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Complete sequence of three different biotypes of *Tomato spotted wilt virus* (wild type, tomato *Sw-5* resistance-breaking and pepper *Tsw* resistance-breaking) from Spain

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Abstract

Tomato spotted wilt virus (TSWV) is worldwide spread and causes production losses in many important horticultural crops such as tomato and pepper. Breeding resistant cultivars has been the most successful method for disease control. So far, only two resistance genes have been found to confer resistance against a wide spectrum of TSWV isolates: *Sw-5* in tomato and *Tsw* in pepper. However, TSWV resistance-breaking isolates have emerged in different countries after a few years of implementation of resistant cultivars. In this paper we report the first complete nucleotide sequence of three TSWV isolates from Spain with different biotypes according to the ability of overcoming resistance: LL-N.05 (wild type, WT), Pujol1TL3 (*Sw-5* resistance breaking, SBR) and PVR (*Tsw* resistance-breaking, TBR). The genome of these TSWV isolates consisted of three genomic segments: L (8913-8914 nt), M (4752-4825 nt) and S (2924-2961 nt). Variations in nucleotide sequences and genomic lengths among the different virus biotypes are reported here. Phylogenetic analysis of the five TSWV open reading frames showed evidences of reassortment between genomic segments of LL-N.05 and Pujol1TL3, which was supported by analysis with different recombination-detecting algorithms. Genetic distances were uncorrelated to biotype, host or geographic location.

Tomato spotted wilt virus (TSWV) is the type member of the genus *Tospovirus* which contains the only plant-infecting members of the family *Bunyaviridae* [23]. TSWV has a wide hosts range including more than 1000 plant species among weed species, ornamental and horticultural crops such as pepper, potato, tobacco, peanut, lettuce and bean [9, 21, 30]. The virus is naturally transmitted by several thrips species (*Thysanoptera: Thripidae)* in a persistent and propagative manner with *Frankliniella occidentalis* (Pergande) being its main vector [7, 31].

TSWV virions are quasi-spherical composed of an outer membrane envelope derived from the host with two viral-coded glycoproteins (G_N and G_C) embedded. Inside there are several copies of the RNA dependent RNA polymerase (RdRp) and nucleoproteins which encapsidate the genome consisting of three negative-sense or ambisense RNA segments: segment L (~9 kb) encodes the RdRp; segment M (~5 kb) encodes the cell-to-cell movement protein (NSm) and a precursor of the surface glycoproteins (G_N/G_C) involved in TSWV transmission by thrips; and segment S (~3 kb) encodes a silencing suppressor (NSs) and the nucleocapsid (N) [23].

TSWV is one of the most harmful viral pathogens, ranking second in the list of the most important plant viruses worldwide [27]. Eradication or control of TSWV has proven to be difficult, being breeding for resistance the most effective. So far, only two genes *Sw-5* introgressed in tomato (*Solanum lycopersicum*) and *Tsw* in pepper (*Capsicum annuum*) have conferred resistance against a wide spectrum of TSWV isolates [21]. However, resistance-breaking TSWV isolates have been reported in several countries after a few years of using these resistant cultivars [16, 19]. Based on the ability of the virus to overcome the resistance conferred by these genes, TSWV isolates are classified in three biotypes: wild type (WT) which cannot infect tomato and pepper with the resistance genes *Sw-5* and *Tsw* respectively; *Sw-5* resistance-breaking (SBR) which can infect *Sw-5* resistant tomato but not *Tsw* resistant pepper; and *Tsw* resistance-breaking (TBR) which can infect *Tsw* resistant pepper but not *Sw-5* resistant tomato. Currently, no natural TSWV isolates infecting both *Sw5*-resistant tomato and *Tsw*-resistant pepper has been reported.

Inoculation in resistant tomato or pepper of reassortants generated between WT and resistance-breaking isolates showed the genetic determinants for overcoming tomato *Sw5*-resistance and pepper *Tsw* resistance were located in the M and S segments, respectively [10, 12]. Comparison of nucleotide and amino acid sequences of the M segment from WT and SBR isolates revealed that *Sw5*-resistance breakdown was related to substitutions C118Y or T120N in

TSWV NSm protein [16], which was demonstrated by transient expression of NSm in *Sw-5* resistant plants by using an heterologous viral system [22]. Analysis of nucleotide sequences of the S segment from WT and TBR isolates suggested several substitutions in TSWV NSs protein could be responsible for *Tsw* resistance-breakdown [19, 29] and transient expression confirmed that NSs is the avirulence protein triggering resistance in pepper cultivars carrying the gene *Tsw* [3, 5].

