A STUDY OF THE MOLECULAR VARIABILITY OF SOME SOUTH AFRICAN ISOLATES OF TOBACCO NECROSIS VIRUS

by

Michael-John Freeborough

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Abstract

The relationship of various tobacco necrosis viruses, isolated from variouscrops and other sources in South Africa was determined. An isolate from avocado was chosen for partial characterisation to confirm that it was a member of the Necrovirus group of plant viruses. TNV was detected in potatoes that exhibited abnormal necrotic foliar symptoms and a D-type TNV was isolated and identified from the Up-to-Date potato variety. Immunoelectroblotting assay grouped the TNV isolates studied into two serotypes (A and D). This result was confirmed by NA hybridization with probes derived to the coat protein of an A- (TNV-Wheat) and a D-type (TNV-Papaya green lesion) isolate. RT-PCR with A and D specific primers did not amplify the coat protein of three A- and D-type TNV isolates which appears to indicate that the detection by PCR with specific primers is too selective to be used for a general test, unless degenerate primers to a more conserved region of the coat protein gene are used. Sequence analysis of the coat protein gene was used to determine the phylogenetic relationship amongst nine TNV isolates examined and also by comparison to three isolates for which the coat protein gene had already been sequenced. Sequence analysis showed high degree of homology amongst the A-type isolates and the D-type isolates, with approximately 45 % homology between the two TNV types.

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General abbreviations

µg	microgram
µL	microlitre
ATCC	American Type Culture Collection
ATP	adenosine-5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BME	β-mercaptoethanol
bp	base pair
cDNA	complimentary deoxyribonucleic acid
CIP	calf intestinal phosphatase
CSPD	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate
ddH_2O	deionised distilled water
DIG	digoxygenin
DMSO	dimethyl-sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
ds	double stranded
DTE	dithioerythretol
DTT	dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
EM	Electron microscopy
et al.	et alia
EtBr	ethidium bromide
g	gram
g	gravitational force
IEB	immunoelectroblotting
IPTG	isopropyl-β-D-thiogalactopyranoside
ISEM	immunosorbant electron microscopy
hrs	hours
kb	kilobase pair
kDa	kilo dalton

L	litre
LA	luria agar
LA-AIX	Luria agar plates with ampicillin, IPTG and X-Gal
M	molar
mg	milligram
min	minutes
mL	millilitre
mM	millimolar
Mr	molecular ratio
NA	nucleic acid
NBT	nitro blue tetrazolium
ng	nanogram
nt	nucleotide
°C	degrees celsius
PCR	polymerase chain reaction
PNK	polynucleotide kinase
RNA	ribonucleic acid
RNase	ribonuclease
RPM	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodiumdodecyl sulphate
SDS-PAGE	sodiumdodecyl sulphate-polyacrylamide gel electrophoresis
sec	seconds
ss	single stranded
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TE	Tris-EDTA buffer
Tris-Cl	Tris(hydroxymethyl)-aminomethane
U	units
UV	ultraviolet
UWGCG	University of Wisconsin Genetics Computer Group
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Abreviations of virus names

BMV	brome mosaic virus
BYDV	barley yellow dwarf virus
CarMV	carnation mottle virus
ChNV	chenopodium necrosis virus
CMV	cucumber mosaic virus
CNV	cucumber necrosis virus
CYSV	carnation yellow stripe virus
LNV	lisianthus necrosis virus
MCMV	maize chlorotic mottle virus
MNSV	melon necrotic spot virus
OCSV	oat chlorotic stunt virus
PLRV	potato leafroll virus
PVX	potat virus X
PVY (necrotic)	potato virus Y (necrotic strain)
PVY (normal)	potato virus Y (normal strain)
RCNMV	red clover necrotic mosaic virus
SBMV	sothern bean mosaic virus
STNV	satellite tobacco necrosis virus
TBSV	tomato bushy stunt virus
TCV	turnip crinkle virus
TMV	tobacco mosaic virus
TNV	tobacco necrosis virus
TYMV	turnip yellow mosaic virus

Chapter 1

Literature review and project outline

Contents

1.1 Introduction

Tobacco necrosis virus (TNV) is the type species of the genus Necrovirus in the family Tombusviridae. This genus also includes chenopodium necrosis virus (ChNV). Carnation yellow stripe virus (CYSV) and lisianthus necrosis virus (LNV) are listed as tentative members of the genus Necrovirus (Lommel, 1995). TNV was first isolated in 1935 from the roots of symptomless *Nicotiana tabacum* plants grown in glass houses in Britain (Smith and Bald, 1935).

TNV is a small icosahedral plant virus with an RNA genome that usually causes localised symptoms in plant hosts. Some strains of TNV however, can cause systemic infections in tulips and bean (Kassanis, 1970a and Appiano and Redolfi, 1993). TNV is generally not associated with economically important diseases of crops, but it does cause the Augusta disease of tulips, bean stipple streak, and ABC disease of potatoes (Kassanis, 1970a), and has been isolated from more than 20 different agricultural crops. TNV has a wide host range infecting more than 88 species of plants from 37 dicotyledonous and monocotyledonous plant families (Price, 1940 and Horvath, 1982), with many cases of virus infection being latent (Horvath, 1982).

The main reasons for studying TNV have been its transmission by zoospores of the chytrid root infecting fungus *Olpidium brassicae* (Teakle, 1962), its ability to act as a helper virus for the replication of satellite tobacco necrosis virus (STNV) (Kassanis and Nixon, 1961) and its physical and chemical stability (Fraenkel-Conrat, 1988).

Different aspects of TNV biology and characteristics have been thoroughly reviewed by Uyemoto (1981), Francki *et al.* (1985) and Fraenkel-Conrat (1988). Since the latest review of Fraenkel-Conrat (1988), sequence data on three TNV isolates have become available and have led to studies into the relationships of TNV with other RNA plant viruses. A review of the sequence data and relationship studies will be presented in this chapter.

1.2 TNV isolates

More than 20 TNV isolates have been identified. Many of the isolates have been isolated from naturally infected roots of different plant species (Fulton, 1950; Kassanis, 1970a). TNV also has been detected and isolated from various agricultural crops; grapevine (Cesati and van

Regenmortel, 1969), apple (Uyemoto and Gilmer, 1971), cucumber (Thomas and Fry, 1972), carrot (Kemp and Barr, 1978), *Pogostemum patchuli* (Gama *et al.*, 1982), strawberry (Tanne, 1984), avocado (Jaffer *et al.*, 1993), tobacco (Zhang *et al.*, 1993), plum and apricot (von Wechmar *et al.*, 1994a), papaya (von Wechmar *et al.*, 1994b) and zucchini (Roggero and Lisa, 1995). TNV also has been isolated from river water (Tomlinson *et al.*, 1983), *Mucor circinelloides* fungus (von Wechmar and Jaffer, 1990; von Wechmar *et al.*, 1990; von Wechmar *et al.*, 1991; von Wechmar *et al.*, 1993), and nutrient feeding solution (Adam *et al.*, 1990).

Disease problems, in which TNV was found to be associated with fungi and/or bacteria, have been studied. No conclusive evidence has yet been put forward to show that these fungi and/or bacteria act as a host to, or vector of the virus, however, an association between TNV and these micro-organisms has been shown (von Wechmar and Jaffer, 1990; von Wechmar *et al.*, 1990; von Wechmar *et al.*, 1991; von Wechmar *et al.*, 1993; Jaffer *et al.*, 1993; von Wechmar *et al.*, 1994a; von Wechmar *et al.*, 1994b and von Wechmar *et al.*, 1994c). This association of TNV with micro-organisms supports the suggestion of Fraenkel-Conrat (1988) that TNV may have evolved from an RNA phage.

Many of the TNV isolates which were compared, vary in biological properties, satellite virus activation and serological specificities (Babos and Kassanis, 1963; Uyemoto *et al.*, 1968; Kassanis and Phillips, 1970).

1.3 Biophysical and biochemical characteristics

Tobacco necrosis virus is a spherical virus with icosahedral symmetry and has a diameter of approximately 28 nm. The nucleic acid comprises approximately 19% of the particle molecular ratio (Mr), the remaining 81% of the virion is protein. The virus has no lipid or carbohydrate component (Lommel, 1995). The Mr of the particle is 7.6×10^6 Da, with a sedimentation co-efficient of 118 S and a buoyant density in CsCl of 1.40 g/cm^3. The thermal inactivation point of TNV is between 85 °C and 95 °C with an isoelectric point of pH 4.5. Longevity *in vitro* is between 7 and 28 days (Kassanis, 1970a). The nucleic acid is linear positive sense single stranded (ss) RNA (Lesnaw and Reichmann, 1969a; Meulewaeter *et al.*, 1990; and Coutts *et al.*, 1991). The entire nucleotide sequence of a TNV-D strain indicated a size of 3759 nucleotides (nt) (Coutts *et al.*, 1991). The 5' end of the TNV RNA has no

covalently linked virion proteins and is uncapped with a ppA... terminus (Lesnaw and Reichmann, 1969b). The 3' end has no poly (A) tail (Coutts *et al.*, 1991). TNV is made up of 180 coat protein monomers in T=3 symmetry to form an icosahedral particle and the coat protein is the only known structural protein (Lesnaw and Reichmann, 1969a; Meulewaeter *et al.*, 1990; Coutts *et al.*, 1991). The coat protein has a Mr of approximately 29 to 30 kDa with between 268 and 277 amino acids (Meulewaeter *et al.*, 1990; Coutts *et al.*, 1991 and Zhang *et al.*, 1993).

Satellite tobacco necrosis virus is spherical with a particle diameter of 17 nm. The Mr of the particle is 1.97 x 10^6 Da, with a sedimentation coefficient of 50 S and aggregates of 169 S, 231 S and 332 S (Kassanis, 1970b). The nucleic acid is linear sense ss RNA of approximately 1240 nt (Danthinne *et al.*, 1991) and comprises 20 % of the particle Mr (Kassanis, 1970b). The capsid is made up of 60 monomer coat protein particles with an approximate Mr of 22 kDa which comprises 80 % of the particle (Kassanis, 1970b).

1.4 TNV transmission

Olpidium brassicae is an obligate parasite of plant roots, and infects plant cells in the cortex of the root tip in the meristematic and cell elongation regions. Teakle (1962) was the first to note that TNV and STNV are transmitted naturally by zoospores of *O. brassicae*. Transmission is dependant on the interaction between fungus race, virus isolate and host species (Kassanis and Macfarlane, 1965; Temmink *et al.*, 1970; Temmink, 1971). Transmission by zoospores of TNV and STNV is very efficient and the virus can be acquired and transmitted from virus solutions that are too dilute to mechanically transmit the virus to host plants (Fry and Campbell, 1966). Temperature and light have an effect on the transmission of TNV. A low temperature of 10-16 °C (Fry and Campbell, 1966) and darkness (Beever and Fry, 1970) resulted in an increase of TNV transmission by *O. brassicae*.

The virus adsorbs to the fungal zoospore plasmalemma and axonemal sheath but the virus adsorbed to the axonemal sheath was shown to be transmitted to plant hosts. The axoneme (flagellum) retracts into the cytoplasm and a cyst wall forms. The zoospores attach to the root epidermal cells by an adhesive substance. The zoospore protoplast penetrates the host cell, leaving the plasmalemma and most other membraneous material outside of the host cell. Approximately 36 hours after penetration a thallus wall forms, but the virus transmission is

thought to occur before the formation of the thallus wall (Temmink *et al.*, 1970; and reviewed by Uyemoto, 1981).

In virus transmission, the virus may be released from the fungal vector into the soil or drainage water (Yarwood, 1960) or from decaying infected roots (Fraenkel-Conrat, 1988). When zoospores are released from infected roots, TNV and STNV particles may bind to the zoospores with a high affinity (Fraenkel-Conrat, 1988). TNV may also have an affinity for other micro-organisms, which would explain the association of TNV with bacteria, yeast morphs and other fungi (von Wechmar *et al.*, 1991; von Wechmar and Jaffer, 1993; von Wechmar *et al.*, 1993; Jaffer and von Wechmar, 1993, Jaffer *et al.*, 1993; von Wechmar *et al.*, 1994a; von Wechmar *et al.*, 1994b and von Wechmar *et al.*,1994c).

1.5 Serological classification

For the sake of brevity in this section, TNV isolates will be referred to with their designation only, ie. TNV-A will be referred to as A.

Tobacco necrosis virus isolates are classified into two major serotypes (A and D) according to the immunological cross-reactivity of the isolates. Each serotype may contain several strains or isolates (Kassanis, 1970a). Babos and Kassanis (1963) determined the serological relationship of seven different TNV isolates. The isolates tested were found to be serologically related, but divided into two serotypes. Isolates A and F were found to be identical. The A-serotype contained isolates A, B, C (cucumber necrosis), F and S (bean stipple-streak). The D-serotype contained isolates D and E. Serological cross-absorbtion tests showed that isolates within each group differed from other isolates in the group, but these differences were much smaller than differences between the two groups. Uyemoto *et al.* (1968) determined the serological relationship of 11 TNV isolates and found it impractical to divide the isolates into only two serotypes. The isolates studied were: CH, A, RO, RE, AC 43, D, AC 42, AC 39, NZ, AC 38, AC 36. TNV B is the main component of RO and isolates A and D were used as references to the work of Babos and Kassanis (1963). Isolates NZ, AC 36 and AC 38 were found to be identical and isolates CH and A were also found to be identical. The isolates could not be grouped into two serotypes because the relative serological relationship between the isolates when tested with different antisera was found to be variable, some isolates shifted between groups. These researchers doubted the significance

of serological classification of TNV, because of the many serologically distinct TNV isolates. The serological relationship of isolates would be variable and would depend on the test isolates and the specific antisera used. Kassanis and Phillips (1970) were however successful in grouping eight TNV isolates into two serotypes. The A-serotype contained isolates A and S, and the D-serotype contained isolates D, E, CT, GV, AC 43 and AC 36.

The specificity of antisera used for serological determination is very important. Kassanis and Phillips inferred that the antisera used by Uyemoto *et al.* (1968) was not specific, due to the injection and bleeding schedule. The propagation host used for amplification of the virus isolates, may also have had an effect on the serological determination of the virus. The serological classification of TNV is ambiguous, because the serological determination was not standardised, the same propagation hosts were not used and the antisera was not prepared in the same way.

The more recent TNV isolates have been grouped according to the serotype designation suggested by Babos and Kassanis (1963), whereby the serological relatedness of the more recent isolates are compared to serotype-A and serotype-D (Thomas and Fry, 1972; Kemp and Barr, 1978, Tomlinson *et al*, 1983; Tanne, 1984; von Wechmar *et al.*, 1990; Adam *et al.*, 1990; Jaffer *et al.*, 1993 and Roggero and Lisa, 1995).

1.6 Satellite activation

The satellite tobacco necrosis virus is not infectious without its helper virus, TNV. STNV has been found to have three serologically distinct isolates, SV-A/SV-1, SV-B/SV-2 and SV-C. TNV isolates can activate the different STNV isolates and this activation of STNV is the basis of a different form of determining grouping of, or relationships between the TNV isolates. TNV's CH, A, RO, RE, AC 43, AC 42 and AC 39 activated SV-A and SV-B. TNV's D, NZ, AC 38 and AC 36 activated SV-C (Uyemoto *et al.* (1968). TNV's A, B, S, E, AC 43 and CT activated SV-1 and SV-2. TNV's D, AC 36 and GV activated SV-C (Kassanis and Phillips, 1970). No correlation between the serological classification of TNV and the activation of STNV strains was determined (Uyemoto *et al.*, 1968 and Kassanis and Phillips, 1970). Kassanis and Phillips (1970) however found a correlation between the activation of STNV and host range. TNV isolates that multiplied with ease in *Phaseolus vulgaris* and *Nicotiana clevelandii* activated SV-1 and SV-2, whilst the TNV isolates that

multiplied with difficulty in these plants activated SV-C. TNV isolates that do not have any natural STNV component can still activate STNV particles (Uyemoto *et al.*, 1968 and Kassanis and Phillips, 1970). TNV isolates that do not have a natural STNV component are easily separated from artificial mixtures of TNV and STNV after inoculation when compared to TNV particles that have a natural STNV component which are not easily separated from the STNV component after inoculation (Uyemoto *et al.*, 1968).

1.7 Cytopathology

The cytopathology of TNV has not been studied intensely because the virus does not produce any useful diagnostic inclusion bodies. Plant cells infected with TNV have a hypersensitive reponse which results in necrosis of the plant cells. Kassanis *et al.* (1970) used the stipple streak strain of TNV (TNV-S) and its associated STNV for cytopathological analysis. This strain can infect *P. vulgaris* plants systemically with only a few necrotic lesions. In light microscopy, granular inclusion bodies can be seen in epidermal cells of plants infected with TNV-S. Electron microscopy showed that the cytoplasmic density increased and a large number of vesicles could be seen and the cytoplasmic membranes were disrupted. TNV and STNV were seen enclosed in vesicles. The spatial arrangement of TNV and STNV showed that the TNV could be found separate from STNV, but STNV was only found with some TNV particles associated. Crystals of STNV always had TNV particles surrounding the crystal. TNV was found to be concentrated, but was not noted in a crystalline array. Other TNV isolates have been shown to form crystalline aggregates in the cytoplasm, as reviewed by Francki *et al.* (1985). The cytopathological effects of TNV isolated from poplar showed that necrotic tissue appeared to be surrounded by a callose-like substance (D'Agostino and Pennazio, 1985). Non-necrotic cells infected with TNV did not have this callose-like substance surrounding the cells. The TNV in the necrotic cells formed a paracrystalline array whereas the TNV in the non-necrotic cells did not crystallize. The TNV isolated by Adam *et al.* (1990) showed that the accumulated TNV protruded from the cytoplasm into the vacuole, but the virus did not crystallize in non-necrotic tissue. Early necrotic tissue indicated that the virus crystallised. Appiano and Redolfi (1993) attributed this to the dehydration of the plant cells. Further cytopathological evidence of TNV was determined with an isolate from poplar, which induced wilting in *P. vulgaris*. Appiano and Redolfi (1993) observed proliferation and vesiculation of the endoplasmic reticulum. Many cells showed signs of plasmolysis with

localised detachment of the plasmalemma from the cell wall. The virus was noted in the cytoplasm as observed by Kassanis *et al*, (1970) and Adam *et al.* (1990). The concentration of the TNV increased over the course of the infection. Electron dense granules were noted in the cytoplasm and were assumed to be made up of protein including some virus coat protein. This supports the finding of D'Agostino and Redolfi (1989) who found that granular bodies found in the intercellular space between cells of leaves infected with TNV contained some viral coat protein. The TNV from poplar is the first reported isolate to crystalise in non-necrotic cells (Appiano and Redolfi, 1993). This crystallization could be promoted by the dehydration of the cells after necrogenesis (D'Agostino and Pennazio, 1985; Appiano and Redolfi, 1993). The cytopathological effects of TNV infection and accumulation of the virus are not unique to the necroviruses, but have been reported with viruses from other groups (Adam, *et al.*, 1990).

1.8 Genome organisation and translation strategy

The genomic organisation of TNV-A and TNV-D strains are similar, 3660 nt of TNV-A have been sequenced (Meulewaeter *et al.*, 1990) and the entire genome (3759 nt) of TNV-D has been sequenced (Coutts *et al.*, 1991). TNV-A contains six open reading frames (ORFs) (Meulewaeter *et al.*, 1990) and TNV-D has 5 ORFs (Coutts *et al.*, 1991). In TNV-A ORF 1 encodes a 22kDa protein with an amber stop codon that allows read-through to ORF 2 coding for an 82 kDa putative RNA-polymerase. ORF 3 codes for a 7.9 kDa protein, ORF 4 codes for a 6.2 kDa protein. ORF 5 codes for a 30 kDa protein shown to be the coat protein, and ORF 6 codes for a 6.7 kDa protein (Meulewaeter *et al.*, 1990).

In TNV-D ORF 1 codes for a 22 kDa protein, the amber stop codon allows read-through to ORF 2 which codes for the 82 kDa putative RNA-polymerase. ORF 3 and ORF 4 both code for a 7 kDa protein and ORF 5 codes for a 29 kDa coat protein (Coutts *et al.*, 1991). Both genomes are compact having a short 5' untranslated region and intercistronic regions (Meulewaeter *et al.*, 1990; Coutts *et al.*, 1991). TNV-D has a long 3' untranslated region (Coutts *et al.*, 1991).

The genome arrangement between the two isolates differs, TNV-A ORFs 2 and 3 overlap by 17 nt, whereas TNV-D has a 34 nt intercistronic region between ORFs 2 and 3. ORFs 3 and 4 have a 3 nt intercistronic region in the TNV-A genome, but no overlap or intercistronic region

is found between ORFs 3 and 4 in the TNV-D genome. TNV-A has a sixth ORF which is absent in TNV-D (Meulewaeter *et al.*, 1990 and Coutts *et al.*, 1991)

The partial nucleotide sequence of TNV-NE (Zhang *et al.*, 1993) had 3 ORFs encoding proteins of 10.7, 6.2 and 30.3 kDa. The ORF coding for the 10.7 kDa protein overlapped an ORF with sequence similarity to the ORF 2 82 kDa protein of TNV-A and TNV-D. The ORF coding for the 10.7 kDa protein could encode for proteins of 10.7, 9.6 and 7.8 kDa depending on the site of initiation. No ORF was noted 3' of the 30.3 kDa coat protein.

TNV-A and TNV-D had two subgenomic RNA species (Condit and Fraenkel-Conrat, 1979; Meulewaeter *et al.*, 1990; Coutts *et al.*, 1991) of approximately 1.6 and 1.3 kb. The translation strategy of the three viruses, A, D and NE, are believed to be similar (Coutts *et al.*, 1991; Zhang *et al.*, 1993). The putative RNA polymerase is expressed from the full length genomic RNA. ORF 3 and ORF 4 are expressed from the 1.6 kb subgenomic RNA in a wheat germ system, and the synthesis of the ORF 4 protein is dependant on the translation efficiency of ORF 3 (Meulewaeter *et al.*, 1992). The major *in vitro* translation product of TNV-A is the coat protein gene and the region upstream of the coat protein promotes internal initiation of translation *in vitro*. *In vivo* this region is functionally inactive and therefore the TNV-A genomic RNA is not required for coat protein synthesis in plants and the coat protein is expressed from the 1.3 kb subgenomic RNA (Meulewaeter *et al.*, 1992).

1.9 Gene products

The 82 kDa read-through protein has not been observed in *in vitro* translation (Salvato and Fraenkel-Conrat, 1977 and Meulewaeter *et al.*, 1990). The protein encoded by ORF 1 has limited amino acid sequence homology with other proteins. Its function as a separate protein or a domain of the 82 kDa readthrough protein is unknown. The 82 kDa readthrough product of ORF 1 and 2 is thought to be an RNA polymerase because of the GDD motif and surrounding conserved regions. The protein also shows sequence homology with the putative RNA polymerase gene in the genus Carmo- and Tombusviruses (Meulewaeter *et al.*, 1990 and Coutts *et al.*, 1991). In the Carmo- and Tombus virus groups expression of ORFs 1 and 2 are required for replication of the virus (Hacker *et al.*, 1992 and Kollar and Burgyan, 1994). ORFs 1 and 2 are both required for the replication of STNV in protoplasts, but the low level of STNV accumulation compared to the levels obtained when STNV is inoculated together

with TNV, may be due to other TNV gene products which may stimulate STNV multiplication. Although the ORF 1 and 2 gene products have been shown to be involved in the replication of STNV, there is no proof that these gene products encode the entire RNA polymerase required for replication of TNV (Andriessen *et al.*, 1995).

The proteins encoded by ORF 3 have limited sequence homology to small proteins encoded by Carmo- and Tombusviruses (Meulewaeter *et al.*, 1990; Coutts *et al.*, 1991 and Zhang *et al.*, 1993). The proteins encoded by ORFs 3 and 4 may have a role in cell-to-cell movement, similar to the 8 kDa and 9 kDa proteins of turnip crinkle virus (TCV). The 7 kDa protein encoded by ORF 3 of TNV-D has been shown to bind ss RNA and DNA *in vitro* (Offei *et al.*, 1995). The protein encoded by ORF 4 has no similarity to other proteins, but has a stretch of hydrophobic residues, which could be a transmembrane domain (Meulewaeter *et al.*, 1990 and Coutts *et al.*, 1991). Offei proposed that both the ORF 3 and 4 proteins may be involved in movement of the virus but the RNA-binding and plasmodesmatal targetting of the movement protein could have been seperated into two proteins.

From *in vitro* translation, the 30 kDa protein was identified as the coat protein of TNV-A (Meulewaeter *et al.*, 1990). The ORF 5 product also had amino acid sequence similarity to the structural coat protein of other small icosahedral viruses. From sequence similarity, the TNV coat protein could be divided into three domains, the R- (Random, N-terminal), a- (arm), and the S (Shell). The coat protein lacks a P- (Protruding) domain (Meulewaeter *et al.*, 1990; Coutts *et al.*, 1991 and Zhang *et al.*, 1993). The regions of the coat protein that determine the serotype and the vector specificity are not known, but the region/s responsible for these functions are likely to be conserved amongst the coat proteins because of the serological relatedness of the TNV isolates and the common vector for transmission (Zhang *et al.*, 1993).

The sequence of the ORF 6 gene product in TNV-A shows no homology to any other protein sequence encoded by other viruses (Meulewaeter *et al.*, 1990).

1.10 Relationship between TNV strains

The TNV-A and -D strains have a very similar genome organisation and translation strategy (Meulewaeter *et al.*, 1990 and Coutts *et al.*, 1991), but they differ in the number of ORFs. TNV-A has a sixth ORF 3' to the coat protein (Meulewaeter *et al.*, 1990), whilst TNV-D has

no ORF 3' to the coat protein (Coutts *et al.*, 1991). TNV-NE is more similar to TNV-D in this respect because it has no detectable ORF 3' to the coat protein (Zhang *et al.*, 1993). The 82 kDa read-through RNA polymerases have only 45 % homology between TNV-A and -D (Coutts *et al.*, 1991). The sequence homology of ORFs 3 and 4 indicates that TNV-NE and TNV-A are more closely related to each other than to TNV-D. The sequence homology of the coat protein indicates that TNV-A has 45 % homology to TNV-D (Coutts *et al.*, 1991), TNV-A has 51 % homology to TNV-NE, TNV-D has 44 % homology to TNV-NE (Zhang *et al.*, 1993).

TNV-A appears to be more closely related to TNV-NE than to TNV-D. The low percentage homology between the isolates indicates that each virus may have evolved in response to a different biological circumstance (Zhang *et al.*, 1993).

1.11 Relationships to other viruses

The Necrovirus group has similarity with other taxa. The taxa to which the Necroviruses are similar varies depending on the criteria used for the comparison. The genome organisation indicates that TNV is most similar to the Carmovirus group. The RNA polymerase indicates that TNV is most similar to the Carmo- and Tombusvirus groups, and the coat protein indicates that TNV is most similar to the Sobemo- and Machlomovirus groups (Lommel, 1995). TNV appears to have varying relationships with viruses from the following groups: Carmo, Tombus, Machlomo, Diantho, Sobemo, Luteo and the unclassified viruses melon necrotic spot virus (MNSV) and oat chlorotic stunt virus (OCSV) (Meulewaeter *et al.*, 1990; Coutts *et al.*, 1991 and Boonham *et al.*, 1995).

1.11.1 Genome organisation

The viral genome organisation of TNV was found to be most similar to the carnation mottle Carmovirus (CarMV) turnip crinkle Carmovirus (TCV) (Meulewaeter *et al.*, 1990 and Coutts *et al.*, 1991), cucumber necrosis Tombusvirus (CNV) (Coutts *et al.*, 1991) and the unclassified OCSV (Boonham *et al.*, 1995). The large 5' ORF codes for the putative RNA polymerase by read-through of the amber stop codon from ORF 1 to ORF 2 (Coutts *et al.*, 1991 and Boonham *et al.*, 1995) . The 3' region of these isolates encode the structural coat protein which is expressed from the smallest subgenomic RNA in all cases except the Tombusvirus group, which expresses the coat protein from the largest subgenomic RNA.

1.11.2 **RNA polymerase**

The RNA polymerase of TNV-A has similarity to CarMV, TCV, CNV, barley yellow dwarf Luteovirus (BYDV), maize chlorotic mottle Machlomovirus (MCMV) and red clover necrotic mosaic Dianthovirus (RCNMV) (Meulewaeter *et al.*, 1990). The RNA polymerase of TNV-D has similarity with CNV, Necrovirus (TNV-A), CarMV and TCV, RCNMV and MNSV. The TNV-A has higher homology to CarMV than to CNV (Meulewaeter *et al.*, 1990), but TNV-D has higher homology with CNV than with CarMV, and also has higher homology to CNV than to TNV-A (Coutts *et al.*, 1991). The Necro-, Tombus-, Carmo-, Diantho and Sobemoviruses lack the characteristic nucleotide-binding site motif (Candresse *et al.*, 1990 and Coutts *et al.*, 1991) that is found in the N-terminal regions of most positive sense unipartite ss RNA viruses. These viruses differ from the Alpha- and Picorna-like supergroups and may have a unique replication strategy (Coutts *et al.*, 1991).

1.11.3 **Coat protein**

TNV-A and TNV-D share 45% identity in the coat protein. TNV-A and -D also have sequence similarity to southern bean mosaic Sobemovirus (SBMV), CNV, TCV, CarMV, tomato bushy stunt Tombusvirus (TBSV), MNSV, RCNMV (Meulewaeter *et al.*, 1990 and Coutts *et al.*, 1991), TNV-NE (Zhang *et al.*, 1993) and OCSV (Boonham *et al.*, 1995). The TNV isolates sequenced are most similar to the SBMV coat protein. The TNV-A and -D coat proteins align with the C and D α helices of SBMV (Meulewaeter *et al.*, 1990 and Coutts *et al.*, 1991). TNV's A, D, NE and SBMV lack the P domain in the coat protein (Meulewaeter *et al.*, 1990; Coutts *et al.*, 1991 and Zhang *et al.*, 1993).

