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Monitoring of Tospoviruses by Real Time Polymerase Chain Reaction

Ralf G Dietzgen
Queensland Department of Primary Industries

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Project VG 00025

MONITORING OF TOSPOVIRUSES BY REAL TIME POLYMERASE CHAIN REACTION

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Horticulture Australia Project VG00025:
Monitoring of Tospoviruses by Real Time Polymerase Chain Reaction

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Purpose of the report:
This 'Final Report' summarises the R&D achievements of project VG00025 addressing milestones outlined in the contract between Horticulture Australia and the Queensland Government through its Department of Primary Industries. The report will provide R&D accountability and awareness, assists in the adoption of research outcomes and can be accessed through http://www.infoscan.com.au/arrip/index.htm.

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MEDIA SUMMARY

Tomato spotted wilt virus (TSWV) causes major losses in vegetable and ornamental crops worldwide. In Australia, lettuce, tomato and capsicum have been the most severely affected crops in recent years. TSWV is difficult to control because it has an extensive host range of more than 900 plant species including many weeds and it is efficiently transmitted by several thrips species.

Sensitive diagnostic assays for TSWV are essential to gain more information about its epidemiology, to conduct large-scale surveys, to monitor success of virus management strategies and as an aid to large-scale routine detection in bulked samples. The DPI Queensland Agricultural Biotechnology Centre (QABC) has developed a highly sensitive and specific molecular diagnostic assay for TSWV, with the potential for automation and high throughput while yielding quantitative data on viral load (ie. extent of infection).

Horticulture Australia Ltd project VG00025, conducted by QABC, the Department of Agriculture for Western Australia and Saturn Biotech Ltd. has validated this TSWV assay to provide a more effective test for growers' samples and to complement and enhance research under way by the national Western Flower Thrips/TSWV projects funded by HAL. The high assay sensitivity enabled (i) reliable virus detection in single and bulked samples of diverse crop species, (ii) identification of virus reservoirs in infected weeds, and (iii) virus detection in single virus-carrying thrips either live or as dead, dry specimens caught in sticky traps. The latter trapped vectors may be used to assist in disease forecasting and decision support over control measures. A similarly sensitive diagnostic assay for the recently discovered capsicum chlorosis tospovirus that infects capsicum varieties with resistance to TSWV and is therefore resistance-breaking was also developed.

The genetic variability of Australian TSWV isolates was found to be low regardless of crop or weed species from which they were obtained across all plant species tested and the diagnostic assay reliably detected all isolates. The new diagnostics tools demonstrated in this project provide a more sensitive alternative to serological assays for TSWV presence in single infected samples. Bulk samples require more extensive extraction methods, but the assay readily detected 1 infected sample in 1000 uninfected ones. The assay will be made available to interested parties through the Northern Australian Diagnostic Network and commercial testing using it may be offered through Saturn Biotech Ltd.

TECHNICAL SUMMARY

Tomato spotted wilt virus (TSWV) causes major losses in vegetable and ornamental crops worldwide. In Australia, lettuce, tomato and capsicum have been most severely affected crops in recent years. TSWV is difficult to control because it has an extensive host range of more than 900 monocotyledonous and dicotyledonous species including many weeds and it is efficiently transmitted by several thrips vector species in the genera *Frankliniella* and *Thrips*.

Sensitive diagnostic assays for TSWV are essential to gain more information about its epidemiology, to provide a more effective test for growers' samples, to conduct large-scale surveys for it, and to monitor success of management strategies and as an aid to large-scale routine detection in bulked samples. The DPI Queensland Agricultural Biotechnology Centre (QABC) has developed a highly sensitive and specific quantitative TaqMan™ real time reverse transcription polymerase chain reaction (RT-qPCR) diagnostic assay for TSWV (Roberts *et al.* 2000), with the potential for automation and high throughput while yielding quantitative data on viral load (ie. extent of infection).

