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Full Length Research Paper

Evaluation of tomato (*Lycopersicon esculentum* Mill.) plants with natural and transgenic resistance against Tomato spotted wilt virus (TSWV) isolates occurring in the Republic of South Africa (RSA)

B. Sivparsad and A. Gubba*

School of Agricultural Sciences and Agribusiness, Discipline of Plant Pathology, University of KwaZulu-Natal, Private Bag X01, Scottsville, Pietermaritzburg, 3201, South Africa.

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Tomato spotted wilt virus (TSWV) infections causes significant economic losses in the commercial production of tomato (*Lycopersicon esculentum* Mill.). This study was undertaken to evaluate tomato with natural and transgenic resistance when inoculated with TSWV isolates occurring in the Republic of South Africa (RSA). The Stevens cultivar which has natural resistance to TSWV conferred by the *Sw-5* gene and the transgenic 13-1 line which expresses the nucleocapsid (N) protein gene of the TSWV-BL isolate were used as test plants. Six TSWV isolates collected from Gauteng, KwaZulu-Natal, North West, Limpopo and Mpumulanga provinces were mechanically inoculated onto test plants. The trial was arranged in a general treatment structure with randomized block design and repeated once. Plants were assessed for TSWV resistance based on a disease severity rating scale and measurements of virion accumulation levels (A_{405}nm) using Enzyme linked immunosorbent assay (ELISA). There were no significant differences among the reactions produced by the six TSWV isolates on the test plants. Although both plants were susceptible to the TSWV isolates from RSA by exhibiting similar high viral accumulation levels, the transgenic tomato line showed milder disease severity than the natural resistant cultivar. Results suggest that transgenic resistance is a more viable approach in the control of TSWV in RSA.

Key words: Tomato spotted wilt virus (TSWV), Republic of South Africa, virus resistance, transgenic, tomato (*Lycopersicon esculentum* Mill.).

INTRODUCTION

Tomato spotted wilt virus (TSWV), classified as a *Tospovirus* in the family Bunyaviridae, is an important pathogen infecting many crops on a worldwide scale. Over 1090 plant species including vegetables, ornamentals, fruit trees and industrial crops are susceptible to TSWV, with tomato (*Lycopersicon esculentum* Mill.) among the species most severely affected (German et al., 1992; Rosellò et al., 1996; Llamas-Llamas et al., 1998; Peters, 2003). Considerable economic losses can occur due to infection rates of up to 90% in commercial crops (Cho et al., 1989). Thrips (order, *Thysanoptera*, family *Thripidae*) are the natural vectors of *Tospoviruses*, with the western flower thrip, *Frankliniella occidentalis*, being the most common vector of TSWV (Sakimuru, 1963; Wijkamp et al., 1993; Ullman et al., 1997).

In the Republic of South Africa (RSA), the emergence of TSWV is a significant problem in crop cultivation (Thompson and van Zijl, 1996). A disease survey conducted by Uys et al. (1996) has ranked TSWV as the most prevalent viral disease infecting tomato (*Lycopersicon esculentum* Mill.) in RSA. The tomato yields in each province in RSA are reduced each year by TSWV (Uys et al., 1996). The huge economic loss caused by TSWV in tomato crops in RSA has spurred interest into research on TSWV disease management.

*Corresponding author. E-mail: GubbaA@ukzn.ac.za Tel: (0027)332605795. Fax: (0027)332606475.

Table 1. Details of the *Tomato spotted wilt virus* (TSWV) isolates used in this study.

Locality	Province	Isolate name	Plant source
Kranskop	KwaZulu-Natal	TSWV-KZN	*Capsicum* sp.
former 'Transvaal'	Limpopo	TSWV-LP	C*apsicum* sp.
Zeekoegat	Gauteng	TSWV-GP	*Solanum tubersosum*
Marble Hall	Mpumulanga	TSWV-MP	*Pisum sativum*
Klerksdorp	North West	TSWV-NW1	*Arachis hypogaea*
Groot Marico	North West	TSWV-NW2	*Pisum sativum*

The control of TSWV has been extremely difficult due to its extensive host range and resistance of the thrip vectors to insecticides (Boiteux and Giordano, 1993). Consequently, resistant cultivars have proven to be the most effective method for controlling TSWV (Fraser, 1990).

