

Field isolates of *Tomato spotted wilt virus* overcoming resistance in capsicum in Australia

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Abstract. In 2002 at Virginia, South Australia, capsicum cultivars having the *Tsw* resistance gene against *Tomato spotted wilt virus* (TSWV) developed symptoms typical of TSWV infection and several glasshouse-grown crops were almost 100% infected. Samples reacted with TSWV antibodies in ELISA. Virus isolates from infected plants induced severe systemic symptoms, rather than a hypersensitive reaction, when inoculated onto capsicum cultivars and *Capsicum chinense* genotypes (PI 152225 and PI 159236) that carry the *Tsw* resistance gene. Isolates virulent towards the *Tsw* gene had molecular and biological properties very similar to standard TSWV isolates, including a hypersensitive reaction in *Sw-5* (TSWV-resistant) tomato genotypes. *Tsw*-virulent isolates were found during surveys at Virginia in 2002 and 2004 in both TSWV-resistant and susceptible cultivars of capsicum and tomato.

Additional keywords: *Capsicum chinense*, *Sw-5* gene, *Tsw* gene.

Introduction

Tomato spotted wilt virus (TSWV; genus *Tospovirus*, Family *Bunyaviridae*) has a wide host range among ornamental, crop and weed species with capsicum (*Capsicum annuum*) and tomato (*Lycopersicon esculentum*) among the species most severely affected (Roselló *et al.* 1996; Gitaitis *et al.* 1998; Persley *et al.* 2006).

The genome of TSWV is composed of three single-stranded RNA segments, large (L), medium (M) and small (S), closely associated with virus-encoded nucleocapsid (N) protein and enveloped in a lipid membrane carrying two types of glycoprotein (Ullman *et al.* 2002). The nucleoprotein and the precursor to the glycoproteins are encoded on the viral complementary strand of the S and M RNAs, respectively. The L RNA is negative sense and encodes the viral RNA-dependent RNA polymerase (Goldbach and Peters 1996).

All tospoviruses are transmitted by thrips in a propagative manner with 11 species being confirmed as vectors of TSWV (Ullman *et al.* 2002; Persley *et al.* 2006). Global spread of the efficient thrips vector *Frankliniella occidentalis* has been a major factor in the increased importance of TSWV in many countries in recent years (Prins and Goldbach 1998).

Effective control of TSWV has proven difficult. In addition to the wide host ranges of both the virus and thrips vectors, the lack of useful resistance in many crop hosts has been

a major limitation in developing effective management strategies (Mumford *et al.* 1996a). Although resistance to TSWV has not been found in *C. annuum* or *L. esculentum*, it does occur in several other *Capsicum* and *Lycopersicon* species (Stevens *et al.* 1994; Boiteux *et al.* 1993; Cebolla-Cornejo *et al.* 2003). Resistance expressed as a hypersensitive response and controlled by the single dominant gene *Tsw* has been found in the *C. chinense* accessions PI 152225, PI 159236 and Panca (syn. CNPH 275) (Black *et al.* 1991; Moury *et al.* 1997; Boiteux and de Avila 1994; Boiteux *et al.* 1993; Boiteux 1995). Commercially available cultivars with this resistance are now grown in several countries including Australia (Roggero *et al.* 2002; Persley *et al.* 2006). The most widely used resistance source in tomato is the *Sw-5* gene, derived from *L. peruvianum* and expressed as a hypersensitive reaction (Stevens *et al.* 1992). Field isolates virulent towards the *Tsw* gene have been found in Louisiana (Hobbs *et al.* 1994), Brazil (Boiteux and Nagata 1992), Italy (Roggero *et al.* 2002) and Spain (Margaria *et al.* 2004). Several workers have generated isolates able to overcome the *Tsw* gene and cause systemic infection following sap inoculation of resistant lines and serial passage of selected isolates (Moury *et al.* 1997; Thomas-Carroll and Jones 2003).

