Generation of envelope and defective interfering RNA mutants of tomato spotted wilt virus by mechanical passage

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During a series of mechanical transfers of tomato spotted wilt virus, two distinct types of mutants were generated. Firstly, a morphologically defective isolate was obtained which had lost the ability to produce the membrane glycoproteins and, as a consequence, was not able to form enveloped particles. Analysis of the genomic RNAs of this isolate suggested that this defect was caused by either point mutations or very small deletions in the medium genomic RNA segment. Secondly, isolates were obtained which had accumulated truncated forms of the large (L) RNA segment. These shortened L RNA molecules most likely represented defective interfering RNAs, since they replicated more rapidly than full-length L RNA and their appearance was often associated with symptom attenuation. Defective L RNAs of different sizes were generated after repeated transfers, and hybridization analysis using L RNA-specific cDNA probes showed that the internal regions deleted varied in length. The presence of defective L RNAs in nucleocapsid fractions as well as in enveloped virus particles indicates that all defective molecules retained the sequences required for replication, encapsidation by nucleocapsid proteins and packaging of the nucleocapsid into virus particles.

Introduction

Repeated passage of RNA viruses between hosts or cell cultures often results in the generation of defective or incomplete virus particles. These defective particles have the same protein components as standard virus, but their genomes differ from those of the original virus as a result of deletions. Replication of defective particles depends on the presence of the standard virus in the inoculum. RNAs that are dependent on a helper virus for replication have frequently been described in animal virus systems (Holland, 1986) but thus far only rarely in plant virus systems (Hillmann et al., 1987). The occurrence of these defective RNAs frequently leads to modulation of the cytopathogenicity of the standard virus (Lazzarini et al., 1981). Typical defective interfering (DI) RNAs exhibiting the properties defined by Huang (1973) have been described for only a few plant viruses, e.g. tomato bushy stunt virus (Hillman et al., 1987; Morris & Knorr, 1990; Knorr et al., 1991), turnip crinkle virus (Li et al., 1989; Cascone et al., 1990) and cymbidium ringspot virus (CyRSV; Burgyan et al., 1989, 1991). These DI RNAs consist of a mosaic of the original genome. Ismail & Milner (1988) isolated DI particles from Sonchus yellow net virus, generated in chronically infected plants. Other types of defective strains of plant viruses have also been observed. They may be generated by point mutations, deletions or, in the case of multipartite genomes, loss of entire genome segments of the parental virus (Black, 1979; Shirako & Brakke, 1984; Shirako & Ebara, 1986).

Defective forms of tomato spotted wilt virus (TSWV) have previously been reported (Le, 1982; Verkleij & Peters, 1983; Resende et al., 1991). TSWV shares many characteristics with bunyaviruses and is now classified as a member of the newly created genus tospovirus within the Bunyaviridae family (de Haan et al., 1989a; Francki, 1991). This virus has a remarkably wide host range and it is the only virus transmitted by thrips species (Cho et al., 1988). Virus particles are enveloped with a roughly isometric morphology and a diameter of 80 to 120 nm. The virus contains four major proteins, a nucleocapsid (N) protein associated with the three genomic RNA segments, two glycoproteins (G1, G2) associated with the viral envelope and a large (more than 200K) protein which has been proposed to represent the viral polymerase (Mohamed et al., 1973; Tas et al., 1977; de Haan et al., 1991). The TSWV genome consists of three, single-stranded RNA molecules denoted small (S) RNA (2916 bases), medium (M) RNA (approximately 5200 bases) and large (L) RNA (8897 bases). The S RNA encodes the N protein and a non-structural protein (NSs) in an ambisense gene arrangement (de Haan et al., 1990). The M and L RNAs are of negative polarity and most likely
encode the membrane glycoproteins (G1 and G2) and the polymerase, respectively (de Haan et al., 1989b, 1991). Ie (1982) noticed that in some isolates, the characteristic enveloped virus particles were absent in cells of infected plants after repeated mechanical transfer. Instead, only small but dense aggregates embedded in the viroplasm were found in the cytoplasm of infected cells. Using immunogold techniques, it was demonstrated that these structures were mainly formed by ribonucleocapsid material (E. W. Kitajima, A. C. de Ávila, R. de O. Resende, R. Goldbach & D. Peters, unpublished results). It was concluded that the lack of glycoproteins led to the formation of non-enveloped nucleocapsids, presumably as a result of mutational defects in the M RNA (Ie, 1982; Verkleij & Peters, 1983).

