACATTGTGCTCACCCAGTCTCCA-3’) and V\textsubscript{L}-3’ (5’-CATCGACCAGGCGTTTGTGATTCACCCAGCTTGGTCCC-3’).

Putative transgenic plants which were positive in NPT II ELISA and PCR tests were mechanically inoculated with a lettuce isolate of TSWV (TSWV-L). The inoculum was prepared by grinding TSWV-infected Gomphrena globosa leaf tissue in ice-cold extraction buffer consisting of 0.1M potassium phosphate buffer + 0.01M Na\textsubscript{2}SO\textsubscript{4} (pH 7.2) (Wang and Gonsalves, 1990). Transformed and non-transformed plants were mechanically inoculated at the 4-leaf stage. Two similarly sized leaves on each plant were dusted with carborundum (320 mesh) and inoculated with 200 μL of inoculum. Plants were maintained in a greenhouse at 25C under daylight conditions for symptom development. The number of necrotic lesions and any systemic infection symptoms were monitored for one month. These plants were also analyzed for TSWV infection by ELISA 10 days after inoculation (Wang and Gonsalves, 1990). Transgenic plants were
characterized by Southern and northern blot analyses as described Sambrook and Russell (2001).

Detection of Expression of scFv in Transgenic Plants

Polyclonal antibodies against TSWV-MAb 8C4D6 (anti-TSWV MAb IgG) were produced in rabbits by Cocalico Biologicals, Inc. (California, USA), and IgGs were purified using the Econo-Pac® Protein A Kit (Bio-Rad, Inc.) following the manufacturer’s protocol. Total soluble protein was extracted from transformed tobacco leaf tissue according to Tavladoraki et al. (1993). ScFv proteins were purified from total soluble protein mixture using affinity columns prepared with purified TSWV-specific polyclonal IgG and magnetizable cellulose/iron oxide particles (Cortex, Inc.).

Expression of TSWV scFv in transgenic plants was examined by ELISA. Microtitre plates (Dynatech) were coated with plant-produced affinity-column purified anti-TSWV scFv antibodies diluted 1:100 in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, 0.02% NaN₃, pH=9.6) at 4°C overnight. The plates were washed with IX PBS-T buffer (8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 150 mM NaCl, 0.02% NaN₃, 3 mM KCl, 0.05% Tween®-20, pH=7.3), and 100 µL anti-TSWV MAb IgG diluted (1 µg/ml) in enzyme buffer (PBS-T + 2% PVP40 + 0.2% ovalbumin) was added to each well. The plates were incubated at 37°C for 3 hours, followed by washing with PBS-T buffer. Goat anti-rabbit IgG alkaline phosphatase conjugate was diluted 1:2000 in enzyme buffer, added to each well, and incubated at 37°C for 3 hours. After washing, p-nitrophenyl phosphate substrate (1 mg/ml) (Sigma #104) in substrate buffer (1 M diethanolamine, 0.02% NaN₃, pH 9.6 with HCl) was added and absorbance at 405 nm was measured with a microtitre plate reader. The TSWV-binding activity of plant-produced TSWV scFv antibodies was determined by ELISA. One hundred microliters of the plant-produced TSWV scFv antibodies were added to wells of microtitre plates coated with purified TSWV (1 mg/mL in coating buffer), and developed as described above.

Characterization of R1 Transgenic Plants

Transgenic tobacco clone #64 R0 plants that were resistant to TSWV systemic infection were allowed to self-pollinate. The resulting seeds were germinated on selective media containing 300 µg/mL kanamycin. The seedlings which survived on this media were further analyzed for the presence of TSWV scFv genes by PCR analysis using TSWV heavy-chain 5’ primer (VH-5’) 5’-ATCGACCATGGAGGCATGC(A/G)(A/C)CTGCAG(C/G)AGTC(A/T)GG-3’ and light-chain 3’ primer (VL-3’) 5’-CATCGACCATGGCCGTTTGATCTCGAGCTTGGTGCC-3’. To determine the resistance to TSWV infection in the R1 generation, seeds from self-pollinated plants of two resistant lines (#52 and #64) and one susceptible line (#32) were germinated on medium containing 300 µg/mL kanamycin and mechanically challenged with TSWV-L at the 4- to 6-leaf stage, together with non transformed plants as controls. The experiments were conducted at the University of Hawaii (UH) and at the USDA-ARS Beltsville Agricultural Research Center, Maryland.

