Disease Resistance in Tobacco and Tomato Plants Transformed with the Tomato Spotted Wilt Virus Nucleocapsid Gene

J. W. KIM, Postdoctoral Research Associate, and S. S. M. SUN, Professor, Department of Plant Molecular Physiology, University of Hawaii, Honolulu 96822; and T. L. GERMAN, Department of Plant Pathology, University of Wisconsin, Madison 53706

ABSTRACT

Kim, J. W., Sun, S. S. M., and German, T. L. 1994. Disease resistance in tobacco and tomato plants transformed with the tomato spotted wilt virus nucleocapsid gene. Plant Dis. 78:615-621.

The nucleocapsid protein (N) gene was cloned from the tomato spotted wilt virus (TSWV) Hawaiian L isolate. The 777-nucleotide N gene had 97 and 99% homology with a tomato (CNPH1) and two lettuce (L3 and BL) isolates, respectively. Leaf disks of *Nicotiana tabacum* cv. Xanthi nc and cotyledons of *Lycopersicon esculentum* cv. VF36 were transformed with *Agrobacterium tumefaciens* harboring a binary vector with a chimeric TSWV N gene cassette. Significantly lower numbers of local lesions and several days delay of symptom development were observed when R_1 transgenic tobacco plants expressing the TSWV N gene were challenged with TSWV. The R_1 transgenic tobacco plants transformed to produce an antisense RNA were as resistant as plants producing sense RNA. Only 50–80% of the transgenic tomato plants became infected after mechanical inoculation with TSWV in the greenhouse. These results demonstrate that expression of the sense or antisense RNA of the TSWV N gene can be used in both tobacco and tomato plants to achieve genetically engineered resistance.

Tomato spotted wilt virus (TSWV) causes important plant diseases in tropical, subtropical, and temperate regions throughout the world which are virtually impossible to control by conventional methods. The host range of TSWV encompasses 500 species in more than 70 plant families that include important ornamental, fruit, and vegetable crops. In commercial vegetable crops, losses can be extremely large due to an infection incidence of 50–90% (1,4–6,18,19,21,25, 36,38).

Accepted for publication 19 February 1994.

TSWV, the type member of the Tospovirus genus in the family Bunyaviridae (18), has 80- to 110-nm, spherical particles bound by a membrane acquired from the host during maturation. Virions are composed of four proteins (ca. 300, 78, 58, and 29 kDa): a putative polymerase (L), two glycosylated membrane proteins (G1 and G2), and the nucleocapsid protein (N), respectively (31,37). The genome consists of three single-stranded RNA molecules of 2.9 kb (S RNA), 4.9 kb (M RNA), and 8.9 kb (L RNA).

The genome organization and gene expression strategy have been established based on the recently published nucleotide sequences of genomic RNAs of the Brazilian CNPH1 isolate (10,11,15,26). The L RNA has a large open-reading frame (ORF) in the viral complementary sense encoding the putative 331.5 kDa polymerase protein (L) that is translated from a subgenomic RNA (10). The M RNA contains two ORFs, one in the viral sense with coding capacity for a 33.6 kDa protein (NSm) and the other in the viral complementary sense that encodes a 124.7 kDa precursor for G1 and G2 (26). The S RNA has one ORF encoding a 52.4 kDa protein (NSs) in the viral sense and another encoding a 28.8 kDa nucleocapsid protein (N) in the viral complementary sense, and both proteins are expressed by means of subgenomic RNAs (11).

Coat protein-mediated protection (CP-MP) has been reported for a number of different positive-strand viruses (2,34). Since it was first demonstrated with tobacco mosaic virus in tobacco (*Nicotiana tabacum* L.), CP-MP has also been demonstrated for TSWV in tobacco, showing that CP-MP can be used for negative-stranded viruses (20,29,33). Although no universal mechanism has been accepted for CP-MP, data derived from work with both positive- and nega-

tive-sense viral systems indicates that the resistance of transgenic plants may be due to expression of RNA rather than the accumulation of coat protein (9,16, 28,39).

We cloned the N gene from the Hawaiian L isolate of TSWV and expressed it as a chimeric nuclear gene in tomato (*Lycopersicon esculentum* Mill.), a systemic host to TSWV, and *N. tabacum* cv. Xanthi nc, a local lesion host to TSWV, to develop agronomically useful resistance and to study the mechanism of resistance to TSWV. Plants expressing the N gene show delayed disease development and reduced viral accumulation.

MATERIALS AND METHODS

Purification of virus and viral RNA. The TSWV Hawaiian L isolate, originally obtained from lettuce, was maintained in *Emilia sonchifolia* (L.) DC. ex Wight and propagated in *Datura stramonium* L. by mechanical inoculation. Eight days after inoculation, TSWV was purified based on the method of Gonsalves and Trujillo (21). The viral genomic RNA was extracted from purified virus preparations by the method of Chomczynski and Sacchi (7).

N gene cloning and sequencing. Two cDNA libraries were generated by a random priming method or by using a synthetic oligonucleotide (5′-ACAACTT TTACGTCATCATG-3′) synthesized based on the sequence of TSWV-CNPH1 S RNA (nt 2,761–2,781) (11). Double-stranded cDNA was synthesized from the purified viral RNA as described by Gubler and Hoffman (22). The blunt-ended cDNA was ligated with *Pst*I linkers and inserted into the *Pst*I site of pBluescript/KS+ (Stratagene, La Jolla, CA). Following transformation of *Escherichia coli* DH5α, colony hybridizations were carried out using the synthetic oligonucleotide as a probe. DNA was isolated from selected clones (35) and sequenced using Sequenase (USB, Cleveland, OH) following the protocols recommended by the manufacturer. The nucleic acid sequence data of both DNA strands were determined using the Genetics Computer Group program of The University of Wisconsin (12).