Project VG00025 was conducted over a 2-year period by QABC, the Department of Agriculture for Western Australia and Saturn Biotech Ltd. to validate this new diagnostic assay by directly comparing it with the established Enzyme-Linked Immunosorbent Assay (ELISA). TSWV was detected in all infected samples collected from eight different ornamental species, nine different horticultural crops, and potato, peanut, ranunculus, chickweed and sowthistle by both RT-qPCR and ELISA. RT-qPCR detected TSWV in a wide range of concentrations in leaf tissues of all 22 different plant species. RNeasy and leaf soak extracts yielded the best results. Bulk samples required more extensive extraction methods, but the assay can then readily detect 1 infected sample in 1000 uninfected ones.

Reliable detection of TSWV in individual thrips vectors is important for forecasting TSWV epidemics in vegetable and ornamental crops. Thus, a duplex TSWV RT-qPCR assay using an internal control primer/probe set for thrips RNA was developed and applied to thrips vector specimens of *Frankliniella occidentalis* (Western Flower Thrips) and *F. schultzei* (tomato thrips) either live or as dead, dry specimen caught in sticky traps. The internal control worked also for *Thrips palmi* (melon thrips) and *T. tabaci* (onion thrips), making this duplex assay applicable to all TSWV vector thrips in Australia. Trizol® solution yielded thrips RNA free of PCR inhibitors reproducibly. The assay was

sufficiently sensitive to detect TSWV in pools of five individuals in which only one individual per group carried the virus. Live thrips specimens are not a prerequisite to successful detection. Thus when trapping takes place in epidemiological studies, the dried, trapped insects can be left for some time before virus testing to see if they are infective (ie. are carrying TSWV).

The genetic variability among Australian isolates of TSWV was low. Direct sequencing of a 587bp region of the nucleoprotein gene (S RNA) of 29 isolates yielded a maximum of 4.3% nucleotide sequence difference. Phylogenetic analysis revealed no obvious groupings of isolates according to geographic origin or host species. TSWV isolates, which break TSWV resistance genes in tomato or capsicum did not differ significantly in the region studied, indicating that a different region of the genome may be responsible for this activity.

TSWV-related viruses in the genus *Tospovirus*, family *Bunyaviridae*, are emerging as increasingly damaging plant pathogens worldwide. Novel or potentially exotic tospoviruses have been identified in Australia in the last couple of years, highlighting the need for diagnostics to identify the other members of this virus group. The use of degenerate primers in *TaqMan* diagnostics directed at conserved regions of the viral genome to detect a broader range of tospoviruses was unsuccessful. We therefore developed an RT-qPCR assay which detects the newly discovered capsicum chlorosis virus (McMichael et al., 2002) and possibly other serogroup IV tospoviruses.

Real time PCR requires sophisticated hardware and technical know-how, which is currently available at facilities at the University of Queensland (UQ School of Molecular and Microbial Sciences) and the WA State Agricultural Biotechnology Centre at Murdoch University. Diagnostic procedures and protocols including sample collection and preparation, setting up of reactions, real time PCR assay and data analysis will be compiled in the form of a manual. The assay will be made available to interested parties through the NADN with technical support from QABC and the UQ facility. Negotiations are in progress to offer commercial TSWV testing through Saturn Biotech Ltd in Western Australia.

TECHNICAL REPORT

INTRODUCTION & LITERATURE REVIEW

Tomato spotted wilt virus (TSWV) is an economically important pathogen in many vegetable and flower crops under field and protected cropping conditions. TSWV infection leads to major reductions in quality and yield of produce and causes millions of dollars in damages each year. Major losses in vegetable and ornamental crops have been reported in Australia, especially in the last 5 years, with tomato, capsicum and lettuce being the most severely affected vegetable crops. TSWV, which was first reported in Victoria in 1915 and occurs in all Australian states, is transmitted propagatively by at least 8 species of thrips in the genera *Frankliniella* and *Thrips* (Wijkamp et al., 1993), four of which are present in Australia. It has an extensive host range including more than 925 susceptible species of monocotyledonous and dicotyledonous plants belonging to 70 botanical families (Latham and Jones, 1997; Peters, 1998; Wilson, 1998). A brief illustrated summary of TSWV is provided at *http://www.apsnet.org/online/feature/tospovirus/Top.html*.

