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Resistance to Tomato Spotted Wilt Virus Infection in Transgenic Tobacco Expressing the Viral Nucleocapsid Gene

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A recombinant plasmid containing the entire tomato spotted wilt virus (TSWV) nucleocapsid gene, with the exception of nucleotides encoding three N-terminal amino acids, was isolated by screening a complementary DNA library, prepared against random primed viral RNA, using a specific monoclonal antibody. The insert contained in plasmid pTSW1 was repaired and amplified by polymerase chain reaction, and the complete nucleocapsid protein gene was introduced into *Nicotiana tabacum* 'Samsun' by leaf disk transformation using *Agrobacterium tumefaciens*. Transgenic plants expressing the viral nucleocapsid protein were resistant to subsequent infection following mechanical inoculation with TSWV as indicated by a lack of systemic symptoms and little or no systemic accumulation of virus as determined by double antibody sandwich enzyme-linked immunosorbent assay. These results further extend the applicability of coat protein-mediated resistance, as previously demonstrated for a number of simple plant viruses composed of a positive-sense RNA genome encapsidated with a single species of coat protein, to a membrane-encapsidated, multi-component, negative-sense RNA virus.

Tomato spotted wilt virus (TSWV) was first described by Samuel *et al.* (1930) as the agent responsible for severe tomato crop losses in Australia. Since then, the virus has been reported from many other regions and is now considered to have worldwide distribution (Ie 1970), causing significant yield losses in a large number of economically important crops. TSWV has an extremely wide host range including at least 370 plant species in 50 botanical families (Cho *et al.* 1987) and is the only plant virus known to be transmitted by thrips (Sakimura 1962). Particles of TSWV are membrane-enveloped, roughly spherical in shape with a mean diameter of 85–100 nm, and are composed of at least three distinct structural proteins when analyzed by SDS-PAGE: an internal nucleocapsid protein (NP) of M_r 28 K and two membrane-associated glycoproteins of M_r 78 K (G1) and M_r 58 K (G2). In addition, small amounts of a high molecular weight protein (L), M_r ~200 K, have been reported (Mohamed *et al.* 1973; Tas *et al.* 1977).

The viral genome is composed of three linear ssRNA molecules designated S RNA (2.9 kb), M RNA (5.4 kb), and L RNA (8.2 kb). The complete nucleotide sequence of the S RNA has recently been determined and shown to contain two open reading frames (ORFs) arranged in a novel ambisense coding strategy (De Haan *et al.* 1990) very similar to that observed for the S RNAs of phlebo- and uukuviruses, two members of the Bunyaviridae family of arthropod-associated animal viruses (Bishop *et al.* 1980). One ORF, located on the viral complementary strand, has been shown to encode the 28-K NP. A second ORF, located on the viral strand, encodes a 52.4-K nonstructural protein (NS_s) of unknown function that has been detected by western immunoblotting and immunogold labeling in TSWV-infected tissue with specific antisera produced against the viral protein expressed using a recombinant baculovirus (Kormelink *et al.* 1991).

Whereas the specific coding properties of the other RNA molecules remain unknown, studies on transmission-defective strains of TSWV have provided evidence that the G1 membrane glycoprotein is encoded on the M RNA (Verkleij and Peters 1983). Partial nucleotide sequence information indicates that the M RNA is of negative polarity (De Haan *et al.* 1989) and that the N-terminal 80 amino acids of the predicted gene product of this segment show 25% similarity with the NSm protein encoded by the M segment of Rift Valley fever virus (phlebovirus genus; Collett *et al.* 1985). Taken together, these data suggest that TSWV should properly be considered a member of the Bunyaviridae within the new genus phytophlebovirus (De Haan *et al.* 1990).

Since the first report of the expression of bacterial genes in plants (Fraley *et al.* 1983), the process of genetic transformation and regeneration, as a mechanism for the expression of novel genes, has become routine for a number of plant species (Gasser and Fraley 1989). The first successful demonstration of genetically engineered resistance to a plant virus was reported by Powell-Abel *et al.* (1986), who found that transgenic tobacco plants expressing the coat protein gene from tobacco mosaic virus (TMV) showed a significant delay in symptom development after inoculation with TMV. This form of coat protein-mediated protection has now been demonstrated for a number of different viruses from at least nine different virus groups (reviewed by Beachy *et al.* 1990). Without exception, the studies described to date have dealt with simple plant viruses whose particles consist of a single species of structural protein encapsidating one or more molecules of positive-sense RNA.

In this report, we describe the production of transgenic tobacco plants that express the NP of TSWV and are re-

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sistant to infection following mechanical inoculation of TSWV.