Construction of plant transformation vectors. The N gene sequence was isolated from the clone pBS-NC4.5 that contained a 1.5-kb insertion. The N gene sequence was excised by *Pst*I and *Pvu*II double digestion and blunt-ended by mung bean nuclease. Plant expression plasmids were derived from the pBI221 expression vector (Clontech, Palo Alto, CA) by replacing the *Sma*I-*Sst*I DNA fragment corresponding to the GUS sequence with the N gene sequence. The *Sst*I site was made blunt-ended with mung bean nuclease, and the vector and the insert sequences were religated. Following transformation of *E. coli* DH5α, both sense and antisense plasmids were selected as pBI221-N46 and pBI221-N112, respectively, based on the direction of N gene sequence with respect to the direction of transcription. The DNA fragment 35S-N-NOS was excised from the expression plasmids by *Eco*RI and *Hin*dIII double digestion. The *Eco*RI site was blunt-ended, followed by *Hin*dIII linker addition. The *Hin*dIII fragment containing the 35S-N-NOS chimeric gene was then ligated into the *Hin*dIII site of the pBI121 plant transformation vector that contains an NPTII kanamycin resistance gene and a GUS reporter gene. This plant transformation vector (pBI121-N46 and pBI121-N112) was transferred from *E. coli* DH5α to *Agrobacterium tumefaciens* LBA4404 by triparental mating (13).

***Agrobacterium*-mediated transformation and regeneration of tobacco.** Leaf disks of *N. tabacum* cv. Xanthi nc were transformed with *A. tumefaciens* carrying sense and antisense constructs of the TSWV N gene; and shoots were regenerated by the method of Horsch et al (23) on a medium consisting of Murashige and Skoog salts and vitamins (32), 3% sucrose, 6-benzyl-aminopurine (1.0 μg/ml), α-naphthaleneacetic acid (0.1 μg/ml), carbenicillin (500 μg/ml), kanamycin (300 μg/ml), and 0.2% phytogel. The transformed shoots were transferred to a hormone-free medium with carbenicillin (500 μg/ml) and kanamycin (100 μg/ml) for rooting, and subsequently transferred to soil.

***Agrobacterium*-mediated transformation and regeneration of tomato.** Cotyledons of *L. esculentum* cv. VF36 were cocultivated with *A. tumefaciens* carrying sense and antisense constructs of the TSWV N gene in the presence of tobacco feeder layer cells based on the method of Fillatti et al (17). Cotyledons were then transferred to 2Z regeneration medium containing Murashige and Skoog salts, Nitsch vitamins, 3% sucrose, zeatin (2 μg/ml), carbenicillin or ampicillin (500 μg/ml), kanamycin (50 μg/ml), and 0.2% phytogel. The shoots were transferred to a hormone-free medium with carbenicillin or ampicillin (500 μg/ml) and kanamycin (50 μg/ml), then transferred to soil.

Southern and Northern analysis. Genomic DNA was isolated from tobacco and tomato leaves as described by Doyle and Doyle (14). The purified DNA (10 μg) was digested with *Hin*dIII, separated by gel electrophoresis, blotted onto Hybond N+ membrane (Amersham, Arlington Heights, IL), and hybridized to a ^{32}P-labeled N gene probe from pBS-NC4.5 following the protocols of the manufacturer.

Total RNA was isolated from tobacco and tomato leaves using the method of Verwoerd et al (40). The purified total RNA (10 μg) was separated by formaldehyde gel electrophoresis, blotted, and hybridized to an N gene probe as described above.

Protein analysis. A leaf disk was collected from individual transformed plants, and double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was performed initially to screen for expression of the N gene using polyclonal rabbit IgG against TSWV. Subsequently, ELISA-positive plants were used for Western immunoblot analysis. Leaf tissues were homogenized with sodium docecyl sulfate-polyacryla-

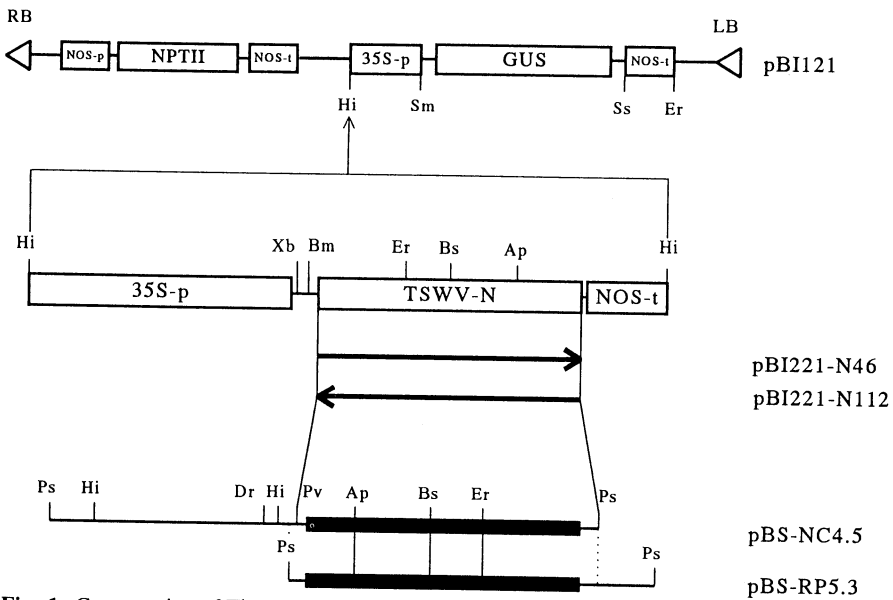


Fig. 1. Construction of Ti plasmid vectors that express TSWV N gene in plants. Steps involved in the construction of these vectors are described in Materials and Methods. RB and LB, right and left border of T-DNA; NOS-p, nopaline synthase promoter; NOS-t, nopaline synthase terminator; NPTII, neomycin phosphotransferase II; 35S-p, cauliflower mosaic virus 35S RNA promoter; GUS, β-glucuronidase; TSWV-N, tomato spotted wilt virus nucleocapsid protein gene. The restriction enzymes are Hi, *Hin*dIII; Sm, *Sma*I; Ss, *Sst*I; Er, *Eco*RI; Xb, *Xba*I; Bm, *Bam*HI; Bs, *Bst*XI; Ap, *Apa*I; Ps, *Pst*I; Dr, *Dra*I; Pv, *Pvu*II.

mide gel electrophoresis (SDS-PAGE) sample buffer, and 50–100 μg of total soluble proteins were separated by 12.5% SDS-PAGE (27) and electroblotted onto nitrocellulose membranes. The immunoblots were reacted sequentially with TSWV IgG and either radiolabeled ^{125}I-protein A (3) or nonradiolabeled protein A (ECL method of Amersham).