TSWV is the type species in the genus *Tospovirus*, family *Bunyaviridae*. Tospoviruses are the only viruses in this virus family that infect plants (Elliott et al., 2000). Their particles are quasi-spherical and enveloped, and have a tripartite single-stranded RNA genome composed of L, M and S RNA. L RNA is of negative sense, whereas M and S RNAs have an ambisense coding strategy (Goldbach and Peters, 1996; Moyer, 1999). S RNA encodes the nonstructural protein NSs in a viral sense and the nucleocapsid protein N in a viral complementary sense. Prior to 1990, TSWV was the only known tospovirus species. Subsequent strong interest in tospoviruses has led to the identification of at least 16 distinct viruses (Table 1) which have been separated into several distinct serogroups (Cortes et al., 1998; Chu et al., 2001). Species are defined based on vector specificity, plant host range, serological relationships of the N protein and their N protein sequence showing less than 90% amino acid identity with that of any other tospovirus species (Elliott et al., 2000). Overseas isolates of TSWV have a high degree (94-100%) of nucleotide sequence identity in the N gene (De Avila et al., 1993; Pappu et al., 1998) and appear to group phylogenetically according to geographic location (Pappu et al., 1998). TSWV occurs in plants as a heterogeneous mixture of isolates that have been distinguished by symptom phenotype. TSWV can rapidly adapt to new or resistant hosts by reassortment of genome segments in such mixed populations (Moyer, 1999).

The first appearance of western flower thrips (WFT, *Frankliniella occidentalis*) in 1993 and its increasingly widespread distribution in Australia, has led to an upsurge in TSWV epidemics in horticultural crops. This is because WFT is its most efficient vector. WFT has now spread to all Australian states and further increases in TSWV epidemics are expected due to future expansion of its range (Cook et al., 1996). The complex of WFT and TSWV has been recognised as high priority disease problem threatening Australian horticulture, which is being addressed through a national management strategy coordinated through Horticulture Australia (eg. Latham and Jones, 1996,1997). However, TSWV is also readily transmitted by onion thrips (*Thrips tabaci*) and tomato thrips (*Frankliniella schultzei*), which are principal vectors in parts of Australia where WFT has not yet become dominant, and also play a role in magnifying the threat in districts where WFT occurs.

Large-scale surveys conducted in 1996-1998 using serological assays found TSWV infecting many vegetable crops, flowering ornamentals and weeds. Single gene resistance (gene *Sw-5*) to TSWV was present in some tomato cultivars, however, resistance breaking virus strains occasionally develop that overcome it (Latham and Jones, 1997, 1998). TSWV-resistant capsicum cultivars (gene *Tsw*) are also available but the resistance in these too is sometimes overcome by resistance-breaking strains (Thomas-Caroll and Jones, 2003). There is apparently no TSWV resistance in commercial lettuce varieties, but wild lettuce has been used as a resistance source in breeding programs to improve this crop. TSWV is the economically most damaging tospovirus in vegetables both nationally and internationally. Until recently, all Australian tospovirus isolates assayed serologically reacted with antisera to TSWV serogroup I. For the first time a serogroup IV tospovirus, capsicum chlorosis virus (CaCV), was recorded in capsicum, tomato and chilli plants in Australia in 1999 (McMichael et al., 2002). CaCV overcame a TSWV resistance gene introgressed into tomato (*Sw-5*) and used in capsicum breeding programs. The complete nucleotide sequence of the nucleocapsid protein indicated that CaCV should be considered as a new tospovirus species, related to other serogroup IV tospoviruses (McMichael et al., 2002). During 2002 another non-TSWV tospovirus, serologically related to *Iris yellow spot virus* (IYSV) was identified in imported tulips grown in open quarantine (B. Coutts and R. Jones, pers. communication), and IYSV itself is being increasingly found damaging onion crops in several states (WA, NSW, VIC).

