Tomato Spotted Wilt Tospovirus Adapts to the TSWV N Gene-Derived Resistance by Genome Reassortment

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ABSTRACT

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Pathogen- and host-derived resistance have been shown to suppress infection by many plant viruses. Tomato spotted wilt tospovirus (TSWV) is among these systems; however, it has easily overcome nearly all host resistance genes and has recently been shown to overcome resistance mediated by the TSWV N gene. To better understand the resistance-breaking mechanisms, we have chosen TSWV N gene-derived resistance (TNDR) as a model to study how plant viruses defeat resistance genes. A defined viral population of isolates TSWV-D and TSWV-10, both suppressed by TNDR, was subjected to TNDR selection by serial passage in an N-gene transgenic plant. The genotype analysis demonstrated that the mixed viral population was driven to form a specific reassortant, $L_{10}M_{10}S_D$, in the presence of TNDR selection, but remained as a heterogeneous mixture in the absence of the selection. A genotype assay of 120 local lesion isolates from the first, fourth, and seventh transfers confirmed the shift of genomic composition. Further analysis demonstrated that the individual L_{10}, M_{10}, and S_D RNA segments were each selected independently in response to TNDR selection rather than to a mutation or recombination event. Following the seventh transfer on the N-gene transgenic plants, TSWV S RNA remained essentially identical to the S RNA from TSWV-D, indicating that no intermolecular recombination occurred between the two S RNAs from TSWV-10 and TSWV-D nor with the transferred N gene. These results support the hypothesis that TSWV utilizes genome reassortment to adapt to new host genotypes rapidly and that elements from two or more segments of the genome are involved in suppression of the resistance reaction.

Additional keywords: genomic marker.

Tomato spotted wilt tospovirus (TSWV), the type member of the genus *Tospovirus* in the family *Bunyaviridae*, has a broad host range covering more than 600 plant species in both monocots and dicots (20). In addition to the large number of natural hosts, it also has been shown that TSWV easily defeats resistance genes and rapidly adapts to the resistant hosts (17,18,41). For example, a tomato line with the Sw^5 resistance gene initially conferred resistance to at least five TSWV isolates (49), but recently was observed to be susceptible to a virulent TSWV isolate (7). It is generally accepted that TSWV exists in nature as a heterogeneous population of stable isolates (5,9,10,37,48,51; J. W. Moyer, *unpublished data*) that may serve as the genetic reservoir for adaptation. However, the underlying genetic mechanisms for the adaptation of TSWV as well as other viruses within the family *Bunyaviridae* to different hosts are poorly understood (5,14,20).

The TSWV genome is distributed on three single-stranded RNA molecules designated as L RNA, M RNA, and S RNA. The L RNA encodes a putative RNA-dependent RNA polymerase (RdRp) in the viral complementary-sense RNA (12). RdRp purified from virions has been shown to synthesize short, double-stranded viral RNAs in vitro, implying that the TSWV virion packages the functional RNA polymerase (2). The M RNA encodes a nonstructural protein (NSm) in the viral-sense RNA and a G1/G2 precursor to two glycoproteins in viral complementary-sense RNA (28,30). The G1/G2 precursor autocatalytically processes to the individual G1 and G2 proteins found in the viral envelope (1). The NSm protein is closely associated with tubular structures in plant and insect cells transiently expressing the NSm protein, suggesting its association with cell-to-cell movement of TSWV (50). The S RNA encodes two proteins, a nonstructural protein (NSs) in the viral-sense RNA to which no biological function has yet been assigned and the nucleocapsid protein (N) in the viral complementary-sense RNA with a role of packaging viral genomic RNAs (13). In addition, both the M RNA and S RNA possess the characteristic A-U–rich intergenic regions (IGR) (13,30).

Transgenic plants transformed with the TSWV N gene vary in their degree of resistance to different TSWV isolates (8,22), which is defined as TSWV N gene-derived resistance (TNDR). In most cases, TNDR was assessed against homologous or closely related TSWV isolates. While some transgenic lines were resistant to closely related TSWV isolates, others were resistant to species other than TSWV (38,52). TNDR was postulated to be either RNA mediated or protein mediated (11,21,27,33,39,40,43,52). Recent evidence suggests that posttranscriptional transgene silencing may be involved in the transinactivation of an invading TSWV viral genome of a specific strain (39,40). Despite the wealth of information about the effect of TNDR on individual isolates, little is known about how TNDR impacts the TSWV population consisting of various isolates.

In a previous study (44), a reassortant, $L_{10}M_{10}S_D$, was isolated from a mixed infection of two isolates, TSWV-D and TSWV-10, on the susceptible plant *Nicotiana tabacum* cv. Burley 21. TSWV-D and TSWV-10 were suppressed by TNDR, but the reassortant $L_{10}M_{10}S_D$ was able to overcome TNDR, although it was isolated from susceptible plants. This observation prompted us to evaluate if TNDR could exert a specific selection pressure on the viral population composed of the two suppressed isolates to direct the formation of a specific reassortant or reassortants. To test this hypothesis, a mixed viral population of the two suppressed isolates, TSWV-D and TSWV-10, was subjected to seven parallel passages on the susceptible and TSWV N-gene transgenic plants. The TSWV genome composition shift of the viral population was monitored with the isolate-specific reverse-transcription polymerase chain reaction (RT-

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PCR) genomic markers (44). The genotypes of 120 local lesion isolates selected from the first, fourth, and seventh passages on both hosts were also assayed as additional evidence for the genome composition shift. The results showed that viral RNA segments from a mixed TSWV population are able to reassort in an appropriate combination that facilitates adaptation to new host plants.

