GENERATION AND CHARACTERIZATION OF MUTANTS OF TOMATO SPOTTED WILT VIRUS

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GENERATION AND CHARACTERIZATION OF MUTANTS OF TOMATO SPOTTED WILT VIRUS

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
Dr. H.C. van der Plas,
in het openbaar te verdedigen
op woensdag 17 maart 1993
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

Generation and characterization of mutants of tomato spotted wilt virus / Renato de Oliveira Resende
[S.I. : s.n.], - III
Thesis Wageningen - With ref. - With summary in Dutch and Portuguese.
ISBN 90-5485-095-7
Subject:headings: tospoviruses

PROPOSITIONS

1. The use of mechanically transmitted isolates of tomato spotted wilt virus for testing resistance levels in breeding programs should be avoided.

2. The envelope-deficient isolate of tomato spotted wilt virus described by Verkleij and Peters (1983) represents a double mutant of this virus.

   Verkleij F. N. & Peters, D. (1983). Characterization of a defective form of tomato spotted wilt virus. *Journal of General Virology* 65, 677-686.

3. For the classification of virus species and strains within the family *Bunyaviridae* the same descriptors should be used.

4. Breeders and virologists should standardize their terminology and definitions of the different types of resistance.

5. The world wide campaigns to prevent AIDS transmission will favour the selection of mild strains of the human immuno-deficiency virus.

6. Assembly of tomato spotted wilt virus particles involves a non-selective RNA packaging process.

   Kitajima, E.W., de Ávila, A.C., Resende, R. de O., Goldbach, R.W. & Peters, D. (1992). Comparative cytological and immunogold labelling studies on different isolates of tomato spotted wilt virus. *Journal of Submicroscopical Cytology and Pathology* 24, 1-14.

   Kormelink, R., de Haan, P., Peters, D. & Goldbach, R. (1992). Viral synthesis in tomato spotted wilt virus-infected *Nicotiana rustica* plants. *Journal of General Virology* 73, 687-693.

   Resende, R. de O., de Haan, P., de Ávila, A.C., Kitajima, E.W., Kormelink,R., Goldbach, R. & Peters, D. (1991). Generation of envelope and defective interfering RNA mutants of tomato spotted wilt virus by mechanical passage. *Journal of General Virology* 72, 2375-2383.

7. Review articles have to be read with caution, since authors of such articles frequently misreport results by not consulting the publications referred to.

8. Taking into account the high population density, the richness of dialects in the Netherlands reveals a low human mobility.

The work described in this thesis was financially supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Wageningen Agricultural University.

Cover layout: P.J. Kostense
Photos: Fotolocatie Binnenhaven, LUW

Aos meus pais

Romeu e Silvinha

e especialmente a minha

Rosane

CONTENTS

PREFACE

I would like to express my sincere gratitude to all people who have been involved in the achievement of this work: To Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG) for the opportunity to do my PhD; Dr Ir Dick Peters and Prof. Dr Rob Goldbach for the stimulating discussions and helpful criticism of de manuscripts; to all my colleagues of the Department of Virology, in particular, our working group (Antonio Carlos de Ávila, Claire Huguenot, Cor Meurs, Elliot Watanabe Kitajima, Frank van Poeldijk, Ineke Wijkamp, Kit Boye, Marcel Prins, Peter de Haan, Richard Kormelink, Wies Smeets) and the (ex)students Edwin van de Vossen, Guido Pennings, René Luyten and Nicole Struijk for their contribution and assistance. I want to thank the Photography Section and the greenhouse staff for the efficient work with the pictures, slides and plants; all my new friends for sharing very nice moments in the last 4 years; Luciana for her help in the final "touching" of the thesis; my family for their far but "very close" support and specially and more important Rosane.

CHAPTER 1 INTRODUCTION

Tomato spotted wilt virus (TSWV), a cosmopolitan plant virus which is spread in nature exclusively by some thrips species. The virus has a wide host range and causes diseases in economically important agricultural vegetable and ornamental crops, predominantly in tropical regions (Peters *et al.*, 1991). Recently, TSWV also became prominent in temperate climate zones, a process which was preceded by the spread of *Frankliniella occidentalis* Perg. over the Northern hemisphere.

TSWV consists of roughly spherical, enveloped particles, ranging in diameter from 70-110 nm, which are covered with surface projections. Among plant viruses, TSWV appears to have a unique genome organization (Fig. 1). The genome of TSWV consists of three single-stranded RNA segments, denoted large (L), medium (M) and small (S). These RNA segments are complexed with nucleocapsid (N) protein to form pseudo-circular nucleocapsid structures.

In the past two years, the complete nucleotide sequence of the genome of a Brazilian isolate (BR-01) has been elucidated. The molecular date obtained showed that TSWV is actually a bunyavirus, being unique in its property to infect plants. In view of its deviant host range and biology, TSWV has recently been placed in a newly created genus (Tospovirus) within the *Bunyaviridae*, a large family of arthropod-borne, enveloped viruses (Francki *et al.*, 1991). The L RNA (8897 nucleotides long) of TSWV has a negative polarity and contains a single open reading frame (ORF) corresponding with a translation product of 331.5 kilodaltons (kD), which may represent the viral transcriptase (de Haan *et al.*, 1991). Expression of this genome segment occurs via the synthesis of a full-length mRNA (Kormelink *et al.*, 1992a). The M and S RNAs both have an ambisense coding strategy, each containing two ORFs. The M RNA is 4821 nucleotides long, encoding a non-structural (NSm) protein with a size of 33.6 kD in viral sense, and a common precursor to the glycoproteins (127.4 kD) in viral complementary sense. The glycoprotein precursor contains a sequence motif (RGD) which is characteristic for cellular attachment domains (Kormelink *et al.*, 1992b).

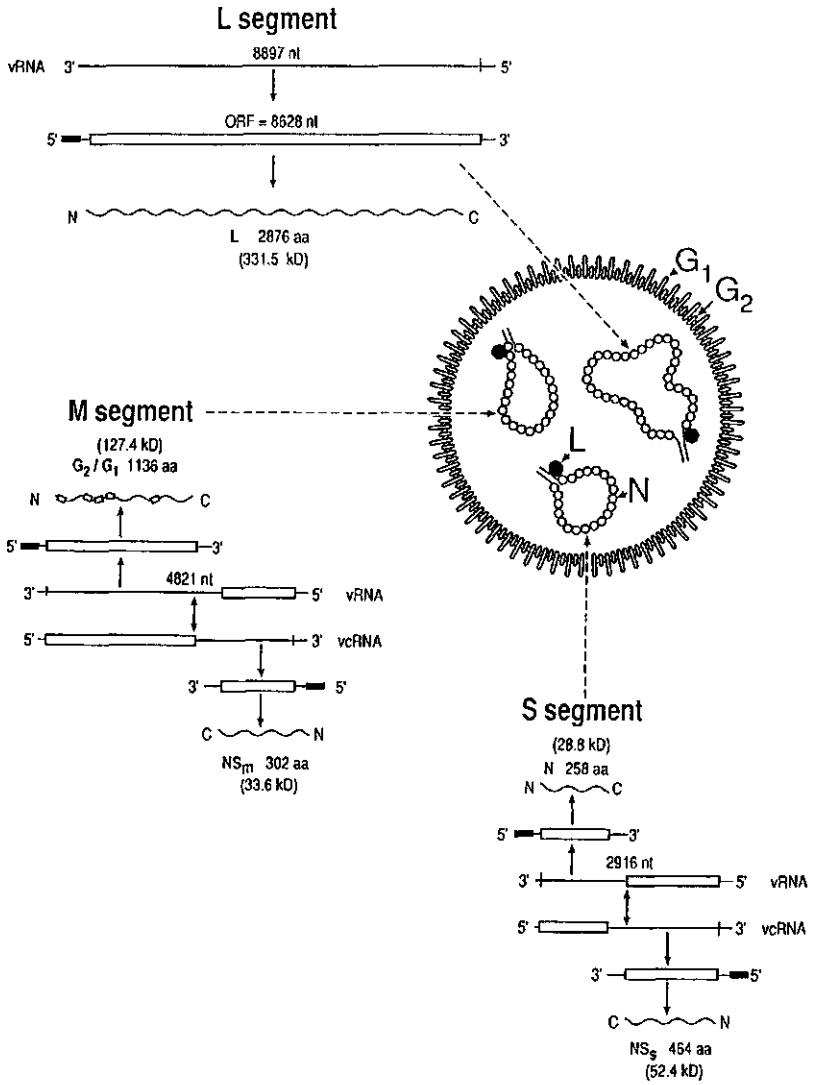

Fig. 1. Structure and strategy of gene expression of tomato spotted wilt virus

The S RNA is 2916 nucleotides long. It encodes a non-structural (NSs) protein of 52.2 kD in viral sense and the nucleocapsid (N) protein in viral complementary sense (de Haan *et al.*, 1990). Both the M and S RNA are translationally expressed via the synthesis of subgenomic mRNAs, transcribed from either viral or viral-complementary strands. The transcription of these RNAs is initiated by a process of of cap-snatching (Kormelink *et al.*, 1992c). The mRNAs produced by M and the S RNA molecules probably terminate in the intercistronic region, at a long stable A-U rich hairpin (de Haan *et al.*, 1990; Kormelink *et al.*, 1992b). All three genomic RNAs have complementary 3' and 5' ends which folded into a stable panhandle structure and which may be involved in the formation of the pseudo-circular nucleocapsids.

The virus particles accumulate in the cavities of the endoplasmatic reticulum (ER) and, most likely, mature by budding of nucleocapsids through the ER membrane (Milne, 1970; Kitajima *et al.*, 1992). Large clusters consisting of granular material, designated viroplasms, are also observed. They often contained complexes of nucleocapsid aggregates with a more dense appearence which are sometimes arranged in chain or string-like structures. These complexes had a lattice periodicity of 5 nm (Ie, 1982) and are not bound by a membrane but are scattered throughout the viroplasms surrounded by ribosomes. In addition, elongated flexible filaments or paracrystalline rods can be found which do not share any antigen associated with virus particles, but immunostain with antiserum against the non-structural protein (NSs) encoded by the S RNA (Kormelink *et al.*, 1991; Kitajima *et al.*, 1992).

In nature, TSWV is exclusively transmitted by thrips in a circulative/propagative manner. The vector seems to acquire the virus only during its larval stage and becomes infective afterwards for its whole lifespan. However, under experimental conditions, it is common practice to maintain the virus by mechanical inoculation onto susceptible host plants. This procedure causes the generation of defective forms of the virus which are characterized by the presence of electron dense structures in the infected cells (Ie, 1982; Verkley and Peters, 1983).

Initially, it was assumed that the electron dense structures represented an early stage in the virus assembly process (Ie, 1982). However, in the course of repeated mechanical transmissions, it was observed that the characteristic TSWV particles completely

disappeared, whereas amorphous masses of the electron dense structures accumulated abundantly in the cytoplasm of infected cells. Therefore, it was concluded that upon serial mechanical transmission the virus had apparently become defective, i.e. lacked the lipid membrane glycoproteins (Ie, 1982).

Verkleij & Peters (1983) studied the morphological defective isolates further. They detected nucleocapsid proteins in infected tissues, but were unable to detect the membrane glycoproteins. The dense masses apparently represent aggregates of viral nucleocapsids, which are not enveloped into mature virus particles. It was concluded that envelopes could not be formed due to the inability of the virus to produce membrane glycoprotein species. The results obtained suggested that the lack of glycoprotein synthesis by the defective forms was caused by large deletions in the M RNA (Verkleij and Peters, 1983).

These morphologically defective forms of TSWV can not be regarded as "defective interfering" particles (DI particles). They are able to replicate autonomously in plant tissues without any helper virus, although they do not contain all the viral structural proteins. However, they do possess one of the properties characteristic for DI particles, i.e. the ability to increase in number at the expense of the intact, wild type particles.

Typical DI particles originate mainly from RNA segments encoding the viral polymerase and comprise the characteristics defined and described by Huang & Baltimore (1970). They are derived from wild type viral genomes, interfere in the replication of the standard virus, and are not infectious. The DI particles possess a defective genome and thus need helper-functions of wild type virus particles for propagation. Since the presence of defective interfering viral genomes is often associated with attenuation of disease symptoms in susceptible hosts, they have been extensively studied in animal viruses (Holland, 1985; Nayak *et al.*, 1990). These studies encompass the mode of origin and structure of defective viruses, the function of defective particles in the interference with standard virus, the evolution of defective viruses and their effect on viral pathogenesis. As defective viral mutants lack one or more genetic functions but are still capable to co-replicate, they may constitute useful tools to study viral genes and viral protein functions. The understanding of these mechanisms, indeed, can provide new ways to elucidate the multiple events which take place in the infection process. Genuine

DI mutants have been described for only a few plant viruses (Morris & Knorr, 1990), but they have not yet been found in TSWV infected systems.

The present study was aimed to characterize defective forms generated by successive mechanical inoculations of TSWV. For this purpose, several TSWV isolates were serially transmitted onto susceptible hosts. The defective virus genomes generated were analyzed on the basis of their biological, serological and molecular properties. Chapter 2 describes the characteristics of defective interfering RNAs generated in different animal and plant cell systems with properties significant for the understanding of the defective genomes generated in TSWV. A rapid and efficient technique to detect the presence of non-enveloped, nucleocapsid aggregates, typical for envelope-deficient mutants, in infected plants by electron microscopy, is described in Chapter 3. Chapter 4 describes the generation of two distinct classes of TSWV mutants, i.e. envelope-deficient mutants and DI RNA mutants, by mechanical passage. The molecular characterization and possible mechanisms involved in the generation of the various DI RNAs and envelope-deficient isolates of TSWV are presented in Chapters 5 and 6, respectively. Finally, Chapter 7 presents the use of DI RNAs to provide engineered protection to TSWV and transgenic DI RNA-producing plant systems to study the genome information required for TSWV replication, encapsidation and packaging into virus particles.

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CHAPTER 2 DEFECTIVE INTERFERING RNAs

DEFECTIVE INTERFERING RNAs ASSOCIATED WITH ANIMAL VIRUSES

Among defective viral genomes, the biologically most important ones are those referred to as "defective interfering" particles (DI particles). They are deletion mutants which have lost essential segments of the viral genome. The deleted sequences may vary from a small number of nucleotides to over 90% of the wild type genome (Lazzarini *et al.*, 1981; Holland, 1985; Nayak *et al.*, 1990). As a result DI genomes are "helper-dependent", that means that they will replicate in cells only when co-infected with the wild type virus from which they are generated; the latter delivering the genetic functions deleted from the DI-genomes. In addition, these genomes suppress the replication of wild type virus by diverging virus-supplied gene products toward DI replication and away from wild type virus replication. Therefore, DI particles interfere with the replication of the wild type virus and are often associated to disease modulation in susceptible hosts.

Phenomena as symptom attenuation and virus interference have well been documented in animal virus systems. Defective interfering RNAs have been described for both positive and negative stranded RNA viruses of animals (Lazzarini *et al.*, 1981; Holland, 1985). Among the negative-strand RNA viruses, DI RNAs of vesicular stomatitis virus, which has a non-segmented RNA genome and influenza virus, having a segmented RNA genome, have been extensively studied and will, therefore, be discussed here in some more detail.

Structure of defective interfering RNAs of vesicular stomatitis virus

Studies of rhabdovirus DI particles have almost exclusively been carried out with the prototype of the rhabdoviruses, vesicular stomatitis virus (VSV). Its DI particles are probably the most studied and best understood of all DI genomes. VSV DI particles, though smaller in length than the parental virus, generally exhibit the same shape and symmetry characteristics as the wild type virus. The smallest VSV-DI particles reported contain an RNA segment corresponding with only 10% of the viral genome, while the

largest ones possess RNA segments of up to about 50-60% of the wild type size (Holland, 1987).

Several classes of RNA can be distinguished among the DI particles of VSV (Holland, 1987). The class I DI RNAs consist of, adjacent to the 5' viral terminus, variable portions of the L gene, and end with a 3' terminus which is complementary to the 5' terminus of the original viral RNA. The 3' and 5' ends can therefore form a stem which varies in size from 45 to about 150 base pairs (Meier *et al.*, 1984; Holland, 1987). Since these panhandle-forming RNAs do not contain transcription-initiation sites in their genome or in their antigenomic plus-strands, they can only be replicated when interacting with parental viral gene products. Most VSV DI RNAs found belong to this class.

The second class of DI particles, having the form of a hairpin, consist of genomic minus RNA sequences which are covalently linked to their antigenomic (plus-sense) complements. In this case the minus strand is formed by a 5' portion of the L gene, including the 5' terminus. Also, this class of DI RNAs can not be transcribed. The third class, which has been found rarely among the DI particles of VSV, consists of genomic RNA of which a large, but internal, portion of the L gene has been deleted. The presence of the 3' terminus allows transcription as well as replication of these DI RNAs. The transcriptase for this replication is supplied by the co-infecting parental virus. The fourth class consists of several types of DI RNAs. They often consist of extensively rearranged genomes, having in common a 3' terminus which is elongated with a complement of the 5' terminus. These bizarre molecules, which can not be transcribed, occur usually in persistent infections and other situations in which earlier-generated DI particle genomes can evolve and rearrange.

The most plausible and widely accepted general model for the generation of these defective genome is the "copy-back" or "copy choice" mechanism (Holland, 1985). This mechanism implies that during replication the polymerase terminates prematurely, moves with the nascent daughter strand to another site on the same or a different template molecule, and resumes elongation of the nascent chain. Termination and resumption of elongation suggests the involvement of internal polymerase recognition sites. However, in most cases, evidence has not been found for sequence specificity at the junction sites

at which the replicase terminate and resume the synthesis.

DI RNAs are involved in persistent VSV infections of cells (Holland, 1987). Their interference can also lead to complete curation of cells from virus infection. DI particles can be generated, replicated and may interfere in animals *in vivo*, although their presence in infected animals or humans has never been demonstrated in field studies.

Most rhabdoviral DI RNAs can not be transcribed. They interfere at the level of virus replication (Holland, 1985, 1987). They do not inhibit the primary transcription of the virus, but suppress viral genome replication. After their efficient primary replication they eventually strongly suppress secondary transcription. Since the reduction of secondary transcription leads to a relative shortage of viral replicase and encapsidation proteins, and since the reduced numbers of virus templates are mainly devoted to transcription (while DI nucleocapsids are devoted only to replication) almost all of the limiting viral replication/encapsidation polypeptides will be shunted to DI genome replication at the expense of wild type replication (Holland, 1987).

Structure of defective-interfering RNAs of influenza viruses

Influenza viruses contain genomes usually divided in eight segments. Influenza virus DI RNAs are consistently formed as soon as the virus is passaged at high multiplicity. They are present in a molar excess over the parental viral RNA segment from which they are derived. In addition, they are even present, although at much lower levels, in clonal stocks of influenza viruses. They will then be amplified during subsequent passages at high multiplicity. The causal reason of the generation of DI RNAs, their apparent function in the biology of virus replication, and their occurrence at high frequency is not well understood.

The influenza virus DI RNAs are substantially different from those of most other negative strand DI RNAs. They arise upon deletion of internal sequences without any major sequence rearrangement. Thus they retain both 3'- and 5'-terminus, and do not have any obvious replicative advantage other than their small size, compared to the progenitor RNAs. Most of the DI segments found arise from one of the three large virus RNA segments, which encode the viral polymerase subunits (P1, P2 and P3). A single segment can give rise to DI RNAs with varying lengths, the smaller ones not always

being a subset of the larger ones (Sivasubramanian & Nayak, 1983; Nayak *et al.*, 1985). These DI RNAs show a wide variation in their sequences at the junction sites with do not show any unique feature when compared with either the parental RNA or other subgenomic RNAs. The DI RNAs studied may initially not be generated from the parental RNA but they may rather represent predominant species that have undergone evolution, survived the selection pressure, and become amplified during subsequent replicative cycles. Consequently, the DI RNAs found in DI virus preparations may not be the final end product of evolution either, as it has been shown that any given DI RNA may further evolve or even disappear during subsequent growth cycles of the virus. In fact, it is reasonable to assume that most DI RNAs studied to date may fall into the intermediate or evolving RNA species (Nayak *et al.*, 1990).

The interference characteristics as defined for rhabdoviruses by Huang (1975, 1977) also hold for the interference mediated by DI particles of influenza virus. Although the precise mechanism of interference is unknown, co-infection of parental virus-infected cells with DI virus particles results in (1) a reduction in the wild type virus yield with consequent amplification of DI viral RNA, (2) overall reduction in the total yield of virus particles as well as of intracellular synthesis of viral macromolecules, and (3) reduction in cytophatic effects caused by standard virus infection.

The mechanism of this interference appears to be complex and may involve multiple steps. Interference may occur either at the level of RNA replication and/or transcription since DI RNAs can fully function as both replicative and transcriptive templates (Chambers *et al.*, 1984; Akkina *et al.*, 1984; Nayak *et al.*, 1990). Interference at the latter level may also affect the production of essential enzymes such as polymerases. Since these DI RNAs are transcribed into mRNAs, some of which are translated into defective proteins, transcriptional as well as translational products may also be involved in DI virus-mediated interference. These DI-specific proteins, which have an amino terminal sequence in common with the polymerase, may affect the formation of functional polymerase complexes and thus, inhibit the total replication and transcription of viral RNAs. Finally, these DI RNAs may interfere in the packaging and assembly process by selectively displacing their parental standard RNA segments from the virion (Akkina *et al.*, 1984; Nayak *et al.* 1985). This means that these DI RNA segments will function as

competitors with standard RNA segments because of their smaller size. They, most probably, do not have an altered structure with an increased affinity for polymerase binding or replicative ability. Therefore, influenza DI-mediated interference as an intracellular process can partly be reversed with increasing multiplicity of standard viruses. The strength of the interference phenomenon is that DI RNAs seem to be generated easily during replication.

The factors affecting the generation and evolution of influenza virus DI RNAs are poorly understood. Sequence analysis rules out the normal eukaryotic splicing mechanism or post-transcriptional processing of nascent RNA in the generation of DI RNAs. It was suggested that aberrant replicative event(s), in which the polymerase complex somehow skips a portion of the RNA template, is (are) involved in the generation of DI RNAs. Sequences which specify either the detachment or reattachment of the polymerase on the plus or minus progenitor RNA strands in the generation of DI RNAs have not been observed (Nayak *et al.*, 1985; Nayak & Sivasubramanian, 1983; Jennings *et al.*, 1983). However, it is still possible that secondary structure(s) in the nucleotide sequence or some higher order structure in the nucleocapsids may be responsible for causing the detachment and reattachment. Two general mechanisms for the introduction of internal deletions have been proposed (Nayak *et al.*, 1985, 1990). The first is the jumping polymerase model, which involves the detachment and reattachment of the polymerase complex from the template. In this model, the polymerase detaches from the template and reattaches downstream on the same template, giving rise to shortened RNA molecules possessing internal deletions. This may occur during the replication of either the plus or the minus RNA strand. This process requires that the nascent RNA strand must remain attached to the polymerase complex when physically detached from the template. As discussed above no unique sequence or an obvious RNA secondary structure at either the junction or the flanking regions is not involved in the detachment and reattachment of the polymerase on the template RNA. Thus, detachment and reattachment may occur either at random or some unique features of RNA in the ribonucleoprotein complex have yet to be identified.

The second mechanism for generating an internal deletion can be described as a process that involves rolling over of the polymerase complex and looping out of the RNA

template (rollover/loop-out model). In this process, the polymerase complex does not completely detach from the template but rolls over, with the attached nascent daughter RNA strand, to a new site on the template that is brought into juxtaposition. The fact that most, if not all, influenza virus DI RNAs are monogenic and not of polygenic origin argues against a complete detachment and reattachment to a new site but would favour this rollover/loop-out model. How the two sites on the RNA template are brought close together so that the polymerase complex can rollover is not clear. One possibility is that such a juxtaposition of two sites of the RNA template is caused by the formation of transient RNA secondary structures during the replication.

This model, in which the polymerase rolls over to a new site without detaching completely, would favor the generation of internally deleted influenza DI RNAs with conserved 5' and 3' termini (Nayak *et al.*, 1985, 1990). Also, this model would explain why the other DI RNA classes e.g. panhandle, mosaic or rearranged DI molecules are either absent or rare in influenza viruses. Taken together, current evidence suggests that rolling over of the polymerase complex and the possibility of multiple contact points within the RNA template are likely to generate 5'-3' internally deleted influenza DI RNAs.

The biological function of influenza DI RNAs remains to be elucidated. They may be generated in natural infections attenuating the pathogenic effects of the wild type virus. Therefore, DI RNAs may prove useful as the basis of an effective influenza vaccine.