Presently, no complete sequence of SBR and TBR isolates are available, although there are partial sequences [16, 19, 29]. The nucleotide sequence of the complete genome has been determined for 17 WT isolates (Table 1): one from Brazil [1, 2, 13], two from China [11], 14 from South Korea [14, 15] and two from Italy [18]. Here, we report the complete sequence of three isolates from Spain, corresponding to WT, SBR or TBR biotypes (Table 1).

Samples from tomato with and without *Sw-5* gene and from pepper with *Tsw* gene were collected in North-Eastern and South-Eastern Spain. To obtain pure WT or resistance-breaking TSWV isolates free of other viruses, the samples were biologically cloned by mechanical inoculation in *Nicotiana glutinosa* to produce local lesions which were used to inoculate *Datura stramonium* [7]. To determine the biotype (WT, SBR or TBR), each TSWV isolate was inoculated in tomato cultivars: 'Verdi' (Fitó), with *Sw-5*, and 'Marmande' (Fitó) without *Sw-5*; and pepper cultivars 'Spiro' and 'Divino' (Seminis), both with *Tsw*; and 'C804' (Fitó), without *Tsw*. For sequencing the complete genome, one isolate of each biotype was selected: LL-N.05, Pujol1TL3 and PVR corresponding to biotypes WT, SBR and TBR, respectively (Table 1). Total RNAs were purified from *D. stramonium* plants infected by these TSWV isolates by a protocol involving phenol/chloroform/isoamyl-alcohol extraction followed by isopropanol precipitation [6]. RT-PCR was performed with conserved primers to amplify the complete S segment (primers S1 / S7), two overlapping fragments comprising the complete M segment (primers M1 / M7 and M6 / M12, and two overlapping fragments comprising the complete L segment (primers 1L_f / 3L_r and 2L_f / 1L_r). For RT-PCR, total RNA (~5 μg) was denatured by heating at 65ºC and chilling quickly on ice. First-strand cDNA synthesis was performed in a 20 μl reaction mixture containing the denatured RNA, first strand buffer, 1 mM DTT, 200 μM each of dNTPs, 0.4 μM of each primer, 40 U of RNAse OUT inhibitor and 100 U of SuperScript™ II Reverse Transcriptase (Invitrogen) and incubating at 42ºC for 50 min. The reaction was inactivated by heating at 70°C for 15 min. Removal of RNA complementary to the cDNA was performed by adding 2 U of E.

coli RNase H (Invitrogen) and incubating at 37°C for 20 min. PCR was performed in a 50 µl reaction containing 5 x iProof HF buffer, 0.2 mM $MgCl_2$, DMSO 3%, 200 µM of each dNTP, 0.2 µM of each specific primer, and 1 U of iProof High-Fidelity DNA polymerase (BIORAD). The PCR conditions were: 1 cycle at 98ºC for 30 s, followed by 5 cycles at 98ºC for 10 s, 60ºC for 20 s and 72ºC for 30 s/Kb, next, 30 cycles at 98ºC for 10 s, 65ºC for 20 s and 72ºC for 30 s/kb, and a final extension step of 72ºC for 10 min. Nucleotide sequences were determined with primers encompassing overlapping regions of about 1 kb covering the whole genome by using a ABI 3130XL Genetic Analyzer (Life Technologies, USA)

The 5'- and 3'- terminal sequences were determined using the 5'/3' RACE Kit 2nd Generation (Roche). To avoid errors associated with RT-PCR and sequencing, each genomic region was amplified and sequenced at least twice in both directions.

The pool of sequences was assembled with Vector NTI [17]. The complete nucleotide sequences of segments L and S of TSWV isolates LL-N.05, Pujol1TL3 and PVR and segment M of PVR were deposited in the GenBank database under accession numbers KP008128-KP008134 and nucleotide sequences of segment M of isolates LL-N.05 and Pujol1TL3 were retrieved from GenBank under accession numbers FM163373 and HM015520 (Table 1).