Virus inoculation of transgenic plants. Self-pollinated R_1 progeny plants from 10 transgenic tobacco lines and four transgenic tomato lines were selected to test the segregation of the N gene using DAS-ELISA, and subsequently were used for TSWV inoculation 4–6 wk after sowing. Inocula were prepared by grinding systemically infected *E. sonchifolia* leaves in phosphate buffer (0.033 M KH_2PO_4, 0.067 M Na_2HPO_4) supplemented with 10 mM sodium sulfite. All inocula were made fresh and kept on ice. Eight to 12 tobacco plants (6–10 fully expanded leaves per plant) were dusted with Carborundum and inoculated with inocula diluted 1:20. The local lesion development was observed for 9 days. Forty to 50 tomato plants were inoculated three times with 1:10 inocula on large leaves during a 2-wk period. Systemic symptom development was observed for 2 mo.

RESULTS

Cloning of TSWV N gene and construction of expression vectors. cDNA synthesis from the total viral genomic RNAs of a TSWV Hawaiian L isolate was carried out using two different primers. Using an oligonucleotide that was synthesized based on the S RNA sequence of the CNPH1 isolate (nt 2,761–2,781), we obtained clone pBS-NC4.5 with a 1.5-kbp inserted. Using the random primed method, we obtained clone pBS-RP5.3 with a 0.9-kbp inserted (Fig. 1). Sequence analysis of these clones revealed that pBS-NC4.5 covers from 1,325 nt to 2,778 nt, and pBS-RP5.3 covers from 1,956 nt to 2,878 nt with respect to the S RNA sequence of the CNPH1 isolate. Clone pBS-NC4.5 includes 160 bases of the NSs ORF, the complete sequence of the intergenic region (502 bases), the N gene ORF, and 15 bases at the 3′ end. Clone pBS-RP5.3 contains the complete N gene ORF with 31 bases at the 5′ end and 95 bases at the 3′ end. The two clones completely overlap with respect to the N gene, yielding a 777-bp ORF that encodes the 29K nucleocapsid (N) protein. To confirm that the clones contained an ORF encoding the TSWV N protein, in vitro transcripts were produced using T7 polymerase after the clones were restricted with *Eco*RV. These transcripts were translated in vitro in a wheat germ system. The results of these experiments demonstrated that the 29K protein from these reactions co-migrated with TSWV N protein during electrophoresis and was

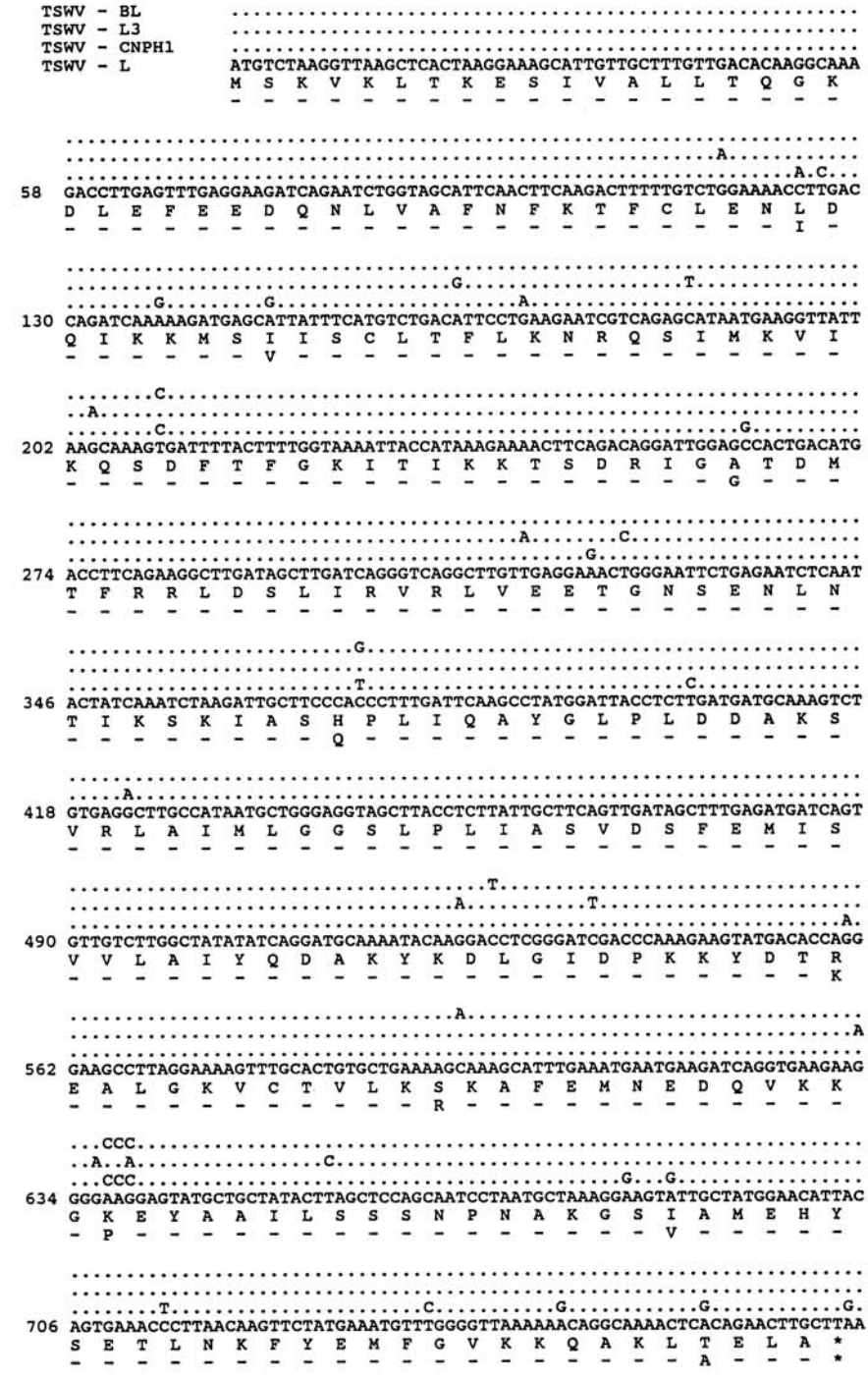


Fig. 2. Comparison of the nucleotide and deduced amino acid sequences of the TSWV N gene of TSWV-L (Hawaiian), CNPH1 (Brazilian), L3 (Bulgarian), and BL isolates. The complete nucleotide and amino acid sequences of the Hawaiian L isolate are shown. Nucleotide and amino acid changes in the other isolates are indicated above and below the corresponding Hawaiian sequences, respectively. Dots indicate identical nucleotides, and a star, termination codons.