MATERIALS AND METHODS

TSWV isolates and host plants. TSWV-D was isolated from dahlia (*Dahlia hybrida*) originating in the Netherlands, and TSWV-10 was isolated from peanut (*Arachis hypogaea*) in the United States. Stock cultures of both isolates were stored as frozen leaves at –80°C, and working cultures were recovered on *N. benthamiana* and maintained on *Emilia sonchifolia* or *N. benthamiana*.

Resistant plants were selected from *N. tabacum* cv. Burley 21 transformed by the binary vector carrying the TSWV N gene from TSWV-D (42). Resistance was expressed as a reduction in local lesion number and no systemic movement beyond the inoculated leaves. R_4 plants were used in all experiments. All experiments were conducted in a greenhouse at 25 to 28°C.

Constitutive or induced TNDR. Susceptible and N-gene transgenic *N. tabacum* cv. Burley 21 at the three-leaf stage were first inoculated with TSWV-D– or TSWV-10–infected leaf tissue from *N. benthamiana* (1:5, wt/vol) in the inoculation buffer (10 mM Tris-HCl, pH 7.8; 10 mM Na_2SO_3; and 0.1% cysteine-HCl). Two weeks postinoculation when plants were at the six-leaf stage, the second and third leaves numbered from the top were challenge-inoculated with TSWV-D, TSWV-10, and the three reassortants. Symptoms on upper noninoculated leaves were recorded 18 days after the challenge inoculation. Five plants were inoculated per treatment in two replicated experiments.

Serial passages to exert the selection on the viral population. The symptomatic leaves of *N. benthamiana* infected by TSWV-D or TSWV-10 were ground in cold inoculation buffer at 1:5 (wt/vol). The serially diluted inocula made from systemically infected *N. benthamiana* leaves by TSWV-D or TSWV-10 were inoculated on