Host-plant resistance to TSWV may be the most promising means of controlling the disease in the long-term (de Haan et al., 1996; Saidi and Warade, 2008). Research has led to the identification and characterization of several genes for TSWV resistance in tomato (Finlay, 1953; Stevens et al., 1992; Roselló et al., 1998). The *Sw-5* gene, first identified in *L. peruvianum,* was found to be the more stable and less isolate specific (Stevens et al., 1992). Therefore, the *Sw-5* gene has been used widely in breeding programs (Cho et al., 1989). However, in the field, plants carrying the *Sw-5* gene still accumulate virus resulting in the development of disease symptoms (Ultzen et al., 1995). In addition, despite offering the most promising natural form of TSWV resistance, TSWV isolates virulent to *Sw-5* have been identified in RSA (Thompson and van Zijl, 1996), Hawaii (Canady et al., 2001; Gordillo et al., 2008), Australia (Latham and Jones, 1998), Spain (Aramburu and Marti, 2003; Margaria et al., 2004) and Italy (Roggero et al., 2002; Zaccardelli et al., 2008). Therefore there is a need to identify and develop new sources of resistance to be incorporated into crop breeding programs.

When host resistance against TSWV is overcome, pathogen-derived resistance (PDR) for virus control may provide a significant alternative to the traditional strategy of using natural resistance genes (Hoffmann et al., 2001). Transgenic resistance to TSWV was first introduced into tobacco by Gielen et al. (1991) with the use of the nucleocapsid (N) gene. Engineered TSWV resistance has been introduced into tomato plants (Kim et al., 1994; Ultzen et al., 1995; Gonsalves et al., 1996). This type of resistance is known as RNA-mediated resistance; where untranslatable constructs containing full length or segments of the N gene are able to confer resistance via a post-transcriptional gene silencing (PTGS) mechanism (Baulcombe, 1996; Pang et al., 1996). Resistance is only effective against viral sequences with a high degree of sequence homology to the transgene (Pang et al., 1996).

It is well known that TSWV shows the capacity to generate new phenotypes more readily than other viruses (Moyer and Qui, 1996; Qui and Moyer, 1999). This attribute makes the development of cultivars that exhibit long-term durable resistance difficult. This study was conducted to evaluate the resistance of transgenic tomato expressing the N gene and natural resistant tomato carrying the *Sw-5* gene to TSWV isolates occurring in RSA.

MATERIALS AND METHODS

Virus isolates

Six TSWV isolates from different geographical locations in RSA were used in this study. Isolates were tentatively named after the province in which they were sourced. Desiccated and freeze-dried TSWV sources from North West, Limpopo and Mpumulanga provinces were provided by Jacolene Meyer (Plant Protection and Research Institute, Agricultural Research Council, Private Bag x134, Pretoria 0001, RSA). Details of TSWV isolates used in this study are given in Table 1.

Test plants

Two accessions of tomato were evaluated for TSWV resistance. Seeds of the transgenic line '13-1' and the natural resistant cultivar 'Stevens' were provided by D. Gonsalves (USDA, Hilo, Hawaii, USA) and imported into RSA for experimental purposes under permit number P0036564 issued by the Directorate Plant Health in the Department of Agriculture, Forestry and Fisheries. The transgenic line had been produced by transferring the N gene of the TSWV-BL (Pang et al., 1992) into a *Tobacco mosaic virus* (TMV) resistant tomato line (G-80) (Gonsalves et al., 1996). The 'Stevens' cultivar confers natural resistance through the expression of the Sw-5 dominant gene (Stevens et al., 1992; Thompson and van Zijl, 1996). The Geneva 80 (G-80) line was used as a susceptible control. Seeds of G-80 were provided by R. Provvidenti (Department of Plant Pathology, Cornell University, Geneva, NY14456, USA). The G-80 line was chosen because it possesses the Tm-2^2 gene which confers resistance to TMV, *Verticillium wilt*, and *Phytophthora infestans* (Race 0) (Provvidenti and Gonsalves, 1995).