Table 1. Numbers of nucleotide (nt) sequence changes and amino acid (aa) changes among TSWV Hawaiian L, Brazilian CNPH1, Bulgarian L3, and Batavia BL isolates

TSWV	L	CNPH1	L3	BL
L	...	22 nt	12 nt	7 nt
CNPH1	7 aa	...	13 nt	21 nt
L3	0 aa	7 aa	...	18 nt
BL	3 aa	8 aa	3 aa	...

selectively immunoprecipitated with antiserum to TSWV (*data not shown*).

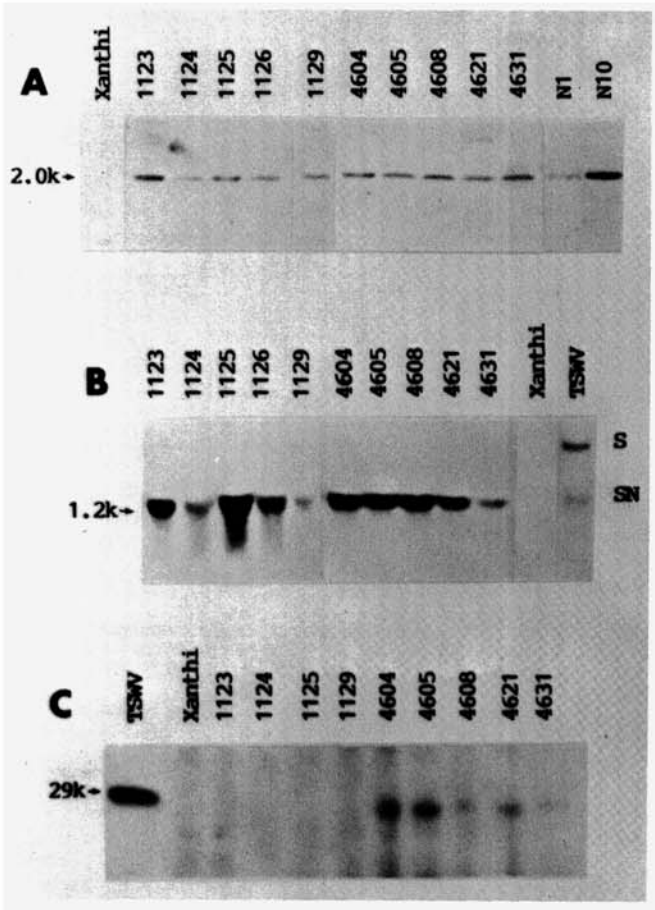
Clone pBS-NC4.5 was chosen for isolation of the N gene. The DNA fragment excised by *Pst*I and *Pvu*II restriction enzymes was inserted into the expression cassette vector, pBI221, to create a chimeric gene containing the 35S promoter from cauliflower mosaic virus (CaMV) and a polyadenylation signal sequence from the nopaline synthase gene (NOS). Both sense and antisense plasmids were selected based on the direction of the N gene with respect to the direction of transcription. The sense plasmid was denoted as pBI221-N46 and the antisense plasmid as pBI221-N112. Ultimately, the 35S-N-NOS chimeric construct excised with *Hin*dIII was inserted into the *Hin*dIII site of the plant transformation vector pBI121 flanked by a kanamycin resistance gene (NPTII) and a GUS reporter gene, resulting in pBI121-N46 (sense) and pBI121-N112 (antisense) (Fig. 1).

Nucleotide sequence analysis. The individual nucleotide sequencing results from the oligomer primed clone (pBS-NC4.5) and the random primed clone (pBS-RP5.3) showed a complete match in the N gene ORF. The sequence of the Hawaiian L isolate was compared to the published sequence of the Brazilian CNPH1 (11), Bulgarian lettuce L3 (30), and BL isolates (33) (Fig. 2). The nucleotide changes among all four isolates scatter evenly over the N gene ORF. As summarized in Table 1, seven to 22 nucleotide base changes resulted in zero to eight amino acid substitutions. The three lettuce isolates (L, L3, and BL) share 99% sequence homology in both nucleotide and amino acid sequences while having 97% homology with the tomato isolate (CNPH1), suggesting that all of these isolates are very closely related. Yet the tomato isolate is clearly divergent from the lettuce isolates (L, L3, and BL).

Expression of N gene in transgenic plants. Leaf disks of *N. tabacum* cv. Xanthi nc and cotyledons of *L. esculentum* cv. VF36 were inoculated with *A. tumefaciens* containing the pBI121-N46 or pBI121-N112 binary vectors. Transformed cells were selected for kanamycin resistance and GUS expression. Forty-six transformed tobacco plants and 54 tomato plants were transferred to soil.

Genomic Southern blot analysis of the tranformed plants revealed that 29 tobacco lines (60%) and 10 tomato lines (19%) were positive for N gene integration. These trangenic plants contained the expected 2-kb size *Hin*dIII fragment harboring the N gene cassette (Figs. 3A and 4A). The copy number of the chimeric N gene, as estimated by genome reconstruction experiments, ranges from one to five for all transgenic plants. Twenty lines of transgenic tobacco had the sense construct, and nine lines had the antisense construct. In tomatoes, only lines expressing the sense construct were obtained. All the transgenic plants were initially assayed histochemically for GUS gene activity. Only 31% of tobacco plants were GUS-positive, but all 10 tomato lines were GUS-positive. The 10 lines of tomato plants and 10 lines of randomly selected tobacco plants were chosen for further study for N gene expression and viral protection assays.

