STELLINGEN

1. In tegenstelling tot de meeste andere gesegmenteerde minstrengs RNA virussen gebruikt TSWV het "prime-and-realign" mechanisme slechts zelden.
   Dit proefschrift.

2. Voor optimale TSWV transcriptie-initiatie dient er complementariteit te zijn tussen de 3'-terminale nucleotiden van cap donor en virale matrijs.
   Dit proefschrift.

3. De bewering van Richmond *et al.* dat het TSWV N eiwit volledig aspecifiek enkelstrengs RNA bindt is prematuur.
   Richmond, K.E., Chenault, K., Sherwood, J.L. & German, T.L. 1998. Characterization of the nucleic acid binding properties of *Tomato spotted wilt virus* nucleocapsid protein. *Virology* **248**, 6-11.

4. De ontdekking van de functie van "tiny RNAs" toont aan dat resultaten gevonden met behulp van cDNA micro-arrays niet per definitie hoeven te correleren met eiwit-expressieniveau's
   Ruvkun, G. 2001. Glimpses of a tiny RNA world. *Science* **294**, 797-799.

5. Een directe herkenning van dubbelstrengs RNA door de Toll-like receptor 3 is niet bewezen.
   Alexopoulou, L., Czopik Holt, A., Medzhitov, R. & Flavell, R.A. 2001. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature* **413**, 732-738.

6. De lipidensamenstelling van het virion-membraan is mede bepalend voor de fysische eigenschappen van een virion.
   Scheiffele, P., Rietveld, A., Wilk, T. & Simons, K. 1999. *Influenza* viruses select ordered lipid domains during budding from the plasma membrane. *Journal of Biological Chemistry* **274**, 2038-2044.

7. Als de "fundamentalisten" in de wetenschap op een zelfde wijze zouden discussiëren als de fundamentalisten in de religie, zouden beduidend minder promovendi de eindstreep halen.

8. Passief roken heeft niet alleen schadelijke gevolgen voor de lichamelijke gezondheid.

Stellingen behorende bij het proefschrift: "Analysis of the transcription initiation mechanism of *Tomato spotted wilt virus*", Danny Duijsings, 2001.

ANALYSIS OF THE TRANSCRIPTION INITIATION MECHANISM OF *TOMATO SPOTTED WILT VIRUS*

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ANALYSIS OF THE TRANSCRIPTION INITIATION MECHANISM OF *TOMATO SPOTTED WILT VIRUS*

Danny Duijsings

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de Rector Magnificus
van Wageningen Universiteit
Prof. Dr. ir. L. Speelman,
in het openbaar te verdedigen
op vrijdag 21 december 2001
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't is een kwestie van geduld...

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CHAPTER 1

INTRODUCTION

Tomato spotted wilt virus (TSWV) is the type species of the genus *Tospovirus* within the large *Bunyaviridae* family [94]. Most viruses belonging to this family are transmitted by arthropods and for all genera but the genus *Tospovirus* they infect animals, including humans, often causing severe and sometimes fatal diseases. The members of the genus *Tospovirus* are exclusively plant pathogens, of which TSWV causes severe crop losses in several economically important food and ornamental crops such as lettuce, tomato, sweet pepper and chrysanthemum. Tospoviruses are transmitted by thrips (*Thripidae*) and by now, nine different thrips species are known to act as a vector. The main vector is *Frankliniella occidentalis* (Fig. 1.1), but also *Thrips tabaci*, *F. schultzei*, *F. intonsa*, *F. fusca*, *F. bispinosa*, *T. palmi*, *T. setosus* and *F. zucchini* can serve as vector insects [110].

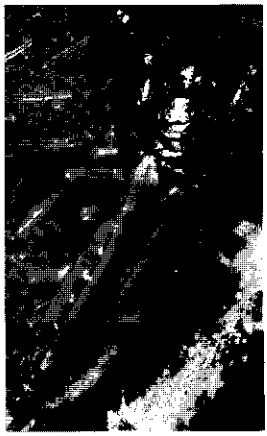

Figure 1.1. Close up of *Frankliniella occidentalis*, an important vector of *Tomato spotted wilt virus*.

Once infected by a tospovirus, symptoms of the plant may vary from chlorosis, stunting and wilting to severe necrosis in leafs and stems. Only few natural resistance genes have been identified in e.g. tomato and pepper [10,11,14,49,92], but these may not be very durable. It has been reported that the tomato SW-5 resistance gene has been broken by TSWV [65]. Hence, TSWV and other tospoviruses remain a problem of main economical impact.

Molecular biology of TSWV

Like for all members of the *Bunyaviridae*, the virion of TSWV is a membrane bound, spherical particle of approx. 80-110 nm in diameter (Fig. 1.2), containing a tripartite RNA genome. The genomic organisation of TSWV is distinct from those of the *Bunyaviridae* as two out of the three RNA segments have an ambisense coding strategy.

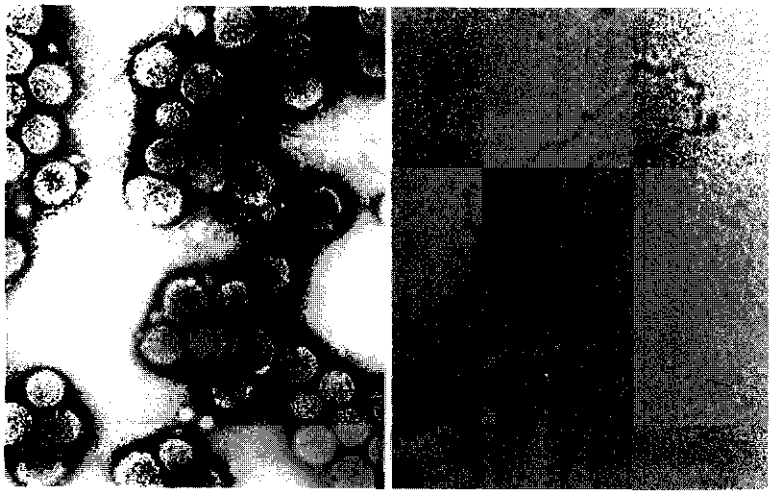

Figure 1.2. Morphology of TSWV virions and nucleocapsids. (A) Electron micrograph of TSWV virions. Particles have a diameter of 80-110 nm. (B) Electron micrograph of TSWV nucleocapsids. Nucleocapsids have a circular appearance due to base pairing between the 5' and 3' termini of their genomic RNA. The RNA is encapsidated by nucleoprotein. Bar represents 100 nm.

Only the 8,997 nt large (L) RNA segment is of complete negative polarity and contains in the viral complementary (vc) strand the open reading frame (ORF) for the L protein (Fig. 1.3) [20]. This large sized (331.5 kDa) protein represents the putative RNA-dependent RNA polymerase and is thought to encompass several enzymatic activities, i.e. transcriptase, replicase and endonuclease [1,57,109]. Several copies of the L protein are present in the virion, associated to the ribonucleoprotein complexes (RNPs) (Fig. 1.2).

The 4821 nt medium sized (M) RNA segment is of ambisense polarity and encodes the cell-to-cell movement protein (NSm) in viral sense (v) and the precursor to the envelope glycoproteins (G1 and G2) in vc sense [56].

The 2916 nt small (S) RNA segment is also of ambisense polarity and encodes a non-structural protein (NSs) in v sense and the nucleoprotein (N) in vc sense [22]. The NSs protein forms crystalline aggregates in the cell and its function in the infection cycle is still unknown [58]. Within mature virus particles, the nucleoprotein (N) is found tightly associated with the viral (anti)genomic RNA strands, thereby forming the viral RNPs. This protein has an affinity to ssRNA [91] and is able to form oligomers [105]. Both capacities are likely important in the (facilitated) formation of RNPs.

All three genomic RNAs have conserved 5' and 3' terminal sequences, of which the ultimate 8 nt are identical in all three RNA segments (5'-AGAGCAAU...; 3'-UCUCGUUA...). In each segment, this terminal complementarity is extended to ~ 65 nt, which enables the genomic RNAs to fold into a panhandle structure [21,22,56].

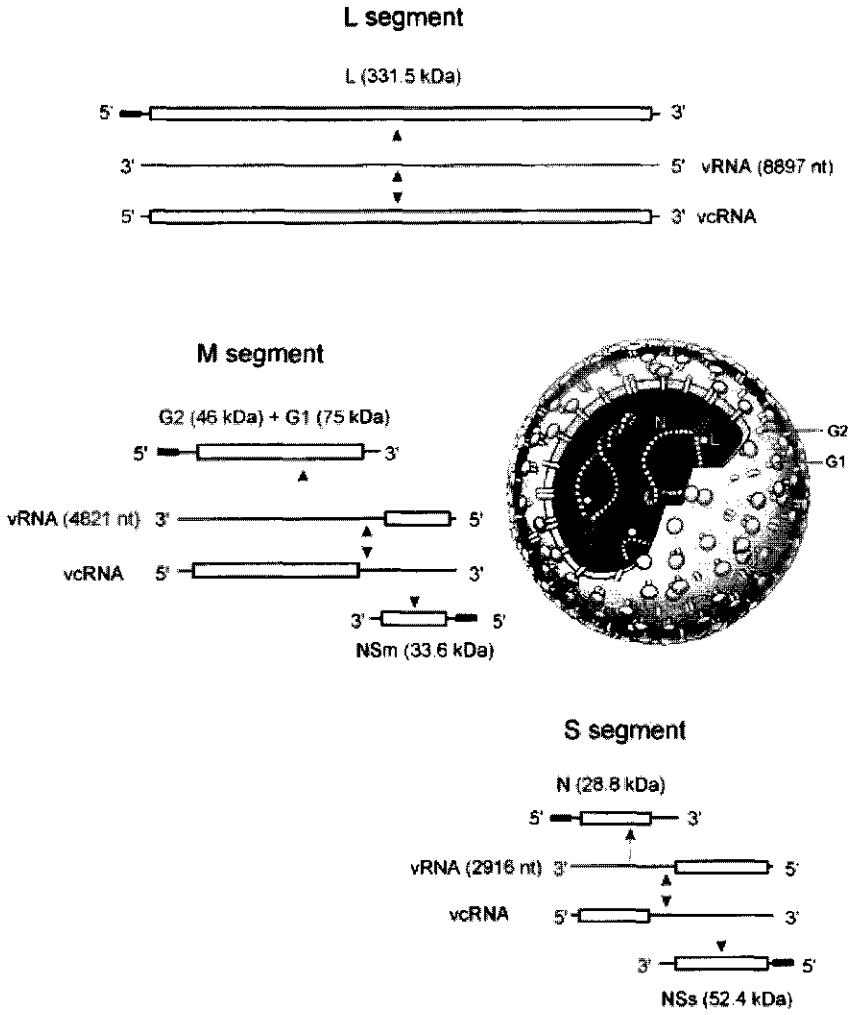

Figure 1.3. Coding strategy of the TSWV genome. vRNA = viral sense RNA; vcRNA = viral complementary sense RNA. Open reading frames (ORFs) are indicated by white boxes, non-templated leader sequences are indicated by black bars.

The genes of TSWV become expressed by the synthesis of mRNAs, which are primed by a capped leader sequence snatched from host mRNAs, a mechanism generally referred to as "cap snatching" [60,109]. The length of the S and M RNA-derived subgenomic mRNAs has been determined to be ~ 1.7 kb and ~ 1.2 kb for the NSs and N mRNAs, resp. and ~1.1 kb and ~3.5 kb for the NSm and G1/G2 mRNAs, resp. [56,57]. These sizes indicate that the subgenomic mRNAs terminate at the hairpin structure of the AU-rich intergenic region [22,56], as found for *Uukuniemi virus* S RNA, a related member of the *Bunyaviridae* [99].

The viral infection cycle

Though many details of the TSWV infection cycle are still unknown, a model for the viral infection cycle can be proposed based on data obtained in recent studies (Fig. 1.4). The infection cycle starts with entry of the virus into a plant cell, either by feeding thrips or, experimentally, by mechanical inoculation. After entry, the lipid envelope should be removed, perhaps by fusion with acidic lysosomes and the RNPs are released into the cytoplasm (Fig. 1.4). These are mainly viral sense RNPs, though minor amounts of the S and M viral complementary (vc) strands have also been found in the virion [57].

Primary transcription of the viral genome by the attached RdRp (L protein) occurs, resulting in the production of (sub)genomic length mRNAs for the L gene, G1/G2 precursor gene and N gene [57,59]. It is proposed that after the level of these viral mRNAs and the resulting translated proteins has increased in the cell, the polymerase complex also starts to replicate the viral genome, i.e. synthesise full length genomic RNAs.

After a single round of replication, vc strands are produced, which serve as templates for (secondary) transcription, resulting in the synthesis of NSs and NSm mRNAs. After replication, both v and vc sense RNAs become enwrapped by nucleoprotein and small amounts of the polymerase will attach, forming RNPs. Mainly the viral sense RNPs are found in new virions, reflecting the *in vivo* ratio between v and vc strands [57].

Prior to particle assembly, part of the RNPs are transported through plasmodesmata by means of tubular structures existing of NSm protein, resulting in viral spread to neighbouring tissues and eventually systemic infection [59,101].

The viral glycoproteins, G1 and G2, are processed from a common glycoprotein precursor by proteolytic cleavage. While they are post-translationally glycosylated, G1 and G2 become targeted to the Golgi apparatus, where particle

assembly takes place [52,53].

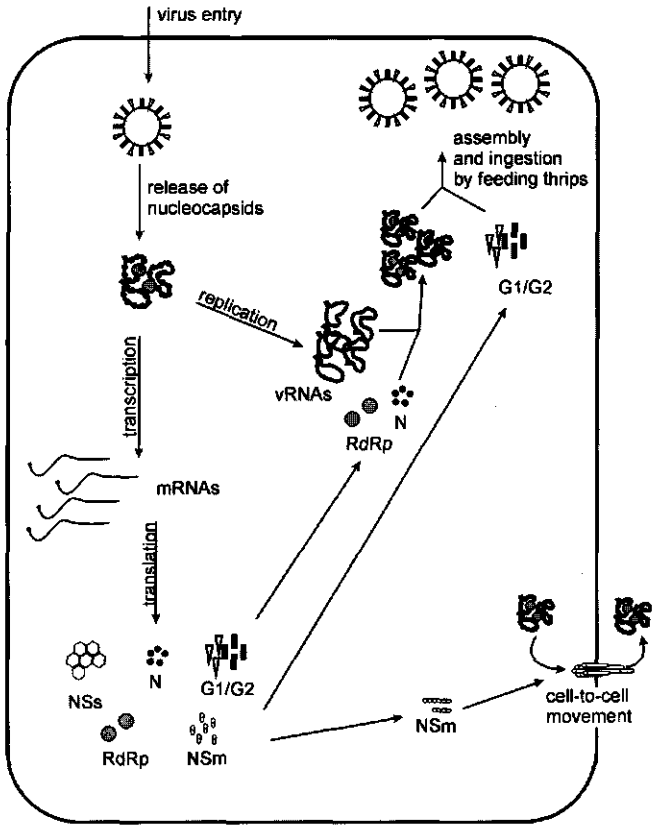

Figure 1.4. The viral infection cycle.

Maturation of virions occurs by enwrapment of RNPs with Golgi membranes containing the viral glycoproteins G1 and G2 [52]. The newly formed virions, enwrapped by a double envelope, hence are referred to as doubly enveloped virus particles (DEV). Fusion of the outer membranes of these DEV or in turn to the ER membrane finally leads to an accumulation of single enveloped particles (SEV) in large vesicles [52,53]. The virions synthesised in this way can be ingested by feeding thrips, by which the cycle starts again.

Transcription of negative strand RNA viruses

Synthesis of viral proteins requires mRNAs that are recognised and accepted by the host cell's ribosomes. As a consequence, a virus has to produce mRNAs provided with a 5'- cap structure. Most RNA viruses, however, replicate in the cytoplasm, whereas the host's capping enzymes are to be found in the nucleus, where cellular transcription

takes place. One way viruses have developed to overcome this problem is by encoding their own enzymes involved in the capping process. For example, the genome of *Vaccinia virus* codes for enzymes, guanylyl transferase and 2-O'-methyltransferase, that can synthesise cap structures at the 5' end of viral mRNAs [72], even though the virus replicates in the cytoplasm.

Segmented negative strand RNA viruses, among which the *Orthomyxoviridae* (like *Influenza A virus*) and the *Bunyaviridae* (like TSWV), have solved the problem by stealing capped leaders from host mRNAs to use these as primers for their own transcription. This mechanism is generally referred to as "cap snatching". During this process, capped host mRNAs are recruited and subsequently cleaved by a virally encoded endonuclease, generally at ~10-20 nt from the cap structure [5,6,15,17,23,25,37,40,47,51,60,64,79,85,90,94,99]. Subsequently, these capped leader sequences are elongated with sequences according to the viral RNA template [64]. Van Poelwijk *et al.* [109] have compared the 3' terminal residue of host-derived leader sequences on the mRNAs of several segmented negative strand and ambisense RNA viruses, as it was expected that endonucleolytic cleavage would take place downstream of this residue (Table 1.1).

Table 1.1. Properties of non-viral leader sequences and cleavage specificities.

Virus	Family	terminal genomic sequence	average leader length (nt)	Cleavage after
TSWV	*Bunyaviridae*	5'-AGAGCAAU..	16	U
GER	*Bunyaviridae*	5'-AGUAGUGUA...	12	U,C
BUN	*Bunyaviridae*	5'-AGUAGUGUA...	14	U
UUK	*Bunyaviridae*	5'-ACACAAAG...	13	C
DUG	*Bunyaviridae*	5'-UCUCAAAG...	10	C
MStV	*Tenuivirus* *	5'-ACACAAAG...	12	A,C
FLUA	*Orthomyxoviridae*	5'-AGCAAAAG...	11	A,G
FLUB	*Orthomyxoviridae*	5'-AGCAGAAG...	11	A,G

Data in this table are derived from 5' RACE analyses of viral mRNAs. Cleavage preferences are based on the identity of the residue preceding the viral genomic sequence and occurring in the majority of the analysed mRNAs. TSWV: *Tomato spotted wilt virus*; GER: *Germiston virus*; BUN: *Bunyamwera virus*; UUK: *Uukuniemi virus*; DUG: *Dugbe virus*; MStV: *Maize stripe virus*; FLUA: *Influenza A virus*; FLUB: *Influenza B virus*. *: *Tenuivirus* is an unassigned genus.

However, as the original host mRNA sequences from which these leaders were derived were unknown, the actual site of endonucleolytic cleavage could not be determined. Hence, the proposed occurrence of base pairing between the capped host RNA and the viral template during cap snatching was not really proven [37,95]. Additionally, for *Influenza A virus* it has been shown that mRNAs containing cap 1 structures (which contain an additional 2-O'-methyl group at the 5' ultimate base) are preferred over identical mRNAs containing a cap 0 structure (which are only methylated at the N7 position of the cap guanine) [7].

Aim and outline of this thesis

As only limited studies on the cap snatching mechanism, shared by all segmented negative strand RNA viruses, have been performed, many of the details involved in this process have remained unresolved. The few studies that have been done mainly refer to *Influenza A virus*, using exclusively *in vitro* approaches moreover. Based on these studies, a base pairing interaction between the cap donor RNA and the viral template has been postulated for successful initiation of transcription, but conclusive evidence was not obtained. Also, the distance between the cap structure and the actual site of endonucleolytic cleavage was expected to influence the efficiency of use of the cap donor RNA.

From earlier data, it was known that TSWV also uses cap snatching as a means to initiate viral transcription [60,109]. It was considered that this virus might have some advantages over the animal-infecting negative strand RNA viruses, as it allows easy *in vivo* studies using co-infection strategies, thus providing information that reflects the natural situation more closely than the *in vitro* approaches used so far. To address the requirements for host mRNAs to serve as a cap donor RNA, specific mRNAs needed to be provided. The capped, genomic RNAs of *Alfalfa mosaic virus* (AMV) were provided *in vivo* by co-infection with TSWV (**Chapter 2**). This approach allowed to investigate the fate of these specific cap donor RNAs during cap snatching *in vivo*. To study the requirements of cap donor RNAs during cap snatching further, mutant AMV RNA 3 and 4 molecules were constructed. Using transgenic tobacco "p12" plants synthesising functional AMV replicase proteins [74,75,103], these mutant AMV RNAs were amplified *in vivo* and presented to TSWV (**Chapter 3**). In this way, primer length and possible base pairing requirements could be determined.

To open future possibilities to unravel the mechanism of cap snatching by reverse genetics, active viral RNPs had to be reconstituted from cloned genomic sequences. To this end, the TSWV RNA-dependent RNA polymerase (L) protein and the

nucleoprotein (N) were cloned and expressed in a *Vaccinia*-T7 expression system along with a reporter template based on the viral S RNA and the reconstituted RNPs assessed for their transcriptase activity. (**Chapter 4**)

In **Chapter 5**, the results of the preceding chapters are discussed in relation to available data on transcription and replication of other members of the *Bunyaviridae* as well as the *Orthomyxoviridae*. Finally, a model for the cap snatching mechanism is proposed.

CHAPTER 2

Alfalfa Mosaic Virus RNAs serve as cap donors for *Tomato Spotted Wilt Virus* transcription during co-infection of *Nicotiana benthamiana*

A modified version of this chapter has been published in a condensed form as:
Duijsings, D., Kormelink, R. & Goldbach, R. 1999. *Alfalfa Mosaic Virus* RNAs Serve as Cap Donors for *Tomato Spotted Wilt Virus* Transcription during Co-infection of *Nicotiana benthamiana*. *Journal of Virology* **73**, 5172-5175.

SUMMARY

The cap snatching mechanism of TSWV was studied by analysing AMV derived cap structures obtained *in vivo* during a mixed infection of TSWV and AMV in *Nicotiana benthamiana*. Using a nested RT-PCR, the 5'ends of TSWV transcripts containing cap structures and short nucleotide sequences derived from the 5'ends of the AMV RNAs were amplified. Cloning and sequencing of the obtained fragments showed that all four AMV RNAs served as cap donors during initiation of TSWV N and NSs gene transcription. In several of the analysed clones, either extra non-templated nucleotides were added at the fusion site of AMV and TSWV sequences or the first nucleotide of the TSWV genomic sequence was lost. A strong preference for endonucleolytic cleavage downstream of a $UUUU^{A}/_{C}$ sequence was observed. The sequence specificity of the endonuclease cleavage is discussed.