The North Adelaide Plains, centred around Virginia, South Australia, is an intensive vegetable production area with many small-holders producing capsicum, tomato,

lettuce, cucurbits and other vegetable crops both in the field and in glasshouses. A severe outbreak of TSWV and western flower thrips occurred in the area in 2000 causing crop failures, particularly in capsicum, tomato and lettuce, with severe financial consequences for growers and the community (Anon. 2000). As part of an integrated management strategy to reduce losses, capsicum cultivars with resistance to TSWV were introduced to the area in 2001. In 2002, several glasshouse-grown crops of the TSWV-resistant cultivar Yatasto developed a high incidence of disease with symptoms suggesting infection by TSWV.

The aims of the research described in this paper were to determine the cause of the disease in the TSWV-resistant capsicum cultivars, determine the distribution and importance of the disease in capsicum at Virginia and investigate the properties of representative isolates of the causal virus. This work was a prerequisite to developing a more effective virus control strategy incorporating resistant cultivars.

Methods

Surveys

In November 2002 and January 2004, capsicum, tomato and lettuce crops, at Virginia, were surveyed for TSWV on a total of 15 properties. Both glasshouse and field sites were inspected. Disease incidence was estimated by counting the number of symptomatic plants from at least four randomly located blocks within the crop with 50 plants per block. Samples were collected from 166 symptomatic plants (122 capsicums, 33 tomatoes, seven lettuce and four sweet basil, *Ocimum basilicum*), stored in plastic bags at 5°C and all were assayed by ELISA for TSWV within 7 days of collection. Of these, 145 samples were positive for TSWV, and 54 of these (30 from capsicum, 19 tomato and five lettuce) were inoculated onto the capsicum genotypes PI 152225 and Aristocrata or Yatasto (all possess the *Tsw* gene conferring resistance to TSWV), and Yolo Wonder (TSWV susceptible) to determine if they were able to systemically infect lines having the *Tsw* resistance gene. A further three capsicum samples were tested on capsicum cultivars Yatasto and Bello Rosso (TSWV susceptible) but not PI 152225. Twenty-six TSWV-positive samples (six *Tsw* capsicums, six *Sw-5* tomatoes, four non-resistant capsicums, eight non-resistant tomatoes and two lettuce) were inoculated onto tomato cultivars Grosse Lisse (TSWV susceptible) and an experimental line (DRW 6189) with the *Sw-5* gene to determine if these isolates were virulent to this gene.

Plants and inoculations

The genotypes of *Capsicum* spp. and tomato used in host range studies are given in Table 1. Sap inoculations were performed by preparing leaf extracts with a cold mortar and pestle and cold 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1% sodium sulphite. Diatomaceous earth and carborundum abrasives were added to the inoculum before applying to leaves of test plants. Plants were rinsed with tap water following inoculation and maintained in a glasshouse.

ELISA

The antibodies used were TSWV (BioRad catalogue number 355–1267); *Watermelon silver mottle virus* (tosopovirus serogroup IV; DSMZ catalogue number AS-0118) and *Cucumber mosaic virus* (CMV)

(BioRad catalogue number 355–1219). ELISA tests for TSWV and serogroup IV were done essentially according to suppliers' instructions and the CMV ELISA as described by Sharman *et al.* (2000). Samples were extracted (1 g/10 mL) in PBS-T-PVP for TSWV and serogroup IV testing, or in the common extraction buffer of Sharman *et al.* (2000) for CMV testing. Assays were carried out in Nunc Maxisorb microtitre plates with reaction volumes of 100 µL for TSWV and CMV, and 50 µL for serogroup IV. Overnight incubations were at 5°C and all other incubations were at room temperature. Absorbance values ($A_{410\text{nm}}$) were measured using a Dynatech MR 7000 ELISA plate reader. Samples were scored as positive when the absorbance values exceeded twice the mean of the appropriate healthy controls.

Electron microscopy

Original and representative samples from surveys and experimental work were examined by electron microscopy. For negative staining of sap preparations, extracts were placed onto nitrocellulose-coated, carbon-stabilised copper grids and contrasted with 2% ammonium molybdate, pH 5.8, prior to examination in a Hitachi H7000 electron microscope.