Sendai virus

Sendai virus, a parainfluenza virus, possesses a non-segmented negative-stranded genome. Upon infection of cells with this virus a variety of DI RNAs has been observed which exhibits extensive deletions and are dependent on wild type virus for replication (Kailash *et al.*, 1983). The majority of Sendai virus DI genomes described so far, are internally deleted molecules, which have been designated "fusion" DI RNA species to indicate that they are products of errors in viral RNA replication deleting the internal genes and fusing the 3'-terminal regions of the first gene, NP, with 5'-terminal regions of the last gene, L, as suggested by Amesse *et al.* (1982). Sofar, among non-segmented

negative-strand RNA viruses, such deletion mutants are almost restricted to Sendai viruses. Studies of the fusion DI genomes of Sendai virus indicate that they are generated by a copy-choice mechanism dependent upon signals that control transcription and replication (Re *et al.*, 1985).

Picornaviruses

For both the Mahoney and Sabin strain of poliovirus type I the occurrence of DI RNAs have been described (Cole *et al.*, 1971; Kajigaya *et al.*, 1985). In both systems the DIs contain internal deletions comprising approximately 15% of the genome. These DI genomes are generated at very low rates after many serial high-multiplicity-of-infection passages. The purified DI particles are able to initiate a normal poliovirus replication cycle but fail to synthesize capsid proteins and therefore cannot produce progeny virions. This observation suggests that the DI RNAs lack the genomic region that encodes the viral capsid proteins. Sequence analysis of several poliovirus DI molecules revealed the precise location and size of the deletions. The size distribution of the deletions varied from 9.6 to 13.2% of the total genome length and span the region in the poliovirus genome between nucleotide positions 1226 and 2705, showing that the rearranged sites occur in a limited area of the approximately 7000 nucleotide long genomic RNA (Hagino-Yamagishi *et al.*, 1990).

All the deletions are introduced in such a manner that the correct open reading frame of the poliovirus polyprotein is maintained downstream of the deletion. This finding strongly suggests that certain non-structural proteins must be provided in cis to support DI RNA replication.

Coronaviruses

Coronaviruses contain a single-stranded, positive-sense RNA genome of approximately 30 kb long. DI RNAs generated by this virus consist of distinct subgenomic-sized polyadenylated RNA. They contain sequences derived from several discontiguous parts of the DI genome (Makino *et al.*, 1985). They are not appreciably detected in virions and are, thus, probably transcribed *de novo* from the viral genome by a discontinuous mechanism. Evidence has been presented that the transcription of

these subgenomic DI RNAs requires a helper virus function, while the replication of the DI genomic RNA does not (Makino *et al.*, 1985).

The DI genomes of mouse hepatitis virus (MHV), the best-studied member of the *Coronaviridae*, have retained both original 5' and 3' termini while multiple internal segments of the standard virus are deleted. During propagation, the MHV DI genomes appear to undergo progressive modifications, notably insertions and deletions, and new DIs emerge only to be succeeded by other, more efficiently replicating species (Makino & Lai, 1989; Makino *et al.*, 1988; de Groot *et al.*, 1992).

De Groot *et al.* (1992) have shown that the genome of a naturally generated MHV DI virus (denoted DI-a) contains a full-length fused ORF encoding a 184K "fusion" polypeptide. Using constructs derived from this genome, they demonstrated that the fitness of these defective molecules and its synthetic derivatives is decreased by nonsense and frameshift mutations, while upon further replication the disturbed ORF becomes restored (de Groot *et al.*, 1992). These results lead to the conclusion that translation of the fusion ORF is indeed required for efficient propagation of DI-a and its derivatives. The precise role of this ORF and the encoded functional proteins remains to be elucidated.

Deletion analysis of murine hepatitis virus DI RNAs have been used to elucidate important signals involved in the coronavirus infection process (Makino *et al.*, 1989, 1990; van der Most *et al.*, 1991, 1992; de Groot *et al.*, 1992).

DEFECTIVE INTERFERING RNAs ASSOCIATED WITH PLANT VIRUSES

For plant viruses, phenomena as symptom attenuation and virus interference have been documented much less extensively than for animal systems. The majority of reports of these phenomena in plants describes interactions between a helper virus and an associated component identified as either a satellite virus or a satellite RNA (Francki, 1985). In contrast, only few reports describe the occurrence of defective viruses or genuine DI RNAs associated with plant virus infections. They include DI-like particles found in plants infected with (negative-strand) rhabdoviruses (Adam *et al.*, 1983; Ismail and Milner, 1988). DI RNA segments have also been reported for wound tumor virus,

a plant reovirus (Nuss, 1988). DI RNAs which meet the criteria of Huang and Baltimore (1970) have been described for plant virus of only two related groups, the tombusviruses and carmoviruses, which have positive-strand RNA genomes (Morris and Knorr, 1990). Within these virus groups DI RNAs have been reported for tomato bushy stunt virus (TBSV) (Hillman *et al.*, 1987; Morris and Knorr, 1990; Knorr *et al.*, 1991), turnip crinkle virus (TCV) (Li *et al.*, 1989; Cascone *et al.*, 1990) and Cymbidium ringspot virus (CyRSV) (Burgyan *et al.*, 1989, 1991).

DI RNAs of Tombusviruses

Biological and molecular characterization of a novel RNA species associated with the cherry strain of TBSV provided the first definitive demonstration of DI RNAs for a plant virus (Hillman *et al.*, 1987). These DI RNAs were found to prevent the development of lethal necrosis as normally caused by TBSV in solanaceous hosts. The DI RNAs were shown to be colinear deletion mutants of the non-segmented virus genome, derived from 5'-proximal, internal and 3'-proximal genomic regions. These DIs, which are dependent on the parent virus for both replication and encapsidation, have been shown to reduce virus accumulation in infected plants (Hillman *et al.*, 1987) and to inhibit genomic RNA replication in single protoplasts (Jones *et al.*, 1990).

De novo generation of defective interfering RNAs of TBSV by high multiplicity passages has been demonstrated (Knorr *et al.*, 1991). They were generated *de novo* in 12 independent isolates of TBSV upon serial passages. Comparison of the nucleotide sequences of 10 cDNA clones from 2 DI populations with a previously characterized TBSV DI RNA revealed that the same four regions of the TBSV genome were strictly conserved in each of the DI RNAs. Each consisted of the 5' leader sequence of 168 bases, an internal region of approximately 200-250 bases from the viral polymerase gene, plus approximately 70 bases from the 3' terminus of the viral p19 and p22 genes, and approximately 130 bases from the 3' terminal noncoding region. The sequences retained by the DI RNAs allow speculation on possible mechanisms for their formation, since both the presence of a certain region of the viral replicase and specific structures of the viral template seems to contribute in the formation of DI RNAs. A hexanucleotide motif 5'-APUAGAA-3' occurs at or near the endpoints of the second and third regions in the

DI RNAs. It is possible that polymerase dissociation and reinitiation take place in these regions, thereby deleting large regions. Several sequences with "strong stop" signals present in the TBSV genome have been also suggested to be involved in the formation of DI RNAs as regions destabilizing the polymerase-RNA complex. Conservation of the same four regions of TBSV sequences in the DI RNA clones suggests that the retained elements are required for RNA replication and possibly also for encapsidation. The maintenance of the 5' and 3' terminus suggests that they are involved in polymerase binding and initiation of replication. The internal regions are more likely to act as sites of recognition for trans-acting factors important for the initiation or regulation of replication.

The most likely mechanism leading to symptom attenuation may be explained by the reduced viral replication resulting from competition between the viral genome and DI RNAs for replicase. It is possible that the selection pressure resulting in symptom attenuation would favour replicative fitness of the DI RNAs through selection of optimal polymerase binding sequences. In addition, the small size and structural stability of DI RNAs might also contribute to their ability to be maintained.

Another tombusvirus, CyRSV, has been shown to support the generation of DI RNAs (Burgyan *et al.*, 1989; 1991). Analysis of these RNAs showed that 3 conserved sequence blocks, A, B, and C, are retained. Block A is composed of the first 164 nucleotides of genomic RNA, including the 5' leader sequence and the initiation codon of the 92 kD protein, block B is 112 nucleotides long and represents the central part of the putative polymerase gene, and block C, 403 nucleotides long, corresponds to 49 nucleotides of the carboxyl terminus of the 22 kD protein gene and the entire non-coding region of 354 nucleotides. Smaller DI RNAs which have also been identified, are formed by essentially the same genomic RNA sequence blocks with a progressive reduction of their size by further deletions inside blocks A and C.

Nucleotide sequences of several of these DI RNAs suggested a possible evolution of smaller from larger molecules. No clear consensus sequences were found when the nucleotide composition of each block was compared, but the initiation sequences showed marked similarities. Most of the DI RNAs had one or two Gs in the first two positions as observed in many recombination sites. These two Gs are part of the consensus

sequences recognized in all replicating molecules of CyRSV, i.e. genomic, subgenomic and satellite RNAs.

A "copy choice" mechanism is suggested to be involved in the generation of CyRSV DI RNAs. In addition, stable secondary structures surrounding the deleted regions and containing hairpin loops where rearrangement sites are located, may also play a role in this process.

DI RNAs of turnip crinkle virus

Turnip crinkle virus (TCV) is a single-stranded, positive sense RNA virus which has been classified as a member of the carmoviruses (Morris and Carrington, 1988). Studies demonstrate that TCV supports the replication of a variety of linear small RNAs such as (i) satellite (sat) RNAs (Simon and Howell, 1986) being small RNAs, which do not function as mRNAs and require a helper virus for replication and propagation; (ii) chimeric RNAs (Simon and Howell, 1986) composed of sat-RNA sequence at the 5' end and viral sequences at the 3' end; and (iii) DI RNAs (Li *et al.*, 1989) derived almost exclusively from parental virus sequences and, unlike other DI RNAs, intensify the symptoms produced in host plants. The DI RNA designated G (346 bases), normally associated with TCV-B isolate, is a mosaic molecule composed of 21 nucleotides of unknown origin at the 5' end, 5' and 3' parts of viral segments and a centrally repeated block of viral sequences (Li *et al.*, 1989). Another DI molecule denoted DI-1 (383 bases) was generated *de novo* following inoculation of plants with material derived from *in vitro* synthesized viral transcripts (TCV-B isolate) and contains the exact 5' and 3' ends of TCV as well as an internal viral sequence (Li *et al.*, 1989). Additional DI RNAs derived *de novo* from the cloned viral inoculum were identified. They contained a colinear mosaic of sequences entirely derived from the genomic RNA consisting of 135 bases of 5' sequence, an internal region of 90 bases from the 3' terminus of the coat protein gene, and 150 bases of the viral 3' terminus (Morris and Knorr, 1990).

Cascone *et al.* (1990) and Zhang *et al.* (1991) demonstrated the occurrence of recombination events between TCV associated RNAs. Analysis of the recombination junctions revealed the presence of conserved sequence motifs. These sequences were proposed to represent putative signals recognized by the viral replicase during generation

of defective interfering and recombinant RNAs in the TCV system. Based on the similarities in sat-RNA C, the right side junction of DI RNA G and the 5' end of TCV, as well as the sequence similarity between the right side junctions of DI-1 RNA and sat-RNA C and the 5' end of the sat-RNAs, a replicase-driven mechanism is proposed. The replicase, while replicating viral or sub-viral minus strands, dissociate from the template along with the nascent plus strand and reinitiate synthesis at one of the internal replicase recognition sequences on the same or different template thereby generating recombinant sat-RNAs or DI RNAs.

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CHAPTER 3 IMMUNO-ELECTRON MICROSCOPICAL DETECTION OF TOMATO SPOTTED WILT VIRUS AND ITS NUCLEOCAPSIDS IN CRUDE PLANT EXTRACTS

Elliot Watanabe Kitajima, Renato de Oliveira Resende, Antonio Carlos de Ávila, Rob Goldbach and Dick Peters.

SUMMARY

Tomato spotted wilt virus (TSWV) particles were identified in thin sections and in crude extracts of leaves from plants infected with different TSWV isolates, using gold labelled protein A and antibodies prepared against purified virus particles or against nucleocapsid preparations. In addition, both in thin sections and in dip preparations aggregates were detected using either gold labelled antiserum. These aggregates were not detected using antisera against envelope proteins or against the non-structural protein NSs. They were the only detectably labelled material in preparations made from plants infected by morphologically defective isolates of TSWV, i.e. isolates that did not produce enveloped particles. The aggregates were discerned in dip preparations as cloudy amorphous structures having a cottonwool boll-like outlook. These "cotton bolls" were interpreted as being the *in vitro* structures of the nucleocapsid aggregates occurring in plant cells infected by isolates maintained by mechanical inoculation and are the only structures in cells infected by morphologically defective isolates (Ie, 1982; Kitajima et al., 1992). The detection of the "cotton boll-like" structures in crude extracts provides an adequate and rapid method to establish the presence of nucleocapsid aggregates in cells of infected plants, irrespective of whether they are infected by morphologically defective isolates or not.

This chapter has been published in a slightly modified version as: Kitajima *et al.* (1992). Immuno-electron microscopical detection of tomato spotted wilt virus and its nucleocapsids in crude plant extracts. *Journal of Virological Methods* 38, 313-322.

INTRODUCTION

Tomato spotted wilt virus (TSWV) is a cosmopolitan plant virus, spread in nature by thrips species and mechanically transmissible under experimental conditions. The virus has a wide host range and causes economically important diseases in vegetable and ornamental crops, mostly in tropical regions (Peters *et al.*, 1991). Recently, due to the spread of *Frankliniella occidentalis* Perg. over the Northern hemisphere, TSWV is also becoming prominent in temperate climate zones, e.g. in the US, Canada and Europe.

TSWV particles, spheroidal in shape with a diameter of 70-120 nm, possess a lipid membrane provided with two viral glycoproteins (G1 of 78 kD and G2 of 58 kD), and contain three genomic ssRNA segments of different sizes (L, M and S, respectively, measuring 8,897, 4,821 and 2,916 nucleotides) that are tightly associated with nucleocapsid (N) proteins of 29 kD. A high molecular weight protein (L) of approximately 200 kD also occurs in the particle (Ie, 1970; Francki & Hatta, 1981; de Haan *et al.*, 1989; Peters *et al.*, 1991).

In infected cells, TSWV particles accumulate after their envelopment in the cisternae of the endoplasmic reticulum system. Amorphous aggregates of moderate density (viroplasm), intermingled with high electron dense complexes with a 5 nm periodicity, as well as inclusions of fibrous material appear in the cytoplasm of infected cells (Francki *et al.*, 1985; Kitajima *et al.*, 1992).

The high electron dense complexes are thought to be aggregates of nucleocapsids which are not enveloped in virus particles. They appear in cells after serial mechanical transfers and are the only structures containing viral antigens in cells infected with morphologically defective isolates (Ie, 1982; Resende *et al.*, 1991a, b). In addition, Kitajima *et al.* (1992) showed that these aggregates of nucleocapsids accumulate, sometimes abundantly, in almost all isolates which have been mechanically transmitted but are still able to form enveloped particles. Understanding the generation of nucleocapsids at the molecular level might shed light on the morphogenesis of the virus, and the molecular requirements of vector transmission (Resende *et al.*, 1991a, b).

Detection of morphologically defective isolates and of the occurrence of nucleocapsid aggregates in cells infected with non-defective isolates, was achieved by electron

microscopic studies in ultrathin sections. Since these studies were time consuming. we developed a method to analyze large numbers of samples for the development of such aggregates in infected cells and for the generation of morphologically defective isolates.

This report describes the successful use of the gold labelled antibody decoration method (Pares & Whitecross, 1982) to establish whether TSWV isolates generate nucleocapsid aggregates and to determine whether either enveloped or morphologically defective TSWV particles, or both, are produced.

METHODS

TSWV isolates

The isolates BR-01, BR-13, NL-04 and SA-04 used in this study were all members of the proposed serogroup I (de Ávila *et al.*, 1992). They were isolated from different hosts in different geographical regions (de Ávila *et al.*, 1991; Kitajima *et al.*, 1992) and maintained by mechanical inoculation, mostly on *Nicotiana rustica* cv 'America' plants. Two lines of the isolate NL-04 were used; one of which formed complete particles, while the other was morphologically defective. Inocula and the samples tested were prepared from systemically infected leaves 10-14 days after inoculation.

Antisera

Antisera were raised in rabbits against purified virus preparations or purified nucleocapsids of the Brazilian isolate BR-01 and the Dutch isolate NL-04, both previously described and belonging to serogroup I (de Ávila *et al.*, 1991; de Ávila *et al.*, 1992).

Protein A-gold (pAg)

This label was prepared following the procedure described by Van Lent & Verduin (1985). Colloidal gold particles of 7 μmin diameter were used in most experiments.

Preparation of leaf extracts

Leaf pieces of ca. 1 cm^2 were triturated in a droplet of phosphate buffer/saline (PBS) containing 1% bovine serum albumin (BSA). The extract obtained was mixed with another droplet of BSA/PBS.

Gold labelling of preparations

Formvar/carbon coated grids were floated on the mixture for 1 min with the membrane side down, 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 and incubated in a droplet of pAg with an OD_{520} concentration of 0.1 for another 10 min, washed with 10 droplets of PBS and 30 droplets of distilled water, and finally negatively stained with aqueous 2% uranyl acetate. The grids were prepared at room temperature and examined in a Philips CM 12 electron microscope.

RESULTS AND DISCUSSION

Cytopathology of TSWV infected cells

Ultrathin sections of TSWV infected plants invariably contain the characteristic virus particles, scattered between the membranes of the endoplasmic reticulum (Fig. 1A). They have a circular profile with a diameter varying from 70 to 110 nm. In addition, in most cells, complexes of dense material occur embedded in viroplasms, which are condensed in several forms of approximately 50-100 nm wide with cross striations of 5 nm (Fig. 1A & B). These nucleocapsid aggregates are thought to represent stacks of nucleocapsids that failed to complete budding, possibly because of an imbalance in the ratio of glycoprotein/nucleocapsid synthesis or of a defective glycoprotein (Verkleij & Peters, 1983; Resende *et al.*, 1991a, b; Kitajima *et al.*, 1992). They often occur in chain-like formations. They have been described in studies on TSWV cytopathology as "irregular, dense and tangled strands" (Kitajima, 1965), "densely staining amorphous mass (Milne, 1970), "densely staining granular material" (Francki & Grivell, 1970), "locally dense striated spots" (Ie, 1971) "amorphous, diffuse masses" (Ie, 1982), dense aggregates (Resende *et al.*, 1991a, b), and have been definitely identified as nucleocapsid aggregates

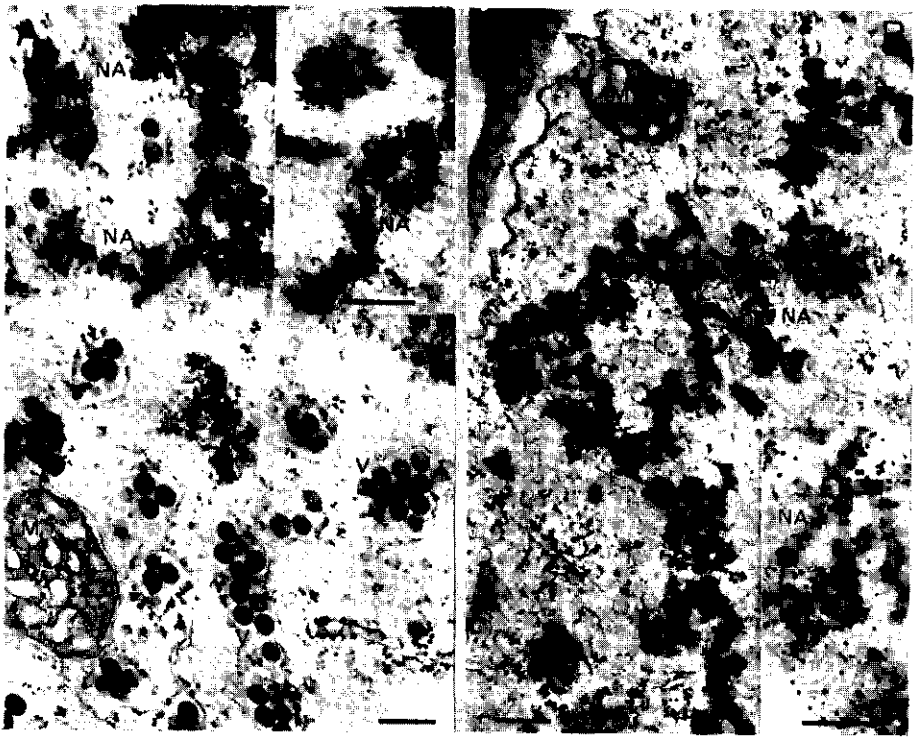

Fig. 1A. Virus particles (V) and nucleocapsid aggregates (NA) in a leaf cell infected by the isolate SA-04. Both structures are immunolabelled by gold particles when sections are incubated with antiserum to BR-01 (insert). M- mitochondrion. **1B** - Nucleocapsid aggregates (NA) in a leaf parenchyma cell of *N. rustica* infected with the morphologically defective form of TSWV isolate NL-04. Enveloped virus particles are absent, but most of the cells contain nucleocapsid aggregates. *In situ* immunogold labelling clearly demonstrates the affinity of the complexes to antibodies directed against BR-01 nucleocapsid protein (insert). Bars represent 0.2 μm.

by Kitajima *et al.* (1992). Immunolabelling experiments carried out on tissue sections clearly showed that these complexes can be tagged with gold using nucleocapsid antiserum (inserts, Fig. 1A & B), but not when antiserum to the envelope glycoproteins or to the S-RNA-encoded non-structural (NSs) protein is used (Kormelink *et al.*, 1991).

Detection of virus particles and nucleocapsid material in dip preparations

TSWV particles can readily be detected in leaf dip preparations of infected plants of several species (Fig. 2A). They exhibit their characteristic morphology as described

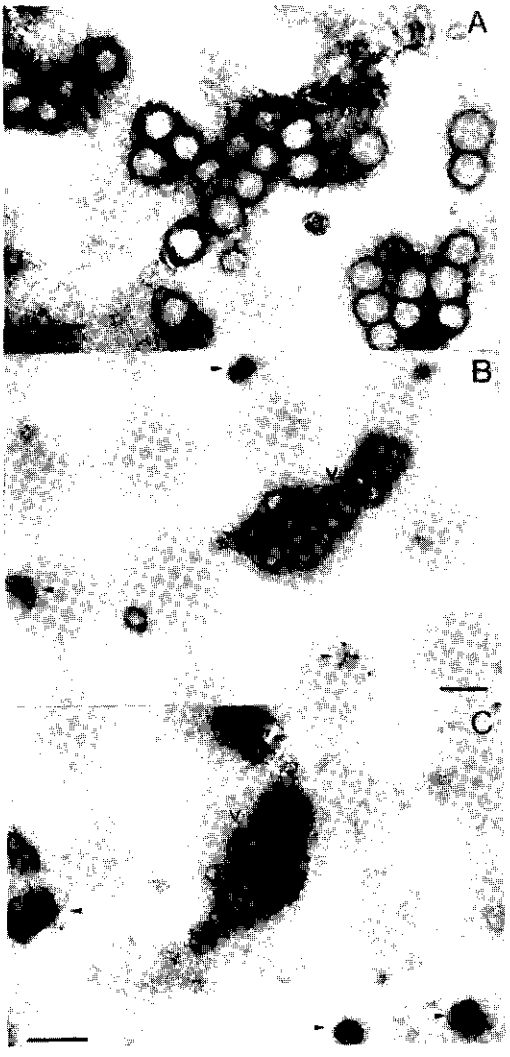

Fig. 2A. *Leaf dip preparation from N. rustica infected with isolate BR-01, negatively stained with uranyl acetate. Numerous spheroidal particles, 70-110 nm in diameter, can be seen among the cell debris.*
2B - Leaf dip preparation treated with antiserum to BR-01 and probed by protein-A gold (pAg). Gold particles are visible on virus particles (V) and also on small amorphous masses (arrowheads), which are believed to represent aggregates of nucleocapsids that failed to acquire an envelope.
2C - A crude sap preparation from NL-04 infected plants, immunolabelled with antibodies against BR-01 nucleocapsid protein. Virus particles (V) are not or sparsely tagged with gold particles, but the nucleocapsid aggregates are consistently labelled (arrowheads). Bars represent 0.1 μm.

above. Eventually, the stain penetrates the particle revealing a fuzzy content, while spikes can then be observed on the envelope. The virus particles might either appear scattered individually or in groups. These groups are sometimes contained in membranous sacs, which probably represent remnants of the endoplasmic reticulum cisternae in which the virus particles aggregate after envelopment (data not shown). A comparison of particles found in leaf dip preparations from plants infected with different isolates did not reveal any significant difference in particle morphology.