MATERIALS AND METHODS

Preparation of cDNA and isolation of recombinant plasmids expressing portions of the TSWV NP gene. A local isolate of TSWV belonging to the "lettuce" serogroup was maintained in *Nicotiana tabacum* L. 'Samsun' and purified by sucrose density gradient centrifugation using the method of Black *et al.* (1963). Viral RNA was prepared from alkaline SDS-treated particles by multiple phenol-chloroform extractions and ethanol precipitation.

All recombinant DNA manipulations were performed essentially as described by Maniatis *et al.* (1982), unless otherwise indicated. Complementary DNA (cDNA) was synthesized from random primed total TSWV RNA using cloned murine Moloney leukemia virus reverse transcriptase (D'Alessio *et al.* 1987). The ds cDNA was rendered blunt-ended by treatment with T4 DNA polymerase, size-fractionated by chromatography over Sepharose CL-4B, and ligated into *Sma*I-digested, bacterial alkaline phosphatase-treated pUC13 (Pharmacia Inc., Piscataway, NJ). Following transformation of competent *Escherichia coli* DH5α cells to ampicillin resistance, bacterial colonies expressing an in-frame fusion product of the *lacZ* α peptide and portions of the TSWV NP protein were detected by immunolabeling (Mackenzie *et al.* 1989) using a specific monoclonal antibody (MAb), 2C5, and ^{125}I-labeled goat anti-mouse IgG.

Preparation of a Ti plasmid construct containing the TSWV NP gene and plant transformation. The insert contained in plasmid pTSW1, which included the entire NP protein gene with the exception of nucleotides encoding three N-terminal amino acids, was amplified by polymerase chain reaction (PCR) and ligated into an intermediate, co-integrate Ti plasmid vector, pCDX1. Vector pCDX1 (Kay *et al.* 1987) is a derivative of pMON178 and contains a duplicated cauliflower mosaic virus (CaMV) 35S promoter upstream from a multiple cloning site and the nopaline synthase polyadenylation signal. The specific primers utilized were:

T1: 5'-GTATCGATCATGTCTAAGGTTAAGCTCACTAAGGAA-3'
T2: 5'-GCTCTAGAGGTACCGTAAAACGACGGCCAGT-3'

Primer T1 contained a *Cla*I restriction endonuclease site plus nucleotides encoding the N-terminal amino acids, Met-Ser-Lys, in addition to 18 nucleotides complementary to the anti-coding strand of the 5' terminus of the pTSW1 insert DNA. Primer T2 was essentially the M13 universal primer with 5' terminal *Xba*I and *Kpn*I restriction endonuclease sites. Approximately 50 ng of pTSW1 plasmid DNA was subjected to 35 cycles of PCR amplification (2 min denaturation at 94° C, 2 min annealing at 55° C, 2 min polymerase extension at 72° C) using 20 pmol of each primer, 20 nmol of each dNTP, 2.0 mM $MgCl_2$, and 2.5 units of *Taq* polymerase in a 100-μl reaction (Perkin Elmer Cetus, Norwalk, CT). Amplified DNA was extracted sequentially with phenol and chloroform/isoamyl alcohol (24:1), precipitated with isopropyl alcohol, washed once with 75% ethanol, and resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.

For preparation of a plus-sense Ti construct (equivalent to the viral complementary strand), PCR-amplified DNA was double-digested with *Cla*I + *Kpn*I, and the gel-purified fragment was subsequently ligated into similarly digested pCDX1 plasmid. This construct was then introduced into the resident disarmed octopine-type plasmid, pTiB6S3SE, carried by strain GV3111SE of *Agrobacterium tumefaciens* (Smith and Townsend) Conn by homologous recombination using a triparental mating procedure, and the resulting clones were isolated by screening for resistance to spectinomycin, kanamycin, and chloramphenicol (Rogers *et al.* 1986).

Leaf disks of *N. tabacum* 'Samsun' were transformed using *A. tumefaciens* carrying the sense construct of the TSWV NP, and shoots were regenerated according to the method of Horsch *et al.* (1985) on medium consisting of Murashige and Skoog salts, sucrose (30 g/l), benzyladenine (1.0 μg/ml), naphthalene acetic acid (0.1 μg/ml), kanamycin (250 μg/ml), carbenicillin (250 μg/ml), and cefotaxime (250 μg/ml). Transformed shoots were subsequently rooted on phytohormone-free medium containing 100 μg/ml of kanamycin and were transferred to soil.