Seeds were germinated in Speedling® 24 trays containing sterilized seedling mix (Growmor, Cato Ridge). Three weeks post germination, seedlings were transplanted into individual pots (12 cm) filled with potting medium (Growmor, Cato Ridge). Irrigation was done three times daily for durations of 5 min. Water was supplemented with soluble fertilizer. Plants were kept at constant temperature of 20 to 25°C in a fibre glass tunnel, University of

KwaZulu- Natal, Pietermaritzburg for the duration of the study.

Trial design

A total of 18 treatments comprising of the three tomato lines (G-80, 13-1 and Stevens) inoculated with the six TSWV isolates was used. The pots were arranged in a general treatment structure with randomized block design. Each treatment comprised of five replicates. The trial was repeated once.

Mechanical inoculation

TSWV inoculum was obtained from fresh leaf material of infected *Nicotiania rustica* L. plants. At least 10 plants served as inoculum source for each block. Mechanical transmission to *N. rustica* was carried out as described by Mandal et al. (2001) with minor modifications. 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 tomato plants that had been previously dusted with carborundum and leaves were rinsed thereafter with chilled distilled water. Four weeks post germination the first two true leaves of the plant were mechanically inoculated. The upper leaves were re-inoculated one week later to ensure maximum disease incidence.

Screening for resistance

Two weeks after the second inoculation, plants were assessed for resistance to TSWV. TSWV infection of plants was confirmed by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using Bio-Rad Phyto-diagnostics commercial ELISA kits (Phyto-diagnostics, France). Samples with absorbance values higher than two-fold the value of the negative control were presumed to be positive for TSWV. The severity of TSWV disease and the amount of virion accumulation was used as an assessment of TSWV resistance.

Disease severity

Disease severity was assessed visually using the rating scale described by Canady et al. (2001). The visual rating scale: 1 = no visible symptoms, 2 = mild chlorosis and limited leaf distortion, 3 = moderate chlorosis, leaf distortion with some plant stunting, 4 = severe chlorosis, leaf distortion and plant stunting, 5 = severe chlorosis, leaf distortion and extreme stunting.

Accumulation of TSWV virions

ELISA was used to determine the amount of virion accumulation in infected plant tissue. Plant tissue was collected from the apical leaves nearest to the growing point using an Eppendorf tube. To obtain a uniform sample size, the Eppendorf tube cap was snapped down on the leaf to punch out a leaf sample of approximately 10 mm in diameter. Care was taken to ensure that the sample did not have excess non-affected tissue. Dead tissue was not tested. The absorbance value at A_{405}nm, which reflects viral particle accumulation in each sample, was read using an Anthos 2001 photometer (Anthos Labtec Instruments, Austria). Expressed sap from uninfected healthy control plants was used as the negative controls. Positive controls, provided in the kit were rehydrated in 1 ml distilled water. Each sample was loaded in duplicate wells and the mean absorbance value for the two duplicate wells was used for analysis.

Statistical analysis

The experiment was repeated once and results were pooled together for statistical analysis. Data was subjected to analysis of variance (ANOVA) using GenStat 7th Edition statistical analysis software program. Mean separations were based on the Fiseur's least significant differences (LSD) at $P<0.05$.

RESULTS

All inoculated test plants tested positive for the presence of TSWV using ELISA. This indicated a disease incidence of 100%.