When extracts from infected plants were treated with BR-01 antiserum and pAg, virus particles were effectively labelled (Fig. 2B). Sometimes, when present inside the membranous sacs, labelling of the particles did not occur. The background contained virtually no label in preparations from healthy or infected plants whatever antiserum was applied.

Virus particles of all isolates tested, including also those which did not belong to serogroup I (de Ávila *et al.*, 1992) reacted, often weakly, with gold-labelled antibodies to complete BR-01 virus, but not with antibodies directed to purified BR-01 nucleocapsids. This observation confirms the results of de Ávila *et al.* (1991) showing that the envelope glycoproteins are more conserved among different serogroups than their nucleocapsid proteins.

Detection of nucleocapsid material in dip preparations

Besides the virus particles, particulate clumps of amorphous material were observed in the dip preparations that were not surrounded by a membrane but tagged with gold particles, using either antiserum to purified BR-01 virus preparations or antiserum to its nucleocapsid protein. These clumps are found in variable amounts and more densely labelled than the enveloped virus particles (Fig. 2B & C and Fig. 3). They have variably spheroidal forms and differ considerably in size, ranging from 50 to 100 nm in diameter to sizes of up to 1 μmor more. These amorphous and cloudy clumps of material have an appearance of "cotton bolls".

In experiments using BR-01 nucleocapsid protein antiserum, virus particles were not or only sparsely labelled (Fig. 2C). This can be explained by the inaccessibility of the nucleocapsid protein in the virus particle as the viral membrane may hinder penetration

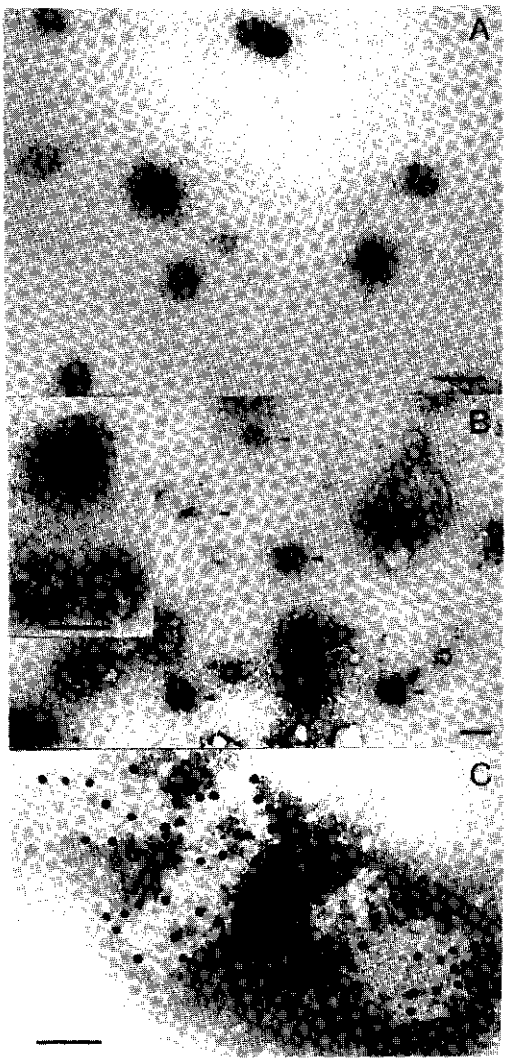

Fig. 3A. Extract from plants infected with a morphologically defective form of the NL-04 isolate of TSWV, immunolabelled with antibodies against whole particles of TSWV-BR-01. Gold labelled nucleocapsid aggregates of various sizes are visible. **3B** - Nucleocapsid aggregates (arrowheads), gold labelled using antibodies against the nucleocapsid protein of isolate BR-01, in an extract of *N. rustica* plants infected by the defective form of BR-13 isolate. Insert shows purified nucleocapsids of isolate BR-01 immunostained with this antiserum. **3C** - A higher magnification of the nucleocapsid aggregates found in dip preparations of extracts of plants infected by a defective form of isolate NL-04. The preparation was immunolabelled using pAg and antibodies against the nucleocapsid protein of isolate BR-01. Bars represent 0.1 μm.

of the gold-antibody complexes into the particle. The labelling of the amorphous structures by this antiserum with a high intensity demonstrates that they contain nucleocapsid protein. Such aggregates were the only labelled structures found in large amounts in the crude extracts of NL-04 and BR-13 lines which are morphologically defective as has been demonstrated by serology and confirmed by electron microscope studies on ultrathin sections by Resende *et al.* (1991a, b) (Fig 3A & B). The nucleocapsid aggregates are also readily detectable in preparations of isolates maintained for long periods by serial mechanical inoculation, such as the more intensively studied lines of the isolates BR-01, NL-04, and ES-01 (Fig. 2B & C), but could also be detected in recently obtained isolates.

Due to the presence of other amorphous structures in dip preparations made from plant extracts the nucleocapsid aggregates can only be identified by gold labelling. They were not found in purified nucleocapsid preparations indicating that the aggregates dissociate into the individual nucleocapsids during their purification, while they remain associated and apparently swell to a certain extent during the preparation of dip preparations.

Immunolabelling permits the unequivocal detection of TSWV particles, even when they occur in relatively low concentrations, and can furthermore be used in leaf dip preparations to demonstrate the development and accumulation of nucleocapsid aggregates.

A time course study showed that the virus particles and "cotton bolls" are detectable 6-8 days after inoculation with complete or defective lines of TSWV in the leaves immediately above the inoculated leaf, coinciding with the time at which positive responses in ELISA were obtained and before the appearance of the first systemic symptoms (data not shown). These results show that immuno-electronmicroscopic detection can be used as early as ELISA to detect TSWV infection and to follow the development of nucleocapsid aggregates in infected cells.

ACKNOWLEDGEMENTS

This study is supported by a post-doctoral fellowship of the Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) and a research fellowship of the Agriculture University, Wageningen to E.W. Kitajima. A.C. de Ávila hold a doctoral fellowship from Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA) and R. de O. Resende from CNPq. The authors express their thanks to Dr J.W.M. van Lent and mr J. Groenewegen for their guidance in immunolabelling work.

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CHAPTER 4 GENERATION OF ENVELOPE AND DEFECTIVE INTERFERING RNA MUTANTS OF TOMATO SPOTTED WILT VIRUS BY MECHANICAL PASSAGE

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SUMMARY

During a series of mechanical transfers of tomato spotted wilt virus (TSWV), 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 deleted 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 indicate that all defective molecules had retained the sequences required for replication, encapsidation by nucleocapsid proteins, and packaging of the nucleocapsid into virus particles.

This chapter has been published in a slightly modified version as: Resende *et al.* (1991). Generation of envelope and defective interfering RNA mutants of tomato spotted wilt virus by mechanical passage. *Journal of General Virology* 72, 2375-2383.

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 the 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, 1985) 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 (TBSV) (Hillman *et al.*, 1987; Morris & Knorr, 1990; Knorr *et al.*, 1991), turnip crinkle virus (TCV) (Li *et al.*, 1989; Cascone *et al.* 1990) and Cymbidium ringspot virus (CyRSV) (Burgyan *et al.*, 1989; Burgyan *et al.*, 1991). These DI RNAs consist of a mosaic of the original genome. Ismail & Milner (1988) isolated DI particles from *Sonchus* yellow net virus (SYNV), 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 case of multipartite genomes, loss of entire genome segments of the parental virus (Black, 1979; Shirako & Brakke, 1984; Shirako & Ehara, 1986).

Defective forms of tomato spotted wilt virus (TSWV) have previously been reported (Ie, 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 (de Haan *et al.*, 1989a; Francki *et al.*, 1991). This virus has a remarkable 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 70 to 110 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 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 S RNA (2916 bases), medium (M) RNA (4821 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 which is also ambisense encodes the membrane glycoproteins (G1 and G2) and another non-structural protein NSm (Kormelink *et al.*,1992). The L RNA is of negative polarity and most likely encode the viral polymerase (de Haan *et al.*, 1991). Ie (1982) noticed that in some isolates, the characteristic enveloped virus particles were absent in cells of infected plants after repeated mechanical transfers. 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 (Kitajima *et al.*, 1992). 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, BR-01, a Brazilian isolate from tomato, and NL-04, a Dutch isolate from chrysanthemum, stored in liquid nitrogen were passaged in two different ways over host plants. Leaves of *Nicotiana rustica* cv America plants systemically infected with BR-01, were used as inoculum sources for mechanical inoculation of *Nicotiana*

tabacum cv Samsun NN plants. 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 days intervals, using systemically infected leaves as 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-Na_2SO_3. After inoculation, the plants were placed in a glasshouse at approximately 22°C (light:dark,16:8 h) for symptom development.

Detection of morphological defective forms by serology and electron microscopy

Each generation of infected plants was tested in 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). The labelling of both ultrathin sections and dip preparations were carried out as described by Kitajima *et al.* (1992). Leaf extracts were assayed by crushing 1 cm^2 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 analyzed by electrophoresis in 1% agarose gels under denaturing conditions (Bailey & Davidson, 1976). After transfer to nitrocellulose membranes the RNAs were hybridized to ^{32}P-labelled DNA probes, directed to the S, M and L RNAs of isolate BR-01 (Maniatis *et al.*, 1982; de Haan *et al.*, 1991). 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 strand-specific L RNA riboprobes produced as described by de Haan *et al.* (1990).

RESULTS

Propagation for morphological defective isolates by serial mechanical transfer

A large number of local lesions was 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 as those shown for BR-01 (originally CNPH$_1$ 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 these 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

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 was declined drastically.

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.

To separate possible defective forms from enveloped forms the isolate NL-04 was subsequently serially passaged in two lines over different hosts (Fig. 2). The line passaged over *N. rustica* resulted in a partial recovery of the original reaction with MAbs G1 to G4 in ELISA. These results showed that the amount of membrane protein increased and suggested 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.

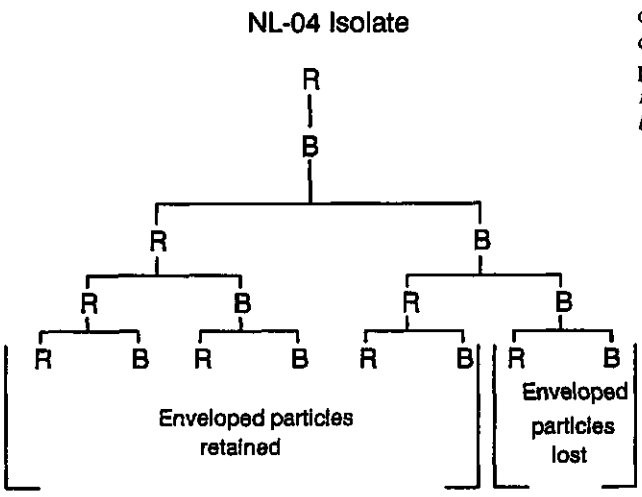

Fig. 2. Schematic representation of repeated mechanical transfers of the NL-04 isolate through different hosts. The isolate was passaged several times in *N. rustica* plants (R) and *N. benthamiana* (B).

A morphologically defective form was found when the isolate NL-04 was passaged over *N. benthamiana* (Fig. 2). The resulting isolate strongly reacted with the 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

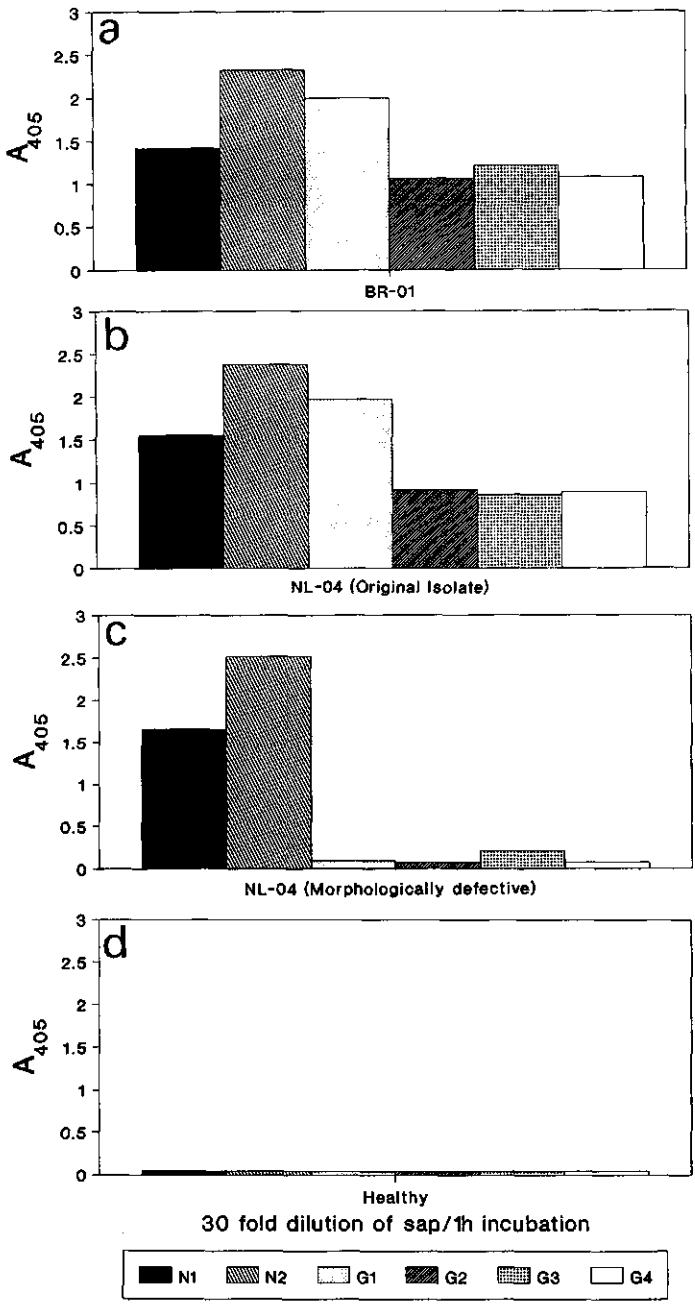

Fig. 3. Characterization of a morphologically defective isolate from TSWV NL-04 (c) and comparison with original NL-04 (b) using a panel of MAbs. The isolate was generated after successive mechanical inoculations onto *N. benthamiana* plants. Isolate BR-01 (a) and sap from health plants (d) were used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

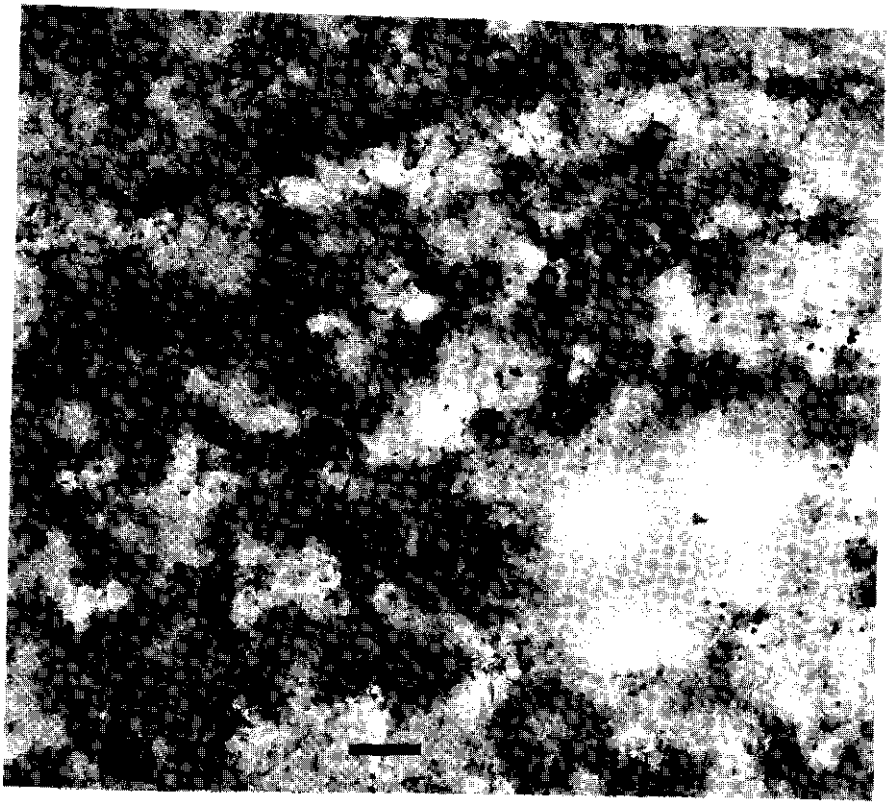

Fig. 4. Electron micrograph of a mesophyll cell of *N. rustica* infected with a morphologically defective isolate of NL-04. Only virus-induced inclusions of dense aggregates (DA), which are specifically tagged with gold-labelled antibodies to the N protein are detectable. The bar represents 0.2 μm.

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 analyzed on agarose gels (Fig. 5*a*). The RNA was blotted onto nitrocellulose membranes and hybridized with S, M and L

RNA-specific cDNA probes. Although the hybridizations with ^{32}P-labelled S and M RNA-specific probes (Fig. 5*b*, *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. 5*d*). 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 tc the full length L RNA deleted L RNA-derived species of different sizes.

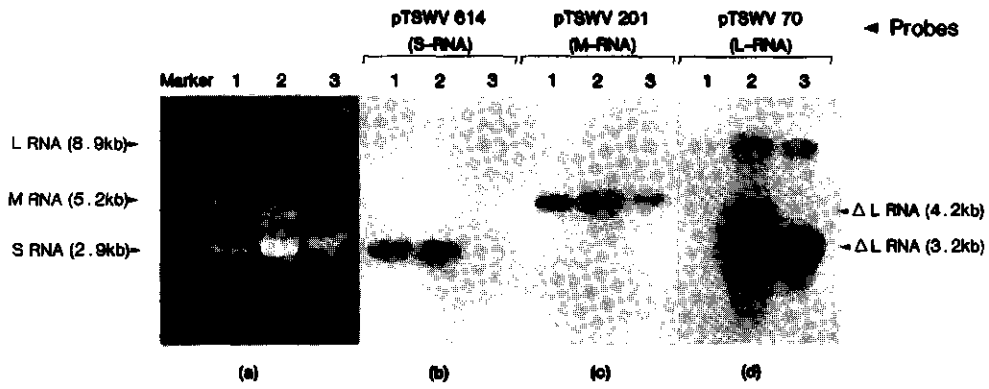

Fig. 5. Northern hybridizations of TSWV nucleocapsid RNA (a) purified from infected *N. rustica* plants (isolate BR-01), using ^{32}P-labelled cDNA probes specific for S-(pTSWV-614) (b), M-(pTSWV-201) (c) and L-RNA(pTSWV-70) (d). The Northern blots contained RNA from isolate BR-01 after three (lane 1),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

To verify whether these deleted 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 ^{32}P-labelled strand-specific L RNA probes. The results

revealed that the deleted L RNA species could be detected in virus (-) sense (Fig. 6*a*) as well as complementary (+) sense (Fig. 6*b*). Detection of complementary-sense segments demonstrated that the deleted L RNA species did replicate during the infection process and did 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

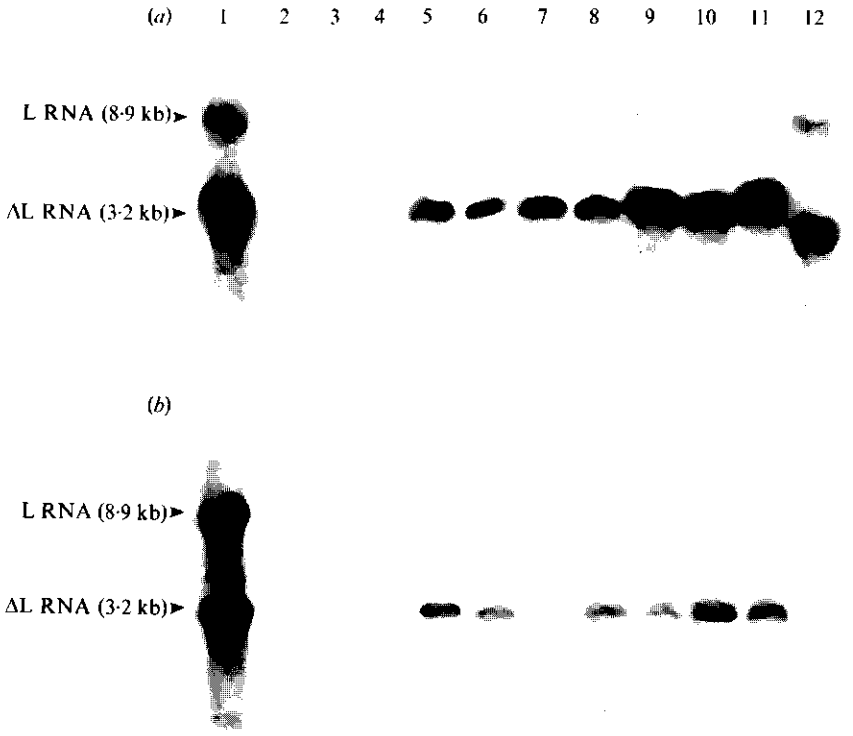

Fig. 6. Time course analysis of TSWV-infected *N. rustica* plants 2 (lane 2), 4 (lane 4), 6 (lane 5), 7 (lane 6), 8 (lane 7), 9 (lane 8), 10 (lane 9), 12 (lane 10) and 14 (lane 11) days p.i. Northern blots containing total RNA extracts were hybridized to ^{32}P-labelled riboprobes, detecting either viral sense L RNA (a) or viral complementary L RNA (b). Nucleocapsid RNA (lane 1), total RNA from healthy plants (lane 2) and purified viral RNA (lane 12) were included as controls.

6 days p.i., more deleted L RNAs than full-length L RNAs had been produced. These results can be interpreted such that the replication of the deleted 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 deleted L RNAs (Fig. 6*a*). Complementary-sense strands of full-length and deleted L RNAs were not detected in virus particles, an observation which strongly suggests that the negative strands of the genomic and deleted L RNAs are exclusively packaged into envelopes (Fig. 6*a*, *b*). When RNAs extracted from nucleocapsid preparations were analyzed, both the negative and positive strands of full-length and deleted forms of L RNA were detected (Fig. 6*a*, *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 similiar as those of normal TSWV isolates. This indicates that large deletions did not occur in any of the three RNA segments of that isolate (Fig. 7*a*).