Enzyme-linked immunosorbent assay (ELISA). Samples of leaf tissue (100–150 mg) from kanamycin-resistant regenerated plants were initially screened for expression of TSWV NP using a double-antibody sandwich (DAS)-ELISA employing two different MAbs, 6C2 and 8H11, each specific for the 28-K NP. Tissue samples were processed through a mechanical leaf press (Pollahne, Wennigsen, Germany) irrigated with 0.4 ml of ELISA blocking buffer (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% bovine serum albumin, 0.05% Tween-20, 0.1% NaN_3) and incubated in microtitre plate wells that had previously been treated with 100 μl of purified MAb 6C2 IgG (10 μg/ml in 50 mM sodium carbonate, pH 9.6, 12–16 hr, 4° C). After rinsing, bound antigen was detected by incubation with alkaline phosphatase-conjugated MAb 8H11 IgG (200 ng/ml in blocking buffer; 90 min) followed by p-nitrophenyl phosphate substrate development (0.5 mg/ml in 10% diethanolamine, pH 9.8; 90 min). The absorbance of each well was measured at 405 nm using a Dynatech MR5000 (Dynatech Laboratories, Chantilly, VA) multiwell plate reader interfaced with an IBM PC/AT microcomputer. Transgenic plantlets that had been transferred to soil and that tested positive by ELISA were subsequently screened by western immunoblotting using MAb 2C5 (Fig. 1B) and ^{125}I-goat anti-mouse IgG.

For the detection of TSWV infection in inoculated plants, a DAS-ELISA procedure employing a polyclonal rabbit anti-TSWV IgG (1:5,000 in 50 mM sodium carbonate, pH 9.6) for trapping and alkaline phosphatase-conjugated MAb 6F3 IgG (200 ng/ml in blocking buffer), specific for the viral membrane-associated G1 glycoprotein (Fig. 1C), was employed. This technique allowed for the specific detection of TSWV without interference from high levels of endogenously expressed viral NP in transgenic plants.

Western immunoblot analysis of TSWV NP expression. The ability of transgenic tobacco to produce TSWV NP was assessed by western immunoblotting using a specific

monoclonal antibody. Samples of leaf tissue (100 mg) were homogenized with 100 μl of SDS-PAGE sample cocktail (4% SDS, 125 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 0.04% bromophenol blue, 20% glycerol) and incubated at 95° C for 5 min. After centrifugation (13,000 × *g*, 5 min), 10-μl aliquots were loaded onto a 12% polyacrylamide gel and after electrophoresis using the buffer system of Laemmli (1970), electroblotted onto PVDF membrane (Bio-Rad Laboratories, Richmond, CA). Immunoblots were labeled sequentially with MAb 2C5 hybridoma culture supernatant (diluted 1/20 in blocking buffer) and ^{125}I-labeled goat anti-mouse IgG, using the protocol previously described (MacKenzie *et al.* 1991).

Virus inoculation of transgenic and nontransformed tobacco plants. A number of independent transgenic lines that expressed significant quantities of viral NP were used to evaluate resistance to TSWV infection. Groups of R1 progeny plants (three- to five-leaf stage), which had been screened for TSWV NP expression by DAS-ELISA, together with similar numbers of nontransformed tobacco plants, were dusted with Carborundum and inoculated with two different concentrations (1:10, 1:20) of a TSWV-infected tissue homogenate. For preparation of the 1:10 homogenate, approximately 2 g of systemically infected leaf tissue, obtained from plants inoculated 2–3 wk previously, was homogenized with 20 ml of 10 mM sodium sulfite using a polytron (Brinkmann Instruments, Westbury, NY; setting 6) and expressed through cheesecloth. After inoculation, the extent of virus infection was monitored both by DAS-ELISA and observation of visual symptoms.

