**An efficient and high fidelity method for amplification, cloning and sequencing of complete
tospovirus genomic RNA segments**

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ABSTRACT

Tospoviruses (genus *Tospovirus*, family *Bunyaviridae*) are responsible for major losses in an extensive range of crops worldwide. New species of these single-stranded, ambisense RNA viruses regularly emerge and have been shown to maintain heterogeneous populations with individual isolates having quite variable biological and virulence characteristics. Most tospovirus phylogenetic studies have focused on analysis of a single gene, most often the nucleocapsid protein gene. Complete genomic RNA segment amplification as a single fragment would facilitate more detailed analyses of genome-wide sequence variability, but obtaining such sequences for a large number of tospovirus isolates using traditional methods of amplification and cloning of small overlapping fragments is tedious, time consuming and expensive. In this study, protocols were optimized to amplify, clone and sequence full-length M- and S-RNA genome segments of *Tomato spotted wilt virus* and *Impatiens necrotic spot virus*. The strategy presented here is straightforward, scalable and offers several advantages over the previously commonplace and overlapping amplicon-based approach. Use of whole genome segments, instead of individual gene sequences or defined portions of genome segments, will facilitate a better understanding of the underlying molecular diversity of tospoviruses in mixed infections.

Keywords: *Bunyaviridae*, *Tospovirus*, *Tomato spotted wilt virus*, *Impatiens necrotic spot virus*, M-RNA, S-RNA.

• 1. Introduction

Members of the genus *Tospovirus* (family *Bunyaviridae*) are plant- and insect-infecting viruses that cause economically significant constraints to horticultural, agronomic and field crops worldwide (German, et al., 1992; Adkins, 2000; Whitfield et al., 2005; Pappu et al., 2009; Riley et al., 2011). *Tomato spotted wilt virus* (TSWV), the type member of the genus, is the most widespread across many continents with a host range of more than 1,000 plant species (Parella et al., 2003). Annual crop losses due to TSWV alone have been estimated at about \$1 billion worldwide (Sherwood et al., 2001). For these reasons, TSWV is by far the most extensively studied among the currently recognized tospovirus species (Adkins, 2000). Other tospoviruses, including *Impatiens necrotic spot virus* (INSV), *Iris yellow spot virus* (IYSV), *Groundnut ringspot virus* (GRSV), *Peanut bud necrosis virus* (PBNV) and *Tomato chlorotic spot virus* (TCSV), have more limited geographic distribution and vary regionally in importance. All tospoviruses are transmitted plant to plant by several thrips species (Thysanoptera: Thripidae) in a persistent, propagative manner, with successful transmission by adults occurring only when the virus is acquired by larvae (Rotenberg et al., 2015 and citations).

The genome of tospoviruses is composed of three single-stranded RNA segments, designated as large (L), medium (M) and small (S) RNAs (Adkins, 2000; Whitfield et al., 2005). The negative sense L-RNA is approximately 9 kb and encodes the RNA-dependent RNA polymerase in the viral complementary (vc) sense RNA. In contrast, the M- and S-RNAs are approximately 5 kb and 3 kb, respectively, and encode structural and non-structural proteins in an ambisense strategy. The M-RNA encodes the movement protein (NSm) in the viral (v) sense

and the glycoprotein precursor ($G_N G_C$) in the *vc* sense. The S-RNA encodes the non-structural silencing suppressor protein (NSs) in the *v* sense and the nucleocapsid (N) protein in the *vc* sense. The three genomic RNAs are each encapsidated by the N protein and packaged into pleomorphic virions of 80 to 120 nm in diameter. The two mature glycoproteins (G_N and G_C), cleaved post-translationally from the glycoprotein precursor, are embedded into host-derived lipid envelope membranes of virions and seen as spike-like projections on the surface of virions.

Full-genome sequence data has been generated for representative isolates of tospoviruses, that together with biological data, enabled recognition of distinct species in the genus *Tospovirus* (King et al., 2011). In addition, several studies have provided insight into the genetic diversity of tospoviruses, with particular emphasis on TSWV (Tsompana et al., 2005; Tentchev et al., 2011; Margaria et al., 2015). The N gene sequence, and to a limited extent partial sequences of other regions of the genome, have been the focus in these studies for elucidating genetic diversity to gain greater insight into viral evolution, fitness and emergence of virulent strains capable of breaking host-gene or transgene-mediated resistance (Margaria et al., 2004). Recently, Lian et al. (2013) have used complete genome sequences to study molecular diversity of TSWV. However, standard RT-PCR methods with sequence-specific primers were used in these studies to amplify defined regions of the virus genome and the amplicons were used for sequencing, assembling virus genome contigs and downstream applications. Thus, these approaches do not generate complete genome segments in a single amplification reaction for comparative genome-wide variability and attributing the source of different sequences derived from distinct strains in mixed infections. The segmented genome of tospoviruses and the propensity for generating viable reassortants leading to altered epidemiological characteristics (Qui and Moyer, 1999; Webster et

al., 2015) and the ‘quasispecies’ nature of viruses with RNA genomes in general, makes it a challenge to generate comprehensive information on genetic diversity and evolution.