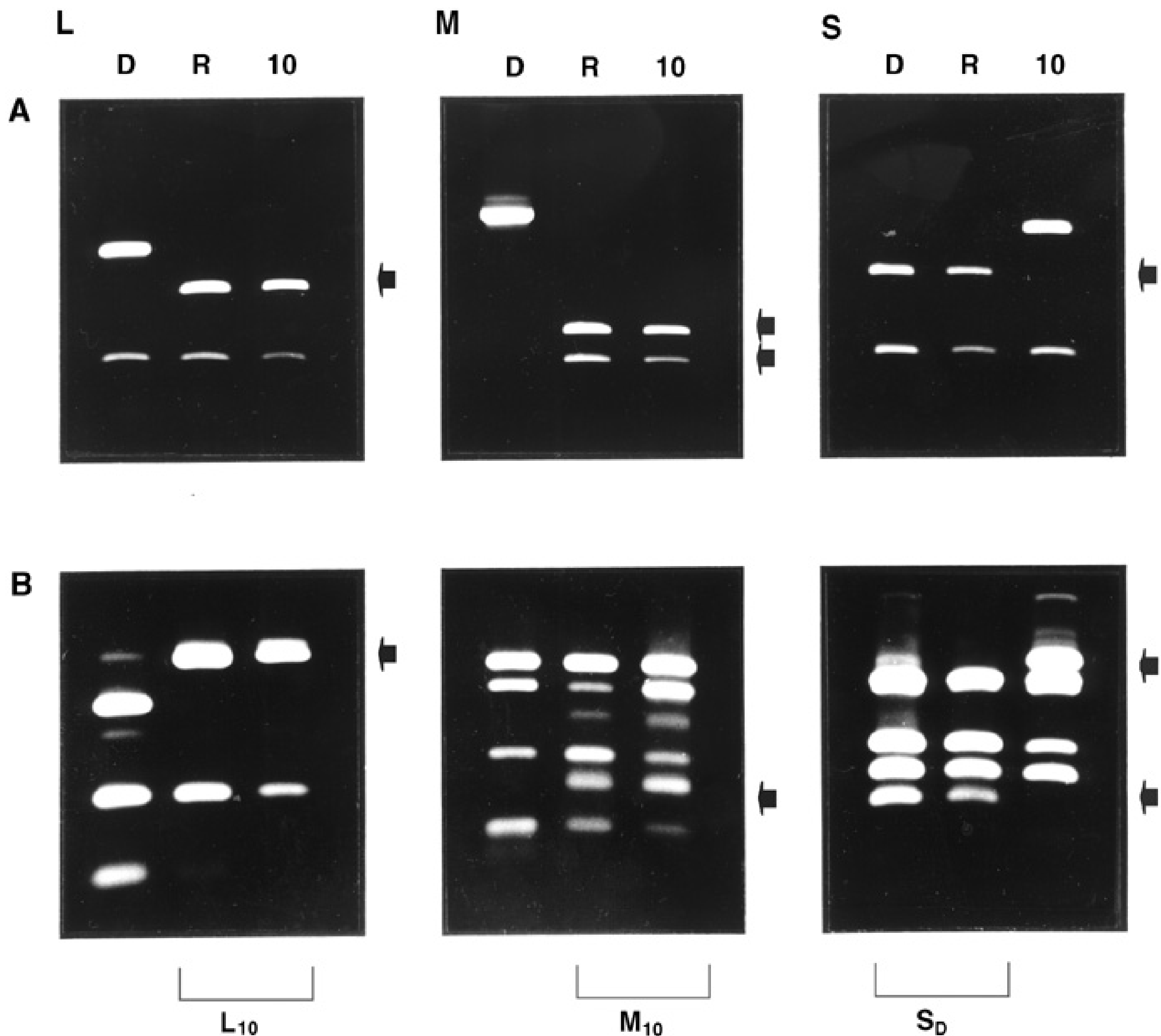

Fig. 1. Authentication of the reassortant genotype $L_{10}M_{10}S_D$ (lane R) isolated from the mixed infection of TSWV-D (lane D) and TSWV-10 (lane 10) as determined by two sets of genomic markers for each of the three genomic RNA segments. L, M, and S indicate panels with markers for the L, M, and S RNAs, respectively. **A,** Reverse-transcription polymerase chain reaction (RT-PCR)–amplified L RNA (nucleotides [nt] 28 to 895), M RNA (nt 66 to 962), and S RNA (nt 70 to 890) fragments were digested with *Taq*I, *Rsa*I, and *Taq*I, respectively. **B,** RT-PCR–amplified L RNA (nt 4,856 to 5,578), M RNA (nt 3,050 to 4,037), and S RNA (nt 1,983 to 2,767) fragments were digested with *Alu*I. Arrows on the right indicated the signature restriction fragments for L_{10}, M_{10}, and S_D.

cucumber (*Cucumis sativus* cv. National Pickling) cotyledons to determine the relative viral concentration. Statistical analysis of local lesion numbers showed that the viral concentration used in the coinoculation was similar for both isolates. The mixed inocula were prepared by mixing equal volumes of each inoculum followed by application to susceptible and N-gene transgenic *N. tabacum* cv. Burley 21 leaves predusted with Carborundum. Inoculum for subsequent serial transfers was prepared from systemically infected tissue of the previous passage on susceptible and N-gene transgenic *N. tabacum* cv. Burley 21. The viral cultures were passed and collected through seven serial transfers. At each passage, *N. benthamiana* was also inoculated. Total RNA was extracted from *N. benthamiana* to determine the presence of genome segments from each parent as described below. A reference stock of systemically infected susceptible and N-gene transgenic *N. tabacum* cv. Burley 21 leaves from each transfer was also stored at –80°C for the subsequent local lesion assay.

Systemically infected tissue from each transfer was inoculated on susceptible *N. tabacum* cv. Burley 21 leaves for lesion production. Twenty local lesion isolates were obtained from the first, fourth, and seventh transfers of systemically infected susceptible and N-gene transgenic *N. tabacum* cv. Burley 21. Individual lesions were excised and ground in 200 μl of inoculation buffer and inoculated onto *N. benthamiana*. The genotype of each local lesion isolate was determined for 20 local lesion isolates derived from three (first, fourth, and seventh) serial transfers on both lines.

Verification of parental origin of genome segment. Total plant RNA was extracted from 100 mg of TSWV-infected *N. benthamiana* leaves using RNA ISOLATER (Genosys Biotechnologies, Inc., The Woodlands, TX) according to the supplier's protocols. First-strand cDNA synthesis, RT-PCR, and restriction enzyme digestion were performed according to the methods previously described (44). The RT-PCR products were purified by Qiagen PCR purification columns (Qiagen, Inc., Chatsworth, CA). Amplified L RNA (nucleotides [nt] 4,856 to 5,578), M RNA (nt 3,050 to 4,037), and S RNA (nt 1,983 to 2,767) fragments were subjected to *Alu*I digestion. Amplified fragments from nt 28 to 895 of the L RNA and from nt 70 to 890 of the S RNA were digested by *Taq*I. The fragment from nt 66 to 962 of the M RNA was subjected to *Rsa*I digestion. Digested products were resolved on a 1.5% agarose gel and stained by ethidium bromide for characterization of the TSWV-D and TSWV-10 genotypes.

Cloning and sequencing strategy. TSWV virion purification and virion RNA extractions were performed as previously described (29,35). Sequence of the 5′ and 3′ termini of the viral RNA was determined by the method of Weng and Xiong (54). The remainder of the S RNA sequence was determined from RT-PCR products. The N gene was amplified from primers S2767 (5′ GCTCTAGAGCCATCATGTCTAAGGTTAAGCTCAC 3′; restriction enzyme site underlined, number denotes the position of the primer in the viral-sense strand) and S1983 (5′ CCCTCGAGGCTTTCAAGCAAGTTCTGCG 3′). The intergenic region was amplified with primers S2083 (5′ GGAAGTATTGCTATGG 3′) and S1282 (5′ CACTTGAATGTCTTCC 3′). The NSs gene was amplified with primers S1507 (5′ CCGGACACAGCAAGATTA 3′) and S70 (5′ CACAGTACCAATAACC 3′).

The RT-PCR–amplified products were cloned into pGEM-T vectors (Promega Corp., Madison, WI). The recombinant plasmids were purified by the WizardPlus Minipreps DNA Purification System (Promega Corp.). The cDNA clones were sequenced by the dideoxynucleotide chain termination method (46) using Sequenase 2.0 (United States Biochemical Corp., Cleveland). The sequences of the NSs open reading frame (ORF) from reassortant $L_{10}M_{10}S_D$ isolated from the susceptible and seventh-transferred viral populations on N-gene transgenic plants and $L_{10+D}M_{10}S_D$ isolated from the seventh-transferred viral population on susceptible plants were determined by the DNA Sequencing Core Laboratory, University of Florida (Gainesville).

RESULTS

Characterization of the TNDR-breaking reassortant. The TNDR-breaking reassortant, $L_{10}M_{10}S_D$, was originally obtained from susceptible *N. tabacum* cv. Burley 21 and assayed on several hosts including N-gene transgenic *N. tabacum* cv. Burley 21 that was transformed by the TSWV N gene (42). The same TSWV genotype was isolated from two additional experiments conducted on susceptible plants. Isolates obtained from each experiment included the parental isolates and two other reassortants in addition to $L_{10}M_{10}S_D$. Only $L_{10}M_{10}S_D$ was capable of sustaining a systemic invasion of the plants displaying TNDR. The parental and other reassortant genotypes caused local lesions but no systemic invasion on the N-gene transgenic plants.

