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Full Length Research Paper

Isolation and molecular characterization of Tomato spotted wilt virus (TSWV) isolates occurring in South Africa

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***Tomato spotted wilt virus* (TSWV), a *Tospovirus*, causes high economic losses in many crops worldwide. A typical Tospovirus was isolated from naturally infected pepper (*Capsicum* sp.) in KwaZulu-Natal (KZN), South Africa (SA). The identity of the virus was confirmed as TSWV using an enzyme-linked immunosorbent assay (ELISA), electron microscopy, and protein analysis. Genetic diversity of TSWV in SA was investigated. A total of six TSWV isolates originating from different regions in SA were used in this study. Total plant RNA, isolated from *Nicotiana rustica* infected with the SA TSWV isolates were subjected to reverse-transcription polymerase chain reaction (RT-PCR) using primers specific to the nucleocapsid (N) gene of TSWV. The amplified 760 bp products was then cloned and sequenced. Nucleotide sequence comparisons of the N gene revealed high similarity (> 90%) between the SA isolates. Phylogenetic analysis based on the multiple alignments of N gene sequences of the SA TSWV and isolates occurring in different geographical locations in the world revealed the branching of TSWV isolates into two distinct clusters designated the American and European groups. The SA isolates showed high sequence similarity with TSWV isolates from Europe. The information generated in this study will be useful in formulating effective control measures using genetic engineering approaches for this economically important virus.**

Keywords: Electron microscopy, molecular characterization, phylogenetic analysis, South Africa, *Tomato spotted wilt virus*, *Tospovirus*.

INTRODUCTION

Tomato spotted wilt virus (TSWV) is an enveloped plant virus that causes significant yield losses in many economically important crops worldwide (German et al., 1992; Mumford et al., 1996). Based on morphological and molecular data, TSWV has been classified as the type member of the genus *Tospovirus* within the *Bunyaviridae*, a large family of mainly arthropod-borne viruses (de Haan et al., 1989; Elliot, 1990; Francki et al., 1991). The economic impact of TSWV is huge mainly due to its extremely broad host range and world-wide distribution (Rosello et al., 1996) . It possesses one of the largest host ranges of any plant virus, with over 1090, plant species in over 100 families cited (German et al., 1992; Peter, 2003). The virus is exclusively transmitted by thrips in a persistent manner (Sakimura, 1963; Paliwal, 1975).

As a typical *Tospovirus* , TSWV virions are spherical enveloped particles, with a diameter of 80-120 nm, covered with surface projections consisting of two glycol-proteins denoted G1 (78K) and G2 (54K) (Mohamed et al., 1973; Tas et al., 1977, German et al., 1992). The genome is characterized by three single-stranded linear segments of RNA, denoted small (S) RNA (2.9 kb), medium (M) RNA (5.4 kb), and large (L) RNA (8.9 kb) (van den Hurk et al., 1977; Mohamed, 1981; de Haan et al., 1990; de Haan et al., 1991). Each RNA segment is associated with nucleocapsid (N) proteins (29K) and a few copies of the large (L) protein to form pseudo-circular nucleocapsid structures (Kormelink et al., 1992; Peters, 2003).

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In South Africa (SA), the emergence of TSWV is a significant problem in crop cultivation (Thompson and van Zijl, 1996). A disease survey has ranked TSWV as the most prevalent viral disease in tomato (*Lycopersicon esculentum*) in SA (Uys et al., 1996).

The use of resistant cultivars provides the most effective and durable way to minimise crop losses due to TSWV infection (Pappu et al., 1998). As natural sources of resistance are limited and often strain-specific, there is a need to look for alternative methods to control the virus. To this end, crops with transgenic expression of the TSWV N gene have been developed (Mackenzie and Ellis, 1992; Pang et al., 1992) . However, the enormous diversity that exists among the isolates of TSWV has had a significant negative impact on these control efforts.

In general, the resistance conferred by the N gene is effective only against viral isolates with a high degree of sequence homology to the transgene. Thus knowledge of the relatedness of N gene sequences of the various isolates of TSWV is essential for the success of genetic engineering resistance strategies (Pappu et al., 1998). However, little or no information is available on the genetic diversity of the virus isolates that occur in SA. Here, we describe the first report of a TSWV isolate occurring in KZN, SA. In order to understand the sequence diversity of TSWV isolates in SA, this study also investigates the sequence divergence of the N gene of the SA TSWV isolates when compared to isolates occurring in different geographical locations in the world.