The three TSWV isolates LL-N.05, Pujol1TL3 and PVR have the typical genome organization of TSWV. The sizes of L, M and S segment were 8913-8914 nt, 4752-4825 nt and 2924-2961 nt, respectively. The length of the open reading frames (ORFs) and 5'- and 3'-untranslated regions (UTRs) were identical or almost identical for the three isolates: RdRP (8640 nt), NSm (909 nt), G_N-G_C (3408), NSs (1404 nt), and N (777 nt), L segment 5'-UTR (33 nt) and 3'-UTR (240-241 nt), M segment 5'-UTR (100 nt) and 3'-UTR (84 nt), and S segment 5'-UTR (88 nt) and 3'-UTR (151). However, the intergenic regions (IR) had different sizes for each isolates: M segment IR had 251, 324 or 280 nt and S segment IR 504, 541 and 531 nt, for LL-N.05, Pujol1TL3 or PVR, respectively. This shows that differences in RNA size were mostly caused by insertions and/or deletions of nucleotide sequences within the IR. Nucleotide positions are showed in Table 2.

The complete sequences of isolates LL-N.05, Pujol1TL3 and PVR were aligned and compared with all complete sequences available for TSWV retrieved from GenBank (Table 1) by using the algorithm CLUSTALW implemented in the program MEGA V6.0 [28]. Nucleotide and amino acid sequence identities for different genomic regions between TSWV isolates were

calculated from the p-distance (proportion of distinct nucleotides between two sequences) with MEGA V6.0 as (1 – p-distance) × 100. Nucleotide identities between isolates LL-N.05, Pujol1TL3 and PVR and the others varied depending of the genomic region and isolates ranging from 85.2 to 100% for the non-coding regions and from 93.1 to 99.3% for the ORFs.

Phylogenetic trees of the nucleotide sequences of the five TSWV ORFs were inferred by the maximum likelihood (ML) method with the nucleotide substitution model best fit for each ORF and 1000 bootstrap replicates to estimate the statistical significance of each node, using the program MEGA V6.0. The phylogenetic relationships of the Spanish TSWV isolates changed for the three genomic segments, but they were very similar for the two ORFs within each segment (Fig. 1), suggesting reassortment but no recombination. Thus, for ORF RdRp (L segment) isolate LL-N.05 was very close to isolate Pujol1TL3 and separated from isolate PVR, whereas for ORFs Nsm and G_N/G_C (M segment) the three isolates were in different clades and for ORFs NSs and N (S segment) LL-N.05 was close to PVR and separated from Pujol1TL3. Interestingly, for the five ORFs the Spanish isolate Pujol1TL3 clustered with the Italian isolate p202/3wt and the Korean isolates TSWV-12 and TSWV-17 indicating a common origin and suggesting that reassortment occurred in the ancestor of these isolates before the virus migrated between these countries. The other Italian isolate p105 was also close to the Spanish isolates (PVR for RdRp and LL-N.05 for the other ORFs). Comparison of all isolates showed no correlation between geographic location and genetic relationships, e.g. for G_N/G_C the Spanish isolate PVR and the Korean were in the same clade separated from the Spanish isolate Pujol1TL3 and the Korean TSWV-12 which were included in another clade (Fig. 1). This suggests long distance migration of some isolates and possibly genetic stability of different TSWV strains which might occupy distinct adaptive peaks as it has occurred in other plant viruses [8, 26]. Potential genetic exchange was analyzed with the algorithms 3SeqBootScan, Chimaera, GENECON, Maxchi, RDP, SiScan, implemented in the RDP4 package [20]. Analysis of concatenated sequences of the three genomic segments L, M and S for each isolate revealed, with at least five algorithms, that isolates LL-N.05 and Pujol1TL3 could result from reassortment with the segment L from an ancestor of the Korean isolate TSWV-12 and the segments M and S from ancestors of the Brazilian isolate BR01 or the Italian isolate p105. A likely reassorment involving the L segment has been also observed for the Italian isolates [18]; reassortment but no recombination were also found between Asiatic and European isolates [29]; and several recombination and reassorment events were detected for the Korean

isolates [15]. Reassorment could represent an adaptive advantage [24] or result from differences of fitness between genomic segments for different TSWV isolates [25].