DAS-ELISA of R_1 progeny of four tomato lines and five tobacco lines with the sense construct, and Northern analysis of R_1 progeny of five tobacco



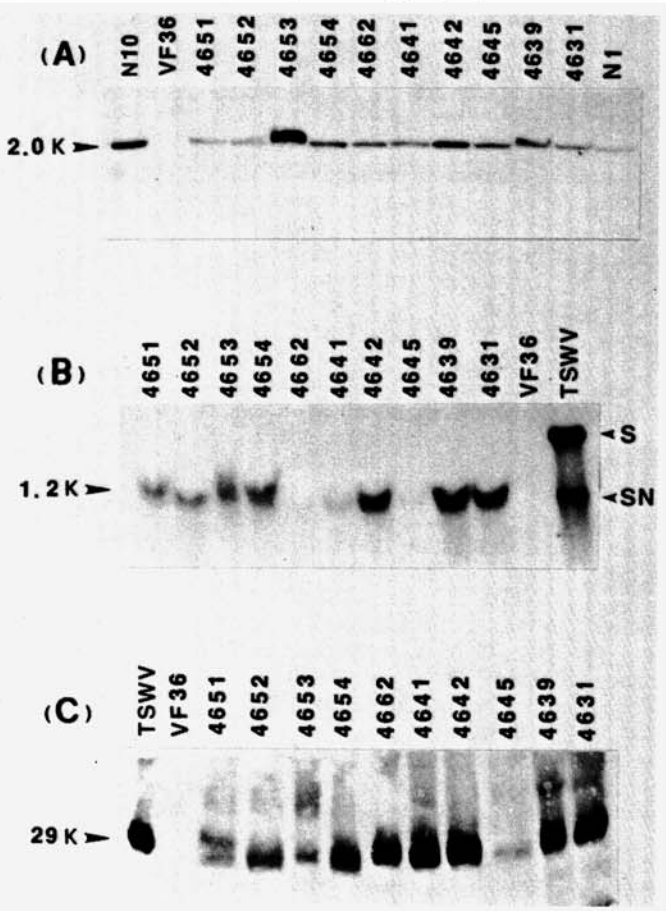


Fig. 3. (A) Southern blot hybridization analysis of transgenic tobacco lines for integration of the chimeric TSWV N gene cassette. One (N1) and 10 (N10) copies of the N gene control were included for copy number estimation. **(B)** Northern blot analysis of N gene transcripts. TSWV, S, and SN indicate the position of TSWV S RNA and N gene subgenomic RNA, respectively. **(C)** Western blot analysis of nucleocapsid protein produced in tobacco lines with sense (4604, 4605, 4608, 4621, and 4631) but not antisense (1123, 1124, 1125, and 1129) constructs. Xanthi is wild type *Nicotiana tabacum* cv. Xanthi nc.

Fig. 4. (A) Southern blot hybridization analysis of transgenic tomato plants for integration of the chimeric TSWV N gene cassette. One (N1) and 10 (N10) copies of the N gene control were included for copy number estimation. **(B)** Northern blot analysis of N gene transcripts. TSWV, S, and SN indicate the position of TSWV S RNA and N gene subgenomic RNA, respectively. **(C)** Western blot analysis of nucleocapsid protein produced in tomato lines with sense constructs.

lines with the antisense construct, indicated that the N gene segregated at a ratio of either 3:1 or 15:1, suggesting that the parent R_0 plants contained one or two loci of the N gene (Table 2).

Total RNA was isolated from transgenic plants and analyzed for N gene messenger RNA (Figs. 3B and 4B). The size of the chimeric mRNA is expected to be the total of the 777-nt N gene ORF, the 43-nt 5′ leader sequence, the 173-nt 3′ untranslated region, and the polyadenylate residues, for a total length of 1.02–1.20 kb. Results showed that all of the N gene mRNAs from individual transgenic plants co-migrated with the N gene subgenomic RNA, indicating a length of 1.2 kb. Results of Northern analysis indicated a difference in N gene RNA levels (Figs. 3C and 4C) among transgenic plants: tobaccos 4604, 4605, 4608, 4621, 1123, and 1125 expressed at high levels and 4631, 1124, and 1129 at low levels; tomato 4651, 4652, and 4654 at high levels and 4662 at low levels.

Expression of TSWV N protein in transgenic plants was also confirmed by Western blot analysis (Figs. 3C and 4C). In tobacco plants expressing TSWV N gene antisense RNA, TSWV N protein was not detected. The plants expressing sense TSWV N RNA produced a 29-kDa protein that reacted to TSWV antiserum and co-migrated with the viral N protein standard. The amount of N protein detected in the R_1 progeny by immunoblot analysis varied significantly among individual plants.

Infection of transgenic tobacco and tomato plants with TSWV. Transgenic R_1 tobacco lines expressing TSWV sense (lines 4604, 4605, 4608, 4621, and 4631) and antisense (lines 1123, 1124, 1125, 1126, and 1129) N gene RNA were challenged by inoculation with TSWV. Six to eight leaves from each plant were inoculated with freshly ground TSWV-infected leaves in buffer (1 g of leaves per 10 ml of buffer), and symptom development was monitored daily up to 9 days in the greenhouse (Fig. 5). In *N. tabacum* cv. Xanthi nc, a local lesion host of TSWV, necrotic localized lesions were clearly visible on inoculated leaves at 3 days after inoculation. Transgenic tobacco plants expressing N protein showed a lower number of localized necrotic lesions initially than did wild-type tobacco plants and required 2–3 days more to express the same number of local lesions as wild-type plants. The levels of N protein in transgenic plants as judged by the intensity of signals from Western blots did not correspond with the degree of protection from TSWV infection. Interestingly, the antisense tobacco plants had fewer localized lesions than did the sense tobacco plants, and again the Northern blot signal level did not correspond with the degree of protection from virus infection.