The placement of TNV in the Carmovirus group has been proposed (Morris and Carrington, 1988). Although the TNV-A and -D have similarities to the Carmovirus group, the viruses also have significant differences. TNV also has significant similarities to other viruses in the RNA polymerase and coat protein genes. The TNV isolates that have been entirely sequenced also show significant differences between themselves and the isolate that has been partially sequenced. These factors must be considered before placing TNV in the Carmovirus supergroup.

1.12 Evolution

The variation in the genome organisation and the percentage homology between the TNV isolates, and other viruses, indicates molecular flexibility between the Necro-, Tombus-, Carmo-, Diantho and Sobemovirus groups, MNSV and OCSV unclassified viruses. This modular evolution involving gene exchange by recombination (Zimmern, 1988) may permit independant evolution of different genes (Meulewaeter *et al.*, 1990). Phylogenetic analysis of the coat protein of small icosahedral RNA plant viruses showed obvious signs of gene exchange between the virus genomes of Tombus-, Carmo-, Necro-, Diantho-, Sobemo-, and Machlomovirus groups and MNSV unclasssified virus (Dolja and Koonin, 1991). This type of evolution suggests that all these viruses are related in some complex way.

1.13 Project outline

Aspects investigated in this thesis were part of a larger ongoing research project on tobacco necrosis virus in Prof. M.B. von Wechmars laboratory. For the thesis the following was done:

A TNV isolated from avocado was partially characterized (Chapter 2) prior to molecular analysis.

In an independent exercise, TNV could be detected in and isolated from potato plants that exhibited abnormal symptoms.

The major aim of this project was to examine several selected TNV isolates by comparison of their coat protein gene sequences. Prior to sequencing the coat protein of a number of isolates the coat protein and the genomic RNA were sized. Immunological assays were used to identify these isolates as TNV by comparison to reference TNV isolates obtained from the American Type Culture Collection and other research laboratories. A relationship study based on the immunoelectroblot assay, was conducted to group the isolates into two serotypes, this grouping was confirmed by nucleic acid (NA) hybridization.

Finally the coat protein sequence data were used to determine phylogenetic relationships amongst the TNV isolates. The degree of sequence conservation amongst isolates within the same serotype and between the two serotypes was also determined.

Chapter 2

Partial characterization of a TNV isolate from Avocado (*Persea americana* Mill) cv. Colin V-33

Contents

2.1 Introduction

In a research program on avocado (*Persea americana* Mill) to determine whether varieties used as an interstock to reduce tree size (Priego *et al.*, 1987) contained any pathogens, and whether these pathogens could cause the dwarfing effect in the recipient avocado varieties, several interstock varieties were examined and tobacco necrosis virus (TNV) was detected in the cultivar Colin V-33 (Jaffer *et al.*, 1993). This finding resulted in an investigation into the TNV detected. The Colin V-33 was introduced as unrooted material and was grafted onto local Edranol avocado seedlings to maintain the variety. TNV had not previously been reported in avocado. The symptoms associated with the presence of TNV in Colin V-33 are shown in Fig. 2.1 A to E. The figure illustrates the progression of abnormal symptoms until the necrotised tissue fragments separate from the living tissue (arrow in Fig. 2.1 E) leaving foliage with a ragged leaf margin ("lace leaf").

The TNV isolate was partially characterised in a preliminary study (M.J. Freeborough, B.Sc. Honours dissertation, 1993, entitled "Analysis of a new strain of tobacco necrosis virus isolated from avocado plants"). Two particles, a larger and a smaller particle were noted. They were shown to be TNV and an associated satellite tobacco necrosis virus (STNV) and will be referred to as TNV-Avo and STNV-Avo. To verify the results obtained in the preliminary study some aspects of the work were repeated for this dissertation.

The work described in this chapter deals with the propagation of TNV-Avo and the purification of the virus by differential centrifugation and rate zonal centrifugation through a sucrose gradient. TNV-Avo was partially characterized by determining some biochemical and biophysical properties and some molecular characteristics of the virus. Immunodetection studies (immunoelectroblots and immunosorbant electron microscopy) were used to positively identify the virus.

Immunological assays were used to determine the relationship of TNV-Avo to other TNV isolates (Chapter 4) and the coat protein gene was seqenced and compared to other TNV isolates for phylogenetic relationship studies (Chapter 5).

Figure 2.1 Symptoms on avocado (*Persea americana*) cv. Colin V-33. A to D show the progression of symptom development. E, young leaves of Colin V-33 tree exhibiting "lace leaf" syndrome. Arrows indicate the abscission of necrotic tissue from the living tissue at the leaf edge.

2.2 Materials and Methods

2.2.1 Virus isolate

TNV-Avo was initially isolated by M.B. von Wechmar from young branches with leaves taken from a *Persea americana* cv. Colin V-33 tree, grafted on an Edranol seedling. The Colin V-33 topgraft exhibited the 'lace leaf' symptom shown in Fig. 2.1 D and E. Preliminary analysis of the virus identified it as TNV by immunosorbant electron microscopy (ISEM) studies utilising anti-TNV-D-type serum (Jaffer *et al.*, 1993). Electron microscopic studies showed the presence of a smaller icosahedral particle which was assumed to be satellite tobacco necrosis virus (STNV-Avo).

TNV-Avo can be detected in avocado leaf concentrates, although the concentration is very low. Therefore several alternate hosts including *Phaseolus vulgaris* cv. Bonus, *Cucurbita pepo* cv. Caserta bush and *Cucumis sativa* cv. White rust resistant, were used to propagate the TNV-Avo. Detached *C. pepo* and *C. sativa* seedlings in the cotyledonous stage were used as the main propagation hosts for TNV-Avo.

No particular effort was made to study the STNV-Avo particle or to determine its immunological relationship to other STNV isolates. Some biophysical and biochemical characteristics of STNV-Avo were determined.

2.2.2 Virus inoculation

For virus inoculation, TNV-Avo refers to the virus mixture isolated from avocado and contains both the TNV and STNV particles. A concentrated preparation of TNV-Avo from infected *C. pepo* cotyledons (four passages in *C. pepo*) was supplied by M.B. von Wechmar for this work.

Detached *C. pepo* and / or *C. sativa* seedlings in the cotyledonous stage were inoculated with the starting material mixed with carborundum (180 grit). Successive inoculations were done with plant sap obtained from infected plant material ground in 0.01 M phosphate buffer (pH 7.0) with carborundum in a mortar with pestle and subjected to low speed centrifugation at 10 000*g* (10 000 RPM for 10 min at 10 ^{0}C in a SS34 rotor in a Sorvall RC-5 superspeed refrigerated centrifuge). Mechanical inoculation was achieved by dipping fingers into the plant sap and rubbing gently, yet firmly over the surface of the detached leaves harvested

from plants grown in sterilised soil in a temperature and humidity controlled plant growth room (Addendum C.1). The leaves were placed on moist paper towel on trays and the trays placed in a plastic bag to maintain a high humidity and thus prevent the leaves from wilting. A beaker was placed in the centre of the tray to ensure that an air space remained between the leaves and the plastic sheeting (Fig. 2.2 D).

2.2.3 Virus purification

The virus was purified from infected detached *C. sativa* or *C. pepo* cotyledons 48 hrs post inoculation. Purification was based on the method of Uyemoto and Gilmer (1971) utilizing differential centrifugation. Unless otherwise stated the buffer used was 0.01 M phosphate (pH 7.0) (Addendum B.1). Infected leaves were reduced to a pulp with carborundum in buffer (100 ml / 50 g), using a mortar and pestle. The sap was filtered through cheesecloth and subjected to low speed centrifugation at 10 000*g* (10 000 RPM, SS 34 rotor in a Sorvall RC-5 refrigerated centrifuge) for 10 min at 10 °C. The supernatant was subjected to ultracentrifugation at 100 000*g* (30 000 RPM, type 35 rotor, Beckman L5/65 ultracentrifuge) for 120 min at 10 °C in order to pellet the virus. The pellet was resuspended in buffer and then centrifuged at 10 000*g* (as before) for 10 min at 10 °C. This concentrated virus preparation was layered on top of a 10 % - 40 % sucrose gradient (Addendum B.3) and centrifuged at 82 000*g* (25 000 RPM, SW 28 rotor, Beckman L5/65 ultracentrifuge) for 150 min at 20 °C. After centrifugation the gradients were observed in the dark with a fiber-optic light. The light scattering viral bands were removed by displacement in an ISCO density gradient fractionator coupled to an ISCO optical unit, or by tube puncture with a syringe and needle. The sucrose solution containing the viral band was diluted 1 in 3 with buffer and subjected to ultracentrifugation at 150 000*g* (35 000 RPM, 60Ti rotor Beckman L6/55 ultracentrifuge) for 120 min at 10 °C. The final viral pellet was resuspended in buffer and then filtered first through a 0.45µm acetate and then a 0.22µm acetate filter (MSI) and stored in a sterile bottle at 4 °C for further analysis. Virus prepared in this manner will be referred to as purified virus.

2.2.4 Antiserum preparation and antisera used

One mL of a 1 mg/mL purified virus preparation was emulsified in 1 mL of Freund's incomplete adjuvant and injected intramuscularly into the thigh muscle of rabbits. Four rabbits were injected with TNV-Avo and four with STNV-Avo. The rabbits were injected weekly for four weeks followed by a booster injection four weeks after the last weekly injection (Rybicki and von Wechmar, 1985). The serum was taken weekly after the fourth injection for several weeks. Other antisera used were prepared by M.B. von Wechmar to a variety of TNV isolates under research (See Addendum A, Table A.3).

2.2.5 Biophysical and biochemical characterization

The characterization of TNV-Avo and STNV-Avo was done with virus preparations purified by rate zonal density gradients in a 10 % - 40 % sucrose gradient (Addendum B.2).

2.2.5.1 *Ultraviolet absorption*

The ultraviolet absorption spectrum of the viruses in the top and bottom light scattering bands were determined in a Beckman DU-64 spectrophotometer. The virus preparations were diluted 1 : 50 in water.

2.2.5.2 *Morphology*

The morphology of the virus was determined by electron microscopy (EM) using negative staining. The virus preparations were adsorbed to carbon coated copper grids, washed and stained with 2 % uranyl acetate (pH 4.2) as described in Addendum B.6.1 (Milne, 1984). The grids were examined on a Jeol 200 CX electron microscope and photographed at 30 000 x magnification.

2.2.5.3 *Relative sedimentation coefficient*

The relative sedimentation coefficients of TNV-Avo and STNV-Avo were determined by rate zonal density gradient centrifugation through a 10 % - 40 % sucrose gradient. Tobacco mosaic virus (TMV), turnip yellow mosaic virus (TYMV) and brome mosaic virus (BMV) were used as standards in reference gradients run in the same rotor. One mL of a 1 mg/mL virus preparation was layered on top of the gradients and centrifuged at 82 000*g* (25 000 RPM) in a SW 28 Beckman swinging bucket rotor for 150 min at 10 °C (Addendum B.2). The distance of migration of the viral bands was plotted on log paper and the linear

relationship between migration distance and the sedimentation coefficient of the known viruses was used to determine the relative sedimentation coefficients of TNV-Avo and STNV-Avo.

2.2.5.4 *Buoyant density*

The buoyant densities of TNV-Avo and STNV-Avo were determined by isopycnic density gradient separation using cesium chloride salt to make a self-forming gradient. Virus samples were layered on a 1.4 g/mL cesium chloride solution and centrifuged at 114 000*g* (35 000 RPM) in a SW 50.1 Beckman swinging bucket rotor for 18 hrs at 20 °C (Addendum B.3). The refractive index of the viral containing band was measured using a hand held Atago refractometer and the buoyant density determined from standard Beckman tables (Griffith, O.M., Techniques of preparative, Zonal, and continuous flow ultracentrifugation, 1979).

2.2.5.5 *Protein molecular weight (Mr) determination*

TNV-Avo and STNV-Avo preparations were disrupted in 62.5 mM Tris-Cl (pH 6.8), 5 % SDS and 5 % BME by heating at 96 °C for 10 min (Addendum B.4.1). The number and the Mr's of the virus proteins were determined by SDS-PAGE on a 12 % gel using the discontinuous buffer system of Laemmli (1970) (Addendum B.4). Pharmacia LMW proteins were used as standards and the gel was stained with PAGE blue (Addendum B.4.1.2). The protein Mr's were determined from the linear relationship between the distance of migration and the log of the Mr of the standards.

2.2.5.6 *Genomic RNA size*

Single-stranded RNA of TNV-Avo and STNV-Avo was isolated from purified virus preparations in 10 mM Tris-Cl (pH 8.25), 1 mM EDTA and 1 % SDS and extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) (Addendum B.7.1). The RNA was stored in ethanol at -20 °C and salt precipitated (Sambrook *et al.*, 1989, Addendum B.7.2). The RNA was run in a formaldehyde denaturing agarose gel (Sambrook *et al.*, 1989, Addendum B.7.1) with RNA Mr markers (Gibco BRL). The gels were stained with EtBr (Addendum B.7.1) and viewed on a 254 nm transilluminator.

2.2.6 Immunological assays

2.2.6.1 *Immunoelectroblotting (IEB)*

Virus preparations were disrupted in 62.5 mM Tris-Cl (pH 6.8), 5 % SDS and 5 % BME by heating at 96 °C for 10 minutes (Addendum B.4.1). Protein disrupts were electrophoresed through 12 % acrylamide gels using the discontinuous buffer system of Laemmli (1970) (Addendum B.4). The proteins were electrophoretically transferred to nitrocellulose membrane (MSI, 0.45 µm pore size) essentially according to the method of Towbin (1979) (Addendum B.5.1). The membranes were probed with polyclonal antisera listed in Table 2.1.

2.2.6.2 *Immunosorbant electron microscopy(ISEM)*

Antisera listed in Table 2.1 were used for ISEM studies to trap and decorate TNV-Avo particles according to the method of Milne (1984) (Addendum B.6.2). The preparations were stained with 2 % uranyl acetate, pH 4.2. The grids were examined in a Jeol 200CX electron microscope and viewed at 30 000 x magnification.

Table 2.1 List of antisera used for identification and serological typing of TNV-Avo in Immunoelectroblot and Immunosorbant electron microscopy assays.

Assays	A-type antisera	D-Type antisera
IEB	Anti-TNV-Bean/1	Anti-TNV-Avo, Anti-TNV-Citr, Anti-TNV-D/RK
ISEM	Anti-TNV-Fu/M.c.	Anti-TNV-D/RK, Anti-TNV-Avo

Refer Addendum A, Table A.3 for antisera details.

2.3 Results

2.3.1 Host range studies and propagation

TNV-Avo could be mechanically transmitted to susceptible detached *C. pepo* and *C. sativa* cotyledons and *P. vulgaris*, *N. benthamiana*, *N. glutinosa*, *N. tabacum* cv. Xanthi and *C. quinoa* leaves (Table 2.2). The virus produced local lesions in infected cotyledons and leaves indicating a non-systemic infecting virus. The local lesion symptoms on the *C. pepo* appeared dark and sunken. The *C. sativa* cotyledons developed pale, sunken local lesions. See Fig. 2.2.

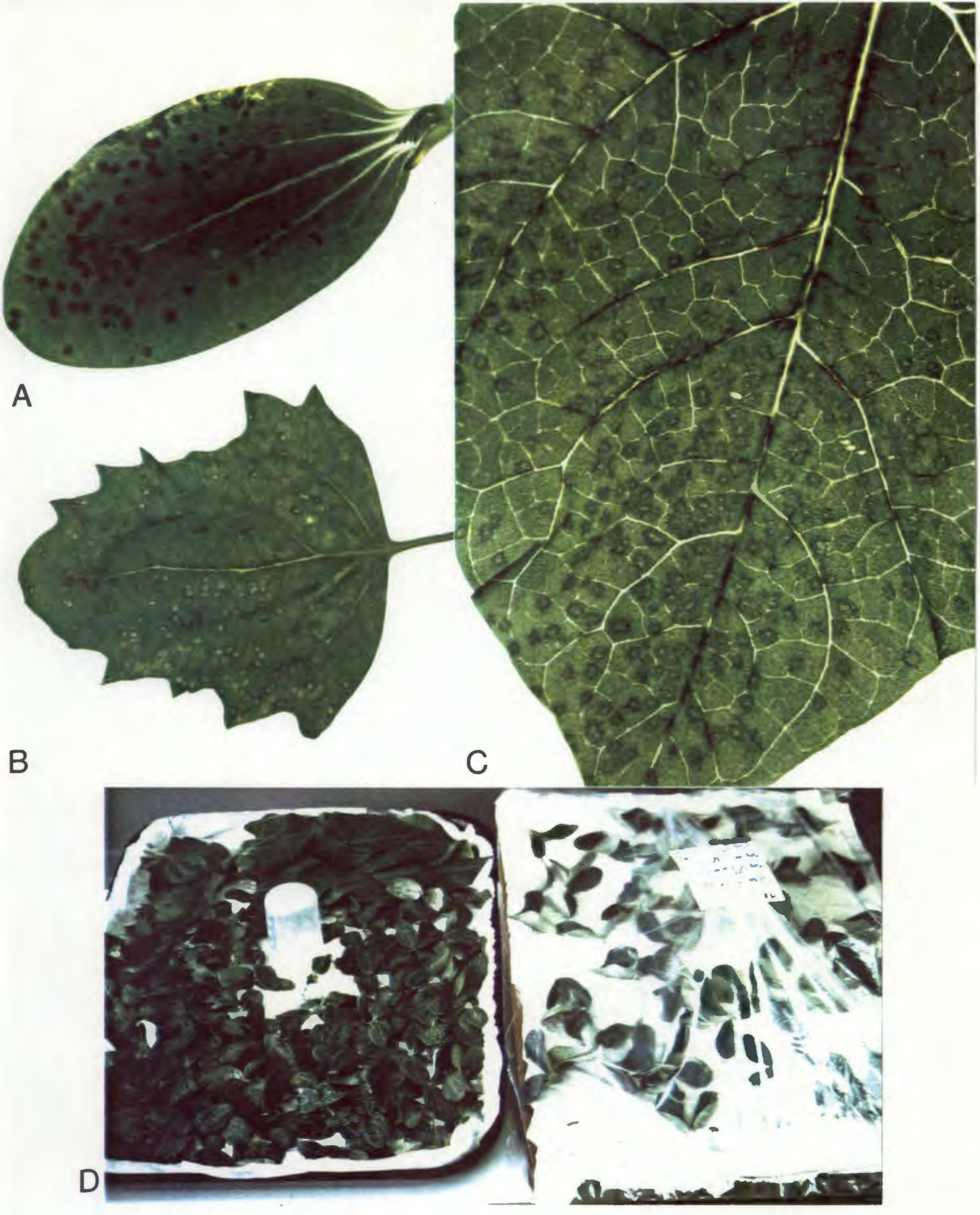


Figure 2.2 Symptoms of TNV-Avo on: A) *C. pepo* cotyledons, B) *C. quinoa* leaf, C) *P. vulgaris* leaf and D) detached *C. sativa* cotyledons and a few other leaves on a tray (left) and a tray with detached *C. pepo* seedlings inside a plastic bag.

Table 2.2 Typical symptoms of the TNV-Avo on selected host plants.

Host	Symptoms
Cucumis sativa cv. Rust resistant	small white, sunken local lesions
Cucurbita pepo cv. Caserta bush	large dark, sunken local lesions
Chenopodium quinoa	small dry local lesions
Phaseolus vulgaris cv. Bonus	large brown local lesions
Nicotiana benthamiana	small, pinprick local lesions
Nicotiana glutinosa	scorch, pin prick local lesions
Nicotiana tabacum cv. Xanthi	scorch, pin prick local lesions
Nicotiana tabacum cv. Soulouk	no symptoms

2.3.2 Virus purification

The purification protocol mentioned was found to be successful if followed to completion in one to two days. The low speed precipitates, and the high speed supernatants were found to contain infectious virus when indicator plants were mechanically inoculated, indicating the loss of some infectious virus in each step of the differential centrifugation procedure. After the first cycle of ultracentrifugation, the virus preparation was still very impure and appeared dark green. Most of these impurities and host proteins were removed in the rate zonal density gradient centrifugation step.

Rate zonal density gradient purification gave two light scattering bands, at approximately 15 mm and 25 mm from the meniscus. When the gradients were fractionated with an ISCO gradient fractionator and the UV absorbance of the gradient was determined, two clear absorbance peaks were observed, with a smaller absorbance peak directly below and adjoining the second peak. These peaks were collected by an ISCO gradient fractionator. The sucrose was removed by diluting the various peaks with three parts buffer and subjecting to ultracentrifugation. The pellets were light brown or light green in colour. Ultraviolet absorption spectra for the light scattering bands showed a peak at 260 nm. The top absorbance peak had a 260/280 ratio of 2.02 and the second absorbance peak had a 260/280 ratio of 2.0.

Electron microscopy of the first absorbance peak, corresponding to the light scattering band at 15 mm, contained mostly the smaller STNV virus particles interspersed with a few larger TNV particles, Fig. 2.3 A. The second absorbance peak, corresponding to the bottom light scattering band at 25 mm, contained mostly TNV particles with a small percentage of the smaller STNV particles. The TNV particles sometimes had an affinity to aggregate into a crystal structure, Fig. 2.3 B. TNV particles in the crystal structure appear to be smaller and have a distinct hexagonal shape when compared to the unattached virus particles. The small third absorbance peak, with no corresponding light scattering band contained an equal mixture of TNV and STNV particles, Fig. 2.3 C.

2.3.3 Antisera

Antibodies to the TNV-Avo and STNV-Avo were prepared. The antibodies were tested by IEB against homologous and heterologous antigens. The TNV-Avo particles were antigenic and good antisera was raised. This can be seen in immunological assays shown in Figs 2.6 B and 2.7 A. The STNV-Avo particles were less immunogenic and poor quality antisera was obtained to the STNV particles. Antibodies to STNV-Avo were prepared twice, with the same result, thus attempts to make antibodies to STNV-Avo were stopped. The antisera were used for IEB and ISEM assays.

2.3.4 Virus morphology

The TNV-Avo particles were icosahedral in shape with a hexagonal outline and a diameter of approximately 28 nm. The particles tended to form a crystal structure (Fig. 2.3 B). The STNV-Avo was also icosahedral in shape, but smaller in size with a diameter of approximately 18 nm (Fig. 2.3 A).

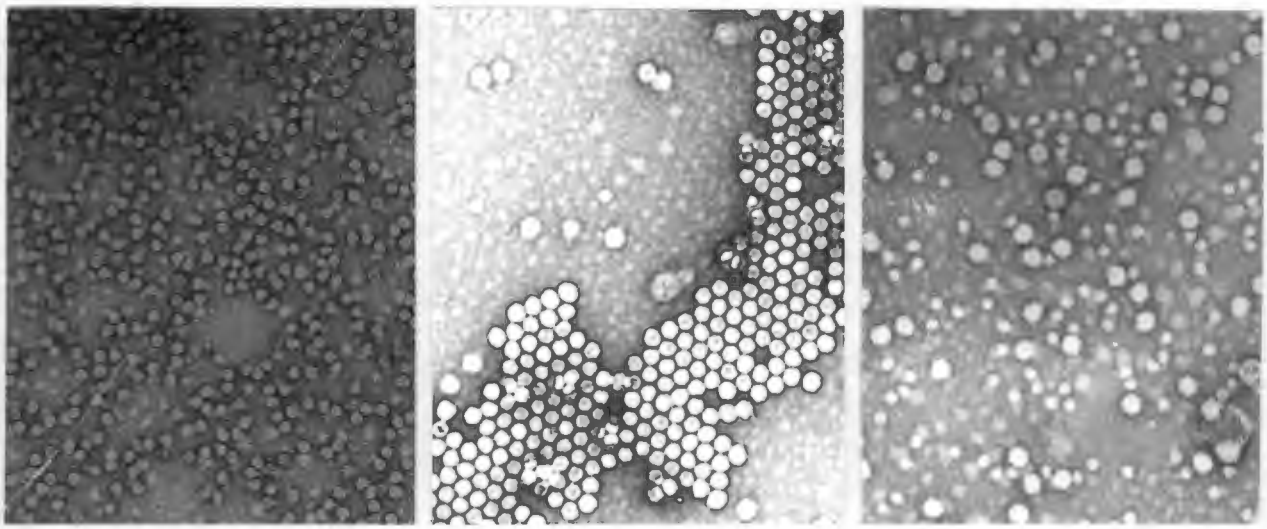


Figure 2.3 Electron micrographs of the TNV-Avo and STNV-Avo virus particles. Micrograph A: Top light scattering band containing mostly STNV particles with a few larger TNV particles, Micrograph B: Bottom light scattering band containing TNV particles in a crystal structure and some free TNV particles, a few smaller STNV particles can be seen. Micrograph C: Small absorbance peak below the light scattering bands with an equal amount of TNV and STNV particles. The particles are negatively stained with 2% uranyl acetate, pH 4.2. The micrographs are at the same magnification, the bar represents 150 nm.

2.3.5 Biophysical and biochemical characteristics

The TNV-Avo and STNV-Avo each had only one major protein product, with a Mr of 31 kDa and 22 kDa respectively and one genomic RNA component, with a size of 3.8 kb and 1.3 kb respectively. The relative sedimentation coefficient, buoyant density, the Mr's of the viral protein (Fig. 2.4 lanes 3 and 4) and the genomic RNA size (Fig. 2.5 lanes 2 and 3) are listed in Table 2.3.

Table 2.3 Biochemical and Biophysical characteristics of TNV-Avo and STNV-Avo.

	TNV-Avo	STNV-Avo
Morphology	Icosahedral	Icosahedral
Approximate particle size[1]	28 nm	18 nm
Coat protein size[2]	31 kDa	22 kDa
Genomic RNA size[3]	3.8 kb	1.3 kb
Relative sedimentation coefficient[4]	118 S	49 S
Buoyant density[3]	1.394 g/ml	1.387 g/ml

1 Average diameter of 100 particles stained with uranyl acetate pH 4.2

2 Average of three determinations

3 One determination only

4 Average of two determinations

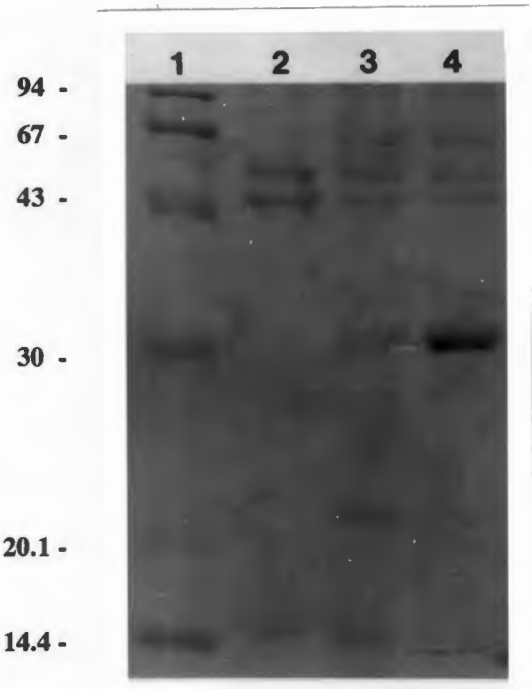


Figure 2.4 SDS-PAGE analysis of the proteins of the TNV-Avo and STNV-Avo viruses. The lanes contain: 1) MWt markers in kDa, 2) TNV-Avo (isolated from *C. sativa*), 3) STNV-Avo (isolated from *C. pepo*), 4) TNV-Avo (isolated from *C. pepo*). The gel was stained with PAGE blue.

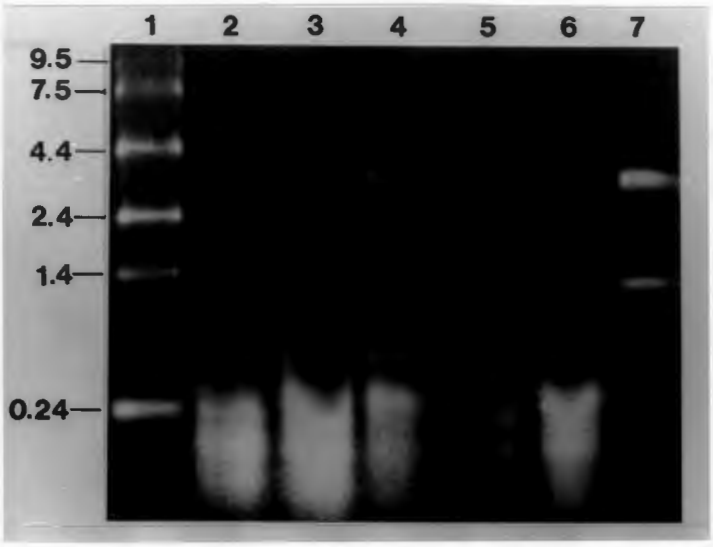


Figure 2.5 Formaldehyde denaturing agarose gel analysis of the genomic RNA extracted from the TNV-Avo and STNV-Avo virus particles. The lanes contain: 1) MWt markers in kb, 2) STNV-Avo (isolated from *C. pepo*), 3) TNV-Avo (isolated from *C. pepo*), 4) TNV-Avo (isolated from *C. sativa*), 5) STNV-Avo (RNase treated), 6) TNV-Avo (RNase treated), 7) TNV-Avo (concentrated preparation from *C. pepo*). The gel was stained with ethidium bromide.

2.3.6 Immunological assay

The TNV-Avo reacted with anti-TNV-D-like antisera in IEB assays. Figure 2.6 illustrates the serological relationship of TNV-Avo (lane 2) and STNV-Avo (lane 3) isolates when compared to TNV-Citr (lane 1) (Addendum A, Table A.1) and TNV-Wheat (lane 4) (Addendum A, Table A.1).

The strong reaction of TNV-Avo antigen with anti-TNV-D-sera (Fig. 2.6 B, C, D) and no reaction with anti-TNV-A sera (Fig. 2.6 A) shows that TNV-Avo is closely related to the TNV D-like strains. The anti-TNV-D/RK serum reacted with the A-like and D-like TNV antigens and thus does not distinguish between the isolates. The STNV-Avo did not react with either the anti-TNV-A or anti-TNV-D sera, indicating that it is serologically distinct from the TNV-Avo.