A different type of mutant recently observed in our studies is characterized by the presence of RNA species in addition to the three genomic segments. Preliminary studies suggest that the appearance of these additional RNA species is associated with attenuation of symptoms in infected plants.

In order to gain more insight into the nature of the morphologically defective (non-enveloped) forms and into the generation of defective RNAs, to understand the mechanism by which they are generated during infection, and their possible involvement in symptom attenuation, we have studied two different isolates of TSWV, before and after sequential inoculation to different host plants.

**Methods**

*Generation and maintenance of defective forms.* Two original isolates, CNPH (recently renamed BR-01), a Brazilian isolate from tomato, and NL-04, a Dutch isolate from chrysanthemum, stored in liquid nitrogen were passed in two different ways through host plants. Leaves of *Nicotiana rustica* cv. America plants systemically infected with BR-01 were used as the inoculum sources for mechanical inoculation of *Nicotiana tabacum* cv. Samsun NN. A large number of local lesions were produced and sequentially transferred at intervals of 3 days to Samsun NN plants. Three types of local lesions differing in appearance were selected and further propagated on *N. rustica* plants. The isolate NL-04 was passaged by mechanical inoculation to *N. rustica* and *N. benthamiana* by serial passage at 12 day intervals, using systemically infected leaves as the inoculum source. The mechanical inoculations were carried out by grinding 1 g of infected leaves in 5 ml of 0-01 m-phosphate buffer pH 7-0, containing 0-01 m-Na2SO4. After inoculation, the plants were placed in a glasshouse at approximately 22 °C (light:dark, 16:8 h) for symptom development.

*Detection of morphologically defective forms by serology and electron microscopy.* Each generation of infected plants was tested by ELISA (TAS-ELISA) using a panel of monoclonal antibodies (MAbs) raised against the isolate BR-01 as described by Huguenot et al. (1990) and de Ávila et al. (1990). Two MAbs were directed against the nucleocapsid protein (MAbs N1 and N2) and four were directed to the glycoproteins (MAbs G1 to G4). Samples were prepared by grinding systemically infected leaves in PBS containing 0-05% Tween 20 at a ratio 1:30 (w/v). The absorbance values were measured 30 min after addition of substrate at room temperature.

*Immunogold labelling.* Polyclonal antisera raised in rabbits against purified virus or against purified nucleocapsid preparations of BR-01 and NL-04 were used in immunogold electron microscopy. Protein A-gold was prepared following the procedure described by van Lent & Verduin (1985). Labelling of both ultrathin sections and ‘dip’ preparations were carried out (E. W. Kitajima, A. C. De Ávila, R. de O. Resende, R. Goldbach & D. Peters, unpublished results). Leaf extracts were assayed by crushing 1 cm2 of leaf material in a droplet of 1% BSA in PBS, and transferring the extracts to another droplet of BSA/PBS. For gold labelling, formvar-carbon-coated grids were floated with the membrane side down, on the extracts for 1 min, and then transferred to another droplet of specific antiserum (usually at a dilution of 1:1000) for 10 min. Grids were washed with 30 droplets of PBS, then in a PBS solution diluted 30-fold, and were finally negatively stained with aqueous 2% uranyl acetate. All manipulations were carried out at room temperature. Grids were examined in a Philips CM12 electron microscope.