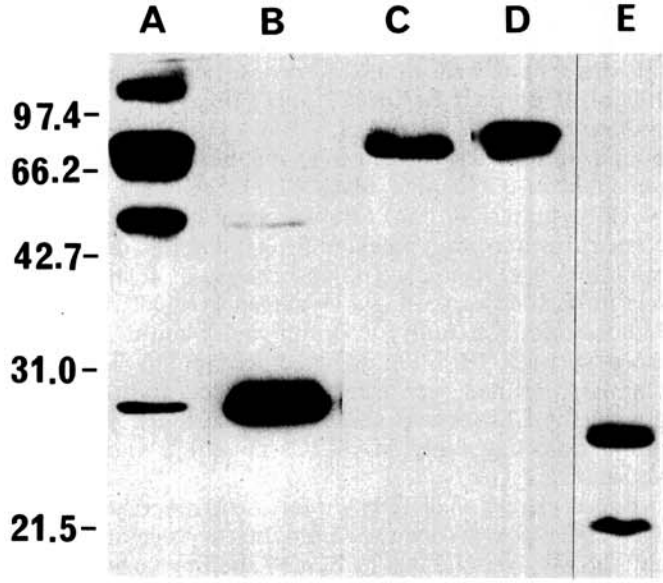

Fig. 1. Western blot analysis of tomato spotted wilt virus (TSWV) structural proteins using rabbit polyclonal or murine monoclonal antibodies (MAb). Samples of purified TSWV (10 μg of protein) were subjected to SDS-PAGE, and immunoblots were labeled with either rabbit anti-TSWV IgG and ^{125}I-protein A (**A**) or two different MAbs, 2C5 (**B**) and 6F3 (**C**), followed by ^{125}I-goat anti-mouse IgG. Labeling of the 78-K membrane-associated glycoprotein (G1) with ^{125}I-concanavalin A is shown in **D**. A similar blot of SDS-soluble proteins from an *Escherichia coli* cell lysate of clone pTSW1 labeled with MAb 2C5 and ^{125}I-goat anti-mouse IgG is shown in **E**. M_r standards (× 10^{-3}) are indicated.

RESULTS

Cloning of the TSWV NP gene. One recombinant plasmid, pTSW1, containing a near full length copy of the TSWV NP gene was isolated by colony screening with MAb 2C5. This MAb had previously been shown, by western immunoblot labeling, to be specific for the NP of the "lettuce" serogroup of TSWV (Fig. 1B). Likewise MAb 6F3 was found to be specific for the viral membrane-associated G1 glycoprotein (Fig. 1C) that could also be labeled with ^{125}I-concanavalin A (Fig. 1D).

A similar blot of total SDS-soluble proteins from an *E. coli* cell lysate of clone pTSW1 showed a major M_r 26,500 band and lesser amounts of an M_r 22,000 polypeptide after labeling with MAb 2C5 (Fig. 1E). The apparent molecular weight of the largest species was significantly less than would be predicted based on nucleotide sequence

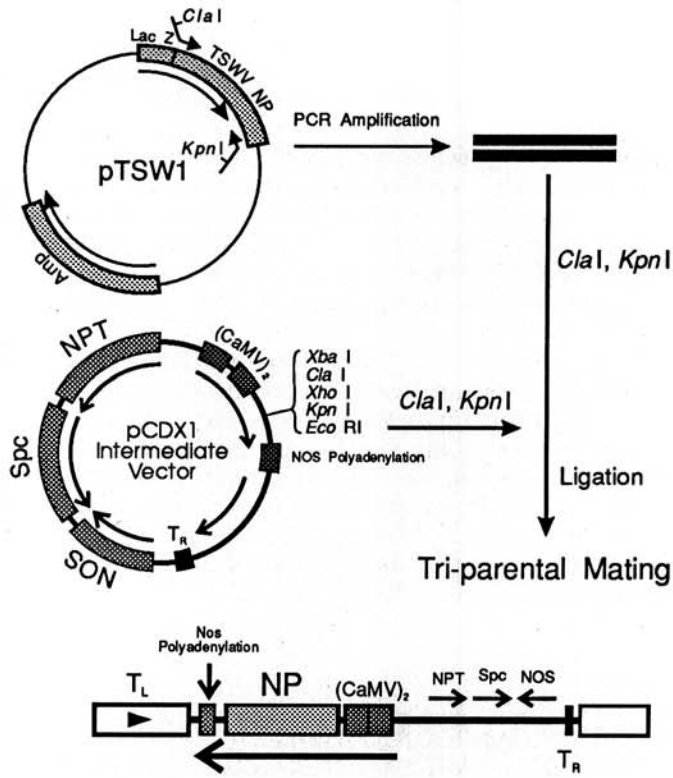

Fig. 2. Construction of a Ti plasmid vector for expression of the tomato spotted wilt virus (TSWV) nucleocapsid protein gene in transformed plants. The insert contained in plasmid pTSW1, which contained the entire nucleocapsid gene with the exception of nucleotides encoding three N-terminal amino acids, was amplified by polymerase chain reaction using primers that included the missing nine nucleotides from the 5′ portion of the gene, as well as compatible restriction endonuclease sites. Amplified DNA was then digested with *Cla*I and *Kpn*I and ligated into the intermediate co-integrate Ti plasmid vector, pCDX1. This construct was then mobilized into *Agrobacterium tumefaciens* strain GV3111SE, containing the disarmed helper Ti plasmid pTIB6S3SE by triparental mating, and clones that had undergone homologous recombination were isolated by screening for resistance to kanamycin, spectinomycin, and chloramphenicol. The left and right T-DNA border sequences are represented by T_L and T_R respectively, and genes for nopaline synthase (NOS), spectinomycin resistance (Spc), and neomycin phosphotransferase (NPT) are indicated. The arrow below indicates the direction of transcription of the TSWV nucleocapsid protein (NP) gene under the control of the duplicated cauliflower mosaic virus $(CaMV)_2$ 35S promoter.

information (below) and may reflect proteolytic degradation, which is known to be a problem with heterologous protein expression in *E. coli*.