In this report, a strategy is described to successfully clone the complete M- and S-RNA genome segments of TSWV and INSV directly from virus-infected samples. This approach is scalable to analyze large numbers of virus-infected samples from different plant species and is broadly applicable for generating sequence coverage for complete genome segments in single and mixed infections of tospoviruses. It also provides an opportunity to gain greater insight into genetic diversity and critically analyze molecular factors underlying the emergence of tospovirus variants with altered epidemiological characteristics.

2. Materials and Methods

2.1. Virus Isolates

TSWV and INSV isolates collected from basil (*Ocimum basilicum*) in a nursery in Richland, WA, were maintained in *Emilia sonchifolia*. Total RNA was isolated from symptomatic tissue using the RNeasy Plant Mini Kit following the manufacturer’s instructions (Qiagen Sciences, Germantown, MD), quantified using a Nanodrop 2000c spectrophotometer (ThermoFisher Scientific, Waltham, MA), and stored at -80°C until use. The presence of each virus in source plants was confirmed by RT-PCR using procedures described previously for TSWV (Adkins & Rosskopf, 2002) and INSV (Poojari and Naidu, 2013).

2.2. Primers

Initially, primers were designed based on consensus sequences of TSWV and INSV available in databases to use for cDNA synthesis and amplification of genomic segments of each virus in several overlapping DNA fragments. Subsequently, primers were designed based on the sequences generated in this study to amplify each RNA segment of individual viruses as single DNA fragments. Primer names, sequences and positions on the respective genome segment of each virus are listed in Table 1.

2.3. cDNA synthesis, RT-PCR and cloning

Total RNA preparations were used as template for first-strand cDNA synthesis using a Superscript III reverse transcriptase kit (Invitrogen Corp, Carlsbad, CA) according to the manufacturer's instructions. A mix of random hexamers and segment-specific primers were used in separate assays for the synthesis of cDNA specific to M- and S-RNA genome segments. Each assay was incubated at 50°C for 60 min and subsequently at 70°C for 15 min to terminate the reaction. Subsequently, cDNA was treated with RNaseH (New England Biolabs, Ipswich, MA), according to the manufacturer's instructions, to degrade RNA in the RNA:cDNA hybrid.

Initially, the single-stranded cDNA served as template to amplify different portions of the M- or S-RNA as several overlapping DNA fragments (Fig. 1 for TSWV and Fig. 2 for INSV). The same cDNA preparation was subsequently used as a template to amplify complete M- and S-RNA genome segments. Specific primer pairs used to amplify DNA fragments covering most of each genome segment as well as complete genome segments are listed in Table 1. For TSWV,

127 primers TS CM1v and TS CM5vc were used to amplify the complete M-RNA segment, whereas
128 primers TS CS1v and TS CS3vc were used to amplify the complete S-RNA segment. For INSV,
129 primers IN NSmv and IN GL3vc were used for amplification of the complete M-RNA segment,
130 whereas primers IN NSsv and utrNvc were used to amplify the complete S-RNA segment. Each
131 50-μL PCR reaction contained 200 to 1000 ng of cDNA template, 200 μM of each dNTP, 0.3 μM
132 each of virus-specific primers, 1X concentration of PrimeSTAR GXL reaction buffer with 1mM
133 Mg^{+2} and 1.25U of high fidelity PrimeSTAR GXL DNA polymerase (Takara Bio Inc, Japan).
134 Aliquots of PCR products were resolved on 1.5% agarose gels containing GelRed Nucleic Acid
135 Stain (Biotium Inc., Hayward, CA) and DNA bands were visualized using the GelDoc XR+ and
136 Image Lab 2.0 software (Bio-Rad Laboratories, Hercules, CA). The remaining PCR products
137 were purified with QIAquick PCR Purification Kit (Qiagen Sciences, Germantown, MD)
138 according to the manufacturer's instructions, mixed with dA-Tailing reaction mixture containing
139 10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl (pH 8.3), 0.2mM dATP, 1U of Taq DNA
140 polymerase (Roche, Mannheim, Germany) and incubated at 72°C for 30min. The PCR products
141 were subsequently cloned into pCR-XL-TOPO[®] vector and the ligated product transformed into
142 One Shot[®] TOP10 chemically competent *Escherichia coli* according to the manufacturer's
143 instructions (Life Technologies, Carlsbad, CA). Colonies of *E. coli* harboring recombinant
144 clones were screened for the presence of inserts of the expected size by colony PCR. Plasmid
145 DNA from positive recombinant clones was purified using the QIAprep Spin Miniprep Kit
146 according to the manufacturer's instructions (Qiagen Inc., Valencia, CA). Concentration and
147 purity was estimated using the Nanodrop 2000c spectrophotometer (ThermoFisher Scientific,
148 Waltham, MA). A minimum of three independent clones for each amplicon were sequenced in