Similarly ISEM assays (Fig. 2.7) show that the TNV-Avo is trapped and decorated with anti-TNV-D antisera; the anti-TNV-A sera (TNV-Fu/M.c.) (Addendum A, Table A.3) trapped very few TNV-Avo particles but did not decorate any TNV-Avo particles. The degree of trap and decoration of TNV-Avo with anti-TNV-Avo serum was much higher than the trap and decoration obtained with the anti-TNV-D/RK serum. Due to the poor antigenicity of the

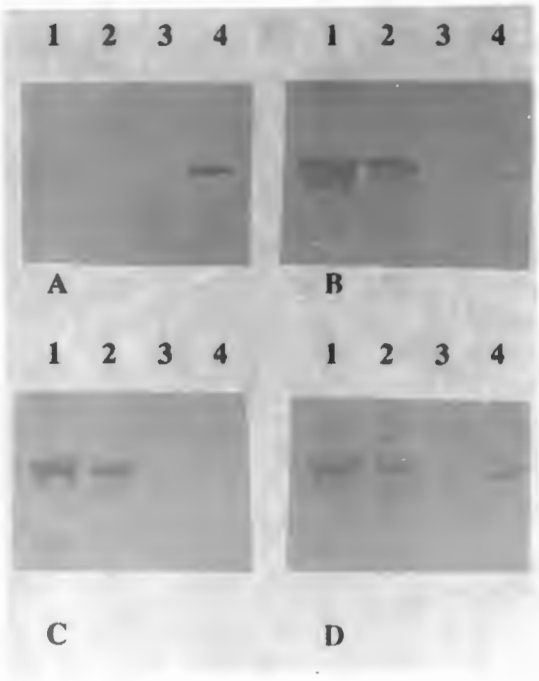


Figure 2.6 Immunoelectroblot analysis of TNV-Avo compared to an A-like and a D-like TNV isolate. The lanes of the blots contain: 1) TNV-Citr, 2) TNV-Avo, 3) STNV-Avo, 4) TNV-Wheat. The blots were probed with the following antisera: A) anti-TNV-Bean/1, B) anti-TNV-Avo, C) anti-TNV-Citr, D) anti-TNV-D/RK.

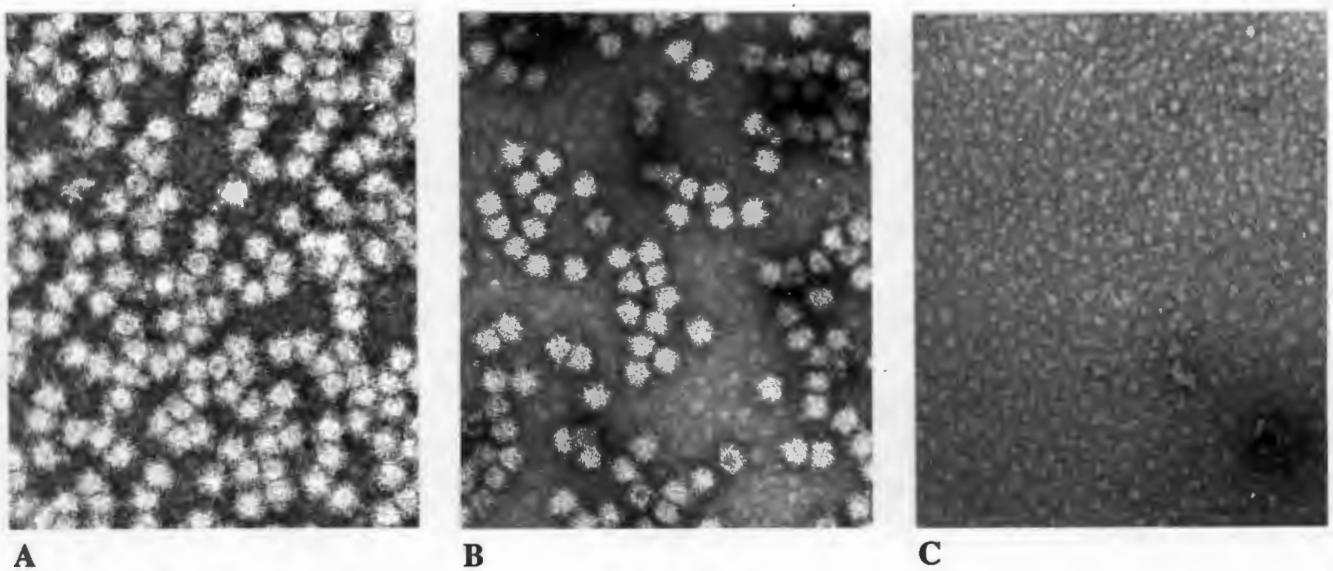


Figure 2.7 Immunosorbant electron micrographs of TNV-Avo particles. The following antisera were used: A) anti-TNV-Avo, B) anti-TNV-D/RK, C) anti-TNV-Fu/M.c.. The particles were negatively stained with 2% uranyl acetate, pH 4.2. The micrographs are at the same magnification, the bar represents 150 nm. ———

smaller STNV-Avo particles, and the failure to prepare high titred antisera to STNV-Avo, immunological assays with the STNV-Avo were not successful.

2.4 Discussion

The virus isolated from the foliage of avocado trees was positively identified as a Necrovirus from the biophysical characteristics, serological analysis and the necrotic local lesion symptoms on inoculated detached leaves. The sedimentation coefficient, buoyant density, number and size of the virus proteins and type and size of the genomic nucleic acid, are similar to the values listed by Kassanis (1970a), Koenig (1988) and Lommel (1995). The virus isolate contained a smaller particle, and from its characteristics was identified as satellite tobacco necrosis virus (Kassanis, 1970b).

The propagation of TNV-Avo was hampered by inoculated detached leaves becoming very soft and 'dissolving' between 24 and 48 hrs. This was thought to be due to the enzymatic action of endophytic bacteria from the original virus source, which may be associated with the virus (Jaffer *et al.*, 1993; von Wechmar, pers comm.). This problem was overcome by removing the bacteria from the virus inoculum by low speed centrifugation before the sap was inoculated onto host plant leaves.

The virus was not entirely pure of all contaminants (see high molecular weight protein bands in Fig. 2.4), however the purification protocol of TNV-Avo and STNV-Avo was found to be adequate for further analysis. Purification of TNV-Avo was achieved by a single cycle of differential centrifugation followed by rate zonal density gradient centrifugation, using low molarity neutral phosphate buffers. The TNV-Avo and STNV-Avo virus particles were not totally separated in the rate zonal centrifugation step. The various absorbance peaks contained STNV-Avo : TNV-Avo in the following approximate ratios: 99:1; 10:90; 50:50. The higher pecentage of STNV in the TNV absorbance peaks can be explained by the tendency of STNV-Avo particles to aggregate during purification (as reviewed by Fraenkel-Conrat, 1988) and thus change their sedimentation rate (Kassanis and Nixon, 1961). The tendency of STNV particles to aggregate would explain the presence of STNV in the TNV bands, because some of the STNV aggregates and the TNV particles might have a similar sedimentation coefficient. The larger TNV particles would not have the same sedimentation coefficient as STNV particles and thus could not sediment in the STNV band. The TNV

contaminants in the STNV band could be due to the removal of the STNV bands from the gradient tube.

Following sucrose gradient centrifugation, the bands were diluted and the virus particles concentrated by ultra centrifugation. The pelleting of the virus might cause TNV particles to form crystal sheets. Large crystal sheets may be damaged or broken up, or might be lost in the low speed centrifugation after the ultracentrifugation concentration. Therfore only small crystal sheets may be seen in electron microscopy (Fig. 2.3 B). TNV crystals were not often seen, but the crystals noted may be due to the specific conditions of the purification procedure for this particular TNV-Avo preparation.

The serological relationship of TNV-Avo fits into the serological relationship proposed by Babos and Kassanis (1963), which divides the Necroviruses into two distinct serological groups. The TNV-Avo was determined to be closely related to the TNV D-like serotypes when examined by IEB and ISEM assays. The TNV-Avo was indistinguishable from the TNV-Citr (see Addendum A, Table A.1) in the IEB assays. Examination of the ISEM data indicated that the TNV-Avo is different from the TNV-D/RK due to the degree of decoration by anti-TNV-Avo and TNV-D/RK sera. The anti-TNV-D/RK serum had antibodies that recognised A- and D-type TNV isolates (Fig. 2.6). This could result in the different degree of decoration by this antisera when compared to decoration tests with the homologous anti-TNV-Avo serum. Due to the unavailability of antisera to STNV-Avo, it was not possible to determine the relationship of the STNV-Avo to known STNV strains. Antibodies prepared to TNV-Avo were determined to be type-specific and only reacted with D-Type TNV isolates. The antisera prepared to the STNV-Avo had a very low titre and did not react with purified STNV-Avo in IEB tests, leading to the assumption that the STNV-Avo is weakly immunogenic.

In conclusion, the virus isolated from avocado cv. Colin V-33 was identified as TNV by partial characterization. The D-like TNV isolate was associated with STNV. Its effect on dwarfing has not been determined and the origin of the virus is uncertain considering the fact that the Colin V-33 was grafted on local Edranol root stock grown from seed.

Chapter 3

Detection and identification of TNV in Potato (*Solanum tuberosum* L.) cvs. Up-to-Date and Buffelspoort-13

Contents

3.1 Introduction

Attempts at diagnosis can be done from preliminary studies such as infectivity assays, host range, symptom expression and mode of transmission. However these diagnostic assays are not reliable methods for virus identification. A more sensitive and less time consuming method of detection and identification of viruses is based on immunological assays, which can be applied to crude plant extracts and purified virus preparations (Hamilton *et al.*, 1981).

For an undergraduate student project dealing with viral diseases of potatoes, certified virus-free (free of potato virus Y and potato leafroll virus) seed potatoes (*Solanum tuberosum* cvs Up-to-Date and Buffelspoort-13) were obtained for use as controls in assays. The control Up-to-Date and Buffelspoort-13 potatoes were sprouted and grown in sterilized soil. The foliage showed abnormal necrosis. To determine whether these potato plants were free of virus, the foliage of the Buffelspoort-13 potato was ground to a fine paste with carborundum (180 grit), using a mortar and pestle and concentrated by one cycle of differential centrifugation. This concentrated preparation was tested in IEB assays with anti-PVY (normal strain), anti-PVY (necrotic strain) and anti-TNV-sera (Fig 3.1). For details of antisera refer to Addendum A, Table A.3. The anti-TNV-Bean/1 serum recognized a protein of approximately 30 kDa in the concentrated Buffelspoort-13 control.

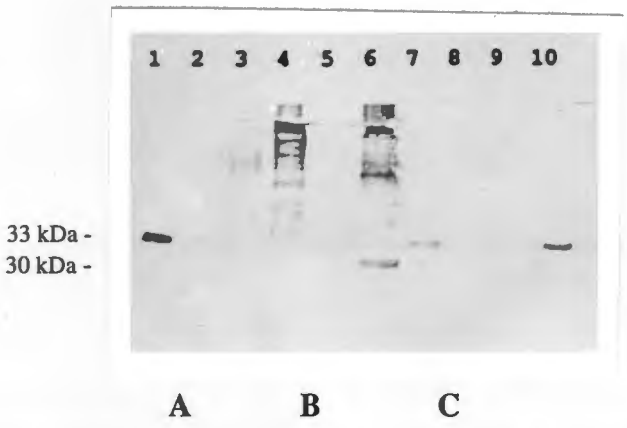


Figure 3.1 Immunoelectroblot assay to identify viruses found in potatoes. The lanes contain the following antigens: 1and7) Potato virus Y (normal strain), 2,5and8) Potato sample, 3,6and9) Buffelspoort-13 potato control, 4) Potato virus X, 10) Potato virus Y (necrotic strain). The following antisera were used to probe the blots: A) lanes 1-3, anti-PVY (normal strain), B) lanes 4-6, anti-TNV-Bean/1, C) lanes 7-10, anti-PVY (necrotic strain).

This was an unexpected result and focussed attention on the control seed potatoes, which exhibited an unexplained abnormal appearance, grown under controlled conditions in plant growth rooms. The leaves of the potato plants had a bronzed colour and the leaf edge became necrotic. The overall appearance of the plants was reminiscent of nutrient stress. An inspection of field grown seed potatoes, grown in the West Coast Sandveld region of the Western Cape, indicated that the plants had similar abnormal appearances as the plants grown in the controlled conditions of the plant growth rooms. Plant and tuber samples of the Buffelspoort-13 and Up-to-Date varieties were taken from the potato fields (Fig. 3.2 A).

In an effort to rule out a possible nutrient stress factor due to the soil used, tubers of the same source were surface disinfected and grown in sterilized vermiculite in canning jars covered with gauze to prevent virus transmission by aphids. A set of control Up-to-Date and Buffelspoort-13 potatoes were surface disinfected for 10 min in 2 % hypochlorous acid. Half the number of each potato variety were grown in canning jars with vermiculite and nutrient solution, the other half of the potatoes were left on the laboratory bench to sprout. The Up-to-Date potatoes sprouted and grew into plants, the Buffelspoort-13 potatoes developed a soft rot and did not sprout. Unidentified culturable organisms were isolated from the Buffelspoort-13 soft rot potatoes and grown on culture media.

The Up-to-Date potatoes grown in the jars with nutrient solution developed symptoms (Fig. 3.2 B) similar to those observed in the field grown Up-to-Date potatoes and those grown in soil in the controlled plant growth rooms. This indicated that an undetected disease was apparently present in these seed potatoes and the symptoms might be caused by the presence of TNV.

The presence of TNV in potatoes has previously been described by Noordam (as quoted by Hooker, 1981), and causes the ABC disease of potatoes (Kassanis, 1970a). The symptoms noted by Noordam however differed from the symptoms observed locally. To verify the result obtained in the student project, further diagnostic work was conducted on different potatoes, from the same source.

This chapter deals with the detection of TNV in a source of the Buffelspoort-13 potato variety and the detection, extraction, transmission and the identification of TNV in a source of Up-to-Date potato variety. Biological and immunological techniques were used to detect and assay

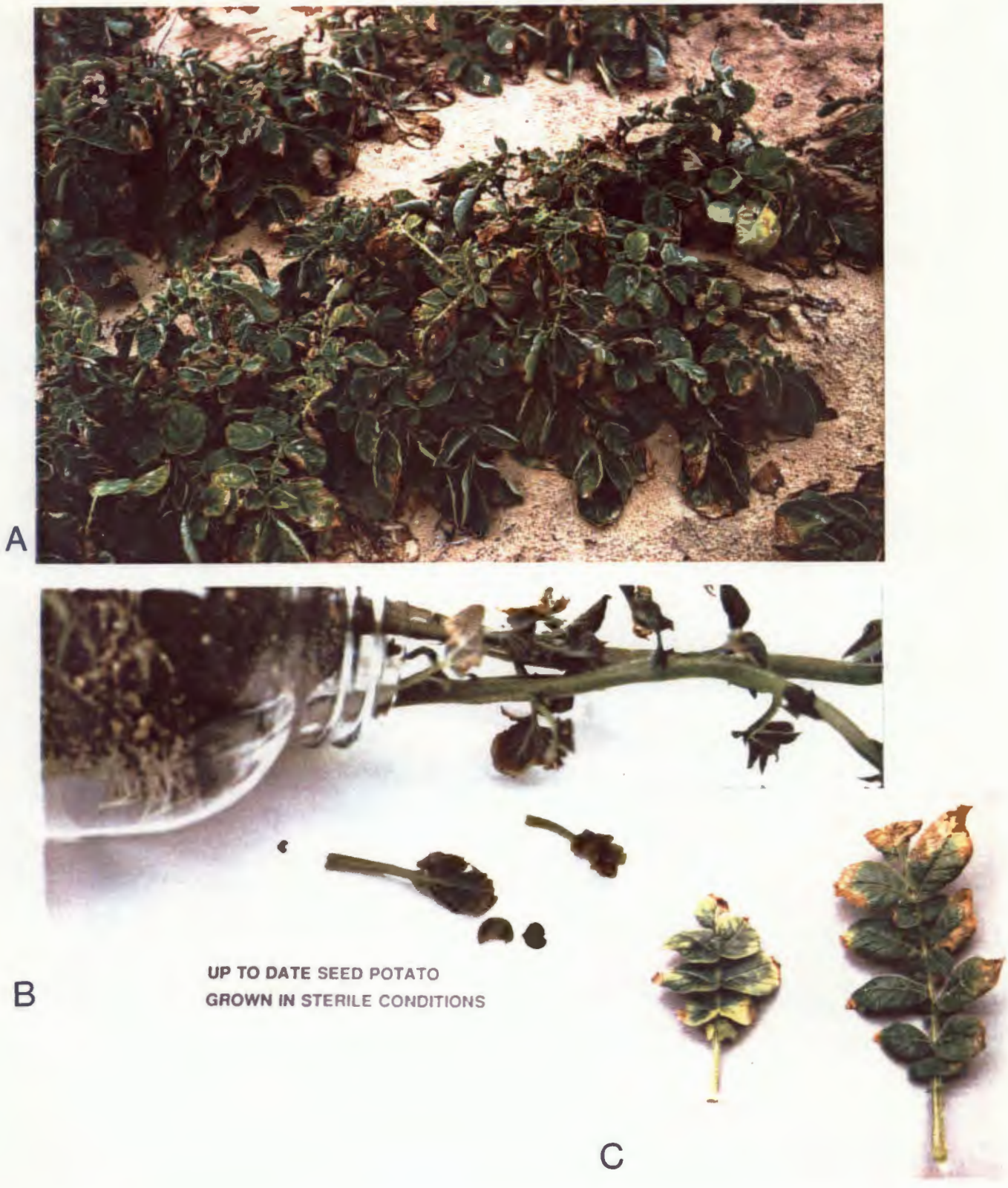


Figure 3.2 A) Buffelspoort-13 potatoes in the field with scorched leaves, B) Up-to Date potato grown in semi-sterile conditions with nutrient solution in a canning jar, C) Details of symptoms noticed on potato foliage.

for the virus. Immunological and nucleic acid hybridization techniques were used to identify the virus. The TNV isolate from the Up-to-Date variety (TNV-Pot/Upd) was subjected to further molecular analysis (see Chapters 4 and 5).

3.2 Materials and methods

3.2.1 Virus isolates

The virus detected in Buffelspoort-13 potatoes will be referred to as TNV-Pot/BP and the virus isolated from Up-to-Date potatoes will be referred to as TNV-Pot/Upd. TNV was initially detected in Buffelspoort-13 seed potatoes, but the TNV identified and studied in this chapter was isolated from the skin of the Up-to-Date seed potato variety.

3.2.2 TNV isolation and propagation

Up-to-Date and Buffelspoort-13 seed potatoes obtained as "virus-free" control potatoes were surface disinfected with 2 % hypochlorous acid for 10 min, germinated and grown in canning jars with vermiculite and Knopps nutrient solution (Addendum C.3). The skin of the Up-to-Date potato tuber was sliced off the tubers with a surgical blade, ground to a fine paste with carborundum (180 grit) and 0.01 M phosphate buffer pH 7.0 (Addendum B.1), using a mortar and pestle, and subjected to differential centrifugation (see Chapter 2 Section 2.2.3). Sterile carborundum (180 grit) was added to the resuspended pellet of the Up-to-Date potato and mechanically inoculated to detached *C. quinoa*, *N. benthamiana*, *N. tabacum* cvs Xanthi and Soulouk, and *Gomphrena globosa* leaves and *C. sativa* and *C. pepo* cotyledons (Addendum A, Table A.2). A few local lesions developed on *C. quinoa* leaves after three to four days. These leaves were used to inoculate a larger number of leaves for virus purification.

3.2.3 TNV-Pot/Upd purification

The *C. quinoa* leaves were covered with an infinite number of necrotic local lesions 48 hours post inoculation (Fig. 3.3). The TNV-Pot/Upd was purified as mentioned in Chapter 2 Section 2.2.3 The light scattering band was removed from the rate zonal sucrose density gradient by tube puncture with a syringe and a needle. Virus purified in this manner will be referred to as purified virus.

3.2.4 Antiserum preparation

Antisera to the TNV-Pot/Upd was made as mentioned in Chapter 2 Section 2.2.4. For details of antisera see Addendum A, Table A.3.

3.2.5 Immunological assay

3.2.5.1 *Tissue blot*

Initial detection of TNV in potato plant tissue was by tissue immunoblots as described by Bravo-Almonacid *et al.* (1992). The stems of near mature field grown Buffelspoort-13 potato plants and potatoes grown in nutrient solution in canning jars, were cut into 2 cm long sections with a surgical blade and the newly cut surface pressed onto nitrocellulose paper (pore 0.45 µm, MSI) for a period of one min and left on the membrane for a further 10 min. The tissue was lifted from the membrane and the membrane left to dry for one hour. The membranes were probed with anti-TNV sera (Table 3.1) and the reaction detected by a colour indicator system (Addendum B.5.2).

3.2.5.2 *Immunoelectroblot (IEB)*

The serological relationship of TNV-Pot/Upd to TNV-Wheat (Addendum A, Table A.1) and TNV-Citr (Addendum A, Table A.1) was determined by IEB assays. The purified TNV-Pot/Upd preparations (Section 3.2.3) were disrupted in 62.5 mM Tris-Cl (pH 6.8), 5 % SDS and 5 % BME (Addendum B.4.1). The denatured proteins were electrophoresed through 12% acrylamide gels using the discontinuous buffer system of Laemmli (1970) (Addendum B.4.1). The proteins were electrophoretically transferred to a nitrocellulose membrane (pore 0.45 µm) by a modification the Towbin (1979) method (Addendum B.5.1). The membranes were probed with polyclonal anti-TNV antisera listed in Table 3.1 and the reaction detected by colour indicator system (Addendum B.5.2).

3.2.5.3 *Immunosorbant electron microscopy (ISEM)*

ISEM was used to positively identify TNV-Pot/Upd. Purified TNV-Pot/Upd (Section 3.2.3) was used in ISEM assays. ISEM was used to: 1) enrich for particles, 2) positively identify the virus isolate, and 3) determine its serotype (Milne, 1984; Addendum B.6.2). The antisera used for ISEM studies are listed in.Table 3.1. The grids were viewed on a Jeol 200 CX electron microscope and photographed at 30 000 x magnification.

Table 3.1 List of antisera used for immunological assays of the TNV-Pot/BP and TNV-Pot/Upd isolates.

Assays	TNV D-type antisera[3]	TNV A type antisera[3]
Tissue blot [1]	Anti-TNV-Avo	Anti-TNV-Bean/1, Anti-TNV-Pas, Anti-TNV-Cel
Immunoelectroblot [2] (IEB)	Anti-TNV-Pot/Upd, Anti-TNV-Citr, Anti-TNV-Pap/GL,	Anti-TNV-Bean/1, Anti-TNV-Pap/A, Anti-TNV-Apt
Immunosorbant electron microscopy [2] (ISEM)	Anti-TNV-Pot/Upd, Anti-TNV-D/RK,	Anti-TNV-PV68, Anti-TNV-Pas

1 Tissue blot assay was used on Buffelspoort-13 potato stems

2 IEB and ISEM were used on purified TNV-Pot/Upd

3 Refer Addendum A, Table A.3 for antisera details

3.2.6 Electron microscopic (EM) analysis by negative stain

To determine the morphology and approximate size of TNV-Pot/Upd, purified preparations (Section 3.2.3) were adsorbed to carbon coated copper grids, and negatively stained with 2 % uranyl acetate pH 4.2 (Milne, 1984; Addendum B.6.1).

3.2.7 Nucleic acid hybridization

The hybridization pattern of RNA or total nucleic acid, extracted from various cultured organisms isolated from Buffelspoort-13 soft rot potatoes and from the skin of Buffelspoort-13 and Up-to-Date potato tubers, were determined by dot blot hybridization assays (Addendum B.9.2.). The source of RNA or total nucleic acid is listed in Table 3.2 and was prepared by phenol/chloroform extraction (Addendum B.7.2) and stored as a salt precipitate in ethanol at -20 °C (Sambrook, 1989; Addendum B.15). The nucleic acid was dot-blotted onto a positively charged nylon membrane (Hybond N^+) using a Schleicher and Schuell minifold apparatus coupled to a water tap vacuum pump (Martin and D'Arcy, 1990; Addendum B.9.2). The membrane was probed with DIG labeled TNV-Wheat (A-like) and TNV-Pap/GL (D-like) coat protein cDNA probes (Addendum B.9.3). For details of the virus isolates used to make cDNA probes, refer to Addendum A, Table A.1.

Table 3.2 Nucleic acids used for dot blot hybridization assays to identify the presence of TNV in Buffelspoort-13 and Up-to-Date potato cultivars.

Nucleic acid	Source
TNV-A control	RNA extracted from purified TNV-Fu/M.c.*
TNV-D control	RNA extracted from purified TNV-Pap/GL*
TNV-Pot/Upd	RNA extracted from purified TNV-Pot/Upd propagated in *C. quinoa* or *N. benthamiana* leaves
Buffelspoort-13 potato	Total nucleic acid extracted from concentrate of Buffelspoort-13 potato skins
Up-to-Date potato	Total nucleic acid extracted from concentrate of Up-to-Date potato skins
Culturable organisms	Total nucleic acid extracted from culturable organisms isolated from Buffelspoort-13 potatoes showing signs of soft rot

* Refer Addendum A, Table A.1 for details of TNV isolates used as controls

3.3 Results

3.3.1 Infectivity assay

The concentrate prepared from ground Up-to-Date potato skin infected detached *C. quinoa* leaves. The symptoms on the *C. quinoa* leaves were necrotic local lesions. Leaves of other host plants inoculated with the sap of the Up-to-Date potato skins showed no disease symptoms. In further infectivity assays using infected *C. quinoa* leaves as inoculum, *G. globosa* leaves and *C. sativa* cotyledons also showed local lesion symptoms. This can be seen in Fig. 3.3.

3.3.2 Virus purification

Purification of TNV-Pot/Upd resulted in a single light scattering band approximately 25 mm from the meniscus in the sucrose rate zonal density gradient. EM analysis of this viral band showed uniform spherical particles of approximately 28 nm in size when stained with 2 % uranyl acetate pH 4.2. TNV-Pot/Upd did not appear to form a crystal structure and no smaller STNV virus particles were seen in negatively stained partially purified virus preparations (results not illustrated).

Figure 3.3 Symptoms of TNV-Pot/Upd on detached leaves of : A) *Chenopodium quinoa*, B) *Gomphrena globosa* and C) *Cucumis sativa*.

3.3.3 Immunological assays

The results of tissue immunoblots, Fig 3.4, shows that antisera raised to TNV-A type isolates reacted more strongly to the tissue blots of Buffelspoort-13 potatoes than antisera raised to the TNV-D type isolates. Normal rabbit antisera was used as a negative control.

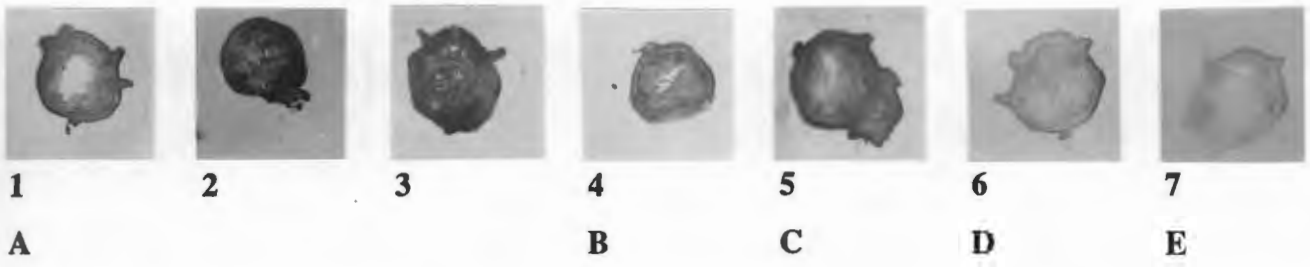


Figure 3.4 Tissue-blots of Buffelspoort 13 potato stems grown from seed potatoes. The blots were probed with the following antisera: A) Anti-TNV-Bean/1, B) Anti-TNV-Cel, C) Anti-TNV-Pas, D) Anti-TNV-Avo, E) Normal rabbit antisera. Blots 1, 4-7 represent stems of Buffelspoort-13 potato plants collected in the field, blots 2 and 3 represent stems of control Buffelspoort-13 potato plants grown in canning jars. Refer to Addendum A, Table A.3 for details of the antisera.

In IEB the anti-TNV-Pot/Upd (Fig 3.5 B) and anti-TNV-Citr sera (Fig 3.5 C), reacted strongly with the TNV-Pap/GL and TNV-Pot/Upd antigens and did not react with the TNV-Wheat antigen. The anti-TNV-Bean/1 sera (Fig 3.5 A) did not react with TNV-Pap/GL and TNV-Pot/Upd antigens but reacted with the TNV-Wheat antigen.

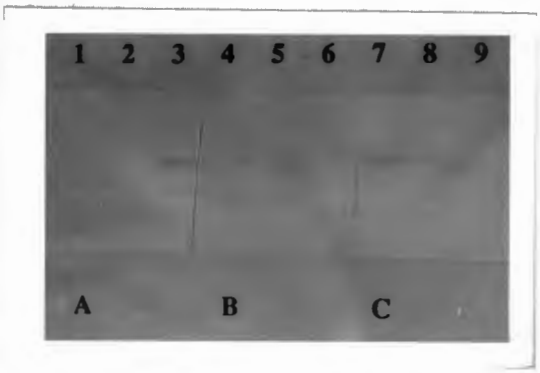


Figure 3.5 Immunoelectroblots to show the relationship between TNV-Pot/Upd, TNV-Pap/GL and TNV-Wheat. The lanes contain: 1,4and7) TNV-Pap/GL, 2,5and8) TNV-Pot/Upd, 3,6and9) TNV-Wheat. The blots were probed with the following antisera: A) Lanes 1-3, anti-TNV-Bean/1, B) Lanes 4-6, anti-TNV-Pot/Upd, C) Lanes 7-9, anti-TNV-Citr.