Reverse transcription PCR and sequencing of the N gene

For TSWV-specific RT-PCR, RNA was extracted from leaf tissue using the Concert RNA reagent (Invitrogen) and cDNA prepared using Superscript II (Invitrogen) as per the manufacturer's instructions with primer S2 UNIV (Mumford *et al.* 1996b). The PCR mix consisted of 2.5 µL of 10× PCR Buffer (Invitrogen), 1.75 mM MgCl₂, 200 µM of each dNTP, 400 nM each of primers S2 UNIV and TSWV.NPF3 (5'-TAAGCAYAACACACAGAAAGCA-3'), 1.5 units of *Taq* DNA polymerase (Invitrogen), 2 µL of cDNA and sterile water to a total reaction volume of 25 µL. PCR cycling parameters were: one cycle at 94°C for 1 min, then 35 cycles at 94°C for 20 s, 56°C for 1 min and 72°C for 1 min, and finally one cycle at 72°C for 3 min. An amplicon of ~1040 bp containing the complete nucleocapsid gene was produced. Excess primers were removed using the QIAquick PCR purification kit (QIAGEN) and the PCR product was directly sequenced with an Applied Biosystems Inc. automated sequencing system. Sequences of the nucleocapsid gene (N) gene of two *Tsw*-resistance breaking strains were aligned with the sequence of a standard isolate of TSWV using CLUSTAL W (Thompson *et al.* 1994).

Insect transmission tests

Virus transmission by thrips was attempted in two separate experiments. Method 1: A healthy colony of *Frankliniella occidentalis* was maintained on plants of *Phaseolus vulgaris*. About 200 of these thrips were transferred to tomato plants infected with *Tsw*-virulent isolate 1438 (originally from capsicum cv. Yatasto, at Virginia) while being caged simultaneously with a pot containing four healthy tomato plants, cv. Grosse Lisse. The thrips were allowed to multiply and spread between the plants in the cage for 27 days. Method 2: About 100 *F. occidentalis* were collected from flowers of TSWV-infected capsicum, cv. Yatasto, plants in a glasshouse crop at Virginia and then released into a cage with 16 healthy young tomato plants, cv. Grosse Lisse, and allowed access to these plants for 14 days. The tomato plants were then sprayed with insecticide, removed from the cage and maintained in a glasshouse to monitor symptom development.

Results

Virus identification

Six diseased samples of TSWV-resistant capsicum, cv. Yatasto, were first received in April 2002 from two

Table 1. Reactions of Tomato spotted wilt virus isolates 1423 and 1438 on *Capsicum* spp., tomato and key diagnostic hosts

Species/genotype	Supplier	TSWV resistance	Host response ^A	
			1423 (non-virulent)	1438 ^B (<i>Tsw</i> virulent)
<i>Capsicum annuum</i>				
cv. Yatasto	Rijk Zwaan Seeds	<i>Tsw</i>	HR/NSS	SR
cv. Aristocrata	Rijk Zwaan Seeds	<i>Tsw</i>	HR/NSS	SR
cv. Merida	Rijk Zwaan Seeds	<i>Tsw</i>	HR/NSS	SR
cv. Percheron	Rijk Zwaan Seeds	<i>Tsw</i>	HR/NSS	SR
cv. Remy	Syngenta Seeds	<i>Tsw</i>	HR/NSS	SR
cv. Yolo Wonder	Yates Vegetable Seeds	Susceptible	SR	SR
cv. Raptor	Syngenta Seeds	Susceptible	SR	SR
cv. Bello rosso	South Pacific Seeds	Susceptible	SR	SR
<i>C. chinense</i>				
PI 159236	AVRDC ^C	<i>Tsw</i>	HR/NSS	SR
PI 152225	AVRDC	<i>Tsw</i>	HR/NSS	SR
AVRDC 00943	AVRDC	<i>Tsw</i> (?)	HR/NSS	SR
<i>Lycopersicon esculentum</i> (tomato)				
cv. Grosse Lisse	Yates Vegetable Seeds	Susceptible	SR	SR
cv. Patrice	Rijk Zwaan Seeds	Susceptible	SR	SR
cv. DRW 6189	Rijk Zwaan Seeds	<i>Sw-5</i>	HR/NSS	HR/NSS
cv. Gaudi	Rijk Zwaan Seeds	<i>Sw-5</i>	HR/NSS	HR/NSS
cv. Stevens	D. McGrath	<i>Sw-5</i>	HR/NSS	HR/NSS
<i>Nicotiana benthamiana</i>			SM/SW	SM/SW
<i>N. glutinosa</i>			LN/Mo/LD	LN/Mo/LD
<i>Datura stramonium</i>			NLL/SM	NLL/SM/SM
<i>Gomphrena globosa</i>			NR/Mo	NR/Mo
<i>Petunia hybrida</i>			NR/Mo	NR/Mo