Nucleotide sequence information was obtained for 200 nucleotides from both the 5′ and 3′ terminal portions of the pTSW1 insert. Comparison with the previously published sequence of the TSWV S RNA (De Haan *et al.* 1990) revealed a >95% identity in these regions and confirmed that this plasmid contained an entire copy of the 28-K protein gene (nucleotide positions 1,888–2,754 numbered from the 5′ end of the S RNA), with the exception of nucleotides encoding three amino acids from the N-terminus.

Expression of TSWV NP in transgenic plants. The TSWV nucleocapsid cistron contained in plasmid pTSW1 was amplified by PCR using specific primers, one of which contained additional nucleotides encoding the three N-terminal amino acids absent in pTSW1, and introduced into the intermediate, co-integrate Ti plasmid vector pCDX1 (Kay *et al.* 1987) (Fig. 2). After transformation and shoot regeneration in the presence of kanamycin, transgenic plants were screened for TSWV NP expression. Approximately 65% of the regenerated R0 plants expressed quantities of viral NP that were detectable by DAS-ELISA, in some cases at sap dilutions of 1/160 or more (Fig. 3).

DAS-ELISA analysis of the R1 progeny plants from five independent transgenic lines (TSW209-13, -21, -25, -27, and -42) revealed that 72% ($x^2 = 0.48$), 68% ($x^2 = 2.61$), 67% ($x^2 = 3.41$), 71% ($x^2 = 0.85$), and 69% ($x^2 = 1.92$), respectively, expressed detectable levels of TSWV NP. These data are consistent with a 3:1 segregation ratio ($x^2_{0.95(1)} = 3.84$) and indicate that the parent R0 plants contained a single expressed copy of the gene.

Expression of TSWV NP in transgenic leaf tissues was also confirmed by western immunoblot labeling. Protein extracts from five different transgenic lines (Fig. 4B,C, D,E,F) as well as nontransformed tobacco (Fig. 4A) were separated by SDS-PAGE, and blots were probed with MAb 2C5 and ^{125}I-labeled goat anti-mouse IgG.

Susceptibility of transgenic tobacco to infection with TSWV. Transgenic tobacco plants expressing the viral NP were challenged by mechanical inoculation with a homogenate prepared from TSWV-infected tissue. Virus replication and spread were monitored in these plants by sampling disks from the upper uninoculated leaves of each plant at various times after inoculation and quantitating viral antigen levels by DAS-ELISA.

Following inoculation of nontransformed tobacco with a 1:20 homogenate of TSWV-infected tissue, measurable quantities of virus could readily be detected in the upper uninoculated leaves after 10 days, and virus titre continued to increase approximately 20-fold to a maximum level at about 3 wk after inoculation (Fig. 5). Systemically infected plants also displayed very pronounced visual symptoms including severe stunting, leaf-curling, and deformation with mosaic and necrotic patches (Fig. 6A, right; C).

The extent of resistance to TSWV infection among various transgenic lines was variable. Lines TSW209-13, -21, and -27 were highly resistant and showed little or no accumulation of virus in the upper uninoculated leaves by 36 days after inoculation with a 1:20 homogenate of TSWV-infected tissue (Fig. 5, TSW209-13 and -21) and were free of any obvious symptoms (Fig. 6A, left; B). In contrast, lines TSW209-25 and -42, which expressed even greater quantities of viral NP, showed a much reduced degree of resistance to TSWV infection. Virus accumulation in the