Electron micrographs in Fig. 3.6 illustrate ISEM results of TNV-Pot/Upd particles when reacted with several antisera. Approximately 70 % of the TNV-Pot/Upd virus particles were trapped and decorated by the homologous anti-TNV-Pot/Upd serum (Fig 3.6 A) and the anti-TNV-Pas-serum (Fig 3.6 B). Anti-TNV-D/RK (Fig. 3.6 C) and anti-TNV-PV68 (Fig. 3.6 D) trapped TNV-Pot/Upd particles, but partially decorated less than 10% of the virus particles.

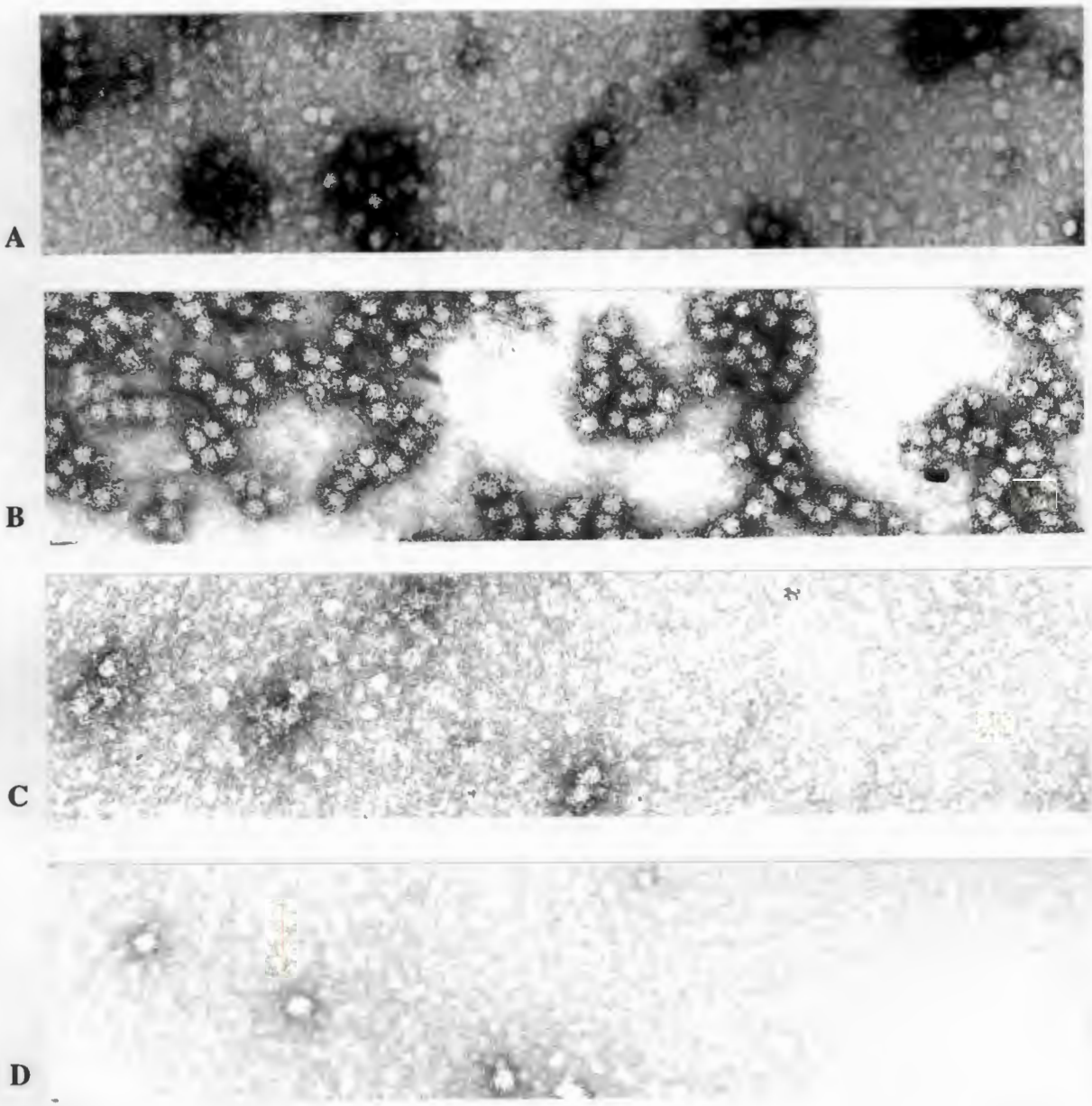


Figure 3.6 Electron micrographs of an ISEM study of TNV-Pot/Upd particles. The samples were trapped and decorated with the following antisera: A) anti-TNV-Pot/Upd, B) anti-TNV-Pas, C) anti-TNV-D/RK, D) anti-TNV-PV68. The grids were stained with 2% uranyl acetate, pH 4.2. All micrographs are at the same magnification and the bar represents 150 nm. ——

3.3.4 Nucleic acid hybridization

RNA prepared from purified TNV-Pot/Upd hybridized very strongly to the cDNA probe derived from the coat protein gene of TNV-Pap/GL (D-like probe). Two different preparations, purified at different times were tested (Fig. 3.7 B, b1, b2 and b3). TNV-Pot/Upd purified from *N. benthamiana* hybridized very weakly to the D probe (Fig. 3.7 B b4). Total nucleic acid extracted from the concentrate of Up-to-Date potato skins, from semi-dormant potatoes, hybridized to the D probe (Fig. 3.7 B, b6). Total nucleic acid extracted from concentrated Buffelspoort-13 potato skins, from semi-dormant potatoes, hybridized

weekly to both the A and D probes (Fig. 3.7 A, b7; B, b7). Total nucleic acid extracted from a bacterial culture isolated from Buffelspoort-13 potatoes hybridized to the A probe (Fig. 3.7 A, c2). Total nucleic acid, extracted from other culturable organisms, isolated from the same potato, in c1, c3 c5, c6 and c7 did not hybridize to the A or the D probe.

3.4 Discussion

TNV was found to be associated with Buffelspoort-13 and Up-to-Date field grown seed potatoes. The virus was also detected in plants grown from the same tuber source in controlled plant growth rooms, and in vermiculite with nutrient solution, thus eliminating the chance that contamination could have been associated with the soil used for growing the potatoes. The source of Buffelspoort-13 seed potatoes examined appeared to be infected with both TNV-A and TNV-D type isolates as seen in cDNA hybridisation blots (Fig. 3.7 c7). The TNV detected and isolated from the Up-to-Date seed potatoes was identified as belonging to the D-type by immunological (Fig. 3.5 and 3.6) and cDNA hybridization assays (Fig. 3.7 b1-4). Potatoes free of the symptoms were not available at the time, and thus Koch's postulates could not be proven, hence it is not known whether TNV is the causative agent for the symptoms noted in the potato foliage. This study was mainly a diagnostic exercise and no attempt was made to determine the frequency in which TNV could be detected in the potato varieties grown in separate fields.

To prove that TNV was present in the potato tubers, and to remove the possibility of TNV contamination of the potting soil (Reviewed by Kassanis, 1970; Uyemoto, 1981 and Fraenkel-Conrat, 1988), Up-to-Date potatoes were surface sterilized and allowed to start sprouting. From the skins of these potatoes a D-like TNV was isolated. This indicates that the TNV was transmitted *in vivo*, and the seed potato tuber had a primary infection. A secondary infection was ruled out from contaminated soil, because potatoes grown in fields, in sterilized soil in plant growth rooms and in nutrient solution, had the same uncharacteristic bronze colour of the foliage and necrotic leaf regions.

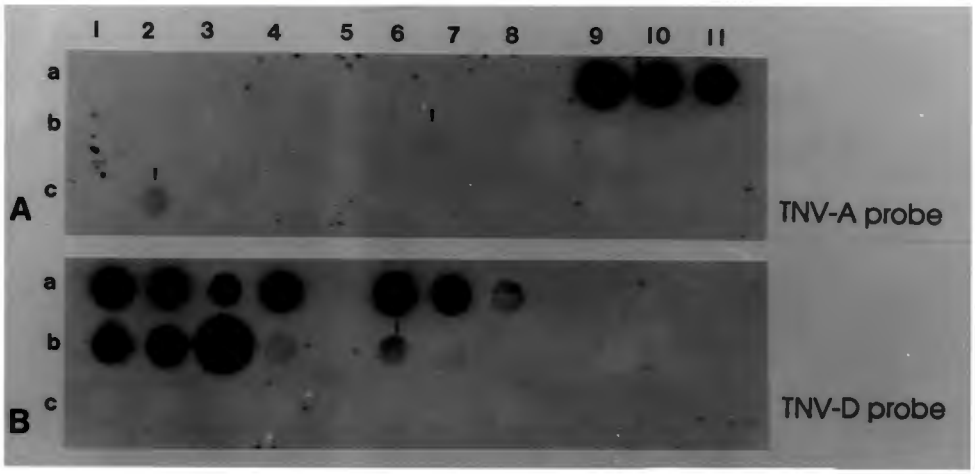


Figure 3.7 cDNA Dot Blot hybridization of genomic RNA isolated from partially purified TNV-Pot/Upd and total nucleic acid extracted from concentrate of potato peels of Buffelspoort-13 and Up-to-Date potato varieties, and culturable organisms isolated from Bufflespoort 13 seed potatoes. Blot A is probed with TNV-Wheat (A-type) coat protein probe; Blot B is probed with a TNV-Pap/GL (D-type) coat protein probe.

Row a1-3 and 6-8	TNV-D control	Row a9-11	TNV-A control
Row b1 and 2	TNV-Pot/Upd isolated from *C. quinoa*	Row b3	TNV-Pot/Upd isolated from *C. quinoa*
Row b4	TNV-Pot/Upd isolated from *N. benthamiana*	Row b6	Concentrate from Up-to-date potato skins
Row b7	Concentrate from Buffelspoort-13 potato skins	Row c1	Culturable organis,s frm BHA to BH broth*
Row c2	Culturable organisms from BHA to luria broth*	Row c3	Culturable organisms from BHA to enriched luria broth*
Row c5	Culturable organsims from luria agar to BH broth*	Row c6	Culturable organisms from luria agar to luria broth*
Row c7	Culturable organisms from luria broth to enriched luria broth*		

* Details of these organisms to be published by M.B. von Wechmar elsewhere. For details of media see Addendum C.2

Characteristics of the TNV-Pot/Upd isolate were similar to the TNV-CN isolate described by Tomlinson *et al.* (1983); both are serologically related to the D-like TNV-strains and both isolates only infect *C. quinoa* and do not infect *Nicotiana* spp in the primary inoculation. cDNA hybridization studies of inoculated *N. benthamiana* showed the presence of TNV RNA, therefore indicating that TNV-Pot/Upd can replicate in *N. benthamiana* without causing any symptoms. A similar silent infection was not noted by Tomlinson *et al.* (1983).

The immunological relationship determined by IEB assays showed that the TNV-Pot/Upd fits into the serological classification of Babos and Kassanis (1963). The TNV-Pot/Upd can be grouped as a D-like isolate. Results from an ISEM study were inconclusive due to some antisera being non-specific. Anti-TNV-D/RK (Chapter 2 Fig. 2.6) anti-TNV-Pas (Chapter 4 Fig . 4.7), and anti-TNV-PV68 (Chapter 4 Fig. 4.5), contain antibodies to A- and D-like TNV isolates. Approximately 70 % of the TNV-Pot/Upd was trap-decorated by the homologous antisera. Anti-TNV-D/RK (see Addendum A, Table A.3) only trapped and decorated very few particles, indicating that very few of the virus particles were similar to the TNV-D/RK. Anti-TNV-Pas trapped and decorated approximately 70 % of TNV-Pot/Upd particles, whereas anti-TNV-PV68 trapped and decorated 10 % of the TNV-Pot/Upd particles. The TNV-Pot/Upd was identified as a D-type TNV isolate on the basis of cDNA hybridisation tests and IEB immunological tests.

The technique of tissue blots has been successfully used to detect PVY and PVX in the leaf tissue of potato plants (Bravo-Almonacid, *et al.*,1992). Initially TNV was detected in Buffelspoort-13 potato stems by tissue blots, however this result was inconclusive as the antisera used were not host-absorbed with healthy potato or plant host proteins before detection, and it is known that the antisera contained antibodies to plant host proteins (von Wechmar, pers comm.). Therefore it is not certain whether the antisera reacted with virus or plant host proteins. Some significance can be placed on the tissue blot results as the cDNA hybridization confirms the results. The A-type antisera had a stronger signal than the D-type antisera in the tissue blots and the nucleic acid extracted from the skin of Buffelspoort-13 potatoes hybridized to the Wheat (A-type) and Pap/GL (D-type) TNV coat protein probes.

TNV-A was also detected in one spore forming unidentified culturable organism (von Wechmar, pers comm.) isolated from surface sterilized Buffelspoort-13 potatoes that developed a soft-rot. Other organisms, cultured on different media, isolated from the same

potatoes did not hybridize to the TNV-A probe (Fig. 3.7). Although this result is also inconclusive, it does draw attention to the possibility that TNV may be associated with culturable organisms as has been shown for avocado (Jaffer *et al.*, 1993), papaya (von Wechmar *et al.*, 1994b) and plum (von Wechmar *et al.*, 1994a).

In conclusion it can be stated that TNV was diagnosed in Buffelspoort-13 and Up-to Date potatoes exhibiting abnormal chlorosis and necrosis in the foliage. It was not proven that TNV was the causative agent of this abnormal appearance.

Chapter 4

Molecular analysis and relationship studies of TNV isolates

Contents

4.1 Introduction

Babos and Kassanis (1963) identified seven strains of TNV and found them to be serologically related. These seven strains could be divided into two distinct groups or serotypes (serotype is defined as: virus strains closely related serologically but distantly related to members of a different serotype) by serological analysis. Uyemoto *et al.* (1968) found it impractical to separate strains of TNV into only two serotypes based on the relative relationship of the test strains to strain-A and strain-D. The relative serological relationship between the 11 strains tested was variable, when tested with different antisera. According to Uyemoto *et al.* this ambiguity in strain relationship made the separation of TNV isolates into two groups (serotype-A and serotype-D) impractical and unreliable, and they therefore redefined serotype as "each isolate or group of serologically identical isolates that can be distinguished serologically from others, regardless of whether the antigenic differences are small or large". Kassanis and Phillips (1970) attributed this variability, observed by Uyemoto *et al.*, to changes in antisera specificity as a result of the immune response and the regimen used to immunize rabbits to obtain antisera. Kassanis and Phillips were able to group eight TNV isolates into the two distinct serotypes (serotype-A and serotype-D) noted previously by Babos and Kassanis. More recent reports identified and characterized TNV isolates according to their serological relationships to TNV strains A and D (Tomlinson *et al.*, 1983; Tanne, 1984; von Wechmar *et al.*, 1990, Adam *et al.*, 1990; Roggero and Lisa, 1995).

The serological relationship studies (Babos and Kassanis, 1963; Uyemoto *et al.*, 1968; Kassanis and Phillips, 1970) were done by tube precipitation and gel immunodiffusion tests to determine titration end points and spur formation. The problem with these tests however is that 1) the antisera must be concentrated, and 2) the assays are unreliable for isolates that readily crystallize. The serological relationship of TNV isolates can now be tested by more sensitive immunological techniques such as: Immunoelectron microscopy (Adam *et al.*, 1990; Tomlinson *et al.*, 1983), ISEM (Jaffer *et al.*, 1993; von Wechmar *et al.*, 1993) ELISA (Roggero and Lisa, 1995) and IEB (von Wechmar *et al.*, 1990).

The availability of sequence data of TNV-A (Meulewaeter *et al.*, 1990) and TNV-D (Coutts *et al.*, 1991) isolates, made it possible to design PCR primers to the coat protein gene of TNV-A and TNV-D. These primers were used to derive cDNA probes to the coat protein gene of TNV-Wheat (A-like) and TNV-Pap/GL (D-like) (see Addendum A, Table A.1).

These probes were used for nucleic acid hybridization analysis of TNV isolates, to determine the molecular grouping amongst the isolates and to compare the grouping to those obtained by immunological analysis. This method has been used before to determine the relationship between Luteoviruses (Martin and D'Arcy, 1990). Nucleic acid hybridization has been used to confirm the grouping of TNV isolates by STNV activation (Meulewaeter *et al.*, 1993). Probes derived to the putative RNA polymerase gene of two isolates that differentially activate different strains of STNV, confirmed the grouping of TNV isolates that could support the replication of different STNV strains.

The work in this chapter is based on differences and similarities found between the coat proteins of some South African TNV isolates. The immunological grouping between these TNV isolates was determined by IEB analysis. This grouping was further tested by determining the nucleic acid hybridization patterns of the genomic RNA with probes derived to the coat protein gene of A-like and D-like isolates. PCR amplification of the coat protein genes, with primers designed to the published TNV-A and TNV-D strains, was also used to group the viruses into A-like and D-like isolates. These techniques made it possible to determine relationships between the various isolates.

4.2 Virus isolates

The tobacco necrosis virus isolates were obtained from various sources (Addendum A, Table A.1) by M.B. von Wechmar (Jaffer *et al.*, 1993, von Wechmar and Jaffer, 1993, von Wechmar *et al.*, 1994a, von Wechmar *et al*, 1994b and von Wechmar, pers. comm.). PV-68 (TNV-A) and PV-67 (TNV-D) TNV type strains (Addendum A, Table A.1) were obtained from the American Type Culture Collection (ATCC) (McDaniel *et al.*, 1993) as lyophilized infected leaf fragments, for comparative studies with the South African TNV isolates. The TNV isolates were propagated by mechanical inoculation on detached host leaves as listed in Addendum A, Table A.1. Figs. 4.1 and 4.2 illustrate typical symptoms of TNV-Wheat and TNV-Pap/GL on different hosts respectively. The risk of mixing and contaminating virus isolates was avoided by working with each isolate in a different time frame. The infected plant material was ground to a fine paste and the virus purified as per Section 2.2.3. The light refracting bands were removed from the sucrose density gradient by a pasteur pipette with a 90° bend at the tip.

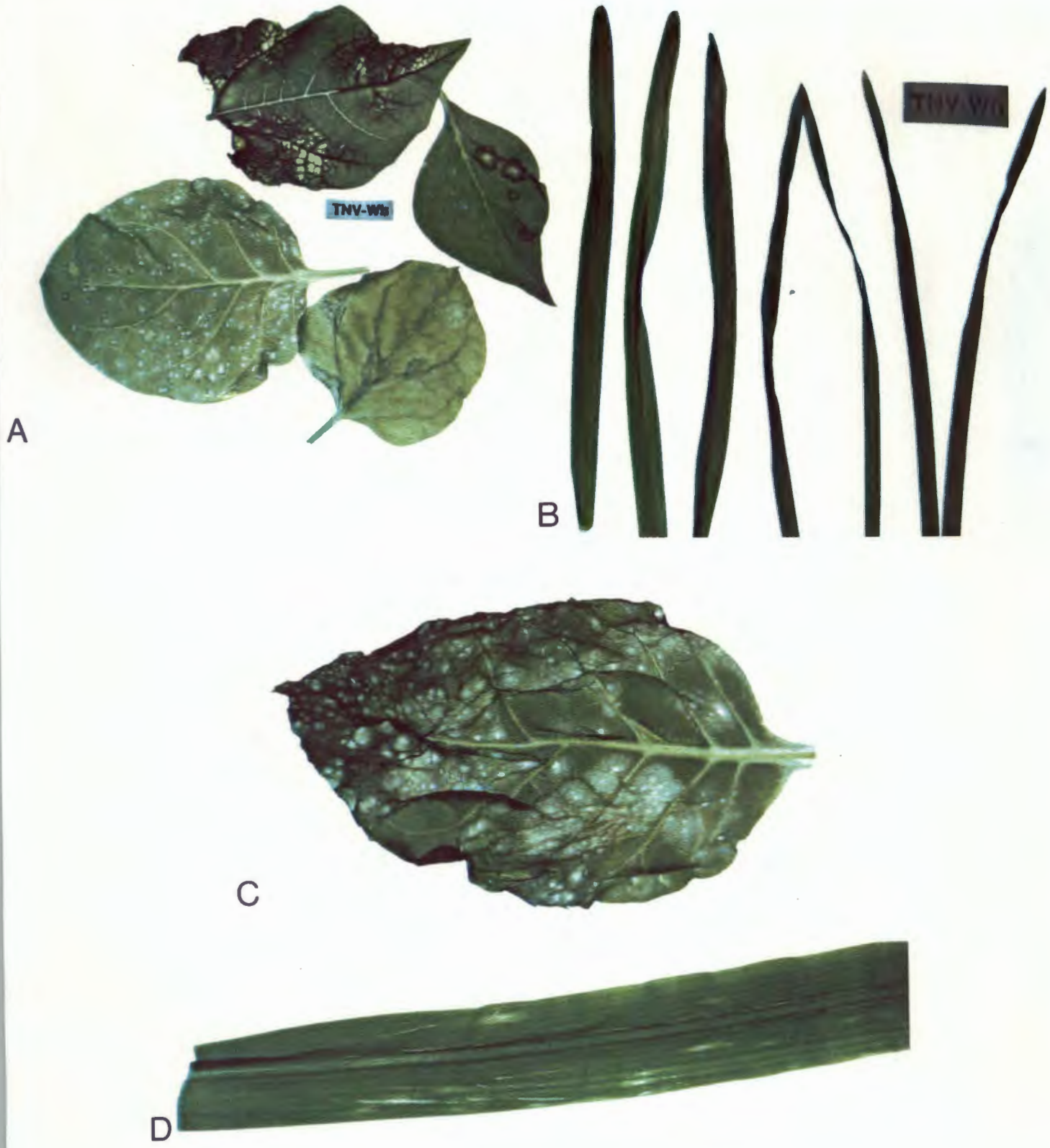


Figure 4.1 Symptoms of TNV-Wheat on different hosts. A) Clockwise from the top: *Phaseolus vulgaris* with typical brown vein pattern spreading from the centre of infection, *Nicotiana clevelandii* with brown lesions, *N. Benthamiana* single lesions spreading along the veins, *N. tabacum* cv. Xanthi with lesions of different size; B) *Hordeum vulgare* cv. Clipper on the left and *Triticum aestivum* cv. Scheepers on the right with elongated necrotic lesions; C) *N. tabacum* cv. Xanthi illustrating drying up of infected leaf three days post-inoculation; D) *Zea mays* cv. Potchefstroom pearl with small necrotic lesions.

Fig. 4.2 Legend on page 51

Figure 4.2 Symptoms of TNV-Pap/GL on different hosts, A) Papaya fruit with green sunken lesions and *Nicotiana benthamiana* exhibiting necrotic lesions and leaf scorch; B) *Phaseolus vulgaris* with brown necrotic lesions; C) *Cucurbita pepo* with necrotic lesions on cotyledons; D) *Chenopodium quinoa* with pinprick necrotic lesions; E) *Cucumis sativa* with necrotic lesions on cotyledons; F) *Nicotiana clevelandii* with brown sunken lesions and G) *Gomphrena globosa* with dry necrotic lesions.

The propagation and purification of the TNV isolates was done by M.B. von Wechmar who studied other aspects of the isolates not discussed here. The concentration of the virus preparations were estimated by ultra-violet absorbance at 260 nm in a Beckman DU-64 spectrophotometer and the virus was diluted to approximately 200 µg/mL for protein disruption (Addendum B 4.1.1) and RNA extraction (Addendum B 7.2). The purified virus used for analysis in this chapter is not necessarily the same sample of purified virus used to immunize rabbits.

4.3 Materials and methods

4.3.1 Antiserum preparation

Antisera used were made by M.B. von Wechmar according to the schedule described in Chapter 2 Section 2.2.4. For details of the antisera used, see Addendum A, Table A.3.

4.3.2 Protein sizing

Purified virus preparations were disrupted in 62.5 mM Tris-Cl (pH 6.8), 5 % SDS and 5 % BME by heating at 96 °C for 10 min (Addendum B 4.1). The Mr of the TNV coat proteins were determined by SDS-PAGE on 11 % - 13 % gradient gels using the discontinuous buffer system of Laemmli (1970) (Addendum B 4.1). Pharmacia LMW proteins were used as standards and the gels were stained with PAGE blue (Addendum B 4.1.2). The protein Mr's were determined from the linear relationship between the distance of migration and the log of the Mr of the standards.

4.3.3 Immunoelectroblotting (IEB)

Denatured TNV proteins were electrophoresed through 12 % acrylamide gels using the discontinuous buffer system of Laemmli (1970) (Addendum B 4.1). The proteins were electrophoretically transferred to a nitrocellulose membrane (pore 0.45 µm, MSI) by a modification of the Towbin (1979) method (Addendum B 5.1). The membranes were probed with polyclonal antisera listed in Table 4.1.

4.3.4 RNA sizing

Single-stranded genomic RNA was isolated from purified virus preparations in 10 mM Tris-Cl, 1 mM EDTA and 1 % SDS and extracted once with phenol : chloroform : isoamyl alcohol (25:24:1) (Addendum B 7.2). The RNA samples were fractionated on a 1 % agarose (Sigma) formaldehyde denaturing gel (Sambrook, 1989; Addendum B 7.1) with standard RNA markers (GibcoBRL).

Table 4.1 List of antisera used for immunoelectroblot analysis of TNV isolates. Plant propagation species, serotype and time after immunization are shown.

Antisera	Propagation species	Serotype	Time after immunization
Anti-TNV-PV68	*N. tabacum* cv. Xanthi	A	7 weeks
Anti-TNV-PV67	*N. tabacum* cv. Xanthi	A*	7 weeks
Anti-TNV-Bean/1	*N. tabacum* cv. Soulouk	A	4 weeks
Anti-TNV-Pap/A	*N. tabacum* cv. Xanthi	A	4 weeks
Anti-TNV-Pas	*N. tabacum* cv. Xanthi	A	5 weeks
Anti-TNV-Citr	*Chenopodium quinoa*	D	4 weeks
Anti-TNV-Pap/GL	*N. benthamiana*	D	6 weeks

* Reported to be a D-type isolate when propagated in cowpea (*Vigna sinensis*) (McDaniel *et al.*, 1993).

4.3.5 RNA Hybridization

RNA electrophoresed on denaturing gels (Addendum B 7.1) was transferred to nylon membranes (Amersham; Hybond N^+) by capillary action with 10 x SSC (Sambrook *et al.*,1989; Addendum B 9.1). The membranes were probed with DIG labeled probes derived from the coat protein gene of TNV-Wheat (A-type) and TNV-Pap/GL (D-type) (Addendum B 10.3). Hybridization was detected by chemiluminescence and autoradiography (Addendum B 9.3) (Boehringer Mannheim Biochemica, The DIG system user's guide for filter hybridisation).

4.3.6 Coat protein gene amplification

Primers were designed to the published sequences of TNV-A (Meulewaeter *et al.*, 1990) and TNV-D (Coutts *et al.*, 1991). The sequence and position of the primers are listed in Table 4.2. The coat protein gene was amplified by reverse transcription polymerase chain reaction (RT-PCR). cDNA was synthesized using the MMLV reverse transcriptase (Boehringer Mannheim) in the presence of the forward and reverse primers from either the TNV-A or -D strains (Addendum B.10.1). cDNA was denatured at 95 °C for 5 min and cooled on ice. *Taq* polymerase (Promega) was added to the tube for PCR amplification (Addendum B.10.2). The PCR products were run on a 1 x TBE agarose gel (Addendum B 8.1) stained with EtBr and viewed on a 254 nm transilluminator.

Table 4.2 PCR primers used in the amplification of the coat protein genes of the TNV isolates indicating sequence position in the relevant TNV strains and the sequences of the primers

Primer	Strain	Position in genome	Sequence
A-5'	TNV-A*	2610-2627	5'-GACATGGCAGGAAAGAAG-3'
A-3'	TNV-A*	3422-3440	5'-GACGTTCATTGTTGGGTTG-3'
D-5'	TNV-D**	2540-2560	5'-CCAAAGCCCTCCTGAAAGACC-3'
D-3'	TNV-D**	3509-3528	5'-CACGCCACAACTCTACTTAC-3'

* Meulewaeter *et al.* (1990) ** Coutts *et al.* (1991)

4.4 Results

4.4.1 Molecular characterizations

The genomic RNA sizes and coat protein Mr's of the TNV isolates are listed in Table 4.3 and illustrated in Figs 4.3 and 4.4. The STNV isolated from avocado has a coat protein of 20.8 kDa and a genomic RNA of 1.3 kb. The TNV isolates have coat protein Mr's ranging from 29.2 kDa to 30.8 kDa, the genomic RNA size is between 3.7 and 3.8 kb.

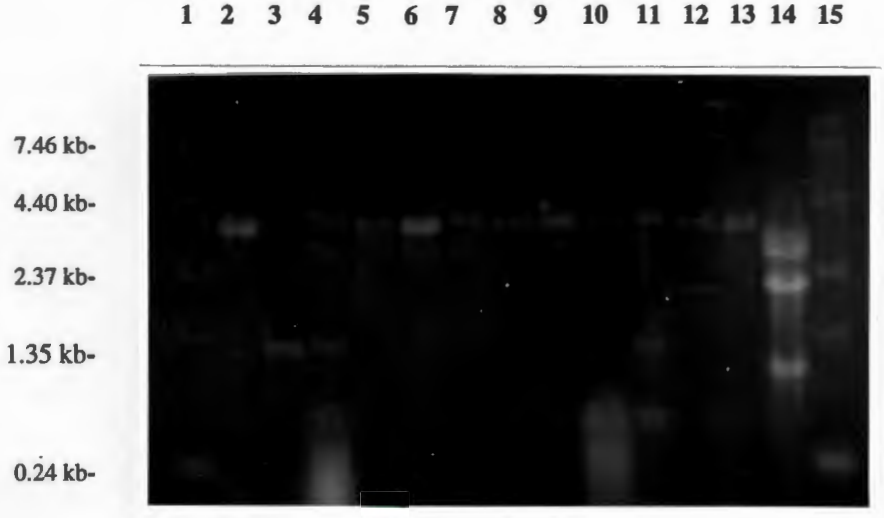


Figure 4.3 Analysis of TNV genomic RNAs by denaturing gel electrophoresis in 1% agarose gels. The lanes contain the following: 1and15) MWt markers, 2) TNV-PV67, 3) STNV-Avo, 4) TNV-Avo, 5) TNV-Citr, 6) TNV-Pap/GL, 7) TNV-Yst/C.o, 8) TNV-Wheat, 9) TNV-Bean/1, 10) TNV-750, 11) TNV-Pot/Upd, 12) TNV-Bean/2, 13) TNV-PV68, 14) BMV.