Transgenic R_1 tomato lines expressing TSWV N protein were also mechanically inoculated with TSWV. To ensure that infection occurred, tomato plants were inoculated three times during a 2-wk period. *L. esculentum* cv. VF36 is a systemic host for TSWV. After the first inoculation, symptom development was monitored for 8 wk (Fig. 6). Three weeks after inoculation, all the control tomatoes showed typical systemic symptoms of TSWV infection. Less than 10% of the transgenic tomatoes showed symptoms at 3 wk; and at 8 wk after inoculation, only 50% of the plants were infected by TSWV. Only 30% of line 4652 became infected. DAS-ELISA of these plants showed that the absence of symptoms corresponded with the lack of virus accumulation.

DISCUSSION

Comparison of the TSWV N gene sequence among four different isolates showed that the tomato isolate (CNPH1) (11) is different from lettuce isolates (L, L3, and BL) (24,33). The amino acid changes in the tomato isolate at numbers 42, 50, 88, 187, 230, and 255 are independent from the amino acid changes of the lettuce isolates at numbers 124 and 199, except that both types of isolates showed a difference at number 213. It has been reported that different isolates of TSWV have different serological relationships against either polyclonal or monoclonal antibodies directed to nucleocapsid proteins (8,41). The above demonstration of amino acid changes between tomato and lettuce isolates could be used to differentiate TSWV isolates in a more defined manner. Interestingly, the tomato isolate uses the opal termination codon (UGA), and the lettuce isolates use an ochre termination codon (UAA).

After inoculation with TSWV, disease development in transgenic plants expressing TSWV N protein was different from that in the control plants. A smaller number of necrotic localized lesions developed in the transgenic tobacco plants compared to the control plants, and there was a 2- to 3-day delay in reaching a similar number of local lesions in some transformed plants compared to control plants. The antisense transgenic tobacco plants developed similar lesion numbers to the sense transgenic tobacco plants when inoculated with TSWV. This is an interesting result because the antisense RNA to the TSWV N gene is the same as the N gene compliment in the viral RNA strand.

The R_2 tobacco plants were analyzed for the segregation of N gene to identify the genetic characteristics of their parent R_1 plants (*data not shown*). The homozygous R_1 plants showed higher expression of N gene mRNA than did heterozygous plants, and the local lesion numbers of the homozygous plants were less than those of the heterozygous plants in tobacco with the sense construct, suggesting a positive correlation between mRNA level and resistance. However, in antisense transformants, there was no relationship between homo/heterozygous tobacco lines and the antisense N gene transcription level.

Four lines of transgenic tomato were challenged with TSWV. At 5 wk after the first inoculation, all of the control plants were infected systemically, but less than 50% of the inoculated plants from three transgenic lines (4651, 4654, and 4664) were infected, and only 23% of line 4652. The nonsymptomatic transformed tomato plants were maintained without observing further virus symptoms for up to 8 wk. High expression of the coat protein is not necessary for the higher protection from virus infection, but low-expressing plants showed lower protection. Since the N gene mRNA is com-

Table 2. Segregation of the TSWV N gene in the progeny of self-pollinated tobacco and tomato plants and chi-square (χ^2) analysis

Transgenic plant	+N[a]	−N[b]	χ^{2c} (3:1)	χ^{2c} (15:1)
Tobacco				
4604	53	22	0.63	
4605	109	8	20.22	0.01
4608	90	10	12.00	1.75
4614	58	17	0.28	
4621	82	39	3.59	
4631	73	27	0.21	
11203	25	0	7.89	1.82
11204	85	15	5.33	11.30
11205	98	21	3.61	
11206	102	23	2.74	
11209	52	17	0.01	
Tomato				
4651	47	19	0.32	
4652	36	9	0.32	
4654	34	13	0.11	
4662	47	22	1.95	

[a] +N = progeny that express the N gene.
[b] −N = progeny that do not express the N gene.
[c] χ^2 of 3.84 is significant at the 0.05 probability level.