*RNA isolation and Northern blot analyses.* Total RNA from healthy and TSWV-infected *N. rustica* plants and from purified nucleocapsid and virus preparations was isolated according to de Vries et al. (1982). Samples of 3 μg of RNA were analysed by electrophoresis in 1% agarose gels under denaturing conditions (Bailey & Davidson, 1976). After transfer to nitrocellulose membranes the RNAs were hybridized to 32P-labelled DNA probes, directed to the S, M and L RNAs of isolate BR-01 (Maniatis et al., 1982; de Haan et al., 1990). The development of infection was monitored by extracting total RNA from BR-01-infected *N. rustica* plants at different periods post-infection (p.i.). The RNAs were resolved in agarose gels, blotted to nitrocellulose membranes and subsequently hybridized to strain-specific L RNA riboprobes produced as described by de Haan et al. (1990).

**Results**

*Propagation of morphologically defective isolates by serial mechanical transfer.*

A large number of local lesions were serially transferred on *N. tabacum* cv. Samsun NN plants. Three types of local lesions which differed in their appearance (necrotic, chlorotic or chlorotic with a white halo) developed on the inoculated leaves. An equal number of each lesion type was used to induce a new generation of local lesions. After four transfers, 18 local lesion lines (six of each type) were selected and propagated on *N. rustica* plants. Analyses of extracts from systemically infected leaves by ELISA using the MAbs N1 and N2 as well as the MAbs G1 and G3, revealed that all local lesion lines gave reactions similar to those shown for BR-01 (originally CNPH1 in Fig. 2 of de Ávila et al., 1990). These results showed that pure morphologically defective forms (defined as isolates which do not contain enveloped virus particles and which do not produce membrane glycoproteins) of BR-01 were not generated by the repeated local lesion transfers.

Electron microscopy studies showed that in the cytoplasm of cells infected with these local lesion lines, considerable amounts of small dense aggregates occurred.
Defective mutants of TSWV

Fig. 1. Electron micrograph of a mesophyll cell from a *N. rustica* plant infected with local lesion line 2 (BR-01), which was obtained after several mechanical passages. Infected cells contain virus-induced inclusions of dense aggregates (DA) and intact virus particles (V). The bar represents 0.2 μm.

Fig. 2. Schematic representation of repeated mechanical transfers of the NL-04 isolate through different hosts. The isolate was passaged several times in *N. rustica* plants (R) and *N. benthamiana* (B).

Fig. 3. Characterization of a morphologically defective isolate from TSWV NL-04 (c) and comparison with normal NL-04 (b) using a panel of MAbs. The isolate was generated after successive mechanical inoculations onto *N. benthamiana* plants. Isolate BR-01 (a) and sap from healthy plants (d) were used as controls. The *A*₄₀₅ was measured 1 h after substrate addition.

Simultaneously with intact virus particles (Fig. 1). Using the Dutch isolate NL-04, the absorbance values obtained in ELISA using MAbs G1 to G4 were much lower than those of BR-01 after four mechanical transfers over *N. rustica*, indicating that for this isolate the amount of membrane protein produced declined drastically. To separate possible defective forms from enveloped forms, this isolate was subsequently serially passaged in two lines through different hosts (Fig. 2). The line passaged through *N. rustica* resulted in a partial recovery of the original reaction with MAbs G1 to G4 in ELISA. These results indicate that the amount of membrane protein increased and that part of the infectious material should consist of enveloped virus particles. This could be confirmed by electron microscopy of ultrathin sections and of 'dip' preparations (data not shown). This partially recovered isolate induced moderate symptoms on *N. rustica*, *N. benthamiana* and tomato.