Since S RNA has been shown to encode the N and NSs proteins (de Haan *et al.*, 1990) and the 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. 7*b*). Deleted L RNAs could not be detected in the morphologically defective isolate (Fig. 7*c*, *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

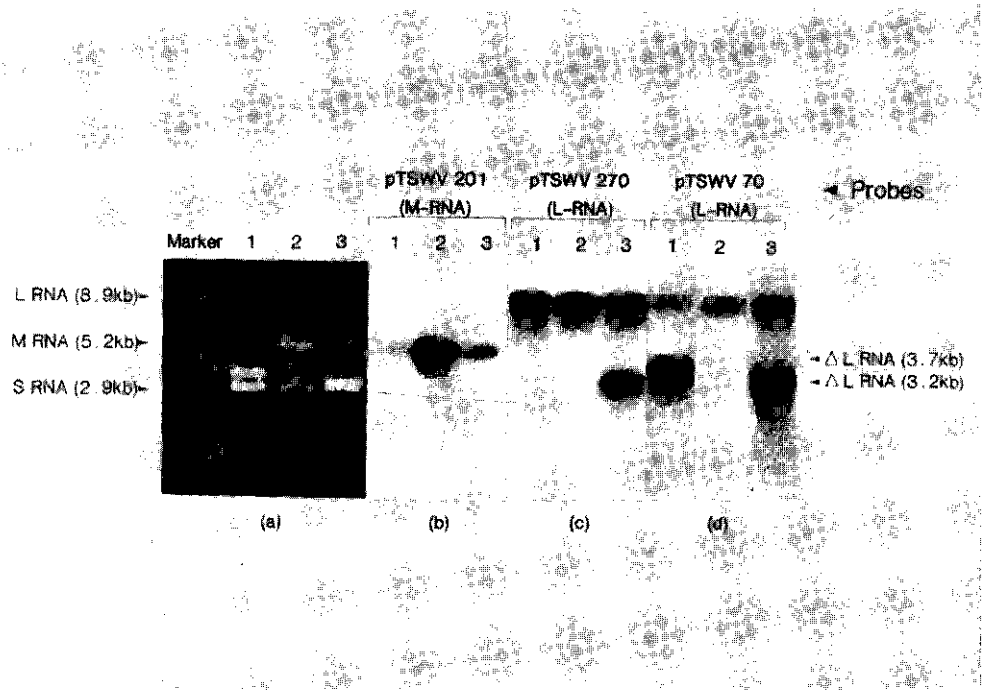

Fig. 7. Northern blot analysis of TSWV nucleocapsid RNA (a) purified from infected *N. rustica* plants using double-stranded cDNA probes specific for M RNA (pTSWV-201) (b), and L-RNA [pTSWV-270 (c) and pTSWV-70 (d)]. Lane 1, NL-04 isolate; lane 2, NL-04 morphologically defective isolate and lane 3, BR-01 isolate.

molecule. It must be noted that the other lines of NL-04 and BR-01 isolates were still able to produce virus particles after serial mechanical passages, but contained besides the S, M and L RNAs of the expected sizes, deleted L RNA molecules (Fig. 7*c*, *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 deleted forms of the L RNA segment. The

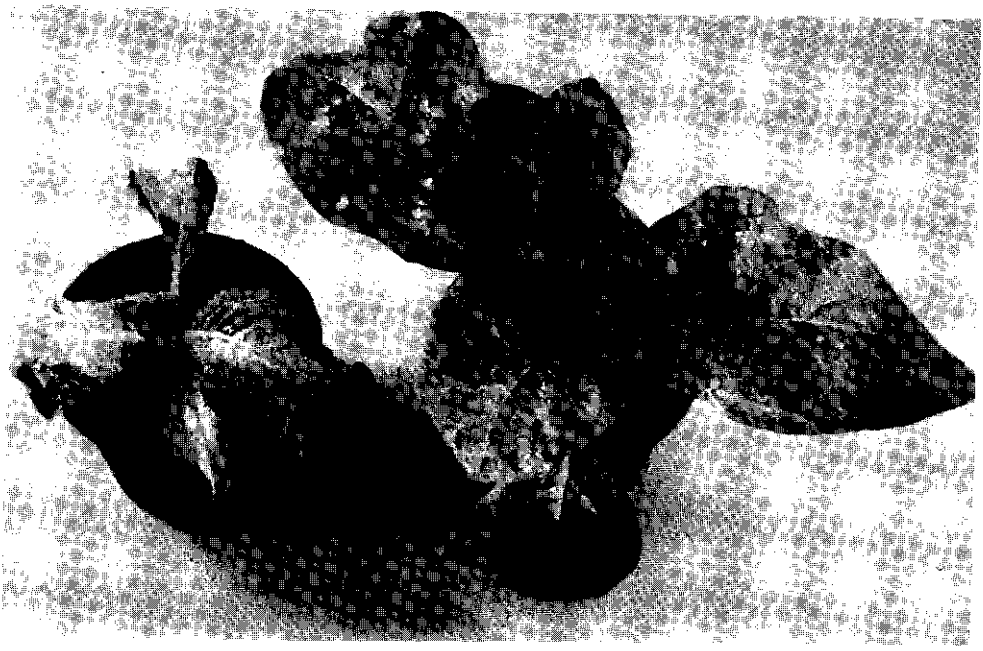

Fig. 8. *N. rustica* plants 12 days after infection with isolate BR-01. The plant on the left side, showing severe symptoms did not contain defective L RNAs, whereas the plant on the right contained high amounts of these RNA molecules.

present data strongly suggest that the deleted 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 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, 1985).

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 multiplicity-of-infection (Nayak *et al.*, 1990). Full-length and deleted 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 and 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 CyRSV genome. DI RNAs which 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 and Milner (1988) also reported DI particles for the plant rhabdovirus SYNV, 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 (Kascsak & Lyons, 1978; David-West &

Porterfield, 1974; 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 and 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. 7*b*, *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, 1985). 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, though deficient in membrane glycoprotein synthesis, showed no large deletions in the M RNA. 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 Ie (1982) in which the M RNAs seemed to be partially deleted (Verkleij & Peters, 1983).

The lack of the 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 tumor 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 M RNA of TSWV seems to possess a mechanism that prevents the occurrence of large deletions in this molecule.

ACKNOWLEDMENTS

The authors, Renato de Oliveira Resende and Ántonio Carlos de Ávila are supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) / Brazil, respectively.

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CHAPTER 5 DEFECTIVE INTERFERING L RNA SEGMENTS OF TOMATO SPOTTED WILT VIRUS RETAIN BOTH VIRAL GENOME TERMINI AND HAVE EXTENSIVE INTERNAL DELETIONS

Renato de Oliveira Resende, Peter de Haan, Edwin van de Vossen, Antonio Carlos de Ávila, Rob Goldbach and Dick Peters

SUMMARY

Defective interfering (DI) RNA molecules derived from the genomic L RNA segment of tomato spotted wilt virus (TSWV) were generated during sequential passage of the virus at high multiplicity. Characterization of DI RNAs from four distinct isolates by Northern blot analysis and sequence determination revealed that both the 5' and 3' genomic termini were retained in these molecules. Each DI RNA contained a single internal deletion of approximately 60% to 80% of the L RNA segment. All DI RNAs studied maintain an open reading frame (ORF) which suggests that these defective molecules should be translatable by ribosomes. Detection of only defective molecules with ORFs indicates either that association to ribosomes or translation is a prerequisite for the selection and maintenance of replicating DI RNAs, or that the truncated proteins produced play a role in their selection or replication. Analysis of the junction sites in the DI RNAs showed that short nucleotide sequences are repeated, one at the release and another at the reinitiation points on the L RNA. One of these is lost during the generation of the DI molecules. A model for TSWV DI RNA generation is proposed in which the viral polymerase can "jump" across the internal sequences from a secondary structure to another containing the repeated sequences, during the replication of the L RNA segment.

This chapter has been published in a slightly modified version as: Resende *et al.* (1992). Defective interfering L RNA segments of tomato spotted wilt virus retain both genome termini and have extensive internal deletions. *Journal of General Virology* 73, 2509-2516.

INTRODUCTION

Tomato spotted wilt virus (TSWV), type species of the genus tospovirus within the Bunyaviridae family, has a genome consisting of three ssRNA segments denoted small (S) (2916 bases), medium (M) (4821 bases) and large (L) (8897 bases) (de Haan *et al.*, 1990, 1991; Kormelink *et al.*, 1992). The S RNA encodes the nucleocapsid (N) protein and a non-structural protein (NSs) in an ambisense gene arrangement (de Haan *et al.*, 1990). The M RNA is also ambisense encoding the membrane glycoproteins (G1 and G2) and another non-structural protein (NSm) (Kormelink *et al.*, 1992). The L RNA is of negative polarity and encodes the putative viral RNA polymerase (de Haan *et al.*, 1991).

Recently, it has been shown that TSWV supports the replication of defective interfering (DI) RNAs formed during serial passage in *Nicotiana rustica* cv America plants (Resende *et al.*, 1991). These DI RNAs are derived from the L RNA genomic segment and contain internal deletions which vary in length. TSWV DI RNA segments resemble those of orthomyxoviruses, which are derived exclusively from the RNA segments encoding the polymerase subunits (Lazzarini *et al.*, 1981; Holland, 1985; Nayak *et al.*, 1990).

In animal-infecting bunyaviruses, Germiston virus DI particles have been detected which contain a shorter RNA component originating from the L RNA segment (Cunninghan & Szilagyi, 1987). Defective L RNA segments have also been found in mosquito cells persistently infected with Bunyamwera virus (Scallan & Elliott, 1992). However, an interfering effect of these molecules has not been demonstrated. More recently, Patel and Elliott (1992) have reported DI L RNA molecules with conserved 5' and 3' termini which arose during attempts to isolate conditional lethal amber nonsense mutants of Bunyamwera virus.

The nucleotide sequences and mechanisms responsible for the generation of the different classes of DI RNA molecules have been studied in several animal virus systems. Several mechanisms, e.g. copy-back, copy-choice, rollover/loop-out mechanisms, have been proposed to be responsible for the generation of DI RNAs, although, for most of the viruses analyzed, the processes by which DI RNAs are formed are not completely

understood (Lazzarini *et al.*, 1981; Perrault, 1981; Holland, 1985; Nayak *et al.*, 1990). In case of plant viruses, e.g. brome mosaic virus, turnip crinkle virus, Cymbidium ring spot virus and tomato bushy stunt virus, the formation of potential double-stranded regions on the RNA, a replicase-driven copy choice mechanism with involvement of secondary structures in the RNA molecule has been proposed to explain the generation of DI RNAs and recombination between viral RNA molecules (Bujarski & Dzianott, 1991; Cascone *et al.*, 1990; Burgyan *et al.*, 1991; Knorr *et al.*, 1991).

To characterize TSWV DI molecules and to identify the nucleotide sequences that might be involved in this event, the recombination sites of the DI RNAs have been studied. In this report, the junction sites and flanking sequences of four different DI L RNAs formed in four different TSWV isolates are presented, and the possible mechanisms involved in the generation of these DI RNA molecules is discussed.

METHODS

Generation and maintenance of DI L RNAs.

Four tospoviruses were used in these study. Three of them, the Brazilian isolate (BR-01) from tomato and two Dutch isolates (NL-04 and NL-11) from chysanthemun and *Impatiens, respectively* belong to serogroup I (de Ávila *et al.*, 1990, 1992), and the fourth (SA-05) is a South African isolate from groundnut belonging to serogroup II. The isolates were serially passaged in *N. rustica* plants by mechanical inoculation for more than 20 generations. Plants were inoculated at 12 days intervals, using an inoculum prepared by grinding 1 g of systemically infected leaves in 5 ml of 0.01 M-phosphate buffer, pH 7.0, containing 0.01 M Na_2SO_3. The inoculated plants were placed in a glasshouse at approximately 22 °C in a light/dark regime of 16/8 h to allow symptom development.

RNA purification and Northern blot hybridization analysis

Nucleocapsid extracts were prepared from *N. rustica* plants infected with isolates containing DI L RNAs (Resende *et al.*, 1991). RNA was extracted from these nucleocapsid preparations and sample of 2 μgRNA were analyzed by electrophoresis in

1% agarose gels under denaturating conditions (Bailey & Davidson, 1976). After transfer to nitrocellulose membranes, the RNA segments were hybridized to ^{32}P-labelled DNA probes directed to the L RNA segment of TSWV isolate BR-01 (Maniatis *et al.*, 1982; de Haan *et al.*, 1991).

Amplification of DNA fragments by the polymerase chain reaction (PCR)

First-strand cDNA was synthesized on RNA segments isolated from nucleocapsid preparations of isolates containing DI molecules. These cDNAs were employed as substrate in the PCR with 4 μg/ml of oligonucleotides (primers) corresponding to the non-deleted regions of the DI L RNA segments. The oligonucleotides used were L1 (identical to nucleotides 1 to 17 of the viral complementary vc RNA strand and containing additional BamHI and XhoI restriction sites), L29 (complementary to nucleotides 876 to 894 of the vc RNA strand), L33 (complementary to nucleotides 6531 to 6548 of the vc RNA strand) and L39 (complementary to nucleotides 8378 to 8396 of the vc RNA strand) (Fig. 1). One microlitre of the RNA sample was first reverse-transcribed for 1 h at 37 °C using 100 units (U) Moloney murine leukaemia virus reverse transcriptase (Gibco-BRL) and subsequently amplified in a final volume of 100 μl containing 2.5 U *Taq* polymerase (Promega) for 30 amplification cycles (1 min denaturation at 94°C, 1.5 min annealing at 54°C and 2 min extension at 72°C). The amplified DNA fragments were analyzed on a 1% agarose gel.

Cloning of the amplified DNA fragments and nucleotide sequencing

The amplified fragments were precipitated with ethanol and resuspended in TE buffer (10 mM TrisHCl, 1 mM EDTA, pH 7.0). The purified DNA fragments were ligated into a T-Vector (Bluescript SK+; Strategene) prepared according to the method of Marchuk *et al.* (1991) using a fragment/T-Vector DNA ratio of 500 ng/100 μg. The nucleotide sequences were determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). Nucleotide and amino acid sequences, and secondary structures were compiled and analyzed using the programs developed by the University of Wisconsin Genetics Computer Group (UWGCG).

RESULTS

Mapping of the deletions of the DI L RNAs

As reported previously, several TSWV isolates have been obtained which contain stable, co-replicating DI RNA molecules. Furthermore, generation of these segments has often been shown to be associated with an attenuation of symptoms on infected plants (Resende *et al.*, 1991).

Nucleocapsid RNA from four of these isolates was purified and analyzed on an agarose gel (Fig. 1*b*). The results show that in addition to the genomic RNA segments (as previously shown by Resende *et al.*, 1991), each isolate contained an L RNA segment-derived DI RNA species which differed in size (Fig. 1*c*, *d* and *e*). These DI RNAs, once established, remained stable in length in subsequent mechanical transfers to plants (data not shown). To map the deleted regions of these DI L RNA segments, three cDNA clones (669, 806 and 70; Fig. 1) copied from full-length TSWV L RNA (BR-01 isolate; de Haan *et al.*, 1991), were labelled with ^{32}P and used in Northern blot hybridization assays (Fig. 1*c*, *d* and *e*).

The hybrization patterns obtained using different cDNA probes indicated that the DI RNAs retain the original L RNA termini, but contain an extensive internal deletion, of approximately 5.4 to 7.0 kb. The presence of these termini (identical as those reported by de Haan *et al.*, 1991) in the DI molecules was confirmed by sequencing of the full-lenght BR-01 DI RNA obtained by PCR using L1 and L63 (complementary to the nucleotides 8881 to 8897 of the vc RNA strand) as primers (data not shown). Hybridization with probe pTSWV-806 showed that the coding region in the L RNA encompassing the conserved polymerase motifs (de Haan *et al.*, 1991) had been deleted from all DI RNA molecules analyzed. This finding indicates that these DI RNA molecules could not encode a functional polymerase protein.

PCR analysis of the defective interfering L RNAs

Based on the hybridization patterns of the DI RNA segments, specific synthetic oligonucleotides were produced to determine, using PCR, more precisely the regions deleted in these defective molecules (Fig. 1*a*). First strand DNA segments were

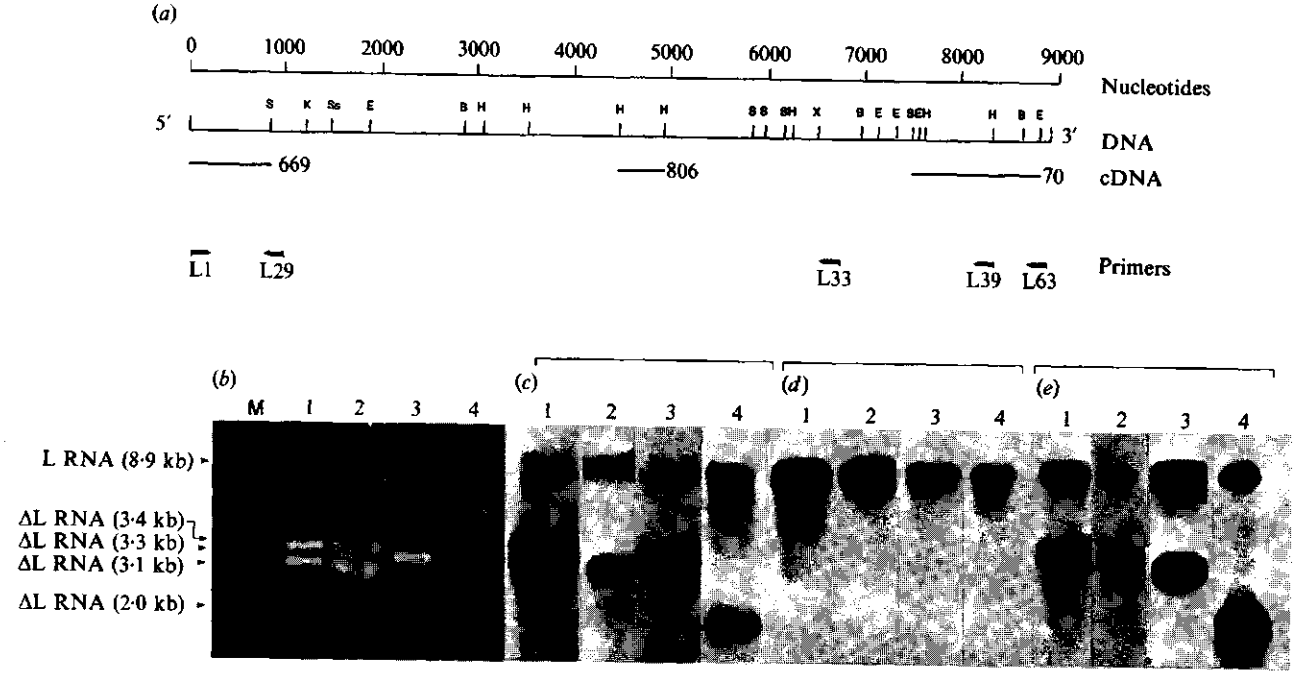

Fig. 1. Mapping of the deletions in the TSWV DI L RNAs. (a) Location of cDNA clones and oligonucleotide primers in the TSWV genome. (b) to (e) Analysis of the RNA of the isolates NL-04, NL-11, BR-01 and SA-05 (b, lanes 1 to 4), and Northern hybridization of these RNAs using three ^{32}P-labelled cDNA probes pTSWV-669 (c), pTSWV-806 (d) and pTSWV-70 (e) specific for the L RNA. The exact location of the primers is given in Methods. Lane M, molecular size markers.

synthesized from viral RNA containing the DI molecules using oligonucleotide L1 (5' end of vc RNA strand) as the primer. For isolates BR-01, NL-04 and NL-11, the PCR reaction was performed using primers L1 and L33 (internal primer), producing DNA fragments with the expected sizes of 750, 1100 and 900 nucleotides, respectively (Fig. 2).

To analyze the defective segments of isolate SA-05, which contained the shortest DI L RNA found, primers L1 and L39 (internal primer) were used to synthesize a predicted DNA fragment of approximately 1600 bases.

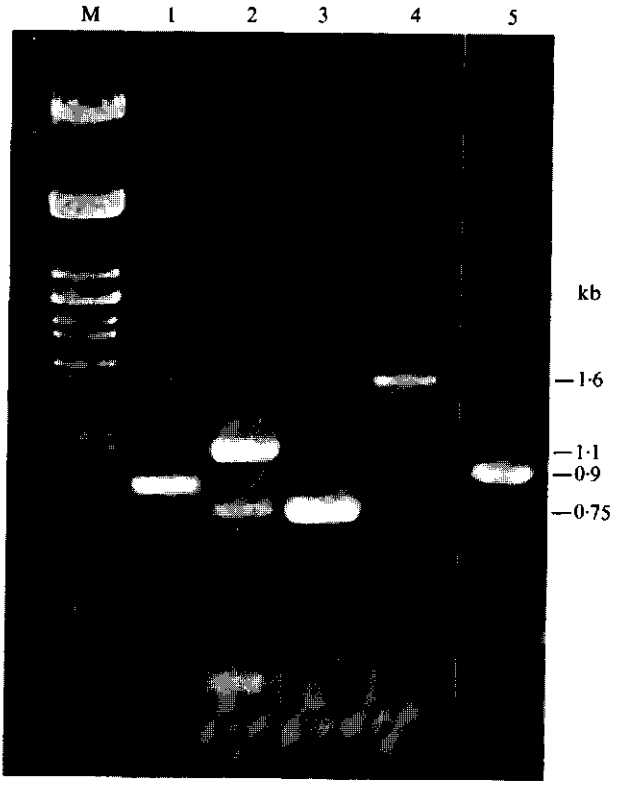

Fig. 2. PCR analysis of the TSWV DI L RNAs. The PCR was performed as described in Methods using oligonucleotides L1 and L33 (Fig. 1) as primers to amplify DI segments of isolates NL-04 (lane 2), BR-01 (lane 3) and NL-11 (lane 5), and oligonucleotides L1 and L39 (Fig. 1) for DI RNA of SA-05 (lane 4). Primers L1 and L29 were used to amplify a BR-01 wilt type L RNA segment as a control (lane 1). The positions of the predicted PCR fragments are shown. Lane M, molecular size markers.

In addition to the desired fragment of isolate NL-04, smaller DNA fragments were also obtained, most likely as a result of non-specific annealing of the primers. The origin of these smaller fragments was not characterized further. All the PCR fragments obtained spanned the deleted regions of the DI L RNA molecules. The size of the PCR fragments also revealed that DI RNAs of isolates BR-01 and NL-11 had longer 3'-terminal regions than those of isolates NL-04 and SA-05. In contrast, the latter two had longer regions at the 5' end than those from BR-01 and NL-11.

Determination of the nucleotide sequences of the junction sites in DI L RNAs

The amplified PCR fragments were cloned in Bluescript T-Vectors and their nucleotide sequences were determined. For each DI RNA, two independent clones were sequenced, and the sequences obtained were aligned to the standard L RNA of wild type isolate BR-01. When compared with this RNA, the sequence and hybridization data showed that all the DI molecules analyzed, as read from the complementary strand, had a shorter 5'-terminal region (including the complete 34 base non-coding sequence and

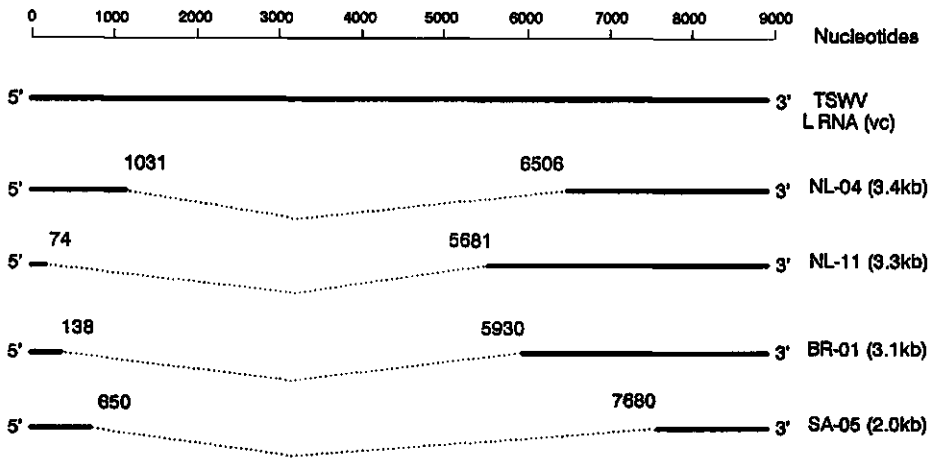

Fig. 3. Schematic representation of the 5'-3' type DI L RNAs of TSWV. The genomic L RNA is presented at the top. The full lines in the DI molecules represent the sequences retained and the dotted lines show the deleted regions. The numbers correspond to the nucleotides present at the junction sites and the size of each DI RNA is indicated.

the translation initiation codon of the L protein gene) and a longer at the 3'-terminal region (Fig. 3). This observation indicates a higher selection pressure for the retention of this region and therefore it may contain sequences essential to select and maintain the DI segments generated. Analysis of the nucleotide sequences of the various DI segments revealed that the position of the junction sites varies and that deletion of internal sequences occurs at random. The recombination sites of the BR-01 DI RNA were

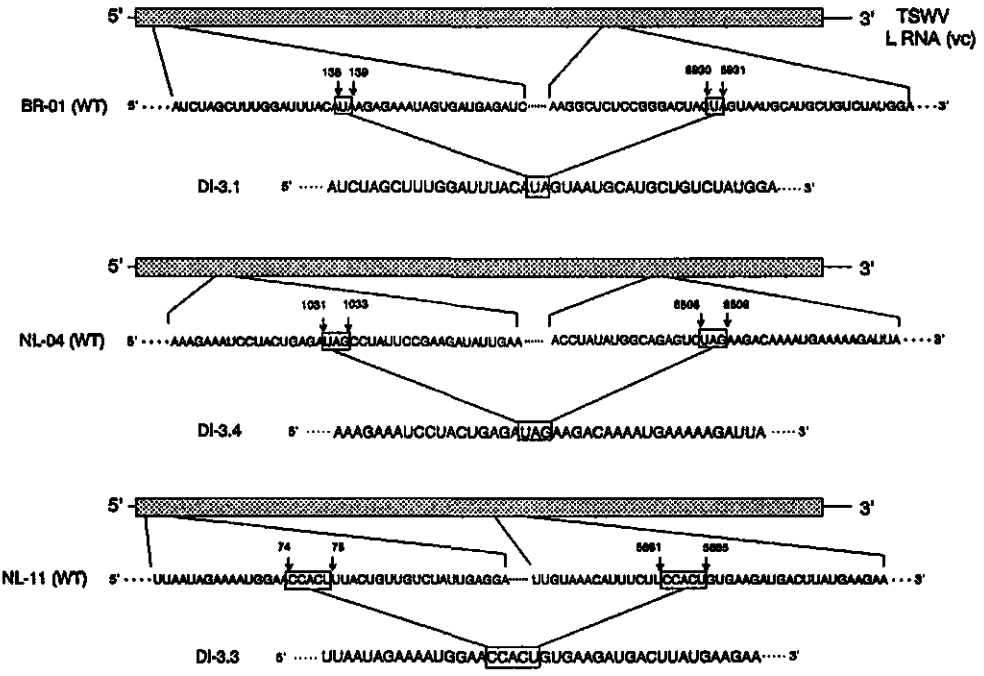

Fig. 4. Junction sites of the TSWV DI L RNAs with the flanking sequences. The location of these flanking sequences in the vc L RNA segment of the BR-01 TSWV isolate (shaded box) is indicated. The repeated nucleotide sequences at the junction sites are boxed.

located at positions 138 and 5930 in the L RNA, yielding a defective molecule of 3105 nucleotides long (Fig. 4). The dinucleotide UA was present on either side of the junction

sequences in the template RNA and those were considered to be release and reinitiation points. One of these UA sequences is lost during the generation of this defective molecule.