both orientations using M13 forward and reverse primers as well as virus genome segment-specific primers(Retrogen Inc., San Diego, CA).

2.4. Sequence analysis

Raw nucleotide sequences of overlapping cDNA clones representing different portions of individual M- and S-RNA segments and those generated for complete genome segments were edited and assembled into contigs using Vector NTI Advance 11 sequence analysis software (Invitrogen Corp, Carlsbad, CA). Multiple alignments of nucleotide and amino acid sequences were made using CLUSTAL W (Thomson et al., 1994) with default settings in MEGA6 software (Tamura et al., 2013). Sequence identities were obtained using Vector NTI Advance 11 program. The M- and S-RNA sequences of TSWV and INSV generated in this study were deposited in GenBank with accession numbers KU179514, KU179515, KX790322 and KX790323.

3. Results

The M-and S-RNA genome segments of TSWV were initially amplified, respectively, in five or three overlapping segments using sequence-specific primers (Table 1; Fig. 1). The cDNA clones specific to each genome segment were then sequenced in both orientations by a ‘genome walking’ approach using progressive sequence-specific primers (Table 1). These sequences were assembled to build a consensus sequence for each genomic RNA. The sizes of the complete M- and S-RNA segments were determined to be 4,767 and 2,952 nt, respectively. Using these newly

determined sequences, we designed specific primers (Table 1) to successfully amplify the complete M- or S-RNA genome segments of TSWV (Fig.1). The nucleotide sequences of clones specific to M-orS-RNAs were determined using the ‘genome walking’ approach described above. The derived nucleotide sequences were 100% and 99.8% identical, respectively, to the corresponding M-and S-RNA sequences generated from overlapping amplicons indicating high fidelity amplification of M-and S-RNAs of TSWV. The genome organization of both RNA segments was in agreement with published reports of TSWV. The M- and S-RNA sequences of the TSWV isolate characterized in this study showed high sequence identities with isolates reported previously from California (Tsompana et al., 2005), with the M-RNA sharing 99.0% identity with isolate CA-4 (accession no. AY744482) and the S-RNA sharing 98.0% identity with isolate CA-2 (accession no. AY744469).

To ascertain if this strategy could be extended to other tospoviruses, we used total RNA from INSV-positive samples to amplify complete M- and S-RNA segments using virus-specific primers. By this approach, the M-RNA was amplified in five overlapping fragments and the S-RNA segment was amplified into two overlapping fragments (Fig. 2). As described above, all clones were sequenced in both orientations using a ‘genome walking approach’ and INSV genome segment-specific primers. The sizes of the complete M- and S-RNA segments were determined to be 4,950 and 3,012 nt, respectively. The complete M-and S-RNA segments were amplified as a single fragment of expected size using segment-specific primers listed in Table 1. The amplicons (Fig. 2) were cloned and sequenced using methods described above. The derived nucleotide sequences were 98.2% and 99.2% identical to the M- and S-RNA sequences, respectively, assembled from the overlapping amplicons. The overall genome organization of M- and S-RNAs was similar to corresponding genome segments of currently published INSV

isolates, with the M-RNA sharing 97.8% identity with the original impatiens isolate (accession no. M74904) and the S-RNA sharing 96.6% identity with isolate NL-07 (accession no. X66972).

4. Discussion

The results presented in this study demonstrate that amplification of complete tospovirus genomic RNAs is a viable and attractive alternative to amplification of overlapping fragments, and offers several advantages for whole genome sequencing. A significant benefit is high throughput processing of a large number of field samples for generating clones representing the complete genomic RNAs by eliminating multiple steps and potential pitfalls associated with cloning and sequencing of overlapping portions of individual genomic RNA segments. In addition, the high fidelity and specificity of the DNA polymerase used minimizes PCR-generated errors, thereby ensuring high quality sequence data generated from complete genome segment clones rather than genome sequence data assembled from independent clones of different regions of each genome segment.