^AAbbreviations: SR, systemic susceptible reaction with chlorotic mottle, chlorotic ring spots and line patterns, leaf distortion and plant stunting; HR, hypersensitive reaction with necrotic local lesions on inoculated leaves; NSS, no systemic symptoms; LN, local necrotic lesions; SM, systemic mosaic; SW, systemic wilt; Mo, systemic mottle; LD, leaf distortion; NR, necrotic ringspots.

^BThe reaction of isolate 1438 was typical of the reaction for isolates 1439, 1440 and 1441 also screened against the same host range.

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properties at Virginia, where almost 100% of crop plants had symptoms of chlorosis, ringspots and mottling on the leaves and distorted fruit with necrotic spots and sunken circular lesions (Fig. 1). All six samples gave strong positive ELISA reactions with antibodies to TSWV. The samples did not react with antibodies to *Cucumber mosaic virus* or tospovirus serogroup IV, which detects *Capsicum chlorosis virus* (McMichael *et al.* 2002). TSWV infection was confirmed by PCR using TSWV-specific primers. Virus particles were not seen in negatively-stained leaf extracts of diseased capsicum plants. However, pleomorphic spherical particles typical of tospoviruses were seen when sap extracts from *Nicotiana tabacum*, inoculated with one of the diseased plants (isolate 1438), were examined by electron microscopy.

Four isolates were maintained on capsicum, cv. Yolo Wonder, and then inoculated to a range of diagnostic

hosts and capsicum and tomato cultivars having the *Tsw* and *Sw-5* resistance genes, respectively. The reactions of these species and accessions are shown in Table 1. The standard TSWV isolate 1423 caused a hypersensitive response on capsicum and tomato genotypes carrying the *Tsw* and *Sw-5* genes, respectively, with no systemic symptoms and no detection of virus from the new growth leaves by ELISA. In contrast, the Virginia isolates (1438, 1439, 1440 and 1441), collected from capsicum cultivars with the *Tsw* gene, did not elicit a hypersensitive response in the capsicum genotypes having the *Tsw* gene and plants developed systemic symptoms typical of TSWV infection. TSWV was readily detected in symptomatic leaves by ELISA. The tomato cultivars having the *Sw-5* gene reacted with a hypersensitive local lesion response to isolates 1438, 1439 and 1423. The reactions of key diagnostic hosts for TSWV were similar for all isolates (Table 1).

PCR, cloning and sequencing

N gene nucleotide sequences of isolates 1438 and 1439 (GenBank accession codes AY818320 and AY818321) were both 97% identical to isolate 1423, which was avirulent to the *Tsw* gene, collected from capsicum in Queensland (GenBank code AY879109). N gene amino acid sequences of isolates 1438 and 1439 were both 96% identical to isolate 1423.