A morphologically defective form was found when the isolate NL-04 was passaged in *N. benthamiana* (Fig. 2). The resulting isolate reacted strongly with MAbs N1 and N2 in ELISA, but negative reactions were obtained when the MAbs directed to the membrane glycoproteins were used (Fig. 3). In dip preparations, structures were observed which reacted with gold-labelled antibodies...
against the nucleocapsid protein (Fig. 4), but which did not react with antibodies to the G1 protein isolated from SDS–polyacrylamide gels (data not shown). Hence these structures most likely represent clusters of non-enveloped nucleocapsids. This morphologically defective isolate was found to induce extremely severe necrosis in different hosts, including tomato, *N. rustica* and *N. benthamiana*.

**Genome characterization of mechanically transmitted TSWV isolates**

Since all the local lesion lines showed a similar serological reactivity in ELISA, only one line was selected and further propagated on *N. rustica* in order to study the effect of repeated mechanical transfers on the genome constitution of TSWV. RNA was purified from plants infected with isolate BR-01 and analysed on agarose gels (Fig. 5a). The RNA was blotted onto nitrocellulose membranes and hybridized with S, M and L RNA-specific cDNA probes. Although the hybridizations with ³²P-labelled S and M RNA-specific probes (Fig. 5b, c) did not reveal abnormalities in the genome of mechanically transferred isolates, hybridization with an L RNA-specific probe revealed the occurrence of smaller L RNA species (Fig. 5d). A line of BR-01 which was mechanically transferred three times contained only full-length L RNA, whereas RNA preparations from lines transferred 20, or more than 30 times, contained in addition to the full-length L RNA truncated L RNA-derived species of different sizes.

To verify whether these truncated forms of L RNA were replicating entities and not breakdown products resulting from the isolation procedure, the replication of these molecules was studied. Total RNA extracts from infected plants were prepared at different times after infection, resolved on agarose gels, blotted to nitrocellulose membranes and hybridized to ³²P-labelled strand-specific L RNA probes. The results revealed that the
**Defective mutants of TSWV**

![Northern hybridizations of TSWV nucleocapsid RNA](image)

**Fig. 5.** Northern hybridizations of TSWV nucleocapsid RNA (a) purified from infected *N. rustica* plants (isolate BR-01), using 32P-labelled cDNA probes specific for S RNA (pTSWV-614) (b), M RNA (pTSWV-201) (c) and L RNA (pTSWV-70) (d). The Northern blots contained RNA from isolate BR-01 after three (lanes 1), 20 (lanes 2) and more than 30 mechanical transfers (lanes 3), respectively.

<table>
<thead>
<tr>
<th>RNA Type</th>
<th>L RNA (8.9 kb)</th>
<th>M RNA (5.2 kb)</th>
<th>S RNA (2.9 kb)</th>
<th>AL RNA (4.2 kb)</th>
<th>AL RNA (3.2 kb)</th>
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<td>Marker</td>
<td>1</td>
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**Fig. 6.** Time course analysis of TSWV-infected *N. rustica* plants 2 (lane 3), 4 (lane 4), 6 (lane 5), 7 (lane 6), 8 (lane 7), 9 (lane 8), 10 (lane 9), 12 (lane 10) and 14 (lane 11) days p.i. Northern blots containing total RNA extracts were hybridized to 32P-labelled riboprobes, detecting either virus-sense L RNA (a) or complementary-sense L RNA (b). N RNA (lane 1), total RNA from healthy plants (lane 2) and purified viral RNA (lane 12) were included as controls.

truncated L RNA species could be detected in the virus (−) sense (Fig. 6a) as well as complementary (+) sense (Fig. 6b). Detection of complementary-sense segments demonstrated that the truncated L RNA species did replicate during the infection process and do not represent breakdown products. The amount of complementary strands was constant during infection, whereas the viral-sense strands accumulated faster and reached higher quantities than full-length genomic L RNA. By 6 days p.i., more truncated L RNAs than full-length L RNAs had been produced. These results can be interpreted such that the replication of the truncated forms interferes with the replication of the genomic L RNA segments.
Analysis of RNA extracted from preparations of enveloped virus particles demonstrated the presence of both the negative strand full-length L RNA and truncated L RNAs (Fig. 6a). Complementary-sense strands of full-length and truncated L RNAs were found to be absent from virus particles, an observation which strongly suggests that the negative strands of the genomic and truncated L RNAs are exclusively packaged into envelopes (Fig. 6a, b). When RNAs extracted from nucleocapsid preparations were analysed, both the negative and positive strands of full-length and truncated forms of L RNA were detected (Fig. 6a, b).