MATERIALS AND METHODS

Virus isolation, detection and maintenance

A total of six TSWV isolates originating from Gauteng (GP), KwaZulu-Natal (KZN), North West (NWP), Limpopo (LP) and Mpumulanga (MP) provinces in South Africa (SA) were used in the study. Desiccated and freeze-dried TSWV sources from North West, Limpopo and Mpumulanga provinces were provided by the Plant Protection and Research Institute (Agricultural Research Council (ARC), Pretoria, SA). The TSWV isolate from KZN was identified and characterized in this study.

Leaf samples showing typical TSWV-like symptoms were collected from five major pepper (*Capsicum* sp.) growing farms in KZN. TSWV infection of these samples was confirmed by direct antibody sandwich (DAS)-enzyme-linked immunosorbent assay (ELISA) as described by Clark and Adams (1977) using a kit from Bio-Rad (Plantest).

Mechanical transmission to *Nicotiana rustica* was carried out using ELISA-positive samples as described by Mandal et al. (2001). The inoculum was prepared by grinding the samples in ice-cold 0.5 M potassium phosphate buffer using a chilled pestle and mortar. The extract was then rubbed onto the primary leaves of plants that had been previously dusted with carborundum and leaves were rinsed thereafter with chilled distilled water. The tests were performed in an insect-proof tunnel and inoculated plants were kept at a constant temperature of 20 - 25^{O}C for the duration of the study.

Electron microscopy

Plant tissues for electron microscope examination were collected at the peak of symptom expression (14 days post inoculation). Pieces of leaf tissue (5 mm x 5 mm) from infected *N. rustica* plants were crushed in a drop of distilled water. Formvar-coated grids were then placed on top of the crude sap for 30 s. Grids were stained with 3% uranyl acetate and observed under a Jeol 100 CX transmission electron microscope (TEM).

For ultra-thin sectioning, leaf slices (2 mm x 2 mm) from chlorotic spots of *N. rustica* infected with TSWV were prefixed for 48 h in 3% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 6.88), washed twice in that buffer and then postfixed with 2% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 6.88) for 1.5 h. After dehydration with a graded ethanol series (10 - 100%) for a minimum of 10 min per solution, the samples were imbedded in Epon-Araldite resin. Ultra-thin sectioning was performed with glass knives mounted on a microtone. Sections were placed on 200-mesh copper grids, stained with 2% uranyl acetate and viewed under a Jeol 100 CX TEM. All images were captured using a digital MegiViewBIII camera.

Purification and analysis of viral proteins

The virus was purified according to the method described by Gonsalves and Trujillo (1986) with modifications as described by Dijkstra and de Jager (1998). *Nicotiana rustica* leaves showing prominent symptoms were used as the virus source during the purification procedure.

Viral proteins of partially purified virus preparations were dissociated and separated by electrophoresis in a 12% sodium dodecyl sulphate (SDS) polyacrylamide gel as described by Laemmli (1970). *Tobacco mosaic virus* (TMV) coat protein was run as a control.

Total RNA isolation and RT-PCR

Total RNA from leaf material of *N. rustica* infected with the various SA TSWV isolates was extracted using the SV Total RNA isolation system (Promega, USA) according to manufacturer's instructions and used as a template for amplification in the RT-PCR.

RT-PCR was performed using a TITANIUM^{TM} One-Step RT-PCR Kit (Clontech, CA). The primer pair (JLS90-46 5'-AGCTAACCATGGTTAAGCTCACTAAGGAAAGC-3' and JLS90-47 5'-AGCATTCCATGGTTAACACACTAAGCAAGCAC-3') derived from the nucleocapsid protein (N) gene sequence of TSWV was used and amplification products of 760 bp were expected (Pang et al., 1992). Amplification was performed in an automated thermal cycler programmed for one cycle of 50^{O}C for 1 h and 94^{O}C for 5 min and 30 cycles of amplification with 30 s of denaturation at 94^{O}C, 30 s of annealing at 65^{O}C and 1 min of extension at 68^{O}C followed by one cycle of final extension for 2 min at 68^{O}C. The RT-PCR products were analyzed by 1.0% agarose gel electrophoresis.