INTRODUCTION

Tomato spotted wilt virus (TSWV) is the type species of the genus *Tospovirus* of the family *Bunyaviridae*. Members of this family are characterised by enveloped virus particles encompassing a tripartite RNA genome of negative and sometimes ambisense polarity. In TSWV both the small (S) and middle (M) RNA segments are of ambisense polarity, encoding the non-structural proteins NSs and NSm in the viral sense (v) orientation and the nucleocapsid protein (N) and the glycoprotein precursor (G1/G2) in the viral complementary sense (vc) orientation, respectively. The genomic termini of all three RNA segments are complementary and contain a stretch of 8 identical and highly conserved nt at the 3' end (sequence: ...AUUGCUCU-3'). Within each individual RNA, the complementarity is extended to ~65 nt by sequences which are not conserved among the RNA segments. The presence of this extended complementarity enables the formation of a panhandle structure [21].
Like all other negative strand RNA viruses with segmented genomes, bunyaviruses use the mechanism of "cap snatching" to initiate transcription of the viral mRNAs [5,6,17,50,79,99]. During this process cap structures are cleaved from host mRNAs by a virally encoded endonuclease and subsequently used to prime transcription of the viral genome, as was firstly described for *Influenza A virus* [61,62,85]. For TSWV it has been shown that the viral mRNAs produced during plant infection contain capped leader sequences of non-viral origin, varying in size between 12 and 21 nt [60]. Furthermore, a preference was found for an endonucleolytic cleavage after a U residue [109].

Although the existence of the cap snatching mechanism has been demonstrated for various segmented, negative stranded RNA viruses, detailed information on this mechanism is still lacking. Some features of this process however have been observed for viruses belonging to different families and thus seem to be communal. Firstly, in all cases, except for *Tacaribe arenavirus* [36], the additional 5'non-viral sequences vary in size between approximately 10 and 20 nt. Secondly, sequence specificity of variable degree has been found at the cleavage site of the host derived cap structure [5,6,50,51]. Thirdly, RNA transcripts have been found lacking the first nucleotide templated by the viral genomic RNA or containing additional sequences that are not templated by the viral genomic RNA, possibly added as a result of polymerase slippage [3,5,6,50,109].

In this report the process of cap snatching of the plant infecting bunyavirus TSWV has been studied in more detail. We have investigated whether TSWV would use *Alfalfa mosaic virus* (AMV) RNAs as cap donors during a mixed infection of

AMV and TSWV in *Nicotiana benthamiana*. Since all four AMV RNAs are U-rich in the region 10-20 nt from their 5' termini, these would fit the previously determined preference for cleavage after a U residue [109]. The results show that all four AMV RNAs can act *in vivo* as cap donors for initiation of TSWV N and NSs mRNA synthesis. Whereas different cleavage sites were found using AMV RNA1, 2 and 4 as cap donors, only one cleavage site was found for RNA3. The sequence specificity for the TSWV endonucleolytic cleavage of AMV cap structures is discussed.

RESULTS

Double infection of AMV and TSWV in *N. benthamiana* plants.

In order to obtain TSWV N and NSs mRNAs containing 5'leader sequences derived from AMV RNAs, it was first necessary to establish a successful double infection of both viruses involved in a common host species for which *N. benthamiana* was selected. By Western immunoblot analysis using antisera against the TSWV nucleoprotein (N) and the AMV coat protein (CP), both viruses were verified to co-replicate to detectable levels (Fig. 2.1) at 15 days p.i. Systemically infected newly formed leaves containing both TSWV and AMV were selected for further analysis of TSWV transcripts.

RT-PCR analysis of TSWV transcripts in singly and doubly infected plants.

As a first step to analyse TSWV N and NSs mRNAs containing AMV specific cap structures, first strand cDNA was synthesised from total RNA extracts of singly and doubly infected *N. benthamiana*, respectively. Subsequently, a nested PCR reaction was performed in combination with PCR primers containing the first 11 nt of the 5'ends of the different AMV RNAs (Fig. 2.2). It was anticipated that TSWV N and NSs mRNAs containing 5' sequences derived from the 5'ends of AMV RNA1, 2, 3 or 4 would be amplified, as schematically shown in Fig. 2.2. Products of the predicted sizes were detected for the N mRNA (~315 bp) and for the NSs mRNA (~420 bp), but only in the doubly infected plants and neither in singly infected plants, nor when *in vitro* mixed RNA extracts from singly infected plants were used (Fig. 2.3, panels A, B and C). Products were obtained with AMV primers A12, A3 and A4, in combination with either of the two TSWV specific primers, indicating that all four AMV (sub)genomic RNAs could serve as cap donors for initiation of TSWV mRNA transcription.

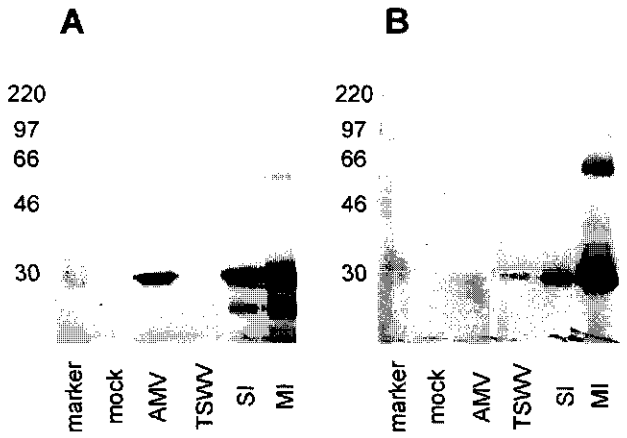

Figure 2.1. Western immunodetection of AMV and TSWV in single and mixed infections of *N. benthamiana*. Panel A: detection with anti-AMV; Panel B: detection with anti-TSWV. Mock: mock infected plants; AMV: AMV infected plants; TSWV: TSWV infected plants; MI: plants infected with mixed inocula of AMV and TSWV; SI: plants infected on separate leaves with AMV and TSWV inocula.

Specific PCR products were always detected in those plants that were co-inoculated on separate leaves with AMV and TSWV inocula, whereas only in a few cases PCR products were obtained from plants that were mixed inoculated on the same leaf. No products of expected size were detected when using RNA extracts from non-infected or singly infected plants, neither with mixed RNA extracts of singly infected plants (Fig. 2.3, panels A, B and C). Sometimes products of deviant sizes were detected in RNA extracts from singly infected plants or in mixed extracts of singly infected plants (Fig. 2.3, panel A, lane 11 and panel C, lanes 10 and 12), probably due to some non-specific cross-annealing of primers. Also, upon amplification with primers A4 and NSs2, two different products were obtained for the doubly infected plants. Only the lower, intense band, which was of the predicted size, was used for further analysis.

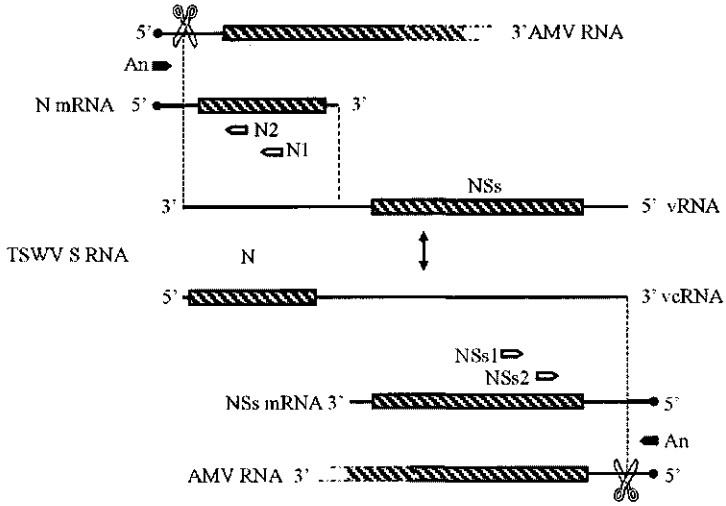

Figure 2.2. Location of primers for RT-PCR on TSWV mRNAs containing 5' terminal AMV sequences. Both the viral (v) and the viral complementary (vc) strand of TSWV S RNA are shown, with open reading frames indicated as dashed bars and caps on AMV RNAs indicated as black circles. An = primer for AMV RNA 1 and 2 (A12), AMV RNA 3 (A3) or AMV RNA 4 (A4). N1 = primer for RT reaction on TSWV N mRNA. N2 = nested primer for PCR on TSWV N mRNA. NSs1 = primer for RT reaction on TSWV NSs mRNA. NSs2 = nested primer for PCR on TSWV NSs mRNA.

Sequence analysis of TSWV N and NSs mRNAs.

In order to determine the precise fusion sites of AMV leader sequences with the TSWV mRNAs, sequence analysis was performed on a series of individually picked clones from different RT-PCR reactions. Sequencing results showed that all clones obtained extra sequences of non-TSWV origin at the 5'end of N and NSs mRNAs, which could be identified as having originated from the 5'termini of AMV (sub)genomic RNAs. These sequences varied in length between 12 and 19 nt, with an average size of 15 nt (Table 2.1). Although there was no difference in the average length of the snatched 5' sequences between the N and NSs mRNAs, clear differences could be seen between the length of the snatched sequences when different AMV RNAs were involved as cap donor.

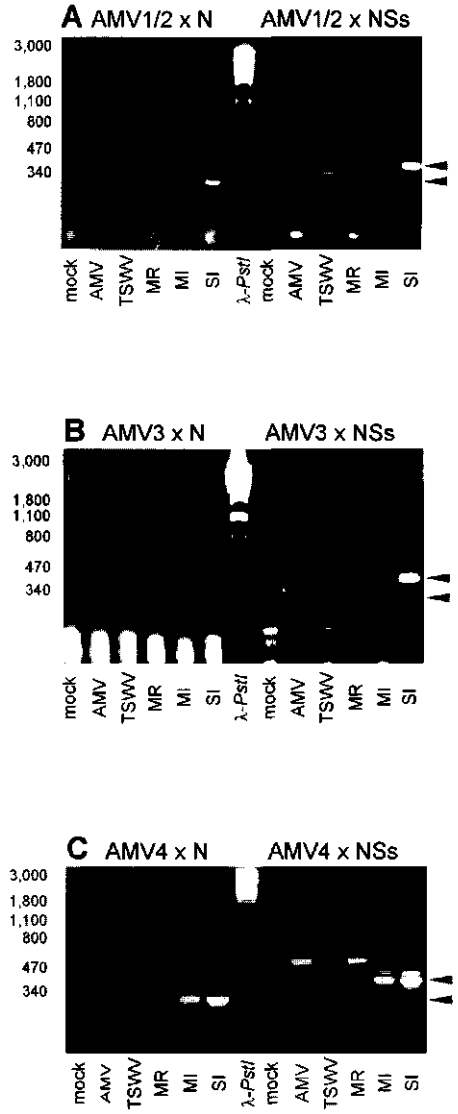

Figure 2.3. RT-PCR products of TSWV N and NSs mRNAs. Panels: PCR products of TSWV mRNAs with AMV RNA1/RNA2 (A), RNA3 (B) and RNA4 (C). Total RNA was isolated from infected *N. benthamiana* at 15 days p.i. RT primers used were N1 and NSs1, PCR primers used were A12 (A), A3 (B) and A4 (C) in combination with N2 and NSs2.

λ-PstI: molecular weight marker; mock: mock-infected plants; AMV: singly AMV infected plants; TSWV: singly TSWV infected plants; MR: *in vitro* mixed RNA of singly AMV and TSWV infected plants; MI: co-infection with mixed inocula; SI: co-infection with both inocula on separate leaves.

Analysis of individual N and NSs mRNAs showed that for RNAs containing 5' cap structures of AMV RNA1, the snatched sequence had a length of 15 nt.
In those cases the endonucleolytic cleavage of the cap donor RNA took place exclusively 3' of a C residue. Only in one of the clones, the first nucleotide of the TSWV sequence, being an A residue, was missing between the cap structure and the TSWV genomic sequence (Table 2.1). When AMV RNA2 was used as a cap donor, the average size of the snatched sequences was 14 nt and no strict sequence preference for endonucleolytic cleavage was observed.
When AMV RNA2 was used as a cap donor, in two of the seven clones the first residue of the TSWV sequence, being an A, was lost (Table 2.1). Strikingly, when AMV RNA3 was used as a cap donor, the endonucleolytic cleavage took place exclusively after the C residue on position +17 (C17) of the AMV sequence (Table 2.1). Moreover, no extra nts were found added or deleted between the AMV and TSWV sequences. This template apparently showed a very strong preference for only one endonucleolytic cleavage site.
When AMV RNA4 was used, the average size of the snatched sequences was 13 nt. As found for AMV RNA1 and RNA3, a preference for a specific endonucleolytic cleavage site was also observed in AMV RNA4 (Table 2.1). However, cleavage in RNA4 occurred preferentially after an A residue, whereas RNA1 and RNA3 were cleaved after a C residue. Of 15 clones analysed, 11 showed a cleavage after A13 of the RNA4 sequence, 3 were cleaved after U12 and 1 was cleaved after C19.
Of the 11 clones that showed cleavage of RNA4 after A13, one clone contained an additional stretch of six nucleotides inserted between the AMV and TSWV sequence (Table 2.1).
This stretch (AGAGCA) was identical to the conserved 5'end of the TWSV S RNA sequence. A similar feature was observed for two clones that were cleaved either at U12 or at A13. In these clones, endonucleolytic cleavage took place either after U12 in the AMV RNA4 sequence, with insertion of an extra AG sequence, or cleavage took place after A13, with only one extra G residue being inserted (Table 2.1).

Table 2.1. 5' terminal sequences of TSWV N and NSs mRNAs containing AMV leader sequences.

AMV leader		TSWV body mRNA	
RNA1			
GUUUUUAUCUUACACACG		*UCUCGUUA*	
GUUUUUAUCUUACACAC	**G**	AGCAAU	1
GUUUUUAUCUUACAC	**A**	GAGCAAU	3
GUUUUUAUCUUAC	**A**	GAGCAAU	2
RNA 2			
GUUUUUAUCUUUUCGC		*UCUCGUUA*	
GUUUUUAUCUUUUCG		AGAGCAAU	1
GUUUUUAUCUUUUC		AGAGCAAU	1
GUUUUUAUCUUUUC	**G**	AGCAAU	2
GUUUUUAUCUUUU		AGAGCAAU	3
RNA 3			
GUAUUA AUACCAUUUUCAAA		*UCUCGUUA*	
GUAUUAAUACCAUUUUC	**A**	GAGCAAU.....	7
RNA4			
GUUUUUAUUUUUAAUUUUCUUCUCGUUA			
GUUUUUAUUUUUAAUUUUC		AGAGCAAU	1
GUUUUUAUUUUUA**A**	GAGCA	AGAGCAAU	1
GUUUUUAUUUUUA	**A**	GAGCAAU	10
GUUUUUAUUUUU	**A**	GAGAGCAAU.....	2
GUUUUUAUUUUU	**A**	GAGCAAU	1

Non-templated nucleotides, which are inserted between the AMV and TSWV sequences, are underlined. Nucleotides that can be derived of either AMV or TSWV RNA are in bold type. Original AMV (5'-3') leader sequences and TSWV (3'-5') terminal sequences are in italic type.

DISCUSSION

In this study it is shown that, during a mixed infection of AMV and TSWV, TSWV is able to use all four AMV RNAs as cap donors for the initiation of viral mRNA synthesis. During the course of our investigation, a similar approach, in which the cap snatching mechanism of *Maize stripe tenuivirus* was investigated *in vivo* by co-infection with *Barley stripe mosaic hordeivirus*, has been published [32], which further validates this *in vivo* approach for future studies on the cap snatching mechanism.

Using a nested RT-PCR assay and subsequent cloning and sequence analysis of the cDNAs obtained, it is demonstrated in this paper that sequences of all four AMV RNAs were present at the 5' ends of both TSWV N and NSs mRNAs. The length of the added AMV sequences varied between 12 and 20 nt. These data correspond nicely with earlier data [60,109], concerning capped RNA primers originating from host mRNAs, where lengths of 12 to 21 nt were found.

Furthermore, both insertions and deletions of nucleotides at the fusion site of the AMV and TSWV sequences could be observed. Insertions and deletions of residues at the fusion sites have also been reported for some animal bunyaviruses, e.g. *Uukuniemi*, *Germiston* and *Snowshoe hare virus* [3,5,6,51]. In our study 35 clones were analysed, of which 3 clones lacked the first nucleotide (A) of the TSWV genomic sequence, while in 2 clones either an additional G or an additional AG was inserted. In one clone even the first six nucleotides of the TWSV genomic sequence were repeated between the AMV and the authentic TSWV sequence. Although the mechanism is still unclear, these insertions might have arisen by polymerase slippage on the template viral RNA, as has been suggested previously [5].

In 49% (17/35) of the studied sequences, the endonucleolytic cleavage of the capped RNA primer donor occurred after a C residue, in 31% (11/35) after an A residue, in 17% (6/35) after a U residue and in the remaining sequence after a G residue. However, when looking at the fusion site of the AMV and TSWV sequences, it can be suggested that for most of the clones that were analysed, the first residue of the TSWV sequence (Table 2.1) might indeed also be the last residue of the AMV leader sequence. In the latter case, annealing of the 3'ultimate residue of the AMV derived capped RNA primer to the 3'ultimate or 3'penultimate residue of the TSWV template might be involved in the initiation of TSWV transcription, similarly to what has been reported for *Influenza A virus* [3]. Using this interpretation, endonucleolytic cleavage then would occur in 74% (26/35) of the studied sequences after an A residue, in 11% (4/35) after a G residue, in 9% (3/35) after a U residue and in 6%(2/35) after a C

residue.

These results seem to contradict our previously reported data [109], indicating a preference (45% occurrence) for cleavage after a U residue and lower incidences for preference for cleavage after A, G or C residues (20%, 15% and 20% respectively). The difference in results obtained may be partly explained by the fact that in the present study a selection is made of AMV RNA primed mRNAs. The number of possibilities for the endonucleolytic cleavage to take place therefore may have been limited and biased, taking into account that no base pairing between the cap donor RNA and the viral template would be involved.

However, when using the second interpretation involving base pairing of the 3'ultimate residue of the host mRNA derived primer to the 3'ultimate or 3'penultimate residue of the TSWV template, it is necessary to know the original sequence of the host mRNA which has been used to prime TSWV transcription. Only then, it is possible to discriminate between host mRNA-derived nucleotides and TSWV body mRNA-derived nucleotides at the fusion site of both sequences.

When using the first model of cap snatching, distance to the cap and identity of a single nt at the cleavage site seem not to be the only two decisive parameters for defining a cleavage site, as AMV RNAs are relatively U-rich in the region of the endonucleolytic cleavage site. However, the previously determined preference for cleavage after a U residue is not seen in the present study.

Comparison of the sequences of N and NSs mRNAs obtained from the mixed AMV-TSWV infection showed that there was a slight preference for cleavage downstream of the nucleotide sequence $UUUU^{A}/_{C}$. These data might suggest that this is either a preferred consensus sequence for cleavage or that the possible cleavage sites surrounding this "consensus" cleavage site are less optimal for the endonuclease, as would be the case when base pairing of the RNA primer to the TSWV template is required. To elucidate in what way this preference for cleavage is obtained, mutational analysis of the cap donor RNA in an *in vitro* cap snatching reaction will have to be performed. In case of the latter, AMV RNA3 would be the most likely candidate to act as a cap donor, as *in vivo* only one cleavage site within this template has been identified. Furthermore, this template contains a U_4CA sequence that seems to have an influence on the preferential cleavage by the endonuclease. Mutation of the nucleotides surrounding the cleavage site itself and/or of the U_4-stretch will have to be performed in order to indicate the influence of specific nucleotides, nucleotide sequences, length and/or structure of the cap donor template on the endonucleolytic activity. Though sequence specificity of the nucleotide preceding the endonucleolytic cleavage site has been demonstrated for some negative stranded RNA viruses,

requirements regarding structure and/or sequences have not been shown, as no mutagenesis of the cap donor template has been performed yet.
For *Influenza A virus* it has been found that the cap structure of the donor RNA is first bound by the polymerase complex, after which the nucleotide preceding the endonucleolytic cleavage site base pairs with the complementary nucleotide in the template RNA, after which the endonuclease is activated and cleavage occurs [96]. This mechanism may suggest that there is a certain distance needed between the cap structure and the endonucleolytic cleavage site, which involves length, three-dimensional structure and specific nucleotide sequence of the cap donor molecule. To elucidate the influence of specific nucleotide sequences at the endonucleolytic cleavage site and structural conformation of the cap donor molecule, further studies will have to be performed, e.g. by mutagenesis of AMV RNA donor sequences that can then be used as specific cap donors in both *in vivo* cap snatching assays by means of mixed infections and *in vitro* transcription assays. In this way, it will be sorted out whether or not base pairing between the cap donor RNA and the viral template RNA is indeed involved in the TSWV cap snatching mechanism.

MATERIALS & METHODS

Western immunoblot analysis.

5 weeks old *N. benthamiana* plants were mechanically inoculated with either TSWV strain BR-01, AMV strain 425 Leiden, or both. In case of double infection with both viruses, plants were inoculated either with both inocula on separate leaves (SI) or with mixed inocula on the same leaves (MI). At 9 and 15 days p.i., systemically infected leaves were harvested and the presence of both viruses was tested. To this end, 400 mg of leaf material was grinded in 1 ml of PBS and 10 µl was applied on a 15% SDS-PAGE gel. After electrophoresis, proteins were semidry blotted onto Immobilon-P PVDF membranes and subsequently scored for presence of TSWV and AMV using antisera against the TSWV nucleoprotein or the AMV capsid protein, according to Kormelink *et al.* [58].

RNA extraction.

Total RNA extracts were made from about 400 mg systemically infected leaf material according to Gurr *et al.* [41].

Primer design.

To obtain first strand cDNA from TSWV N and NSs mRNAs, primer N1

(GGAATGTCAGACATG, identical to nt 2595-2609 of the viral S RNA) and primer NSs1(GGGCAGGAGACAAAACC, complementary to nt 439-455 of the viral S RNA sequence) were designed. PCR primers N2 (CCCGGATCCGTTCGATGTTTT CCAGAC, identical to nt 2634-2651) and NSs2 (CCCGGATCCGATAGTGCCAGA ACAGAG, complementary to nt 384-367) were designed for use in a nested PCR reaction.

AMV primers were designed to match the first 11 nucleotides of the different AMV RNAs (A12: CCCGGATCCGTTTTTATCTT; A3: CCCGGATCCGTATTAATACC; A4: CCCGGATCCGTTTTTATTTT). Based on the 5' terminal homology of AMV RNA1 and 2, primer A12 was designed to recognise AMV RNA1 and 2 simultaneously.