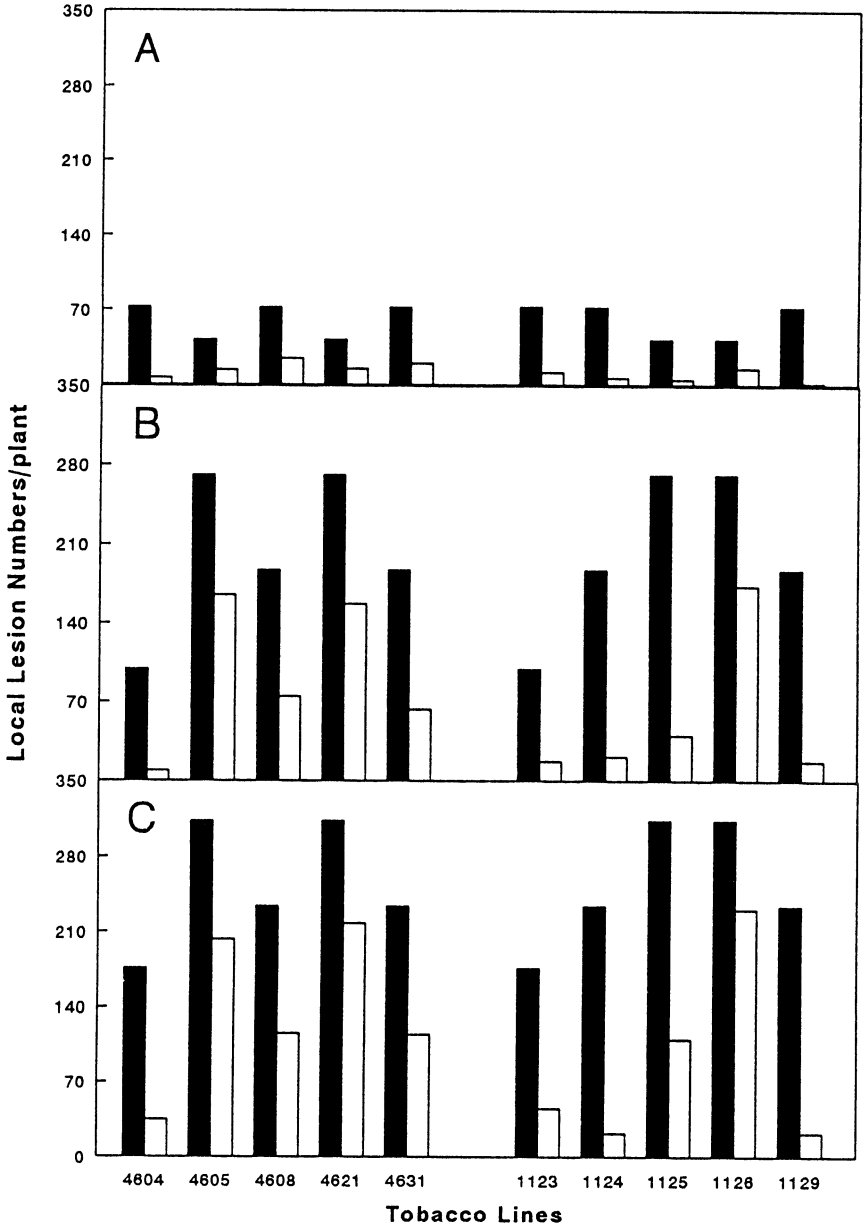


Fig. 5. TSWV protection assay of transgenic tobacco lines. Total numbers of necrotic local lesions were counted at **(A)** 3 days, **(B)** 5 days, and **(C)** 7 days after inoculation. Black bars represent control plants, and white bars represent transgenic tobacco plants. Five tobacco lines with the sense construct (4604, 4605, 4608, 4621, and 4631) and five antisense constructs (1123, 1124, 1125, 1126, and 1129) were tested.

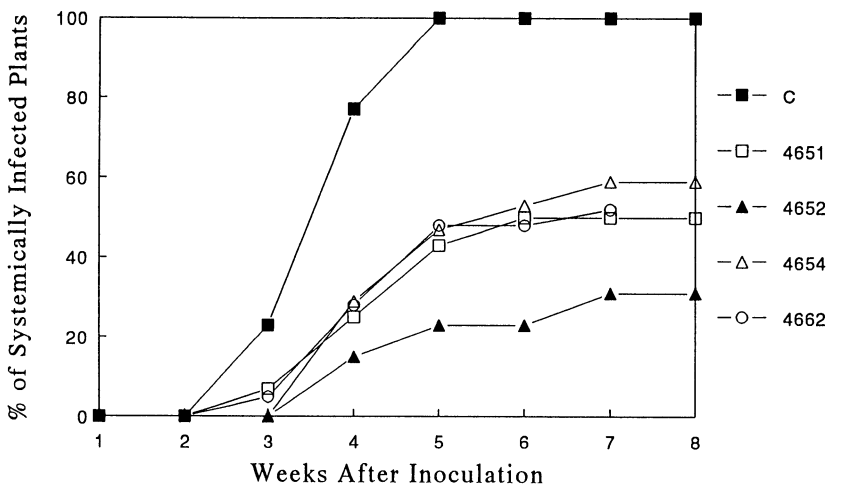


Fig. 6. TSWV protection assay of transgenic tomato lines. Four lines of transgenic tomato (4651, 4652, 4654, and 4662) and wild type VF36 plants were inoculated three times with TSWV, and development of systemic symptoms was observed for 8 wk.

plementary to the 3-prime end of the TSWV S RNA, it could act by inhibiting transcription from the viral template. Our results are consistent with the RNA-mediated resistance described for TSWV in systemically infected tobacco (9).

We demonstrate here that a major vegetable crop, tomato, expressing the TSWV N gene is resistant to TSWV infection in greenhouse conditions. Field trials of these tomatoes will be required to demonstrate the practical nature of this genetically engineered resistance. Previous reports on N gene mediated protection of tobacco plants from TSWV infection covered exclusively the protection responses of systemic hosts (20,29). This study documents similar protection responses in a local lesion host.

ACKNOWLEDGMENTS

This work is supported by the U.S. Department of Agriculture under CSRS Special Research Grant No. 89-34135-4609, managed by the Pacific Basin Advisory Group (PBAG), and published as Journal Series No. 3898 of the Hawaii Institute of Tropical Agriculture and Human Resources. We thank Liang Rasachack for excellent technical assistance.

LITERATURE CITED

1. Allen, W. R., and Matteoni, J. A. 1988. Cyclamen ringspot: Epidemics in Ontario greenhouse caused by the tomato spotted wilt virus. Can. J. Plant Pathol. 10:41-46.
2. Beachy, R. N., Loesch-Fries, S., and Tumer, N. E. 1990. Coat protein-mediated resistance against virus infection. Annu. Rev. Phytopathol. 28:451-474.
3. Burnette, W. N. 1981. "Western Blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112:195-203.
4. Cho, J. J., Mau, R. F. L., German, T. L., Hartmann, R. W., Yudin, L. S., Gonsalves, D., and Provvidenti, R. 1989. A multidisciplinary approach to management of tomato spotted wilt virus in Hawaii. Plant Dis. 73:375-383.
5. Cho, J. J., Mau, R. F. L., Gonsalves, D., and Mitchell, W. C. 1986. Reservoir weed hosts of tomato spotted wilt virus. Plant Dis. 70:1014-1017.
6. Cho, J. J., Mau, R. F. L., Mitchell, W. C., Gonsalves, D., and Yudin, L. 1987. Host list of tomato spotted wilt virus (TSWV) susceptible plants. Univ. Hawaii Coll. Trop. Agric. Hum. Resour. Res. Ext. Ser. 078.
7. Chomczynski, P., and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.
8. de Avila, A. C., Huguenot, C., Resende, R. de O., Kitajima, E. W., Goldbach, R. W., and Peters, D. 1990. Serological differentiation of tomato spotted wilt virus. J. Gen. Virol. 71:2801-2807.
9. de Haan, P., Gielen, J. J. L., Prins, M., Wijkamp, I. G., van Schepen, A., Peters, D., van Grinsven, M. Q. J. M., and Goldbach, R. 1992. Characterization of RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants. Bio/Technology 10:1133-1137.
10. de Haan, P., Kormelink, R., Resende, R. de O., van Poelwijk, F., Peters, D., and Goldbach, R. 1991. Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. J. Gen. Virol. 71:2207-2216.
11. de Haan, P., Wagemakers, L., Peters, D., and Goldbach, P. 1990. The S RNA segment of tomato spotted wilt virus has an ambisense character. J. Gen. Virol. 71:1001-1007.
12. Devereux, J., Haeberli, P., and Smithies, O. 1984. A comprehensive set of sequence analysis