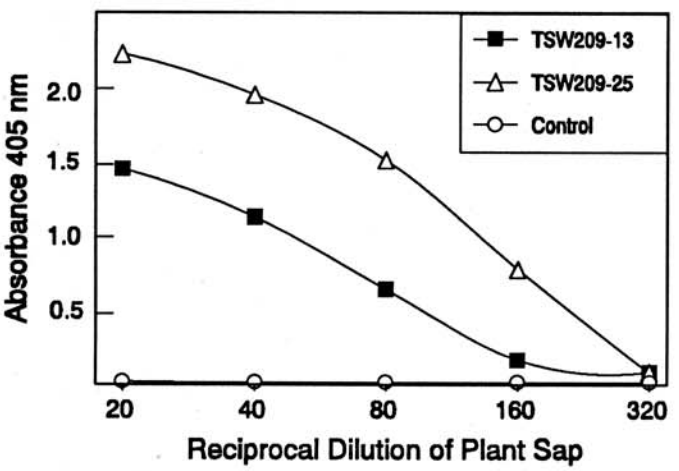

Fig. 3. Detection of heterologously expressed tomato spotted wilt virus (TSWV) nucleocapsid protein in individual transgenic plants by double antibody sandwich (DAS)-ELISA employing two specific monoclonal antibodies (MAbs). Microtitre plate wells, previously coated with MAb 6C2 (10 μg/ml), were incubated with 100 μl vol of serially diluted leaf tissue sap from transgenic lines TSW209-13 (■) and TSW209-25, (△) and a nontransformed control tobacco, (○). Bound antigen was detected by incubation with alkaline phosphatase-conjugated MAb 8H11 (200 ng/ml) and p-nitrophenyl phosphate substrate development.

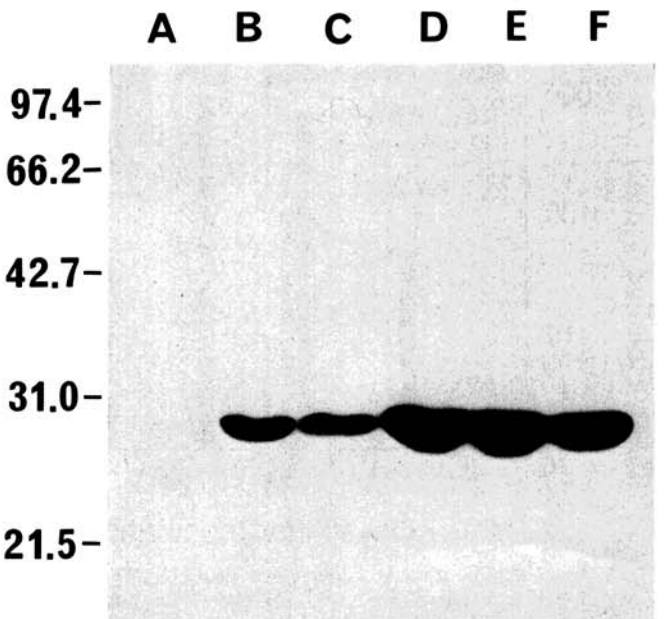

Fig. 4. Western blot analysis of tomato spotted wilt virus (TSWV) nucleocapsid protein expression in five independent transgenic tobacco lines. Total SDS-soluble protein extracts (8–10 μg) of leaf tissue from nontransformed control tobacco (**A**) or transgenic tobacco lines TSW209-13, -21, -27, -25, and -42 (**B, C, D, E,** and **F**, respectively) were subjected to SDS-PAGE. Immunoblots were labeled with monoclonal antibody 2C5, specific for the 28-K nucleocapsid protein, followed by ^{125}I-goat anti-mouse IgG and overnight autoradiography. M_r standards ($\times 10^{-3}$) are indicated.

upper leaves of these latter plants was initially delayed by 6–7 days, but did eventually rise to similar levels as those observed in nontransformed control plants by 3–4 wk following inoculation (Fig. 5, TSW209-25). When challenged with a higher level of inoculum (1:10), approximately 61% of the TSW209-25 and 87% of the TSW209-42 plants displayed obvious symptoms and tested positive for TSWV by DAS-ELISA by 3 wk following inoculation, as compared to 8, 11, and 12%, respectively, for lines TSW209-13, -21, and -27 (Table 1).

DISCUSSION

We have successfully demonstrated that heterologous expression of TSWV NP in transgenic tobacco plants can confer significant resistance to virus infection. Five independent transgenic lines that expressed readily detectable levels of NP, as determined by western immunoblotting and DAS-ELISA, were evaluated for resistance to TSWV infection following mechanical inoculation. Three of these lines, TSW209-13, -21, and -27, showed high levels of resistance, whereas transgenic plants from lines TSW209-25 and -42 displayed a much lower degree of resistance. Whereas previous studies have generally indicated that the extent of coat protein-mediated resistance is proportional to the level of coat protein expression (Loesch-Fries *et al.* 1987; Powell *et al.* 1990), the results presented here together with previous data obtained by using transgenic potato that expresses the potato virus Y coat protein (Lawson *et al.* 1990) and transgenic tobacco that expresses the coat protein gene from strain C of cucumber mosaic virus (Quemada *et al.* 1991) suggest that this is not always the case. It is likely that factors other than coat protein accumulation, such as tissue or cell type specific expression, or subcellular localization, may be important determinants of resistance.