The specificity of resistance of the transgenic plants was examined using the R1 plants. Five R1 plants from each of the transgenic lines #32, #52, and #64, which were shown by PCR to harbor the scFv genes, and 5 non-transgenic plants were mechanically challenged with TSWV as described above. Assays of resistance to the TSWV isolates TSWV-B, (a TSWV isolate from begonia), TSWV-O, (a TSWV isolate from orchids), Tobacco mosaic virus (TMV), and Cucumber mosaic virus (CMV) were performed on R1 plants. TSWV-L, TSWV-B and TSWV-O were maintained in Gomphrena globosa. TMV was maintained in N. tabacum ‘Turk’ and CMV was maintained in cucumber (Cucumis sativus). The inoculum of each virus was prepared by grinding virus-infected leaf tissue in extraction buffer consisting of 0.1M potassium phosphate buffer + 0.01M Na₂SO₄ (pH 7.2). The resistance was determined in non-inoculated leaves 10 days after plant inoculation by ELISA. The experiment was repeated twice.
RESULTS

Analysis of scFv Gene and TSWV-Resistant Transgenic Plants

The TSWV scFv gene was cloned and sequenced (Fig. 1). It is composed of 768 nucleotide residues, which encode a protein of 256 amino acids. Transgenic tobacco plants were produced with the gene construct, pTSWVsFv (Fig. 2). A total of 120 putatively transformed plants were analyzed for NPT II expression using ELISA and PCR. Of the 120 plants tested, 85 plants expressed NPT II at levels that were detectable by ELISA. PCR analyses with the VL-3’ and VL-5’ oligonucleotides of the genomic DNA isolated from these 120 putatively transformed and 4 non-transformed tobacco plants revealed that 48% of the transformed plants contained the expected 320-bp sequences from the TSWV scFv gene. The remaining 52% of these plants and all the non-transformed plants did not contain these sequences.

All 120 putatively transformed R0 plants were transplanted into soil and challenged with TSWV-L at the 4- to 6-leaf stage by mechanical inoculation. Virus replication and movement in these plants were monitored daily by observing the number of necrotic lesions and any systemic symptoms produced for one month after inoculation. Representative results from seven R0 lines are shown in Table 1. Three patterns of response were noted: susceptibility to TSWV-L infection, delayed TSWV-L symptom expression after challenge, and resistance to TSWV-L systemic infection. All susceptible plants developed systemic symptoms on non-inoculated upper leaves within one month after TSWV-L challenge. Delayed TSWV-L systemic symptom development occurred in some transformed plants at least two weeks later than in non-transformed plants. Resistant plants never developed any TSWV-L symptoms on non-inoculated leaves (Fig. 3). Transgenic R0 clones #64 and #52 showed the highest resistance to TSWV-L systemic infection, whereas R0 lines #56 and #61 showed delayed symptom expression after inoculation with TSWV-L (Table 2). No resistance was found in any of the other 116 lines after mechanical inoculation with TSWV-L.

Southern and Northern Blot Analyses

Southern blot analyses of genomic DNA isolated from two resistant lines (#52 and #64) and one line (#61) that showed delayed systemic infection contained a DNA fragment of about 1700 bp which hybridized to a probe prepared from the V\text{L} fragment of the TSWV-L specific MAb gene. This product was not detected from susceptible plants or non-transformed plants (Fig. 4A).

Transcription of the TSWV scFv gene in transgenic tobacco plants was analyzed in northern blots using mRNA isolated from R0 plants that showed either resistance to systemic infection or delayed systemic symptom expression. These analyses revealed an 800-bp fragment present in the two resistant lines #52 and #64 that hybridized to the probe prepared from the V\text{L} fragment of the TSWV-L specific MAb gene. This fragment was not detectable in mRNAs from non-transformed plants (Fig. 4B), nor was it detectable in lines #56 and #61, which showed delayed symptom development (data not shown).

Expression of TSWV-scFv in Plants

The expression levels of TSWV scFv in transgenic plants were examined by ELISA using TSWV-L MAbs for comparison. Two transgenic plants (#56 and #61), which had delayed symptom development phenotypes, produced only small amounts of scFv antibodies (4 pg/g leaf tissue) and three plants (#8, #32 and #76) produced antibodies in the range of 6-9 pg/g leaf tissue, although these plants were susceptible to TSWV infection. Clones #52 and #64, which showed resistance to TSWV-L systemic infection in R0 lines, expressed the highest levels of scFv antibodies, approximately 12 pg/g leaf tissue. TSWV scFv was undetectable in all non-transformed plants (Fig. 5). Furthermore, the antigen binding activity of plant-produced antibodies was similar to that of TSWV-MAb as shown by ELISA (Fig. 6).
Characterization of R1 Transgenic Plants

The results of mechanical challenge by TSWV-L showed that 26 of the 28 R1 plants from line #64 were resistant to TSWV-L systemic infection while the susceptible line #32 and all non-transformed plants were susceptible to infection by TSWV-L in experiments conducted at the University of Hawaii. TSWV-L infection was confirmed by ELISA. In line #64, although the inoculated leaves were TSWV-L positive, all non-inoculated leaves were TSWV-L negative. Similar resistance to TSWV-L systemic infection was also observed in R1 plants of transgenic line #64 at the USDA facility in Beltsville, MD. However, resistant line #52 reacted differently to TSWV-L challenge at the two sites. At the University of Hawaii, 34 of 40 plants (82%) of R1 plants showed systemic resistance to TSWV-L infection, but at the USDA laboratory, no systemic resistance was found. The experiment was repeated four times at UH and twice at USDA.