programs for the VAX. Nucleic Acids Res. 12:387-395.
13. Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Nat. Acad. Sci. USA 77:7347-7351.
14. Doyle, J. J., and Doyle, J. L. 1990. Isolation of plant DNA from fresh tissue. Focus 12:13-15.
15. Elliott, R. M. 1990. Molecular biology of the bunyaviridae. J. Gen. Virol. 71:501-522.
16. Farinelli, L., and Malnoë, P. 1993. Coat protein gene-mediated resistance to potato virus Y in tobacco: Examination of the resistance mechanisms—Is the transgenic coat protein required for protection? Mol. Plant-Microbe Interact. 6:284-292.
17. Fillatti, J. J., Kiser, J., Rose, R., and Comai, L. 1987. Efficient transfer of a glyphosate tolerance gene into tomato using A binary *Agrobacterium tumefaciens* vector. Bio/Technology 5:726-731.
18. Francki, R. I. B., Fauquet, C. M., Knudson, D. L., and Brown, F. 1991. Classification and nomenclature of viruses. Pages 281-283 in: Fifth report of the international committee on taxonomy of viruses. Arch. Virol. Supp. 2.
19. German, T. L., Ullman, D. E., and Moyer, J. W. 1992. Tospoviruses: Diagnosis, molecular biology, physiology, and vector relationships. Annu. Rev. Phytopathol. 30:315-348.
20. Gielen, J. J. L., de Haan, P., Kool, A. J., Peters, D., van Grinsven, M. Q. J. M., and Goldbach, R. W. 1991. Engineered resistance to tomato spotted wilt virus, a negative-strand RNA virus. Bio/Technology 9:1363-1367.
21. Gonsalves, D., and Trujillo, E. E. 1986. Tomato spotted wilt virus in papaya and detection of the virus by ELISA. Plant Dis. 70:501-506.
22. Gubler, U., and Hoffman, B. J. 1983. A simple and very efficient method for generating cDNA libraries. Gene 25:263-269.
23. Horsch, R. B., Fry, J. E., Hoffman, N. L., Eichholtz, D., Rogers, S. G., and Fraley, R. T. 1985. A simple and general method for transferring genes into plants. Science 227:1229-1231.
24. Ivanova, E. M., Breyel, E., and Adam, G. 1991. Cloning and sequencing of the S RNA from a Bulgarian isolate of tomato spotted wilt virus. J. Gen. Virol. 72:461-464.
25. Iwaki, M., Honda, Y., Hanada, K., Tochihara, H., Yonaha, T., Hokama, K., and Yokoyama, T. 1984. Silver mottle disease of watermelon caused by tomato spotted wilt virus. Plant Dis. 68:1006-1008.
26. Kormelink, R., de Haan, P., Meurs, C., Peters, D., and Goldbach, R. 1992. The nucleotide sequence of the M RNA segment of tomato spotted wilt virus, a bunyavirus with two ambisense RNA segments. J. Gen. Virol. 73:2795-2804.
27. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.
28. Lindbo, J. A., and Dougherty, W. G. 1992. Pathogen-derived resistance to a potyvirus: Immune and resistant phenotypes in transgenic tobacco expressing altered forms of a potyvirus coat protein nucleotide sequence. Mol. Plant-Microbe Interact. 5:144-153.
29. MacKenzie, D. J., and Ellis, P. J. 1992. Resistance to tomato spotted wilt virus infection in transgenic tobacco expressing the viral nucleocapsid gene. Mol. Plant-Microbe Interact. 5:34-40.
30. Maiss, E., Ivanova, L., Breyel, E., and Adam, G. 1991. Cloning and sequence analysis of the S RNA from a Bulgarian isolate of tomato spotted wilt virus. J. Gen. Virol. 72:461-464.
31. Mohamed, N. A., Randles, J. W., and Francki, R. I. B. 1973. Protein composition of tomato spotted wilt virus. Virology 56:12-21.
32. Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
33. Pang, S.-Z., Nagpala, P., Wang, M., Slightom, J. L., and Gonsalves, D. 1992. Resistance to heterologous isolates of tomato spotted wilt virus in transgenic tobacco expressing its nucleocapsid protein gene. Phytopathology 82:1223-1229.
34. Powell Abel, P., Nelson, R. S., De, B., Hoffman, N., Rogers, S. G., Fraley, R. T., and Beachy, R. N. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738-743.
35. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 1.29-1.30.
36. Stobbs, L. W., Broadbent, A. B., Allen, W. R., and Stirling, A. L. 1992. Transmission of tomato spotted wilt virus by the western flower thrips to weeds and native plants found in southern Ontario. Plant Dis. 76:23-29.
37. Tas, P. W. L., Boerjan, M. L., and Peters, D. 1977. The structural proteins of tomato spotted wilt virus. J. Gen. Virol. 36:267-279.
38. Ullman, D. E., Cho, J. J., Mau, R. F. L., Hunter, W. B., Westcot, D. M., and Custer, D. M. 1992. Thrips-tomato spotted wilt virus interactions: Morphological, behavioral and cellular components influencing thrips transmission. Pages 195-239 in: Advances in Disease Vector Research, vol. 9. Springer-Verlag, New York.
39. Van der Vlugt, R. A. A, Ruiter, R. K., and Goldbach, R. 1992. Evidence for sense and RNA-mediated protection to PVY^N in tobacco plants transformed with the viral coat protein cistron. Plant Mol. Biol. 20:631-639.
40. Verwoerd, T. C., Dekker, B. M. M., and Hoekema, A. 1989. A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res. 17:2362.
41. Wang, M., and Gonsalves, D. 1990. ELISA detection of various tomato spotted wilt virus isolates using specific antisera to structural proteins of the virus. Plant Dis. 74:154-158.