The genotype of the TNDR-breaking reassortant was validated by the genomic markers to confirm that the L and M RNAs were derived from TSWV-10 and the S RNA was from TSWV-D. The genotype was first defined by the genomic markers in the amplified L RNA (nt 4,856 to 5,578), M RNA (nt 3,050 to 4,037), and S RNA (nt 1,983 to 2,767) fragments (Fig. 1A) and was further authenticated by the second set of genomic markers in the regions of the L RNA (nt 28 to 895), M RNA (nt 66 to 962), and S RNA (nt 70 to 890) fragments (Fig. 1B). This result additionally demonstrated that no large intermolecular recombination had occurred resulting in an exchange of two major portions of the L RNA nor of the two ORFs on the M and S RNAs.

The constitutive expression of TNDR. The TNDR phenotype is characterized by plants that express local lesions induced by TSWV-D and TSWV-10, albeit to a lesser extent than on susceptible plants, but systemic invasion is inhibited. To gain further insight into the nature of TNDR, experiments were conducted to determine if the ability to overcome TNDR was due either to overcoming a constitutively expressed resistance or to a failure to initiate an interaction induced by the viruses. The TNDR plants were inoculated with TSWV-D and TSWV-10 and then challenged 14 days

TABLE 1. The phenotypes of parental and reassortant isolates on resistant (N-gene transgenic) and susceptible *Nicotiana tabacum* cv. Burley 21 to determine if the resistance is inducible or constitutively expressed

Host plants	Preinoculum	Challenge inoculation	Local lesion[a]	Systemic infection
Resistant[b]	TSWV-10 or TSWV-D	TSWV-D	+	0/20[c]
		$L_{10}M_{10}S_D$[d]	++	13/20
		$L_{10}M_DS_D$	+	1/20
		$L_DM_{10}S_D$	+++	2/20
		TSWV-10	+	3/20
		Mock	–	0/20
	None	TSWV-D	+	0/10
		$L_{10}M_{10}S_D$	++	9/10
		$L_{10}M_DS_D$	+	0/10
		$L_DM_{10}S_D$	+++	0/10
		TSWV-10	+	1/10
		Mock	–	0/10
Susceptible	TSWV-10	None	ND[e]	10/10
	TSWV-D	None	ND	10/10
	None	TSWV-D	+++	3/10
		$L_{10}M_{10}S_D$	+++	10/10
		$L_{10}M_DS_D$	++	5/10
		$L_DM_{10}S_D$	+++	5/10
		TSWV-10	++	5/10
		Mock	–	0/10

[a] The number of local lesions per leaf was measured by averaging the lesion number counted in a 20-cm² leaf area of 10 leaves. The lesion number was categorized into three classes: + = 1 to 10; ++ = 11 to 50; and +++ = over 50.
[b] Resistant: Tomato spotted wilt virus (TSWV) N gene-derived resistant *N. tabacum* cv. Burley 21. Susceptible: *N. tabacum* cv. Burley 21.
[c] Data were combined from the preinoculation treatments with the two suppressed isolates.
[d] Subscript refers to the viral origin (isolate) of that segment: TSWV-D or TSWV-10.
[e] Not determined.

later with the TNDR-breaking reassortant, $L_{10}M_{10}S_D$, two other reassortants, $L_{10}M_DS_D$ and $L_DM_{10}S_D$, as well as the two suppressed parental isolates, TSWV-D and TSWV-10. Thirteen of twenty plants preinoculated with the suppressed isolates and subsequently challenged with the TNDR-breaking reassortant $L_{10}M_{10}S_D$ were systemically infected. Nine of ten mock-inoculated plants expressed systemic symptoms when challenged with $L_{10}M_{10}S_D$. Seventeen of twenty plants displayed only local infections when the preinoculated plants were challenged with the reassortants $L_{10}M_DS_D$ and $L_DM_{10}S_D$ or the two suppressed parental isolates. Reassortant $L_DM_{10}S_D$ generated the largest number of local lesions on the inoculated leaves but was unable to systemically infect the N-gene transgenic plants. The two parental isolates and all three reassortants were able to systemically infected susceptible *N. tabacum* cv. Burley 21 (Table 1). These results demonstrated that the ability of the reassortant $L_{10}M_{10}S_D$ to overcome TNDR is not due to a failure to trigger the resistance reaction in the host; thus, TNDR is constitutively expressed.

The effect of TNDR on the genomic composition of a mixed viral population. To determine if TNDR could impose a selection pressure that would alter the genomic composition of the TSWV viral population, mixed infections of TSWV-D and TSWV-10 were analyzed at comparable intervals through serial passages on either susceptible or N-gene transgenic plants. The origin of the genomic RNA segments was determined following each passage. The L RNAs from both TSWV-D and TSWV-10 coexisted in the viral population from the first to the seventh passage on susceptible plants. However, the L RNA from TSWV-10 became the dominant genomic segment following the fifth transfer on the N-gene transgenic plants (Fig. 2, panel L). The M RNA from TSWV-10 was