Cloning, sequencing and phylogenetic analysis

The RT-PCR products was cloned into the cloning vector pCR®2.1 according to manufacturer's instructions (Invitrogen, CA). pCR®2.1 with the ligated insert was used to transform TOP10F' cells according to manufacturer's instructions (Invitrogen, CA). Plasmid DNA of independently transformed bacterial cells was purified using the QIAprep Spin Miniprep Kit (Qiagen, Germany). Recombinant clones were identified by EcoR1 restriction endonuclease digestion and selected clones were sequenced at Inqaba Biotechnical Industries (Pty) Limited, P.O. Box 14356, Hatfield 0028, SA.

The BLAST program (Altschul et al., 1990) was used to obtain related TSWV N gene sequences from GenBank (Benson et al., 1996) . Sequence comparisons were done using the MegaAlign pro-gram of DNASTAR software (Madison, WI). Multiple alignments

were done using CLUSTALX (Thompson et al., 1997). The sequences were manually verified and edited to ensure optimal alignment using BioEdit Sequence Alignment Editor version 6.0 (Hall, 1999). Phylogenetic analysis was performed with the PHYLIP package version 3.65 (Department of Genetics, University of Washington, Seattle). Distance and similarity matrices were calculated with the DNADIST program according to the Jukes-Cantor model. Phylogenetic trees were constructed using the neighbour- joining algorithm in the NEIGHBOR program. Bootstrap analysis (1000 replications) using the SEQBOOT program (Felsenstein, 1985) was used to estimate the confidence of tree topologies and the CONSENSE program was used to generate the majority rule consensus tree. The TREEVIEW program was used to generate the rooted tree (Page, 1996).

RESULTS

ELISA and symptomatology

TSWV was positively identified in leaf samples collected from the pepper growing farms in KZN using ELISA. *N. rustica* plants inoculated with crude sap from ELISA-positive samples exhibited typical TSWV-like symptoms. Chlorotic spots, followed by necrotic spots and vein necrosis were observed on uninoculated leaves.

Electron microscopy

Under the TEM, typical spherical TSWV-like particles (80 - 110 nm in diameter) were observed in negatively stained preparations of crude leaf sap (Figure 1a) from symptomatic *N. rustica* leaves. A few particles appeared distorted giving a characteristic 'dumbbell' form. There was clear evidence of the presence of an outer viral envelope, a key distinguishing feature of tospoviruses.

In negatively stained ultra-thin sections of infected plant material, typical mature virus particles measuring 100 nm in diameter were observed in clusters surrounded by enveloping membranes, which may be the cisternae of the endoplasmic reticulum (Figure 1b). These structures were present only in the cytoplasm and not in nuclei, chloroplasts or mitochondria. Dense amorphous masses known as viroplasms measuring 30 nm in diameter occurred in tight association with mature virion clusters. All structures are typical of TSWV cytopathology. No comparable structures were found in leaf sections of healthy control plants.

Analysis of viral protein

Viral protein migrating as a 29K band, which correspond to typical TSWV nucleocapsid (N) proteins was observed after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (data not shown).

RT-PCR

An amplification product of 760 bp was detected after agarose electrophoresis of RT-PCR products of total RNA isolations from leaf material infected with the SA TSWV isolates. The positive control yielded an expected 1100 bp band whilst negative control showed no products (Figure 2).

Phylogenetic analysis

The nucleotide sequences reported in this paper have been deposited into GenBank. The details of the sequences used in the analysis are presented in Table 1. Although all TSWV isolates used in the comparison revealed a nucleotide identity of greater than 90%, a distinct evolutionary clustering pattern was observed. The phylogenetic tree (Figure 3) derived from the TSWV N gene sequences grouped the isolates into two distinct clusters, one composed of isolates from Bulgaria, Czech Republic, Germany, Italy and SA (European group) whilst the other cluster comprised of isolates from Japan, California, North Carolina, Hawaii, Spain and Colorado (American group). With the exception of the Japan and Spain isolates, the observed clustering pattern is in accordance with the findings of Silva et al. (2001), who tentatively designated the clusters as 'European' and 'American' groups.

DISCUSSION

We have confirmed, based on positive ELISA reactions, characteristic symptomatology, electron microscopy, protein banding and phylogenetic patterns, that the virus isolated from peppers in KZN is TSWV. This is the first report of TSWV on peppers in KZN.