The nucleotide sequences of both NL-04 and NL-11 DI RNA showed that these isolates are very similar, if not identical, to the corresponding regions in the L RNA of isolate BR-01, thus allowing a precise determination of the junction sites (similar identity between the nucleotide sequences of the S RNA segments of two TSWV isolates belonging to serogroup I has also been found (de Haan *et al.*, 1990; Maiss *et al.*, 1991).

The recombination sites of NL-04 are located at positions 1031 and 6506, resulting in an DI RNA segment of 3424 nucleotides long. In this molecule, a UAG triplet sequence was present at the release and reinitiation sites. As in the DI L RNA of BR-01, one of these triplets has been lost during the generation of the DI molecules of NL-04. The DI segments of isolate NL-11 contain only a short portion of the 5'-terminal sequence. Since the recombination sites were located at nucleotide 74 and 5681, the segment generated was 3290 nucleotides long. In this isolate a repeated pentanucleotide sequence, CCACU, was found at the junction sites, one of which was also lost during the generation of the DI RNA molecule.

It was not possible to determine precisely the position of the junction site in the 2.0 kb DI L RNA of isolate SA-05, because the partially sequenced SA-05 DI L RNA showed approximately 75% nucleotide sequence identity with the L RNA of isolate BR-01. Based on differences in nucleotide sequence identity, this isolate has been proposed as a novel species within the genus tospovirus (de Ávila *et al.*, 1992). Nevertheless, alignment of the sequenced regions of this DI molecule with the corresponding parts of TSWV L RNA allowed the positions of the recombination sites to be estimated as being either at or around nucleotide positions 650 and 7680 (Fig. 3).

Maintenance of open reading frames (ORFs) in the DI L RNAs

Determination of nucleotide sequences surrounding the junction sites showed that all TSWV DI molecules characterized seem to posses an ORF, which may encode proteins considerably smaller than the original polymerase protein (Fig. 5). The ORF of isolate NL-04 starts at the same position (the AUG start codon at position 34) as that

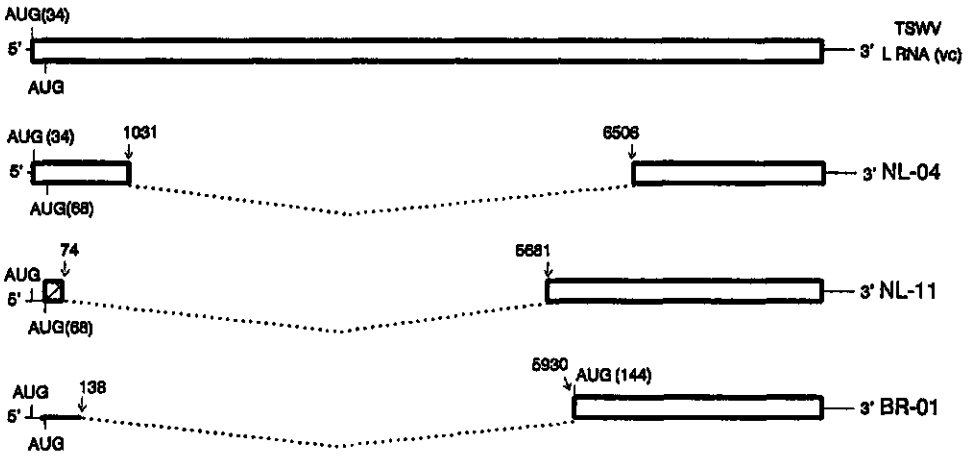

Fig. 5. Schematic representation of the ORFs present in the TSWV DI L RNAs. The wild type ORF is presented at the top. The open boxes in the three DI RNAs indicate encoded amino acid sequences identical to those in the L protein and the shaded box the novel sequences encoded by the ORF generated by the NL-11 DI RNA. The numbers with an arrow indicate the position of the nucleotides at the junction sites; the position of the AUG start codon of each ORF is given in parentheses.

of the wild type L RNA and continues in the same frame after the junction site. In this case, the ORF is 3184 bases long, corresponding to a primary translation product of approximately 120 kD, and thus has the same N and C termini as the functional polymerase protein. The DI molecules of isolate BR-01 and NL-11 contain the original start codon of the L protein, but the ORFs terminate immediately downstream from the junction sites in both DI RNAs and therefore can encode only small polypeptides. However, both DI RNAs contain a large ORF. The ORF of the DI RNA of isolate NL-11 starts at position 68 in a wild type no-sense reading frame but continues in the original frame after the junction site, encoding a putative protein of approximately 100 kD. The ORF in the DI RNA of isolate BR-01 starts immediately after the junction site at an AUG triplet located at position 144 (Fig. 5), and its predicted protein has an M_r of 90 kD. All DI RNA-encoded proteins have the same C-terminal sequence, corresponding to the last 717 amino acids of the viral polymerase.

DISCUSSION

Analysis of four naturally generated TSWV DI L RNAs by hybridization studies, PCR cloning and sequence determination revealed that these defective molecules belong to the 5'-3' class of DI RNAs (Lazzarini *et al.*, 1981; Holland, 1985). They retain both genomic termini and have one extensive internal deletion of approximately 5.4 (60%) to 7.0 (80%) kb of the standard genomic L RNA. Stable DI RNAs shorter than 2.0 kb have not been identified. The existence of DI RNAs exclusively larger than 2.0 kb suggests that extensive regions of both genomic terminal are required to maintain these RNA molecules during their subsequent replication. DI RNA species of the 5'-3' type containing large deletions have commonly been found with influenza and Sendai viruses (Amesse *et al.*, 1982; Re *et al.*, 1985; Nayak *et al.*, 1990). In the case of influenza virus, DI molecules measuring less than 100 nucleotides have been detected and characterized. These DI RNAs originated from the polymerase (PB2)-encoding RNA segment, of which up to 84% is deleted (Sivasubramanian & Nayak, 1983; Jennings *et al.*, 1983; Nayak *et al.*, 1990).

The presence of the 5' and 3' ends with the complete non-coding regions of the wild type L RNA implies that DI molecules of TSWV possess the essential signals for virus replication, transcription and possibly those for other unknown functions. The presence of these signals suggests that interference and selective pressure could act at different stages of virus replication.

The existence of an ORF in all DI segments analyzed may indicate that they encode a protein. These ORFs, which start with initiation codons conform to Kozak's rules (Kozak, 1978), encode proteins which have the original amino acid sequences in the C-terminal region of the polymerase protein. Formation of these proteins may be a prerequisite for the survival of the DI L RNAs. If the scanning model for translation (Kozak, 1989) holds for TSWV RNA translation, the strong AUG codons in the ORFs of the DI molecules may compete efficiently with the normal start codon in the wild-type L RNA. Alternatively, the synthesis of TSWV DI L RNA-specific protein may affect the formation of functional polymerase and, owing to their common C-terminal sequences, may consequently inhibit the replication and transcription of the wild type DI L RNA

protein. Patel and Elliott (1992) detected novel polypeptides in mouse L cells infected with Bunyawera virus preparations containing DI particles. This observation suggests that these polypeptides may be encoded by the DI L RNAs generated. Also, defective influenza virus PB2 segments encoding truncated polymerase subunits have been observed (Nayak *et al.*, 1990). These polypeptides could not be detected in virus particles, but appear to be associated with nucleocapsid complexes. Nayak *et al.* (1990) have proposed that these DI RNA-encoded proteins possibly interfere with virus assembly by selectively inhibiting the incorporation of wild type RNA segments into the virion. Interference of TSWV DI L proteins with the virus assembly, therefore suppressing the yield of standard enveloped virus particles, can not be excluded.

The almost identical nucleotide sequences of TSWV isolates belonging to serogroup I (de Ávila *et al.*, 1990, 1992) allowed us to determine precisely the junction sites of various DI RNAs. Analysis of the nucleotide sequences surrounding the recombination sites of the TSWV DI L RNAs revealed the involvement of identical sequences UA, UAG and CCACU at the release and reinitiation points of BR-01, NL-04 and NL-11 DI RNAs, respectively. The occurrence of such junction sites is a remarkable feature of TSWV DI L RNAs as they have not been described for most of other DI systems in either positive-strand or negative-strand RNA strand viruses (Lazzarini *et al.*, 1981; Holland, 1985; Re *et al.*, 1984; Romanova *et al.*, 1986; Morris & Knorr, 1990; Nayak *et al.*, 1990). Meier *et al.* (1984) have proposed the involvement of repeated sequences in the generation of internally deleted Sendai virus DI genomes; however, in this case, these sequences were located not at the junction sites but in the region flanking the recombination sites. In the TBSV, the presence of a hexanucleotide motif at or near the endpoints of two conserved regions of DI RNAs has also been observed (Knorr *et al.*, 1991).

The different repeated sequences found in TSWV DI RNAs do not support the existence of general consensus sequences at which recombination occurs. They neither resemble the consensus splice sequences of eukaryotic mRNAs, nor the GAA and CAA splice sequences observed for influenza virus mRNAs (Nayak *et al.*, 1985; Jennings *et al.*, 1983). Also, significant similarities with the sequence motifs proposed to be involved in the generation of DI molecules and viral RNA recombinants in turnip crinkle virus have

not been found (Cascone *et al.*, 1990). Nevertheless, repeated sequences as UA, UAG and CCACU at the junction sites in the template RNA may enable the polymerase to jump, from one site to another on the L RNA molecule during virus replication. This hypothesis suggests the involvement of the copy-choice mechanism in the generation of TSWV DI RNAs. This mechanism, in which the polymerase has to detach completely from a minus- or plus-strand RNA and reinitiate synthesis at a specific signal on the same or a different molecule has been proposed to explain the generation of viral RNA recombinants and DI molecules in different systems (Lazzarini *et al.*, 1981; Perrault, 1981; Re *et al.*, 1985; Cascone *et al.*, 1990).

Since more than two of these short identical nucleotide sequences are present in the L RNA molecule, it is unlikely that the ones used specify either detachment from or reattachment to the template RNA. It is likely that, in addition to the repeated sequences, secondary structure(s) in the L RNA or even some higher order structures in the RNA-nucleocapsid protein complexes is responsible for the jump of the polymerase. This hypothesis is supported by the observation that stable computer-predicted secondary structures surrounding the deleted regions at the 5' end of genomic L RNA can be formed (Fig. 6). These possible secondary structures consist of hairpins with the repeated sequences located in the end loop. The repeated sequences at the release sites were found in umparied sequences occurring in the stems. These conformations, together with high order structures, may expose the nucleotides at these recombination sites allowing the polymerase to jump. This mechanism also explains why only DI RNAs of the 5'-3' type seem to be generated. Similar predicted secondary structures have also been observed in Cymbidium ring spot virus, in which the sequences surrounding the recombination sites formed extensive and stables structures in the interior or hairpin loops (Burgyan *et al.*, 1991). The involvement of secondary structures in the generation of DI and/or viral recombinant molecules has also been suggested for vesicular stomatitis virus (Yang & Lazzarini, 1983; Meier *et al.*, 1984), poliovirus (Kirkegaard & Baltimore, 1986; Kuge *et al.*, 1986) and mouse hepatitis virus (Baric *et al.*, 1987; Makino *et al.*, 1988). No sequence homology between different recombination sites of these viruses have been found.

As observed for all TSWV DI RNAs characterized, one of the repeated sequences

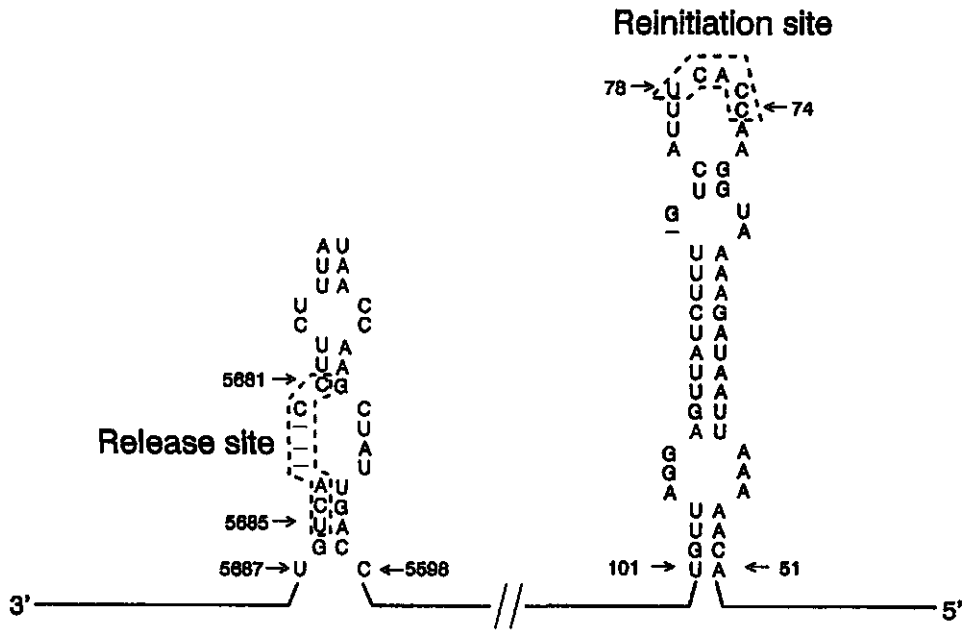

Fig. 6. The secondary structure at the junction sites of DI L RNA of isolate NL-11. These structures were predicted by computer analysis using up to 500 nucleotides flanking the release (nucleotides 5281 to 5785 and reinitiation (nucleotides 1 to 478) sites on the L RNA fused into a segment. The repeated pentanucleotides of the release and reinitiation sites are boxed. The nucleotide positions are indicated in the vc L RNA.

present at the release and reinitiation sites is lost during these polymerase error events. This observation may be explained by the annealing of the anti-sequences present at the release sites to the second set of repeated nucleotides, after which the replication is reinitiated and by which the second sequence is lost. In recombinant RNA molecules of brome mosaic virus, the formation of heteroduplex structures at both sites of the junctions suggest that, as in poliovirus, local hybridizations, rather than primary sequences, may assist the RNA polymerase to switch among the RNA templates (Bujarski & Dzianott, 1991; Romanova *et al.*, 1986).

The understanding of the mechanisms of generation of the defective molecules described in this paper, as well as their involvement in the attenuation of symptom expression during virus infection, may shed light on the replication process of tospoviruses and thereby of bunyaviruses in general. The replicative advantages of these

DI RNAs will allow us to investigate the virus infection process and to study the minimal genome information required for virus replication, encapsidation and packaging (Resende *et al.*, 1991). Furthermore, symptom attenuation effects of the DI RNAs may provide new ways to protect host plants against the devastating effects of TSWV infection.

ACKNOWLEDMENTS

Renato de Oliveira Resende and Antonio Carlos de Ávila are supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA)/Brazil, respectively.

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CHAPTER 6 POINT MUTATIONS IN THE G2/G1 GENE OF THE M RNA SEGMENT GENERATE ENVELOPE-DEFICIENT ISOLATES OF TSWV AND INSV

Renato de Oliveira Resende, Richard Kormelink, Antonio Carlos de Ávila, Rob Goldbach and Dick Peters

SUMMARY

Upon serial mechanical transmission tospovirus isolates tend to loose the capacity to produce enveloped particles. To investigate the genetic nature of this defect, partial nucleotide sequences of the M RNA segments of several envelope-deficient and wild type tospovirus isolates were compared. These sequence comparisons revealed that accumulation of point mutations in the gene coding for the common precursor to both glycoproteins G2 and G1 (G2/G1 gene) may be the causal event leading to the morphological defect. Comparison with a non-defective isolate (NL-07) of *Impatiens* necrotic spot virus (INSV) showed that the envelope-deficient isolate US-01 of INSV acquired two nucleotide insertions in the M RNA, causing early frame-shifts in the G2/G1 gene and consequently in the loss of the putative signal sequence in the precursor protein. In isolate NL-04 of TSWV the defect appeared to be caused by multiple point mutations in the gene, while no distortion of the reading frame was detected in the region sequenced. Several point mutations were found clustered in the sequences neighboring a U-rich stretch not far downstream of the translational start codon of the G2/G1 gene. A frame-shift mutation in isolate BR-01 of TSWV also mapped in this stretch, suggests that this region is a mutational hot spot.

This Chapter has been submitted for publication in a slightly modified version as: Resende *et al.* 1993. Accumulation of point mutations in the G2/G1 gene of envelope-deficient tospovirus isolates. *Journal of General Virology.*

INTRODUCTION

Tospoviruses like tomato spotted wilt virus (TSWV), produce during infection numerous enveloped virions, which accumulate in the cisternae of the endoplasmic reticulum system (Ie, 1971, Kitajima *et al.*, 1992a). Some TSWV isolates, when serially transmitted by mechanical inoculation to tobacco plants loose their capacity to produce the characteristic particles and generate only electron-dense aggregates, embedded in the viroplasm, located in the cytoplasm of infected cells (Ie, 1982). These mechanically transmitted isolates are considered to represent morphologically defective forms of TSWV, lacking the lipid envelope though still being infectious (Ie, 1982). Further analysis revealed that these morphologically defective isolates failed to produce detectable amounts of the envelope glycoproteins G1 and G2 (Verkleij & Peters, 1983), while the dense aggregates in infected cells turned out to represent aggregates of non-enveloped nucleocapsids (Kitajima *et al.*, 1992a, b).

Recently, a morphologically defective isolate was obtained after serially mechanical passages of a Dutch isolate, NL-04, over *Nicotiana benthamiana*, which also failed to produce both envelope glycoproteins (Resende *et al.*, 1991). Northern analysis revealed that the M RNA, encoding both glycoproteins (Kormelink *et al.*, 1992), did not acquire deletions of detectable size (Resende *et al.*, 1991). It was, therefore, concluded that the defectiveness was caused either by small deletions or single point mutations in the gene encoding the common precursor to both glycoproteins.

Since the nucleotide sequence of the M RNA of two tospoviruses, i.e. TSWV and *Impatiens* necrotic spot virus (INSV), has been elucidated (Kormelink *et al.*, 1992; Law *et al.*, 1992), the genetic nature of the morphological defectiveness of the envelope-deficient isolates can now be identified by comparing their M RNA sequences with those of wild type isolates. To this end, it was decided to determine and compare two different, but distinct, regions in the M RNA of both the wild type and the morphological defective NL-04 isolate, which could be diagnostic for the genetic instability of the M RNA during mechanical transmission.

The M RNA segment encodes, in ambisense arrangement, both the common (127 kD) precursor to both glycoproteins (denoted G2/G1) and a 32 kD non-structural

protein (NSm). To follow the potential acquisition of point mutations in the G2/G1 gene, the part encoding the N-terminal region of the G2/G1 precursor was determined. Since it was anticipated that NSm was not involved in the envelope-deficiency an equal-sized part (approximately 500 nucleotides) of the NSm gene was sequenced as control. These regions were compared in two combinations of a wild type and a envelope-deficient tospovirus isolate, i.e. TSWV isolate NL-04 and its defective derivative, and INSV isolate NL-07 (wild type) and the American isolate originally referred to as I-type (Law & Moyer, 1990; Law *et al.*, 1992), and here denoted INSV isolate US-01, which has lost its capacity to form envelopes to a large extend (Vali & Gildow, 1992; Urban *et al.*, 1991). Furthermore also a serially passaged line of TSWV isolate BR-01, which only produced a limited number of enveloped particles, was included in this study.

METHODS

Generation and maintenance of envelope-deficient isolates of TSWV

Two tospoviruses isolates, the Dutch TSWV isolate NL-04 from chrysanthemum (de Ávila *et al.*, 1990) and the Dutch INSV isolate NL-07 from *Impatiens* (de Ávila *et al.*, 1992) were used in this study. The original isolates were maintained in liquid nitrogen and inoculated to *N. rustica* and *N. benthamiana* plants, respectively, when necessary to prepare purified virus samples. A morphologically defective form of NL-04 isolate was generated by serial passage of this isolate at 12 day intervals onto *N. benthamiana*, using systemically infected leaves as inoculum source (Resende *et al.*, 1991).

Amplification of DNA fragments by polymerase chain reaction (PCR)

Nucleocapsid RNA from original and envelope-deficient isolates was used for cDNA synthesis. First-strand cDNA to the N-terminal part of the glycoprotein precursor gene was synthesized using oligonucleotide ZUP035 (dGAATATATGACACCATTG), identical to nucleotides 4307 to 4324 and nucleotides 4445 to 4462 of the TSWV (BR-01) and INSV (US-01) M RNA, respectively (Fig. 1). Subsequently, the DNA segments were amplified in PCR reactions using oligonucleotide M1 (dGAGCAATCAGTGCAAA), complementary to the 3' end of both M RNAs, as second primer. Similarly, part of the

NSm gene was synthesized and PCR amplified using oligonucleotides ZUP082 (dGCAGAAAACAATACATGATG), complementary to nucleotides 468 to 487 of the TSWV M RNA as first primer and oligonucleotide ZUP013 (dCCCTGCAGGATCC AGAGCAATCAGTGCA) identical to nucleotides 1 to 15 of the M RNA and containing additional BamHI and PstI sites). First strand cDNA was performed according to Gubler & Hoffman (1983) employing 1 µlof the RNA samples, and PCR amplification was performed using Super Taq polymerase (Sphaero Q) according to the manufacturer for 30 cycles (1 min denaturation at 94 °C, 1.5 min annealing at 54 °C and 1 min extension at 72 °C). The amplified DNA fragments were analyzed on 1% agarose gel.

Cloning and nucleotide sequence analysis of the amplified DNA fragments

The amplified fragments were cloned into a T-Vector (Bluescript SK+; Stratagene) prepared according to the method of Marchuk *et al.* (1991) DNA sequencing was performed by the dideoxynucleotide chain termination method (Sanger *et al.* 1977). Nucleotide and amino acid sequences were compiled and analyzed using the programs developed by the University of Wisconsin Genetics Computer Group (UWGCG).

RESULTS

Cloning and analysis of M RNA sequences

An envelope-deficient TSWV NL-04 isolate was generated after serial passages in *N. benthamiana* (Resende *et al.*, 1991). ELISA experiments using monoclonal antibodies (MAbs) revealed that the defective isolate reacted strongly with MAbs directed against the nucleocapsid protein, but weakly with only one of four MAbs directed to the membrane glycoproteins, demonstrating that this isolate produced hardly or no glycoproteins (Fig. 3, Chapter 4). Large deletions did not occur in the M RNA of this isolate, as Northern blot analysis showed that the M RNA of this non-enveloped isolate had the same mobility as that of the parental virus (Fig. 7, Chapter 4). Hence, envelope-deficient mutants seems to arise by either single point mutations or small deletions in the M RNA segment.

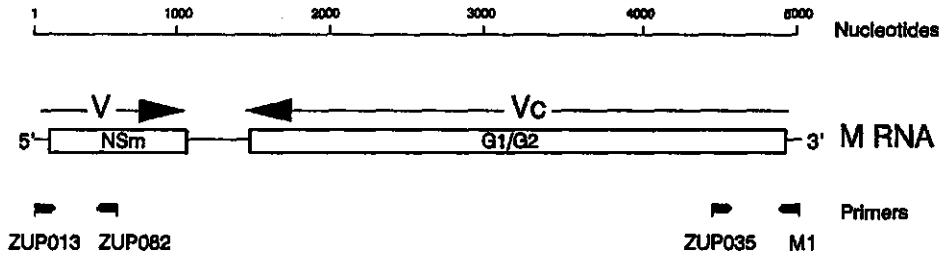

Fig. 1. Schematic representation of the M RNA structure of TSWV and location of oligonucleotides used as primers in PCR reactions. The genes for NSm and the G2/G1 precursor are in an ambisense arrangement located in the viral (V) and viral complementary (Vc) strand, respectively.