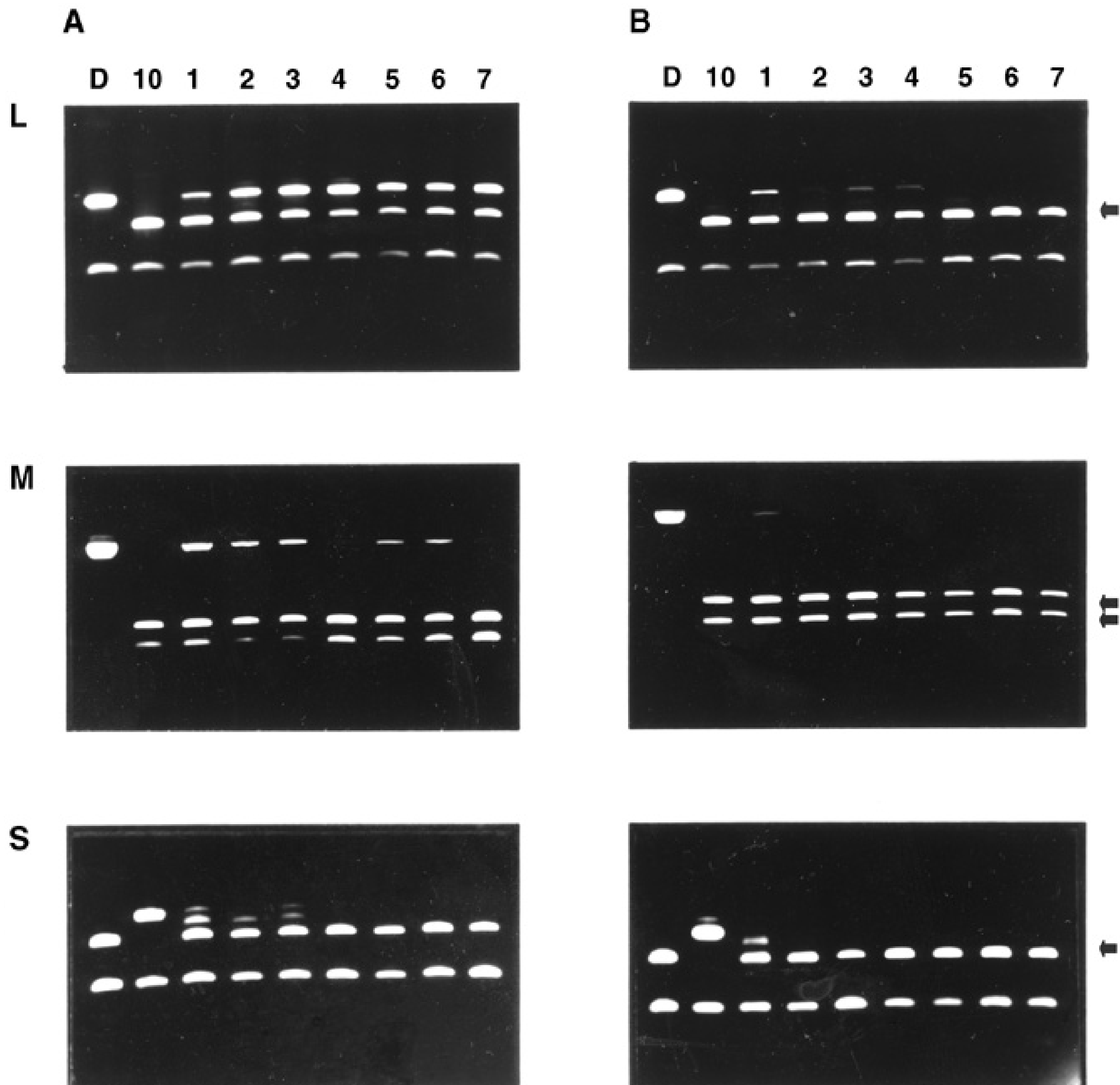

Fig. 2. The viral genomic composition shift in a mixed viral infection of TSWV-D and TSWV-10 during seven serial passages on the susceptible *Nicotiana tabacum* cv. Burley 21 and the TSWV N gene-derived resistance (TNDR) *N. tabacum* cv. Burley 21. **A,** Mixed tomato spotted wilt virus (TSWV) infection on the susceptible *N. tabacum* cv. Burley 21. **B,** Mixed TSWV infection on the TNDR *N. tabacum* cv. Burley 21. L, Reverse-transcription polymerase chain reaction (RT-PCR)–amplified L RNA (nucleotides [nt] 28 to 895) fragment digested by *Taq*I; M, amplified M RNA (nt 66 to 962) fragment digested by *Rsa*I; and S, amplified S RNA (nt 70 to 890) fragment digested by *Taq*I. Lanes D and 10 contain the reference markers for three genomic segments from the two parental isolates, TSWV-D and TSWV-10. Lanes 1 to 7 indicates the seven serial passages. Arrows on the right indicated the signature restriction fragments for L_{10}, M_{10}, and S_D.

dominant, although the M RNA from TSWV-D could be detected following the seventh transfer on susceptible plants. In contrast, the M RNA from TSWV-D was below the limit of detection by the third transfer on the N-gene transgenic plants (Fig. 2, panel M). Unlike the L and M RNAs, the S RNA from TSWV-10 was less competitive (44) and could not be detected on either susceptible or N-gene transgenic plants following the fourth and third transfers, respectively (Fig. 2, panel S).

The viral genome segment composition detected in total RNA extracts were confirmed by analyzing 20 local lesion isolates selected randomly from each of the first, fourth, and seventh transfers of the mixed viral population on both susceptible and N-gene transgenic plants (Table 2). The genomic makeup of each of the 20 local lesion isolates from the seventh transfer of the mixed viral population passed through the N-gene transgenic plants was $L_{10}M_{10}S_D$. This genotype was obtained in 5 of 20 local lesion isolates in the first transfer and 8 of 20 local lesion isolates after the fourth transfer on the N-gene transgenic plants.

In contrast, 14 of 20 local lesion isolates from the seventh transfer of the population passed through susceptible plants were $L_{10+D}M_{10}S_D$. The same genotype was detected in 7 of 20 local lesion isolates in the first transfer and 10 of 20 local lesion isolates in the fourth transfer on susceptible plants (Table 2).