Thrips transmission

Two of four tomato plants developed TSWV symptoms following exposure to viruliferous western flower thrips allowed access to tomato plants infected with the *Tsw*-virulent isolate 1438. Twelve of 16 tomato plants developed TSWV symptoms following exposure to western flower thrips collected from infected capsicum plants at Virginia. In both experiments, TSWV infection was confirmed by ELISA



Fig. 1. Symptoms of *Tsw*-virulent *Tomato spotted wilt virus* isolate (a) on leaves and (b) fruit of capsicum cultivar with *Tsw* gene showing chlorotic line patterns and ring spots on leaves and sunken necrotic lesions on fruit.

TSWV survey

In November 2002 and January 2004, the incidence of tomato spotted wilt in the crops surveyed was generally less than 5% although one mature crop of capsicum, cv. Yatasto, in 2002 had an incidence of ~30%.

TSWV was confirmed by ELSIA in 103 capsicum, 31 tomato, seven lettuce and four sweet basil plants, from the 166 samples tested. Of the 57 TSWV positive isolates tested for *Tsw* virulence, 16 were from capsicum cvv. Yatasto and Remy and these all caused systemic symptoms when inoculated onto PI 152225 and capsicum cvv. Aristocrata, Yatasto and Yolo Wonder (Table 2). *Tsw*-virulent strains were not exclusively associated with capsicums with the *Tsw* gene and were also identified in eight of 17 capsicums without the *Tsw* gene and two of 13 tomatoes without the *Sw-5* gene.

All 26 TSWV-positive isolates (of which six each were from *Sw-5* tomatoes and *Tsw* capsicums) inoculated onto tomato, cv. Grosse Lisse, induced typical systemic symptoms including chlorotic mottle, chlorotic ring spots and line patterns, and plant stunting. The same isolates induced only a typical hypersensitive response on the tomato cultivar DRW 6189 (*Sw-5* gene) indicating that none of the TSWV isolates tested were virulent to the *Sw-5* gene. Eight out of the 26 isolates tested were virulent towards the *Tsw* gene in capsicum, suggesting that this virulence was specific to the *Tsw* gene.

Testing of capsicum cultivars with reputed TSWV resistance

All nine commercially available and pre-release TSWV-resistant cultivars with the *Tsw* gene developed severe systemic symptoms when inoculated with isolate 1438. These cultivars developed only a local hypersensitive reaction following inoculation with a standard isolate 1423. The five named cultivars are listed in Table 1.

Discussion

TSWV displays a high level of biological diversity and the capacity to generate new strains more readily than

Table 2. Screening of *Tomato spotted wilt virus* isolates collected from South Australian crops for virulence to *Tsw* gene following manual inoculation onto capsicum cultivars with the *Tsw* gene

Source and no. of TSWV isolates tested	No. of isolates with virulence to <i>Tsw</i> gene ^A
16 capsicums with <i>Tsw</i> gene	16
17 capsicums without <i>Tsw</i>	8
6 tomatoes with <i>Sw-5</i> gene	1
13 tomatoes without <i>Sw-5</i> gene	2
5 TSWV susceptible lettuce	0

^A *Tsw*-virulent TSWV isolates systemically infected capsicum cultivars with the *Tsw* gene while those without virulence to *Tsw* only systemically infected non-resistant capsicum cv. Yolo Wonder.

almost any other plant virus (Moyer and Qiu 1996). The biological diversity of Australian isolates of TSWV, including the capacity to overcome resistance genes, was clearly demonstrated some 50 years ago (Best and Gallus 1953; Finlay 1953). In recent work, Latham and Jones (1998) and Thomas-Carroll and Jones (2003) isolated variants from diverse sources able to overcome resistance genes in tomato (*Sw-5*) and capsicum (*Tsw*) following serial passage of isolates through resistant genotypes by sap inoculation. Resistance-breaking isolates were obtained from only some wild isolates and then only a small number of plants per isolate/genotype combination developed a susceptible systemic reaction. In our work, we have identified field isolates of TSWV that caused severe disease in resistant capsicum cultivars grown at several locations at Virginia, South Australia. Cultivar Yatasto (*Tsw* gene) was first grown in the area in 2001 and a high incidence of TSWV was found in crops in 2002. The rapid adaptation of TSWV to a resistance gene is likely to have occurred through selection of a variant virulent towards the *Tsw* gene from the heterogeneous virus population present in the area and the subsequent rapid dispersal by vectors in crops of the resistant cultivar grown in glasshouses. An alternative source of the variant may have been genome reassortment, which has been shown to be a mechanism for overcoming host resistance to TSWV (Qiu *et al.* 1998; Qiu and Moyer 1999). The rapid dispersal of the *Tsw*-virulent strain was most likely aided by monoculture of the resistant cultivar in glasshouses where control of western flower thrips was often unsatisfactory and plants were grown with a high incidence of virus for periods of up to 9 months, thus providing an excellent environment for virus spread.