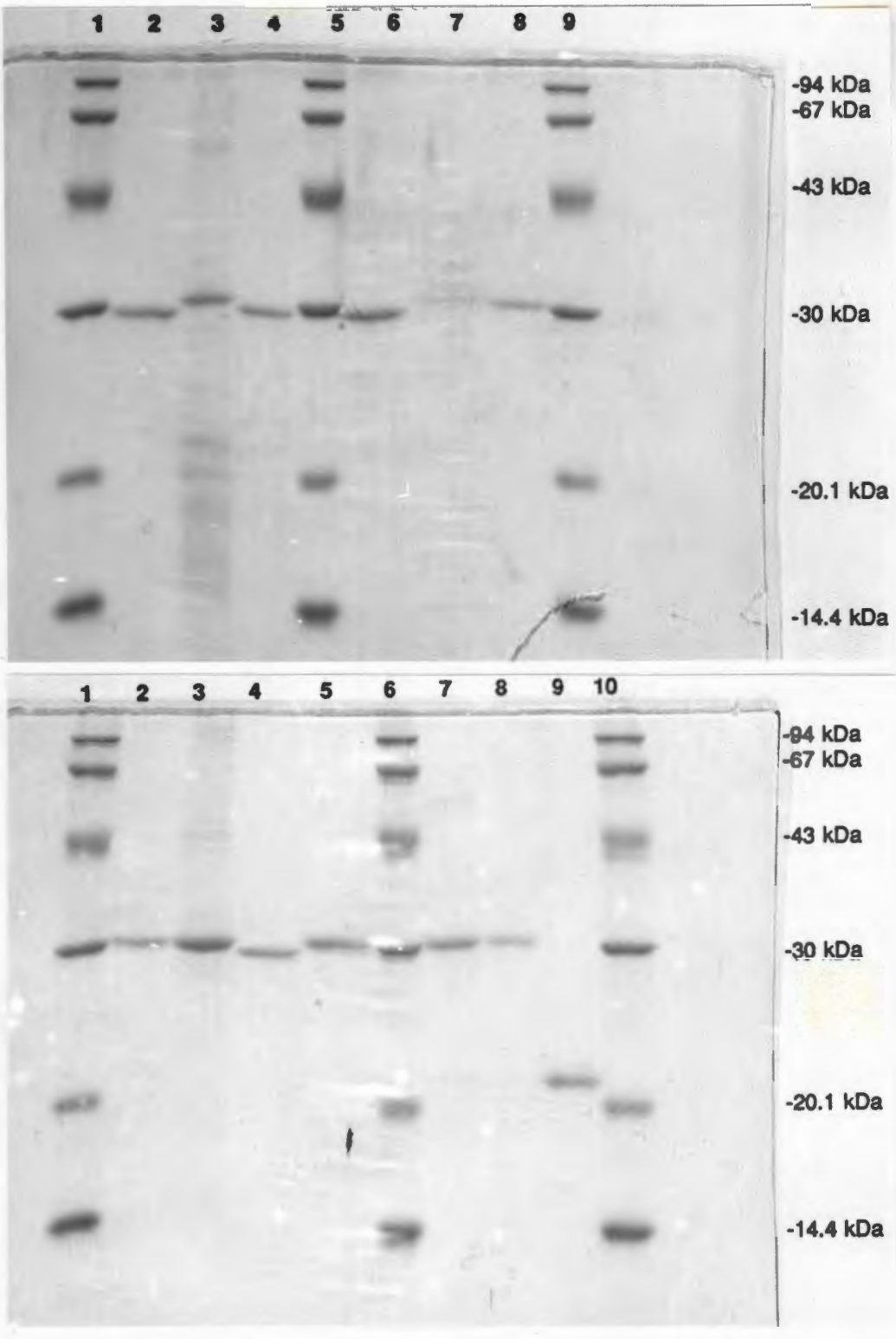


Figure 4.4 SDS-PAGE analysis of TNV isolates, indicating the coat protein Mr. The lanes in gel A contain: 1,5,9) MWt markers, 2) TNV-Wheat, 3) TNV-750, 4) TNV-Bean/1, 6) TNV-Bean/2, 7) TNV-Tulip, 8) TNV-Yst/C.o. Lanes in gel B contain: 1,6,10) MWt markers, 2) TNV-Pot/Upd, 3) TNV-Pap/GL, 4) TNV-Cel, 5) TNV-Citr, 7) TNV-Bact/Ps, 8) TNV-Avo, 9) STNV-Avo.

Table 4.3 List of the coat protein Mr (kDa) and the genomic RNA size (kb) for the TNV isolates listed.

Virus isolate	Serotype	Genomic RNA size	Coat protein Mr
STNV-Avo	-	1.3	21.5
TNV-Avo	D	3.8	30.6
TNV-Citr	D	3.7	30.4
TNV-Pap/GL	D	3.7	30.4
TNV-Yst/C.o.	D	3.8	30.8
TNV-Pot/Upd	D	3.8	30.4
TNV-750	D	3.8	30.5
TNV-Bact/Ps	D	NT	30.3
TNV-Wheat	A	3.7	29.2
TNV-Bean-1	A	3.7	29.2
TNV-Bean-2	A	3.7	29.2
TNV-Tulip	A	NT	30.8
TNV-Cel	A	NT	29.2
ATCC-PV68	A	3.7	29.2
ATCC-PV67	A	3.7	29.2

NT Not Tested

4.4.2 Immunological analysis of TNV isolates

The results of IEB analysis with selected polyclonal antisera are illustrated in Figs. 4.5, 4.6 and 4.7 and listed in Tables 4.4 and 4.5. Figure 4.5 illustrates the reaction of TNV coat protein antigens to antisera raised to TNV isolates obtained from the ATCC. Coat proteins from the following TNV isolates react strongly with the anti-TNV-PV67 and anti-TNV-PV68 sera: -PV67, -PV68, -Wheat, -Bean/1 and -Bean/2. TNVs -750 and -Pot/Upd react weakly to both antisera whereas TNVs -Avo and -Citr react weakly only to anti-TNV-PV68. TNVs -749, Pap/GL, Yst/C.o., STNV-Avo and BMV do not react with the antisera.

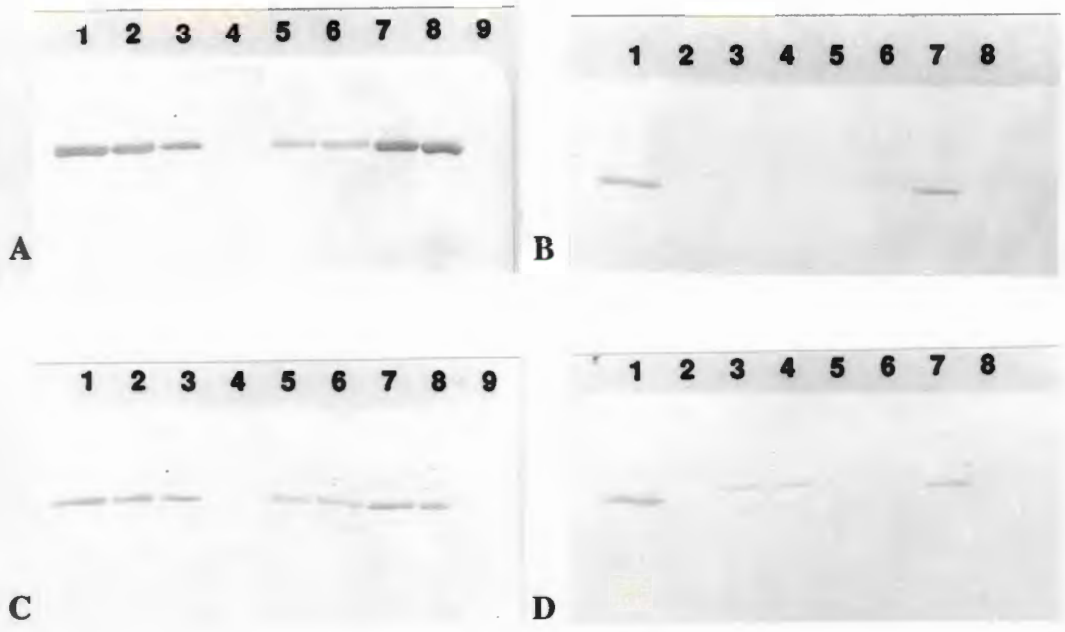


Figure 4.5 Immunoelectroblot analysis of local TNV isolates with antisera raised to TNV-PV67 and TNV-PV68. The lanes contain the following TNVantigens: Blots A and C, 1) -PV68, 2) -Wheat, 3) -Bean/1, 4) -749, 5) -750, 6) -Pot/Upd, 7) -Bean/2, 8) -PV67, 9) BMV. Blots B and D, 1) -PV68, 2) STNV-Avo, 3) -Avo, 4) -Citr, 5) -Pap/GL, 6) -Yst/C.o, 7) -PV67, 8) BMV. Blot A and B were probed with anti-TNV-PV67 and blots C and D were probed with anti-TNV-PV68 sera. The protein bands are approximately 30 kDa in size.

Figure 4.6 illustrates the antigenic reaction of the virus isolates when tested with antisera raised to South African TNV isolates. Anti-TNV-Bean/1 and anti-TNV-Pap/A sera have strong reactions with TNVs -PV67, -PV68, -Wheat, -Bean/1 and -Bean/2. There is no reaction with TNVs -749, -750, -Pot/Upd, -Avo, -Citr, -Pap/GL or -Yst/C.o. antigens. The anti-TNV-Pap/GL and anti-TNV-Citr sera have a strong reaction with TNVs -Avo, -Citr, -Pap/GL, -Yst/C.o. -750 and -Pot/Upd. The anti-TNV-Pap/GL has a weak reaction with: -PV68, -PV67, -Wheat, -Bean/1 and -Bean/2 antigens, and a weaker reaction with the -749 antigen. Anti-TNV-Citr does not react with TNVs -PV68, -PV67, -Wheat, -Bean/1, -Bean/2 and -749 antigens. None of the antisera recognized the coat proteins of the BMV or the STNV-Avo.

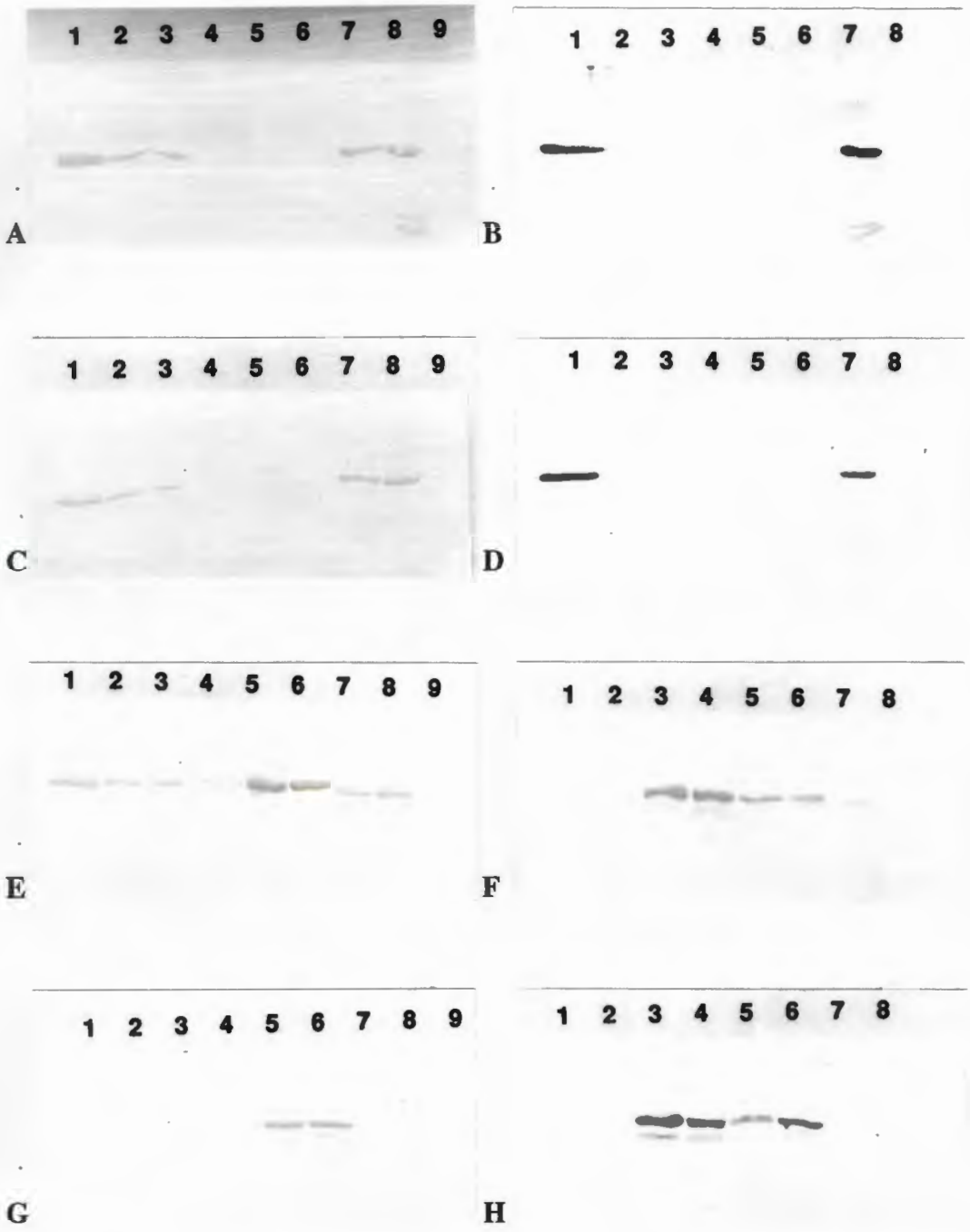


Figure 4.6 Immunoelectroblot analysis of TNV isolates with antisera to South African TNV isolates. The blots contain the following TNV antigens: Blots A, C, E and G, 1) -PV68, 2) -Wheat, 3) -Bean/1, 4) -749, 5) -750, 6) -Pot/Upd, 7) -Bean/2, 8) -PV67, 9) BMV. Blots B, D, F and H: 1) -PV68, 2) STNV-Avo, 3) -Avo, 4) -Citr, 5) -Pap/GL, 6) -Yst/C.o, 7) -PV67, 8) BMV. Blots A and B were probed with anti-TNV-Pap/A, blots C and D were probed with anti-TNV-Bean/1, blots E and F were probed with anti-TNV-Pap/GL, and blots G and H were probed with anti-TNV-Citr sera. The protein bands are approximately 30 kDa in size.

Figure 4.7 illustrates the antigenic reaction of some TNV isolates tested with antisera to TNV-Pas and TNV-Pap/GL. The anti-TNV-Pas serum has a strong reaction with TNVs -Wheat, -Bean/1, -Bean/2 and -Cel, and a weak reaction with TNVs -750, -Tulip, -Yst/C.o., -Pot/Upd, -Pap/GL, -Citr, -Bact/Ps and -Avo. The anti-TNV-Pap/GL serum reacted strongly with TNVs -750, -Yst/C.o., -Pot/Upd, -Pap/GL, -Citr, -Bact/Ps and -Avo, but reacted weakly with TNVs -Wheat, -Tulip, -Bean/1, -Bean/2 and -Cel. Both antisera did not react with: STNV-Avo, BMV, CMV, TMV and the MWt markers.

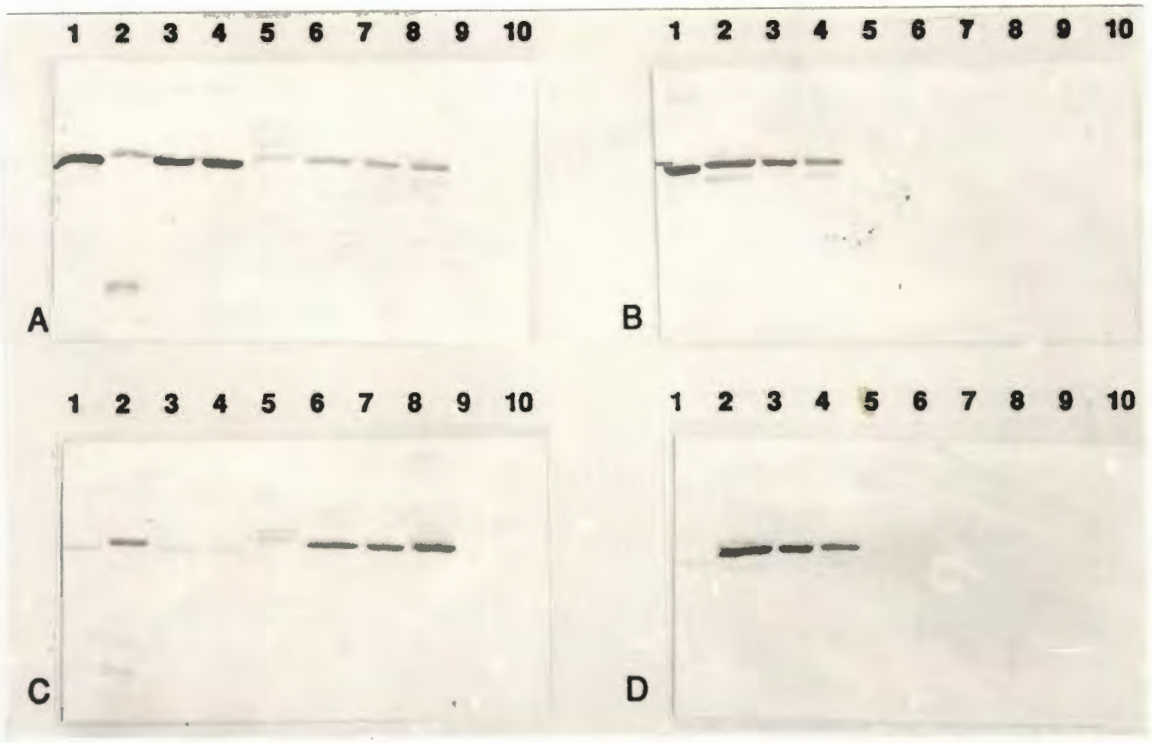


Figure 4.7 Immunoelectroblot analysis of TNV isolates with antisera to South African TNV isolates. Some isolates not studied in detail are compared to isolates illustrated in Figs 4.5 and 4.6. The lanes contain the following antigens: Blot A and C: 1) TNV-Wheat, 2) -750, 3) -Bean/1, 4) -Bean/2, 5) -Tulip, 6) -Yst/C.o, 7) -Pot/Upd, 8) -Pap/GL, 9) MWt markers. Blot B and D: 1)TNV-Cel, 2) -Citr, 3) -Bact/Ps, 4) -Avo, 5) STNV-Avo, 6) BMV, 7) CMV, 8) TMV, 9) MWt Markers. Blots A and B were probed with anti-TNV-Pas and blots C and D were probed with anti-TNV-Pap/GL sera. The protein bands are approximately 30 kDa in size.

Table 4.4 The reaction of coat protein antigens of TNV isolates to selected TNV antisera. The table summerises the results illustrated in Figs 4.5 and 4.6.

	TNV-Antisera					
TNV-Antigen	PV68	PV67	Bean/1	Pap/A	Pap/GL	Citr
PV68	++	++	++	++	+	-
PV67	++	++	++	++	+	-
Wheat	++	++	++	++	+	-
Bean/1	++	++	++	++	+	-
Bean/2	++	++	++	++	+	-
749	-	-	-	-	+	-
750	+	+	-	-	++	++
Pot/Upd	+	+	-	-	++	++
Avo	+	-	-	-	++	++
Citr	+	-	-	-	++	++
Pap/GL	-	-	-	-	++	++
Yst/C.o.	-	-	-	-	++	++
STNV-Avo	-	-	-	-	-	-
BMV	-	-	-	-	-	-

Table 4.5 Reaction of the coat protein antigens of TNV isolates to anti-TNV-Pas and anti-TNV-Pap/GL-sera as illustrated in Fig. 4.7.

TNV-Antigens	TNV-Antisera	
	Pas	Pap/GL
Wheat	++	+
Bean/1	++	+
Bean/2	++	+
Cel	++	+
Augusta	+	+
Yst/C.o.	+	++
Pot/Upd	+	++
Pap/GL	+	++
Citr	+	++
Bact/Ps	+	++
Avo	+	++
STNV-Avo	-	-
BMV	-	-
CMV	-	-
TMV	-	-
MWt Markers	-	-

++ - Strong positive signal
+ - Positive signal
- - Negative signal

4.4.3 Nucleic acid hybridization

The relationship between the TNV isolates at the genomic level was determined by nucleic acid hybridization under stringent conditions with probes derived to the coat protein genes of TNV-Wheat and TNV-Pap/GL. The results for the nucleic acid hybridization of the genomic RNA is illustrated in Fig. 4.8 and listed in Table 4.6. The ATCC isolates -PV67 and -PV68, and other TNV isolates -Wheat, -Bean/1 and -Bean/2 hybridized strongly to the probe derived from the coat protein of TNV-Wheat. The signal intensities were similar. The genomic RNA of TNVs -Avo, -Citr, -Pap/GL, -Yst/C.o., -750 and -Pot/Update hybridized to the probe derived from the coat protein of TNV-Pap/GL. The -Citr, -Pap/GL, and -Yst/C.o. signals are approximately equal in intensity, the -Avo, -750 and -Pot/Upd have a weaker signal. The STNV-Avo and BMV genomic RNA's did not hybridize to the TNV coat protein probes.

Table 4.6 Nucleic acid hybridization analysis of TNV genomic RNA of different isolates with cDNA probes derived to the coat protein gene of TNV-wheat and TNV-Pap/GL isolates.

	TNV probe	
TNV Isolate	Wheat probe	Pap/GL probe
PV68	+++	-
PV67	+++	-
Wheat	+++	-
Bean/1	+++	-
Bean/2	+++	-
Avo	-	+
Citr	-	++
Pap/GL	-	++
Yst/C.o.	-	++
750	-	+
Pot/Upd	-	+
STNV-Avo	-	-
BMV	-	-

+++ - Very strong positive signal
++ - Strong positive signal
+ - Positive signal
- - Negative signal

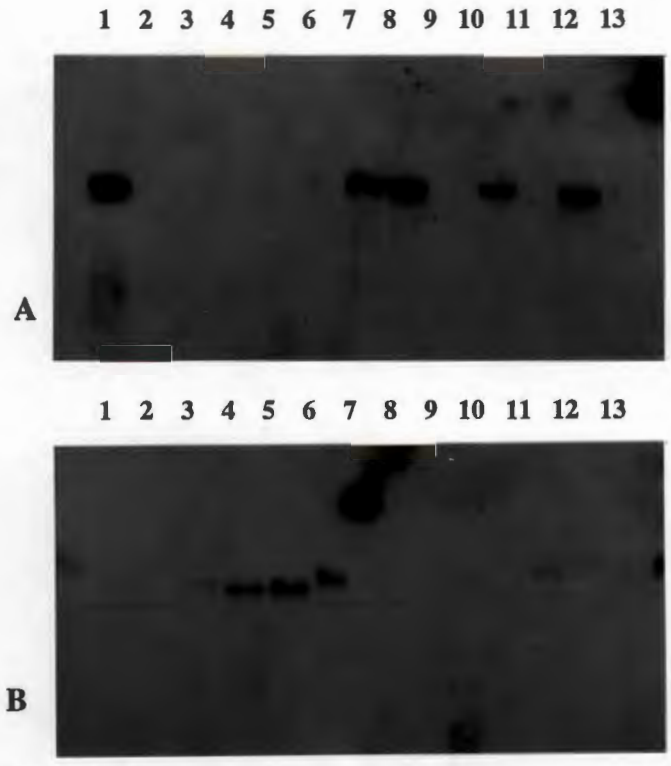


Figure 4.8 Northern blot analysis of genomic RNAs extracted from TNV isolates. cDNA Probes derived to the coat protein gene of TNV-Wheat (blot A) and TNV-Pap/GL (blot B) were used. The same RNA samples and volumes were used for Fig. 4.3. The lanes in blots A and B contain RNA from the following TNV isolates: 1) -PV67, 2) STNV-Avo, 3) -Avo, 4) -Citr, 5) -Pap/GL, 6) -Yst/C.o, 7) -Wheat, 8) -Bean/1, 9) -750, 10) -Bean/2, 11) -Pot/Upd, 12) -PV68, 13) BMV.

4.4.4 PCR amplification

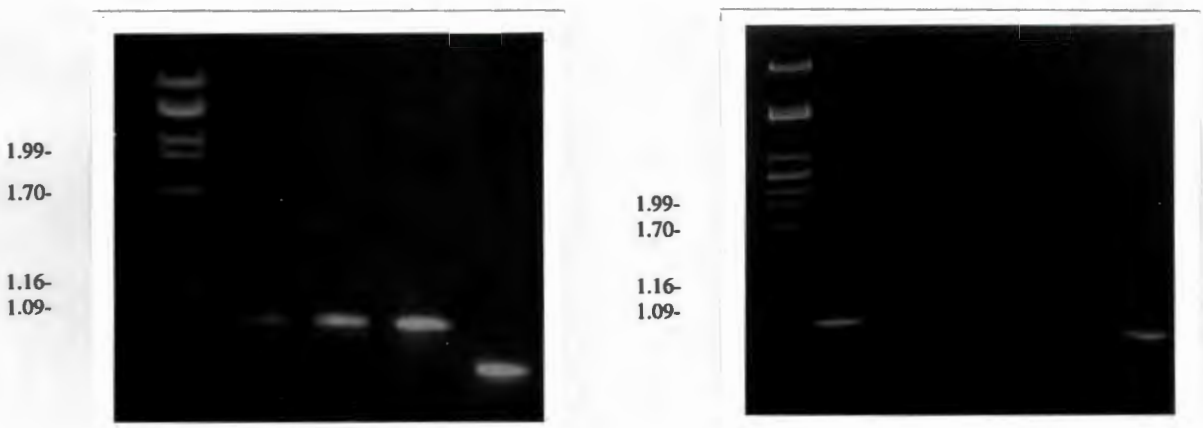
The coat protein genes of the TNV isolates amplified by RT-PCR with TNV-A and TNV-D specific primers is listed in Table 4.6. An illustration of the size of the PCR products is shown in Fig 4.9. The coat protein gene of TNVs -Wheat, -Bean/1, -750 and -781 were amplified by the primers designed to the TNV-A strain. The coat protein gene of TNVs -Avo, -Citr, -Pap/GL, -Yst/C.o. and -750 were amplified by primers designed to the TNV-D strain. The coat protein gene of TNVs -Bean/2, -Pot/Upd and -Bact/Ps were not amplified by the primers designed to either the TNV-A or the TNV-D strains. Amplification of the coat protein of the following TNV isolates was not attempted: -PV67, -PV68, -Cel and -Tulip.

Table 4.7 PCR amplification of the coat protein gene of the TNV isolates with primers designed to the TNV-A or the TNV-D coat protein regions.

TNV Isolate	A-Primer	D-Primers
Avo	-	+
Citr	-	+
Yst/C.o.	-	+
Pap/GL	-	+
750	+	+
Wheat	+	-
Bean/1	+	-
Yst/H.u.	+	-
Pot/Upd	-	-
Bean/2	-	-
Bact/Ps	-	-

+ - Positive amplification

- - Negative amplification



A B

Figure 4.9 Analysis of the RT-PCR amplification of coat protein genes of TNV isolates. The lanes in gel A contain: 1) λ-Pst marker, 2) -Citr, 3) -Pap/GL, 4) -750/D and 5) -Yst/H.u. The lanes in gel B contain: 1) λ-Pst marker,2) -Pap/GL, 3) -Pot/Upd with D specific primers, 4) -Pot/Upd with A specific primers, 5) -Bact/Ps with A specific primers, 6) -Bact/Ps with D specific primers, 7) -Avo.

4.5 Discussion

On the basis of IEB analysis with the available antisera, the TNV isolates studied in this chapter appear to fall into the classification system proposed by Babos and Kassanis (1963), dividing the isolates into two serotypes, A and D, each containing specific isolates. The viruses in each group appear to be serologically similar but can be differentiated from other isolates by host range, symptom expression, protein Mr, RNA size and PCR amplification. Nucleic acid hybridization with A- and D-probes confirmed the grouping of the TNV isolates into A- and D- serotypes. The A-like isolates include: TNV-PV67, TNV-PV68, TNV-Wheat, TNV-Bean/1, TNV-Bean/2 and TNV-Cel. The D-like isolates include: TNV-Avo, TNV-Citr, TNV-Pap/GL, TNV-Yst/C.o., TNV-Pot/Upd, TNV-750 and TNV-Bact/Ps. The TNV-Tulip isolate appeared to be serologically different from both the A- and D- type isolates as the virus antigen had an equally weak reaction with the A- and D-type antisera (Fig. 4.7). It is not known whether TNV-Tulip is a different serotype or whether the virus preparation was poor, because nucleic acid hybridization studies were not done.

The Mr of the coat proteins were found to vary between 29.2 kDa and 30.8 kDa, the TNV D-like isolates all had a coat protein Mr greater than 30 kDa and the TNV A-like isolates had a coat protein of approximately 29.2 kDa. The genomic RNA size was approximately 3.7 kb or 3.8 kb. The values correspond well with published data (Uyemoto, 1981, Fraenkel-Conrat, 1988).