All primers except N1 and NSs1 were designed with additional 5'*BamHI* linker sequences.

RT-PCR amplification.

First strand cDNA was synthesised from 10 µg of total RNA extract using primer N1 or NSs1 and Superscript reverse transcriptase according to the manufacturer's procedures (GIBCO BRL).

PCR amplification was performed on 1 µl of the synthesised first strand cDNA using 100 pmol of AMV primer A12, A3 or A4 in combination with 100 pmol of TSWV primer N2 or NSs2 in a 100 µl PCR reaction (5 min. at 94°C; 5 cycles of 30 sec. at 94°C, 30 sec. at 25°C, 30 sec. at 72°C; 35 cycles of 30 sec. at 94°C, 30 sec. at 55°C, 30 sec. at 72°C; and 7 min. at 72°C). Amplified fragments were analysed by electrophoresis on a 2% agarose gel. PCR products were purified from gel, cloned into a pBluescript-SK(-) vector (Stratagene) and clones selected were sequenced using standard T3 and T7 sequencing primers.

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CHAPTER 3

In vivo analysis of the TSWV cap snatching mechanism: single base complementarity and primer length requirements

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SUMMARY

Requirements of capped leader sequences for use during transcription initiation by *Tomato spotted wilt virus* (TSWV) were tested providing mutant *Alfalfa mosaic virus* (AMV) RNAs as specific cap donors in transgenic *Nicotiana tabacum* plants expressing the AMV replicase proteins. Using a series of AMV RNA3 mutants modified in either the 5' non-translated region or in the subgenomic RNA4 leader, sequence analysis revealed that cleaved leader lengths could vary between 13 and 18 nucleotides. Cleavage occurred preferentially at an A residue, suggesting a requirement for a single base complementarity with the TSWV RNA template, which could be confirmed by analyses of host mRNAs used *in vivo* as cap donors.

INTRODUCTION

Segmented, negative strand RNA viruses share the transcription initiation mechanism generally referred to as "cap snatching". During this process, a ^{7m}G-capped host mRNA is recruited by the viral transcriptase complex and subsequently cleaved by a virally encoded endonuclease. The resulting capped leader RNA is used to prime transcription on the viral genome, as most extensively described for *Influenza A virus* [8,15,23,85,106].

Yet, knowledge of the requirements for sequence specificity, length and structure of a suitable donor RNA has remained rather limited. Commonly, cap donor RNAs are cleaved at a distance of around 15 nt from the cap structure, though variation in length occurs between 10 to 20 nt [5,6,15,16,23,31,38,40,47,48,50,51, 57,60,79,90,97,99,109]. Exceptions have been reported for members of the *Arenaviridae* (*Tacaribe virus*) [36,89] and *Nairovirus* genus (*Dugbe virus*) [51], which use relatively short (1-4 nt and 5-16 nt, resp.) non-viral leader sequences. For many of these viruses, sequence analyses of their mRNAs have shown a nucleotide preference at the 3' end of the non-viral leader, assumed to reflect a sequence preference for cleavage by the viral endonuclease. For example, in case of *Dugbe virus*, endonucleolytic cleavage has been proposed to take place exclusively after a C residue [51], whereas for *Bunyamwera virus* a strong preference for cleavage after a U residue has been proposed (80% of the mRNAs studied) [50]. However, most mRNAs analysed in these cases were produced *in vivo*, hence the particular mRNAs that were used to provide these capped leader sequences (the cap donors) remained unknown. Therefore, it is still unknown whether cleavage of the cap donor indeed has taken place immediately after the assumed 3' end of the capped leader sequence or whether this cleavage has taken place further downstream, e.g. 1 or 2 nucleotides, which would be complementary to the 3' terminal residues of the viral template.

For several viruses, *in vitro* studies have provided information about leader length requirements and have suggested that base pair interactions can contribute to alignment of the capped leader RNA sequence to the viral template RNA [18,37,42,43,45,95]. For *Hantaan virus*, an additional "prime-and-realign" mechanism has been proposed to explain data obtained from transcription initiation studies [37]. During this "prime-and-realign" mechanism, transcription initiation starts with a capped leader RNA that base pairs to the viral template RNA and becomes extended for a few nucleotides only. Subsequently, it is released from the viral RNA template and realigns backward by virtue of the terminal sequence repeats. Only then is progressive elongation of the nascent mRNA chain thought to take place. The reason

for a "prime-and-realign" mechanism is still unclear; however, it is proposed to account for the viral RNA polymerase to initiate *Hantaan* (anti)genome and mRNA synthesis with GTP [37]. Meanwhile, the occurrence of a "prime-and-realign" mechanism has been proposed for several other negative strand RNA viruses, e.g. *Germiston virus* and *LaCrosse virus* [6,25], in order to explain the presence of repetitive sequences within the heterogeneous sequences at the 5' end of the viral mRNAs.

Tomato spotted wilt virus (TSWV) is the representative of the *Tospovirus* genus within the *Bunyaviridae*, a family of negative strand RNA viruses with a tripartite genome. TSWV is the first plant virus for which the occurrence of cap snatching has been investigated [60,109]. These studies have shown the presence of non-viral leader sequences, 12 to 21 nt in length, at the 5' ends of viral subgenomic mRNAs. Recent findings have demonstrated that during a co-infection of *Nicotiana benthamiana* with TSWV and *Alfalfa mosaic virus* (AMV), all capped (sub)genomic RNAs of the latter (positive strand RNA) virus can act *in vivo* as cap donors for TSWV [27], as likewise was shown to occur for the *tenuivirus Maize stripe* during a co-infection with the *hordeivirus Barley stripe mosaic* [32]. Cleavage of the AMV leaders preferentially took place at an A residue. The AMV capped leader sequences found at the 5' ends of TSWV N and NSs mRNAs varied in length, even when originating from the same AMV RNA molecule, except for RNA 3, where obviously a single cleavage site was used. Apparently the capped leader sequence of AMV RNA 3 matched strict requirements resulting in a unique cleavage site. However, alignment of the original AMV RNA 3 sequence and also those of RNA 1, 2 and 4, to the chimaeric AMV-TSWV mRNA sequences did not allow identification of the cleavage site as the position of this site is dependent on the possible need for complementarity between snatched leader and template RNA (Fig. 3.1). In order to test for leader length preference, cleavage specificity and a possible base pairing requirement, mutant AMV RNAs were analysed for their ability to act as cap donors in a manipulable *in vivo* system. To this end, transgenic (p12) tobacco plants expressing the AMV p1 and p2 replicase subunits were used, allowing *in vivo* replication of AMV RNA3 molecules specifically mutated in their leader sequences [74,75,103].

Here it is shown that mutant AMV RNAs provided in this way can be tested as cap donors during TSWV transcription initiation. These analyses are furthermore complemented by testing selected host mRNAs as cap donors. Altogether, the *in vivo* observations indicate that for successful transcription initiation a single base complementarity between the snatched leader and the viral template RNA is required.

Fig.1

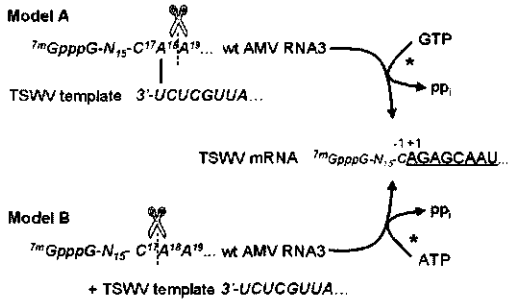

Figure 3.1. Possible models for the cap snatching mechanism.
Model A: Endonucleolytic cleavage occurs 3' of an A residue within the cap donor RNA (e.g. wt AMV RNA3), which subsequently base pairs with the 3' ultimate residue of the viral template RNA. The capped RNA primer is elongated according to the viral template, with a G (complementary to the 3' penultimate residue of the template) being the first incorporated residue during elongation. Model B: The cap donor RNA is cleaved (non-)specifically at a certain distance of the cap structure and is elongated according to the viral template, with an A (complementary to the 3' ultimate residue of the template) being the first incorporated residue during elongation.

RESULTS

Use of mutant AMV RNA 3 leader sequences for transcription initiation by TSWV.

From previous studies [27], it was known that all four AMV RNAs are accepted as cap donors for TSWV transcription initiation and that cleavage of the AMV leaders preferentially took place at an A residue. These results were in support of a possible base pairing requirement between snatched leader and template RNA, but still left room for an alternative explanation (Fig. 3.1) [27]. To collect further evidence that the preferential cleavage at A residues indeed reflects the requirement for base pairing, two sets of experiments were performed. Firstly, it was investigated whether the length of the snatched leader RNA strictly co-varies along with the position of an available A residue. Secondly, it was tested whether leader sequences with a G instead of an A residue at the preferred cleavage site would be accepted as cap donors but now by base pairing with the penultimate residue (C) of the TSWV template RNA.

To this end, specific AMV RNA3 leader mutants were presented to TSWV using transgenic (p12) tobacco plants expressing functional AMV p1 and p2 replicase subunits. As reported before [74,75,103], such plants support the replication and systemic spread of AMV RNA3, even when provided as CaMV 35S promoter driven cDNA clones. To test the feasibility of this *in vivo* system, first a wt construct of

AMV RNA3 was co-inoculated along with TSWV and 7 days later chimaeric AMV-TSWV mRNAs were rescued from systemically infected top leaves using RT-PCR (Fig. 3.2 A).

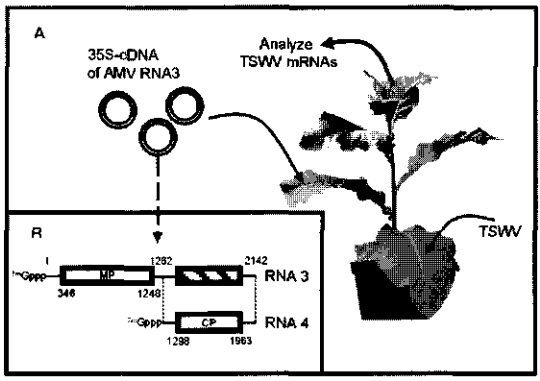

Figure 3.2. *In vivo* amplification of (mutant) AMV RNA3 and rescue of chimaeric TSWV mRNAs. (A) Transgenic *N. tabacum* p12 plants expressing the AMV replicase proteins were mechanically inoculated with a 35S promoter driven plasmid containing the AMV RNA3 segment. Subsequently, the plants were inoculated with TSWV strain BR-01. Newly formed leaves showing symptoms of TSWV infection were harvested at 7 days post infection and total RNA isolated from these leaves was analysed for the presence of AMV leader containing TSWV mRNAs. (B) Schematic drawing of the AMV RNA3 segment. The genomic RNA3 segment (transcribed from a 35S driven plasmid) is replicated by the p1 and p2 replicase proteins in the p12 plant. Subgenomic AMV RNA4 is subsequently transcribed from this RNA segment by the p1 and p2 replicase proteins.

This analysis demonstrated that AMV RNA3 molecules accumulating in p12 plants were indeed used as cap donors for TSWV mRNA synthesis (Fig. 3.3 A, lane WT). Sequence analyses of several independent clones (Table 3.1) showed that the junction site found between the AMV RNA3 leader and the TSWV mRNA sequence completely matched the sequence data from earlier experiments during which *N. benthamiana* plants were co-inoculated with TSWV and AMV [27].

As a next step, to investigate whether cleavage specifically would take place at an A residue, single point mutants (Table 3.1) were made at nt positions 17 or 18 and subsequently used in co-inoculation experiments with TSWV on p12 plants. It was anticipated that a change of residue C17 into an A would result in a -1 shift of the cleavage site, the resulting AMV leader sequence within the TSWV mRNA thus becoming 1 nucleotide shorter. Similarly, a change of A18 into a C would result in a +1 shift of the endonucleolytic cleavage site to still meet the supposed cleavage

specificity for an A residue. In this case, the added AMV leader sequence of the TSWV mRNA would increase in size with one nt residue, nt residue A19 now being the residue at which cleavage would take place. Sequence data from independent RT-PCR clones (Fig. 3.3 A + Table 3.1), collected from co-inoculations of these AMV RNA 3 mutants and TSWV on p12 plants, indeed showed a -1 shift when nt C17 was changed into an A (mutant C17A), and a +1 shift in case nt A18 changed into a C (mutant A18C). The leader length thus shifted along with the position of an available A residue, demonstrating that cleavage specificity (at an A) determines leader length and supporting the model in which a single base complementarity between leader and template RNA is required (Fig. 3.1).

Table 3.1. AMV RNA3 mutant leader sequences in TSWV mRNAs.

Mutan	Sequence	After p12 amplification	Chimeric TSWV mRNAs	No. of clones
WT	$^{7m}GpppG\text{-}N_{15}\text{-}C^{17}A^{18}A^{19}$..	WT	$^{7m}GpppG\text{-}N_{15}\text{-}C^{17}A^{18}$GAGCAAU...	10
C17A	$^{7m}GpppG\text{-}N_{15}\text{-}A^{17}A^{18}A^{19}$..	C17A + A18C	$^{7m}GpppG\text{-}N_{15}\text{-}A^{17}$GAGCAAU...	4
			$^{7m}GpppG\text{-}N_{15}\text{-}C^{17}C^{18}A^{19}$GAGCAAU...	2
C17G	$^{7m}GpppG\text{-}N_{15}\text{-}G^{17}A^{18}A^{19}$..	A18C	$^{7m}GpppG\text{-}N_{15}\text{-}C^{17}C^{18}A^{19}$GAGCAAU...	1
C17U	$^{7m}GpppG\text{-}N_{15}\text{-}U^{17}A^{18}A^{19}$..	Unstable[a]	ND	ND
A18C	$^{7m}GpppG\text{-}N_{15}\text{-}C^{17}C^{18}A^{19}$..	A18C	$^{7m}GpppG\text{-}N_{15}\text{-}C^{17}C^{18}A^{19}$GAGCAAU...	7
A18G	$^{7m}GpppG\text{-}N_{15}\text{-}C^{17}G^{18}A^{19}$..	A18C	$^{7m}GpppG\text{-}N_{15}\text{-}C^{17}C^{18}A^{19}$GAGCAAU...	8
A18U	$^{7m}GpppG\text{-}N_{15}\text{-}C^{17}U^{18}A^{19}$..	A18C	$^{7m}GpppG\text{-}N_{15}\text{-}C^{17}C^{18}A^{19}$GAGCAAU...	9

TSWV mRNAs primed by (mutant) AMV RNA3 leaders were amplified using nested RT-PCR. Individual clones from these reactions were sequence analysed.

[a]Not amplified in the p12 system; ND, not done.

To demonstrate more conclusively that base pairing was involved, two point mutants were made in which G residues were introduced (mutants C17G and A18G, Table 3.1), which would potentially lead to cleavage at G and base pairing with the penultimate nucleotide (C) of the TSWV template. Cloning and sequence analysis of progeny AMV RNA revealed that these mutants rapidly converted into mutant A18C. As a consequence, RT-PCR clones obtained from co-inoculation of these AMV RNA3 mutants with TSWV invariably showed an endonucleolytic cleavage pattern for the AMV leader sequence identical to the pattern observed with mutant A18C (Table 3.1).

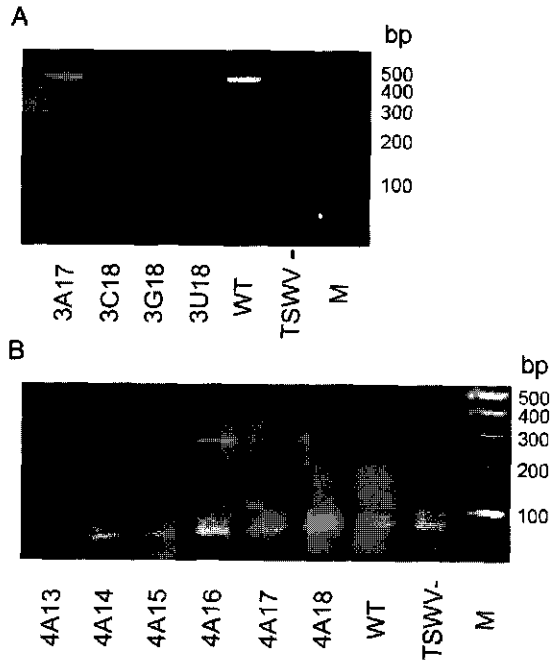

Figure 3.3. RT-PCR products of TSWV mRNAs containing (mutant) AMV RNA leaders.
(A) TSWV NSs mRNAs containing (mutant) AMV RNA3 leaders. Total RNA was isolated from infected *N. tabacum* p12 at 7 days p.i. and TSWV NSs mRNAs containing AMV RNA3 derived leaders were RT-PCR amplified using primer NSs1 (RT) and primers NSs2 and A3 (PCR). Products of ~420 bp represent TSWV NSs mRNAs. 3A17: AMV RNA3 mutant 3A17; 3C18: AMV RNA3 mutant 3C18; 3G18: AMV RNA3 mutant 3G18; 3U18: AMV RNA3 mutant 3U18; wt: wild type AMV RNA3; TSWV-: uninfected, wild type AMV RNA3 containing plants; M: 100 bp molecular marker. (B) TSWV N mRNAs containing (mutant) AMV RNA4 leaders. Total RNA was isolated from infected *N. tabacum* p12 at 7 days p.i. and TSWV N mRNAs containing AMV RNA4 derived leaders were amplified using primer N1 (RT) and primers N2 and A4 (PCR). Products of ~315 bp represent TSWV N mRNAs. 4A13: AMV RNA4 mutant A13; 4A14: AMV RNA4 mutant A14; 4A15: AMV RNA4 mutant A15; 4A16: AMV RNA4 mutant A16; 4A17: AMV RNA4 mutant A17; 4A18: AMV RNA4 mutant A18; wt: wild type AMV RNA4; M: 100 bp molecular marker.

Also, additional AMV RNA3 mutants tested, i.e. C17U and A18U, rapidly converted into mutant A18C (Table 3.1), indicating restrictions in the mutability of the AMV RNA3 leader.

Mutant AMV RNA4 leaders confirm donor sequence and length requirements for priming TSWV transcription.

As further mutagenesis of its leader led to genetic instability of AMV RNA3, we next presented modified AMV RNA4 leaders to TSWV infected p12 plants to confirm the

cleavage specificity at an A residue and to test for leader length preference and base pairing requirement. AMV RNA4 has been used as cap donor before in several *in vitro* studies with other negative strand RNA viruses [7,35,80,85,106] and *in vivo* with TSWV [27]. AMV RNA4 is a subgenomic mRNA (Fig. 3.2 B), its leader residing internally in genomic RNA3 and not containing any *cis*-replication signals. Therefore, RNA4 leader mutants might be more stable than RNA3 leader mutants. The wild type AMV RNA4 contains only two A residues at position 13 and 14 within a U-rich context (Table 3.2). Hence, only a minimal modification would be required to obtain a set of mutants with a single A residue at different positions within an oligo(U)-stretch running from position 12 to 18 (Table 3.2, mutants denoted as A12 through A18). Additionally, a mutant was made lacking any A residue between position 12 and 18 (mutant RNA4-noA). These mutants would allow us to test whether the length of the snatched leader sequence would precisely co-vary with the position of the A residue in this leader and provide information about leader length preference. First, the fitness and genetic stability of the AMV RNA4 mutants were tested by inoculation of the cDNA constructs harbouring these mutations on p12 plants and sequence analysis of progeny RNA4. All mutants, except RNA4-noA and A12, were relatively stable. Progeny RNA from mutant RNA4-noA could not be recovered by RT-PCR and no coat protein production was observed, indicating that this mutant was not viable (data not shown). RT-PCR and sequence analysis of several independent clones showed that mutant A12 was unstable, rapidly converting into wt RNA4 as well as other mutant sequences (A15-A17). For most of the other mutants, minor amounts of another mutant sequence were found in addition to the expected mutant genotype after a single round of replication in p12 plants (Table 3.2).

Having tested the fitness and stability of the AMV RNA4 mutants, these were provided as cap donors during a co-infection with TSWV on p12 plants. RT-PCR cloning of TSWV N mRNAs obtained from these co-infection experiments (Fig. 3.3B), and subsequent sequence analyses of the 5' capped leader sequences indicated that all of the RNA4 mutants with an A residue at different positions in the AMV RNA 4 leader could serve as cap donors (Table 3.2).

Sequence data from several clones of TSWV N mRNAs, obtained from co-inoculation experiments with AMV RNA4 mutants, showed a shift of the snatched leader length along with the position of the A residue within the oligo(U) track. A number of the AMV RNA4 mutants produced a polymorphous population of RNA4 molecules (Table 3.2), the differently sized capped leaders found on the resulting TSWV mRNAs reflecting this variation.

Table 3.2. AMV RNA4 mutant leader sequences in TSWV mRNAs.

Mutant	Sequence	After p12 amplification	Chimeric TSWV mRNAs	No. of clones
WT	^{7m}*GpppG-N$_{11}$-A^{13}A^{14}UUUUC..*	WT	^{7m}*GpppG-N$_{11}$-A^{13}*GAGCAAU...	1
			^{7m}*GpppG-N$_{11}$-A^{13}A^{14}*GAGCAAU...	9
A12	^{7m}*GpppG-N$_{10}$-A^{12}UUUUUUC..*	A15,A16		
		A17, WT	ND	ND
A13	^{7m}*GpppG-N$_{10}$-UA^{13}UUUUUC..*	A13, A16	^{7m}*GpppG-N$_{11}$-A^{13}*GAGCAAU...	0
			^{7m}*GpppG-N$_{11}$-UUUA^{16}*GAGCAAU...	1
A14	^{7m}*GpppG-N$_{10}$-UUA^{14}UUUUC..*	A14, A16	^{7m}*GpppG-N$_{11}$-UA^{14}*GAGCAAU...	0
			^{7m}*GpppG-N$_{11}$-UUUA^{16}*GAGCAAU...	3
A15	^{7m}*GpppG-N$_{10}$-UUUA^{15}UUUC..*	A15, A16	^{7m}*GpppG-N$_{11}$-UA^{14}*GAGCAAU...	1
			^{7m}*GpppG-N$_{11}$-UUA^{15}*GAGCAAU...	2
			^{7m}*GpppG-N$_{11}$-UUUA^{16}*GAGCAAU...	3
A16	^{7m}*GpppG-N$_{10}$-UUUUA^{16}UUC..*	A16	^{7m}*GpppG-N$_{11}$-UUUA^{16}*GAGCAAU...	5
			^{7m}*GpppG-N$_{11}$-UUUA^{16}*GCAAU...	1
A17	^{7m}*GpppG-N$_{10}$-UUUUUA^{17}UC..*	A17	^{7m}*GpppG-N$_{11}$-UUUUA^{17}*GAGCAAU...	2
A18	^{7m}*GpppG-N$_{10}$-UUUUUUA^{18}C..*	A18	^{7m}*GpppG-N$_{11}$-UUUUUA^{18}*GAGCAAU..	4
noA	^{7m}GpppG-N$_{10}$-UUUUUUUC..	Unstable[a]	ND	ND
G15	^{7m}GpppG-N$_{10}$-UUUG^{15}UUUC..	G15	^{7m}GpppG-N$_{11}$-UUG^{15}AGCAAU...	1

TSWV mRNAs primed by (mutant) AMV RNA4 leaders were amplified using nested RT-PCR. Individual clones from these reactions were sequence analysed.