Current diagnostic assays for TSWV include sap inoculation to susceptible indicator plants, electron microscopy, enzyme-linked immunosorbent assay (ELISA) and tissue blot immunoassay (TBIA). ELISA is most widely used and commercial companies (eg. Bioreba http://www.bioreba.ch, and Loewe http://www.loewe-info.com) and international

antisera collections (*http://www.dsmz.de/plvirus/general/f_sera.htm*) provide tospovirus antisera or diagnostic ELISA kits for either, selected tospoviruses or broad spectrum tospovirus mixtures. Recently, the Central Science Laboratory, UK (CSL, *http://pdiag.csl.gov.uk/*) developed Pocket Diagnostic kits for TSWV and INSV for direct quick on-site field diagnostics, and these are available in Australia.

Table 1: Tentative and confirmed tospovirus species recognised by the ICTV*

TOSPOVIRUS	ALT. NAME	ACRONYM	SERO-GROUP
Tomato spotted wilt virus		TSWV	I
Groundnut ringspot virus		GRSV	II
Tomato chlorotic spot virus		TCSV	II
Impatiens necrotic spot virus		INSV	III
Watermelon silver mottle virus		WSMoV	IV
Groundnut bud necrosis virus	Peanut bud necrosis	GBNV	IV
Watermelon bud necrosis virus		WBNV	IV
Groundnut yellow spot virus	Peanut yellow spot	GYSV	
Zucchini lethal chlorosis virus		ZLCV	
Chrysanthemum stem necrosis virus		CSNV	
Iris yellow spot virus		IYSV	
Groundnut chlorotic fan-spot virus	Peanut chlorotic fan-spot	GCFSV	
Physalis severe mottle virus		PhySMV	
Gloxinia tospovirus			
Melon yellow spot virus		MYSV	
Thailand tomato tospovirus			

*ICTVdB *http://www.ncbi.nlm.nih.gov/ICTVdb/* (October 2002)

Nucleic acid-based diagnostic assays, especially those involving the polymerase chain reaction (PCR), are increasingly being used for diagnostics of plant viruses (Dietzgen, 2002). Reverse transcription-polymerase chain reaction (RT-PCR) followed by gel-based detection has been reported for the identification of TSWV and the discrimination of tospoviruses. This uses primers designed from sequences in the L and S RNAs (Mumford et al., 1996; Dewey et al., 1996). Both species-specific primers and primers with specificity for members of more than one serogroup have been used, and restriction enzyme digests of PCR products have enabled differentiation of virus species (Mumford et al., 1996; Dewey et al., 1996). Immunocapture and design of primers from conserved