To investigate the genetic changes in such defective isolates specific regions of their M RNA, e.g. G2/G1 gene where most likely the defects were expected, were cloned and analyzed. First-strand cDNA to the G2/G1 ORFs of the tospovirus isolates studied was synthesized using oligonucleotide ZUP035 as primer (Fig. 1). The cDNA was subsequently PCR-amplified using the ZUP035 and primer M1 (complementary to the 3' end of vRNA strand). The resulting DNA fragments had an expected size of approximately 515 nucleotides for TSWV isolate NL-04 and its envelope-deficient derivative, and of approximately 528 nucleotides for INSV isolate NL-07 (Fig. 2). To analyze the NSm ORFs, the internal primer ZUP082 was used for first-strand synthesis. Subsequently, PCR was performed using this primer in combination with primer ZUP013 (corresponding with the 5' end of vRNA strand) to synthesize fragments of 487 nucleotides for isolate NL-04 (and its enveloped-deficient derivative) and 475 for INSV isolate NL-07 (Fig. 2).

The amplified PCR fragments were cloned in Bluescript T-Vectors and their nucleotide sequences were determined. For each isolate and each region compared, three different clones obtained from two independent PCR reactions were sequenced, and the sequences obtained aligned to the published M RNA nucleotide sequences.

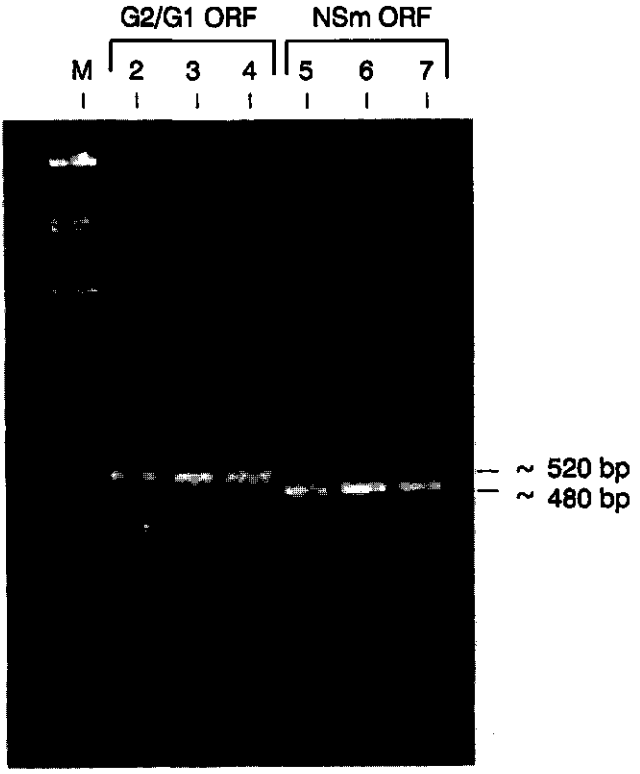

Fig. 2. PCR analysis of the M RNA ORFs. The PCR was performed as described in Methods, using oligonucleotides ZUP035 and M1 (Fig. 1) as primers to amplify part of the G2/G1 ORF of isolates NL-04 wild type (lane 2), NL-04 envelope-deficient (lane 3) and NL-07 (lane 4). For amplifying sequences of the NSm ORF the oligonucleotides ZUP082 and ZUP013 (Fig. 1) were used in the PCR of NL-04 wild type (lane 5), NL-04 envelope-deficient (lane 6) and NL-07 (lane 7). The approximated positions of the predicted PCR fragments are shown. Lane M, molecular size markers.

Accumulation of point mutations in the envelope-deficient NL-04 isolate

Nucleotide sequence determination of the 515 bases long PCR fragment corresponding to the 5' part of the G2/G1 ORF in the M RNA of NL-04 showed that this sequence is almost identical to that of isolate BR-01 (Kormelink *et al.*, 1992), containing only two silent mutations at position 4441 and 4732 (data not shown). Comparison of the sequenced region of the envelope-deficient isolate of NL-04 demonstrated that its G2/G1 ORF initiates at the same AUG start codon (position 4747)

as the wild-type NL-04 (Fig. 3). However, when downstrean sequences in the glycoprotein precursor gene of the envelope-deficient isolate were analyzed, seven point mutations, not present in the original wild type NL-04, were detected (Fig. 4). Remarkably, four of these point mutations were clustered in a stretch of 15 nucleotides neighboring a uracil-rich region between positions 4435 and 4450. Two of these nucleotide changes caused amino acid substitutions, i.e. the U to C replacement at position 4440 corresponding to the substitution of cysteine by arginine, and the C to U replacement at position 4449 causing a change of alanine into threonine. The other two nucleotide substitutions, at positions 4435 (C replaced by U) and 4450 (A replaced by G) were silent point mutations. Additional silent mutations in the envelope-deficient isolate were observed at positions 4354 (G was replaced by A), 4384 (A was replaced by G) and 4399 (an A was replaced by a G).

From this 515 nucleotide-long region sequenced, 431 nucleotides were located in the G2/G1 ORF of NL-04. Hence, this part of the G2/G1 ORF accumulated seven point mutations during serial passages, corresponding to a mutation frequency of 1.60 %. Sequence analysis of a similar size stretch (487 nucleotides) at the 5'-terminal region of the M RNA, encompassing part of the NSm ORF (386 nucleotides), demonstrated that this sequence acquired 4 point mutations, including 2 within the NSm ORF (mutation frequency of NSm ORF 0.51%) which were silent (data not shown). Although stop codons or frame-shift mutations were not detected in the sequenced part of the G2/G1 ORF, the occurrence of two non-conserved amino acid substitution (cysteine to arginine; alanine to threonine) indicate that the G2/G1 ORF has undergone major genetic changes, probably leading to blocking of production of functional glycoproteins.

Analysis of the M RNA of TSWV isolate BR-01

For the second isolate of TSWV studied, the Brazilian isolate BR-01, completely envelope-deficient lines have not been produced. However, it was observed that also this isolate produced decreasing numbers of enveloped particles, and increasing amounts of free nucleocapsids, upon serial mechanical passages. It was thus expected that this isolate would consist of a heterologous population of M RNAs containing either unaltered or mutated G2/G1 ORFs. To verify this, different cDNA clones of isolate BR-01 M RNA

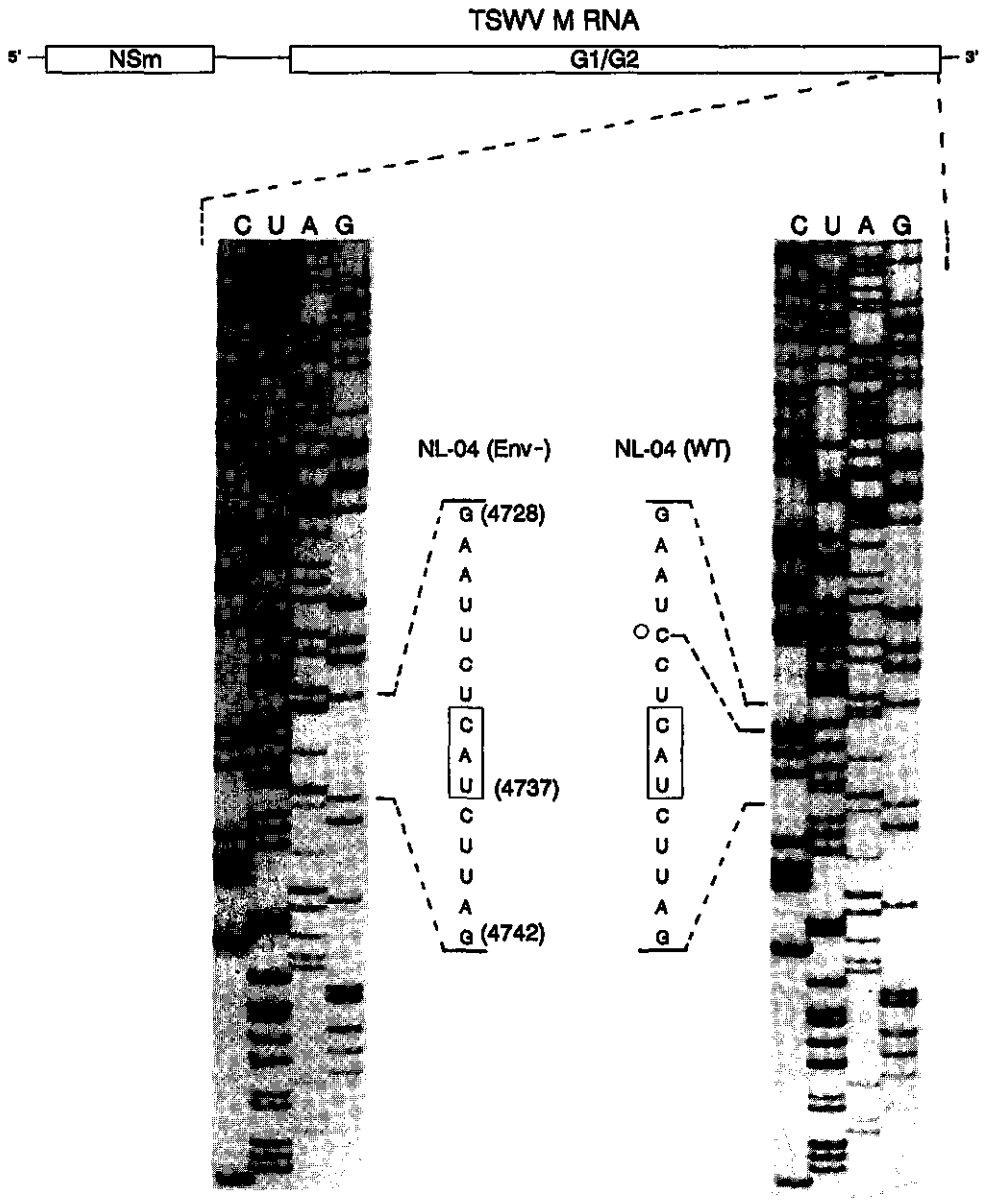

Fig. 3. Nucleotide sequence comparison of the start codon positions of the G2/G1 ORFs of the wild type and envelope-deficient isolates of NL-04. The wild type ORF is presented at the top. The nucleotide positions correspond to the published M RNA sequence of TSWV (Kormelink *et al.*, 1992). The single differences between in NL-04 wild type and BR-01 are indicated.

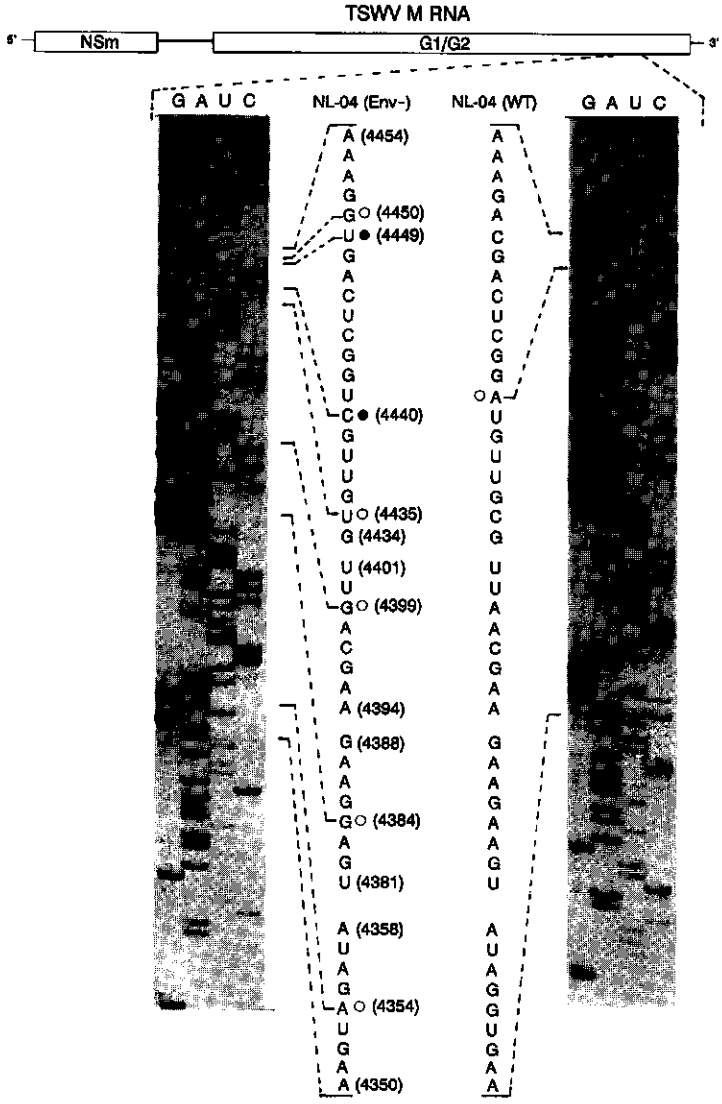

Fig. 4. Nucleotide sequence comparison of the G2/G1 ORFs of NL-04 wild type and envelope-deficient isolates of TSWV. The wild type ORF is presented at the top. The nucleotide positions indicated correspond to the published M RNA sequence of TSWV (BR-01 isolate) (Kormelink *et al.*, 1992). The point mutations in the envelope-deficient isolate are presented, open circles corresponding to silent mutations and closed circles with point mutation causing amino acid changes. The single differences between in NL-04 wild type and BR-01 are indicated.

encompassing the G2/G1 ORF were analyzed. Indeed a cDNA clone was identified, containing an insertion of an extra U at position 4438, in the same uracil-rich region in which most point mutations were acquired in the defective NL-04 isolate. This insertion caused a frame shift in the G2-G1 ORF (data not shown), and consequently, a premature termination of the precursor protein.

Characterization of the morphological defectiveness of INSV

Comparison of the published sequence of an American isolate of INSV (Law *et al.*, 1992) with that of TSWV (Kormelink *et al.*, 1992) revealed the absence of a signal peptide sequence at the start of the G2/G1 ORF in the INSV sequence (Fig. 5). Since the American isolate (here denoted isolate INSV-US-01) apparently is almost incapable to produce enveloped particles (Law & Moyer, 1990; Urban *et al.*, 1991; Vali & Gildow, 1992; Law *et al.*, 1992) it was interesting to study whether this defectiveness could be correlated to the lack of this signal sequence. To this end, the M RNA of a second (Dutch) isolate of INSV, denoted INSV NL-07, which is able to produce enveloped particles (de Ávila *et al.*, 1992; Kitajima *et al.*, 1992a) was partially sequenced. The sequence data obtained indeed demonstrated the presence of a putative signal sequence, encoded by the 5'-proximal part of the G2/G1 ORF (Fig. 5 and 6).

The INSV NL-07 M RNA sequence was almost 100% homologous to the corresponding region in the M RNA of the INSV isolate US-01 (Fig. 5), whereas a homology of approximately 56 % was found with the TSWV isolate BR-01. Nucleotide sequence alignment revealed that the defective INSV isolate US-01 contained an extra G residue at position 4884 and an extra C residue at position 4852 (Fig. 5). As a consequence the G2/G1 ORF in the US-01 isolate started at position 4803, while that of NL-07 started already at position 4884. Comparison of the predicted translation products of both ORFs revealed that the NL-07 glycoprotein precursor is collinear with that of US-01 isolate, but contained 27 additional amino acids at its N-terminus (Fig. 5 and 6). These extra amino acids in NL-07 are indeed highly hydrophobic and correspond with the putative signal sequence for the translocation of TSWV G2-G1 precursor across the ER membrane (Kormelink *et al.*, 1992).

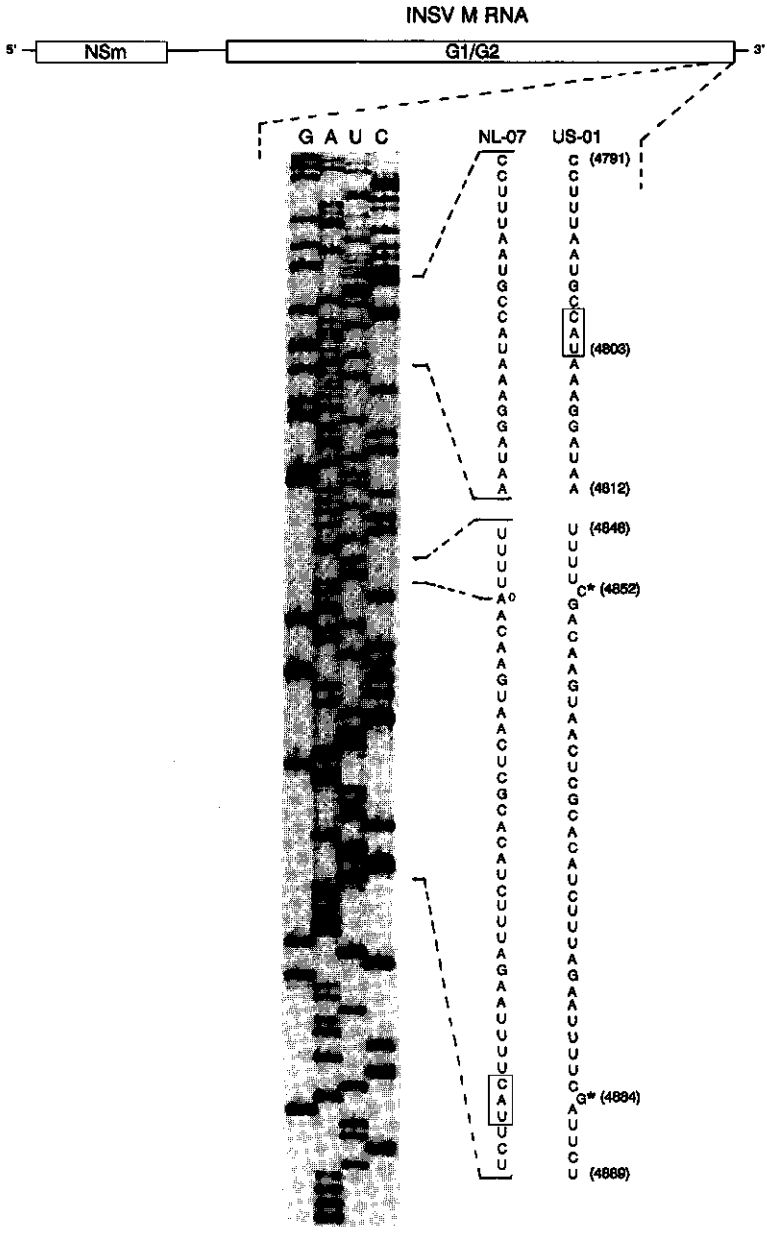

Fig. 5. Nucleotide sequence comparison of the G2/G1 ORFs of the NL-07 and US-01 isolate of INSV. The nucleotides numbering corresponds the US-01 isolate. The AUG start codons and their respective positions are boxed in both sequences. The extra nucleotides present in isolate US-01 are marked with an asterisk. The silent point mutation in the NL-07 isolate is marked with an open circle.

```
      1                                                          50
Nl-07  MKILKMCELL VKISVCTLVV TSVILSFMAL KETDAKIHVE RGDHPEIYDE
US-01  .......... .......... .......MAL KETDAKIHVE RGDHPEIYDE
BR-01  MRILKLLELV VKVSLFTIAL SSVLLAFLIF RATDAKVEII RGDHPEIYDD

     51                                                         100
Nl-07  AYYDRSVD.. ..HKNEILDT LAEMLQNATG KTLRPTRDTQ TVLANNEVPQ
US-01  AYYDRSVD.. ..HKNEILDT LAEMLQNATG KTLRPTRDTQ TVLANNEVPQ
BR-01  SAENEVPTAA SIQREAILET LTNLMLESRT PGTRQIREEK STIP......

    101
Nl-07  SPSGLSSTPT TISVMDLPNP CLNASSLTCS IKGVSTFNVY YQVESNGVIY
US-01  SSSGLSSTPT TISIMDLPNP CLNASSLTCS IKGVSTFNVY YQVESNGVIY
BR-01  .ISAEPTTQK TISVLDLPNN CLNASSLKCE IKGISTYNVY YQVENNGVIY
```

Fig. 6. Sequence comparison of the N-termini of the predicted G2/G1 ORF translation products of isolates TSWV-BR-01, INSV US-01 and INSV NL-07. The terminal hydrophobic signal sequences present in TSWV and NL-07 are showed in bold. The potential cleavage site (TDAK) and the characteristic cellular attachment domains (RGD) observed in all three isolates are underlined. A conserved region in the amino terminal sequences is boxed. The amino acid changes between US-01 and NL-07 isolates are in *italic*. Dots were introduced to obtain the best alignment.

This finding brings the genetic organization of the INSV M RNA in accordance to that of TSWV, and indicates that the ORF in the US-01 isolate of INSV, has undergone some point mutations, which resulted in loss of the signal sequence, and consequently in the incapability of this isolate to form envelopes.

As proposed for the TSWV glycoprotein precursor, this signal sequence would be cleaved from the glycoprotein precursor at the conserved amino acid residues (TDAK). The RGD motif observed in the glycoprotein precursor of both TSWV isolate BR-01 and INSV isolate US-01, was also present in that of NL-07, at the N terminus immediately downstream of the hydrophobic signal sequence. In addition to the presence of this signal sequence seven nucleotide changes were observed in the NL-07 glycoprotein precursor, downstream of this sequence. Two of these mutations resulted in amino acid changes (Fig. 6). At position 4557, a T was replaced by a C which results

in a change of isoleucine into valine, and at position 4593 an A was substituted by a G causing a change of serine to a proline. Four silent mutations were observed at positions 4501 (C was replaced by a T), 4756 (G by an A), 4837 (G by an A) and 4853 (G by an A). Another nucleotide substitution was present in the non-coding region at position 4950. Hence, these results suggest that the US-01 isolate of INSV has acquired several mutations in the G2/G1 gene at similar frequency as observed in the defective NL-04 isolate of TSWV. This high mutation frequency may also contribute for the production of dysfunctional glycoproteins.

DISCUSSION

Comparison of the M RNA sequences of some TSWV and INSV isolates revealed that the accumulation of point mutations in the G2/G1 ORF in M RNA may be the causal event leading to the generation of envelope-deficient isolates during mechanical transmission.

The mutations found thus far, seem to cause two different types of defects concerning glycoprotein synthesis. First, deletion of a transport signal sequence in the G2/G1 gene, as observed for INSV US-01 isolate, will result in a precursor protein that cannot be transported through membranes. Second, the replacement of amino acids without obvious distortion of the ORF, as observed for NL-04, may result in dysfunctional glycoproteins which do not become inserted in the viral membrane. In this latter case, however, it can not be excluded that stop codons are introduced more downstream in the non-sequenced part of the G2/G1 ORF, which would then lead to premature termination.

Comparison of the glycoprotein precursors of the NL-07 and US-01 isolates of INSV showed that the G2/G1 ORF of the US-01 isolate lacks a hydrophobic sequence of 27 amino acids at the N-terminus (Fig. 6) of the NL-07 isolate, and which probably represents a signal sequence for translocation across the endoplasmatic reticulum (Kormelink *et al.*, 1992). The deletion of this signal sequence in the US-01 isolate is caused by insertion of two nucleotides which prevent translational initiation at the correct AUG codon at position 4886. However, deletion of the signal sequence does not

necessarily prevent the synthesis of a (truncated) glycoprotein precursor, since indeed some glycoprotein production has been observed (Law & Moyer, 1990; Urban *et al.*, 1991; Law *et al.*, 1992). Possibly the downstream AUG codon at position 4803 has been used as alternative translational start codon. However, the isolation of a defective G2/G1 gene does not exclude the possibility that the American isolate contains also M RNA segments which encode a functional glycoprotein precursors. This could also explain the presence of low numbers of enveloped virus particles found in US-01-infected cells.

In contrast to the American isolate the Dutch isolate of INSV (NL-07) produces high numbers of enveloped particles (de Ávila *et al.*, 1992; Kitajima *et al.*, 1992a). Its predicted G2/G1 ORF starts approximately 71 nucleotides prior to that of the reported US-01 ORF, the difference corresponding with a hydrophobic signal sequence of 27 amino acids. The presence of putative signal peptides has also been observed in the published sequences of the glycoprotein precursors of four members of the genus bunyaviruses, i.e. Bunyamwera, Germiston, La Crosse and snowshoe hare virus (Elliott, 1990; Gerbaud *et al.*, 1992). Depending on the virus, these sequences contain 13 to 21 amino acid residues.

In the envelope-deficient NL-04 isolate of TSWV the morphological defect seems not to be caused by the deletion of hydrophobic signal sequences. Its G2/G1 ORF starts at the same AUG codon as found for both wild type isolate NL-04 and BR-01 (Kormelink *et al.*, 1992) and specifies a hydrophobic N-terminal stretch of 30 amino acid residues.