The impact of TNDR on the S RNA. We previously demonstrated that the S RNA from TSWV-D has a competitive advantage over the S RNA from TSWV-10 on susceptible hosts and that the competitiveness is associated with the absence of a 33-nt duplicate sequence in the IGR (44). Here, we examined the entire S RNA to determine if the observed changes were specifically related to the adaptation to a resistant host. After seven passages of the mixed viral population through the N-gene transgenic plants, the S RNA in the viral population was contributed solely by the TSWV-D isolate. The entire S RNA of reassortant $L_{10}M_{10}S_D$ (designated as $L_{10}M_{10}S_D$-TB-7 reassortant), which was isolated following the seventh transfer on the N-gene transgenic plants, was sequenced and compared with the S RNAs from the two parental isolates, TSWV-D (GenBank accession no. AF020660) and TSWV-10 (GenBank accession no. AF020559). The S RNA of $L_{10}M_{10}S_D$-TB-7, although of identical length (2,955 nt), was similar but not identical to the S RNA from TSWV-D. The nucleotide sequence comparison revealed that C_{51} of the S RNA from TSWV-D was substituted with U in the 5′ untranslated region (UTR) and G_{1712} was substituted with A in the IGR of $L_{10}M_{10}S_D$-TB-7. The IGR of $L_{10}M_{10}S_D$-TB-7 had 535 nt analogous to that of the S RNA from TSWV-D. Both the 3′ UTR and N ORF of the S RNA from reassortant $L_{10}M_{10}S_D$-TB-7 were identical to those of the S RNA from TSWV-D.

The NSs ORF was the most variable between the two S RNAs from TSWV-D and reassortant $L_{10}M_{10}S_D$-TB-7. Substitutions in the NSs ORF in the S RNA from TSWV-D following seven passages through the N-gene transgenic plants occurred in the following locations: N_{158} to D, P_{233} to S, I_{311} to T, V_{370} to E, R_{408} to Q, and R_{450} to G. To determine the linkage of those substitutions with the adaptation of TSWV-D S RNA to the N-gene transgenic plants, the NSs ORFs were sequenced in the reassortant $L_{10}M_{10}S_D$ previously isolated from susceptible hosts and from an isolate with the mixed genotype $L_{10+D}M_{10}S_D$ (designated as $L_{10+D}M_{10}S_D$-B-7) following seven transfers through susceptible plants. The four amino acid substitutions of P_{233} to S, V_{370} to E, R_{408} to Q, and R_{450} to G occurred in the NSs ORFs of $L_{10}M_{10}S_D$-TB-7, $L_{10+D}M_{10}S_D$-B-7, $L_{10}M_{10}S_D$, and TSWV-10 S RNA. T_{311} was conserved in the NSs ORFs from $L_{10}M_{10}S_D$-TB-7, $L_{10+D}M_{10}S_D$-B-7, and $L_{10}M_{10}S_D$. D_{158} was unique to the NSs ORF of $L_{10}M_{10}S_D$-TB-7 (Fig. 3). Although there were changes in the nucleotide sequence of the S RNA following passages through the resistant hosts, none were associated solely with the TNDR-breaking phenomenon. This is consistent with the preliminary mapping, indicating that determinants of the ability to overcome resistance are located on TSWV genomic RNAs other than the S RNA.

DISCUSSION

Heterogeneity and rapid adaptability are two prominent phenotypic characteristics that distinguish TSWV from the majority of plant viruses (5,36). The heterogeneity of the natural viral population provides a potential genetic reservoir for the adaptation of TSWV to a broad range of hosts. In a previous report, we presented evidence for the genome reassortment and the basis of interisolate competition among genome segments (44). We also isolated a resistance-breaking reassortant in the absence of selection pressure. The hypothesis tested in this study was that genome reassortment is one of the genetic mechanisms TSWV utilizes to adapt to resistant plants. To test this hypothesis, we chose TNDR as a model to study the pattern of reassortment of TSWV genomic segments in a defined population in the presence or absence of TNDR. This experimental system, in which the only difference between the resistant plant and the susceptible plant is the presence of the TSWV N gene in the resistant plant genome, permitted us to determine the extent to which the adaptation of the virus might be due to mutation, recombination between the viral genes, recombination with the transgene, or whether reassortment might play a significant role in the adaptation. Constitutive expression of TNDR (Table 1) reduced the possibility that resistance could be overcome by point mutations in the viral genes commonly associated with a failure of induced resistance. Most importantly, this system permitted us to assay the influences of selection by the TSWV-derived N transgene on the viral population.

The results showed that TNDR could drive the mixed population of TSWV isolates to form a specific reassortant, $L_{10}M_{10}S_D$. The verification of the reassortment genotypes by two genomic markers on each of the genomic RNAs (Fig. 1) indicated that major recombination events had not occurred. The full-length S RNA sequence from the seventh transfer of the viral population on the N-gene transgenic plants ruled out recombination between the two S RNAs from TSWV-10 and TSWV-D, as well as with the TSWV N transgene. Other TSWV isolates ($L_{10}M_DS_D$, $L_DM_{10}S_D$, or $L_{10}M_{10}S_{10}$) were restricted to the local infection on the N-gene transgenic plants, which provided additional circumstantial evidence that point mutations in viral proteins on any individual segments of $L_{10}M_{10}S_D$ are unlikely to be responsible for breaking TNDR. The results strongly support the hypothesis that TSWV utilizes genomic reassortment as a genetic tool to adapt to the N-gene transgenic plants.