[a]Not amplified in the p12 system; ND, not done.

Despite this variation, the leader length always shifted along with the position of the A residue. Although the numbers were not statistically significant, the sequence results from the subset of different RNA4 molecules obtained after p12 amplification pointed towards a preference for molecules containing an A residue at position +16 (Table 3.2), as was found when using host mRNAs as cap donors [109].

The strict co-variance of leader length with the position of the A residue in both AMV RNA3 and RNA4 indicates that endonucleolytic cleavage of the leader takes place 3' of this A residue to allow base pairing with the first U residue of the TSWV template RNA. However, alternatively the results could be explained by an endonuclease activity that cleaves cap donor RNAs 5' of the A residue (Fig. 3.1 B), with the A residue in the TSWV mRNA being the first nucleotide incorporated during capped primer elongation within the transcription process. This scenario seems less likely in view of the sequence data obtained using wild type AMV RNA4 as a cap donor. The leader of this RNA contains two A residues (at positions 13 and 14) and

sequence data of TSWV mRNAs containing leaders derived from RNA4 show that residue A13 is preferentially retained, which does not fit a cleavage mechanism meant to recruit a leader without a 3' terminal A residue.

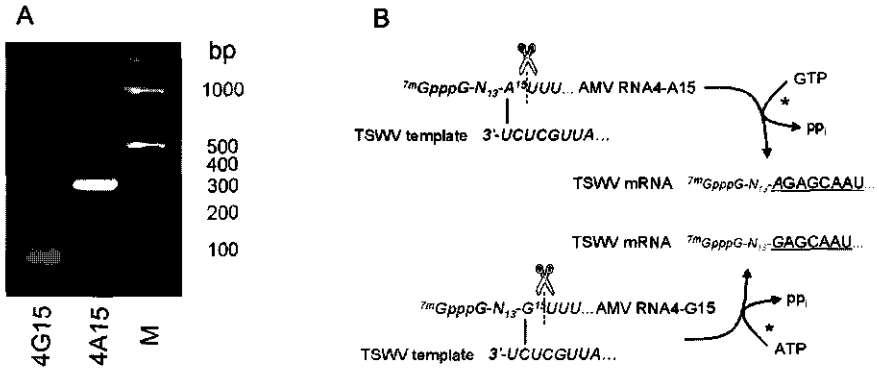

Figure 3.4. Transcription initiation at the ultimate and penultimate base of the TSWV template RNA. (A) RT- PCR amplification of TSWV N mRNAs containing AMV RNA4 leaders mutated at nt 15. Total RNA was isolated from infected *N. tabacum* p12 at 7 days p.i. and TSWV N mRNAs containing AMV RNA4 derived leaders were amplified using primer N1 (RT) and primers N2 and A4 (PCR). Products of ~315 bp represent TSWV N mRNAs. 4G15: AMV RNA4 mutant G15; 4A15: AMV RNA4 mutant A15; M: 100 bp molecular marker. (B) Model for transcription initiation on the penultimate residue. The cap donor RNA is cleaved by the viral endonuclease 3' of an A residue and base pairs to the 3' ultimate U residue of the viral template RNA. Elongation takes place according to the viral RNA, with a G residue being the first residue incorporated during elongation. Alternatively, if no A residue is available at an optimal distance of the cap structure, a G residue may be used in base pairing to the 3' penultimate C residue of the viral template. Subsequently, elongation will take place with an A residue being the first residue incorporated. The resulting viral mRNA will miss the ultimate A residue present at the 5' end of the viral genome.

The results with the A13-A18 mutants indicated that the leader sequence of RNA4 could be modified to a certain extent without complete loss of *in vivo* RNA3 or RNA4 amplification in p12 plants. Therefore, to substantiate the requirement of a single base pairing between leader and TSWV template during the cap snatching process, AMV RNA4 mutant G15 was made. This mutant lacked any A residue and contained a single G at position 15 within an oligo-(U) stretch (Table 3.2). Only a low RT-PCR signal was obtained for TSWV mRNAs primed with G15 leaders (Fig. 3.4 A), but the single clone obtained supported the base pairing model as the mRNA sequence lacked the 5' terminal A residue of the TSWV template. This could only happen by cleavage of the mutant RNA4 leader 3'of residue G15 and base pairing to the penultimate C of the TSWV template (Fig. 3.4 B).

Recognition of host mRNAs during cap snatching.

The low recovery of TSWV mRNAs primed with the AMV RNA4 mutant G15 (Fig. 3.4 A) could be due to both decreased efficiency of priming on the penultimate residue and decreased replication of this mutant in p12 plants. To circumvent the second complication we extended our *in vivo* studies to host mRNA leaders used by TSWV for transcription initiation. Two different host (*N. tabacum*) genes from the GenBank database were selected for which the transcription initiation sites were unequivocally known (Table 3.3), the first gene (polyubiquitin; ubi.U4, GenBank acc. nr. X77456) with a predictable cleavage site in view of the single A residue at position 17, the other gene (S-adenosylmethionine decarboxylase; SAM-DC, GenBank acc. nr. AF033100) potentially giving a more complicated picture, as its mRNA contained multiple possible cleavage sites between positions 12 and 21 (Table 3.3).

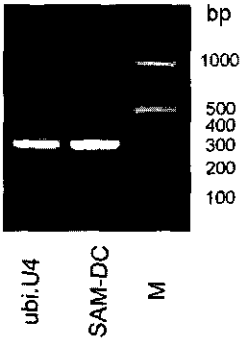

Figure 3.5. RT-PCR detection of TSWV mRNAs initiated with specific host leaders. TSWV N mRNAs containing host leaders were amplified from total RNA, isolated from infected *N. tabacum* p12 at 7 days p.i. TSWV N mRNAs containing host leaders were amplified using primer N1 (RT) and primers N2 and UbiU4-1 or SAMDC-1 (PCR). Products of ~315 bp represent TSWV N mRNAs. Ubi.U4: RT-PCR product of TSWV N mRNAs containing ubi.U4 derived leaders; SAM-DC: RT-PCR product of TSWV N mRNAs containing SAM-DC derived leaders; M: 100 bp molecular marker.

Using a specific RT-PCR primer for the leader of the polyubiquitin mRNA, chimaeric TSWV mRNAs could indeed be obtained from total RNA of TSWV infected tobacco plants (Fig. 3.5). Sequence analysis of several clones revealed that the ubi.U4 mRNA was exclusively cleaved at residue 17 (Table 3.3), confirming the results obtained with the AMV leaders. The SAM-DC mRNA appeared also to be cleaved at a single site, but now after a G residue at the preferred nt position 16. All mRNAs containing the SAM-DC leader lacked the terminal A residue of the authentic TSWV sequence (Table 3.3) and this result could only be explained by endonucleolytic cleavage

downstream of G16 and subsequent base pairing to the penultimate C residue of the viral template. The use of the SAM-DC leader therefore indicates the plasticity of the cap snatching mechanism of TSWV: position seems more important than nucleotide identity, as long as the 3' terminal nucleotide of the snatched leader can base pair with the ultimate or penultimate residue of the viral template.

Table 3.3. Host leader sequences in TSWV mRNAs.

Host gene	Obtained sequence of TSWV mRNAs	No. of clones
ubi.U4	$^{7m}GpppAUCCUUUGAUUUCUCUA^{17}UUCUC...$	
	$^{7m}Gppp\text{-}N_{16}\text{-}A^{17}$GAGCAAU...	4
SAM-DC	$^{7m}GpppAUGGAGUCGAAAGGUG^{16}GUAAAAAC...$	
	$^{7m}Gppp\text{-}N_{15}\text{-}G^{16}$AGCAAU...	11

TSWV mRNAs primed by host mRNA leaders were amplified using nested RT-PCR. Individual clones from these amplifications were sequence analysed.

From the co-infection studies with AMV [27], the data obtained using mutant AMV RNA3 and RNA4 donors, and with the two host mRNAs, the conclusion can be drawn that for cap snatching by TSWV, capped leaders are preferentially cleaved behind an A residue, which moreover should preferentially occur at or close to position 16 from the cap. Knowing now that a single base complementarity is a prerequisite for accepting the leader of a mRNA as cap donor, previously obtained sequence data on cloned TSWV mRNAs containing host derived leaders [109] can now be used to validate our conclusions. In 80% of the sequences obtained, the host derived leader sequences fit with a mechanism whereby cleavage occurred after an A residue at a distance of 13 to 21 nt (average length: 16 nt). For the other 20%, the leaders fit with cleavage after a G residue (at a distance of 14 to 22 nt from the cap, average length 17 nt), allowing priming by base pairing at the penultimate C residue of the TSWV template, a minor alternative also found with the AMV leaders.

DISCUSSION

Cap snatching as a general mechanism for transcription initiation among different segmented negative strand RNA viruses has been studied both by *in vivo* and *in vitro* methods. While some of the data obtained in these experiments suggested that

a complementarity or base pairing between the donor RNA and the viral template might be required [25,37,51], other data disagreed with this view [43, 63]. However, both the *in vivo* and *in vitro* methods used to study the cap snatching process contained some disadvantages. The *in vivo* methods were based mainly on analysis of viral mRNAs containing host derived sequences obtained through 5' RACE amplification, which implied that it was virtually impossible to determine the sequence of a given cap donor RNA before its use in the transcription initiation. The influence of a specific sequence within the cap donor and the exact endonucleolytic cleavage site therefore remained unknown, nor could any alternative cleavage sites within the same cap donor be identified. The *in vitro* methods on the other hand allowed the possibility of supplying cap donors with precisely known features in the cap snatching mechanism [7,35,79,106], but the conditions under which it would take place might not have reflected the *in vivo* situation at all.

The approaches described in this paper combine the advantage of natural, *in vivo* conditions with the use of known and even mutable leaders. Specific mutant AMV leaders can easily be generated and inoculated mechanically either as DNA constructs or as *in vitro* transcripts on transgenic ("p12") plants, to become amplified by the AMV p1 and p2 replicase proteins to high levels throughout the plant.

Following this *in vivo* approach, combined with the *in vivo* analyses of two selected host mRNAs as cap donors, we could demonstrate that a single base complementarity is required for a capped leader RNA to successfully prime on the viral template. This base pairing should occur preferentially at position +16 of the donor RNA, though all positions between nt 13 and nt 18 can be used with different efficiencies (Fig. 3.3 B). Furthermore, this base pairing not only can occur with the 3' ultimate A residue of the viral template (apparently the most optimal scenario), but also with the penultimate G and even the antepenultimate A residue, as was observed for an AMV RNA4 A16 leader-primed mRNA, which lacked the first 2 nucleotides of the authentic TSWV sequence (Table 3.2). When evaluating sequences of host leader primed TSWV mRNAs from an earlier study [109], a preference for cleavage at position 16 could be observed with priming on the 3' ultimate residue of the viral template, as well as a possible priming of the used leaders to the 3' penultimate residue.

Evidence for realignment of the recruited capped RNA primer on the viral template, resulting in repeated insertions of the first few nucleotides of the viral genomic sequence between leader sequence and (authentic) viral RNA sequence, has been hardly monitored in our studies. Only in one occasion (Table 3.2; AMV4 A15) an extra AG dinucleotide insertion was found. Such repeated sequences have been

observed more frequently with some animal infecting viruses (e.g. *Germiston, Hantaan, Bunyamwera, Dugbe, Influenza A* and *B virus*) [6,37,50,51,95], as well as with the plant infecting *Tenuiviruses* [32,47,97]. The inserted sequences have been explained first for *Hantaan virus* as being the result of a "prime-and-realign" mechanism [37]. The low frequency of insertions of 5' terminal viral sequences between leader sequence and viral sequence [27,109] suggests that initiation of transcription for TSWV occurs at the 3' ultimate A residue, rather than at the antepenultimate A residue. Therefore, a "prime-and-realign" mechanism seems not be favoured as a means for initiation of transcription. Future experiments may reveal why for TSWV repeats of the 5' terminal nucleotides are rarely seen within the viral mRNAs.

Though specific nucleotide composition and distance of the base pairing residue from the 5' end within the leader play an important role in the efficiency for use as a cap donor, the effects of specific secondary and tertiary structures within the leader have not been investigated yet. If secondary and tertiary structures indeed occur within the 5' end of the leader during the cap snatching process, these may influence the physical distance between cap structure and possible cleavage sites, thereby altering the optimal site for endonucleolytic cleavage. It is likely though that the viral polymerase complex disrupts these secondary and tertiary structures within the 5' end of the leader. The viral nucleoprotein, a ssRNA binding protein [91] that is part of the viral transcriptase complex, may play a role in this.

In summary, the combined analyses of mutated AMV RNAs and host mRNAs have led to an improved insight in the requirements on length and specific nucleotide composition of cap donors during TSWV transcription initiation. Moreover, it has resolved the base pairing requirement during cap snatching, which may hold for all segmented negative strand RNA viruses.

MATERIAL & METHODS

Host plants.

Transgenic *Nicotiana tabacum* cv. Samsun NN plants expressing AMV replicase proteins P1 and P2 (referred to as p12 plants) were used for *in vivo* replication of wild type and mutant AMV RNA3 and RNA4 from cloned cDNAs as described previously [75].

Construction of plasmids.

Plasmid pCa32T, which contains a cDNA copy of the wild type AMV RNA3 flanked by the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (nos) terminator [74], was used as a source to create mutants of AMV RNA3 and RNA4. Mutant AMV RNA3 constructs which contained a point mutation at either position 17 or 18 of the AMV RNA3 sequence were made by amplifying pCa32T using primers α35S (CTCTCCAAATGAAATGAACTTCC, complementary to the 35S promoter) and A3/*D*17 (GTATTAATACCATTTT*D*AAAATATTCCAATTC, identical to nt 1-32 of the AMV RNA 3 sequence;D=A,G,T) or A3/B18 (GTATTAATACCATTTT C*B*AAATATTCCAATTC; B=C,G,T) and the Expand Long template PCR system (Roche). Amplified PCR fragments were purified using the High Pure PCR purification kit (Roche), restriction enzyme digested with *Dpn*I (to destroy input template DNA) and ligated using T4 DNA ligase (Promega). Individual clones were verified by sequence analysis. Point mutants of AMV RNA3 at nt 17 are referred to as C17A (in which nt 17 was changed from the wt C residue into an A residue), C17G and C17U. Point mutants of AMV RNA3 at nt 18 are referred to as A18C (where nt 18 was mutated into a C residue), A18G and A18U. Similarly, point mutants in the subgenomic promoter region of AMV RNA4 were derived from pCa32T using primers A4/rev (AAAATAAAAACGGCCCATTACCG, complementary to nt positions 1250-1272 of the AMV RNA3 sequence) and A4/A12 through A4/A18 (ATTTTTTCTTTCAAATACTTCCATCATGAG; TATTTTTCTTTCAAATACTTC CATCATGAG; TTATTTTCTTTCAAATACTTCCATCATGAG; TTTATTTCTTTC AAATACTTCCATCATGAG; TTTTATTCTTTCAAATACTTCCATCATGAG; TT TTTATCTTTCAAATACTTCCATCATGAG; TTTTTTACTTTCAAATACTTCCAT CATGAG), A4/noA (TTTTTTTCTTTCAAATACTTCCATCATGAG) and A4/G15 (TTTGTTTCTTTCAAATACTTCCATCATGAG) (all identical to nt positions 1273-1302 of the wt RNA3 sequence). Point mutants of AMV RNA4 are referred to as A12 (containing an A residue at nt 12 of the wt RNA4 sequence and U residues at nt 13 and 14), A13, A14, A15, A16, A17, A18 and G15 (Table 3.2). Likewise, mutant RNA4-noA was made, containing a poly(U)-track between nt 12 and 18 of the wt RNA4 sequence.

Inoculation of p12 plants.

Transgenic *Nicotiana tabacum* cv. Samsun NN plants expressing AMV replicase proteins P1 and P2 (p12 plants) were grown under greenhouse conditions and mechanically inoculated with 35S-cDNA constructs and TSWV strain BR-01 as described previously [27,74,103].

Analyses of AMV-TSWV mRNA sequences.

TSWV N mRNAs containing capped 5' nucleotide sequences derived from AMV RNA3 and RNA4 were detected and cloned into the pGEM-T vector (Promega) as described previously [27]. Briefly, total RNA was isolated from systemically infected leaf material as described by Gurr and McPherson [41]. First strand cDNA was synthesised from this total RNA and a nested PCR amplification was subsequently performed on the synthesised first strand cDNA using a primer identical to the first 11 nt of the AMV RNA leader sequences. PCR products obtained were purified using the High Pure PCR purification kit (Roche) and cloned into pGEM-T (Promega) according to the manufacturer's procedures. Sequence analysis of the obtained clones was performed using the Sanger dideoxy method (Amersham-Pharmacia).

Analyses of host leader sequences in TSWV mRNAs.

TSWV N mRNAs containing capped 5' leader sequences derived from different host (*N. tabacum*) genes were detected by nested RT-PCR and analysed by sequence determination as described above. Shortly, amplification on first strand cDNA material was performed with a nested TSWV primer in combination with a primer specific for the first 11 nt of the 5' end of different host genes. Host genes chosen were a polyubiquitin gene (GenBank acc.no. X77456, with corresponding primer UbiU4-1 (*CCCGGATCC*ATCCTTTGATT)) and a S-adenosylmethionine decarboxylase (SAMDC) gene (GenBank acc.no. AF033100, with corresponding primer SAMDC-1 (*CCCGGATCC*ATGGAGTCGAA)).

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CHAPTER 4

Rescue of ambisense tospoviral ribonucleoproteins from cloned cDNAs.

This chapter has been submitted as:
Duijsings, D., van Knippenberg, I., de Haan, C.A.M., Rottier, P.J.M., Goldbach, R. & Kormelink, R. Rescue of ambisense tospoviral ribonucleoproteins from cloned cDNAs.

SUMMARY

Using the *Vaccinia*-T7 expression system in mammalian cells a transcriptionally active ribonucleoprotein (RNP) complex derived from the ambisense S RNA segment of tomato spotted wilt virus (TSWV) was obtained. The sensitivity of the system was improved by replacing the antisense NSs open reading frame, which served as a marker gene for transcription, by the firefly luciferase (*luc*) gene. Five different TSWV L clones were compared to achieve optimal *luc* expression. Replacement of the NSs gene indicated that only the viral L and N genes are essential for transcription of the TSWV genome segment. RT-PCR analysis of the viral NSs mRNAs synthesised in this system showed the presence of host leader sequences at their 5' ends, indicating that these were produced through the genuine, TSWV-specific cap snatching mechanism.

INTRODUCTION

Tomato spotted wilt virus (TSWV) is the representative of the plant infecting *Tospovirus* genus within the family *Bunyaviridae*, a family of enveloped negative strand RNA viruses. The tripartite genome encompasses a large (L) RNA segment of complete negative polarity and a medium (M) and small (S) RNA segment of ambisense polarity.

The L RNA encodes the putative RNA-dependent RNA polymerase (RdRp) [20,104] and is thought to harbour several enzymatic activities, e.g. transcriptase, replicase and endonuclease activities. The ambisense M RNA contains the open reading frame (ORF) for the viral movement (NSm) protein in the viral (v) sense orientation. This protein is a unique feature for the plant infecting viruses, needed for movement from cell to cell through plasmodesmata and therefore no homologue is present in the related animal infecting *Bunyaviridae* [59]. In viral complementary (vc) sense, the M RNA encodes a common precursor for the two envelop glycoproteins G1 and G2. The ambisense S RNA encodes in v sense orientation for a non-structural protein (NSs), the function of which still is unknown, and in vc sense orientation for the viral nucleoprotein (N). Genomic RNA segments are tightly enwrapped by the N protein and small amounts of the viral polymerase, forming transcriptionally active (infectious) ribonucleoprotein complexes (RNPs) [1,94,107]. In the virion, the RNPs are surrounded by a lipid membrane derived from the Golgi apparatus containing both the G1 and G2 glycoproteins [52,53].

Whereas the role of various TSWV gene products in the infection cycle has been studied and partly unravelled over the past few years, e.g. the movement protein [59,101] and the glycoproteins [53], studies on the viral transcription and replication have been limited and were primarily focussed on the cap snatching mechanism [1,26,27,109]. Like the animal infecting members of the *Bunyaviridae*, the plant pathogenic TSWV uses cap snatching to initiate transcription on the genomic RNA, a process during which capped leader sequences are cleaved from suitable donor mRNAs in order to be used as primers for viral mRNA synthesis [26,27,109]. Recently, studies in this respect have revealed a base pairing requirement and leader length preference for cap donor RNAs in order to be used during transcription initiation *in vivo* [26]. More detailed studies on genome replication and transcription, especially for the ambisense RNA segments, are still being hampered though due to the lack of a suitable reversed genetics system, which would allow the introduction of specific mutations in coding and non-coding viral genomic sequences in order to study

their effects *in vivo*.

Since the late eighties, different approaches have been developed to achieve this goal for other negative strand RNA viruses [13,19,28,81,82,83,98]; of these, the most widely used is the reconstitution of active RNP complexes from cDNA clones using a *Vaccinia*-T7 expression system [30,34,115]. The development of this system has not only lead to identification and analysis of *cis*-acting sequences and genes involved in transcription and replication, but even to the rescue of infectious virus particles for several segmented negative strand RNA viruses like *Influenza A* and *Bunyamwera virus* [9,84]. Here we have followed a similar strategy for the *in vivo* reconstitution of TSWV RNPs. By expressing the TSWV N protein and L protein in murine OST7-1 fibroblasts and the ambisense S RNA segment as a template, viral transcriptase activity was detected by monitoring the expression of the antisense ORF from this ambisense RNA. Transcripts were shown to contain capped, host derived leader sequences, confirming that the reconstituted S RNPs were faithfully transcribed in a TSWV-governed fashion.

RESULTS

Translatability of cloned TSWV genes.