The nucleotide sequences of six TSWV isolates from SA are reported here. This is the first phylogenetic analysis of TSWV in SA. No genetic variation among TSWV isolates in SA was found (> 90%). This result indicated that TSWV in SA has not evolved at a molecular level. Evolution is a process in which genetic variation in a population changes occur over time (Snustad and Simmons, 1997). It could be that since the emergence of TSWV in SA dates back as early as 1939 (Thompson and van Zijl, 1996), insufficient time has passed for evolutionary forces to sift and shape mutational variation in SA TSWV populations. However, given the necessary length of evolutionary time, natural selection will allow viral mutants with impaired survival and replication abilities to decrease in frequency, and mutants with superior survival and replication abilities to increase in frequency (Snustad and Simmons, 1997). It may be that the generation of new resistant varieties could impose selection pressure that could favor the selection of variants with the ability to overcome current resistance strategies for control. Therefore, population genetic studies should be carried out on SA TSWV populations to investigate the systemic and random forces of evolution, and in doing so; attempts can be made to elucidate the fundamental

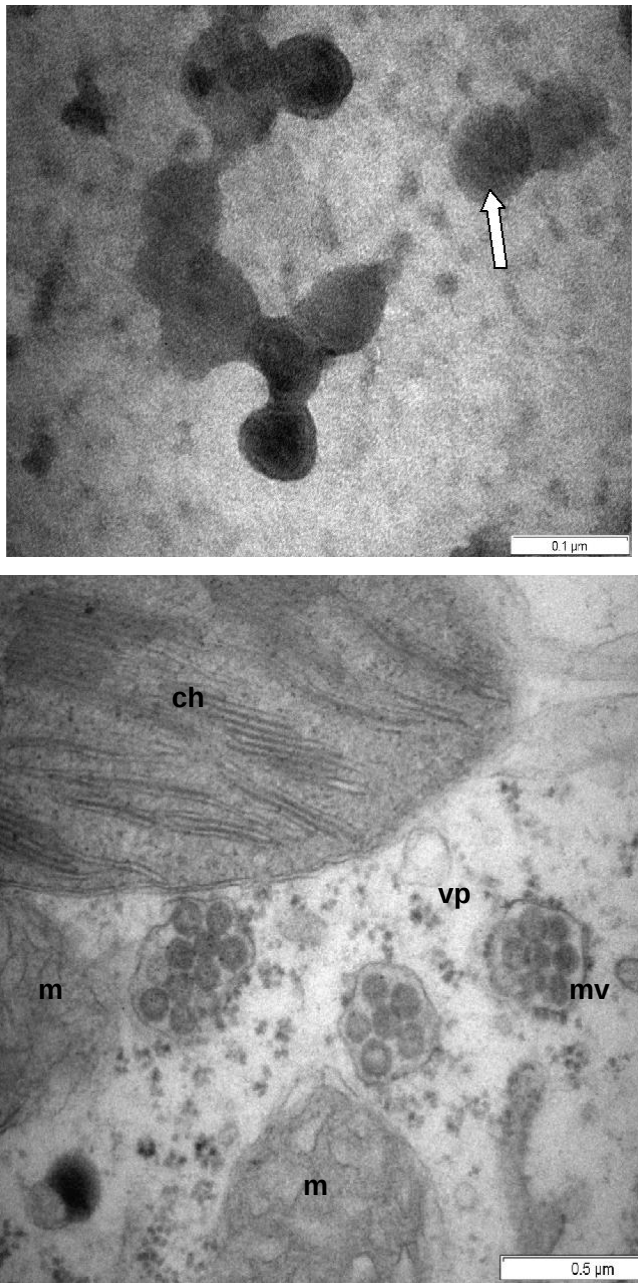

Figure 1. Transmission electron micrographs of *tomato spotted wilt virus* (TSWV) particles in (a) negatively stained crude leaf sap and (b) negatively stained ultra-thin leaf sections from symptomatic *Nicotiana rustica* plants. The characteristic outer-envelope of TSWV is visible in a. Arrow indicates the presence of a 'dumbbell-like particle. An abundance of mature TSWV virion particles (mv), housed in membrane-bound cisternae of the endoplasmic reticulum. vp = viroplasm inclusion bodies, ch = chloroplast, m = mitochondrion.

mechanisms that will cause genetic changes in TSWV populations over time.