The virus concentration was estimated at 200µg/ml. From these virus preparations, protein disruption and RNA extraction was done. The different propagation hosts of the virus isolates affected the amount of host protein present in the purified preparation. The isolates also had different stabilities, therefore the virus concentrations were found not to be equal. The results obtained in IEB with TNV-Pap/GL and TNV-Yst/C.o. indicates that these two virus isolates had lower protein concentrations in the disrupts (Fig. 4.6). The RNA bands shown in denaturing gel electrophoresis (Fig. 4.3) are not equal in intensity, due to the concentration of virus for RNA extraction, and RNA degradation in some isolates. Because of the variation in the concentrations of virus preparations used for coat protein disruption and RNA extraction, the results of IEB and nucleic acid hybridization are qualitative, and can only be used to determine broad group divisions, i.e. serotype divisions. Quantitative results establishing

specific relationships between isolates within the serological groups could not be determined from these results.

The PV67 and PV68 TNV isolates obtained from the ATCC appear to be serologically similar. Both isolates react strongly to anti-PV67, anti-PV68, anti-TNV-Pap/A and anti-TNV-Bean/1 sera. This indicates that PV67 and PV68 are serologically A-type isolates. This is an unexpected result as PV67 is reported to be a TNV-D serotype (McDaniel *et al.*, 1993) and PV68 is reported to be a TNV-A serotype. A possible explanation for this could be that the PV67 isolate contained both an A-type and a D-type TNV isolate, and propagation in *N. tabacum* cv. Xanthi preferentially amplified the A-type isolate (von Wechmar, pers comm.). It is possible for more than one strain of TNV to simultaneously infect a plant as is illustrated in Chapter 3 in which an A-type and a D-type TNV were detected in Buffelspoort-13 potatoes, as illustrated by nucleic acid hybridization analysis.

The anti-TNV-PV67 and anti-TNV-PV68 sera have strong reactions with the A-like isolates and have weak reactions with some D-like (750 and Pot/Upd) isolates. The weak reaction could be due to cross reactivity or the presence of some D-like antibodies in the antisera. The signal strength of the TNV-PV67 and TNV-PV68 antigens are comparable with anti-PV68 serum in the two blots (Fig. 4.5 C and D) but are not comparable with anti-PV67 serum (Fig. 4.5 A and B).

The anti-TNV-Pap/GL and anti-TNV-Pas sera appear to react with A- and D-type TNV isolates. This indicates that the antisera could have: 1) antibodies to both TNV-A and -D type isolates, or 2) the antibody cross reacts with A- and D-type TNV antigens. Anti-TNV-Pap/GL serum has a strong reaction with D-like isolates and a weak reaction with A-like isolates (Figs 4.6 E and F and 4.7 C and D). Anti-TNV-Pas serum has a strong reaction with A-like isolates and a weak reaction with D-like isolates (Fig. 4.7 A and B). This cross reactivity could possibly be avoided if the purified virus preparations used for rabbit immunization had been started from single local lesions and had been propagated in one particular host only. The anti-TNV-Citr serum has a very specific and strong reaction with the D-type isolates (Fig. 4.6 G and H). The anti-TNV-Pap/A and anti-TNV-Bean/1 sera both have very specific and strong reactions with the A-type isolates (Fig. 4.6 A - D).

Antisera that cross-reacted with virus isolates of both groups were bled later than the antisera that did not have any cross-reaction. This antisera would no longer be in the primary immune response and the specificity of the antibodies would be lower although the antibody titre would be higher (van Regenmortel, 1982).

The nucleic acid hybridization signal strengths of the isolates that hybridized to the A-probe were similar, indicating a high degree of homology between these isolates at the RNA sequence level. The variation in the signal strength of the isolates that hybridized to the D-probe could be due to sequence variation amongst these isolates, or due to the uneven concentration of the RNA that was run in the denaturing agarose gels.

The size difference in the A and D coat protein PCR products were due to the design of the primers. The A primers were designed to amplify an approximately 800 bp fragment and the D primers were designed to amplify an approximately 1000 bp fragment

PCR amplification of the coat protein gene of the TNV isolates identified some isolates that appear to differ from the other isolates in the same serological grouping. The coat protein of TNV-Bean/2, an A-type isolate, TNV-Pot/Upd, a D-type isolate could not be amplified. The coat protein of TNV-750 was amplified by both the A and D primers. The anomaly with TNV-750 can be explained by virus preparations that had been propagated in different hosts. The RNA from which the A-type coat protein was amplified, was propagated in and purified from *C. quinoa* leaves, whilst the RNA from which the D-type coat protein was amplified, and the virus preparation used for IEB and nucleic acid hybridization, was propagated in and purified from *N. tabacum* cv. Xanthi leaves.

Immunological determination and cDNA hybridization of TNV isolates divided the TNV isolates studied into two serologically distinct groups. RT-PCR amplification of the virus isolates within each serotype further divided the isolates into groups that could and could not be amplified with specific primers. This indicates that within each serotype, further divisions and groupings may be found and more accurate relationships between the TNV virus isolates may be determined.

Chapter 5

Molecular cloning and sequencing of TNV CP genes

Contents

5.1 Introduction

Tobacco necrosis virus isolates have been shown to be serologically related (Babos and Kassanis, 1963; and Kassanis and Phillips, 1970) but divided into two distinct, yet related serotypes, A and D. The coat protein is the only structural protein produced by TNV and is also the major gene product produced *in vitro* (Salvato and Fraenkel-Conrat, 1977, Meulewaeter *et al.*, 1990). Hence the serological reactivity of TNV is dependant on this protein. For these reasons, several coat protein genes, of TNV isolates, shown to fall into this classification scheme in Chapter 4, were sequenced to determine the variability amongst the South African TNV isolates and the conservation of sequence amongst isolates in the same serotype.

The coat protein gene sequences of three TNV isolates have been reported: TNV-A/Mw (Meulewaeter *et al.*, 1990), TNV-D/Ct (Coutts *et al.*, 1991) and TNV-Ne (Zhang *et al.*, 1993). Sequence heterogeneity was reported for the TNV-A/Mw and TNV-Ne strains. Six out of nine amino acid changes in the TNV-A/Mw sequence (Meulewaeter *et al.*, 1990), and eight out of nine in the TNV-Ne sequence (Zhang *et al.*, 1993) were located in the coat protein gene. This indicates that some sequence variation in the coat protein gene can occur without a loss of function, structure, shape and antigenicity. The deduced coat protein amino acid sequence of these TNV isolates show significant differences. The coat protein amino acid sequence similarities between the TNV isolates are: A-NE 51%, A-D 45%, D-NE 44% (Zhang *et al.*, 1993).

The coat protein of many small icosahedral, ss RNA virions are divided into 4 domains: R-Random (amino-terminal), a-arm, S-shell and P-Projecting (carboxy-terminal). The TNV isolates have been shown to share the highest sequence homology in the S-domain and the lowest sequence homology in the R- and a-domains (Coutts *et al.*, 1991 and Zhang *et al.*, 1993). The TNV coat proteins sequenced previously do not have a P-domain, which is believed to be responsible for the antigenic properties of tomato bushy stunt virus (TBSV), turnip crinkle virus (TCV), cucumber necrosis virus (CNV) and carnation mottle virus (CarMV). It is therefore infered that the antigenic properties of TNV could lie in either the R-, a- or S-domains.

This chapter reports on the preparation of a cDNA copy of the coat protein gene of a selection of TNV isolates, by RT-PCR, which was then cloned into a suitable vector and sequenced. The sequence variation between the A- and D-like TNV isolates was determined. The genomic RNA sequences and the deduced amino acid sequences were grouped to determine phylogenetic relationships between the A- and D-like TNV isolates.

5.2 TNV isolates

The following TNV isolates were used for cDNA and PCR amplification of the coat protein gene: -Avo, -Citr, -Pap/GL, -Yst/C.o., -750, -Pot/Upd, -Bact/Ps, -Wheat, -Bean/1, -Bean/2 and -Yst/H.u. These viruses were propagated in hosts listed in Addendum A, Table A.1, and purified by differential centrifugation (Addendum B.1) and rate zonal density gradient centrifugation (Addendum B.2). (See Chapter 2, Section 2.2.3). TNV-Yst/H.u. was not included in the immunological study of Chapter 4 as it had not been isolated at the time of that study. It has since been shown to group with the A-like isolates (von Wechmar, unpublished results). In this chapter TNV-A/Mw refers to the TNV sequenced by Meulewaeter *et al.* (1990) and is the A strain studied by Kassanis, TNV-D/Ct refers to the TNV sequenced by Coutts *et al.* (1991) and is the D strain studied by Kassanis (1970a) and TNV-Ne refers to the TNV sequenced by Zhang *et al.* (1993).

5.3 Materials and methods

5.3.1 RNA isolation

Viral RNA was extracted from the TNV isolates after differential centrifugation (Addendum B.1) and rate zonal centrifugation (Addendum B.2). Single stranded genomic RNA was isolated from virions dissociated in 1 % SDS, 1 mM EDTA and 10 mM Tris-Cl (pH 8.25) by phenol / chloroform extraction (Addendum B.7.2) and stored as an ethanol precipitate at -20 °C (Addendum B.15).

5.3.2 cDNA synthesis

The coat protein gene from the TNV isolates were amplified by a combination of cDNA reverse transcription and PCR amplification in a single tube RT-PCR reaction. 0.3 -1.0 µg of RNA was used for amplification. The primers used are listed in Table 4.2. Briefly: cDNA was synthesized using the MMLV reverse transcriptase (Boehringer Mannheim) (Addendum B.10.1) in the presence of the forward and reverse primers designed from the TNV-A/Mw or

-D/Ct strains. The cDNA was denatured at 95 °C for 5 min and cooled on ice. *Taq* polymerase (Promega) was added to the tube for PCR amplification on a University PCR thermal cycler (Addendum B.10.2). The PCR products were run on a 1 x TBE agarose gel and visualised on a 254 nm UV transilluminator (Addendum B.8).

5.3.3 Cloning

Taq polymerase possesses a template independant terminal transferase which adds a single nucleotide to the 3' ends of the PCR product. Thus blunt end cloning of PCR products is inefficient. To overcome this, the PCR products were blunted with klenow (Boehringer Mannheim) (Sambrook *et al.*, 1989) (Addendum B.11.1) and then run on a 1 x TAE agarose gel (Addendum B.8) for purification by Geneclean (Bio101) (Addendum B 11.2). To reduce the background of blunt-end cloning in the bluescript SK (Stratagene) vector, the 5' phosphate of the vector blunt ends were removed by calf intestinal phosphatase (Sambrook *et al.*, 1989) (Addendum B.11.3). The blunted PCR products were ligated into the EcoRV site of the bluescript SK vector (Addendum B.11.4). Competent cells were prepared (Chung and Miller, 1988) and the TNV-coat protein recombinant vectors were transformed into competent *Escherichia coli* strain JM105 (Departmental culture collection) by heat shock (Addendum B.11.5) and grown at 37 °C overnight on LA-AIX plates (Addendum B.11.5).

5.3.4 Clone characterisation

Clones were screened on LA-AIX (Addendum B.11.5) plates by insertional inactivation of the β-galactosidase gene. A selection of white colonies showing insertional inactivation of the β-galactosidase gene of each TNV-coat protein were picked off and grown in 5 ml of LB-Amp for small scale plasmid preparation (Addendum B.14.1). Restriction enzyme digestion of the plasmid DNA with ClaI (Boehringer Mannheim) (Addendum B.12) and agarose gel electrophoresis (Addendum B.8) were used to determine the approximate size of the insert. Clones of the correct size were selected for large scale plasmid DNA purification by Nucleobond (Macherey-Nagel) (Addendum B.14.2).

5.3.5 Subcloning

Partial restriction enzyme maps were determined using the following restriction enzymes: *Bam*HI, *Bst*XI, *Cla*I, *Eco*RI, *Eco*RV, *Kpn*I, *Mlu*I, *Nae*I, *Nde*I, *Pst*I, *Pvu*I, *Pvu*II, *Sca*I, *Xba*I and *Xmn*I. The cloned PCR products were cut into smaller fragments (Addendum B.12) of between 300 and 500 bp and run on a 1 x TAE gel (Addendum B.8). Fragments of the appropriate size were cut out of the gel and purified by Geneclean (Bio101) (Addendum B.11.2) and ligated into bluescript SK (Addendum B.11.4). Recombinant vectors were transformed into competent *E. coli* JM105 or DH5α cells by heat shock (Addendum B.11.5) and grown on LA-AIX (Addendum B.11.5) plates overnight. Clones of the correct size were selected by the method decribed in Chapter 5, Section 5.3.4.

5.3.6 Nucleotide sequencing

The nucleotide sequence was determined by the dideoxy chain termination method of Sanger *et al.* (1977). Cycle sequencing reactions, with fluorescent labelled Cy5-Far Red primers (Forward- 5'-CGCCAGGGTTTTCCCAGTCACGAC-3'
Reverse- 5'GAGCGGATAACAATTTCACACAGG-3'), were done using the Sequitherm™ cycle sequencing kit (Epicentre Technologies) (Addendum B.16.1.1), or Thermosequenase™ cycle sequencing kit (Amersham Life Science) (Addendum B.16.1.2) and run on an ALF-Express automated sequencer (Pharmacia) (Addendum B.16.2 and B.16.3). Double-stranded DNA used in sequencing reactions was purified by Nucleobond (Machery-Nagel) (Addendum B.14.2).

5.3.7 Sequence annalysis

Generated sequences were analysed by the University of Wisconsin Genetics Computer Group (UWGCG) package of programs (Deveraux *et al.*, 1984) Version 8 (1994). Pairwise alignment of sequences was obtained using the GAP program (Needleman and Wunsch, 1970) of UWGCG. Multiple sequence alignments of the generated TNV sequences were made by progressive pairwise alignments using the PILEUP program (Feng and Doolittle, 1987) of UWGCG. ClustalW (Version 1.5 and 1.6) (Thompson *et al.*, 1994) and TREEVIEW (Version 1.2) (Page, 1996) were used to align sequences and draw dendrograms for phylogenetic relationship studies.

5.4 Results

5.4.1 RT-PCR amplification of the coat protein

Amplification of the coat protein gene from the TNV isolates yielded approximately 10 µg of ds DNA. The TNV-D primers amplified the coat protein from the following TNV isolates: -Avo, -Pap/GL, -Citr, -750, -Yst/C.o.. The TNV-A primers amplified the coat protein from the following TNV isolates: -Wheat, -Bean/1, -750, -Yst/H.u.. The coat protein gene of TNVs -Pot/Upd and -Bean/2 were not amplified by PCR using either the TNV-D primers, the TNV-A primers, or any combination of the primers. The sequences amplified with the TNV-D primers were approximately 1000 bp in size, whilst the sequences amplified with the TNV-A primers were approximately 850 bp in size (See Chapter 4 Fig. 4.9).

5.4.2 cDNA cloning and clone characterization

Transformation of competent *E. coli* JM105 cells with the recombinant vector yielded mostly blue colonies with some white colonies, thus indicating that the calf intestinal phosphatase reaction was inefficient. White colonies containing the correct size insert were selected for restriction enzyme manipulation and subcloning. Bacterial colonies containing the various subcloned PCR fragments encompassing the entire coat protein gene of the various TNV isolates were selected for sequencing.

5.4.3 Sequence analysis

Sequence analysis of the TNV clones amplified with the D-specific primers indicated the clones to be 989 bp in length, and TNV clones amplified with the A-specific primers were 831 bp in length.

From PCR amplification and nucleotide sequencing analysis, TNVs -Avo, -Yst/C.o., -750/D, -Citr, -Pap/GL and -D/Ct can be grouped as TNV D-like isolates. TNVs -Bean/1, -Wheat, -750/A, -Yst/H.u., and -A/Mw can be grouped as TNV A-like isolates.

The TNV D-like coat proteins were 268 amino acids in length and had a Mr of approximately 29.1 kDa. The TNV A-like coat proteins were 276 amino acids in length with a Mr of approximately 29.8 kDa. The percentage similarity and identity of the deduced coat protein amino acid sequences of the South African TNV isolates sequenced and the TNV-A/Mw (Meulewaeter *et al.*, 1990), TNV-D/Ct (Coutts *et al.*, 1991) and TNV-Ne (Zhang *et al.*, 1993)

strains is shown in Tables 5.1 and 5.2. TNV-Citr and TNV-Pap/GL have 100% amino acid identity. The amino acid percentage similarity and percentage identity amongst TNVs -Avo, -Yst/C.o., -750/D, -Citr and -Pap/GL is higher than between each virus and the TNV-D/Ct. With TNVs -Bean, -Wheat, -Yst/H.u. and -750/A the amino acid percentage similarity and percentage identity is higher amongst these isolates as compared to the TNV-A/Mw type strain. The amino acid percentage identity between TNV A-like and TNV D-like isolates is approximately 45 to 46%. The TNV D-like isolates have approximately 45% amino acid identity to TNV-Ne and the TNV A-like isolates have approximately 51% amino acid identity to TNV-Ne. These results compare favourably to those obtained by Zhang *et al.* (1993).

The amino acid sequences of TNV-Citr and TNV-Pap/GL are identical and show no variation from the consensus coat protein amino acid sequence of the TNV D-like isolates. TNV-750/D has one amino acid change, TNV-Yst/C.o. and TNV-Avo each have two amino acid changes, and TNV-D/Ct has eight amino acid changes from the consensus coat protein amino acid sequence of the TNV D-like isolates. TNV-Bean, TNV-Yst/H.u., TNV-750/A and TNV-Wheat each have one amino acid change, and TNV-A/Mw has five amino acid changes from the consensus coat protein amino acid sequence of the TNV A-like isolates. The deduced amino acid sequence of the TNV A-like, TNV D-like and TNV-Ne isolates shows little homology at the 5'- end of the coat protein and high homology at the 3'- end of the coat protein (Fig. 5.1).

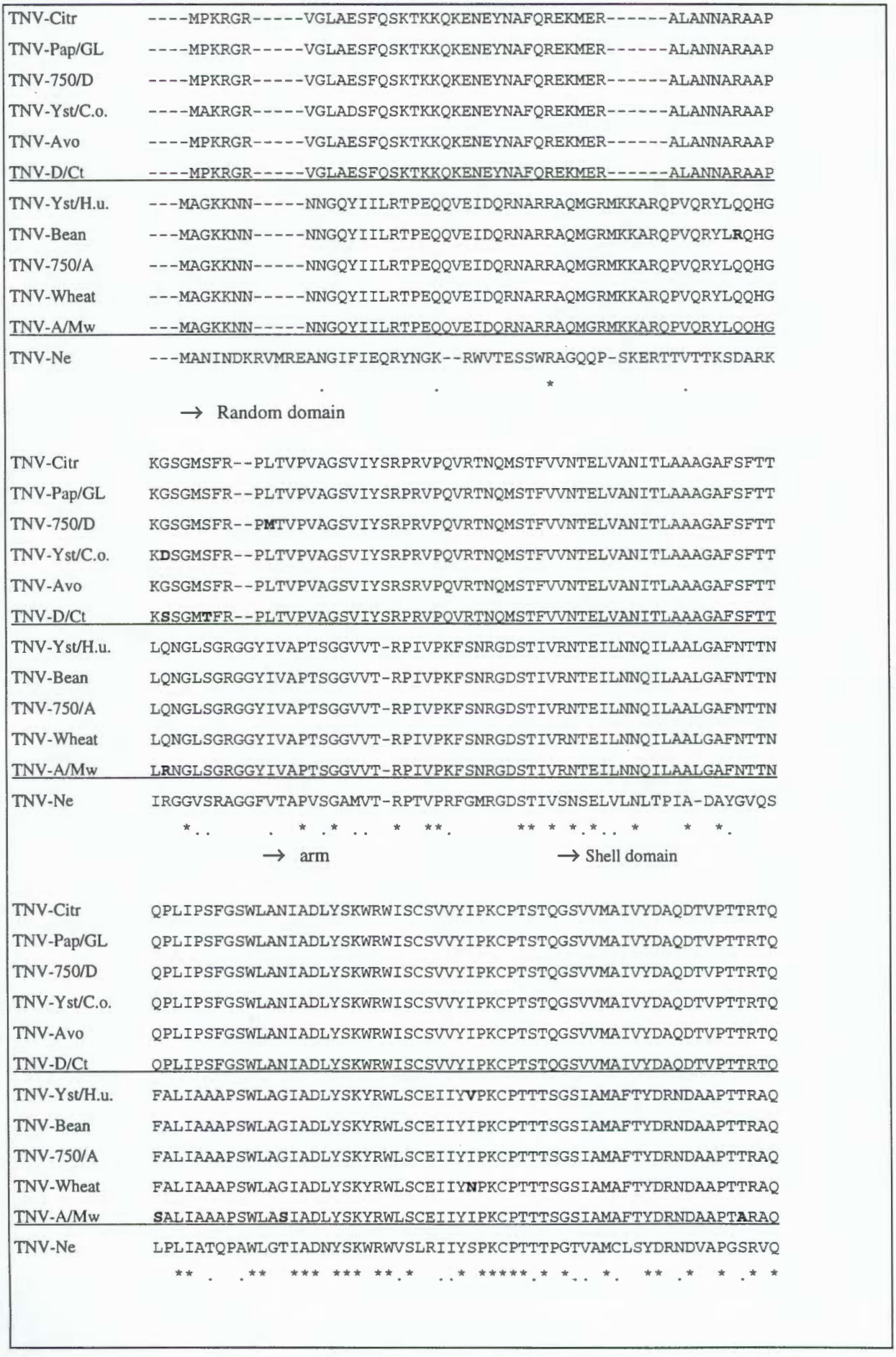
The nucleotide sequence shows that TNV-Citr has no base changes from the consensus coat protein nucleotide sequence of the TNV D-like isolates, TNV-Pap/GL has one base change, TNV-Yst/C.o. and TNV-750/D each have five base changes, TNV-Avo has 11 base changes and TNV-D/Ct has 51 base changes from the consensus sequence coding for the coat protein of the TNV D-like isolates. TNV-Yst/H.u. has two base changes, TNV-Bean/1, and TNV-750/A each have 3 base changes, TNV-Wheat has five base changes and TNV-A/Mw has 33 base changes from the consensus sequence coding for the coat protein of TNV A-like isolates.

Table 5.1 Percentage similarity of the coat protein amino acid sequences of various TNV isolates. The values were determined by the algorithm of Needleman and Wunsch used by the GAP program in UWGCG.

	Avo	Yst/C.o.	750/D	Citr	Pap/GL	D/Ct	A/Mw	Bean	Wheat	Yst/H.u.	750/A	Ne
Avo	-	98.881	98.881	98.881	98.881	97.388	60.821	61.567	60.821	61.194	60.821	58.113
Yst/C.o.		-	100	100	100	98.134	63.806	62.687	61.940	62.313	61.940	58.491
750/D			-	100	100	98.507	61.194	61.940	61.194	61.567	61.194	58.491
Citr				-	100	98.507	61.194	61.940	61.194	61.567	61.194	58.491
Pap/GL					-	98.507	61.194	61.940	61.194	61.567	61.194	58.491
D/Ct						-	60.448	61.194	62.264	62.642	62.264	58.491
A/Mw							-	98.551	98.551	98.913	98.551	68.248
Bean								-	99.275	99.638	99.275	67.883
Wheat									-	99.638	99.275	68.248
Yst/H.u.										-	99.638	68.248
750/A											-	67.883
Ne												-

Table 5.2 Percentage identity of the coat protein amino acid sequences of various TNV isolates. The values were determined by the algorithm of Needleman and Wunsch used by the GAP program in UWGCG.

	Avo	Yst/C.o.	750/D	Citr	Pap/GL	D/Ct	A/Mw	Bean	Wheat	Yst/H.u.	750/A	Ne
Avo	-	97.761	98.507	98.881	98.881	95.896	44.776	45.552	44.776	44.776	44.776	44.151
Yst/C.o.		-	98.507	98.881	98.881	96.269	46.269	46.269	45.552	45.552	45.552	44.906
750/D			-	99.627	99.627	96.642	45.522	46.269	45.522	45.522	45.522	44.906
Citr				-	100	97.015	45.522	46.269	45.522	45.522	45.522	44.906
Pap/GL					-	97.015	45.522	46.269	45.522	45.522	45.522	44.906
D/Ct						-	44.776	45.522	45.283	45.283	45.283	44.906
A/Mw							-	97.826	97.826	97.826	97.826	51.460
Bean								-	99.275	99.275	99.275	51.095
Wheat									-	99.638	99.275	51.095
Yst/H.u.										-	99.275	51.095
750/A											-	50.730
Ne												-


TNV-Citr ----MPKRGR-----VGLAESFQSKTKKQKENEYNAFQREKMER------ALANNARAAP
TNV-Pap/GL ----MPKRGR-----VGLAESFQSKTKKQKENEYNAFQREKMER------ALANNARAAP
TNV-750/D ----MPKRGR-----VGLAESFQSKTKKQKENEYNAFQREKMER------ALANNARAAP
TNV-Yst/C.o. ----MAKRGR-----VGLADSFQSKTKKQKENEYNAFQREKMER------ALANNARAAP
TNV-Avo ----MPKRGR-----VGLAESFQSKTKKQKENEYNAFQREKMER------ALANNARAAP
TNV-D/Ct ----MPKRGR-----VGLAESFQSKTKKQKENEYNAFQREKMER------ALANNARAAP
TNV-Yst/H.u. ---MAGKKNN-----NNGQYIILRTPEQQVEIDQRNARRAQMGRMKKARQPVQRYLQQHG
TNV-Bean ---MAGKKNN-----NNGQYIILRTPEQQVEIDQRNARRAQMGRMKKARQPVQRYLRQHG
TNV-750/A ---MAGKKNN-----NNGQYIILRTPEQQVEIDQRNARRAQMGRMKKARQPVQRYLQQHG
TNV-Wheat ---MAGKKNN-----NNGQYIILRTPEQQVEIDQRNARRAQMGRMKKARQPVQRYLQQHG
TNV-A/Mw ---MAGKKNN-----NNGQYIILRTPEQQVEIDQRNARRAQMGRMKKARQPVQRYLQQHG
TNV-Ne ---MANINDKRVMREANGIFIEQRYNGK--RWVTESSWRAGQQP-SKERTTVTTKSDARK
→ Random domain
TNV-Citr KGSGMSFR--PLTVPVAGSVIYSRPRVPQVRTNQMSTFVVNTELVANITLAAAGAFSFTT
TNV-Pap/GL KGSGMSFR--PLTVPVAGSVIYSRPRVPQVRTNQMSTFVVNTELVANITLAAAGAFSFTT
TNV-750/D KGSGMSFR--PMTVPVAGSVIYSRPRVPQVRTNQMSTFVVNTELVANITLAAAGAFSFTT
TNV-Yst/C.o. KDSGMSFR--PLTVPVAGSVIYSRPRVPQVRTNQMSTFVVNTELVANITLAAAGAFSFTT
TNV-Avo KGSGMSFR--PLTVPVAGSVIYSRSRVPQVRTNQMSTFVVNTELVANITLAAAGAFSFTT
TNV-D/Ct KSSGMTFR--PLTVPVAGSVIYSRPRVPQVRTNQMSTFVVNTELVANITLAAAGAFSFTT
TNV-Yst/H.u. LQNGLSGRGGYIVAPTSGGVVT-RPIVPKFSNRGDSTIVRNTEILNNQILAALGAFNTTN
TNV-Bean LQNGLSGRGGYIVAPTSGGVVT-RPIVPKFSNRGDSTIVRNTEILNNQILAALGAFNTTN
TNV-750/A LQNGLSGRGGYIVAPTSGGVVT-RPIVPKFSNRGDSTIVRNTEILNNQILAALGAFNTTN
TNV-Wheat LQNGLSGRGGYIVAPTSGGVVT-RPIVPKFSNRGDSTIVRNTEILNNQILAALGAFNTTN
TNV-A/Mw LRNGLSGRGGYIVAPTSGGVVT-RPIVPKFSNRGDSTIVRNTEILNNQILAALGAFNTTN
TNV-Ne IRGGVSRAGGFVTAPVSGAMVT-RPTVPRFGMRGDSTIVSNSELVLNLTPIA-DAYGVQS
→ arm
→ Shell domain
TNV-Citr QPLIPSFGSWLANIADLYSKWRWISCSVVYIPKCPTSTQGSVVMAIVYDAQDTVPTTRTQ
TNV-Pap/GL QPLIPSFGSWLANIADLYSKWRWISCSVVYIPKCPTSTQGSVVMAIVYDAQDTVPTTRTQ
TNV-750/D QPLIPSFGSWLANIADLYSKWRWISCSVVYIPKCPTSTQGSVVMAIVYDAQDTVPTTRTQ
TNV-Yst/C.o. QPLIPSFGSWLANIADLYSKWRWISCSVVYIPKCPTSTQGSVVMAIVYDAQDTVPTTRTQ
TNV-Avo QPLIPSFGSWLANIADLYSKWRWISCSVVYIPKCPTSTQGSVVMAIVYDAQDTVPTTRTQ
TNV-D/Ct QPLIPSFGSWLANIADLYSKWRWISCSVVYIPKCPTSTQGSVVMAIVYDAQDTVPTTRTQ
TNV-Yst/H.u. FALIAAAPSWLAGIADLYSKYRWLSCEIIYVPKCPTTTSGSIAMAFTYDRNDAAPTTRAQ
TNV-Bean FALIAAAPSWLAGIADLYSKYRWLSCEIIYIPKCPTTTSGSIAMAFTYDRNDAAPTTRAQ
TNV-750/A FALIAAAPSWLAGIADLYSKYRWLSCEIIYIPKCPTTTSGSIAMAFTYDRNDAAPTTRAQ
TNV-Wheat FALIAAAPSWLAGIADLYSKYRWLSCEIIYNPKCPTTTSGSIAMAFTYDRNDAAPTTRAQ
TNV-A/Mw SALIAAAPSWLASIADLYSKYRWLSCEIIYIPKCPTTTSGSIAMAFTYDRNDAAPTARAQ
TNV-Ne LPLIATQPAWLGTIADNYSKWRWVSLRIIYSPKCPTTTPGTVAMCLSYDRNDVAPGSRVQ

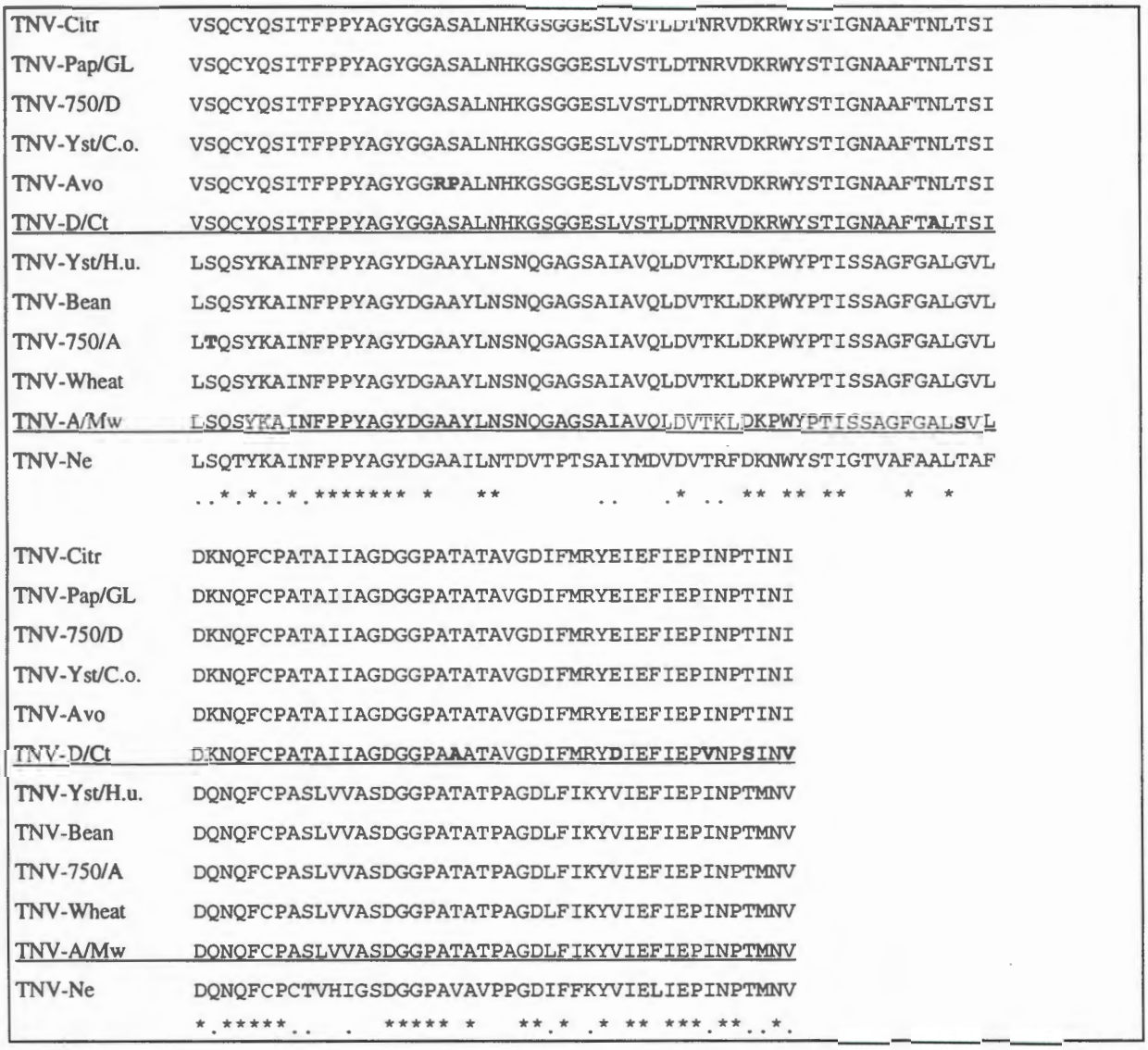


Figure 5.1 Multiple sequence alignment of the deduced amino acid sequence from the coat protein of the TNV isolates. The first six isolates are D-like, the next five isolates are A-like. The underligned TNV-D/Ct indicates the end of the TNV D-like isolates, the underligned TNV-A/Mw indicates the end of the TNV A-like isolates. TNV-D/Ct, TNV-A/Mw and TNV-Ne sequences were previously published. The asterisk (*) indicates identity in the amino acid sequence. The period (.) indicates similarity in the amino acid sequence. The dash indicates gaps introduced to give optimal alignment. Bold letters indicate amino acid residues that vary from the consensus sequence of the TNV D-like and TNV A-like groups. The start of the Random, arm and Shell domains are indicated by an arrow.