Prior to *in vivo* reconstitution of a functional viral RNP, translatable N and L genes had to be obtained. As the *Vaccinia virus*-T7 RNA polymerase expression system was selected for this purpose, cDNAs of the N and L genes were made by RT-PCR (Materials & Methods) and cloned into the T7 promoter controlled transcription vector pTUG3 [111]; these plasmids are referred to as pTUG-N and pTUG-L, respectively.

Translatability of these genes was verified by transfection into OST7-1 cells (murine cells transgenically expressing T7 RNA polymerase) concomitantly infected with MVA-T7 (attenuated *Vaccinia virus* containing a copy of the T7 RNA polymerase gene). Whereas the nucleoprotein could easily be detected by Western immunoblotting, immunoprecipitation and immunofluorescence (Fig. 4.1 B and Fig. 4.3), the L protein was only detected by immunofluorescence (Fig. 4.1 A). As an antiserum against a 60 kDa C-terminal fragment of the L protein was used [108], it was assumed that the immunofluorescence signal correlated with expression of the full length L ORF.

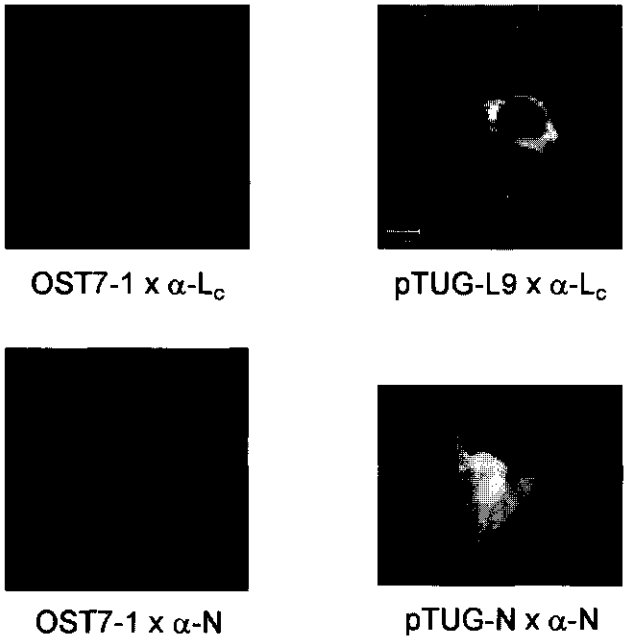

Figure 4.1. Immunofluorescence detection of TSWV proteins in OST7-1 cells.
TSWV proteins presumably involved in transcription and replication were expressed in murine OST7-1 fibroblasts using a *Vaccinia*-T7 expression system. Expression of the L protein from pTUG-L9 could be demonstrated using a polyclonal antiserum (α-L_C) directed against a C-terminal 60 kDa fragment of the L protein (A). Expression of the N protein from pTUG-N could be demonstrated using a polyclonal antiserum (α-N) directed against isolated virus particles of TSWV strain BR-01 (B). Specificity of detection was verified by absence of staining in control cells.

Testing for transcriptase activity.

To establish whether the expressed L protein was a functional RdRp, its transcriptase activity had to be demonstrated on a viral RNA template. To this end, a full-length DNA copy of the ambisense S RNA was constructed by RT-PCR and cloned in a pUC19 plasmid containing a T7 promoter-terminator cassette, resulting in plasmid pTOS-S (Fig. 4.2). The S RNA copy in this construct was flanked by a hepatitis delta ribozyme to obtain RNA transcripts with authentic 5' and 3' termini, as described in detail in Materials and Methods. When this RNA associates *in vivo* with a functional RdRp and nucleoprotein, active viral RNPs are reconstituted and only then transcription of the antisense NSs ORF occurs, leading to expression of its protein.
As a first attempt, only pTUG-L and pTOS-S were used in a co-transfection. It was anticipated that the N protein could directly be translated from the TOS-S RNA, capped by *Vaccinia* enzymes. Indeed, transfection of MVA-T7 infected OST7-1 cells

with pTUG-L and pTOS-S led to the expression of the L and N proteins as well as the NSs protein, the presence of the latter being indicative for TSWV transcriptase activity (Fig. 4.3, lane 3). The amounts of NSs protein detected, however, always were low, probably due to background reactivity of the used antiserum. Remarkably, upon addition of pTUG-N the amount of N protein increased but the NSs protein could no longer be detected.

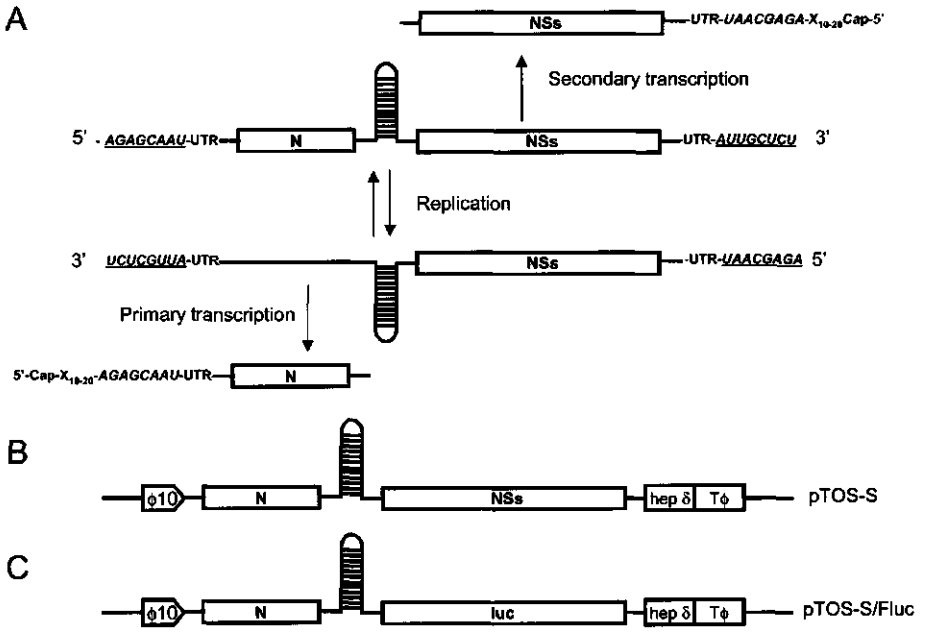

Figure 4.2. Expression strategy of the TSWV S RNA segment. A. The viral genomic RNA strand (v) is released into the cell upon infection and primary transcription of the N subgenomic mRNA occurs. The mRNA contains a 5' cap structure and leader sequence of ~10-20 nt of non-viral origin. The v strand is also replicated into a vc strand, which in turn is the template for replication of new viral sense genomic RNA segments that can be packaged into newly formed virions. Alternatively, it can be transcribed yielding NSs mRNAs. (B) Reporter construct pTOS-S was based on the viral complementary sense S RNA. A cDNA copy of the S RNA was cloned into a T7 transcription cassette and flanked by a hepatitis delta ribozyme sequence to provide the T7 transcript with authentic TSWV termini. The T7 transcript is capped by *Vaccinia* capping enzymes and therefore simultaneously acts as a mRNA for expression of the N protein. (C) pTOS-S/Fluc was derived from pTOS-S, where the NSs ORF was replaced by the firefly luciferase gene. ϕ10: bacteriophage T7 ϕ10 gene promoter; hep δ: hepatitis delta ribozyme; Tϕ: bacteriophage T7 ϕ10 gene terminator; luc: firefly luciferase gene.

Use of luciferase as a marker for transcriptase activity.

To allow for easier and quantifiable detection of transcriptase activity, the pTOS-S template was modified by replacing the NSs gene by the firefly luciferase (*luc*) gene. The construct obtained in this way (pTOS-S/Fluc) was used in co-transfection experiments with either or both pTUG-L and pTUG-N. *Luc* expression was only detected in those cases in which the pTOS-S/Fluc reporter construct was co-transfected with pTUG-L.

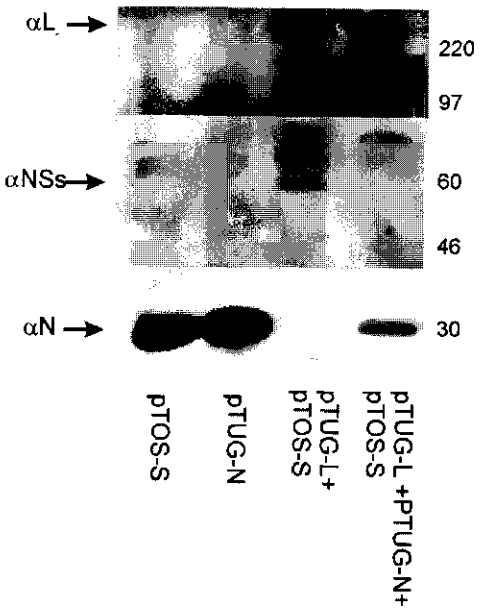

Figure 4.3. Detection of transcription activity. OST7-1 cells were infected with MVA-T7 and subsequently transfixed with pTUG-L, pTUG-N, pTOS-S and combinations of these. At 24 hours post infection (p.i.), the presence of TSWV proteins was verified using Western immunoblotting and antisera directed against a C-terminal (60 kDa) part of the L protein (top panel), the NSs protein (middle panel) or the N protein (bottom panel). Lane 1: transfection with pTOS-S; lane 2: transfection with pTUG-N; lane 3: transfection with pTUG-L and pTOS-S; lane 4: transfection with pTUG-L, pTUG-N and pTOS-S. Expression of the NSs protein, indicative for TSWV transcription, was only observed in co-transfection of pTUG-L and pTOS-S. Molecular marker sizes are indicated on the right.

During construction of the pTUG-L template, several individual cDNA clones of the L gene were synthesised in order to circumvent any problems arising from possible RT-PCR errors in the extremely long ORF. Using luciferase as a marker gene now, the individually picked L clones could each be assayed for their capacity to

produce a transcriptionally active L protein. Activity levels varied between the different pTUG-L clones, clone pTUG-L9 giving the highest *luc* expression (Fig. 4.4). This L clone replacement analysis actually demonstrates that the L RNA segment encodes the viral transcriptase, which was previously only indirectly shown based on the conserved sequence motifs in the L protein [104] and on results with other *Bunyaviridae* [28,69].

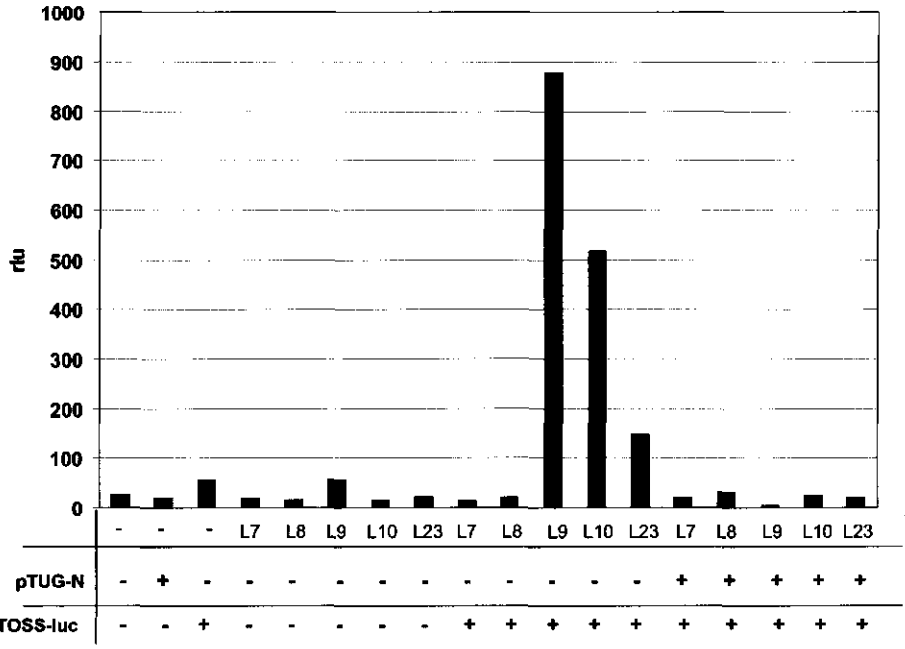

Figure 4.4. Transcription activity of individual TSWV L clones. OST7-1 cells were infected with MVA-T7 and subsequently co-transfixed with pTOS-S/Fluc and different, individually selected clones of the L RNA. Luciferase activity was assayed at 24 hours p.i. Significant luciferase activity was only observed when pTUG-N was omitted, and varied between the different L clones from very high to background levels.

Again, addition of pTUG-N reduced *luc* expression to background levels (Fig. 4.4), as previously observed using the NSs gene as a reporter (Fig. 4.3). Based on the results in Fig. 4.4, pTUG-L9 was selected for all further experiments. To reach maximal *luc* expression levels, increasing amounts of the RdRp expressing construct (pTUG-L9) versus the reporter construct (pTOS-S/Fluc) were added. Maximal expression levels were found using equimolar amounts of both constructs (Fig. 4.5). To analyse whether replacement of the NSs protein would have any influence on transcription levels, additional transfection of a translatable pTUG-NSs construct was

performed. Preliminary data indicated that *luc* expression levels did not significantly alter in the presence of NSs, suggesting that this protein likely does not play a role during transcription of TSWV.

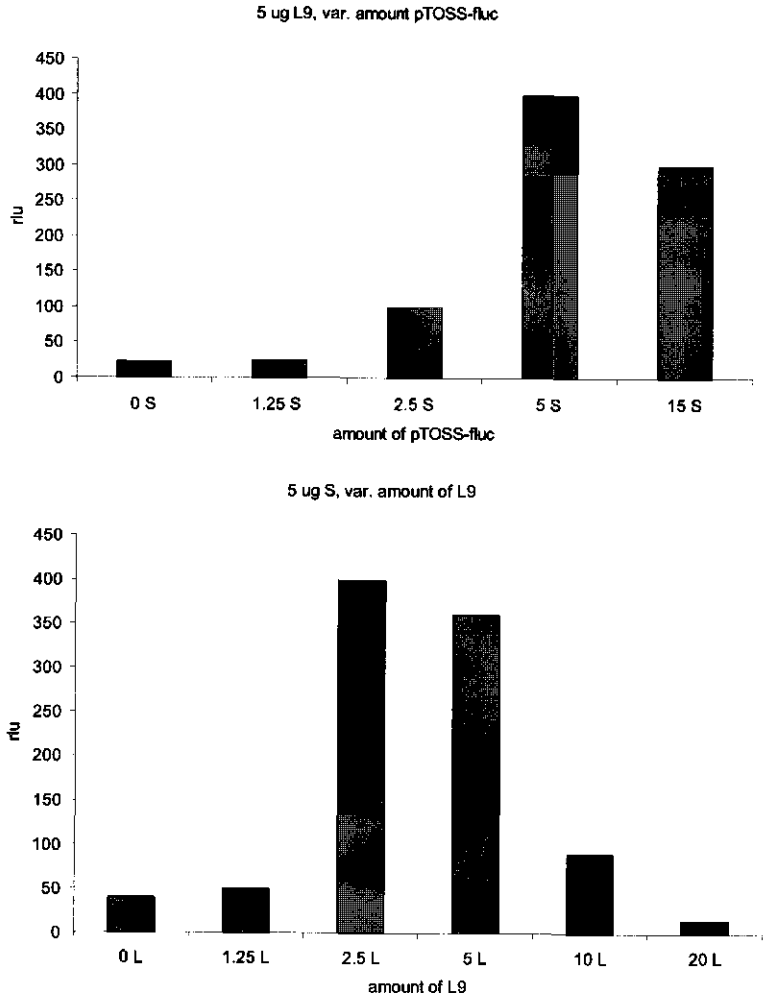

Figure 4.5. Optimisation of *luc* expression levels. Subconfluent monolayers of OST7-1 cells in 35 mm culture dishes were infected with MVA-T7 and subsequently co-transfixed with varying concentrations of pTUG-L and pTOS-S/Fluc and lysates were assayed 24 h.p.i. for luciferase activity.

Requirement of N protein for transcription.

To establish whether or not the N protein was required for primary transcription of the S RNA template, a mutant of pTOS-S/Fluc was made, in which the start codon of the

N ORF was deleted (pTOS-S/Fluc-$\Delta N^{(ATG)}$, see Materials & Methods). After co-transfecting pTOS-S/Fluc-$\Delta N^{(ATG)}$ and pTUG-L, cells were tested for luciferase activity at 24 hours p.i. No luciferase activity was detected (Table 4.1), indicating that both the nucleoprotein and RdRp are required for transcriptase activity. Like in previous experiments, the extra addition of pTUG-N again resulted in total loss of *luc* expression.

Table 4.1. Effect of N protein on primary transcription.

	luciferase activity in #rlu
control cells	5
pTUG-L9 + pTOS-S/Fluc	3092
pTUG-L9 + pTOS-S/Fluc-$\Delta N^{(ATG)}$	0
pTUG-L9 + pTUG-N + pTOS-S/Fluc-$\Delta N^{(ATG)}$	0

Luciferase activity was assayed at 24 hours p.i. and is given in # of relative light units (rlu).

Time course analysis of reporter gene expression.

To further substantiate that ongoing transcription of the reconstituted ambisense S RNP took place, a time course analysis was performed and samples were tested for the presence of N protein and *luc* activity. The N protein could be detected at 9 hours p.i. and accumulated upon longer expression (Fig. 4.6, panel B). Luciferase activity could be detected as early as 6 hours p.i. and increased until a maximum was reached at 18 hours p.i. (Fig. 4.6, panel A). At 18 hours p.i., the luciferase activity decreased again. Consistently, in different experiments the amount of N protein drastically increased between 21 and 24 hours p.i.

TSWV specific mRNAs contain host derived leader sequences.

To demonstrate that reporter gene expression was indeed the result of genuine, TSWV L protein governed transcription and not of translation of genomic RNA strands capped by *Vaccinia virus* enzymes, experiments were performed to show the presence of host leader sequences at the 5' ends of TSWV S-RNA specific transcripts, indicative for "cap snatching" [26,27,109]. During the latter process, capped host mRNAs are recruited by the viral polymerase complex and subsequently cleaved by an encompassing endonuclease activity, generally at a distance of 10-20 nt from the cap structure. The capped RNA primer obtained is subsequently used to prime synthesis of viral mRNA [26,27]. Previously we have shown that by nested RT-PCR,

TSWV transcripts provided with specific host derived leader sequences could be amplified from infected cells [26,27]. Using the same strategy, the presence of murine mRNA leader sequences at the 5' ends of TSWV mRNAs, synthesised in the *Vaccinia*-T7 expression system, was investigated. To this end two host (*M. musculus*) mRNAs with precisely known 5' leader sequences were selected. These mRNAs coded for the TATA-box binding protein (TBP; GenBank acc.no. D86619) and S-adenosylmethionine decarboxylase (AMD1; GenBank acc.no. AB025024).

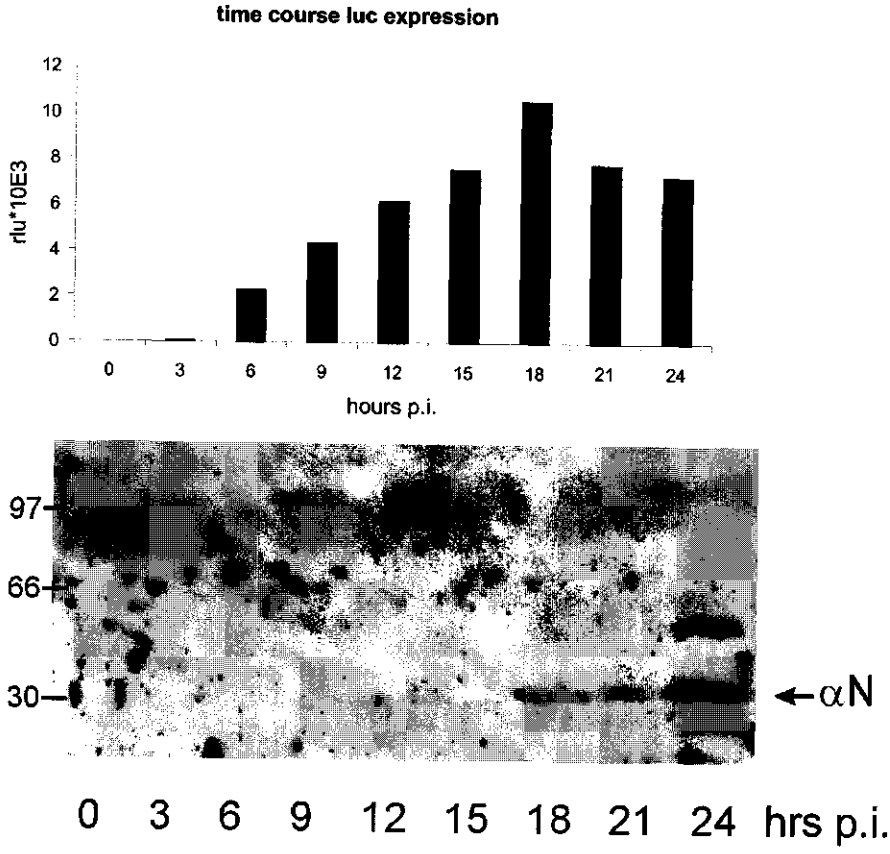

Figure 4.6. Time course expression of S RNA ORFs. OST7-1 cells were infected with MVA-T7 and subsequently co-transfixed with pTUG-L and pTOS-S/Fluc. Samples were assayed at different time points post transfection for luciferase activity (A) and for presence of N protein (B). N protein was detected using Western immunoblotting and ECL detection. Size markers (in kDa) are indicated at the left side of the Western blot.

As both TBP and AMD-1 mRNAs matched the cap donor requirements [26], it was expected that they would be used as cap donors during TSWV transcription initiation. To test for this, primers were designed for the 5' terminal 11 nt of the TBP and AMD-1 leaders, which were used in combination with NSs-gene specific primers in a nested RT-PCR [27]. Fragments of the expected size (~ 420 bp) could be amplified from total RNA of OST7-1 cells co-transfixed with pTUG-L9 and pTOS-S (Fig. 4.7). The presence of host leader sequences in viral mRNAs thus suggests that expression of luciferase was the result of transcription by the TSWV RdRp.