Plants from lines TSW209-25, -27, and -42 that tested negative for TSWV at 3 wk following inoculation were essentially symptomless and identical in appearance with TSW209-13 plants (i.e., Fig. 6A, left; B). By comparison, TSW209-25 plants that tested positive eventually developed the same severity of symptoms as shown by nontransformed control plants infected with TSWV. Transgenic plants that became infected after TSWV inoculation did not appear to develop milder or attenuated symptoms compared to nontransgenic controls.

Previous examples of engineered coat protein-mediated resistance (reviewed by Beachy *et al.* 1990) have involved viruses with positive-sense RNA genomes encapsidated by a single species of capsid protein. It is significant that a similar strategy can also be employed effectively with a membrane-enveloped, multicomponent, negative, and ambisense RNA genome virus such as TSWV. While the underlying mechanism of coat protein-mediated resistance is not fully understood, it has been likened to the phenomenon of classical cross-protection (Fulton 1986) in which it is believed that the presence of coat protein from the

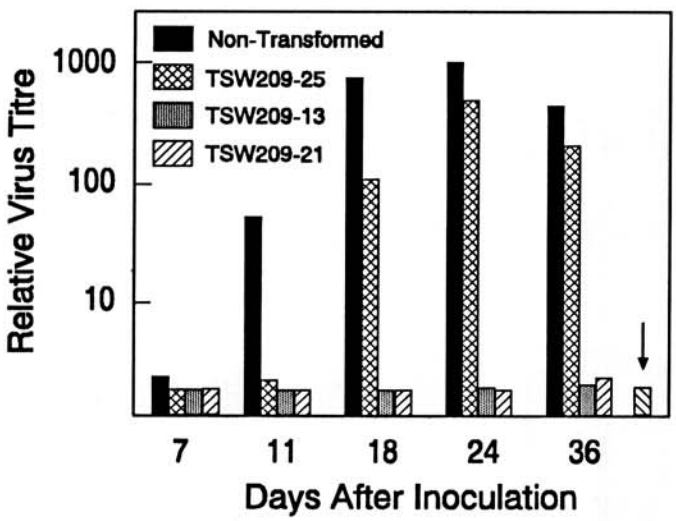

Fig. 5. Accumulation of tomato spotted wilt virus (TSWV) in the upper leaves of transgenic lines (TSW209-13, -21, and -25) or nontransformed control tobacco at various times after inoculation with a 1:20 homogenate of TSWV-infected tissue. Duplicate samples of leaf tissue (150 mg) were obtained from the upper uninoculated leaves, homogenized with 0.5 ml of buffer, and serial twofold dilutions were assayed for the presence of TSWV by DAS-ELISA as described in Materials and Methods, using alkaline phosphatase-conjugated monoclonal antibody 6F3, specific for the viral membrane-associated G1 glycoprotein. The relative virus titre was defined as the equivalent reciprocal sap dilution giving half-maximal response. The bar designated by the arrow shows the background value obtained from uninoculated control plants.