Resistance specificity of the transgenic plants was examined using R1 plants. Five R1 plants from each of the transgenic lines #32, #52, and #64, which were shown by PCR to have the scFv genes, and non-transgenic plants were challenged with different TSWV strains (TSWV-B and TSWV-O), TMV and CMV. The virus titers in non-inoculated leaves were determined by ELISA ten days after mechanical inoculation. Results from these assays (data not shown) showed that all plants tested were susceptible to these viruses, indicating that resistance in these transgenic plants is specific to TSWV-L.

DISCUSSION

This study demonstrates that functional antibodies against plant viruses can be produced in tobacco plants and that transgenic tobacco plants expressing these anti-virus antibodies are resistant to infection by the homologous virus. No protection was found when plants were challenged with the unrelated virus species, as also shown in similar studies by Tavladoraki et al. (1993). Moreover, we not only obtained transgenic plants which showed delayed TSWV symptom development, but also identified two lines which were resistant to systemic infection of TSWV.

We have examined the integration into the host genome and transcription of TSWV scFvs in transgenic tobacco plants using PCR, Southern, and Northern blot analyses. The results of Southern blot hybridization and PCR showed that a unique 1700-bp fragment that hybridized to the scFv probe was present in genomic DNA and that a 320-bp PCR product was present in some transgenic plants, but was absent in non-transformed plants. TSWV scFv mRNA (800-bp) was detected in two resistant lines (#52 and #64), but not in others that showed delayed symptom development, or in susceptible lines. Transcription levels of this gene were very low; the mRNA transcripts of the scFv gene could not be detected when total RNA was used in Northern blot analysis. Total RNA had to be enriched for messenger RNAs before the scFv mRNA could be detected in Northern blot analyses. Because the two lines that had the highest transgene mRNA production levels were also the most resistant to TSWV-L infection, it appears that the expression level of TSWV scFv is an important factor in the virus resistance of these plants.

The expression of plant-produced TSWV-scFv proteins and the antigen-binding specificity of plant-produced TSWV-scFv antibodies were confirmed by ELISA analyses. The results indicate that the plant-produced TSWV-scFv antibody maintains the antigenic specificity of the murine TSWV MAb8C4D6. However, the production level of TSWV-scFv proteins was very low (≤ 12 pg/g leaf tissue). Bioassays for resistance to TSWV infection revealed that the plants which expressed the highest levels of TSWV-scFv also showed the greatest resistance to TSWV-L systemic infection (clones #52 and #64). Low cytoplasmic accumulation of scFv protein has also been reported from other studies (Fiedler et al., 1995).

The resistance to TSWV infection is inherited by the R1 generation and is specific to the TSWV-L strain. No resistance was found to other TSWV strains or other viruses. These results further suggest that the resistance is due to expression of the TSWV-scFv gene, and also indicates that TSWV-scFv genes are heritable. The mechanism by which
plant-produced single-chain antibodies confer resistance is unknown. The binding of the single-chain antibody to the Ca\textsuperscript{2+} binding site on the coat protein may interfere with a critical step in the uncoating of the virus or in the assembly of virions (Tavladoraki et al., 1993).

This antibody-mediated protection principle may be useful for protecting plants against plant viruses. A major segment of the genes for the IgG V\textsubscript{L} and V\textsubscript{H} protein subunits are very conserved and their sequences are known. This makes engineering of the cDNA relatively straightforward. Monoclonal antibodies are produced in abundant amounts, which make them relatively easy to isolate and characterize. Recently, several other labs have been evaluating this antibody-mediated protection strategy to control different plant viruses (Fecker et al., 1996; Zimmermann et al., 1998; and Aebig et al., this issue). In the next few years, more information will be available regarding the usefulness of this approach for control of plant viruses.