Considering the geographical prevalence of phylo-genetic patterns of the N and NSm proteins, Silva et al. (2001) proposed the evolution of two geographical distinct sequence variants in the natural population of TSWV. The authors suggest that although TSWV is widespread, it might have originated in the American continent due to the high homology of N and NSm proteins with other Tospovirus species from the American continent. The clusters were tentatively designated as 'American' and 'European' groups. With the exception of

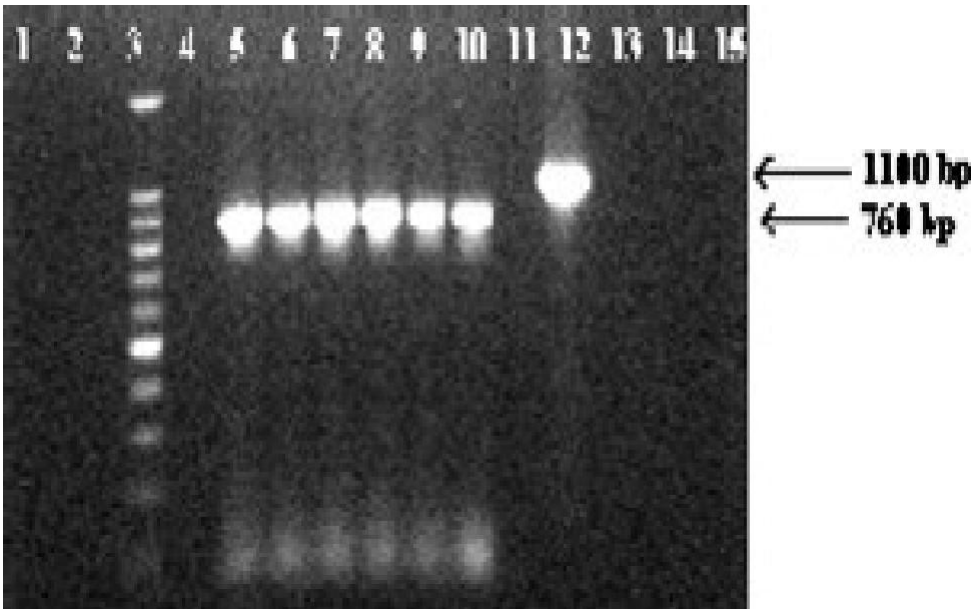

Figure 2 Agarose gel showing the reverse-transcription polymerase chain reaction (RT-PCR) product of total plant RNA extracted from *Nicotiana rustica* infected with *tomato spotted wilt virus* (TSWV) isolates from South Africa. Lanes 1, 2, 4, 11 and 15 are blank. Lane 3 shows the molecular weight marker. Lanes 5-10 shows the 760 bp amplification products of the total RNA from KwaZulu-Natal (KZN), Limpopo (LP), North West (NW1), North West (NW2), Mpumulanga (MP) and Gauteng (GP) TSWV isolates respectively. Lane 12 shows the MS2 viral RNA (1100 bp) positive control reaction. Lanes 13 and 14 show the negative control reactions which contain water and total plant RNA from healthy uninfected plants respectively.

Table 1. Sources of *tomato spotted wilt virus* (TSWV) N gene sequences used in this study.

Designation	Origin	Reference	Source of sequence
TSWV-BULG	Bulgaria	Heinze et al., 2003	GenBank, AJ418779
TSWV-CZECH	Czech Republic	Heinze et al., 2001	GenBank, AJ296599
TSWV-GER	Germany	Heinze et al., 2003	GenBank, AJ418781
TSWV-ITALY	Italy	Ciuffo et al., 2005	GenBank, AY848922
TSWV-JAPAN	Japan	Takeda et al., 2002	GenBank, AB088385
TSWV-GP	SA, Gauteng	This study	This study, EF059705
TSWV-KZN	SA, KwaZulu-Natal	This study	This study, DQ834847
TSWV-LP	SA, Limpopo	This study	This study, EF059704
TSWV-MP	SA, Mpumulanga	This study	This study, EF059706
TSWV-NW1	SA, North West	This study	This study, EF059702
TSWV-NW2	SA, North West	This study	This study, EF059703
TSWV-SPAIN	Spain	Tsompana et al., 2005	GenBank, AY744480
TSWV-CAL	USA, California	Tsompana et al., 2005	GenBank, AY744474
TSWV-COL	USA, Colorado	Tsompana et al., 2005	GenBank, AY744475
TSWV-BL	USA, Hawaii	Pang et al., 1992	GenBank, L20953
TSWV-NC	USA, North Carolina	Tsompana et al., 2005	GenBank, AY744477
RiftValley[a]	-	Giorgi et al., 1991	GenBank, NC002045