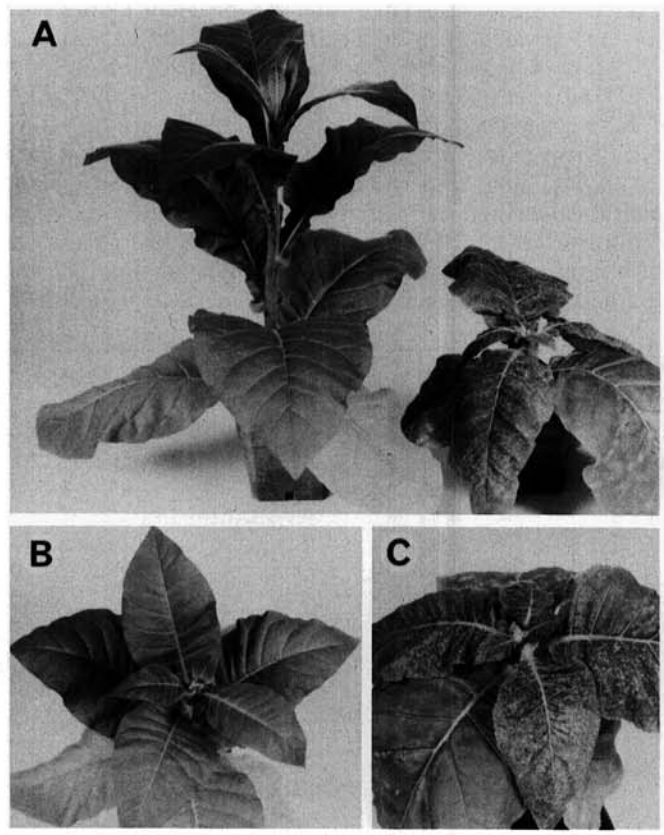

Fig. 6. Comparison of symptom development in transgenic TSW209-13 (**A**, left) or nontransformed control tobacco (**A**, right) following inoculation with tomato spotted wilt virus (TSWV). Plants were mechanically inoculated with a 1:20 homogenate prepared from the upper leaves of a TSWV-infected plant. By 3 wk after inoculation, control plants were severely stunted (**A**, right; **C**) and showed the classic symptoms of systemic TSWV infection (mosaic, leaf distortion, and necrotic patches), whereas transgenic plants (**A**, left; **B**) were free of obvious symptoms.

first virus acts to inhibit the uncoating of the second challenge virus or that it encapsidates the RNA of the challenging strain, thereby preventing its replication. In this regard, it is interesting to note that conventional cross-protection has also been described for TSWV by Best (1954), who found that plants inoculated with a mild strain of the virus were protected against subsequent systemic infection by a severe strain.

Studies with other viruses have suggested that coat protein-mediated resistance involves inhibition of virus uncoating (Osbourn *et al.* 1989), and in some cases there may also be at least partial re-coating of infectious viral RNA (Hemenway *et al.* 1988; MacKenzie and Tremaine 1990). Additionally, resistance may involve inhibition of long-distance transport of the viral infectious agent through transgenic phloem tissues (Wisniewski *et al.* 1990; MacKenzie *et al.* 1991).

Whereas the mechanisms involved in TSWV replication and transport throughout the infected plant are not well understood, its similarity to members of the Bunyaviridae may allow these latter viruses to serve as useful model systems for TSWV replication. The ambisense gene arrangement of TSWV S RNA suggests that the two ORFs are translated from separate subgenomic mRNAs in a manner similar to that of the ambisense S RNA of Punta Toro phlebovirus (Ihara *et al.* 1985). In the latter case, it is believed that genomic S RNA serves as a template for the production of nucleocapsid mRNA as well as full-length viral complementary (antigenome) RNA, which in turn acts as a template for both the NS_s mRNA and for new genome synthesis. The presence, in TSWV-infected tissue, of two subgenomic mRNA species derived from the S RNA, a 1.7-kb fragment encoding the NS_s protein, and a 1.2-kb fragment encoding the NP support this expression strategy (De Haan *et al.* 1990). Encapsidation of anti-genome or subgenomic RNA by endogenously expressed NP in transgenic plants could result in inhibition of TSWV replication.

To determine which, if any, of these possible mechanisms are responsible for the resistance observed in transgenic plants expressing TSWV NP, further elucidation of the mechanism(s) of TSWV replication are required.

Table 1. Susceptibility of transgenic and nontransformed control plants to tomato spotted wilt virus (TSWV) infection

	Fraction of plants systemically infected 21 days after inoculation[a]	
Plant line	**1:10 Inoculum[b]**	**1:20 Inoculum**
Nontransformed control	13/13 (100%)	15/15 (100%)[c]
TSW209-13	1/12 (8.3%)	0/12 (0%)[d]
TSW209-21	3/28 (10.7%)	0/32 (0%)
TSW209-25	8/13 (61.5%)	4/10 (40%)
TSW209-27	2/16 (12.5%)	1/16 (6.3%)
TSW209-42	14/16 (87.5%)	9/16 (56.3%)

[a]Duplicate samples (150 mg) of leaf tissue obtained from the upper uninoculated leaves of each plant were homogenized with 0.5 ml of ELISA blocking buffer and assayed for the presence of TSWV by DAS-ELISA, using a polyclonal antiserum for trapping and alkaline phosphatase-conjugated MAb 6F3, specific for the G1 viral membrane glycoprotein, for detection. In all cases, a positive ELISA result could be correlated with the appearance of visual symptoms.

[b]Nontransformed control plants or transgenic R1 progeny plants (three- to five-leaf stage) that had previously been screened for nucleocapsid protein expression were mechanically inoculated with either a 1:10 (1 g of tissue per 10 ml) or 1:20 homogenate of TSWV-infected tissue prepared in 10 mM sodium sulfite.

[c]Average DAS-ELISA result for positively infected plants was $A_{405nm} = 2.15 \pm 0.39$. The cut-off value for a positive DAS-ELISA result was three times the mean of a healthy control sample.

[d]Average DAS-ELISA result was $A_{405nm} = 0.03 \pm 0.02$.

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