It is significant that most of the heterogeneities introduced by passages on the N-gene transgenic plants were in the NSs ORF.

TABLE 2. The genotype analysis of 120 local lesion isolates randomly selected from the first, fourth, and seventh serial transfers of a mixed viral population initiated with TSWV-D and TSWV-10 isolates coinoculated onto resistant (N-gene transgenic) and susceptible *Nicotiana tabacum* cv. Burley 21 plants

	Serial transfers					
	1st		4th		7th	
Host plants	Composition	No.[a]	Composition	No.	Composition	No.
Resistant[b]	$L_{10}M_{10}S_D$[c]	5	$L_{10}M_{10}S_D$	8	$L_{10}M_{10}S_D$	20
	$L_{10+D}M_{10}S_D$	5	$L_{10+D}M_{10}S_D$	8		
	$L_{10+D}M_{10}S_{D+10}$	6	$L_{10+D}M_{10}S_{D+10}$	2		
	$L_{10}M_{10}S_{D+10}$	4	$L_{10}M_{10}S_{D+10}$	2		
Susceptible[b]	$L_{10}M_{10}S_D$	2	$L_{10}M_{10}S_D$	1	$L_{10+D}M_{10}S_D$	14
	$L_{10+D}M_{10}S_D$	7	$L_{10+D}M_{10}S_D$	10	$L_{10+D}M_{10}S_{D+10}$	5
	$L_{10+D}M_{10}S_{D+10}$	7	$L_{10+D}M_{10}S_{D+10}$	8	$L_{10+D}M_{10+D}S_{D+10}$	1
	$L_{10+D}M_{10}S_{10}$	2	$L_{10+D}M_{10+D}S_D$	1		
	$L_{10+D}M_{10+D}S_D$	1				
	$L_{10}M_{10}S_{D+10}$	1				

[a] The number of isolates possessing that particular genome composition.

[b] Resistant: Tomato spotted wilt virus (TSWV) N gene-derived resistant *N. tabacum* cv. Burley 21. Susceptible: *N. tabacum* cv. Burley 21.

[c] Subscript refers to the origin of that segment: TSWV-D or TSWV-10. Some isolates contained segments from both parents, e.g., L_{10+D}. It is not known if this is due to packaging or a mixture of isolates.

Four amino acid substitutions in the NSs ORF, P_{233} to S, V_{370} to E, R_{408} to Q, and R_{450} to G, occur in reassortants with the genotype of $L_{10}M_{10}S_D$ and $L_{10+D}M_{10}S_D$ after the coinfection of TSWV-D with TSWV-10. Since the NSs ORF of TSWV-10, which is not involved in the resistance breaking, also has those four amino acids (S_{233}, E_{370}, Q_{408}, and G_{450}), those amino acid substitutions could not be linked to the resistance breaking. The significance of the unique T_{311} in the NSs ORF of $L_{10}M_{10}S_D$ and the unique D_{158} in the NSs ORF of $L_{10}M_{10}S_D$-TB-7 in the determination of particular phenotypes needs further investigation.

The mechanism of TNDR is not precisely known. The resistance phenotype is characterized by chlorotic lesions on the inoculated leaf; however, systemic movement of suppressed isolates is inhibited. This would imply that the inserted TSWV N gene might interfere with the systemic movement. Posttranscriptional transgene silencing has been implicated in the suppression of the invading TSWV genome in transgenic plants (39). The silencing of the transgene transcripts and viral RNAs was proposed to be mediated by the homology-dependent RNA degradation in the cytoplasm (4,15,23). Viral coat protein-mediated resistance has been postulated to operate by transinactivation of viral replication, virion disassembly and assembly, and interference with host components (4). In addition, a highly specific, inducible resistance in the transgenic plants expressing coat protein was reported for tobacco etch potyvirus (31). In other systems such as replicase-derived resistance, two independent mechanisms, the inhibition of viral replication and the blockage of systemic movement, were reported (25). The N transgene used for the development of transgenic plants originated from the S RNA of TSWV-D. The TNDR-breaking reassortant, $L_{10}M_{10}S_D$, also had the S RNA from TSWV-D. Therefore, TNDR in our investigation may not meet the rigorous conditions of the homology-dependent RNA degradation model (4), but is more likely explained by a modification of the model which also accommodates a blockage of systemic movement.