[a]*Rift Valley Fever Virus* (*Bunyaviridae*, Phlebovirus) was used as the outgroup

the Japan and Spain isolates, our phylogenetic analysis (Figure 3) is in agreement with the classification proposed by Silva et al. (2001). One cluster comprised of isolates form Bulgaria, Czech Republic, Germany, and Italy (European group) whilst the other cluster comprised of isolates from Japan, California, North Carolina, Hawaii,

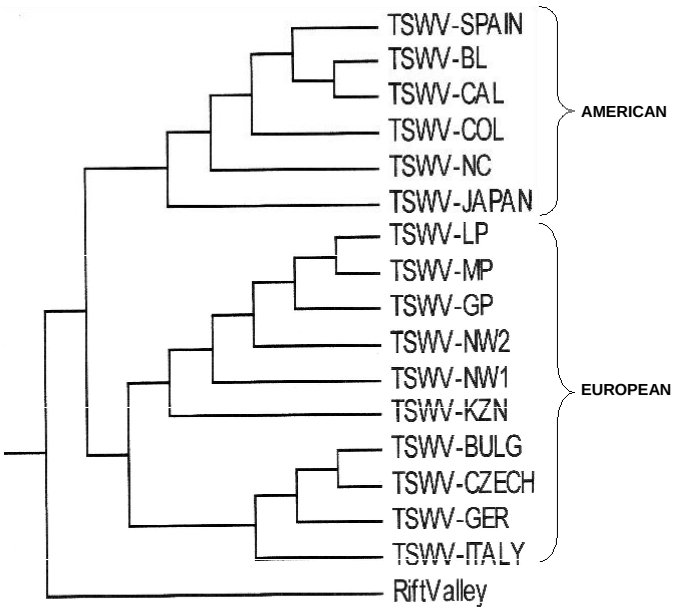

Figure 3. Phylogram based on nucleocapsid gene sequences of *Tomato spotted wilt virus* (TSWV) isolates. The TSWV isolate from South Africa (SA) sequenced in this study was compared with TSWV isolates reported from other parts of the world. Sources and designations given to each TSWV isolate used in the study were given in Table 1. The Rift Valley Fever Virus was used as an outgroup. Vertical and horizontal lengths of the branches are arbitrary.

Spain and Colorado (American group). The SA isolates has high sequence similarity with members of the European group. The Japan and Spain isolates formed tight associations with American group though having originated in Asian and European countries respectively. This could be explained by plant material exchanges and/or introduction of efficient thrips vectors into these distinct geographical regions (Silva et al., 2001).

Although the SA isolates form a cluster with the European isolates, they formed a distinct clade within the cluster. The grouping of the SA isolates apart from the European isolates may reflect that these isolates represent a divergent TSWV group that has not yet acquired phenotypic or genotypic differences that allow its discrimination by serology and molecular methods. This distinct clustering among the SA isolates could be a representative evolutionary trend of TSWV in Africa. To confirm this speculation, TSWV isolates from the rest of the African continent need to be characterized.

Discrimination among TSWV isolates is essential to improve existing control strategies. These include the generation of N gene transgenic plants resistant to TSWV infection. It has been demonstrated that depending on the isolate used as a source of the N gene to develop the transgenic plant, the resistance against different *Tospovirus* species varies from non-detectable to a high degree of protection (Gielen et al., 1991). Therefore, in order to devise effective and sustainable strategies to control TSWV, knowledge of the diversity of the virus becomes indispensable (Dewey et al., 1995).

In view of the world-wide spread of TSWV, its ability to infect a large host range and the variety of ecological niches in which it exists, an extended survey of TSWV isolates occurring in Africa is being undertaken. Knowledge on the occurrence and diversity of TSWV isolates in the continent will be used as a basis for developing transgenic plants with resistance to infection by the virus.

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