ACKNOWLEDGEMENTS
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Literature Cited


Table 1. Comparison of resistance responses of several lines of R0 *Nicotiana tabacum* ‘Turk’ transformed with a TSWV scFv construct and a nontransformed line (NT) mechanically challenged with TSWV-L.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Line 1</th>
<th>Line 2</th>
<th>Line 3</th>
<th>Line 4</th>
<th>Line 5</th>
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</tr>
</tbody>
</table>

1 Number of TSWV-free plants after one month/total number of plants tested in each trial.

Table 2. Reactions of transgenic *Nicotiana tabacum* ‘Turk’ R0 plants inoculated with TSWV-L. Means and standard deviations from 3 experiments.

<table>
<thead>
<tr>
<th>Plant lines</th>
<th>64</th>
<th>52</th>
<th>56</th>
<th>61</th>
<th>8</th>
<th>76</th>
<th>32</th>
<th>NT</th>
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<tbody>
<tr>
<td>L.L. ^2</td>
<td>2 ± 1.7</td>
<td>6 ± 3.0</td>
<td>3 ± 1.2</td>
<td>7 ± 1.5</td>
<td>14 ± 6.4</td>
<td>6 ± 1.5</td>
<td>6 ± 0.7</td>
<td>23 ± 2.1</td>
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<tr>
<td>D.S.A. ^3</td>
<td>7 ± 2.1</td>
<td>7 ± 2.8</td>
<td>7 ± 2.0</td>
<td>6 ± 2.3</td>
<td>6 ± 2.3</td>
<td>7 ± 2.0</td>
<td>7 ± 2.0</td>
<td>5 ± 0.6</td>
</tr>
<tr>
<td>D.S.I. ^4</td>
<td>NS</td>
<td>NS</td>
<td>20 ± 1.0</td>
<td>22 ± 0.6</td>
<td>9 ± 0.6</td>
<td>11 ± 4.4</td>
<td>8 ± 2.0</td>
<td>5 ± 0.6</td>
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<tr>
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<td>R</td>
<td>R</td>
<td>D</td>
<td>D</td>
<td>S</td>
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</table>

^1 Lines are identified by number; NT is nontransformed

^2 L. L. = number of local lesions, necrotic lesions were counted beginning 4 days after inoculation.

^3 D.S.A. = days until local symptoms appearance.

^4 D.ted; plants were observed for one month. NS = no systemic infection observed

^5 R = resistant; S = susceptible; D = delayed systemic infection.
Fig. 1. Sequence of the scFv gene construct of TSWV-MAb 8C4D6 which recognizes the nucleoprotein of TSWV. The complete amino acid sequence of the scFv gene contains 256 amino acid residues. The linker sequence is underlined.
Fig. 2. Development of scFv gene construct for plant transformation. The scFv gene together with the double 35S promoter, AMV enhancer, and the NOS terminator were subcloned into the plant transformation vector, pBI121 at the HindIII and EcoRI sites replacing the GUS gene construct. Tobacco (*Nicotiana tabacum* ‘Turk’) leaf discs were transformed with *Agrobacterium tumefaciens* strain EHA105 containing pTSWVsFv.

Fig. 3. Reaction of *Nicotiana tabacum* ‘Turk’ plants after inoculation with TSWV-L. Plant 64 (left), transformed with the scFv construct, never developed symptoms on non-inoculated leaves whereas a nontransformed plant (right) developed systemic symptoms on the non-inoculated leaves.
Fig. 4. Southern (A) and northern (B) blot analyses. Genomic DNA from two resistant lines (#52 and #64) and one delayed symptom expression line (#61) contained a DNA fragment of about 1700-bp that hybridized with the scFv gene probe (A). This product was not detected from other lines or non-transformed plants. Northern blot analysis (B) revealed an 800-bp fragment present only in the resistant lines #52 and #64. Lanes 1 and 2 are line #52 (1 and 3μg purified mRNA, respectively); lanes 3 and 4 are line #64 (1 and 3μg purified mRNA, respectively).

Fig. 5. Expression of scFv in plants. Polyclonal antibodies against TSWV-MAb 8C4D6 were produced and the purified IgG and magnetizable cellulose/iron oxide particles were used to prepare affinity columns. ScFv proteins produced in transgenic plants were purified on these columns from total soluble proteins extracted from transformed tobacco leaves. Lines #52 and #64, which showed resistance to TSWV-L systemic infection in R0, expressed the highest level (about 12 pg/g leaf tissue) of scFv. The scFv was undetectable in all non-transformed plants.
Fig. 6. Specificity of scFv to TSWV. Plant-produced scFv antibodies were added to wells of microtiter plates coated with purified TSWV. The plant-produced TSWV scFv antibodies have the same antigen-binding specificity as TSWV monoclonal antibodies (TSWV-MAb) produced in mice. NT = non-transformed plant. TSWV-MAb = anti-TSWV murine monoclonal antibodies. Bars represent means of two experiments.