The specific selection per se is unable to cause genome reassortment. The reassortment of the RNAs from two suppressed isolates under TNDR selection to form a specific combination, which is capable of defeating the resistance in the absence of any con-

```
1 MSSSVYESII QTRASVWGST ASGKAVVDSY WIHELGTGSP LVQTQLYSDS RSKSSFGYTA  60
2 ---------- ---------- ---------- ---------- ---------- ----------
3 ---------- ---------- ---------- ---------- ---------- ----------
4 ---------- ---------- ---------- ---------- ---------- ----------
5 ---------- ---------- ---------- ---------Q ---------- ----------

1 KVGNLPCEEE EILSQHVYIP IFDDIDFSIN IDDSVLALSV CSNTVNTNGV KHQGHLKVLS 120
2 ---------- ---------- ---------- ---------- ---------- ----------
3 ---------- ---------- ---------- ---------- ---------- ----------
4 ---------- ---------- ---------- ---------- ---------- ----------
5 ---D------ ---------- ---------- ---------- ------A--- ----------

1 PAQLHSIGST MNRSDITDRF QLQEKDIIPN DRYIEAANKG SLSCVKEHTY KIEMCYNQAL 180
2 ---------- ---------- ---------- -------D-- ---------- ----------
3 ---------- ---------- ---------- ---------- ---------- ----------
4 ---------- ---------- ---------- ---------- ---------- ----------
5 ---------I -S-------- ---------- V--------- ---------- ----------

1 GKVNVLSPNR NVHEWLYSFK PSFNQVESNN RTVNSLAVKS LLMSAENNIM PNSQAFVKAS 240
2 ---------- ---------- ---------- ---------- ---------- --S-------
3 ---------- ---------- ---------- ---------- ---------- --S-------
4 ---------- ---------- ---------- ---------- ---------- --P-------
5 ---------- ---------- -N-------- ---------- ---------- --S-------

1 TDSHFKLSLW LRVPKVLKQV SIQKLFKVAG DETNKTFYLS IACIPNHNSV ETALNISVIC 300
2 ---------- ---------- ---------- ---------- ---------- ----------
3 ---------- ---------- ---------- ---------- ---------- ----------
4 ---------- ---------- ---------- ---------- ---------- ----------
5 ---------- ---------I ---------- ---------- ----T----- ------T---

1 KHQLPIRKCK TPFELSMMFS DLKEPYNIVH DPSYPQRIVH ALLETHTSFA QVLCNNLQED 360
2 ---------- T--------- ---------- ---------- ---------- ----------
3 ---------- T--------- ---------- ---------- ---------- ----------
4 ---------- I--------- ---------- ---------- ---------- ----------
5 ---------- A--------- ---------- ---------- ---------- ---S------

1 VIIYTLNNYE LTPGKLDLGE RTLNYSEDIC KRKYFLSKTL ECLPSNTQTM SYLDSIQIPS 420
2 ---------E ---------- ---------- ---------- -------Q-- ----------
3 -------K-E ---------- ---------- ---------- -------Q-- ----------
4 ---------V ---------- ---------- ---------- -------R-- ----------
5 --------HE -------I-- --------AY ---------- ----T--Q-- ----------

1 WKIDFARGEI KISPQPVSVA KSLLKLDLSG IEKKESKIPE TYASGSK 467
2 ---------- ---------- ---------G ---------- -------
3 ---------- ---------- ---------G V--------- -------
4 ---------- ---------- ---------R ---------- -------
5 ---------- -----SI--E ---------G -------VK- A------
```

Fig. 3. The predicted NSs amino acid sequence comparison of TSWV-D, TSWV-10, reassortant $L_{10}M_{10}S_D$-TB-7 (from the seventh transfer of viral population on the TSWV N gene-derived resistance [TNDR] *Nicotiana tabacum* cv. Burley 21), $L_{10+D}M_{10}S_D$-B-7 (from the seventh transfer of viral population on the susceptible *N. tabacum* cv. Burley 21), and $L_{10}M_{10}S_D$ (from the mixed infection of TSWV-D and TSWV-10 on the susceptible *N. tabacum* cv. Burley 21). Rows 1, TNDR-breaking reassortant $L_{10}M_{10}S_D$ isolated from the susceptible *N. tabacum* cv. Burley 21; rows 2, $L_{10}M_{10}S_D$-TB-7; rows 3, $L_{10+D}M_{10}S_D$-B-7; rows 4, TSWV-D; and rows 5, TSWV-10. Identical amino acids are indicated by dashes.

comitant alteration in the N gene, provides support for the hypothesis that overcoming TNDR is a cooperative event involving two or more elements. It is probable that the capacity of the reassortant $L_{10}M_{10}S_D$ to break TNDR results from the concerted interaction among viral proteins resident on separate viral RNAs contributed by each of the original viruses. That neither parent alone is capable of overcoming TNDR is further support of this hypothesis. The resistance may be broken directly by the cooperative effect of viral proteins that permits systemic movement or by the suppression of the resistance response such as has been proposed for suppression of posttranslational transgene silencing (3,6,26).

Preliminary genetic mapping of the determinants of the resistance-breaking phenotype and of systemic movement (44) also supports the involvement of genome segments other than the S RNA. The mechanism of driving the reassortment of the genome RNAs into an "adapted" combination could include the preferential selection of segments by the resistant hosts or that the RNAs from one parental isolate versus the corresponding RNAs from the other parental isolate are preferentially acceptable template for the RdRp. Thus, an equally significant question is does the host influence the RdRp complex of TSWV to favor one template over the other in response to the selection? A previous observation suggested that the S RNA from TSWV-D was more competitive than the corresponding S RNA from TSWV-10 (44) and the individual RNAs rather than the overall genome was specifically selected in response to the selection (Fig. 2 and Table 2). Although details of how the RdRp complex actively interacts with their templates have not been elucidated, these observations suggest the possible role of the viral RdRp complex in the genetic reassortment.

The possibility of generating new viruses on the viral pathogen-derived resistant plants is still controversial (16,34,45). In this study, the tendency of TNDR to drive the mixed viral population to form a novel resistance-breaking isolate (Fig. 2 and Table 2) supports the speculation that the potential risk of generating a new virus exists on the transgenic plant as it does with the natural, host-derived resistance. It is known that new TSWV isolates can be generated on host-derived resistant plants (7,41). The emergence of the novel isolate results from the genome reassortment indirectly influenced by TNDR rather than from the direct rearrangement between the viral genome and the transgene, which provides evidence for an additional mechanism for adaptation of plant viruses (19,24,32,47). Mixed infections of various TSWV isolates occur commonly on host plants, as evidenced by the existence of heterogeneous populations in the field (5,9,10,37,48,51,53; J. W. Moyer, *unpublished data*). Therefore, the opportunities for TSWV isolates to adapt to new host genotypes are greatly increased. Since resistant plants have the potential to drive viral populations to generate a resistance breaking isolate(s), we suggest that transgenic plants should be assessed using mixed infections of genetically compatible isolates to test the efficiency of resistance and to predict the possibility of inducing new isolates.

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