The genetic distances among TSWV isolates were uncorrelated to biotypes (resistance-breakdown ability) or plant hosts (Fig. 1). Thus, for ORF NSm (where the mutations responsible for the change of biotype WT to SBR are produced), there are WT isolates with lower nucleotide identity between them (e.g. p202/3wt and BR01, 91.6% identity) than the SBR isolate Pujol1TL3 with other WT isolates (e.g. p202/3wt, 98.9% identity). Likewise, for the ORF NSs (where the mutations responsible for change of biotype WT to TBR occurs) there are WT isolates with lower nucleotide identities (e.g. p105 and TSWV-10, 92.3% identity) than those between the TBR isolate PVR and some WT isolates (e.g. p105, 97.5% identity). This is in agreement with other studies which analyzed the nucleotide sequences of ORFs NSm or NSs from many TSWV isolates [16, 19, 29]. The probable cause is that resistance-breakdown seems to have occurred several times independently and involve a few nucleotide changes as for other plant viruses [4]. With respect to plant hosts, some TSWV isolates were more closely related to isolates from other plant species than isolates from the same plant host. E.g isolate TSWV-4 from pepper was closely related to isolate TSWV-10 from *Stellaria aquatica*, and isolate TSWV-16 from tomato, but distantly related to isolate CY-CN1 from pepper (Fig. 1). This could be due to that TSWV has similar fitness in different hosts or that only a few nucleotide changes are involved in host adaptation (fitness increase).

The complete genome sequences of TSWV isolates with different ability to overcome resistance in tomato and pepper are useful for further studies to understand emergence and evolution of TSWV adaptation to new hosts genotypes which can be applied in breeding programs to develop durable and efficient resistance cultivars against TSWV.

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Figures and tables

Figure 1. Unrooted maximum likelihood phylogenetic tree of the five ORFs (RdRp, NSm, G_N/G_C, NSs and N) of Tomato spotted wilt virus (TSWV) isolates whose complete genome has been sequenced (Table 1). Bootstrap values higher or equal to 50% are indicated in the nodes. TSWV isolates sequenced here are shadowed.