Disease severity

There were no significant differences among the reactions produced by the six TSWV isolates on the Stevens cultivar and 13-1 line. Between the two resistant plants, the Stevens cultivar proved more susceptible to all six TSWV isolates with a mean disease severity of 2.9. The transgenic 13-1 line proved resistant to infection with a mean severity of 1.75. The susceptible G-80 line showed a high disease severity with an average mean of 4.78 (Table 2 and Figures 1 and 2).

Accumulation of TSWV virions

All six TSWV isolates showed no significant differences in the amount of virion accumulation in the Stevens cultivar and 13-1 line. The Stevens cultivar and 13-1 line also showed no significant differences in the amounts of virion accumulation with absorbance means of 0.444 and 0.413 respectively. The susceptible G-80 line showed a high virion accumulation with an absorbance mean of 1.828 (Table 2 and Figure 3).

DISCUSSION

Results of this study showed no significant differences in the reactions produced by the six RSA TSWV isolates on the test plants evaluated. All isolates showed the same degree of virulence in that they produced comparable disease severity reactions and virion accumulation levels in all lines evaluated. Therefore, this degree of aggressiveness is a phenotypic trait that is common to all RSA TSWV isolates used in this study. This phenotypic uniformity among isolates is reflected genotypically in the phylogenetic analysis of these isolates (Sivparsad and Gubba, 2008). The phylogenetic analysis showed that N gene sequence homologies of 99-100% of RSA isolates indicated that TSWV in RSA has not evolved on a molecular level. The uniformity of reactions produced by RSA TSWV isolates on test plants confirms the findings of the phylogenetic analysis.

Table 2. ANOVA of disease severity and virion accumulation in the evaluation of natural (Stevens) and transgenic (13-1) resistant tomato (*Lycopersicon esculentum* Mill.) plants against *tomato spotted wilt virus* (TSWV) isolates from the Republic of South Africa.

	TSWV isolate	
Test plant	**Severity[x]**	**Virion accumulation (A_{405}nm)[y]**
TSWV-GP		
G-80	4.7[a]	1.79[a]
13-1	1.6[b]	0.40[b]
Stevens	2.9[c]	0.48[b]
TSWV-KZN		
G-80	4.8[a]	1.87[a]
13-1	1.7[b]	0.39[b]
Stevens	2.8[c]	0.47[b]
TSWV-LP		
G-80	4.8[a]	1.95[a]
13-1	1.6[b]	0.40[b]
Stevens	2.7[c]	0.45[b]
TSWV-MP		
G-80	4.8[a]	1.84[a]
13-1	1.6[b]	0.40[b]
Stevens	2.7[c]	0.40[b]
TSWV-NW1		
G-80	4.8[a]	1.88[a]
13-1	1.6[b]	0.40[b]
Stevens	2.7[c]	0.43[b]
TSWV-NW2		
G-80	4.8[a]	1.78[a]
13-1	1.7[b]	0.47[b]
Stevens	2.9[c]	0.40[b]
F-test		
Test plant	<0.001	<0.001
Isolate	0.350	0.431
Test plant * Isolate	0.700	0.139
l.s.d	0.316	0.166
s.e.d	0.155	0.081
cv%	6.1	11.2

-means followed by a different letter in the same column are significantly different at P = 0.05; [x] disease severity assessed from visual rating scale of 1-5; [y] TSWV virion accumulation measured by absorbance (A_{405}nm).

All lines evaluated tested positive for the presence of TSWV by ELISA. Disease incidence of 100% would indicate that neither cultivar Stevens (natural) nor 13-1 (transgenic) line exhibited immunity to TSWV isolates from RSA. However, resistance encompasses a wide variety of host-pathogen interactions. Moreover, a resistant phenotype reduces the growth, replication or disease-producing activities of the pathogen. Disease symptoms are less severe on resistant hosts than on susceptible hosts (Pataky and Carson, 2004). The G-80 line was used as a susceptible control as its phenotype was unable to restrict the replication and disease-producing