Analysis of the nucleotide sequence of the defective NL-04 strongly suggests that the defect was generated by the accumulation of point mutations in the G2/G1 ORF without distortion of the reading frame. Four of the seven point mutations detected were found clustered in a stretch of 15 nucleotides neighboring a U-rich region located approximately 300 nucleotides downstream of the start codon. This region might be highly susceptible to mutations. A frame-shifted mutation in the BR-01 isolate maps in the same region and supports this hypothesis. The observed point mutations in the defective NL-04 isolate were not generated by PCR errors during amplification cycles, since they were found at the same position in various clones, obtained from independent PCR reactions.

The accumulation of mutations in the G2/G1 ORF as detected in the first 431

nucleotides, may have been accompanied with the introduction of one or more stop codons more downstream in the non-analyzed part. This would result in the synthesis of truncated, dysfunctional glycoproteins, by which envelope formation will be bloked. ELISA experiments using monoclonal antibodies revealed that the NL-04 envelope-deficient isolate strongly reacts with MAbs against the nucleocapsid protein, but only weakly with one of the MAbs directed to the membrane glycoproteins (Figure 3 in Chapter 4). Enveloped particles were not detected by electron microscopical analyses of plant tissues infected with this defective isolate, even after extensive search (Kitajima *et al.*, 1992).

It is likely that only complete virus particles will be transmitted by thrips vectors. Since a low accumulation of envelope particles of the US-01 isolate of INSV occurs in infected cells, it may be expected that this isolate will be transmitted at a low rate. Preliminary experiments, comparing the transmission efficiencies of NL-07 and US-01 by *Frankliniella occidentalis*, confirm this hypothesis (Wijkamp, personal communication). Similar results were observed with the NL-04 isolates. In contrast to the wild type isolate, no vector transmission has been obtained sofar of the NL-04 envelope-deficient isolate (Wijkamp, personal communication). These observations indicate that morphologically defective isolates of TSWV, though still able to infect plants, have lost their vector transmissibility (Ie, 1982; Verkleij & Peters, 1983; Resende *et al.*, 1991).

ACKNOWLEDGMENTS

The authors thank Marcel Prins for the helpful discussions during the course of the present work. The authors Renato de Oliveira Resende and Antonio Carlos de Ávila are supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and by Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA)/Brazil, respectively.

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CHAPTER 7 ENGINEERED PROTECTION TO TOMATO SPOTTED WILD VIRUS IN TRANSGENIC TOBACCO PLANTS TRANSFORMED WITH DI L RNA COPIES

Renato de Oliveira Resende, Peter de Haan, Marcel Prins, Jan J.L. Gielen, Rob Goldbach and Dick Peters

SUMMARY

Recently, high levels of protection against tomato spotted wilt virus (TSWV) have been obtained by transforming tobacco with viral nucleoprotein (N) gene sequences, and this protection was demonstrated to be primarily RNA-mediated. Here it is tested whether engineered protection to TSWV can also be obtained by transforming tobacco plants with defective interfering (DI) L RNA copies. Transgenic plants expressing viral DI RNA sequences were obtained, showing upon virus inoculation, a pronounced delay in symptom expression in 9 out of 20 lines tested, while in 3 of these 9 lines (VC4, VC7 and V7) part of the progeny plants was completely protected. Since the type of protection obtained appeared to be immunity rather than tolerance, it is proposed that the protection observed in the DI-expressing transgenic plants is based on anti-sense inhibition.

INTRODUCTION

Genetic transformation of plants has become a useful approach to design non-conventional forms of plant resistance to viral infections. For a growing number of virus-plant combinations, insertion of viral sequences in the plant genome has been shown to result into protection (i.e. absence or delay of disease symptoms) to infections by the corresponding virus. Among the various approaches reported, coat protein-mediated resistance is the most widely applied approach (Beachy *et al.*, 1990; Hull & Davies, 1992). Also, cloned DNA-copies of symptom-attenuating satellite RNAs have been used to convert susceptible plants into resistent or tolerant ones (Baulcombe *et al.*, 1986; Gerlach *et al.*, 1987).

Recently, genetic transformation has also been applied to create plant resistance to tomato spotted wilt virus (TSWV), the type species of the genus tospovirus, within the arthropod-borne family Bunyaviridae.

High levels of protection against TSWV have been obtained by transforming tobacco plants with viral nucleoprotein (N) gene sequences (Gielen *et al.*, 1991). This resistance can be explained either by the accumulation of N protein in the transgenic plants and subsequent disregulation of transcription and replication of incoming viral RNA by altering the mode of the viral polymerase, or by the presence of N gene transcripts in the transgenic plants giving rise to anti-sense inhibition of the viral replication (Gielen *et al.*, 1991).

Recent results have shown that the N-gene mediated resistance to TSWV is mainly, if not completely, the consequence of the accumulation of N-specific RNA transcripts in transgenic plants, which is in favour of the second explanation (de Haan *et al.*, 1992). Also, this N-gene mediated resistance to TSWV seems to be higly specific. Plants showing high levels of resistance were not protected to other tospovirus species, although they share considerable nucleotide sequence homology (between 55 and 80 %) in their N gene to TSWV (de Ávila *et al.*, 1992).

Another approach for obtaining engineered resistance to tospoviruses may be based on transformation of host plants with DNA copies of defective interfering (DI) RNAs. DI RNAs are readily generated by tospoviruses upon repeated mechanical transfers and,

therefore, available for potential approaches towards engineered protection. These DI molecules interfere strongly with the replication of the wild type virus genome resulting in less severe, attenuated symptoms in host plants (Resende *et al.*, 1991, 1992).

Characterization of the DI RNAs revealed that they are exclusively derived from the (viral polymerase-encoding) L RNA segment by deletion of approximately 5.4 kilobases (60%) to 7.0 kilobases (80%) of the genomic L RNA, while both genomic termini are retained (Resende *et al.*, 1992). The presence of both the 5' and 3' termini in the DI molecules of TSWV indicate that these regions possess the essential signals for genome replication, transcription, and possibly other regulatory functions (Resende *et al.*, 1992).

Due to these features, transgenic production of DI RNAs may create protection to tospoviruses. Such transcripts may interfere with the replication of the virus and also attenuate symptoms even though the transformed plants are not resistant. Since it is assumed that the polymerase gene of tospoviruses is more conserved than the other viral genes like the N gene, transgenic plants producing TSWV DI molecules may also exhibit broader protection to other tospoviruses.

In addition, plants transformed with DI L RNAs may also be a powerful tool to study the genome information required for viral replication, encapsidation and packaging into virus particles.

To test the potential of DI RNAs to obtain engineered protection to TSWV infection a full-length DNA copy of a symptom-attenuating DI RNA derived from the isolate BR-01, was cloned and transferred to tobacco using the *Agrobacterium tumefaciens* gene transfer system. This chapter summarizes the results obtained with this approach.

METHODS

Virus and DI RNA isolation.

TSWV isolate BR-01, originating from Brazil, was maintained in *Nicotiana rustica* cv America plants by mechanical inoculation. The DI L RNA selected for the experiments described in this paper, was generated and characterized as reported earlier (Resende *et al.*, 1991, 1992). Viral RNA was purified from nucleocapsid extracts containing DI molecules according to de Vries *et al.* (1982).

Construction of DI RNA expression vectors and plant transformation.

All manipulations involving DNA or RNA were performed according to standard procedures (Ausubel *et al.*, 1990). First-strand cDNA synthesized on the RNA segments was employed as substrate in PCR using 4 μg/mlof the L RNA terminal primers L1 and L63. Primer L1 contained the first 17 nucleotides of the viral complementary (vc) L RNA strand and additional BamHI and XhoI restriction sites, while primer L63 was complementary to the last 17 nucleotides of this strand and also contained additional BamHI and SalI sites (Fig. 1a, in Chapter 5). The PCR products were cloned as BamHI fragments into the vector pZU119 (Fig. 1). This pUC derived vector contains the cauliflower mosaic virus (CaMV) 35S promoter sequences, the 5'-untranslated leader sequence (Omega) of TMV and the transcription-termination sequences from the nopalin synthase (nos) gene of the *A. tumefaciens* Ti plasmid (Gielen *et al.*, 1991; de Haan *et al.*, 1992). Using site directed mutagenesis this vector was modified replacing the PstI site by a BamHI site.

The recombinant transformation vectors were mobilized into *A. tumefaciens* strain LBA4404 by a triparental mating using pRK2013 as a helper plasmid (Ditta *et al.*, 1980). Transformation of tobacco leaf discs was done essentially as described by Horsch *et al.* (1984). *Nicotiana tabacum* cv SR1 was used as recipient in the transformation experiments. Transgenic tobacco plants were grown under certificated greenhouse conditions (PKII), according to the legislation imposed by the Dutch administration (Voorlopige Commissie Genetische Modificatie: VCOGEM).

Southern and Northern blot analysis.

Total DNA was extracted according to Ausubel *et al.* (1990). Total RNA was isolated from plants (Verwoerd *et al.*, 1989), electrophorezed and blotted onto Hybond N membranes (Amersham). Southern and Northern blots were hybridized with a random-primed alpha-^{32}P-dATP-labelled DI-specific probes. Hybridization and washing of the blots was done according to the manufactures recommended method at 65°C.

Analysis of resistance to TSWV.

Progeny plants of self-pollinated original transformants were mechanically inoculated

ca. 6 weeks after sowing when they were in their four-leaves stage. Approximately 15 plants of each transgenic line and 15 non-transgenic SR1 control plants were used in virus challenging experiments, using carborundum powder as abrasive, with approximately 5 μgTSWV/plant as inoculum. The inoculated plants were monitored daily for the development of local and/or systemic symptoms. Periodically, leaf-samples were taken and analyzed by ELISA to assay the virus concentrations in infected plants.

RESULTS

Construction of transgenic tobacco lines expressing DI L RNA molecules

Using PCR and primers L1 and L63, a full-length DNA copy of expected size (3107 nucleotides) was amplified (data not shown). This DI cDNA copy, which contained the 5' and 3'-untranslated region of the L RNA and part of the polymerase ORF was cloned in both orientations in the cloning vector pZU119, as to aim the transgenic production of viral sense (vRNA) and viral complementary (vcRNA) RNA transcripts, respectively (Fig. 1). Due to the fact that DI RNAs of TSWV seem to maintain an open reading frame and therefore still may encode truncated polymerase proteins (Resende *et al.*, 1992), the expression vector also contained the 5'-untranslated leader (Omega) from tobacco mosaic virus (TMV). As this leader sequence is known to function as a translational enhancer (Gallie *et al.*, 1987), any DI-mediated protein production is expected to be enhanced.

The distinct cassettes were cloned as SalI-SstI fragments in the binary transformation vector pBIN19 (Fig. 1). The pBIN19 derived transformation vectors pTSWV DI-vRNA and pTSWV DI-vcRNA were mated into the non-oncogenic *A. tumefaciens* strain LBA4404 by a triparental mating using pRK2013 as a helper plasmid. The resultant recombinant *A. tumefaciens* strains were checked for the integrity of the DI copy by Southern blot analysis (data not shown). Each recombinant *A. tumefaciens* strain was used to transform approximately 80 leaf discs of tobacco cv SR1. The transformants were selected for resistance to kanamycin and rifampicin (100 μg/ml). The positive transformants were rooted, potted in soil and subsequently transferred to the greenhouse. Except for one transformant, all transgenic tobacco plants showed a normal

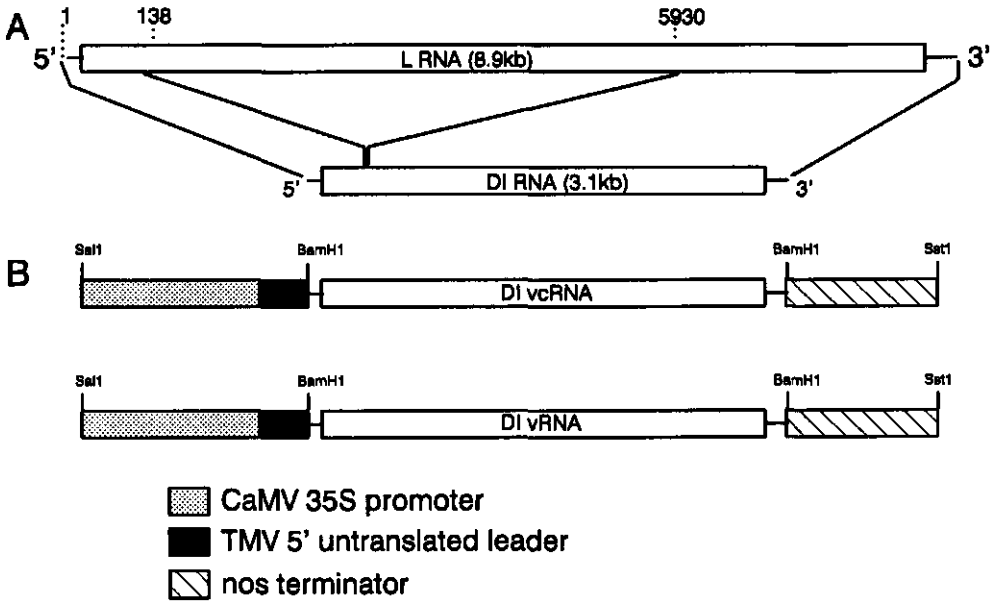

Fig. 1. Panel A - Structure of the TSWV defective interfering RNA denoted DIa (3.1 kb). The wild type L RNA segment is presented on top with the respective nucleotide positions (numbered from the 5' end of the vcRNA) indicated at the junction sites of the DI molecule. **Panel B** - Schematic representation of the chimeric gene cassettes TSWV DI-vcRNA and TSWV DI-vRNA. The double-hatched box represents CaMV 35S promoter sequences, the hatched box *nos* terminator sequences and the black box the 5'-untranslated leader sequence of TMV.

phenotype and set seed after self-pollination.

Virus protection in DI transgenic plants

Thirteen S1 progeny pTSWV DI-vRNA transformed plant lines (numbered V1 to V13) and seven S1 progeny pTSWV DI-vcRNA transformed lines (numbered VC1 to VC7) were obtained. To determine whether these S1 transgenic tobacco lines were protected to TSWV infection, 15 six-week old plants of each line were mechanically inoculated with wild type BR-01 isolate which was free of DI RNAs as verified by Northern blot hybridization. As controls, an equal number of SR1 non-transgenic tobacco plants were also challenged with the same inoculum.

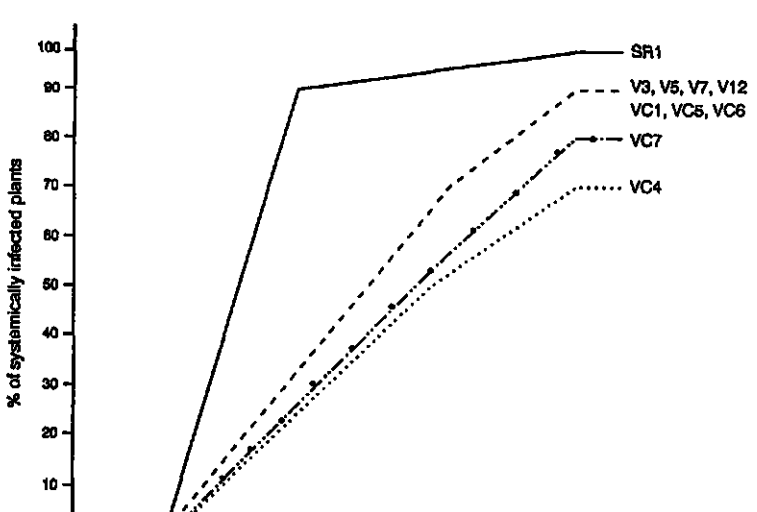

Fig. 2. Development of systemic disease symptoms in transgenic DI-RNA-expressing S1 tobacco plants upon mechanical inoculation with TSWV. Control plants consisted of non-transgenic tobacco SR1 plants. Plants were inoculated six weeks after sowing with approximately 5 μg of virus and subsequently monitored for the appearance of systemic symptoms. The panel shows the representatives of lines V3, V5, V7, V12, VC1, VC5 and VC6 which had a pronounced delay in symptom expression, and lines VC4 and VC7 of which part of the progeny plants were completely protected.

After inoculation, plants were monitored daily for the appearance of local and systemic symptoms. Similar to the control plants, the transgenic lines showed thecharacteristic necrotic lesions in the inoculated leaves. However, when the transgenic DI-containing lines were analyzed for the development of systemic symptoms they showed reduced susceptibility to TSWV infection as compared to the SR1 control plants. Eight days post infection 13 out of 14 control plants died to the TSWV infection, the single deviant control plant developed symptoms within the next 12 days. Of the transformants 4 out of the 13 DI-vRNA lines and 4 out of the 7 DI-vcRNA lines developed only mild or no symptoms at all in most of their progeny plants eight days post infection. Continued observation revealed that the progeny plants of lines V3, V5, V7, V12, and the lines VC1, VC5, and VC6 exhibited a pronounced delay in symptom development (Fig. 2). In spite of this, 20 days post infection approximately 90 % of

Fig. 3. Symptom development in inoculated tobacco plants of lines VC4 and VC7. The plants were inoculated with approximately 5 µg TSWV isolate BR-01 and photographed 20 days post infection. From left to right, TSWV control tobacco SR1 inoculated, TSWV control tobacco SR1 non-inoculated, TSWV-inoculated plants of VC4 and VC7 lines.

progeny plants developed symptoms.

Highest protection levels were observed in lines VC4 and VC7, of which approximately 30 and 20% respectively of the DI-expressing progeny plants appeared completely protected, remaining virus-free during their further development (Fig. 2).

Examples of protected plants of these two lines are shown in Figure 3. Protection was also observed in line V-7, in which approximately 15% of the progeny plants remained healthy. DAS-ELISA experiments using polyclonal antibodies directed to the nucleoprotein demonstrated that TSWV was only present in plants showing symptoms. Virus could not be found in any of the protected, symptom-less plants.

Transcriptional analysis of transgenic lines

Part of the progeny plants were tested for the integration of the DI L RNA copies into the plant genome. Total DNA was extracted from the tobacco plants and digested with BamHI to release the full-length size DI molecule. Southern blot results using a DI-specific probe (^{32}P-labelled full-length DI probe) demonstrated that most of the progeny plants of all transgenic lines tested contained the DI copies. An example of the hybridization patterns obtained is shown in Figure 4.

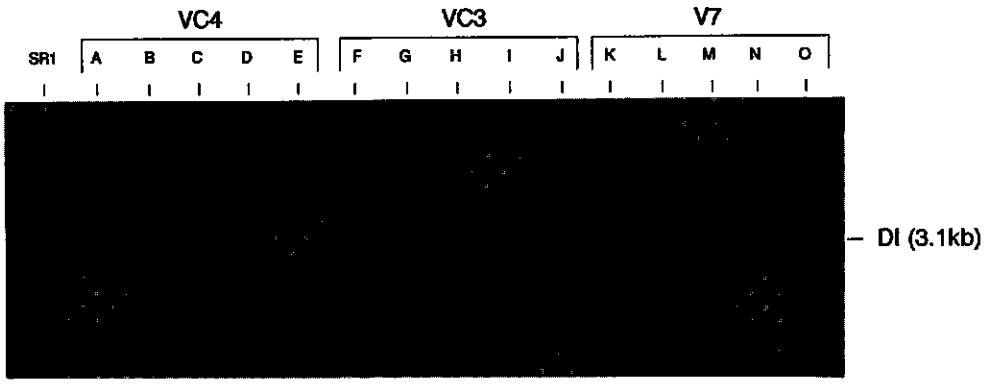

Fig. 4. Southern blot analysis of 15 S1 progeny plants of lines VC4 (lanes A to E), VC3 (lanes F to J) and V2 (lanes K to O). Total plant DNA was extracted, digested with BamHI and hybridized with a ^{32}P-labelled full-length DI as probe. SR1, total DNA from tobacco control plants. The size of the full-length DI of isolate BR-01 is shown.

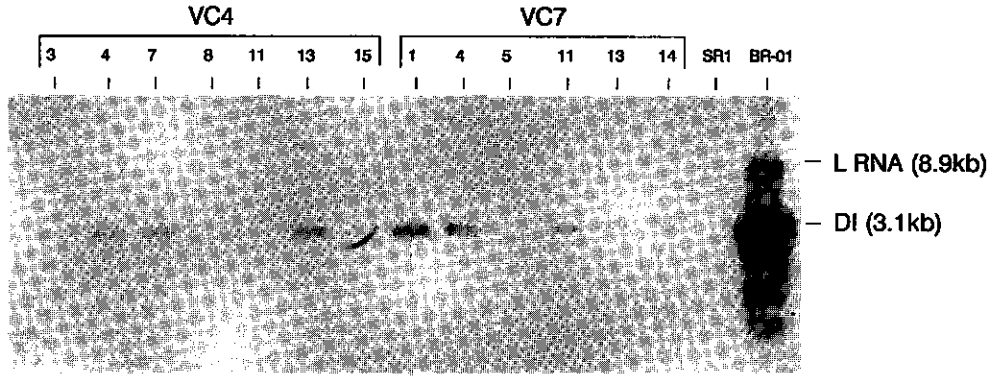

Fig. 5. Northern blot analysis of 14 S1 progeny plants of the resistent lines VC4 and VC7, using a ^{32}P-labelled pTSWV L 70 DNA fragment as probe. The plant numbers are indicated. The sizes of full-length L RNA and DI of isolate BR-01 are shown. SR1, non-transgenic control plants. BR-01-DI, purified nucleocapsid containing DI molecules.

To examine the transgenic production of viral DI transcripts, total RNA was extracted from progeny plants of each transgenic line and electrophoresed in a 1% agarose gel under denaturating conditions. After transfer to nitrocellulose membranes, the RNAs were hybridized to a ^{32}P-labelled pTSWV L-70 probe directed to the 3'-terminal region of L RNA.

Northern blot analysis demonstrated that DI-specific RNA transcripts of the expected size were detectably produced in at least 7 out of the 20 transgenic lines tested. Taking the non-viral sequences at both ends into account (promoter sequences and poly A tail), transcripts of approximately 3300 nucleotides long were expected. All protected progeny plants of lines VC4 and VC7 indeed produced transcripts of this size (Fig. 5). However, 2 out of 15 plants of these two RNA-expressing lines were not protected. For line V7, which showed delayed symptom expression and partial resistance, as well as for the other VC and V transgenic lines tested, preliminary Northern analyses indicate different levels of transgenic transcription (data not shown). Further experiments are required to characterize these transgenic lines more definitively.

DISCUSSION

The results described in this paper indicate that transgenic plants expressing viral DI RNA sequences show upon virus inoculation, a pronounced delay in symptom expression in 9 out of 20 lines tested while in case of three lines (VC4, VC7 and V7) part of the progeny plants were completely protected. There are at least two possible explanations for the antiviral activity of the transgenic transcripts, i.e. antisense blocking of viral RNA replication which would lead to "immunity" and, secondly, symptom suppression due to co-replication of the transgenically expressed DI RNA molecules may result in "tolerance". Since the transgenic DI sequences contain both genomic termini of the L RNA, including the putative replication and transcriptional initiation signals, transgenic plants expressing DI-specific transcripts indeed may exhibit anti-sense activity i.e. blocking of viral RNA multiplication by direct RNA-RNA interactions leading to resistance to virus infection. The second possibility would imply that the transgenic DI transcripts co-replicate with infecting viral RNA, thus interfering with the replication of

wild type genomic RNAs and consequently, conferring tolerance to TSWV infection.

Since the type of protection obtained seems to be "immunity" rather than "tolerance" (virus could not be detected in plants showing no symptoms), it is likely that the protection observed in the DI-expressing transgenic plants is based on anti-sense inhibition.

This observation is in agreement with the findings that have been reported for other plant viruses. For instance, in case of potato virus X and cucumber mosaic virus, transgenic expression of a negative-sense coat protein gene transcript has been shown to result in both delay of symptom expression and partial resistance, but the latter phenomenon only at low virus inoculum titer (Hemenway *et al.*, 1988; Cuozzo *et al.*, 1988). Furthermore, the previously reported N-gene mediated resistance to TSWV may be based on anti-sense blocking as well since the protection observed turned out to be, to a major degree, based on transcriptional expression of the transgene (de Haan *et al.*, 1992).

Taking into account that the polymerase gene is expected to be more conserved among tospoviruses than the other genes, the use of DI RNAs, which are derived from these genes, may confer a broader protection to other tospovirus species.

Northern blot studies on challenged, protected plants are required to verify whether the DI-specific transcripts, though containing extensive non-viral sequences at both ends, have indeed been unable to replicate in the transgenic plants. To obtain a genuine "DI-mediated" protection and not only anti-sense inhibition, transgenic DI-cDNA cassettes should probably be provided with a (upstream) trimmed CaMV promoter and a (downstream) ribozyme sequence (e.g. a hepatitis delta type ribozyme) as to obtain transgenic DI transcripts without any extra, non-viral nucleotides which may block their potential replicative ability.