The *Tsw*-virulent strain had similar properties to standard isolates of TSWV in terms of host range, serological reactions, vector transmission, and nucleotide and amino acid sequence of the N gene. The major distinction was the capacity to cause systemic infection of *Capsicum* lines and cultivars carrying the *Tsw* gene for TSWV resistance. This strain did not overcome resistance in several tomato lines and cultivars carrying the *Sw-5* gene following manual inoculation. Although there are phenotypic and genetic similarities with TSWV resistance in capsicum and tomato, the *Tsw* and *Sw-5* genes are distinct and the ability of TSWV isolates to overcome the resistance conferred by these genes maps to different TSWV genome segments (Jahn *et al.* 2000; Hoffmann *et al.* 2001). Virulence to the *Tsw* gene mapped to the S RNA segment and virulence towards the *Sw-5* gene in tomato to the M RNA.

The *Tsw*-virulent strain was found only in cultivars Yatasto and Remy (*Tsw* gene) in a survey at Virginia in November 2002. In 2004, the *Tsw*-virulent strain was recovered from both TSWV-resistant and -susceptible cultivars. The frequency of these isolates in susceptible cultivars was generally higher on farms where TSWV-

resistant cultivars were currently grown or had previously been grown. These data and observations suggest that the *Tsw*-virulent isolates are able to compete in the field with standard isolates in the absence of the *Tsw* gene.

The strain described here is similar in several respects to the *Tsw*-virulent strain reported from Italy (Roggero *et al.* 2002). Both are virulent towards the *Tsw* gene only and infect a wide range of *Capsicum* genotypes carrying this gene. Both strains are transmitted by *F. occidentalis* and have caused high disease levels in capsicum crops with the *Tsw* gene grown in glasshouses. Although there are several reports on the ability of TSWV isolates to overcome resistance in capsicum lines following manual inoculation, the only reported instances of serious field infection of *Tsw* genotypes are from Italy (Roggero *et al.* 2001, 2002), Spain (Margaria *et al.* 2004) and Australia (this work). Although *Tsw*-virulent isolates detected following serial manual inoculation of TSWV-resistant genotypes may compete effectively in field situations, it is also likely that some will be defective, having lost the envelope and associated glycoproteins during successive mechanical transfers, thus reducing or preventing transmission by thrips (Moury *et al.* 1997; Sin *et al.* 2005)

All capsicum cultivars tested for resistance to TSWV were susceptible to the *Tsw*-virulent strain, indicating that a common source of resistance, the *Tsw* gene, was present in all and the cultivars' genetic background had little, if any, influence on the expression of the resistance gene.

Virus-resistant cultivars are an important component of TSWV control in both capsicum and tomato. This resistance is currently largely dependent on the *Tsw* gene in capsicum and the *Sw-5* gene in tomato. The continued commercial value of these genes will be prolonged if they are used as part of integrated disease management strategies which reduce virus inoculum levels and populations of thrips vectors rather than relying on resistance as the major means of control (Jones 2004). There is also an urgent need to find and use new sources of resistance to TSWV and other tospovirus species infecting tomato and capsicum (Canady *et al.* 2001; Cebolla-Cornejo *et al.* 2003).

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