Although results presented in this study demonstrate the applicability of this approach for two distinct tospovirus species, this strategy can be readily extended for simultaneous amplification of all complete genomic RNA segments of other tospovirus species. Additionally, amplification of complete genomic RNAs can be achieved directly from infected field samples collected from a wide variety of plant hosts and from a diverse set of virus genotypes. The consensus sequences generated using this approach embody a more accurate representation of the *in vivo* genetic diversity than the previously commonplace and indirect approaches of using greenhouse inoculated plant tissues in concert with amplification of partial or overlapping

genome fragments. The exactness of the final sequence is especially important when evaluating population genetics and evolutionary structure using advanced statistical and computational methods that can be easily skewed by minor errors. In addition, this new approach allows for significant reductions in time and resources necessary to complete the process, facilitating more thorough and efficient future studies on tospoviruses.

In contrast to overlapping amplicon-based approaches (Lian et al., 2013), our strategy is advantageous in investigating the possible occurrence of mixtures of distinct sequence variants of a tospovirus species for understanding the genetic structure of field populations within natural hosts. Since sequence divergence varies across different portions of the tospovirus genome segments, whole genome-based methods offer additional insight for understanding the underlying molecular differences between corresponding genome segments of virus strains co-infecting a single host. Likewise, amplification of complete genome segments of the M- and S-RNAs of distinct tospoviruses co-infecting a single plant host can be achieved to examine the dynamics of mixed virus infections and spread of individual viruses by thrips vectors. On a broader scale, simultaneous analyses of complete genome segments from an infected sample can provide more reliable information about the evolutionary mechanisms, genome segment reassortments and epidemiological dynamics of viruses with segmented genomes.

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Table 1.

Primers used for sequencing complete M- and S-RNAs of *Tomato spotted wilt virus* (TSWV) and *Impatiens necrotic spot virus* (INSV).

Virus	RNA ^a	Primer name ^b	Nucleotide sequence ^c	Location ^d
TSWV	M	TS CM1v	AGAGCAATCAGTGCRTCAGAAATATACC	1-28
		TS CM1vc	CTTTTATCTTTATCAGCTCTGGGTGAATC	801-773
		TS CM2v	TACAAGAARGGGGTTTCTTTTGGTAG	716-741
		TS CM2vc	CTGGTTTAGARCAAATATCAGATATTCC	2057-2030
		TS CM3v	TAACTTGCAATGAAATGAATGG	1511-1533
		TS CM3vc	GATTATACAGGAAGCTCTTTRGG	3006-2984
		TS CM4v	GAGATTGTTCTATCATRTATCTATATTCCC	2775-2805
		TS CM4vc	TTGATAGCTTTGTGTGATCTATTTCCC	3708-3683
		TS CM5v	TGGAAAATATTTCCATAAGCARTTTATRAGC	3568-3598
		TS CM5vc	AGAGCAATCAGTGCAAACAAAACC	4767-4743
	S	TS CS1v	AGAGCAATTGTGTCATAATTTTATTCWTAATC	1-32
		TS CS1vc	CCGCTTAAATCAAGCTTTAACAAAG	1437-1413
		TS CS2v	CAAAGGAAATATTTCTTTCAAAAAC	12581284
		TS CS2vc	TGCACTGTGCTGAAAAGCAAAGC	2222-2200
		TS CS3v	GCTGGAGCTAAGTATAGCAGC	2136-2156
		TS CS3vc	AGAGCAATTGTGTCAATTTTATTCAAACC	2952-2924
INSV	M	IN NSmv	AGAGCAATCAGTGCATCAAAATTATATC	1-28
		IN NSmvc	GATATCTCTATCTTGTTATCAGTAAC	978-953
		IN MIgrv	TGCAAGTTAATCATACCTAAAGGC	851-874
		IN MIgrvc	GTTGCCACAATGTGTAGAGGCTA	1600-1578
		IN GL1v	TTTAATCATATACCGCTGAAGTCTAG	1446-1471
		IN GL1vc	TTGTGGGAGCAAAGTGTTCCTAG	2556-2533
		IN GL2v	ATTTAATCTAGCTATGTTTCCCGCAT	2390-2415
		IN GL2vc	GTTTGGTAACCCATGAATGCTC	3711-3690
		IN GL3v	ATCCTATCAAAGTTTCTGTGACTGC	3505-3529
		IN GL3vc	AGAGCAATCAGTGCAAACAAAATC	4950-4926
	S	IN NSsv	AGAGCAATTGTGTACGAACAACC	1-24 ^e
		IN NSsvc	GTAAACAACTTTACTTAGGAACAAG	1467-1442
		IN SIgrV	ATTGGAAATTTGACTATGCTGC	1339-1360
		IN SIgrvc	TACAGCATGTTTAATGCTAC	2125-2106
		IN Ngv	ATGCATTTAACACACACAAAGC	2032-2054
		IN Ngvc	GCAGATCAAGATGAACAAAGC	2873-2853
		IN utrNv	GAGCCAGATCTAAAGCCAAAAAAG	1182-1205
		IN utrNvc	AGAGCAATTGTGTACGAATATAAC	3012-2988