In addition, the transgenic DI-expressing plants should be challenged with a range of different virus concentrations and ultimately using viruliferous thrips to evaluate the degree of protection obtained with these defective molecules.

ACKNOWLEDGMENTS

The authors wish to thank Dr. A.C. de Ávila for many fruitful discussions, Nicole Struijk and Alie van Schepen for technical assistance in the course of this work. The author Renato de Oliveira Resende is supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)/Brazil.

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CHAPTER 8 CONCLUDING REMARKS

In nature, tospoviruses like tomato spotted wilt virus (TSWV) are exclusively transmitted by thrips species (Sakimura, 1962) producing numerous enveloped virions during infection, which accumulate in the cisternae of the endoplasmatic reticulum system (Kitajima, 1965; Milne, 1970; Ie, 1971). Under experimental conditions however, it is common practice to maintain the virus by mechanical inoculation onto susceptible host plants.

Repeated passages of animal viruses, certainly at high inoculum densities results in the generation of defective mutants which co-replicate with the wild type virus interfering in their multiplication (Lazzarini *et al.*, 1981). This phenomenon is reported for only a few plant viruses (Morris & Knorr, 1990). As defective viral mutants lack one or more genetic functions but are still capable to co-replicate, they may constitute useful tools to study viral genes and viral protein functions.

This thesis reports studies aimed to generate and characterize defective forms of TSWV by their biological, serological and genetic properties. The understanding of these characteristics indeed, can help the elucidation the multiple events which take place during the infection process, and ultimately provide new ways to control the virus.

The first attempts to characterize TSWV defective forms showed that non-enveloped virus isolates are generated upon mechanical inoculation (Ie, 1982). These mechanically transmitted isolates were considered to represent morphologically defective forms of TSWV, lacking the lipid envelope though still being infectious (Ie, 1982). Further analysis revealed that these morphologically defective isolates failed to produce detectable amounts of the envelope glycoproteins G1 and G2 (Verkleij & Peters, 1983).

The results reported in this thesis show that during a series of mechanical transfers of TSWV, actually two distinct types of mutants are generated (Chapter 4). Firstly, starting with a wild type, the Dutch isolate of TSWV NL-04, a morphologically-defective isolate was obtained, which had lost its ability to produce the membrane-glycoproteins and, as a consequence, was not able to form enveloped particles. The appearance of such isolates could be followed by ELISA tests and readily detected by electron

microscopy (Chapter 3 and 4). Secondly, starting from various TSWV wild type-isolates, defective mutants were obtained that had accumulated deleted forms of the large (L) RNA segment that most likely represented defective interfering (DI) RNAs, since they replicated more rapidly than full-length L RNA and their appearance was often associated with symptom attenuation in host plants (Chapter 4).

After elucidation of the nucleotide sequence of the M RNA of two tospoviruses, i.e. TSWV and *Impatiens* necrotic spot virus (INSV) (Kormelink *et al.*, 1992; Law *et al.*, 1992), the genetic nature of the morphological defectiveness of the envelope-deficient isolates could be identified by comparing their M RNA sequences with those of wild type isolates (Chapter 6). Comparison of (partial) M RNA sequences of several TSWV and INSV isolates revealed that the accumulation of point mutations in the G2/G1 ORF in this RNA may have been the causal event that led to the generation of envelope-deficient isolates during mechanical transmission. It was found that an envelope-deficient isolate (US-01) of INSV had acquired an extra nucleotide in this gene, causing a frame-shift and consequently the loss of the putative signal peptide of the glycoprotein precursor (Chapter 6). In this case, the envelope deficiency may be explained by a blockage of the trans-membrane transport, and hence further maturation of the glycoprotein precursor. For the isolate NL-04 of TSWV the morphological defect seems to be caused by the accumulation of point mutations in the glycoprotein precursor rather than deletion of the hydrophobic signal sequence. The accumulation of point mutations in these isolates may either result in dysfunctional glycoproteins which do not become inserted in viral envelopes, or in stop codons though not detected in the sequenced part of the G2/G1 gene, but which are possibly present (further downstream), in the non analyzed part of this gene. The presence of these stop codons would then lead to premature termination of the G2/G1 precursor.

The generation and characterization of the envelope-deficient isolate of TSWV NL-04 has already lead to two important conclusions, with respect of the possible involvement of the glycoproteins during the tospovirus infection cycle. First, it has been observed that this defective-isolate is still able to infect leaf tissues at a similar rate as to that of wild type infection. Thus the glycoproteins (and lipid envelope) are neither essential for cell-to-cell movement of the virus, nor for long-distance transport in the plant. The

conclusion seems therefore to justify that tospoviruses, during the development of infection, are transported mainly as free nucleocapsid complexes. Secondly, the presence of lipid enveloped (and glycoproteins) appeared to be essential for successful thrips transmission, since sofar, the envelope-deficient isolate of NL-04 fails to show vector transmissibility (Wijkamp, personal communication). This finding strongly suggests a role of G1 and/or G2 glycoproteins in the virus-vector interactions. It is noteworthy in this context, that the primary structure of these proteins contain a so-called "cell attachment site (RGD) at the N-terminus of G2 (Kormelink *et al.*, 1992) which may be involved in the recognition of a receptor in the thrips midgut.

The results reported in this thesis also show that, tospoviruses generate DI RNAs during sequential passages of the virus at high multiplicity of infection (Chapters 4 and 5). DI RNAs have frequently been described in either positive or negative-stranded animal virus systems (Holland, 1985; Nayak *et al.*, 1990), but have been reported for only few positive-strand plant viruses (Morris & Knorr, 1990). Therefore, the DI RNAs of TSWV represent the first fully characterized DI molecules among negative-strand RNA viruses infecting plants. The DI molecules are found to interfere strongly with the replication of the wild type virus genome resulting in less severe, attenuated symptoms in host plants (Chapter 4). Characterization of the DI RNAs occurring with four distinct tospovirus isolates revealed that they are exclusively derived from the (viral polymerase-encoding) L RNA segment (therefore represent "typical" DI RNAs, Holland, 1985; Nayak *et al.*, 1990) by deletion of approximately 5.4 (60%) to 7.0 (80%) kilobases of the standard genomic L RNA while both genomic termini are retained. The presence of both 5' and the 3' termini in the DI molecules of TSWV indicate that these regions possess the essential signals for genome replication and possibly other regulatory functions (Chapters 4 and 5). Furthermore, these defective molecules are able to be encapsidated and incorporated into enveloped particles (Chapter 4).

Short repeated nucleotide sequences were identified at the junction sites of various DI molecules and a possible model for their generation is proposed, in which the viral polymerase can jump across secondary structures where these repeated internal sequences are located.

Due to their replicative advantage over wild type RNAs, the TSWV DI RNAs may

constitute a powerful tool to study the genome information required for viral replication, encapsidation and packaging into virus particles, and to unravel the RNA replication process. Infectious transcripts from cloned DI DNA copies, provide a useful approach among positive-strand RNA viruses infecting either animal or plant systems (Makino *et al.*, 1991; van der Most *et al.*, 1992; Hagino-Yamagishi *et al.*, 1990; Burgyan *et al.*, 1992; Morris & Knorr, 1990). For negative-strand RNA viruses, however, the results have been very limited thus far. Recently, with the development of the vaccinia T7-expression system (Fuerst *et al.*, 1987) infectious rhabdovirus VSV-DI transcripts could be produced and has opened new ways for using these defective molecules (Pattnaik *et al.*, 1992). This system, however, requires the expression of all viral replicatory proteins, an approach which is not available yet for TSWV.

Alternatively, transgenic DI-expressing plant systems could be used, not only to study the viral RNA replication process, but also as an approach towards engineered protection of host plants against virus infection. Due to their features, transgenetically expressed DI transcripts could potentially protect plants against the disease symptoms of TSWV infection even though the transformed plants are only "tolerant", rather than "immune". The results described in Chapter 7 demonstrate that engineered protection to TSWV (as reported using the nucleoprotein gene, Gielen *et al.*, 1991; de Haan *et al.*, 1992) can also be obtained by transforming tobacco plants with defective interfering (DI) L RNA copies. Transgenic plants expressing viral DI RNA sequences were obtained, which upon virus inoculation, showed a pronounced delay in symptom expression in 9 out of 20 lines tested, while in 3 of these 9 lines (VC4, VC7 and V7) part of the progeny plants was completely protected. Since the type of protection obtained appeared to be immunity rather than tolerance, it is proposed that the protection observed in the DI-expressing transgenic plants is based on anti-sense inhibition and not in the ability of the DI transcripts to co-replicate along with the infecting virus. Northern analyses on challenged, protected plants are required to verify whether the DI-specific transcripts, though containing extensive non-viral sequences at both ends, have indeed been unable to replicate in the transgenic plants. To obtain genuine "DI-mediated" protection and not only anti-sense inhibition, transgenic DI-cDNA cassettes should probably be provided with a trimmed CaMV promoter (upstream) and a ribozyme sequence (e.g. a hepatitis

delta type ribozyme) downstream as to obtain transgenic DI transcripts without any extra, non-viral nucleotides which may block their potential replicative ability.

Since DI RNAs of tospoviruses may be generated in any host system that permits the growth of the wild type virus, it is likely that they are not only generated under laboratory conditions, but also in natural infections. The presence of such molecules in enveloped particles (Chapter 4), indeed indicates that tospovirus DI RNAs can potentially be transmitted by thrips vectors and hence, "survive" in nature. If DI RNAs indeed do occur in natural infections, their presence during virus infection may serve to attenuate the pathogenic effects of the parental virus infection. Their occurrence may also have been advantageous for the evolution of tospovirus and for the establishment of their current, impressive host range.

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SAMENVATTING

Het bronsvlekkenvirus van de tomaat, dat in het Engels "tomato spotted wilt virus" (TSWV) wordt genoemd, komt nagenoeg over de gehele wereld voor. Dit virus heeft een groot waardplantenbereik en kan-met name in de tropen en subtropen-aanzienlijke schade in een groot aantal gewassen veroorzaken, zoals bijvoorbeeld in aardnoot, erwt, paprika, sla, tabak, tomaat, etc.

Het genoom van TSWV bestaat uit drie enkelstrengs, lineaire RNA segmenten, die S (small), M (medium), en L (large) RNA genoemd worden. Deze RNA segmenten zijn in nucleocapside eiwitten gewikkeld en vormen aldus drie pseudo-circulaire nucleocapsiden, die door een lipide membraan (envelop) omgeven zijn. De virusdeeltjes hebben een diameter van 70 - 110 nm. Het L RNA, dat codeert voor het transcriptase, heeft een negatieve polariteit. De beide andere RNA strengen zijn ambisense van karakter; dwz dat een gedeelte van elke streng een positieve polariteit bezit en het andere gedeelte een negatieve.

Het M RNA codeert voor een precursor-eiwit waaruit de beide glycoproteinen, G1 en G2, ontstaan, die aan de buitenzijde van het virusdeeltje met de lipidemembraan geassocieerd zijn. Het S RNA tenslotte, codeert o.a. voor het nucleocapside (N) eiwit. Op grond van deze, en andere moleculaire eigenschappen is TSWV ondergebracht in een nieuw genus, Tospovirus genaamd, binnen de Bunyaviridae, een grote familie die verder alleen uit zoogdier-infecterende virussen bestaat.

Onder natuurlijke omstandigheden wordt het virus uitsluitend door tripsen overgebracht. Onder experimentele omstandigheden kan het virus echter ook mechanisch overgebracht worden. Gedurende deze mechanische overdracht treden vaak fenotypische veranderingen op, zoals de afwezigheid van complete virusdeeltjes in de geinfecteerde cellen en het optreden van verzwakte symptomen op toetsplanten. Het veranderen van fenotypische eigenschappen treedt veelvuldig op bij virussen met een negatief-strengs RNA genoom, wanneer zij onder een hoge infectiedruk geinoculeerd worden. Analyse van dergelijke isolaten leert dat het genoom van deze virussen op verschillende wijzen gemuteerd kan zijn, waarbij vaak grote inwendige deleties optreden.

Dit proefschrift beschrijft een studie om de veranderingen in het genoom van die TSWV isolaten te bestuderen die fenotypische veranderingen hebben ondergaan. Karakterisering van deze defecte stammen zal bijdragen tot een grotere kennis in de moleculaire genetica van TSWV en een beter inzicht verschaffen in de processen die bij de vermenigvuldiging van het virus en de infectie van planten plaatsvinden. Vergroting van de kennis over deze defecte isolaten is tevens van belang omdat zij mogelijk een functie kunnen vervullen in het beschermen van planten tegen infecties met TSWV.

Door herhaalde passage van een aantal TSWV isolaten in *Nicotiana rustica* en *Nicotiana benthamiana* bleek het aantal met een lipide membraan omhulde deeltjes in de plant af te nemen en de hoeveelheid vrije, membraan-loze nucleocapsiden in de plant toe te nemen. Dit werd als een duidelijke aanwijzing gezien voor het defect raken van deze isolaten (Hoofdstuk 3). Gedurende deze passages werden twee typen mutanten verkregen, nl. een "membraan-deficiënt" isolaat dat niet meer in staat was om morfologische complete virusdeeltjes te vormen en "defective-interfering" mutanten (Hoofdstuk 4). Met behulp van immunologische methoden kon in planten geinfecteerd met de membraan-deficiënte mutant geen virale glycoproteinen aangetoond worden. Achterwege blijven van de productie van deze eiwitten kan verklaren waarom er geen complete virusdeeltjes konden ontstaan. Bij analyse van het M RNA van deze mutant en van het oorspronkelijke virus, het isolaat NL-04, werden geen waarneembare verschillen in hun lengte gevonden, hetgeen suggereert dat het defect veroorzaakt was door puntmutaties of, op zijn hoogst, door zeer kleine deleties.

Inderdaad werd in het gen dat codeert voor de gemeenschappelijke precursor van de G1 en G2 eiwitten een accumulatie van puntmutaties gevonden (Hoofdstuk 6). Deze mutaties zouden aanleiding kunnen geven tot defecte glycoproteinen die niet meer functioneel zijn in de vorming van de lipide membraan rond de nucleocapsiden. Een morfologische defect isolaat uit de Vereniging Staten van Amerika van het *Impatiens* necrotic spot virus (ISNV), ook een lid van het Tospovirus genus, kon eveneens verklaard worden door een mutatie in dit gen, waarmee de codering voor de signaalsequentie verloren is gegaan (Hoofdstuk 6). Uit het bestaan van deze morfologische defecte stam van NL-04 kunnen reeds enige conclusies getrokken worden ten aanzien van de functies van de glycoproteinen. Allereerst wijst het vermogen van

deze stam om de plant systemisch (even snel als het wild-type isolaat) te infecteren er op dat TSWV in de vorm van vrije nucleocapsiden wordt getransporteerd, zowel van "cel tot cel" (door plasmodesmata) als "over lange afstand" (door de gehele plant). Anderzijds tonen preliminaire experimenten (Wijkamp en Resende, niet gepubliceerd) aan dat voor de overdracht door tripsen de membraan en glycoproteinen nodig zijn. Verondersteld mag worden dat evolutionair gezien de membraan reeds verloren zou zijn indien deze membraan niet vereist was voor overdracht door insecten.

Bij analyse van het RNA van de "defective interfering" mutanten werd steeds een extra RNA segment gevonden. Dit segment bleek uit gedeleteerde vormen van het L RNA te bestaan, waarbij de lengte van het gedeleteerde deel per isolaat zeer sterk verschilde. Omdat deze defecte RNAs voorkomen, in hoeveelheden groter dan die van het L RNA èn het optreden van minder heftige symptomen, dienen deze defecte RNA moleculen als "defective interfering" (DI) RNA moleculen beschouwd te worden. Hun aanwezigheid in gezuiverde virus suspensies en nucleocapsidefracties betekent eveneens dat zij nog steeds de sequenties bezitten die nodig zijn voor replicatie en assemblage in virusdeeltjes. Hybridisatie-experimenten lieten zien dat ze inderdaad nog de oorspronkelijke 5' en 3' terminale sequenties bezitten. Bovendien kunnen alle nader gekarakteriseerde DI moleculen potentiëel het carboxy-terminale deel van het virale transcriptase coderen. Dit eiwit zou de verzwakking van de symptomen bewerkstelligen (Hoofdstuk 5).

Aangezien infecties van planten met TSWV, dee DI RNAs beveitten aanleiding geeven tot aanzienlijke verzwakking van de ziektesymptomen zouden gevoelige waardplanten wellicht beschermd kunnen worden tegen TSWV infecties door inbouw van DNA copiën van deze DI RNAs. Transformatie van tabak met een volledige copie van een 3,1 Kb DI RNA leverde inderdaad een aantal lijnen op, waarvan de nakomelingen deels beschermd bleken te zijn tegen TSWV infectie (Hoofdstuk 7). In de beschermde planten werd echter geen virusvermeerdering waargenomen, zodat geconcludeerd mag worden dat deze bescherming op immuniteit berust en niet op tolerantie. Deze conclusie zou inhouden dat de transcripten van de copiën niet als natuurlijke (replicerende) DI RNAs hebben gefunctioneerd, maar naar alle waarschijnlijkheid als anti-sense RNAs.

RESUMO

Tomato spotted wilt virus (TSWV), denominado no Brasil como vira-cabeça do tomateiro, é encontrado em quase todas as regiões tropicais e sub-tropicais do mundo, infectando plantas de grande valor econômico, como tomate, pimentão, alface, ervilha, amendoim, fumo, etc. Também no Brasil, o vira-cabeça é um dos principais vírus de hortaliças e causa grandes danos nestas culturas.

O TSWV é formado de partículas esféricas, com o diâmetro variando entre 70-110 nm, circundadas por uma membrana de lipídeos denominada envelope e associadas a este, o vírus contém duas glicoproteínas (G1 e G2). O genoma do vírus é constituído de três RNAs de fita simples, denominados S (small), M (medium) e L (large). Estes RNAs são encapsidados pela proteína N codificada pelo S RNA formando os nucleocapsídeos que são pseudo-circulares. O L RNA apresenta polaridade negativa e codifica a polimerase do vírus. Os RNAs M e S apresentam um caráter "ambisense". O M RNA codifica o precursor das glicoproteínas (G1 e G2) e uma proteína não-estrutural (NSm); o S RNA codifica a proteína do nucleocapsídeo (N) e uma segunda proteína não-estrutural (NSs).

Baseado em suas características morfológicas e moleculares o vira-cabeça é classificado no recém-criado gênero tospovirus, pertencente à família Bunyaviridae.

Em condições naturais o TSWV é transmitido por oito espécies de tripes, entretanto, em condições experimentais, o vírus é usualmente propagado via transmissão mecânica. Este procedimento causa mudanças fenotípicas no vírus, tais como, acúmulo de partículas incompletas nas células infectadas e atenuação de sintomas nas plantas hospedeiras. Tais mudanças são frequentemente observadas nos vírus de RNA negativo, quando transmitidos sob alta pressão de inóculo. Análises destes isolados têm mostrado que o genoma apresenta diferentes tipos de mutações.

Este trabalho descreve a geração e caracterização de formas defectivas do TSWV, associadas às mudanças das propriedades biológicas, serológicas e genotípicas do vírus. A caracterização dos isolados defectivos contribui para elucidar os múltiplos eventos que ocorrem durante o processo de infecção, e, em última análise, proporciona o

desenvolvimento de novas alternativas de controle.

Após a transmissão mecânica de vários isolados de vira-cabeça em *Nicotiana rustica* e *Nicotiana benthamiana*, observou-se um acúmulo de partículas incompletas do vírus (agregados de nucleocapsídeos) nas plantas infectadas, e, simultaneamente, um decréscimo das partículas contendo a membrana de lipídeos. A presença destes isolados foi detectada através do teste ELISA e técnicas de imuno-detecção em microscopia eletrônica (Capítulo 3).

Durante sucessivas passagens do TSWV por inoculação mecânica, dois tipos distintos de mutantes foram gerados: isolados morfologicamente defectivos, os quais não produzem partículas completas e isolados contendo moléculas deletadas do L RNA, que interferem na replicação do vírus original, sendo denominados "defective-interfering" RNAs (Capítulo 4).

Partindo-se de um isolado original holandês (NL-04 do TSWV), um mutante morfologicamente defectivo foi obtido, o qual perdeu a habilidade de produzir glicoproteínas. A análise do M RNA deste mutante não mostrou diferenças significativas no tamanho deste RNA, indicando que o defeito morfológico está relacionado com mutações de ponto ou pequenas deleçoes no M RNA. Tais mutações foram detectadas no gene que codifica o precursor das glicoproteínas G1 e G2 (Capítulo 6), que podem causar a produção de glicoproteínas não-funcionais, e, consequentemente, levar a não formação do envelope do vírus. Um segundo isolado morfologicamente defectivo, originado dos USA apresentou também mutações de ponto no mesmo gene, causando a deleção de uma sequência-sinal responsável pela transporte das glicoproteínas nas células (Capítulo 6). Este isolado (US-01) pertence ao grupo Impatiens necrotic virus, uma outra espécie dentro do gênero tospovirus.

Através da caracterização destes isolados defectivos, concluiu-se que as glicoproteínas (e a membrana de lipídeos) parecem não ser essenciais ao transporte do vírus de uma célula a outra, bem como para o transporte à longa distância na planta. Esta conclusão parece justificar que tospovirus, durante o ciclo infectivo, são transportados basicamente na forma de nucleocapsídeos. A presença da membrana de lipídeos (e glicoproteínas), entretanto, parece ser essencial à transmissão do vírus por tripes pois, até o presente momento, tais isolados defectivos não apresentaram transmissão pelo vetor. Estas

observações sugerem que as proteínas G1 e/ou G2 estejam diretamente envolvidas na interação vírus-vetor.

A análise do genoma dos mutantes denominados "defective interfering" RNAs mostraram que estes isolados contêm um segmento extra de RNA, além dos tres RNAs que formam o genoma do vírus. Estes segmentos extras são originados exclusivamente do L RNA através da deleção de 60 a 80% da molécula original. Os RNAs defectivos são eficientemente replicados durante a infecção do vírus, interferindo na multiplicação do L RNA, e, consequentemente, causam significativa atenuação dos sintomas em plantas. Devido as suas características, tais RNAs são denominados "defective interfering (DI) RNAs. Estes DI RNAs são encontrados em partículas completas do vírus assim como em nucleocapsídeos purificados, indicando que estas moléculas defectivas contêm as sequências genéticas essenciais para serem encapsidadas pela proteína do nucleocapsídeo e incorporadas no envelope do vírus (Capítulo 4). Hibridizações com sondas biológicas e sequenciamento genético destes DI RNAs demonstraram, de fato, que tais moléculas retêm os terminais 5' e 3' do L RNA, onde importantes sequências genéticas parecem estar localizadas. Verificou-se também que os DI RNAs, apesar de defectivos, ainda são capazes de codificar proteínas, as quais podem estar envolvidas com o fenômeno de interferência (Capítulo 5).

Devido às caracteristicas de interferência na replicação do vírus e atenuação de sintomas em plantas, os DI RNAs constituem-se potencialmente numa alternativa de controle da infecção do TSWV. Com este objetivo plantas de fumo foram geneticamente trasformadas com uma cópia de DNA de um dos DI RNAs caracterizados. Análise das progenes provenientes das plantas transgênicas, demonstraram que parte destas plantas adquiriram resistência à infecção do TSWV (Capítulo 7). O tipo de proteção obtida parece ser imunidade e não tolerância, pois o vírus não pode ser detectado nas plantas resistentes. Estes resultados parecem indicar que as moléculas dos DI RNAs transcritas nas plantas transgênicas são capazes de bloquear a replicação do TSWV durante o processo de infecção.

CURRICULUM VITAE

Renato de Oliveira Resende was born on 5 December, 1959 in Lavras, Minas Gerais, Brazil. He graduated in Agronomy at the Faculty of Agricultural Sciences of Lavras in 1982, and obtained his master degree in Phytotechnology (with thesis on Plant Tissue Culture) in 1985.

In 1985, he started his career at the Minas Gerais Agricultural Research Corporation (EPAMIG) with the task to breed potatoes resistant to virus infections in the National Potato Breeding Program. Since January 1987, he has worked on an Indexing Program for seed-potato production in the Minas Gerais State (a joint project of Secretariat of Agriculture - Minas Gerais, ESAL and EPAMIG).

From November 1988 until March 1993 he worked for his PhD at the Department of Virology, Wageningen Agricultural University. His project aimed to characterize mutants of TSWV and the results obtained in this study are presented in this thesis.

He will pursue his career as a virologist in Brazil.

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