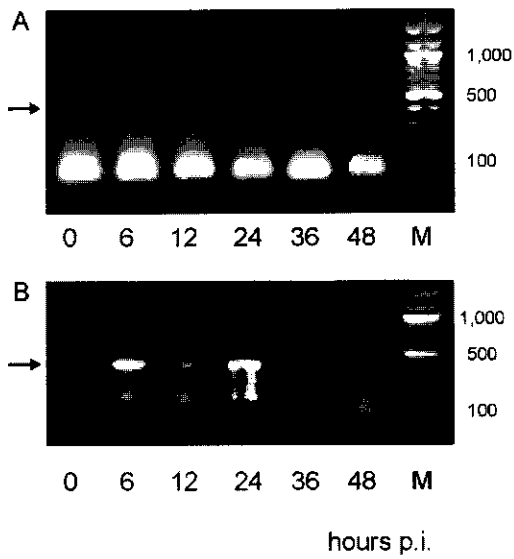

Figure 4.7. Use of host mRNAs as cap donors for TSWV transcription. Total RNA was isolated from MVA-T7 infected OST7-1 cells co-transfixed with pTUG-L and pTOS-S at various time points and the presence of NSs mRNAs containing specific capped host leader sequences was detected using RT-PCR. (A) NSs mRNAs containing leader sequences derived from AMD-1 (S-adenosylmethionine decarboxylase) mRNAs. (B) NSs mRNAs containing leader sequences derived from TBP (TATA-box binding protein) mRNAs. PCR fragments of ~420 bp represent NSs mRNAs and are indicated by arrows.

DISCUSSION

In this report a reverse genetics system has been set up and used to study the transcription of a naturally occurring ambisense RNP, the TSWV S RNP. Natural ambisense RNA segments occur in a limited number of plant- and animal-infecting viruses classified within the *Bunyaviridae*, *Arenaviridae* or *Tenuiviruses* [94,100]. The unique genome organisation of ambisense RNA molecules requires the presence of specific *cis*-acting sequences that are absent in negative strand RNA molecules, e.g. a bi-directional transcription termination signal within the intergenic region and promoter sequences for both replication and transcription at the 3' end of the viral and viral complementary strand (Fig. 4.2).

To obtain transcriptionally active ambisense S RNPs, the TSWV L protein was co-expressed with the N protein and a reporter RNA template derived from the viral S RNA. The L protein had been regarded as the putative RNA-dependent RNA polymerase [1,20,104] and it can be concluded from the experiments described here that the L protein indeed is the transcriptase, as expression of different clones of the L protein in our system resulted in different levels of reporter gene expression. The differences in transcriptase activity and consequently in reporter gene expression levels probably either are caused by point mutations that have arisen by means of RT-PCR errors or reflect a natural polymorphism. To our knowledge, the TSWV RdRp is the largest non-processed protein that has been expressed in the *Vaccinia*-T7 system so far. This enzymatic activity could only be demonstrated when the RdRp was co-expressed with the nucleoprotein, indicating that the latter is required to obtain an active transcriptase complex. Swapping the NSs gene by the luciferase gene further improved the sensitivity of the system and demonstrated the feasibility of reverse genetics with this system.

The time course analysis (Fig. 4.6) showed a decrease of the *luc* activity between 18 and 21 hours p.i. and a (combined) sudden increase of nucleoprotein accumulation between 21 and 24 hours p.i., suggesting a transition from primary transcription (of the *luc* gene) to replication and secondary (N gene) transcription (Fig. 4.2). The sudden increase in nucleoprotein synthesis between 21 and 24 hours p.i. thus would reflect translation of secondary transcripts combined with the already present transcription of the capped TOS-S RNA. A similar effect of high levels of N protein on reduction of marker gene expression (NSs or luciferase) was seen when pTUG-N was added to pTUG-L and pTOS-S or pTOS-S/Fluc. In these cases, the addition of pTUG-N led to high levels of N protein expression (Fig. 4.3 and 4.4) and a decrease

of marker gene expression below detection levels. These data are all in support of the hypothesis that a pool of free (soluble) N protein may trigger a switch from transcription to replication [87].

To further substantiate that the marker gene expression indeed was caused by translation of TSWV mRNAs rather than TSWV antigenomic RNAs capped by *Vaccinia* enzymes, the presence of non-viral leader sequences at the 5' end of these mRNAs was demonstrated, indicative for "cap snatching". Transcription initiation by the recombinant L protein thus resembles the process seen during a natural infection and suggests that the L protein encompasses the endonuclease activity needed to generate capped RNA primers. The availability of a transcriptionally active ambisense TSWV RNP, reconstituted from cloned cDNAs, now enables extended *in vivo* analyses of the transcription initiation mechanism, both of the *cis*- and *trans*-acting factors. For *Influenza A virus*, it has been shown that secondary structures within the terminal sequences of the genomic RNA template are involved in the transcription initiation process [67]. The system described here will allow identification of possible similar structures in the terminal sequences of the TSWV genomic RNA and if these play a role in transcription initiation (and thereby direct the polymerase complex towards cap snatching). Moreover, the intergenic hairpin structure will be analysed for its involvement in bi-directional transcription termination. Such analyses will likely shed more light on the *cis*-acting sequences involved in replication and transcription of these peculiar ambisense RNA molecules.

MATERIALS & METHODS

Viruses and cells.

Recombinant *Vaccinia virus* MVA-T7 [102,115] was used for T7 RNA polymerase driven expression of cDNA constructs. Murine OST7-1 cells [30] were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL), supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml gentamycin. TSWV isolate BR-01 was used for construction of DNA copies of the full-length ambisense S RNA, the N gene and the L gene.

Construction of Plasmids.

Full-length copies of the viral complementary S RNA strand were generated by means of RT-PCR amplification. In brief, the cloning procedure was as follows: first strand cDNA was synthesised from 100 ng purified TSWV nucleocapsid RNA, using primers p18 (AGAGCAATTGTGTCAGAATTTTGTTCATAATCAAACCTCACT-

TAGAAAATCACAATACTG, identical to nt 1-60 of the S RNA) and p17 (AGAGC-AATCGTGTCAATTTTGTGTTCATACCTTAACACTCAGTCTTACAAATCATC ACAT, complementary to nt 2855-2916 of the S RNA) using Superscript Reverse Transcriptase (GIBCO BRL) according to the manufacturer's procedures. Subsequently, the 5' half of the viral complementary S RNA (containing the N ORF) was PCR amplified using primers p07 (GGACCAATT**TGGCCA**AATTTGGG, identical to nt 1702-1724 of the S RNA) and p09 (CCCGCGGCCGC**GGATCC**TAA-TACGACTCACTATAGAGAGCAATCGTGTCAATTTTGTGTTCATACCTT, complementary to nt 2885-2916 of the S RNA and containing a *Bam*HI restriction site (bold) and a T7 promoter sequence (underlined)). The 3' end half of the viral complementary S RNA (containing the NSs ORF) was PCR amplified using primers p08 (CCCAAATT**TGGCCA**AATTGGTCC, complementary to nt 1702-1724 of the S RNA) and p10 (CAGCGGGCGCCAGCGAGGAGGCTGGGACCATGCCGGCC-AGAGCAATTGTGTCAGAATTTT, identical to nt 1-22 of the S RNA and fused to the 3' terminal 40 nt of the hepatitis delta ribozyme sequence (underlined)). For incorporation of the remaining hepatitis delta ribozyme sequence, a re-amplification was performed on this PCR fragment using primers p08 and p03 (CCC**GGATCC**-GTCCCATCCGCCATTACCGAGGGGACGGTCCCCTCGGAATGTTGCCCAGC CGGCGCCAGCGAG, containing the remaining hepatitis delta ribozyme sequence and an additional *BamHI* restriction site (bold)). Both PCR fragments were cloned separately into pGEM-T (Promega Inc.) according to the manufacturer's procedures, resulting in plasmids pGEM-T-ϕ10-N and pGEM-T-NSs-hδ, respectively. After excision of the inserts from plasmids pGEM-T-ϕ10-N and pGEM-T-S-hδ by restriction with *BamHI* and *Msc*I, the fragments were cloned into the *BamHI* site of pUC-Tϕ (modified pUC19, containing a T7 terminator sequence) by triple end ligation. The plasmid obtained thus contained a copy of the full length TSWV S RNA, was referred to as pTOS-S (Fig. 4.2) and was verified by restriction and sequence analyses.

In order to insert a firefly luciferase marker gene, a deletion mutant of pTOS-S was made, denoted pTOS-SΔNSs, lacking the NSs gene but containing a restriction site for convenient cloning instead. Therefore, pTOS-S was re-amplified using primers p71 (CATAAACACTTGAAG**CCATGG**TGGTTATTGGTACTGTGTTC, complementary to nt 67-107 of the S RNA and containing a *Nco*I site (bold) at the position of the start codon of the NSs ORF) and p72 (GTTAAGGAAGCGTATG**CTCGAG** GATCAAAATAATCTTGCTTTGTCCAGC, identical to nt 1451-1499 of the S RNA and containing a *Xho*I site (bold) at the stop codon of the NSs ORF). Amplified PCR fragments were column purified using the High-Pure PCR purification kit (Roche),

restriction enzyme digested with *Dpn*I (to destroy input template DNA), followed by *Nco*I and *Xho*I digestion. The firefly luciferase gene, excised from pSP-luc+ (Promega, Inc.) by *Nco*I and *Xho*I, was ligated into pTOS- SΔNSs, resulting in a plasmid onwards referred to as pTOS-S/Fluc. Plasmid pTOS-S/Fluc-$\Delta N^{(ATG)}$, in which the start codon of the N ORF was deleted, was made by PCR mutagenesis on pTOS-S/Fluc using primers p083 (GATGATCGTAA-AAGTTGTTATATGC, identical to nt 2764-2788 of the S RNA) and p084 (TCTA-AGGTTAAGCTCACTAAGG, complementary to nt 2739-2760 of the S RNA).

The ORF coding for the TSWV L protein was RT-PCR amplified using primers L1 (CCCTA**GGATCC**ATGGTCATCCAGAAAATACAAAAATTAATAGAAAATGG AACCAC, identical to nt 34-77 of the L RNA and containing a *BamHI* site (bold)) and L2 (CCCTA**GGATCC**AATTTAATCTGTGTCTTCTTCTTCATCAAGCT-CATCTTC, complementary to nt 8626-8665 of the L RNA and containing a *BamHI* site (bold)), and subsequently cloned into the *Bam*HI site of pTUG3 [111], resulting in plasmid pTUG-L. The ORF coding for the TSWV N protein was RT-PCR amplified using primers p19 (CCC**GGATCC**ATGTCTAAGGTTAAGCTCACTAAGG, complementary to nt 2739-2763 and containing a *BamHI* site (bold)) and p15 (CCC**GGATCC**TCAAGCAAGTTCTGCGAGTTTTG, identical to nt 1987-2008 and containing a *BamHI* site (bold)), and cloned into the *Bam*HI site of pTUG3, resulting in plasmid pTUG-N. The ORF for the TSWV NSs protein was cloned into pTUG as a *BamHI* fragment from pAc33DZ1/NSs [58], resulting in pTUG-NSs.

Transfection of plasmids and infection with MVA-T7.

Subconfluent monolayers of OST7-1 cells, grown in DMEM without FBS or gentamycin in 35 mm culture dishes, were inoculated at 37°C with *Vaccinia virus* MVA-T7 at a multiplicity of infection (moi.) of 10. Plasmid DNA was diluted in 100 µl of serum free DMEM. For each µg of DNA to be transfixed, 5 µl LipofectACE transfection reagent (GIBCO BRL) was simultaneously diluted in 100 µl of serum free DMEM. Both DNA and LipofectACE dilutions were mixed after 5 min., and left at room temperature for another 45 minutes. At 1 hour post infection (p.i. t = 1 hr), medium containing *Vaccinia virus* was removed from the dishes, and the cells washed once with serum-free DMEM. The DNA-LipofectACE mixture was added to the cells and left for incubation for 10 min at 37°C and 5% CO_2. After this, 800 µl of serum free DMEM was added and incubation was continued at 37°C and 5% CO_2. At t = 6 hr p.i., 1 ml of DMEM containing 20% FBS and 100 µg/ml gentamycin was added and the incubation was continued at 37°C and 5% CO_2 until cells were collected for

further analysis, mostly 24 hrs post transfection (p.t.).

Western immunoblot analyses.

Cells were washed once with phosphate buffered saline (PBS) and subsequently harvested in 250 µl of 1x Cell Culture Lysis Reagent (1xCCLR: 25 mM Tris-phosphate pH 7.8, 2 mM DTT, 2 mM 1,2-diamino-cyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100 ;Promega Inc.). Lysates were cleared in microcentrifuge tubes by centrifugation for 30 sec at 14,000 x g and room temperature. To 10 µl of these lysates, 2 µl of 6x SDS loading buffer (6x SLB: 0.35 M Tris-HCl pH 6.8, 10.28% SDS, 36% glycerol, 0.6 M DTT, 0.012% bromophenol blue; [15]) was added and the sample applied on 12,5% SDS-PAGE. After electrophoresis, proteins were semi-dry blotted onto Immobilon-PVDF membrane for immunoblot detection. Antisera specific for the TSWV N and NSs proteins, in combination with the ECL detection system (Amersham-Pharmacia), were used for visualisation of proteins according to the manufacturer's procedures.

Immunofluorescence analyses.

Cells were grown on 10 mm cover slips to a density of ~ 50% in 35 mm culture dishes. Infection and transfection was performed as described above. After 24 hrs of incubation, cells attached to the cover slips were washed once with PBS and subsequently fixed for 10 min in 96% ethanol. Cells were blocked in PBS-3% BSA (Sigma) for 30 min and subsequently incubated for 1 hr in PBS-1% BSA containing 1:500 diluted (crude) rabbit antiserum directed against a C-terminal 60 kDa fragment of the TSWV L protein [108]. Cells were washed 3 times for 15 min with PBS and incubated with PBS containing 1:100 diluted goat-anti-rabbit fluoresceine isothiocyanate (GAR-FITC; Nordic) labelled IgM for 1 hr at room temperature. Cells were washed 3 times for 15 min with PBS, embedded in Citifluor and analysed by UV fluorescence microscopy (Zeiss LSM510).

Measurement of luciferase activity.

Luciferase activity of cell lysates was measured in a TD 20/20 luminometer (Turner Designs) using the luciferase assay system (Promega) according to the manufacturer's protocols. Briefly, cells were washed once with PBS-0 (PBS lacking Ca^{2+} and Mg^{2+}) and subsequently lysed in an appropriate amount of 1xCCLR. The lysates were cleared of cell debris by centrifugation for 30 sec in a microcentrifuge. An aliquot of 20 µl cell lysate was mixed briefly with 100 µl of luciferase assay reagent and emitted light was immediately measured in the luminometer for 15 sec.

RT-PCR detection of TSWV mRNAs containing capped host leaders.

TSWV N mRNAs containing capped 5' leader sequences derived from different host (*M. musculus*) genes were detected by nested RT-PCR and analysed by sequence determination as described earlier [26,27]. In brief, total RNA was extracted at various time points from OST7-1 cells which had been infected with MVA-T7 and transfixed with pTUG-L9 and pTOS-S using Trizol (GIBCO BRL) according to the manufacturer's procedures. First strand cDNA was synthesised from 10 μg of this total RNA using AMV RT (Promega) and primer NS1 (GGGCAGGAGACAAAACC, complementary to nt 439-455 of the S RNA). PCR amplification on first strand cDNA material was performed with nested primers NS2 (CCC**GGATCC**GATAGTGCC-AGAACAGAG, complementary to nt 367-384 of the S RNA and containing a *BamHI* site (bold)) in combination with a primer specific for the first 11 nt of the 5' end of different host genes. Host genes chosen were the genes for the TATA-box binding protein (GenBank acc.no. D86619, with corresponding primer TBP-1 (CCC**GGATCC**CATCAGATGTG, containing a *BamHI* site (bold)) and for S-adenosylmethionine decarboxylase (AMD-1) (GenBank acc.no. AB025024, with corresponding primer AMD-1 (CCC**GGATCC**GCTTACACAGT, containing a *BamHI* site (bold)). Expected RT-PCR products of ~420 bp represented TSWV NSs mRNAs.

CHAPTER 5

GENERAL DISCUSSION

Novel insights in the TSWV transcription mechanism

In this thesis, the transcription initiation mechanism of *Tomato spotted wilt virus* (TSWV) has been investigated. Like for all segmented negative strand RNA viruses, TSWV uses cap snatching to provide its subgenomic mRNAs with cap structures. During this process, capped host mRNAs or, in case of mixed infections, viral RNAs (Chapter 2) are recruited and their leaders subsequently used to function as primers for transcription. For TSWV, it is shown that cleavage of the leader RNA takes place at a distance of ~12-21 nt from the cap.

Using a series of AMV RNA4 point mutants, it could be shown that a preference exists for cleavage at a distance of 16 nt from the cap structure. Cleavage takes place 3' of a purine residue (preferably after an A, but also, though less efficiently, after a G), after which this residue pairs to complementary residues in the TSWV genomic template (sequence: 3'-UCUCGUUA...) (Chapter 3). Base pairing occurs mostly with the 3' ultimate U residue (3' terminal nucleotide of the capped primer is an A) and to a lesser extent with the penultimate C (3' terminal nucleotide of the primer is a G) (Chapter 3). Very infrequently, capped primers containing a 3' terminal A residue are observed to base pair to the 3' antepenultimate U residue of the viral template (Chapter 3).

Once the capped primer base pairs to the viral template RNA, it is elongated to ultimately result in the synthesis of a subgenomic mRNA. Additional insertions of a 5' terminal sequence between host leader and viral sequence, a feature explained as a result of a "prime-and-realign" mechanism [37], are rarely observed; this is in contrast to what is seen for the other segmented negative strand and ambisense RNA viruses, where extra insertions are seen more frequently [6,37,50,51,95].

In order to further identify and characterise *cis*-acting sequences and *trans*-acting factors of TSWV required for transcription (initiation) and replication, an *in vivo* system has been developed (Chapter 4) in which active TSWV S RNPs are reconstituted from cloned cDNAs by means of a *Vaccinia*-T7 expression system. The possibility to synthesise transcriptionally active (recombinant) viral RNPs now opens the way for site-directed mutagenesis of the viral genome. In order to shed more light on the TSWV transcription initiation mechanism, the results on cap snatching as described in this thesis will be discussed and compared in the following paragraph to what is known for other segmented negative strand RNA viruses.

Cap snatching as a common transcription initiation mechanism among segmented negative strand RNA viruses.

I. Cleavage site specificity in cap donor RNAs.

All segmented negative strand and ambisense RNA viruses share the cap snatching mechanism to initiate transcription of their mRNAs (Table 5.1). For different members of the *Orthomyxoviridae* and *Bunyaviridae*, this mechanism has been studied to some extend, in most detail for *Influenza A virus*.

Table 5.1. Overview of the genomic organisation and cap snatching of segmented negative strand RNA viruses.

Family	Genus	# RNA segments + polarity	Cap snatching demonstrated
Orthomyxoviridae	*Influenzavirus A*	8 (negative sense)	+
	Influenzavirus B	8 (negative sense)	+
	Influenzavirus C	7 (negative sense)	+
	Thogotovirus	6 (negative sense)	+
Bunyaviridae	*Bunyavirus*	3 (L,M,S; negative sense)	+
	Hantavirus	3 (L,M,S; negative sense)	+
	Nairovirus	3 (L,M,S; negative sense)	+
	Phlebovirus	3 (L&M negative sense, S ambisense)	+
	Tospovirus	3 (L negative sense, M&S ambisense)	+
Arenaviridae	*Arenavirus*	2 (both ambisense)	+
Not specified	*Tenuivirus*	4-6 (1,5,6 negative sense, 2,3,4 ambisense)	+
Not specified	*Ophiovirus*	3 (negative sense)	-

Adapted from Van Regenmortel *et al.*, Virus Taxonomy, 7th Ed. [110]

For this virus, it has been shown that the joint activities of the PA, PB1 and PB2 proteins are required, that together form the *Influenza* viral polymerase complex. In this complex, subunit PB2 binds the ^{m7}Gppp-cap structure on host mRNAs, for which tryptophan residues 537 and 564 have been shown to be involved [46,68] (Fig. 5.1 A). Endonucleolytic cleavage is then mediated by subunit PB1, for which three acidic

residues (aspartic acid and glutamic acid) at positions 508, 519 and 522 have been shown to be essential [68].

Activation of the endonuclease requires the presence of a loop within the hairpin or hook structure in the 5' vRNA arm as well as base-paired 5' and 3' terminal genomic sequences downstream of this structure (Fig. 5.1 B) [16,42].

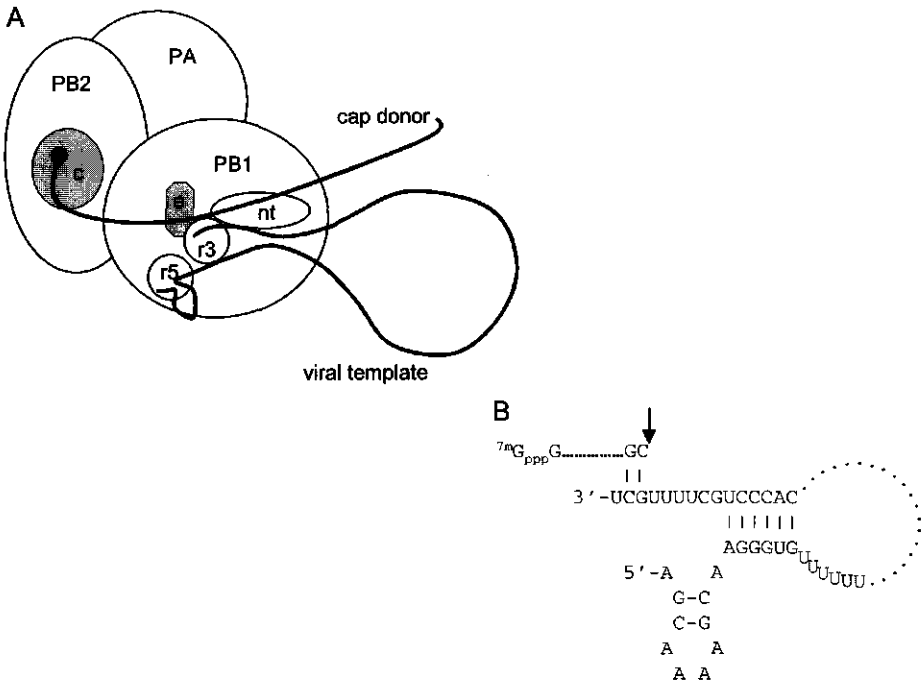

Figure 5.1. The polymerase complex of *Influenza A virus*. A. Schematic representation of the *Influenza A virus* polymerase complex. The viral template RNA is bound at its 5' end and 3' end by the 5' RNA binding (r5) and 3' RNA binding (r3) domains on the PB1 subunit of the complex, res. The cap-binding domain (c) on the PB2 subunit binds the ^{7m}G-cap structure of the cap donor RNA. Cleavage occurs by the endonuclease (e) domain on the PB1 subunit. Subsequently, the capped RNA primer base pairs with the template RNA and is elongated by the nucleotidyl transferase domain (nt) on the PB1 subunit. The PA subunit is thought to interact with both the PB1 and PB2 subunit. B. For *Influenza A virus*, base pairing between the capped primer and viral template preferably occurs at the penultimate and/or antepenultimate residue of the template. The 5' end of the viral template folds into a stem-loop structure, which has been shown to be essential for endonuclease activity. Arrow indicates the position of endonucleolytic cleavage.