Generation of defective L RNAs was consistently associated with symptom attenuation. Plants infected by an isolate containing only full-length L RNA segments usually developed severe symptoms, showing severe necrosis within approximately 10 days p.i., whereas a mild mosaic or mottling was induced in plants infected with isolates containing defective L RNA species in addition to the complete genome (Fig. 8).

RNA analyses of the morphologically defective isolate, which is not able to produce enveloped particles and which lacks the ability to synthesize the membrane glycoproteins (Fig. 3 and 4), showed that it contained three segments with sizes similar to those of normal TSWV isolates. This indicates that large deletions did not occur in any of the three RNA segments of that isolate (Fig. 7a).

Since S RNA has been shown to encode the N and NSs proteins (de Haan et al., 1990) and L RNA the viral transcriptase (de Haan et al., 1991), it can be assumed that, by analogy to other bunyaviruses, the glycoproteins are encoded by the M RNA. Northern blot hybridization experiments using an M RNA-specific cDNA probe (pTSWV-201) unequivocally demonstrated that the M RNA of the non-enveloped isolate was the same size as the M RNA of enveloped virus isolates (Fig. 7b).

Truncated L RNAs could not be detected in the morphologically defective isolate (Fig. 7c, d). Hence, the morphological defect of envelope-deficient isolates does not appear to be associated with the occurrence of deleted forms of L RNA, but rather with the occurrence of point mutations or small deletions in the M RNA that do not influence the size of this molecule. Furthermore, isolates of NL-04 and BR-01 which were still able to produce virus particles after serial mechanical passages contained, besides the S, M and L RNAs of the expected sizes, only truncated L RNA molecules (Fig. 7c, d) which could not be detected in the original isolates.

Discussion

We have demonstrated that after serial mechanical transmissions of TSWV, two distinct classes of mutants are generated: firstly, envelope-deficient mutants that most probably have accumulated point mutations or small deletions in the M RNA, and secondly, isolates that have accumulated truncated forms of the L RNA segment. The present data strongly suggest that the truncated L RNAs of TSWV represent DI RNA molecules, which accumulate more rapidly than full-length L RNA (Fig. 6) and may modulate (attenuate) the symptom expression of TSWV in different host plants (Fig. 8).

The time course analyses clearly indicate that the defective L RNA molecules are preferentially replicated during infection. They seem to interfere with the replication of the full-length L RNA, thereby reducing its amount. Since full-length L RNA molecules are always present in small amounts, it can be concluded that they are required for virus infection and act as 'helpers' for the defective molecules. This is not unexpected since L RNA encodes the putative RNA polymerase of the
Fig. 8. N. rustica plants 12 days after infection with isolate BR-01. The plant on the left showing severe symptoms did not contain defective L RNAs, whereas the plant on the right contained high amounts of these RNA molecules.

virus (de Haan et al., 1991). TSWV seems to resemble the orthomyxoviruses, which also produce DI RNA molecules exclusively derived from the RNA segments encoding the polymerase subunits (Lazzarini et al., 1981; Nayak et al., 1985; Holland, 1986).