Figure 1

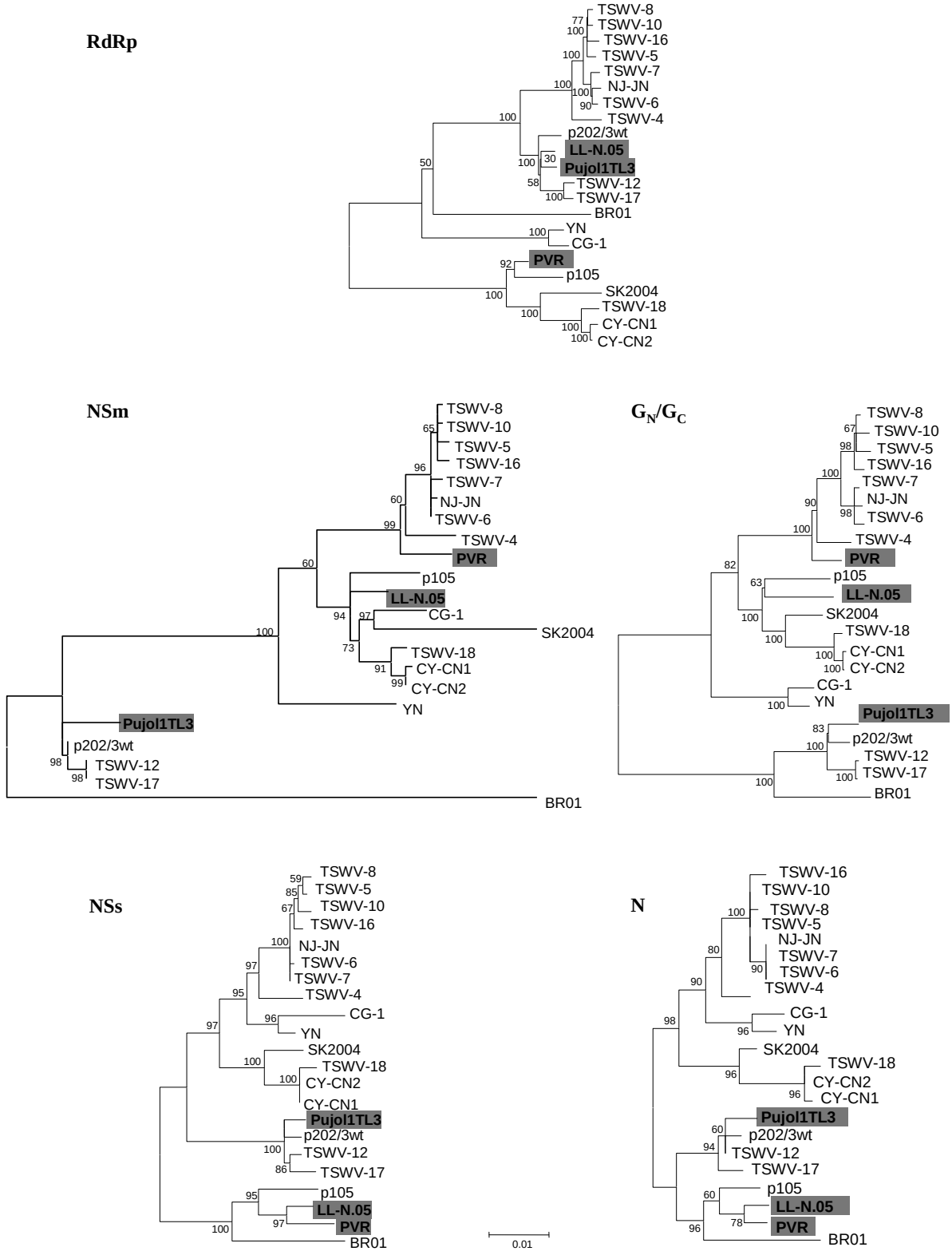
RdRp
TSWV-8
TSWV-10
TSWV-16
TSWV-5
TSWV-7
NJ-JN
TSWV-6
TSWV-4
p202/3wt
LL-N.05
Pujol1TL3
TSWV-12
TSWV-17
BR01
YN
CG-1
PVR
p105
SK2004
TSWV-18
CY-CN1
CY-CN2
NSm
TSWV-8
TSWV-10
TSWV-5
TSWV-16
TSWV-7
NJ-JN
TSWV-6
TSWV-4
PVR
p105
LL-N.05
CG-1
SK2004
TSWV-18
CY-CN1
CY-CN2
YN
Pujol1TL3
p202/3wt
TSWV-12
TSWV-17
BR01
G_N/G_C
TSWV-8
TSWV-10
TSWV-5
TSWV-16
TSWV-7
NJ-JN
TSWV-6
TSWV-4
PVR
p105
LL-N.05
SK2004
TSWV-18
CY-CN1
CY-CN2
CG-1
YN
Pujol1TL3
p202/3wt
TSWV-12
TSWV-17
BR01
NSs
TSWV-8
TSWV-5
TSWV-10
TSWV-16
NJ-JN
TSWV-6
TSWV-7
TSWV-4
CG-1
YN
SK2004
TSWV-18
CY-CN2
CY-CN1
Pujol1TL3
p202/3wt
TSWV-12
TSWV-17
p105
LL-N.05
PVR
BR01
0.01
N
TSWV-16
TSWV-10
TSWV-8
TSWV-5
NJ-JN
TSWV-7
TSWV-6
TSWV-4
CG-1
YN
SK2004
TSWV-18
CY-CN2
CY-CN1
Pujol1TL3
p202/3wt
TSWV-12
TSWV-17
p105
LL-N.05
PVR
BR01

Table 1. TSWV isolates whose genomes have been completely sequenced

Isolate	Origin	Biotype[a]	Host	GenBank accession number		
				L segment	M segment	S segment
LL-N.05	Spain	WT	Tomato	KP008128	FM163373	KP008129
Pujol1TL3	Spain	SBR	Tomato	KP008130	HM015520	KP008131
PVR	Spain	TBR	Pepper	KP008132	KP008133	KP008134
p105	Italy	WT	Pepper	KJ575620	KJ575621	DQ376178
p202/3wt	Italy	WT	Pepper	KJ575619	HQ830188	HQ830187
BR01	Brazil	WT	Tomato	NC_002052	NC_002050	NC_002051
YN	China	WT	Tomato	JF960237	JF960236	JF960235
CG-1	China	WT	Lettuce	JN664254	JN664253	JN664252
SK2004[b]	South Korea	WT	NP[b]	AB190813	AB190818	AB190819
NJ-JN	South Korea	WT	Tomato	HM581934	HM581935	HM581936
CY-CN1	South Korea	WT	Pepper1	HM581937	HM581938	HM581939
CY-CN2	South Korea	WT	Pepper2	HM581940	HM581941	HM581942
TSWV-4	South Korea	WT	Pepper	KC261947	KC261948	KC261949
TSWV-5	South Korea	WT	*Stellaria aquatica*	KC261950	KC261951	KC261952
TSWV-6	South Korea	WT	*Stellaria media*	KC261953	KC261954	KC261955
TSWV-7	South Korea	WT	Pepper	KC261956	KC261957	KC261958
TSWV-8	South Korea	WT	*Lactuca indica*	KC261959	KC261960	KC261961
TSWV-10	South Korea	WT	*Stellaria_aquatica*	KC261962	KC261963	KC261964
TSWV-12	South Korea	WT	Lettuce	KC261965	KC261966	KC261967
TSWV-16	South Korea	WT	Tomato	KC261968	KC261969	KC261970
TSWV-17	South Korea	WT	*Stellaria media*	KC261971	KC261972	KC261973
TSWV-18	South Korea	WT	*Chrysanthemum*	KC261974	KC261975	KC261976