For *Influenza virus*, cleavage takes place 3' of a purine residue, preferably after a G residue and mostly around 10-13 nt from the 5' end. For TSWV also a cleavage preference for purines seems to exist, preferably after an A residue between nt positions 12 and 21 from the 5' end, with an optimum for position 16. These preferences may indicate a physical separation of the cap binding site and the catalytic site of the endonuclease within the viral polymerase, a suggestion that also has been made to explain the leader length preference of *Influenza A virus* [24,46].

Interestingly, mRNAs of *Thogoto virus*, another member of the *Orthomyxoviridae*, are initiated with the ^{m7}Gppp-cap structure almost directly linked to the 5' ultimate (A) residue [2,113]. This remarkable difference in leader length preference between *Thogoto* and *Influenza* transcription cannot be explained by differences in the viral RNA template, as *Thogoto* polymerase complexes can accept *Influenza* vRNA-like templates during transcription and replication [66,67,114]. Rather closely located cap binding and endonuclease domains may cause the snatching of short leaders by *Thogoto virus*.

II. Need for complementarity between cap donor and viral template.

After cleavage of the cap donor, the capped host leader obtained is used to prime transcription on the viral genome. The co-infection experiments described in Chapter 3 demonstrate that for TSWV, priming occurs by base pairing of the 3' ultimate residue(s) of the capped RNA primer to the 3' ultimate residue of the viral RNA. Base pairing to the penultimate or antepenultimate residues also occurs, though this happens with a lower frequency: when specific cap donor RNAs containing either an A (A15) or a G (G15) residue at nt position 15 were offered (Chapter 3), RT-PCR amplification of viral mRNAs containing A15-derived primers yielded relatively more product than those containing G15-derived primers.

Cleavage site specificity for TSWV therefore likely is dictated by the base pairing requirement. Base pairing has also been suggested to occur during transcription initiation for several other negative strand RNA viruses, like *Influenza* A and *Hantaan* virus [24,37,43,54,78]. For *Influenza virus*, *in vitro* transcription even can be primed by di- (ApG and GpC) and trinucleotides (ApGpC) [45], as long as these are complementary to nt positions 1-3 of the 3' terminus of the viral RNA.

Though newly synthesised viral mRNAs (based on their 5' terminal sequence) would appear to be very suitable to serve as cap donors, it is unlikely that they are being cannibalised to initiate another round of transcription. Indeed, for *Influenza A virus*, the polymerase complex is able to recognise viral mRNAs and to selectively protect these from being re-used for cap snatching, a function which resides in the

NS1 protein [96]. For TSWV, such a protein has not been identified yet. For the majority of the segmented negative strand RNA viruses, no clear evidence has been presented yet on the requirement for base pairing during transcription. Hence, endonuclease cleavage specificity for most of these viruses has been assumed to reside on the first non-viral residue upstream of the viral sequence within the mRNA (Table 5.2, column 5, "-bp").

Table 5.2. Properties of non-viral leader sequences and cleavage specificities.

Virus	terminal genomic sequence	av. leader length (nt) + rep./- rep.	"prime-and-realign" frequency	Cleavage after (- bp)	(+bp)	Ref.
TSWV	5'-AGAGCAAU..	16/16	~ 10%	U	A^1,G^2	[26,27,109]
LAC	5'-AGUAGUGU..	13/13	~ 10%	U	A^2,G^1	[25]
GER	5'-AGUAGUGU...	13/12	~ 60%	U,C	A^2,G^1	[6,112]
BUN	5'-AGUAGUGU...	15/14	~ 80%	U	A^1,G^2	[50]
UUK	5'-ACACAAAG...	14/12	~ 50%	C	A^2,C^1	[99]
DUG	5'-UCUCAAAG...	11/9	~ 95%	C	U^2,C^1	[51]
RSV	5'-ACACAAAG...	14/13	~ 50%	A	A^2,C^1	[97]
MStV	5'-ACACAAAG...	13/13	~ 15%	A,C	A^2,C^1	[32,47]
FLUA	5'-AGCAAAAG...	12/10	~ 50%	A,G	A^2,G^1	[95]
FLUB	5'-AGCAGAAG...	12/10	~ 50%	A,G	A^2,G^1	[95]

Data in this table are derived from 5' RACE analyses of viral mRNAs. Average lengths are given including and excluding the additional inserted repeat sequences due to the "prime-and-realign" mechanism. Cleavage preferences are given based on initiation without a required base pairing (- bp) and with base pairing requirement (+ bp). [1] most frequent cleavage site; [2] alternative cleavage site. TSWV: *Tomato spotted wilt virus*; LAC: *LaCrosse virus*; GER: *Germiston virus*; BUN: *Bunyamwera virus*; UUK: *Uukuniemi virus*; DUG: *Dugbe virus*; RSV: *Rice stripe virus*; MStV: *Maize stripe virus*; FLUA: *Influenza A virus*; FLUB: *Influenza B virus*.

III. The "prime-and-realign" mechanism.

For all segmented negative strand and ambisense RNA viruses, small insertions of the 5' terminal sequence are observed with different frequencies between the host leader sequence and the authentic viral sequence. These inserted sequences are the result of a "prime-and-realign" mechanism, which first has been described for *Hantaan virus* [37].

During transcription initiation of this virus, host leader sequences are cleaved at a distance of 7 to 18 nt from the cap structure, generally 3' of a G residue [37]. The 3'

terminal G residue of the capped primer base pairs with the 3' antepenultimate C residue of the viral template, after which the primer is extended for another 3 nt. The primer is then released from the template, realigns backward and only then becomes elongated to yield a mature mRNA. The result of this "prime-and-realign" mechanism is a viral mRNA containing short nucleotide stretches identical to the viral 5' terminal sequence between the host leader and the authentic viral sequence (Fig. 5.2). A similar mechanism is also thought to be responsible for *Hantavirus* genome replication. As the genomic sequence of *Hantavirus* starts with a 5' U residue, replication would have to initiate with incorporation of a U residue. However, for all viral RNA polymerases studied so far [37], replication starts with the incorporation of ATP or GTP, leaving a triphosphate group at the 5' end of the viral RNA. *Hantavirus* replication is thought to initiate with incorporation of GTP, which base pairs with the 3' antepenultimate residue of the template RNA. After extension for 3 nt, the tetranucleotide primer obtained releases from the template, realigns and is further elongated. The additional G residue present at the 5' end of the nascent genomic RNA chain is then cleaved off, leaving a monophosphate (U) at the 5' end of the chain (Fig. 5.2). This slippage of the polymerase on the viral template has been explained as a way to maintain genome integrity and restore any damaged genome ends [37].

Based on our data for TSWV, i.e. base pairing requirement for capped leaders and the possible occurrence of "prime-and-realign", the reported mRNA leader sequences of other segmented negative strand RNA viruses were reassessed to determine the genuine endonucleolytic cleavage site and possible insertions within the 5' non-viral sequence (Table 5.2, column 4 and 6). Reassessment of the cleavage sites within these viral mRNAs shows that the initial base pairing of the recruited host leader occurs most frequently with the 3' penultimate residue of the template RNA and not with the ultimate residue (Table 5.2, column 6). Cleavage in these cases occurs 3' of a G residue (for the *Orthomyxoviridae* and the genus *Bunyavirus*) or 3' of a C residue (for the genus *Tenuivirus* and the genera *Phlebovirus* and *Nairovirus*).

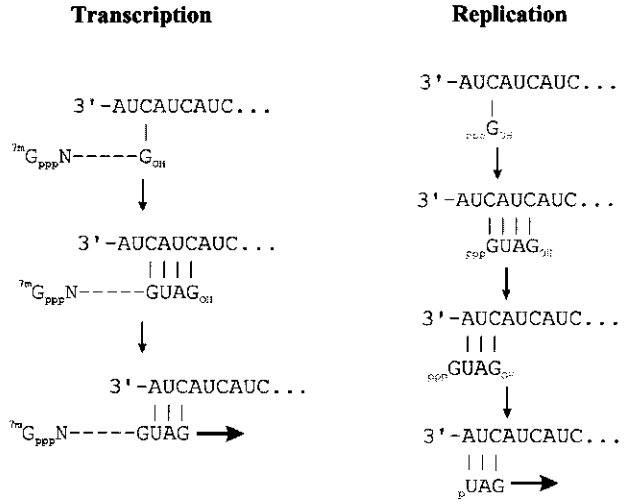

Figure 5.2. The "prime-and-realign" model. During transcription of *Hantaan virus* (left panel), a capped RNA primer, derived from a host mRNA leader through cap snatching and containing a 3' terminal G residue, base pairs to the 3' antepenultimate residue of the viral template. The primer is extended with three residues and released from the template RNA. The extended primer then realigns to the template by virtue of the terminal (UAG) repeats, re-anneals and is subsequently elongated according to the template. During *Hantaan* replication (right panel), the initial GTP base pairs with the 3' antepenultimate residue of the viral template and is extended for another three nucleotides. This tetranucleotide primer is released and realigns, resulting in a 5' non-base pairing G residue. This residue is cleaved off, possibly by the same viral endonuclease that catalyses the cap snatching reaction, to result in a 5' terminal monophosphate and a genomic RNA starting with a U residue. Adapted from Garcin *et al.* [37].

For *Bunyamwera virus*, a slight preference for cleavage 3' of an A residue can be seen [50], resulting in a preferred base pairing to the 3' ultimate residue of the viral template, but only a minimal difference in cleavage preference can be noticed (57% of the clones analysed were cleaved 3' of an A vs. 43% cleavage 3' of a G). The exception to this rule is TSWV, where the vast majority of the analysed mRNAs contained capped leader sequences that were originally cleaved 3' of an A residue and therefore have base paired with the 3' ultimate residue of the viral template. Additional insertions of the terminal sequence repeats due to a "prime-and-realign" mechanism are rarely seen for TSWV (1-3 of the 20 mRNAs analysed using 5' RACE [109], 3-4 of the 109 mRNAs analysed using specific RT-PCR [26,27]) and lack of the 5' terminal genomic residue within the viral mRNA is also infrequent (4-6 of the 20 mRNAs analysed using 5' RACE [109], 4-5 of the 109 mRNAs analysed using specific RT-PCR [26,27]). For most of the other segmented negative strand

RNA viruses including *Influenza virus* [95], "prime-and-realign" occurs more frequently during transcription initiation (up to 95%; Table 5.2, column 4).

For *Hantaan virus*, the "prime-and-realign" mechanism has not only been proposed as a process to initiate replication besides transcription [37]. This explains the presence of a monophospate-U residue at the 5' end of *Hantaan* genomic RNAs. For TSWV, genomic RNAs contain a triphosphate-A residue at their 5' ends [21], therefore "prime-and-realign" is unlikely to play a role during replication of TSWV.

Reverse genetics of negative strand RNA viruses.

I. The Vaccinia-T7 reconstitution system.

Identification and characterisation of the *cis*-acting sequences and *trans*-acting factors involved in TSWV transcription would greatly benefit from a system that allows mutagenesis of these elements. For a long time genetic manipulation of negative strand RNA viruses has lagged behind that of positive strand RNA viruses. The main reason for this drawback has been a mere technical one: whereas for positive strand RNA viruses the RNA itself is infectious and functions as a translatable messenger, for negative strand RNA viruses the isolated viral genomic or antigenomic RNA is only infectious when associated with several viral proteins, among which the viral nucleoprotein and the polymerase, into a ribonucleoprotein (RNP) complex. So in order to enable the application of reverse genetics on a negative strand RNA virus, not only the transcription of a cloned cDNA is important but additionally, the assembly of viral genomic RNA and proteins into a functional RNP is crucial.

Since the late eighties, different approaches have been developed to overcome this problem. The first one is based on *in vitro* reconstitution of a transcriptionally active RNP, as successfully demonstrated by Luytjes *et al.* [70]. In this study, a cDNA clone was made from the *Influenza A virus* RNA 8 segment, coding for the NS1 protein. The NS1 gene was replaced by a foreign (chloramphenicol acetyltransferase, CAT) gene and transcripts of this reporter construct were mixed *in vitro* with purified RNA polymerase proteins. Partially reconstituted RNPs obtained in this way were transfixed along with helper *Influenza A virus*, which enabled the amplification, expression and even packaging into virions of the recombinant RNA. However, the main disadvantage of this system was that it required the presence of a helper virus, thereby making it impossible to perform mutagenesis on *trans*-acting signals that would allow identification of domains on e.g. the polymerase proteins. Moreover, due to the absence of a selection pressure, the recombinant RNP can be outcompeted during replication by the original viral RNPs.

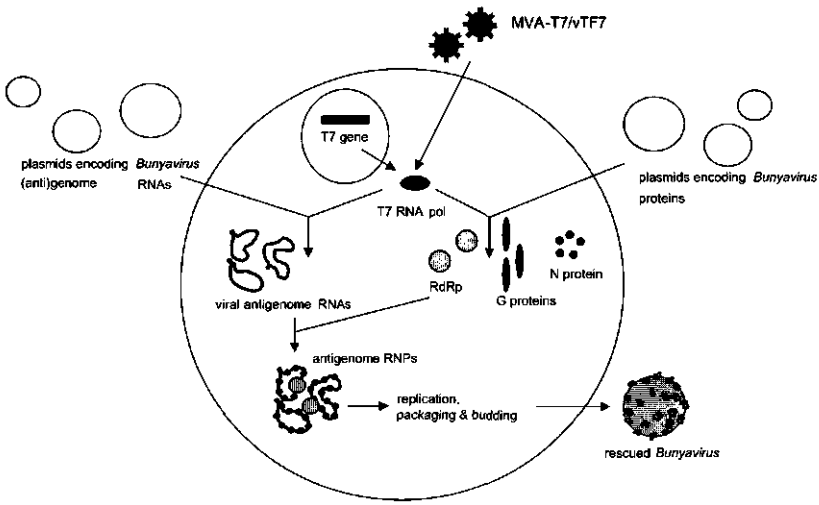

Figure 5.4. Rescue of a *Bunyavirus* (e.g. TSWV) from cloned cDNAs in the *Vaccinia*-T7 system. Mammalian cells are infected with a *Vaccinia virus* recombinant expressing bacteriophage T7 RNA polymerase (vTF7 or MVA-T7). Subsequently, plasmids containing cDNA copies of the viral (anti)genomic RNAs and of the polymerase (RdRp), glycoproteins (G) and nucleoprotein (N) genes are transfixed into these cells. (Anti)genome ribonucleoproteins are reconstituted, after which replication and transcription by the bunyaviral RdRp takes place. Genomic & antigenomic RNPs are subsequently packaged into new virions.

A second approach, which has been used more widely in several systems, is based on *in vivo* reconstitution of an active RNP complex entirely from T7 RNA polymerase driven cDNA constructs using the *Vaccinia* -T7 system [34,115], as first shown for *Vesicular Stomatitis virus* [81,82,83], and soon after that for several other viruses of the *Mononegavirales*, e.g. *Rabies* [19], *Sendai* [13] and *Measles virus* [98]. In 1995, this approach has been successfully applied for the first time for a segmented, negative strand RNA virus, *Bunyamwera virus* [28]. In the *Vaccinia*-T7 system, cDNA clones coding for all viral proteins (or only the viral proteins involved in transcription and replication) and for one or all viral RNA segments, controlled by the T7 RNA polymerase promoter, are transfixed into mammalian cells, which are subsequently infected with a recombinant *Vaccinia virus* expressing T7 RNA polymerase (Fig. 5.4). The viral polymerase and nucleoprotein are expressed to high levels in the *Vaccinia*-T7 system and associate with the genomic (reporter) RNA template that is co-expressed, thus reconstituting active RNPs or (in case copies of all genomic RNAs are provided) even virions (Fig. 5.4).

A disadvantage of the *Vaccinia*-T7 system is that infection of mammalian cells with *Vaccinia virus* will lead to cytopathological effects (CPE) and ultimately to cell death. This problem has partially been overcome by using a recombinant, attenuated strain of *Vaccinia virus* (Modified *Vaccinia* Ankara-T7 RNA polymerase recombinant, MVA-T7 [102,115]), which displays less CPE and thus allows prolonged expression periods. Mutagenesis of both *cis*- and *trans*-acting factors has become possible by the application of this rescue system.

II. Reconstitution of active TSWV S RNPs.

To be able to further unravel the transcription (initiation) and replication processes, a system was required that would express the 331.5 kDa polymerase protein along with the nucleoprotein and a reporter RNA template. As the *Vaccinia*-T7 system was used successfully for these purposes with other segmented negative strand RNA viruses, including a member of the animal-infecting *Bunyaviridae* [28], this system was used to reconstitute active TSWV RNPs *in vivo*. The reporter template used in our studies was based on the ambisense S RNA segment. Although it shares several features with RNA segments of complete negative polarity, ambisense RNA segments in addition hold some factors that are unique for their coding strategy. For example, the promoter sequences in the viral genomic termini of negative sense RNA segments are organised in such a way that the viral sense RNA is both transcribed and replicated, while the viral complementary RNA only needs to serve as a template for replication. For the ambisense TSWV S (and M) RNA segments, both viral sense and complementary sense RNAs serve as templates for transcription and replication. Moreover, the intergenic region located between the two ORFs acts as a bi-directional transcription terminator. Using the *Vaccinia*-T7 system and the TSWV S RNA as a reporter construct, transcriptionally active TSWV S RNPs were reconstituted from cloned cDNAs (Chapter 4), now opening the possibility to investigate the *trans*-acting factors and *cis*-acting signals involved in the transcription initiation mechanism *in vivo*.

Trans- acting factors to be analysed, first of all, are the RdRp and N protein. Analysis of (point/deletion) mutants of the L protein may give information on which domains of the 331,5 kDa protein are essential for specific enzymatic activities, e.g. endonuclease or nucleotidyl transferase. These RdRp mutants, furthermore, may be assessed for their affinity to genomic viral RNA, recruitment of capped host mRNAs and interaction with the N protein.

The *Vaccinia*-T7 system demonstrated that the nucleoprotein is involved in viral transcription and replication (Chapter 4). Point mutants of the nucleoprotein, in which the previously identified domains involved in protein-protein interactions [105]

or RNA binding [91] are mutated, can be expressed in the *Vaccinia*-T7 system and the influence of the mutations on viral transcription and replication can be determined.

Apart from the protein factors involved, the *cis*-acting viral sequences in the 5' and 3' termini can be identified, shedding light on their function as promoters for both transcription and replication, as discussed below.

III. Cis-*acting elements in viral genomic termini.*

The activity of the promoters for transcription and replication, which are located in the 5' and 3' terminal sequences, can differ between the various RNA segments of segmented negative strand and ambisense RNA viruses. For *Bunyamwera virus*, reporter (CAT) gene activity can only be observed when both 5' and 3' terminal sequences are present in the viral RNA template [28,29]. Templates containing the 5' and 3' terminal sequences of the L and M RNA segments showed significantly higher CAT activity than the original template containing the termini of the S RNA segment [29]. Templates containing 5' and 3' terminal sequences derived from different RNA segments did not show any reporter activity, but this could be restored if at least the first 18 terminal nt were complementary (only the terminal 11 nt are identical between all RNA segments; Table 5.3). Based on the number of base pairing residues in the termini of the *Bunyamwera* S, M and L RNA segments, it can be hypothesised that the amount of complementarity between the 5' and 3' terminal sequence determines promoter strength with respect to transcription (Table 5.3) [29].

For TSWV S, M and L RNAs, no major difference in size of the double stranded panhandle is observed, when compared to *Bunyamwera virus*.

Table 5.3. Terminal sequences of *Bunyamwera virus* and TSWV.

Segment	5' terminal sequence	3' terminal sequence
BUN S	AGUAGUGUACUccacacuacaaacu---uaaguuuuaggugg	AGCACACUACU
BUN M	AGUAGUGUACUaccgauacaucaca---guuuuguuaucggu	AGCACACUACU
BUN L	AGUAGUGUACUccuacauauagaaa---uuuucuuauguagg	AGCACACUACU
TSWV S	AGAGCAAUugugucagaauuuuguu---aacacaaaauugacac	GAUUGCUCU
TSWV M	AGAGCAAUcagugcaucagaaauau---gguuuuuguuugcacu	GAUUGCUCU
TSWV L	AGAGCAAUcagguaacaacgauuuu---uguuuuaguuguaccu	GAUUGCUCU

Sequences given are of viral sense RNA segments. Complementary terminal sequences are underlined; identical terminal sequences between different RNA segments are in capitals. BUN:

Bunyamwera virus; TSWV: *Tomato Spotted Wilt Virus*.

The *Vaccinia*-T7 reconstitution system will allow synthesis of template RNAs containing the S, M or L RNA termini, flanking a luciferase reporter gene in order to easily assay for promoter strength within these termini.

The ambisense TSWV S and M RNA segments contain an AU-rich sequence in their intergenic region, which enables the formation of a stable hairpin structure [22,56]. The length of the TSWV subgenomic mRNAs [55], as likewise found for the related *Uukuniemi virus* [99], suggests that this intergenic hairpin is involved in bi-directional transcription termination. Mutations altering the length or amount of complementarity in this hairpin (altering its stability) will determine whether it indeed acts as a transcription termination signal and whether its stability partially determines the balance between transcription and replication. Also, transcription for *Uukuniemi virus* terminates after the intergenic hairpin [99], while for *Punta Toro virus* [48], it terminates within the intergenic hairpin. Mapping the 3' ends of the viral mRNAs derived from (mutant) S RNA segments will determine which of these models applies for TSWV.

IV. Reconstitution using RNA pol I-based templates.

Whereas the *Vaccinia*-T7 system has been used successfully for reverse genetics on several negative strand RNA viruses, the system has some disadvantages that may hamper studies on the transcription and replication of an ambisense RNA. First of all, the *Vaccinia*-T7 system is based on the use of recombinant *Vaccinia virus*. Cells will eventually die during expression due to the *Vaccinia* infection, thus rescue of complete recombinant virus particles in this system is difficult. As a result, reconstituted virions in the *Vaccinia*-T7 system generally have to be passed to uninfected cell cultures in order to obtain high titres.