The presence of defective L RNAs in nucleocapsids as well as in enveloped virus particles indicates that these defective molecules have retained all sequences required for replication, encapsidation with the N protein and packaging of the nucleocapsids into enveloped particles. DI RNAs retaining the same set of properties have also been reported for influenza virus after successive passages at high m.o.i. (Nayak et al., 1990). Full-length and truncated L RNA molecules are present in different amounts in virus particles, indicating that these RNAs are not being packaged in equal molar amounts. Since defective L RNAs are also enveloped it may be expected that these molecules have the potential to be transmitted by thrips vectors. This implies that defective L RNAs may also be present in field isolates, but their presence has not yet been demonstrated.

The existence of defective L RNA molecules in TSWV isolates consistently leads to an attenuation of symptoms upon infection. This phenomenon is well known for animal viruses (Cave et al., 1985), but has been described for only a few plant virus systems where DI-like species were detected and fully characterized. In tomato bushy stunt virus, symptom-modulating DI RNA species were generated, and in this system collinear deletion mutants of the monopartite helper virus genome are found which consist of virus-derived fragments of variable sizes (Hillman et al., 1987; Morris & Knorr, 1990; Knorr et al., 1991). DI RNAs of CyRSV were detected (Burgyan et al., 1989, 1991) which interfered with symptom expression. Sequence analysis revealed that these defective molecules originated from different regions of the CyRSV genome. DI RNAs derived almost exclusively from helper virus sequences have also been found in association with a natural isolate of turnip crinkle virus. However, unlike other DI RNAs, they intensify the symptoms of their helper virus (Li et al., 1989). Ismail & Milner (1988) also reported DI particles for the plant rhabdovirus Sonchus yellow net virus, which are generated in chronically infected plants and form shorter particles. The presence of DI RNAs in some animal-infecting bunyaviruses has been suggested in a few instances, although most of them have not been demonstrated directly (Kacsak & Lyons, 1978; David-West & Porterfield, 1984; Verani et al., 1984). Interfering particles containing mainly full-length S segments rather than RNA molecules with internal deletions, a characteristic of typical DI particles, have been described by Elliott & Wilkie (1986). In Germinston bunyavirus-infected cells, Cunningham & Szilagyi (1987) observed more typical DI RNAs, i.e. deletion-containing RNAs derived from the L RNA segment which encodes the viral RNA polymerase.

For TSWV, defective L RNAs of different sizes are generated after repeated mechanical transfer, although we can not rule out the possibility that these defective molecules were already present in the original isolates in non-detectable amounts. Differential hybridization using L RNA-specific probes (Fig. 7b, c) indicates that the deleted internal regions differ in length, suggesting that deletions may have occurred in different positions in the L RNA as also observed for other viruses (Holland, 1986). Studies are now in progress to sequence the different defective TSWV L RNAs to elucidate the mechanisms involved in their generation during infection.

A morphologically defective isolate was obtained after inoculation onto N. benthamiana, but not in N. rustica. This observation suggests that some hosts interfere in the process by which defective TSWV genes are generated or preferentially replicated or expressed. However, further studies are required to confirm the interactions between some hosts and the virus in the generation of these defective isolates. This defective isolate, although deficient in membrane glycoprotein synthesis, showed no large deletions in the M RNA (the M genome segment most likely codes for these proteins). The unaltered electrophoretic mobility of the M RNA in the morphologically deficient isolate of TSWV suggests that the genetic defect concerns point mutations or only small deletions in the M RNA. This morphologically defective isolate differs from those isolated by le (1982) in which the M RNAs seemed to be partially deleted (Verkleij & Peters, 1983).
The lack of virus envelope and membrane proteins may result in the loss of transmissibility by thrips, a restriction which may then prevent the occurrence of such mutants under natural conditions. Similar results were observed in repeated sap transmission of wound tumour virus, although the loss of insect transmissibility in that case was accompanied by the loss of one or more complete genome segments (Black, 1979).

Regarding the isolate being studied here, it is remarkable to note that even although the translation products (envelope glycoproteins) do not seem to be required for virus multiplication in plants, the mRNA of TSIV seems to possess a mechanism that prevents the occurrence of large deletions in this molecule.

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