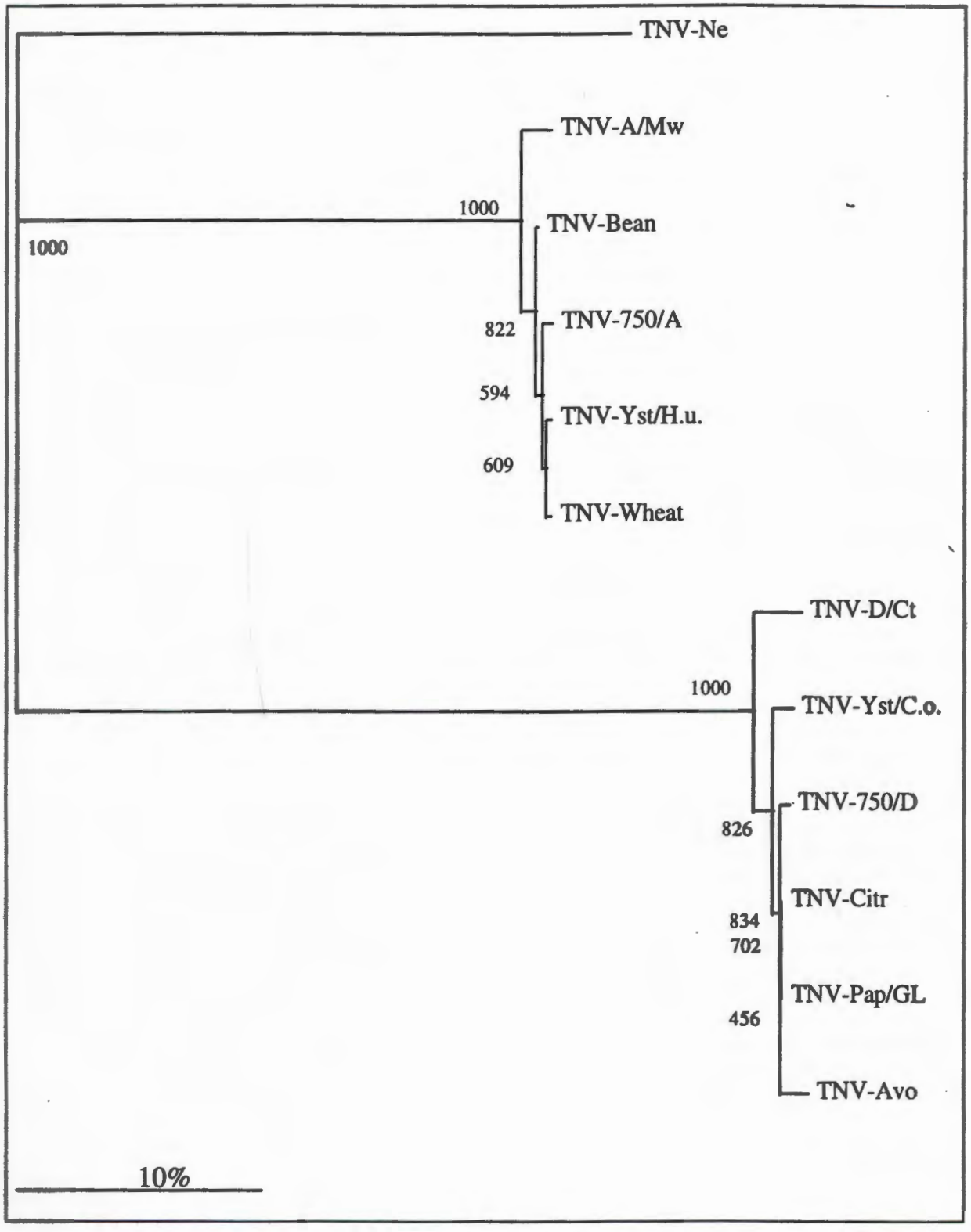


Figure 5.2 Dendrogram of the relationship of the TNV isolates based on pairwise homology scores of the multiple aligned deduced amino acid sequences of the TNV coat protein. The scale at the bottom represents the horizontal distance for 10% difference. The numbers indicate the scores of the bootstrapreplicates of the nodes (out of 1000).

The dendrogram in Fig. 5.2 shows the relationship between the TNV isolates based on the deduced amino acid sequence. The TNV A-like isolates and the TNV D-like isolates clearly cluster into different groups. The TNV-Ne appears to be more closely related to the TNV A-like group than to the TNV D-like group (see Table 5.2), but obviously does not cluster with either of these groups.

5.5 Discussion

Seven of the 11 TNV isolates studied were amplified by RT-PCR with either TNV-A (-Wheat, -Bean/1 and -Yst/H.u.) or TNV-D (-Avo, -Citr, -Pap/GL and -Yst/C.o.) specific primers, one isolate (-750) was amplified with both A- and D-specific primers and two isolates were not amplified with either of the A- or D-specific primers (-Bean/2 and -Pot/Upd). TNV-750 RNA obtained from virus propagated in different host plants was amplified by the A-specific primers (*C. quinoa*) and the D-specific primers (*N. tabacum* cv. Xanthi). Two of the 11 TNV isolates were not amplified, indicating significant sequence variation, in these isolates, in the regions to which the primers were designed. Due to the failure to obtain cDNA clones of the coat protein gene of TNV-Pot/Upd TNV-Bact/Ps and TNV-Bean/2, the RNA and amino acid sequences could not be determined.

The coat protein gene of the TNV D-like and TNV A-like isolates are conserved with the D-like isolates having more than 95 % amino acid identity, and the A-like isolates having more than 97 % amino acid identity. The A-like isolates have only 45 % amino acid identity to the D-like isolates, therefore the A- and D-like isolates form completely different groups in phylogenetic analysis. The TNV-Ne isolate has 51 % amino acid identity with the A-like TNV group and 45 % amino acid identity with the D-like TNV group. The TNV-D/Ct type strain has a lower amino acid percentage identity to the other TNV D-like isolates (Table 5.2) indicating sequence variation from TNVs -Avo, -Yst/C.o., -750/D, -Citr and -Pap/GL. Likewise the TNV-A/Mw type strain has a lower amino acid percentage identity to the other TNV A-like isolates (Table 5.2) indicating sequence variation from TNVs -Bean, -Wheat, -Yst/H.u. and -750/A.

Very little amino acid similarity and identity was noted in the R- and a- domains between the TNV D-like, TNV A-like and TNV-Ne coat proteins. There was a higher percentage amino

acid similarity and identity in the S-domain of the coat proteins from amino acids 79, 87 and 89 in TNV D-like, TNV A-like and TNV-Ne respectively (Fig. 5.1).

The phylogenetic relationships shown in Fig. 5.2 based on the amino acid sequence of the coat protein indicates that TNV-Yst/C.o., TNV-750/D, TNV-Citr, TNV-Pap/GL and TNV-Avo appear to more related with respect to each other than to the TNV-D/Ct. TNV-Bean/1, TNV-750/A, TNV-Yst/H.u. and TNV-Wheat appear to be more related with respect to each other than to the TNV-A/Mw. This sequence variation in the TNV isolates could most likely be due to different evolutionary pressures on the viruses in the different geographical regions.

Because of the low number of amino acid substitutions within the A-like and D-like groups it is not clear whether any region of the coat protein is more susceptable to mutation than any other region. Although there is a risk of PCR amplification introducing mutations, the length of the sequences analysed and the variable degree of differences between the sequences could mean that the amino acid substitutions reflect the heterogeneity of the A-like and D-like TNV groups.

A better phylogenetic relationship dendrogram may have been obtained if the coat protein nucleotide sequences of the TNV isolates was used, because the nucleotide sequence contains more base substitutions than amino acid substitutions in the protein.

Discussion

This is the first detailed study of tobacco necrosis virus (TNV) isolated from avocado. Biophysical and biochemical characteristics identified the virus as a member of the Necrovirus family of plant viruses. Immunological analysis showed that the virus could be characterized as a D-type TNV isolate. The TNV was found to be associated with a satellite tobacco necrosis virus. The D-type TNV-Avo differs from the Kassanis D which was not found to have any natural STNV component.

Immunological and nucleic acid hybridization assays were used to detect TNV in potatoes that exhibited abnormal symptoms. A- and D-like TNV isolates were detected in two potato cultivars. The Buffelspoort-13 potato cultivar was found to be infected with both an A- and a D-type TNV. The Up-to-Date potato cultivar was found to be infected with only a D-type TNV. The unavailability of virus free potato plants prevented the fulfillment of Koch's postulates and thus TNV was not proven to be the causative agent of the abnormal symptoms observed in potato plants.

This is the first report in which new isolates of TNV, isolated from plants and other sources in South Africa were grouped into serotypes by immunological assays and confirmed by NA hybridization. Results obtained from immunoelectroblots were variable depending on the specificity of the antisera. The TNV isolates were divided into two serotypes, A and D. The A isolates examined included TNVs -Bean/1, -Bean/2, -Wheat, -Yst/H.u. and -Cel. The D isolates examined included TNVs -Avo, -Citr, -Pap/GL, -Pot/Upd, -750, -Yst/C.o. and Bact/Ps. The grouping of TNV isolates into two serotypes is in agreement with the findings of Babos and Kassanis (1963) and Kassanis and Phillips (1970), but differs from the findings of Uyemoto *et al.* (1968), who were unable to group TNV isolates into only two serotypes. The findings of Uyemoto *et al.* are most likely due to cross-reactivity of the antisera used, as shown for some antisera in this work, which cross-reacted with both A- and D-type TNV isolates. Anti-TNV sera that were specific for either the A- or D-type serotypes, however made the division of TNV into the A and D serotypes possible. Nucleic acid hybridization of the TNV isolates to cDNA probes derived from the coat protein gene of an A- (TNV-Wheat) and a D-type (TNV-Pap/GL) isolate, confirmed the division of TNV into two serotypes, A and D.

The genomic RNA of the TNV isolates was approximately 3.7 to 3.8 kb in size. SDS-PAGE indicated that the D like isolates had a coat protein greater than 30 kDa in size, and the A like

isolates had a coat protein of approximately 29.2 kDa in size. Sequence analysis of the coat protein genes indicated that the D like isolates had a coat protein of approximately 29.1 kDa, and the A like isolates had a coat protein of approximately 29.8 kDa. This discrepancy in the coat protein size of TNV-D obtained by SDS-PAGE and from sequence analysis cannot be explained at present, but could be due to incomplete denaturation of the coat protein by SDS and BME, or due to some form of post-transcriptional processing of the D-like coat proteins.

PCR amplification of the coat protein genes, with primers designed to two specific TNV isolates, showed variability within the serotypes. TNV-Bean/2, an A-type isolate, coat protein could not be amplified with the A specific primers, whereas TNVs -Bean/1, -Wheat, -750 and -Yst/H.u. could be amplified. TNVs -Pot/Upd and -Bact/Ps are both D-type isolates, but their coat proteins could not be amplified with the D-specific PCR primers, whereas TNVs -Avo, -Citr, -Pap/GL, -750 and -Yst/C.o. coat proteins could be amplified with the primers. The TNV-750 isolate coat protein was amplified with both the A and D specific primers, but the isolate from which the A coat protein was amplified was propagated in *Chenopodium quinoa* and the isolate from which the D coat protein was amplified, was propagated in *Nicotiana tabacum* cv. Xanthi. The reason that the coat protein of some TNV isolates failed to amplify, would most likely be due to significant sequence variation at the site of primer binding, with respect to the strains from which the primers were designed.

Different plant hosts appear to select different TNV isolates as shown by TNV-750 and the PV67 isolate obtained from the ATCC. Therefore the propagation host species is important for further analysis of different TNV isolates.

Sequence analysis of the coat protein genes of the TNV isolates of which the coat proteins were amplified, shows high homology within the A- and D-type isolates. Sequence homology was greater than 97.8 % amongst the A-type isolates and greater than 96.2 % amongst the D-type isolates. The A-type isolates appear to be more conserved than the D-type isolates. The South African A- and D-type isolates are highly conserved and show less sequence variation amongst virus isolates in the same group than to the Kassanis A strain (sequenced by Meulewaeter *et al.* 1990) and Kassanis D strain (sequenced by Coutts *et al.* 1991).

The phylogenetic relationship studies of the TNV coat proteins shows that the TNV isolates divide into three specific groups, the A-type, the D-type and TNV-Ne, and the TNV-Ne isolate being more related to the A-type group than to the D-type group. Within the A-type

group the Kassanis A appears to be distantly related to the South African A-type isolates. Within the D-type group the Kassanis D appears to be distantly related to the South African D-type isolates. This indicates that the South African TNV isolates had different evolutionary pressures which caused sequence divergence when compared to the British TNV isolates.

The association of TNV with culturable microorganisms as reported by von Wechmar (Jaffer *et al.*, 1993; von Wechmar and Jaffer, 1990; von Wechmar *et al.*, 1990; von Wechmar *et al.*, 1991; von Wechmar *et al.*, 1993; von Wechmar *et al.*, 1994a; von Wechmar *et al.*, 1994b and von Wechmar *et al.*, 1994c) was observed with TNV identified in Buffelspoort-13 potatoes. However, the exact association of the TNV with the unidentified microorganism was not determined. (The examination of the association of TNV with other microorganisms including yeast and bacteria is in progress and will be published M.B. von Wechmar, pers comm).

The coat protein appears to be more conserved in the Shell-domain than in the Projecting- and arm-domains. Therefore degenerate primers designed to this conserved region could give rise to a very sensitive and relatively quick diagnostic technique for the detection and identification of TNV in diseased crops by RT-PCR analysis.

To study the divergence and sequence similarity of TNV isolates, it may be better to focus on the putative polymerase gene coded by ORFs 1 and 2, as this gene may have more sequence variation, because the polymerase would be responsible for the satellite activation of TNV and the variation in host range and symptom expression noted amongst TNV isolates.

In conclusion, it appears that the serological division of South African TNV isolates into two serotypes is justified and follows the same pattern as observed by Babos and Kassanis (1963). These serotypes most likely contain strains with variable sequence homology. The coat protein sequence of South African TNV isolates, have high homology compared to the published A and D strains and vary by only 2 - 4 %. Other TNV isolates that were shown to be serologically A- or D-types, but of which coat protein genes were not amplified by PCR, could have significantly more sequence variation. The TNV coat proteins of the different isolates have a highly conserved Shell domain with some sequence variation, and this could most likely be the domain which confers the serological specificity of TNV isolates.

Addendum A

Lists of viruses, plants and antisera

Table A.1 TNV isolates, their origin, serotype and hosts in which they were propagated for extraction

Virus	Origin	Serotype	Propagation host*
TNV-749**	Pulverised rock	D	*C.quinoa*
TNV-750**	Pulvarised rock.	D and A	*C. quinoa*
TNV-Avo	*Persea americana* cv. Colin V-33, young shoots with "lace leaf" symptoms.	D	*C. pepo* (Caserta bush), *C. sativa*
TNV-Bact/Ps**	Culturable Organisms from Rock	D	*C. pepo* cv Caserta bush
TNV-Citr	*Cucumis zeiherii* weed in *C. papaya* orchard, Mpumulanga.	D	*C. quinoa*
TNV-D/RK	Lyophilised infected *Chenopodium quinoa* obtained from Renate Koenig, BBA, Braunshweig.	D	*C. sativa*
TNV-Pap/GL	*Carica papaya* cv. Sunrise solo; sunken green lesions on fruit.	D	*N. benthamiana*
TNV-Pot/BP	*Solanum tuberosum* cv. Buffelspoort-13, Foliage; Sandveld, Western Cape	D and A	Not isolated
TNV-Pot/Upd	*Solanum tuberosum* cv. Up-to Date, tuber skin; Sandveld, Western Cape	D	*C.quinoa*
TNV-Yst/H.o.**	Yeast lodged in rock.	D	*N. benthamiana*
TNV-Bean/1	*Phaseolus vulgaris* cv. Canalino; Cedara, Kwazulu-Natal. Plants infected with *Uromyces appendiculatus.*	A	*N. tabacum* cv. Xanthi

Table A.1 continued over the page

TNV-Bean/2	*Phaseolus vulgaris*, cv. CNC; Carltonville, Gauteng. Plants infected with *Uromyces appendiculatus*	A	*N. tabacum* cv. Xanthi
TNV-Cel	*Apium graveolens* cv. Self blanching grown in TNV contaminated soil	A	*N. benthamiana*, *N. tabacum* cv. Soulouk
TNV-Fu/M.c.	*Mucor circinelloides* isolated from *Zea mays*, soil and water.	A	*N. tabacum* cv. Xanthi
TNV-PV67	Lyophilised *Vigna sinensis* obtained from the ATCC permit no. 14/2/2/1(9/93/100)	A (D)	*N. tabacum* cv. Xanthi
TNV-PV68	Lyophilised infected *Phaseolus vulgaris* obtained from the ATCC permit no. 14/2/2/1(9/93/100)	A	*C. pepo* cvs Caserta bush and Long green bush
TNV-Wheat	*Triticum aestivum* cv. Betta, Diseased seedlings; Eastern Free State	A	*Triticum aestivum* cv. Scheepers
TNV-Yst/H.u.**	Yeast lodged in Rock	A	*N. benthamiana*, *N. tabacum* cv. Xanthi
TNV-Tulip***	*Tulipa* sp. imported ornamental variety	unknown	*C. quinoa*

* Refer Table A.2 for full names.

** Details of the exact origins of these isolates will be published by Prof. M.B. von Wechmar elsewhere

*** TNV-Tulip was difficult to propagate and therefore was not studied in detail

Except for the potato TNV isolate, all the isolates examined were made available from Prof. M.B. von Wechmar's collection of TNVs.

Table A.2 Plant hosts used for virus propagation or as indicator hosts

Latin name	Common name
Apium graveolens	Celery
Carica papaya	Papaya
Chenopodium quinoa	
Cucumis sativa cv. Rust resistant	Cucumber
Cucurbita pepo cv. Long green bush	Squash
Cucurbita pepo cv. Caserta bush	Squash
Gomphrena globosa	
Hordeum vulgare cv. Clipper	Barley
Nicotiana benthamiana	
Nicotiana clevelandii	
Nicotiana glutinosa	
Nicotiana tabacum cv. Soulouk	
Nicotiana tabacum cv. Xanthi	
Passiflora sp.	Passion fruit
Persea americana cv. Colin V-33	Avocado
Phaseolus vulgaris cv. Bonus	Bean
Solanum tuberosum	Potato
Triticum aestivum cv. Scheepers	Wheat
Tulipa sp.	Tulip
Vigna sinensis	Cowpea
Zea mays cv. Potchefstroom pearl	Maize

Table A.3 Antisera used in immunological assays

Antisera	Serotype	Virus host	Assay
TNV-Avo[1]	D	*C. pepo* (CB)	IEB, ISEM, Tissue blot
TNV-Citr[1]	D	*C. quinoa*	IEB
TNV-D/RK[1]	D /A	*C. sativa*	IEB, ISEM
TNV-Pap/GL[1]	D /A	*N. benthamiana*	IEB
TNV-Pot/Upd[1]	D	*C. quinoa*	IEB, ISEM
TNV-Apt[2]	A	*C. quinoa*	IEB
TNV-Bean/1[1]	A	*N. tabacum* cv. Soulouk	IEB, Tissue blot
TNV-Cel[1]	A	*N. benthamiana*	Tissue blot
TNV-Fu/M.c	A	*P. vulgaris*	ISEM
TNV-Pap/A[3]	A	*N. tabacum* cv. Xanthi	IEB
TNV-Pas[4]	A /D	*N. tabacum* cv. Xanthi	IEB, ISEM
TNV-PV67[1]	A / D	*N. tabacum* cv. Xanthi	IEB
TNV-PV68[1]	A / D	*P. vulgaris*	IEB, ISEM

1 For details of virus isolates refer Table A.1

2 TNV-Apt isolated from bacillus type bacteria cultured from rock (Jaffer and von Wechmar, 1993).

3 TNV-Pap/A isolated from culturable organisms from *Carica papaya* (von Wechmar *et al.*, 1994b).

4 TNV-Pas isolated from *Passiflora* sp.

Addendum B

Standard methods

This section has been arranged such that the method has been given first followed by the recipes for the buffers and reagents at the end of each section.

B.1 Virus extraction and purification

Unless otherwise stated all steps in the virus purification procedure were carried out in 0.01 M phosphate buffer at room temperature.

Viral samples were purified from infected leaves by differential centrifugation after thorough grinding in a mortar and pestle. Clarification of the viral sample was achieved by low speed centrifugation at 10 000 RPM in an SS34 type rotor or 9 000 RPM in a GSA type rotor in a Sorvall RC-5 superspeed refrigerated centrifuge for 10 min at 10 °C. The supernatent was then concentrated by ultracentrifugation at 30 000 RPM in a type 35 or 50.2 Ti rotor in a Beckman ultracentrifuge model L5-65 / L6-55 for 120 min at 10 °C.

Phosphate buffer

Solution A: 0.5 M KH_2PO_4

Solution B: 0.5 M K_2HPO_4

0.01 M Phospate (pH 7.0): 7.7 mL soln A; 12.3 mL solution B to a final volume of one L

B.2 Rate zonal density gradient centrifugation

Preformed sucrose gradients were made by adding 17.5 mL 10 % sucrose and 17.5 mL of 40 % sucrose solution to separate chambers of a gradient maker. The solutions were mixed by mechanical stirring and the gradient made in nitrocellulose or ultraclear tubes using a peristaltic pump. Two mL of the virus preparation was layererd on top of the preformed 10 % - 40 % sucrose gradient. The tubes were then placed in rotor buckets and loaded on a SW28 swinging bucket rotor and centrifuged at 25 000 RPM for 150 min at 20 °C. Gradients were observed in the dark with a fibre-optic light source shining into the gradient from the top and the viral band(s) removed by tube puncture with a syringe and 18 gauge hypodermic needle, or by siphoning the band out with a pasteur pipette with a tip bent at a right angle.

The sucrose was removed from the virus solution by diluting one part sucrose containing the viral band to three parts 0.01 M phosphate buffer and pelleting the virus by ultracentrifugation at 35 000 RPM in a 60 Ti rotor for 120 min at 4 °C. The pellets were resuspended, centrifuged at 10 000 RPM for 10 min in a SS34 rotor (Sorval RC-5 superspeed centrifuge) at 10 °C and filtered through a 0.45 µm followed by a 0.22 µm acetate filter (MSI).

Rate zonal density gradient reagents

10 % sucrose: 10 % (w/v) made in 0.01 M Phosphate buffer pH 7.0

40 % sucrose: 40 % (w/v) made in 0.01 M Phosphate buffer pH 7.0

B.3 Isopycnic density gradient centrifugation

0.1 mL of purified virus preparations were added to 4.7 mL of a 1.4 g/mL solution of caesium chloride in nitrocellulose tubes. The tubes were placed in rotor buckets and loaded on a SW 50.1 swinging bucket rotor. The gradients were centrifuged at 44 000 RPM for 20 hrs at 20 °C. The gradients were observed in the dark, using a fibre-optic light from the top. The light scattering bands were removed by tube puncture with a syringe and 18 gauge hypodermic needle. The refractive index was determined with an Atago hand held refractometer, and the buoyant density determined from Beckman standard tables (Griffith, O.M., Techniques of preparative, Zonal, and continuous flow ultracentrifugation, 1979).

B.4 Protein methods

B.4.1 SDS-PAGE

Protein samples were denatured in 62.5 mM Tris-Cl (pH 6.8), 5 % SDS, 5 % BME by mixing the virus sample in an equal volume of disruption buffer and heating for 10 min at 96 °C. BRL low molecular weight markers were used as molecular weight standards. Disrupted proteins were electrophoresed through polyacrylamide gels using the discontinuous buffer system of Laemmli (1970). Large polyacrylamide gradient gels (11 % - 13 %) were run in a verticle slab gel apparatus (Hoefer SE 600, Hoefer Scientific Instruments, San Fransisco) and small polyacrylamide gels (12 %) were run in a verticle slab gel apparatus ("mighty small", Hoefer Scientific Instruments, San Fransisco).

Large gels (160mm long X 140 mm wide X 1.5 mm thick) were run overnight at 90V C/V at 4 °C with constant stirring. Small gels (80 mm long X 70 mm wide X 1.5 mm thick) were run at 25 mA C/C for approximately 120 min.

SDS-PAGE reagents

Resolving gel buffer:	1M Tris-Cl, pH 8.8
Stacking gel buffer:	1 M Tris-Cl, pH 6.8
Acrylamide solution:	40% Acrylogel BDH (39:1 acrylamide : bis)
Electrophoresis buffer:	25 mM Tris;192 mM glycine; 0.1 % SDS
Disruption buffer:	125 mM Tris-Cl pH 6.8; 10 % (v/v) β-mercaptoethanol; 15 % (v/v) glycerol; 0.01 % (w/v) bromophenol blue.
Ammonium persulphate:	10 % (w/v)

B.4.1.1 **SDS-PAGE gel preparation**

Stock	Resolving gel				Stacking gel	
	11%	12%	13%	12%	4%	
	Large	Large	Large	Small	Large	Small
Acrylamide:	5.5	6.0	6.5	3	1	0.5
Water:	9.2	8.7	6.2	4.4	6.4	3.2
Resolving gel buffer:	5	5	5	2.5	-	-
Stacking gel buffer:	-	-	-	-	2.5	1.25
10% (w/v) SDS:	0.2	0.2	0.2	0.1	0.1	0.05
100% Glycerol:	-	-	2	-	-	-
10% APS:	100µL	100µL	100µL	125µL	100	50µL
TEMED:	10µL	10µL	10µL	12.5µL	10	5µL
	20	20	20	10	10	5

All volumes in mL unless stated.