A second problem, which especially complicates the rescue of *Orthomyxoviridae* in the *Vaccinia*-T7 system, is transfection efficiency. To successfully rescue e.g. *Influenza A virus* from cloned cDNAs, a minimum of 12 different plasmids has to be transfected into the same cell: eight plasmids containing cDNA copies of all genomic RNAs, three plasmids for the expression of three subunits of the polymerase complex and one for expression of the viral nucleoprotein. For TSWV, however, this complication seems of less importance, as only 5 constructs (three encoding the S, M and L RNA segments and two expressing the RdRp and nucleoprotein) are needed in order to reconstitute virions.

A third problem, which is also encountered during reconstitution of active TSWV S RNPs in the *Vaccinia*-T7 system, is that *Vaccinia* capping enzymes are able

to provide all RNAs in the cell with cap structures. Thus, not only viral subgenomic mRNAs that are capped by means of the TSWV cap snatching mechanism will be translated, but also replicated genome segments, subsequently capped by the *Vaccinia* guanylyl transferase and 2-O'-methyltransferase. The level of reporter gene expression, therefore, will depend on viral transcription and replication as well, as RNA molecules resulting from the latter are capped too.

During the course of the research described in this thesis, an alternative system has become available for *Influenza A virus* to overcome these problems, i.e. the RNA pol I/pol II system. In this system, both proteins and RNA segments are directly expressed from cDNA clones by cellular RNA polymerases [76,77]. The polymerase proteins and nucleoprotein in this system were expressed from a *cytomegalovirus* or chicken β-actin promoter containing cDNA construct and therefore transcribed by the cellular RNA polymerase II complex, yielding translatable mRNAs. Genomic RNA segments were cloned in a cellular RNA polymerase I promoter-terminator cassette. The resulting *Influenza* genomic RNA transcripts therefore are not capped and contain authentic 5' and 3' termini. This system was the first in which recombinant *Influenza A virus* could be rescued entirely from cloned cDNAs [77] without the need for an additional helper virus or recombinant *Vaccinia virus*. The system was further adapted by combining the RNA pol I and RNA pol II cassette into a single construct (Fig. 5.5), minimizing the number of constructs that need to be transfected into cells and thus greatly increasing rescue efficiency [44].

The use of an RNA pol I/ pol II system for TSWV next to the already existing *Vaccinia*-T7 system will open the possibility to assess primary and secondary transcription of its ambisense RNA segments. A recombinant S RNA segment has already been made in which the N gene is replaced by the *Renilla* luciferase gene and the NSs gene by the firefly luciferase gene (Fig. 5.6 and unpublished results). Easy assaying of expression of both reporter genes will be possible due to high sensitivity of detection of both luciferase activities.

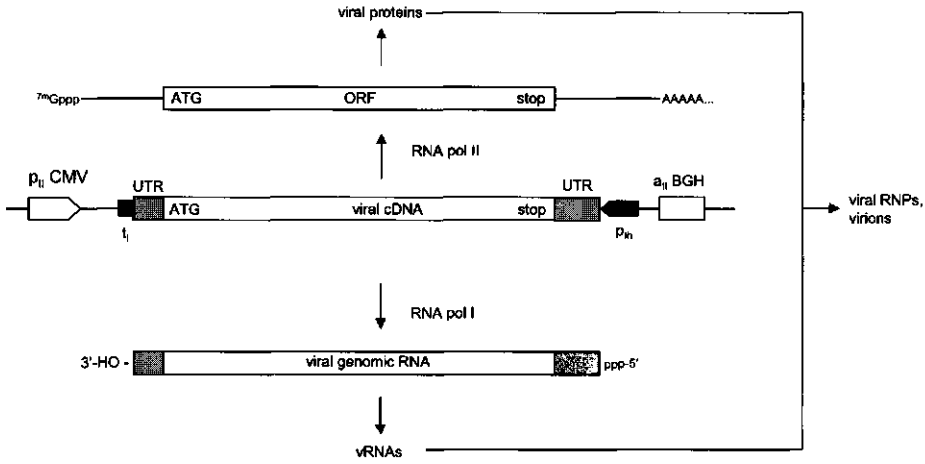

Figure 5.5. The RNA pol I - pol II rescue cassette. A cDNA copy of the viral genome sense RNA segments is cloned between an RNA polymerase I promoter (p_{Ih}) and terminator (t_I). This entire cassette is cloned in an inverse orientation between a *cytomegalovirus* promoter (p_{II} CMV) and bovine growth hormone polyadenylation signal (a_{II} BGH). After transcription by RNA pol I, transcripts containing authentic viral genome termini with 5'- triphosphate and 3'-hydroxyl groups are obtained. Alternatively, transcription by RNA pol II using the p_{II} CMV promoter and the a_{II} BGH poly(A) signal will yield capped, polyadenylated transcripts that are translated into viral proteins. The viral proteins and genomic RNA segments obtained will associate into RNPs and eventually result in rescued virions. Adapted from Hoffmann *et al.* [44].

Mechanistic view of *Tospovirus* transcription.

Transcription initiation of *Tomato spotted wilt virus* is based on the use of a cap snatching mechanism. Capped, non-viral leader sequences are present at the 5' ends of subgenomic mRNAs, with sizes ranging from 13 to 21 nt (average size of 16 nt) (Chapters 2 & 3) [60,109]. The cap-binding site and the endonucleolytic cleavage site likely are spatially separated on the viral polymerase and this physical distance is suggested to determine the preferred leader length of 16 nt, as proposed for *Influenza A virus* [24,68].

Base pairing between the capped primer and viral template RNA occurs at one of the first three 3' terminal residues (5'-AGAGCA..., 3'-UCUCGU...) (Chapter 3). To conform this need for complementarity, cleavage takes places 3' of an A or a G residue in the cap donor RNA [26,27]. This cleavage solely after purine residues resembles the cleavage specificity of the endonucleases of the *Orthomyxoviridae* and of the genera *Bunyavirus* and *Hantavirus*.

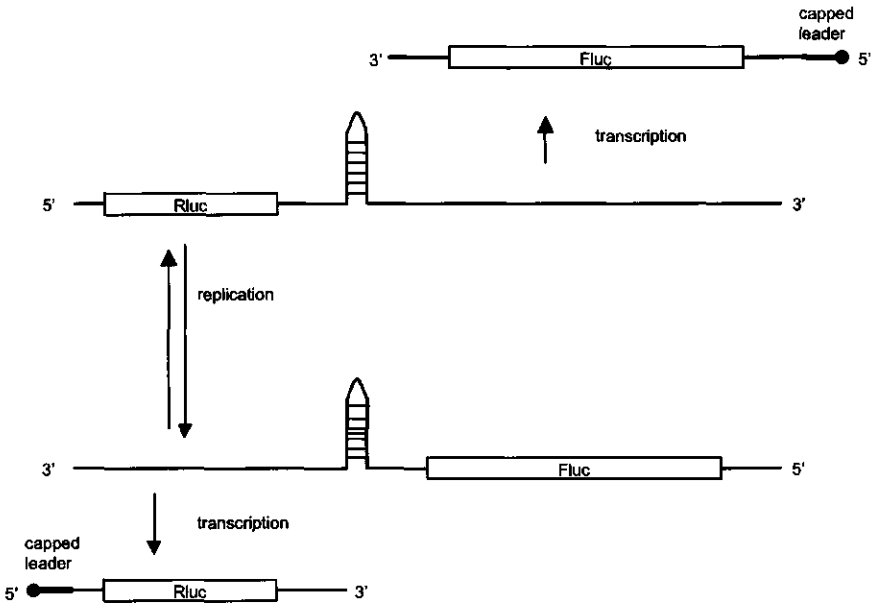

Figure 5.6. The pTOSS-DUAL template. This template is derived from the TSWV S RNA segment, in which the N gene is replaced by *Renilla* luciferase and the NSs gene is replaced by the firefly luciferase.

In contrast to the latter genera of the *Bunyaviridae*, the genomic terminal sequence of TSWV contains a dinucleotide, rather than a trinucleotide repeat and as such, it resembles the genera *Phlebovirus* and *Nairovirus* more closely. Additional insertions of residues between capped primer sequence and the authentic viral genomic sequence are rarely seen for TSWV, whereas they appear more frequently in the other genera. Initial base pairing between the 3' ultimate nucleotide of the capped primer and the template occurs primarily at the 3' ultimate residue of the viral RNA, thereby not favouring a "prime-and-realign" model that would involve internal initiation at the (ante)penultimate residue and subsequent release and realigning.

In vitro studies [1,107] have shown that the presence of divalent metal ions is required for TSWV polymerase activity, as measured by the incorporation of ^{32}P-rCTP into TCA-precipitable material. Both Mn^{2+} and Mg^{2+} were required for optimal activity, however, upon Mg^{2+} depletion, incorporation decreased to ~ 67%. Lack of Mn^{2+} resulted in a drop of incorporation to background levels. The incorporation measured, however, does not necessarily represent genuine TSWV transcription, as no difference in incorporation could be measured upon addition or omission of a suitable

cap donor RNA (AMV RNA4). Therefore, the metal ion requirement found may not reflect the need for these divalent ions in the catalytic site of the TSWV endonuclease, but rather in the nucleotidyl transferase.

Remarkable in that case is that the TSWV nucleotidyl transferase would have a higher activity using Mn^{2+} as a cofactor than when using Mg^{2+}. The highest activity, however, is reached when both Mg^{2+} and Mn^{2+} are used, which would suggest that the nucleotidyl transferase is based on a two-metal ion mechanism, but requires a third ion to complete its catalytic function. Recently, an article has been published on the bacteriophage ϕ6 RNA-dependent RNA polymerase [12], describing a similar mechanism requiring two Mg^{2+} ions and a Mn^{2+} ion for complete polymerase function. Here, two Mg^{2+} ions act together with aspartic acid residues as the catalytic centre for polymerisation, while a Mn^{2+} ion is thought to recruit the NTPs that are used as building blocks for the newly formed RNA strand, possibly, by attraction of the phosphates of the NTP to the Mn^{2+} ion. A similar mechanism for TSWV would explain why both divalent ions (Mn^{2+} and Mg^{2+}) are needed for the polymerisation reaction.

Based on the data presented and discussed in this thesis, a model for TSWV transcription initiation is proposed. As a first step, the viral transcription complex starts with the recruitment and subsequent cleavage of a capped host leader using the cap snatching mechanism. Cleavage takes place at a distance of 12-21 nt from the cap structure, with a preferred distance of 16 nt, and occurs 3' of a purine residue (preferably A). The viral endonuclease involved in cleavage of the host leader is probably based on a two-metal ion mechanism.

The capped primer then base pairs with the 3' ultimate residue or 3' penultimate residue of the viral template (depending on the 3' ultimate residue of the recruited primer) and is subsequently elongated (Fig. 5.7). Possibly, the 3' end of the capped primer and the 3' end of the viral template are brought into close proximity due to interactions with the polymerase complex, facilitating a correct base pairing.

A "prime-and-realign" mechanism as described for *Hantaan virus* occurs only rarely for TSWV. Transcription for the ambisense RNA segments likely terminates in or around the intergenic hairpin structure that acts as a bi-directional transcription terminator, thus yielding the mature mRNAs that are translated into the viral proteins.

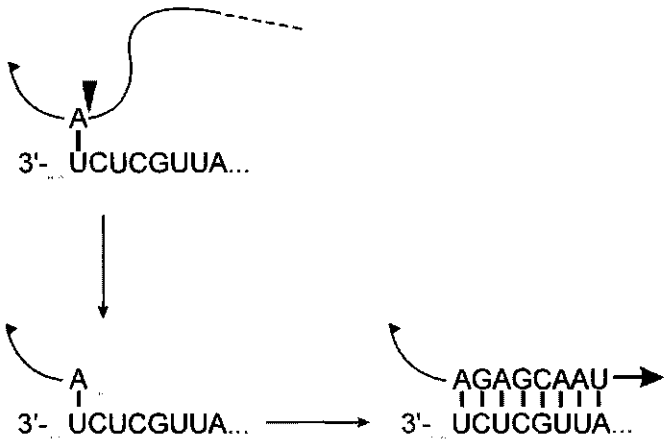

Figure 5.7. Mechanistic view of TSWV transcription initiation. A capped host mRNA, the cap donor, is recruited by the viral polymerase complex and its 3' terminal residue of the primer base pairs with the 3' ultimate (U) or penultimate (C) residue. Subsequently, it is cleaved by the viral endonuclease. This cleavage occurs preferably downstream an A or (less efficiently) a G residue and at a distance of 12-21 nt from the cap structure, with an optimal cleavage at 16 nt. Subsequently, the capped primer is elongated according to the template to eventually yield the mature viral mRNA.

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SUMMARY

Genome replication and transcription of *Tomato spotted wilt virus* (TSWV, genus *Tospovirus*) follows in most aspects the general rules for negative strand RNA viruses with segmented genomes. One common feature is the occurrence of "cap snatching" during transcription initiation. During this process, capped leader sequences of suitable host mRNAs are recruited and cleaved to serve as primers for transcription.

At the start of the research project described in this thesis, the occurrence of cap snatching during transcription initiation of TSWV was established, but the requirements for host leaders to function as cap donors were unknown. In order to identify these requirements under conditions that resembled a natural infection most closely, *Alfalfa mosaic virus* (AMV) RNAs were presented and tested as specific cap donors during a co-infection with TSWV of *Nicotiana benthamiana* (Chapter 2). Thus it was found that all four AMV RNAs can be used by TSWV as cap donors. Of these AMV RNAs, only RNA 3 was cleaved exclusively at one position, i.e. at nucleotide A17, indicating that a cleavage preference existed. However, as the 5' ultimate residue of the TSWV genomic sequence in these mRNAs could be either derived from the AMV leader or represent the first templated nucleotide, the actual cleavage site, i.e. 5' or 3' of the A17 residue, could not be established. In case the first viral residue originated from the capped leader, it would implicate a requirement for a single base pairing between the cap donor RNA and the viral template during transcription initiation.

To confirm whether such single complementarity was required, a series of AMV RNA3 and RNA4 mutants, modified in their leader sequences, was offered as cDNA constructs to transgenic *N. tabacum* p12 plants expressing the AMV replicase proteins and tested as cap donors during a co-infection with TSWV. RT-PCR amplification and sequence analyses of chimaeric AMV-TSWV mRNAs strongly supported a single base pairing requirement between cap donor and viral template and a cleavage preference for nucleotide position 16 from the cap structure. Base pairing not only occurred with the 3' ultimate residue of the viral template, but in some cases also with the penultimate or even antepenultimate residues, indicating that a G-C base pairing instead of an A-U base pairing could also occur. These findings were further tested and substantiated by investigating TSWV mRNAs with leaders derived from known host mRNAs (Chapter 3).

While the co-inoculation approaches using (mutant) AMV RNAs allowed the analysis of cap donor requirements for transcription initiation on TSWV genomic RNA, it was not suitable for the identification and characterisation of the viral proteins involved,

such as the nucleoprotein (N) and RNA-dependent RNA polymerase (RdRp), or of important *cis*-acting sequences within the viral RNA template. Therefore, a *Vaccinia virus*-T7 expression system was developed, which enabled reconstitution of active viral RNPs entirely from cloned cDNAs (Chapter 4). Using this system, the 331.5 kDa RdRp was successfully expressed in OST7-1 murine fibroblasts along with the N protein and a reporter construct resembling the viral S RNA. By replacing the S RNA-encoded NSs gene for the sensitive luciferase reporter gene, functional RdRp activity was demonstrated with the expression of luciferase *in vivo*.

In Chapter 5, the results obtained are discussed in relation to available data for other segmented negative strand RNA viruses. Finally, a model for TSWV transcription initiation is proposed.

SAMENVATTING

Ondanks dat het driedelig RNA genoom van het tomatenbronsvlekkenvirus (Engels: *Tomato spotted wilt virus*, afgekort TSWV), behorende tot het genus *Tospovirus* binnen de familie *Bunyavirida*, een ambisense karakter heeft, ondergaat dit virus in principe dezelfde transcriptie- en replicatieprocessen als de gesegmenteerde, minstrengs RNA virussen.
Een belangrijk proces daarbij is "cap snatching", waarmee het virus de transcriptie start. Tijdens dit proces worden van een cap-structuur voorziene "leader"-sequenties van geschikte gastheer mRNA's herkend en vervolgens gekliefd door een viraal endonuclease, zodat ze kunnen dienen als primers in de virale transcriptie-initiatie.
Bij de aanvang van het in dit proefschrift beschreven onderzoek was reeds bekend dat "cap snatching" ook tijdens de transcriptie-initiatie van TSWV voorkomt. Echter, de criteria waaraan een gastheer mRNA moet voldoen om als cap-donor te kunnen dienen waren niet bekend. Om deze criteria onder de meest natuurlijke condities te bepalen zijn de (sub)genomische *Alfalfa mosaic virus* (AMV) RNA moleculen, welke in het bezit zijn van een 5'-cap structuur, getoetst als cap-donor tijdens een meng-infectie met TSWV in *Nicotiana benthamiana* (Hoofdstuk 2). Deze analyses toonden aan dat alle 4 (sub)genomische AMV RNA moleculen als cap-donor kunnen fungeren. Tevens bleek dat AMV RNA3 slechts op één positie werd gekliefd, nl. op positie A17, wat er op duidde dat klieving van dit RNA door strikte criteria wordt bepaald. Onbeantwoord bleef daarbij de vraag of de klieving plaatsvond aan de 5' kant van nucleotide A17 danwel aan de 3' kant van dit residue. Immers, A17 kon enerzijds afkomstig zijn van de AMV RNA3 "leader" en anderzijds het eerste nucleotide zijn dat aan het "leader" RNA gekoppeld wordt met het virale RNA. In het eerste geval zou er sprake zijn van een noodzaak voor basenparing tussen de afgeknipte leader en de over te schrijven TSWV matrijs.
Om deze vraag te kunnen beantwoorden werden AMV RNA3 en RNA4 "leader" mutanten gemaakt en aangeboden aan transgene tabaksplanten waarin de mutant RNA's door transgeen tot expressie gebracht AMV replicase gerepliceerd werden. Door deze planten vervolgens met TSWV te infecteren konden deze AMV mutanten worden getoetst als cap-donor (Hoofdstuk 3). RT-PCR analyses en daarop volgende bepaling van de nucleotidenvolgorde van chimere AMV-TSWV mRNA's wezen op de noodzaak voor een enkele basenparing tussen cap-donor en virale RNA matrijs. Leaders met een basenparend nucleotide op positie 16 (geteld vanaf de 5'-cap structuur) bleken daarbij het meest gebruikt te worden. Tevens bleek dat de basenparing niet alleen mogelijk was met het terminale residue van de virale RNA

matrijs (middels een A-U basenparing), maar ook, in mindere mate, met het tweede residue (middels een G-C basenparing). Het laatste vond echter plaats met een lagere efficiëntie. Deze waarnemingen werden bevestigd door de acceptatie van een aantal geselecteerde gastheer mRNA's als cap-donor te toetsen (Hoofdstuk 3).

De menginfectieproeven met mutant AMV RNA's leverden weliswaar goede informatie op met betrekking tot de eisen die gesteld worden aan de leader tijdens het cap-snatching proces, maar leenden zich niet voor analyse van de eiwitten (*trans* factoren) die betrokken zijn bij dit proces, waaronder het virale RNA-afhankelijk RNA polymerase (RdRp) en het N eiwit, alsmede analyse van de eigenschappen waar de termini van het te transcriberen TSWV RNA aan moeten voldoen. Hiertoe werd een *Vaccinia*-T7 systeem onwikkeld, waarmee *in vivo* transcriptioneel actieve TSWV ribonucleoproteïne (RNP) deeltjes konden worden gereconstitueerd uitgaande van cDNA klonen (Hoofdstuk 4).

Middels dit systeem werden het 331,5 kDa RdRp en het 29 kDa N eiwit succesvol tot expressie gebracht in OST7-1 muizenfibroblastcellen. Door bovendien een DNA kopie van het TSWV S RNA aan te bieden, waarin het NSs gen was vervangen door een luciferase merkergen, kon vervolgens transcriptie-activiteit van het gereconstitueerde RdRp worden aangetoond.

Tenslotte worden in Hoofdstuk 5 de behaalde resultaten besproken in relatie tot beschikbare gegevens over *in vitro* / *in vivo* transcriptie-replicatie systemen van andere gesegmenteerde minstrengs RNA virussen. Een model voor TSWV transcriptie-initiatie wordt besproken.

NAWOORD

Na dik vier jaar ligt er dan toch uiteindelijk een boekje! Dat boekje is er niet vanzelf gekomen, het cliché dat je nooit alleen promoveert is zeker waar. Het nawoord van een proefschrift is dan ook de plek bij uitstek om al die mensen te bedanken die elk op hun eigen manier geholpen hebben bij het tot stand komen van dit boekje.

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CURRICULUM VITAE

Daniël Mathias Johannes Maria Duijsings werd geboren op 21 september 1974 te Heerlen. In 1992 behaalde hij het Gymnasium B diploma aan het Stella Maris College te Meerssen. In datzelfde jaar werd aangevangen met de studie Scheikunde, specialisatie biomoleculaire wetenschappen, aan de Katholieke Universiteit Nijmegen. Tijdens deze studie werd een hoofdvaksstage gevolgd bij de vakgroep Biochemie FNW (Katholieke Universiteit Nijmegen) onder begeleiding van prof. dr. W.J. van Venrooij en dr. G.J.M. Pruijn. Een nevenrichtingsstage werd gevolgd bij de afdeling Medische Microbiologie van het Academisch Ziekenhuis Nijmegen St. Radboud onder begeleiding van dr. W.J.G. Melchers en dr. G. Zoll. Op 27 januari 1997 werd het doctoraal diploma behaald. Van 1 februari 1997 tot mei 2001 was hij werkzaam als Onderzoeker in Opleiding (OIO) aan de Leerstoelgroep Virologie van Wageningen Universiteit onder begeleiding van prof. dr. R.W. Goldbach en dr. ir. R.J.M. Kormelink. Het onderzoek dat in deze periode is uitgevoerd, werd gefinancierd door de Nederlandse organisatie voor Wetenschappelijk Onderzoek (NWO), divisie Chemische Wetenschappen, en staat beschreven in dit proefschrift.
Per 1 mei 2001 is hij werkzaam als post-doctoraal onderzoeker aan de hoofdafdeling Biochemie, Celbiologie en Histologie van de Faculteit Diergeneeskunde, Universiteit Utrecht, onder begeleiding van dr. K.J. Teerds, dr. M. Houweling en dr. A.B. Vaandrager.

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