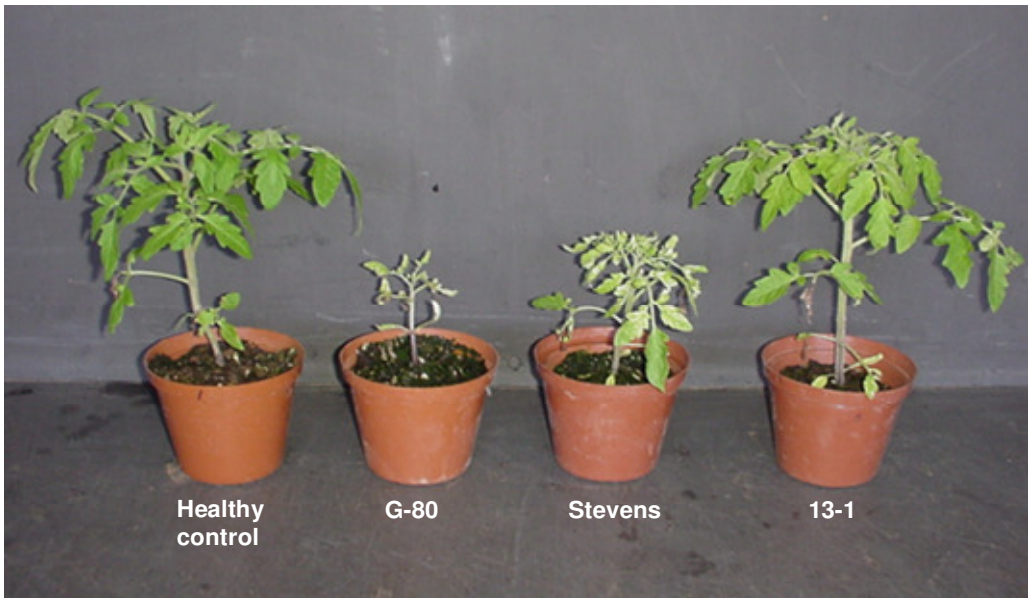

Figure 1. Differential response of natural (Stevens) and transgenic (13-1) resistant tomato (*Lycopersicon esculentum* Mill.) plants against *tomato spotted wilt virus* (TSWV) isolates from the Republic of South Africa. The Geneva 80 (G-80) line was used as a susceptible control. Healthy control was mock inoculated with inoculation buffer.