^a Genomic RNA segment amplified, with 5' and 3' terminal sequences targeted for full segment coverage.

^b v, viral sense and vc, viral complementary sense.

^c Sequences based on available complete TSWV and INSV M- and S-RNA segments in GenBank. R=A or G, W=A or T.

^d Primer locations based on TSWV and INSV M- and S-RNA sequences from this study (KU179514, KU179515, KX790322 and KX790323).

^e Primer location based on accession DQ425096.

Figure Legends

Figure 1. Amplification of *Tomato spotted wilt virus* (TSWV) M- and S-RNAs. **A**, TSWV M-RNA genome organization showing non-structural (NSm) and glycoprotein precursor ($G_N G_C$) coding regions. Diagram above genome depicts amplification of the complete genome segment using primers specific to M-RNA termini (●). Diagram below genome depicts amplification of overlapping fragments with internal (◆) and termini-specific (●) primers. **B**, Agarose gel electrophoresis of complete M-RNA (lanes 1-2) and overlapping M-RNA fragments (lanes 3-12) amplified by RT-PCR. Total RNA from TSWV-infected (lanes 1, 4, 6, 8, 10 and 12) and non-infected (lanes 2, 3, 5, 7, 9 and 12) plants was used as template in RT-PCR assays. M, 1 kb plus DNA marker. **C**, TSWV S-RNA genome organization showing non-structural (NSs) and nucleocapsid (N) coding regions. Diagram above genome depicts amplification of the complete genome segment using primers specific to S-RNA termini (●). Diagram below genome depicts amplification of overlapping fragments with internal (◆) and termini-specific (●) primers. **D**, Agarose gel electrophoresis of complete S-RNA segment (lanes 1-2) and overlapping S-RNA fragments (lanes 3-8) amplified by RT-PCR. Total RNA from TSWV-infected (lanes 1, 4, 6 and 8) and non-infected (lanes 2, 3, 5 and 7) plants was used as template in RT-PCR assays. M, 1 kb plus DNA marker.

Figure 2. Amplification of *Impatiens necrotic spot virus* (INSV) M- and S-RNAs. **A**, INSV M-RNA genome organization showing non-structural (NSm) and glycoprotein precursor ($G_N G_C$) coding regions. Diagram above genome depicts amplification of the complete genome segment using primers specific to M-RNA termini (●). Diagram below genome depicts amplification of

330 overlapping fragments with internal (◆) and termini-specific (●) primers. **B**, Agarose gel
331 electrophoresis of complete M-RNA (lanes 1-2) and overlapping M-RNA fragments (lanes 3-12)
332 amplified by RT-PCR. Total RNA from INSV-infected (lanes 1, 4, 6, 8, 10 & 12) and non-
333 infected (lanes 2, 3, 5, 7, 9 & 11) plants was used as template in RT-PCR assays. M, 1 kb plus
334 DNA marker. **C**, INSV S-RNA genome organization showing non-structural (NSs) and
335 nucleocapsid (N) coding regions. Diagram above genome depicts amplification of the complete
336 genome segment using primers specific to S-RNA termini (●). Diagram below genome depicts
337 amplification of overlapping fragments with internal (◆) and termini-specific (●) primers. **D**,
338 Agarose gel electrophoresis of complete S-RNA segment (lanes 1-2) and overlapping S-RNA
339 fragments (lanes 3-6) amplified by RT-PCR. Total RNA from INSV-infected (lanes 1, 4 & 6)
340 and non-infected (lanes 2, 3 & 5) plants was used as template in RT-PCR assays. M, 1 kb plus
341 DNA marker.

Figure 1

Figure 2