[a]Biotypes: WT: wild type. SBR: tomato Sw-5 resistance-breaking. TBR: pepper Tsw resistance-breaking.

[b]The name and host of this isolate is not published (NP). The isolate was named in this work as SK2004.

Table 2 Conserved primers among TSWV isolates used for RT-PCR and sequencing

Segment	Primers[a]	Polarity	Sequence 5'-3'	Position[b]
L	1L_F	+	agagcaatcaggtaacaacg	1 - 20
	1b	-	gtttggttatttatgctact	1564-1583
	2a	+	atgcaaacactcaaagaatcaaat	911-934
	R1000	-	tttctttctttgtatttgct	1000-1019
	Bf	+	attattaacaagtttcggga	1501-1520
	2b	-	cattacgaaatagatatctgccac	2825-2848
	Br	-	atgctagtatttgctgtggt	2302-2321
	3a	+	caggtacatgactaaagaaa	2268-2287
	Pol3	+	taagcacaaatgccaagcc	2888-2906
	Cf	+	gagaaaaatagattggtgga	3472-3491
	Pol4	-	aaggtcatagagctcctctg	3692-3711
	2Ld	+	gagcactttctacgttatctttggacaca	4025-4053
	Cr	-	gtttgttgagcttttcttag	4285-4304
	3L_r	-	tctaatactatcattcacttcaccaggaagca	4889-4920
	6a	+	gtgataagagatttttgaacaa	5053-5075
	Df	+	gatagacgaggatgctgttt	5415-5434
	5b	-	cttttgaccttaacactctatct	5634-5656
	7a	+	gaacttagagaaaatactggacac	6369-6392
	Dr	-	cagcagtgtccagtattttc	6378-6397
	Ef	+	gaagaacaagatgaatcagg	6706-6725
	6b	-	catagttccagagacataatttcc	6724-6747
	8a	+	ggatttaatgacacagtagaa	7402-7422
	Er	-	ctgaaaaaagaggactatac	7467-7486
	7b	-	gatcatgatccaatcttattctcc	7778-7801
	1L_R	-	agagcaatcaggtacaactaaaacatataa	8868-8897
M	M1	+	agagcaatcagtgcatcagaaatatacctattatac	1-36
	M2	+	gtagatacaaaccatcatatctcaaactgg	365-394
	M3	-	tctttatcagctctgggtgaatcac	771-795
	M4	+	caaggtgagacaaatccataggtggcc	1335-1361
	M5	-	tgatgagtatgctcatgaagaacaac	1638-1663
	M6	+	caggatcattcaagtttgcaatatttccag	2268-2297
	M7	-	cttattggggatgtgaagaagcttgg	2566-2591
	M8	+	gatgttaaccctaaagagcttcctg	3029-3053
	M9	-	gtctcaaatgcccatgtctatggctc	3348-3373
	M10	+	gttataggataattatcttgtgtc	4130-4153
	M11	-	ccagaggtttatgatgattctgctgag	4579-4065
	M12	-	agagcaatcagtgcaaacaaaaaccttaatcc	4790-4821
S	S1	+	agagcaattgtgtcataattttattc	1-26
	S2	-	gaacctgtgcaaaagatgtgtgag	1113-1135
	S3	+	tcctggaagatagactttgccag	1195-1217
	S4	-	ccaaatttggccaaaattgtccctttc	1697-1723
	S5	+	atttaacacactaagcaagcacaagc	1898-1923
	S6	-	cattacagtgaaactcttaacaagttc	2038-2064
	S7	-	agagcaattgtgtcaattttat	2895-2916
	S8	+	gatcgagatgtgctataatcaagc	601-624

[a]Shadowed primers were used for RT-PCR whereas the rest, for sequencing.

[b]Positions are referenced to TSWV isolate BR01 (Table 1).