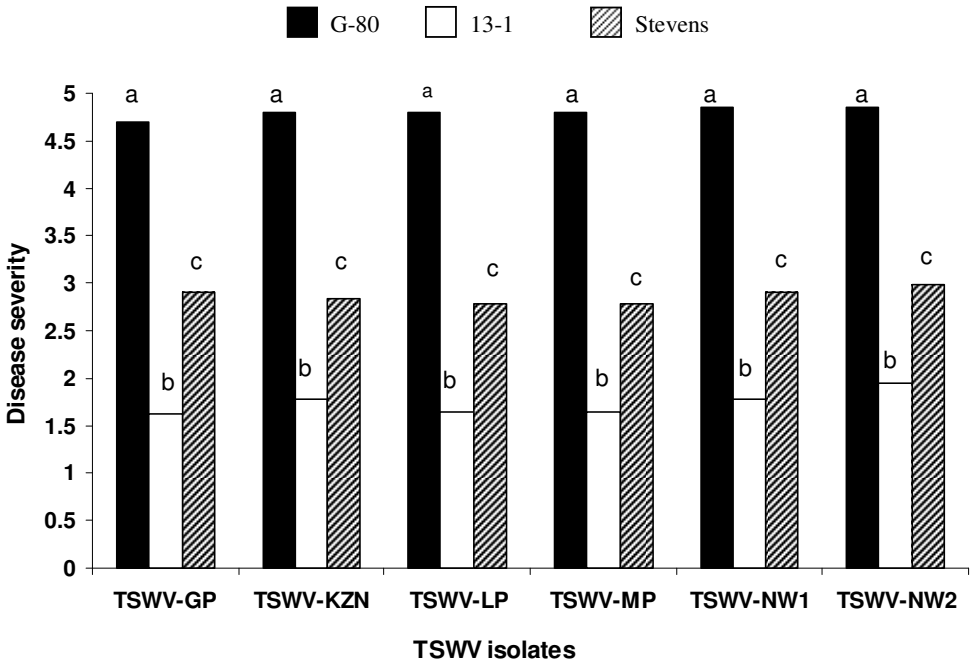

Figure 2. Disease severity reactions in the evaluation of natural (Stevens) and transgenic (13-1) resistant tomato (*Lycopersicon esculentum* Mill.) plants against *tomato spotted wilt virus* (TSWV) isolates from the Republic of South Africa.

properties of TSWV. Therefore, symptoms produced on G-80 were severe (disease severity mean of 4.78) and virion accumulation levels was high (absorbance value mean of 1.828). Resistant reactions produced on Stevens and 13-1 plants varied in degree and kind. The Stevens cultivar proved more susceptible to infection by RSA TSWV isolates by exhibiting disease severity reactions in the range of 2-3, whilst the transgenic 13-1 line showed

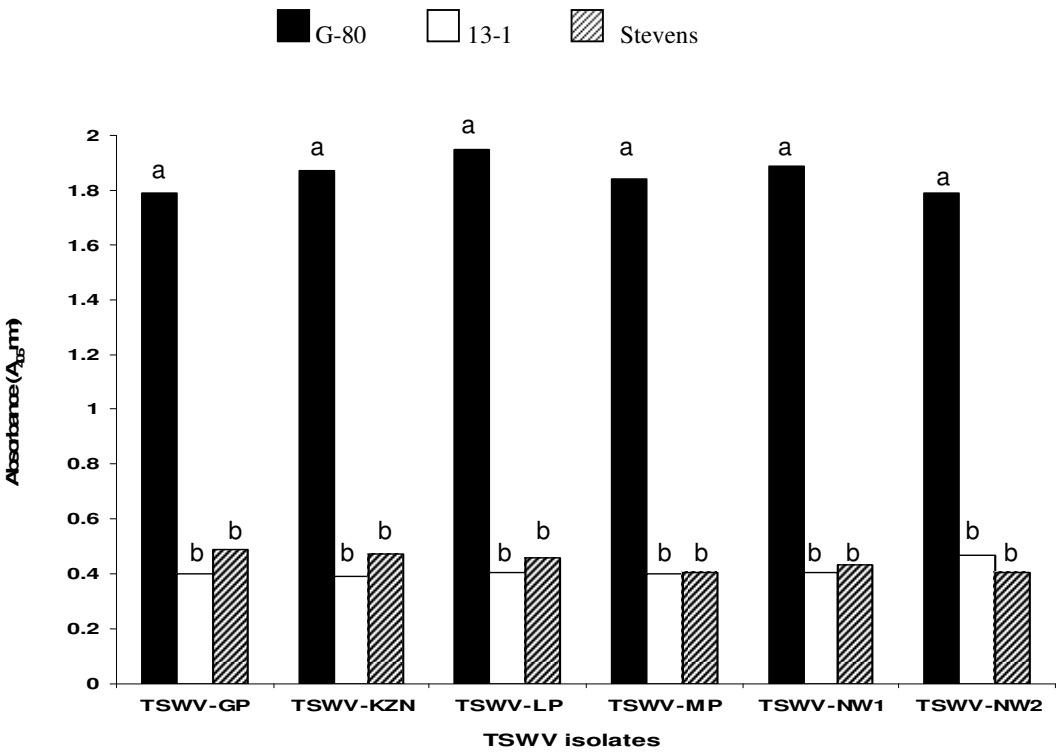

Figure 3. Measurements of virion accumulation (A_{405}nm) in the evaluation of natural (Stevens) and transgenic (13-1) resistant tomato (*Lycopersicon esculentum* Mill.) plants against *tomato spotted wilt virus* (TSWV) isolates from the Republic of South Africa.

milder disease in the range of 1-2. The difference in host reaction is the basis for the differentiation of the two plants into categories of resistance. The Stevens cultivar can be seen as moderately resistant whilst the 13-1 line is seen as tolerant resistant.