For gradient gels, 14.4 mL of the 11 % and 13 % gel mixture were placed in separate tubes of a gradient maker. The gels were mixed at low speed and poured via a peristaltic pump.

B.4.1.2 PAGE blue stain

The gels were stained in stain solution for 2-16 hrs with gentle agitation at room temperature. The gels were destained in several changes of destain solution for eight hours followed by a two hour final destain in 10 % acetic acid. The gels were sealed in plastic bags before being photographed.

Stain Reagents

Stain:	1 % (w/v) Page blue 83 (BDH, UK) dissolved in 30 % (v/v) methanol, 10 (v/v) % acetic acid
Destain:	30 % (v/v) methanol, 10 (v/v) % acetic acid
10 % acteic acid:	10 % (v/v) acetic acid

B. 5 Immunological assays

B. 5.1 Immunoelectroblotting

Immunoelectroblotting was performed essentially according to the method of Towbin (1979) using a semi-dry blotting apparatus (Omeg Scientific, SA) with slight modifications. After electrophoresis, nitrocellulose membrane (Pore 0.45 µm, MSI) presoaked in transfer buffer and the polyacrylamide gels were placed between presoaked filter paper. The sandwich was placed between nappy liners (Johnson & Johnson) soaked in transfer buffer, and placed in a semi-dry blotting apparatus. The nitrocellulose was closest to the anode. Transfer was done for 2 hrs at 0.8 A constant voltage. The nitrocellulose membrane was allowed to dry overnight before immuno detection.

B.5.2 Tissue blot

Tissue blotting was done essentially according to the method of Bravo-Almonacid *et al.* (1992). The potato stems were cut with a surgical blade, the newly cut surface was pressed onto Nitrocellulose membrane (pore 45 µm, MSI) and left for 10 min. The membranes were left to dry for one hour before immuno detection.

B.5.3 Immuno detection

After transfer of the proteins to the nitrocellulose membrane, the membranes were placed in blocking buffer to allow non-specific protein binding sites to be blocked. Anti-TNV antibodies were appropriately diluted (1 : 50 - 1 : 100) in blocking buffer and the membranes were allowed to incubate for two hrs at room temperature. The blots were washed three times for five minutes in wash buffer. The blots were incubated in a dilution of goat anti-rabbit gamma globulin linked to alkaline phospahatase (GAR-AP) (1:1000) in blocking buffer and incubated for 90 min at room temperature. The blots were washed as before and incubated in fresh substrate buffer containing 0.33 mg/mL nitro blue tetrazodium (Sigma); 0.17 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate (Sigma); 0.33 % (v/v) N,N-dimethyl formamide. The membranes were left in the substrate for 5 - 10 min and then washed in water and left to dry before being photographed.

Immunoassay reagents

Transfer buffer:	25 mM Tris; 192 mM glycine; 20% (v/v) methanol
Phosphate buffered saline (PBS):	10 mM phosphate; 150 mM NaCl pH 7.4
Blocking buffer:	4.5 % (w/v) Fat free milk powder in PBS
Wash buffer:	0.1 % Tween 20 in blocking buffer
Substrate Buffer:	100 mM Tris; 100 mM NaCl; 5 mM $MgCl_2$
Nitro blue tetrazodium:	75 mg/mL (w/v) in 70% dimethyl formamide
5-bromo-4-chloro-3-indolyl phosphate:	50 mg/mL (w/v) in 100 % dimethyl formamide

5.4 Goat anti-rabbit alkaline phosphatase conjugation

Goat anti-rabbit gamma globulin (GAR-IgG) was conjugated to alkaline phosphatase (Boehringer Mannheim) by gluteraldehyde cross linking as described by Clark and Adams (1977).

B.6 Electron Microscopy

B.6.1 Negative stain

Virus peparations were stained according to the method of Milne (1984). Carbon coated copper grids are floated on a drop of purified preparation of virus for 30 min. The grid was washed under a slow stream of water and blotted dry. The grid was then stained by placing it on a drop of 2 % (w/v) uranyl acetate (pH 4.2) for two minutes and blotted dry. The grid was viewed on a Jeol 200CX electron microscope.

B.6.2 Immunosorbant electron microscopy

This technique was done according to the method of Milne (1984). Carbon coated copper grids were floated on a 1 : 1000 dilution of antisera for 30 min, washed and blotted dry. The grids were then placed on a purified preparation of virus for 30 min, washed and blotted dry. The grids were then placed on a 1 : 10 dilution of the same antisera for 15 min, washed and blotted dry. The grids were stained on a drop of 2 % (w/v) uranyl acetate (pH 4.2) and viewed on a Jeol 200 CX electron microscope.

B. 7 RNA methods

B. 7.1 Denaturing RNA gel electrophoresis

RNA sizing was determined in formaldehyde denaturing agarose gels according to the method of Sambrook *et al.* (1989). Five µL RNA was denatured in 17,5 % formaldehyde and 50 % formamide by heating to 60 °C for 10 min. RNA was electrophoresed in a 0.8 % agarose (Sigma) gel with 1 x electrophoresis buffer and 6 % (v/v) formadehyde. The agarose was dissolved in water and cooled to 50 °C before the electrophoresis buffer and formaldehyde were added. The gels were run at 80 V, washed in Milli-Q water (Millipore systems) for 30 min, stained in 10 µg/mL ethidium bromide for 30 minutes and destained in several changes of water over a 30 min period. RNA bands were visualised on a 254 nm UV transilluminator and photographed.

Denaturing RNA gel reagents

Electrophoresis buffer:	20 mM N-(3-morpholino)popanesulphonic acid (MOPS); 5 mM Sodium acetate; 1 mM EDTA, pH 7.0
Formaldehyde:	Deionised over a mixed-bed resin
Formamide:	Deionised over a mixed-bed resin
Loading buffer:	50 % (v/v) glycerol; 0.4 % (w/v) bromophenol blue; 1mM EDTA
Et Br:	1 % (w/v)

All reagents were made with designated RNase free chemicals and Milli-Q (Millipore water systems) water in baked glassware to reduce contamination by RNases.

B.7.2 Phenol/chloroform extraction of total nucleic acids

Genomic viral RNA and total NA was extracted from purified virus preparations, concentrated plant tissue or bacterial cells. The specimen was mixed with an equal volume of dissociation buffer, and extracted by phenol/chloroform.

An equal volume of buffered phenol : chloroform : isoamyl alcohol (25:24:1) was added to the sample preparation. The mixture was vortexed and placed on ice for five min. The mixture was centrifuged in a desk top microfuge (Eppendorf centrifuge 5415 C) for 10 min. The top aqueous phase was removed and mixed with an equal volume of buffered phenol : chloroform : isoamyl alcohol (25:24:1) and vortexed. The mixture again centrifuged and the top aqueous phase removed. The nucleic acid was stored with Na-acetate pH 5.5 to 120 mM and 2.5 volumes of 96 % ethanol at -20 °C. The NA was precipitated by centrifugation and washed with ice-cold 70 % ethanol, air-dried and resuspended in RNase free water.

Phenol/chloroform extraction reagents

Dissociation buffer:	2 % (w/v) SDS, 2 mM EDTA, 20 mM Tris pH 8.25
Buffered phenol:	Tris buffered, containing 8-Hydroxyquinoline
Chloroform:	Chloroform isoamylalcohol mixed in a ratio of 24:1

B.8 DNA gel electrophoresis

Agarose gel electrophoresis was performed in submerged horizontal slab gels. Analytical gels (200 mm long X 150 mm wide) were cast and run in custom made perspex apparatus. Gels were electrophoresed at 40 V overnight or 100 V for 4-6 hrs. Hoefer "mini-gel" apparatus (model HE 33, Hoefer Scientific Instruments, San Fransisco) (75 mm long X 50 mm wide) were used for quick runs and electrophoresed at 100 V for 1-2 hrs. DNA was sized against λ DNA digested with PstI.

Tris-borate-EDTA was used for analytical gels, while tris-acetate-EDTA was used for gels from which DNA bands were to be excised for purification by Geneclean (Bio101). Agarose (0.8 % to 1.5 %) was dissolved in TBE or TAE by heating. The gels were cooled to approximately 50 °C before pouring and run in the appropriate buffer. Ethidium bromide (0.5 μg/mL) was added to the running buffer for visualisation of the nucleic acid on a 254 nm transilluminator. When DNA was to be recovered the gels were visualised on a 365 nm transilluminator.

DNA gel electrophoresis reagents

Tris-EDTA:	10 mM Tris-Cl, 1 mM EDTA, pH 7.6
Tris -borate-EDTA:	89 mM Tris; 89 mM Boric acid; 2 mM EDTA
Tris-actetate-EDTA:	40 mM Tris-acetate, 2 mM EDTA pH 8.0
Loading buffer:	10 % (w/v) SDS, 0.02 % Bromophenol blue, made up in TE buffer
EtBr:	1 % (w/v)

B.9 Nucleic acid hybridisation

B.9.1 Northern blot hybridisation

Northern blot hybridisation of the genomic RNA was detected with digoxygenin labelled DNA probes. After electrophoresis of the TNV ss genomic RNA in a denaturing formaldehyde gel (B 5.2.1), the gel was soaked two times for 15 min in 20 x SSC (B 9.3). The RNA was transferred to a positively charged nylon membrane (Hybond N^+, Amersham) by capillary action in 10 x SSC at 4 °C overnight (Sambrook *et al.*, 1989). The gel was placed on a layer of filter paper covering a 5 cm wick of tissue paper (both presoaked in 10 x SSC), the nylon membrane which in turn was placed on five pieces of filter paper (both presoaked in

10 x SSC), was placed on top of the gel. A 10 cm thick wad of dry tissue paper was placed on top of the filter paper and covered by a glass plate. The ends of the tissue paper wick was placed 10 x SSC and a two kg weight placed on top of the glass plate above the assembly of tissue paper, filter paper, gel, membrane, filter paper and covered with a glass plate. The RNA was fixed to the membrane by UV-crosslinking the membrane on a 254 nm transilluminator for 3 min each side. The hybridization and detection procedures were according to the manufacturers instructions (Boehringer Mannheim Biochemica, The DIG system user's guide for filter hybridisation) (B 9.3).

B.9.2 **Nucleic acid dot-blot hybridisation**

RNA or total NA was spotted onto nylon membrane (Hybond N^+; Amersham, UK) presoaked in 10 x SSC (B 9.3) under a water vacuum in a Schleicher & Schuell minifold dot blot apparatus. The nucleic acid was fixed to the membrane by UV crosslinking both sides of the membrane for five min. Hybridisation and detection was according to the manufacturers instructions.

B.9.3 **Nucleic acid hybridisation and detection**

Detection was done according to the manufacturers instructions (Boehringer Mannheim Biochemica, The DIG system user's guide for filter hybridisation). The membranes were placed in a plastic bag and 25 mL of hybridisation solution added, and incubated at 68 °C for 90 min, the DIG-labelled probes were heat denatured at 96 °C for 10 min and 5 µL of the probe (B 10.3) was added to the appropriate membrane and incubated overnight at 50 °C. The membranes were washed twice for 5 min each at room temperature in wash solution 1, followed by two washes for 15 min each at 65 °C in wash solution 2. Subsequent steps were performed at room temperature. After post-hybridisation washes the membranes were equilibrated in buffer 1 for 1 min and then blocked in buffer 2 for 40 min. The membranes were then incubated in anti-DIG-alkaline phosphatase diluted 1 in 10 000 in buffer 2 for 30 min. The membranes were removed from the antibody solution and washed twice for 15 min each in buffer 1. The membranes were placed in buffer 3 for 2 min, and then in a 1 in 200 dilution of CSPD in buffer 3 in the dark for 5 min. The membranes were blotted dry on paper towel and placed in a plastic bag and incubated at 37 °C for 15 min. The membranes were then exposed to AGFA-Curex X-ray film for 16 hours.

Nucleic acid hybridisation reagents

	Transfer solutions
20 x SSC:	3 M NaCl; 0.3 M Na-citrate pH 7.0
	Hybridisation solutions
N-lauroylsarcosine:	10 % (w/v) filter sterilised
SDS:	10 % (w/v) filter sterilised
Blocking reagent:	10 % blocking reagent; 150 mM NaCl; 100 mM Maleic acid pH 7.5
High SDS hybridisation buffer:	7 % SDS; 50% (v/v) deionised formamide; 5 x SSC; 2 % blocking reagent; 50 mM Na-phosphate pH 7.0; 0.1 % N-lauroylsarcosine
	Wash solutions
Wash solution 1:	2 x SSC; 0.1 % SDS
Wash solution 2:	0.1 x SSC; 0.1 % SDS
	Detection solutions
Buffer 1:	150 mM NaCl; 100 mM Maleic acid pH 7.5
Buffer 2:	1 in 10 dilution of blocking reagent in Buffer 1
Buffer 3:	100 mM diethanolamine pH 10; 50 mM $MgCl_2$

B.10 RT-PCR

B.10.1 cDNA synthesis

RNA was added to 50 µL of 1 x PCR buffer containing 8 picomoles of forward primer, 8 picomoles of reverse primer, 10 % (v/v) DMSO, 0.015 mM $MgCl_2$ and 0.2 mM each dNTP. 20 units of MMLV reverse transcriptase (Boehringer Mannheim) was added, and the reaction mix incubated at 42 °C for 60 min. The reaction mix was heated to 96 °C for 5 min, to denature the single stranded RNA - cDNA hybrid, and flash cooled on ice.

B.10.2 PCR amplification

2.5 units of *Taq* polymerase (Promega) was added to the cDNA synthesis mix. 50 µL of sterile mineral oil was overlayed on the reaction mix to prevent evaporation. 35 cycles of 96 °C for 90 sec, 52 °C for 60 sec and 72 °C for 60 sec were used for PCR amplification on a University PCR thermal cycler.

PCR buffer

10 x PCR buffer:	500 mM KCl; 100 mM Tris-Cl (pH 9.0); 1% Triton X-100

B.10.3 DIG labelling PCR amplification

To the cDNA synthesis mix (B 10.1), 2.5 U *Taq* polymerase (Promega) and a 10 x dilution of DIG-dUTP-labelling mix was added for PCR amplification.

DIG labelling mix

10 x DIG-dUTP label mix:	1 mM dATP; 1 mM dGTP; 1 mM dCTP; 0.65 mM dTTP; 0.35 mM DIG-dUTP, pH 7.5

B.11 Cloning strategy

B.11.1 Polynucleotide kinase / klenow reaction of the PCR product

The 5'-phosphate group was added to the PCR product after amplification by PNK by the following method. 20 µL of PCR product (concentration unknown) was added to 30 µL of 1 x PNK buffer containing 20 U PNK and 30 mM ATP. The reaction was incubated at 37 °C for 60 min and the enzyme heat inactivated by heating to 65 °C for 5 min. 0.8 mM each dNTP and 8 U klenow (Boehringer Mannheim) was added to the reaction mix and incubated at room temperature for 20 min. The DNA was removed by phenol/chloroform extraction (Addendum B.12).

PNK buffer

10 x PNK:	500 mM Tris-Cl; 100 mM $MgCl_2$; 50 mM DTT; 1 mM EDTA, pH 7.6

B.11.2 Geneclean

PCR products and DNA fragments were removed from TAE gels and purified using the Geneclean II Kit (Bio101 Inc) as per the manufacturers instructions. Briefly the procedure is as follows: PCR products are run on a TAE gel, stained with EtBr and viewed on a transilluminator. Samples were run in duplicate lanes, with one lane shielded from the UV light. The position of the PCR band or restriction fragment was marked, and the band in the unexposed lane cut out from the gel and placed in an 1.5 mL eppendorf tube. The gel fragment was weighed, three times volume of 6M NaI was added and the gel was melted at 50 °C for 5 min. Five µL glassmilk was added and the solution shaken vigorously and placed on

ice for 5 min. The eppendorf was centrifuged at 10 000 RPM for 30 sec. The supernatant was removed and the pellet washed three times with NEW wash solution. The NEW wash solution was removed and the glassmilk pellet was resuspended in 10 µL TE buffer and placed at 50°C for 5 min. The eppendorf tube was centrifuged at 10 000 RPM for 30 sec and the TE buffer removed and placed in a new eppendorf tube.

B.11.3 **Removal of the 5' phosphate group of the vector**

10 µg of pSK was digested with 20 U of *Eco*RV restriction enzyme in a 20 µL volume containing 1 x Buffer B (B 12). The restriction enzyme was removed by phenol/chloroform extraction and the DNA was salt precipitated in ethanol. The DNA was resuspended in 100 µL containing 3 U CIP (Boehringer Mannheim) and 1 x CIP dephosphorylation buffer. The reaction mix was incubated at 37 °C for 30 min, after which a further 3 U of CIP was added to the reaction mix and incubated at 37 °C for 30 min. The CIP was removed by phenol / chloroform extraction (B.13) and the DNA was salt precipitated in ethanol (B.15). The DNA was resuspended in 50 µL.

CIP buffer

10 x CIP buffer:	500 mM Tris-Cl; 1 mM EDTA pH 8.5

B.11.4 **Ligations**

Ligations were performed in 20 µL volumes in 1 x ligation buffer containing 1 U of T4 ligase (Boehringer Mannheim) at 15 °C overnight. The vector : insert ratio was approximatey 1 : 3 and the DNA concentration was kept at approximately 600 ng.

Ligase buffer

10 x ligase buffer:	660 mM Tris-Cl; 50 mM $MgCl_2$; 10 mM DTE; 10 mM ATP, pH 7.5

B.11.5 Transformations

E. coli were made competent by the rubidium chloride protocol of Chung and Miller (1988). Five µL of the ligation mix was transformed into 100 µL competent DH5α or JM105 *E. coli* cells by placing cells on ice for 10 min and then heat shocking at 37 °C for 5 min. 0.9 mL luria broth was added to the cells and left to incubate for 1 hour at 37 °C. The cells were plated on LA-AIX plates for overnight incubation at 37 °C.

Transformation media

Luria Broth (LB):	10 g Tryptone; 5 g Yeast extract; 5 g NaCl, made to one L
LA-AIX plates:	10 g tryptone, 5 g Yeast extract, 5 g NaCl 15 g agar made to one L and autoclaved. Cool to 50°C, add ampicillin to 100µg/mL and pour plates. Spread 40 µl of X-Gal (20 mg/mL in dimethylformamide) and 4 µl IPTG (200 mg/mL) on each plate and allow to dry.

B.12 Restriction enzyme digestion

B.12.1 Plasmid DNA

Small- and large scale DNA preparations were used in restriction enzyme digests. The restrictrion enzymes were obtained from Boehringer Mannheim and Amersham. Boehringer Mannheim buffers were used for digests, the restriction enzyme buffers are listed below. All digests were done at 37 °C for 60-120 min except XmnI, which was done at 65 °C. Between 1 and 5 µg of DNA was digested using 2 U of enzyme per µg of DNA in a 20 µL or 50 µL volume.

Multiple digests were done in the same buffer and at the same time if the temperature allowed. The percentage buffer activity was read off tables and the amount of restriction enzyme was increased to give approximately 100 % activity. If buffer and temperature did not allow, the digest was done sequentially using the buffer with the lowest salt concentration first. DNA digests were run on 1 x TBE or TAE gels (Addendum B 8.1).

B.12.2 λ DNA

λ DNA was digested with *Pst*I restriction enzyme in 1 x buffer H to give 28 fragments of the following size in bp: 15, 72, 87, 94, 150, 164, 200, 211, 216, 247, 264, 339, 448, 468, 514, 805, 1093, 1159, 1700, 1986, 2140, 2443, 2560, 2838, 4507, 4759, 5077 and 11497.

Composition of Boehringer Mannheim restriction enzyme buffers

Buffer components	Final concentration (mM)				
	A	B	L	M	H
Tris-acetate:	33	-	-	-	-
Tris-Cl:	-	10	10	10	10
Mg-acetate:	10	-	-	-	-
$MgCl_2$:	-	5	10	10	10
K-acetate:	66	-	-	-	-
NaCl:	-	100	-	50	100
DTE:	-	-	1	1	1
DTT:	0.5	-	-	-	-
2-Mercaptoethanol:	-	1	-	-	-
pH at 37 °C:	7.9	8.0	7.5	7.5	7.5

B.13 Phenol / chloroform extractions

Phenol / chloroform extraction was used to remove enzymes after enzymatic manipulations of DNA. The DNA was made to 200 µL in TE buffer, and an equal volume of phenol / chloroform / isoamylalcohol (25:24:1) was added. The mixture was emulsified by vigorous shaking on a vortex, and placed on ice for 5 min. The DNA contained in the aqueous phase was separated by centrifugation at 10 000 RPM for 5 min and removed. The nucleic acid was precipitated by salt precipitation in ethanol (B.15).

B.14 Plasmid DNA preparation

B.14.1 Small scale plasmid DNA preparation

Small scale plasmid DNA preparation were done by the alkaline lysis method of Ish-Horowicz & Burke (1981). 5 mL cell cultures were grown in LB-Amp (LB containing 100 µg/mL ampicillin) at 37 °C overnight with continuous shaking. 1.5 mL of cell culture was centrifuged at 10 000 RPM in a microfuge, the cell pellet drained and resuspended in 200 µL of Solution 1, and left for 10 min at room temperature. 400 µL of Solution 2 was added, well mixed by inverting four times before being placed on ice for 10 min. 300 µL of Solution 3 was added, well mixed, and placed on ice for 10 min. The mixture was centrifuged at 10 000RPM for 10 min and 0.8 mL of the supernatant removed and placed in a clean eppendorf tube. The DNA was precipitated with 0.8 times volume of isopropanol. After centrifugation, the DNA pellet was washed with 70 % ethanol, dried and resuspended in TE buffer.

Small scale plasmid DNA preparation reagents

Solution 1:	250 mM Tris-Cl; 500 mM Glucose; 100 mM EDTA, pH 8.0
Solution 2:	200 mM NaOH; 1 % SDS
Solution 3:	3 M K-acetate; 2 M Acetic acid
TE buffer:	10 mM Tris-Cl; 1 mM EDTA, pH 7.6

B.14.2 Large scale plasmid DNA preparation

Large scale plasmid DNA preparation was done using the Nucleobond AX PC-Kit 100 (Macherey-Nagel) according to the manufacturers instructions. 100 mL cultures were grown in LB-Amp at 37 °C overnight with continuous shaking. The cells were harvested by centrifugation at 5 000*g* for 5 min, resuspended in 4 mL S1 and incubated at room temperature for 5 min. 4 mL S2 was added, the suspension mixed by inversion and incubated at room temperature for 5 min. 4 mL S3 was added, mixed by inversion and incubated on ice for 10 min. The suspension was centrifuged at greater than 10 000*g* for 40 min at 4 °C and the supernatant removed and loaded on a NUCLEOBOND AX-100 cartridge equilibrated with 2 mL of N2. After runthrough of the supernatant, the cartridge was washed twice with 4 mL of N3. The plasmid DNA was eluted with 2 mL of N5, the first 10 drops of eluate were not collected. The plasmid DNA was precipitated with 0.8 volume of isopropanol and centrifuged at 10 000RPM for 15 min. Isopropanol was removed, and the DNA pellet washed twice with

70 % ethanol, dried and resuspended in an appropriate volume of TE buffer. The concentration of the DNA was estimated by UV absorbance at a wavelength of 260 nm.

Nucleobond reagents

S1:	50 mM Tris-Cl; 10 mM EDTA; 100 µg/mL RNase A, pH 8.0
S2:	200 mM NaOH, 1 % SDS
S3:	2.6 M K-acetate, pH 5.2
N2:	100 mM Tris, 15 % ethanol; 900 mM KCl, pH 6.3 with H_3PO_4
N3:	100 mM Tris; 15 % ethanol; 1150 mM KCl, pH 6.3 with H_3PO_4
N5:	100 mM Tris; 15 % ethanol, 1000 mM KCl, pH 8.5 with H_3PO_4

B.15 Salt precipitation in ethanol

DNA and RNA were precipitated from solution by the addition of Na-acetate pH 5.5 to 120 mM and 2.5 volumes of ice-cold 96 % ethanol followed by centrifugation at 10 000 RPM for 10 min at 4 °C. The DNA pellet was washed with 70 % (v/v) ethanol, dried and resuspended in an appropriate buffer.

Salt for nucleic acid precipitation

3 M Na-acetate pH 5.5: pH with glacial acetic acid

B.16 Nucleotide sequencing

B.16.1 Cycle sequencing

B.16.1.1 Sequitherm cycle sequencing (Epicentre Technologies)

The protocol followed was as per the manufacturers instructions. 3 µL of A, C, G, or T termination mix was alliquoted into 200 µL PCR tubes. In separate eppendorf tubes, 1.5 µg of DNA was diluted in 17 µL of Milli-Q water containing 2.5 µL of 10 x sequencing buffer, 2.1 pmol primer, and 1 µL Sequitherm DNA polymerase. The reagents were well mixed. 3.8 µL of this mix was aliquotted into each of the A, C, G, or T termination mixes. The PCR tubes were placed in a Hybaid thermal cycler with a heated lid. The following temperature cycle was used: 1 cycle of 93 °C for 5 min; 30 cycles of 93 °C for 30 sec, 55 °C for 30 sec, 70 °C for 60 sec; and 1 cycle of 93 °C for 30 sec, 55 °C for 30 sec 70 °C for 5 min. The tubes were placed on ice, and 3 µL Stop buffer (Pharmacia Autoread kit) was added.

Sequitherm reagents

10 x sequencing buffer:	500 mM Tris-Cl; 25 mM $MgCl_2$, pH 9.3
G termination mix:	0.03 mM ddGTP, 45 mM dATP, dCTP, dTTP and 7-deaza-dGTP
C termination mix:	0.03 mM ddCTP, 45 mM dATP, dGTP, dTTP and 7-deaza-dCTP
A termination mix:	0.03 mM ddATP, 45 mM dGTP, dCTP, dTTP and 7-deaza-dATP
T termination mix:	0.03 mM ddTTP, 45 mM dGTP, dCTP, dATP and 7-deaza-dTTP
Pharmacia Stop:	As per manufacturer

B.16.1.2 Thermosequenase cycle sequencing (Amersham Life Science)

The method followed was as per the manufacturers instructions. 2µL of A, C, G, or T termination mix was added to 200 µL PCR tubes. In separate eppendorf tubes, 1 µg of DNA was diluted to 22 µL in water containing 2.1 pmol of primer. 5.1 µL of the DNA mix was aliquotted into each termination mix tube, mixed and placed in a Hybaid thermal cycler with a heated lid. The following temperature cycle was used: 1 cycle of 93 °C for 5 min; 30 cycles of 93 °C for 30 sec, 55 °C for 30 sec, 70 °C for 60 sec; and 1 cycle of 93 °C for 30 sec, 55 °C for 30 sec 70 °C for 5 min. The tubes were placed on ice and 4 µL of Stop (Pharmacia autoread) added.

Thermosequenase reagents

G termination mix:	As per manufacturer
C termination mix:	As per manufacturer
A termination mix:	As per manufacturer
T termination mix:	As per manufacturer
Pharmacia Stop:	As per manufacturer

B.16.2 Gel preparation

Acrylamide gel mix was made by mixing 19g Urea (ICN or Pharmacia) in a 50 mL volume of milli-Q water containing: 1.5 x TBE, and 0.5 % (v/v) long ranger acrylamide mix (Pharmacia). The gel mix was stirred slowly for 30 min and filtered through a Millex 0.45 µm vinyl filter. 25 µL TEMED and 250 µL 10 % Amps were added to the acrylamide gel mix. The gel mix was carefully poured between the plates with two 25 mL syringes to avoid bubbles forming in the gel. The acrylamide was left to polymerize for 2 hours.

Sequence gel reagents

10 x TBE:	1 M Tris; 830 mM Boric acid; 10 mM EDTA, pH 8.3
Long Ranger:	As per manufacturer 50 % stock
10 % Amps:	10 % (w/v)

B.16.3 Setting up the Pharmacia ALF-Express automated sequencer

The DNA sequencing samples were heat denatured at 96 °C for 5 min and 4 µL of each sample loaded into the wells. The gel was run in 0.6 % TBE buffer. The ALF-Express was run with the following settings:

Volts	1000
mAmps	60
Watts	25
Temperature	55 °C
Sample time	2 sec
Running time	720 min

The data was processed with the Pharmacia software package AM V 3.02, which controls and evaluates the sequence data generated by the Pharmacia ALF-Express automated sequencer.

Addendum C

Plant growth conditions and culture media for micro-organisms

C.1 Plant growth room conditions

Plants were grown in heat sterilised soil in growth rooms with controlled conditions of approximately 70 % relative humidity, temperature between 18 °C and 24 °C and 14 hours of light (VHO Growlux fluorescent light tubes) and 10 hours of dark.

C.2 Culture media

C.2.1 Luria broth

Tryptone:	10 g
Yeast extract:	5 g
NaCl:	5 g

Make up to one L and autoclave.

2.2 Kings B media

Protease peptone (Difco):	20 g
K_2HPO_4:	1.5 g
$MgSO_4.7H_2O$:	1.5 g
Glycerol:	15 mL

Make up to one L and autoclave.

2.3 Enriched luria

Tryptone:	10 g
Yeast extract:	10 g
NaCl:	5 g
Glucose:	5 g

Make up to one L and autoclave.

For solid media, 15 g of agar was added to each L of liquid media before autoclaving. The media was cooled to approximately 50 °C and poured into petrie dishes.

C.2.4 **Potato dextrose media**

C.2.4.1*Potato dextrose (liquid)*

Potato, peeled and sliced: 200 g

Dextrose: 20 g

The potatoes were adjusted to a L volume and autoclaved. The solid matter was strained out and the dextrose added. The volume was adjusted to 1 L and autolcaved a second time.

C.2.4.2*Potato dextrose (agar)*

Potato dextrose (Difco) 39 g

Make up to one L and autoclave. Cool to approximately 50 °C and pour into petrie dishes.

C.3 Knopps solution for plants

250 mg KNO_3: 250 mg

$Ca(NO_3)$ 2:1 g

$MgSO_4.7H_2O$ 250 mg

KH_2PO_4: 250 mg

Make up to 1 L and autoclave (Riker & Riker, 1936).

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