Both test plants showed a decline in virion accumulation when compared to the level seen in the susceptible G-80 line. This is indicative of the mechanisms that govern both *Sw-5* and N gene mediated resistance. In natural resistance, it was proposed that reduced virion accumulation levels may be due to the inhibition of virion disassembly in initially infected cells (Baulcombe, 1996). In the case of N gene mediated resistance, the reduction in virion accumulation levels in due to post-transcriptional gene silencing (PTGS) (Pang et al., 1992).

The varied disease severity reactions produced on the Stevens and 13-1 plants was not reflected in the levels of virion accumulation. Both test plants exhibited similar levels of virion accumulation despite showing significantly different disease severity reactions. Although the 13-1 line showed the same level of virion accumulation, it exhibited milder disease symptoms than seen on the Stevens cultivar. Absorbance readings detect levels of N protein which indirectly show the amount of virion accumulation. Research has shown that transgenic resistance conferred by the N gene is RNA-mediated (Baulcombe, 1996). Therefore transgenic plants expressing intact N gene showing resistance to homologous or closely related TSWV isolates is due to the presence of the N gene transcript and not the N protein. A high amount of N protein detected by ELISA that would cause disease in the Stevens cultivar does not result in severe disease in the transgenic 13-1 line.

Generally, resistance to TSWV from tomato containing the *Sw-5* gene has been non-isolate dependent and stable (Bioteux and Giordano, 1992). However in this study, the Stevens cultivar which contains the *Sw-5* gene was overcome by the RSA TSWV isolates. TSWV isolates that overcome the *Sw-5* gene have also been identified in several locations around the world. In RSA, Thompson and van Zijl (1996) reported that four samples of symptomatic Stevens plants from the Klapmuts district of the Western Cape were positive for TSWV. The isolates were referred to as the JF strains. At the time, they found that the JF strains were not able to establish themselves and spread beyond the original field in which they were isolated from. The ability of the RSA TSWV isolates reported in this study to overcome the Stevens cultivar, extend these initial reports, by suggesting that the JF isolates might have spread from the Western Cape to other provinces in RSA. Considering the current rapid expansion of one of its major vectors (*Frankliniella occidentalis*) and the recent upsurge of agricultural

activity in RSA, it is not surprising that these strains have spread and established themselves in other provinces in RSA.

The tolerant phenotype observed in transgenic 13-1 line is indicative of a milder form of plant infection in which the virus has still accumulated without causing significant damage to the plant. This phenotype is of commercial value as it reduces economic losses that result from severe symptom expression. Results indicate that resistance conferred by the N gene is homology-dependent (Baulcombe, 1996). The transgenic 13-1 line was transformed with N gene from the TSWV-BL isolate. Sequence comparisons reported by Sivparsad and Gubba (2008), revealed a >90% homology between the N genes of the RSA isolates and the TSWV-BL isolate.

Field evaluations will be more useful in this study than mechanical inoculation of tomato cultivars in evaluating TSWV resistance. The potential for multiple introductions of a virus across an entire leaf surface during the mechanical inoculation procedure may impart a higher level of disease pressure than natural thrips inoculation. As a result, mechanical inoculations may overwhelm slight variations in disease resistance mechanisms, which may be more apparent under field conditions (Garcia et al., 2000). In addition, field environments present a more diverse array of challenging TSWV strains that occur as a result genomic reassortment in nature.

Information presented here clearly illustrates the devastating effects of TSWV infection on tomato. Data suggests that transgenic resistance is a more viable approach to disease control when natural host-resistance is overcome. The knowledge of the uniform virulence among TSWV strains from RSA together with information on disease severity on transgenic and natural resistant cultivars could be used as a tool to make strategic decisions in integrated control programs for TSWV.

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