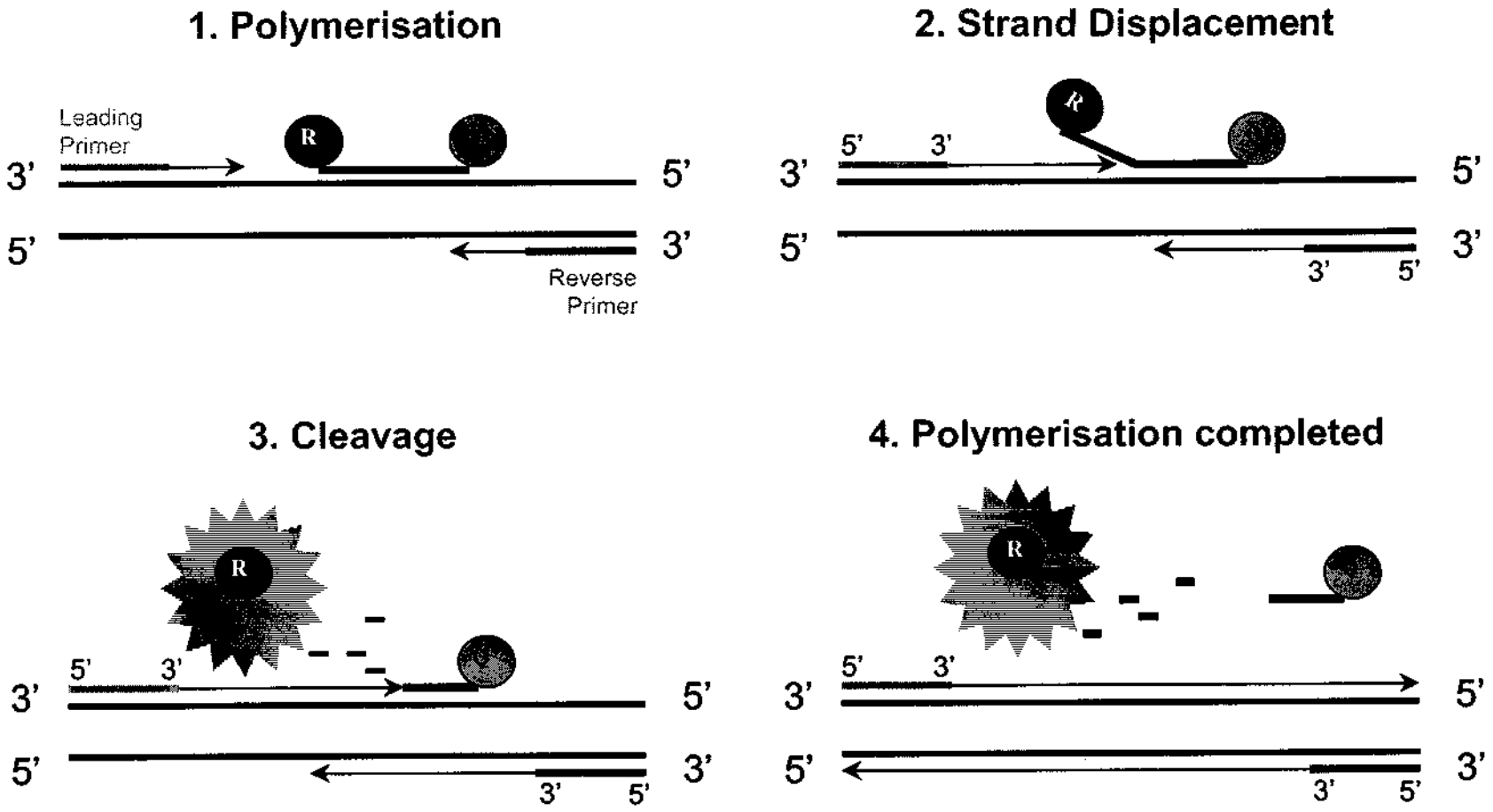
regions has enabled amplification of the entire N gene of TSWV from peanut tissue (Pappu et al., 1998). Degenerate primers targeting conserved motifs in the L RNA or S RNA have been used to detect a range of tospoviruses belonging to five different serogroups (Chu et al., 2001; Okuda and Hanada, 2001).

Real time quantitative PCR (qPCR) allows the increase of specific PCR amplicons to be monitored during each successive amplification cycle. The optimal amplicon length is about 50-150 base pairs. A pair of PCR primers and an internal hybridisation oligonucleotide probe provide dual specificity. TaqMan™ (PE Biosystems) chemistry utilises the 5'→ 3' nuclease activity of *Taq* DNA polymerase to cleave a specific probe, which is labelled with a 5' reporter (eg. FAM) and a 3' quencher (TAMRA) fluorescent dye, during strand elongation of the target sequence and hence generates a detectable fluorescent signal during amplification (Fig. 1). QABC has developed and laboratory-tested such a RT-qPCR assay which detect a range of Australian isolates of TSWV (Roberts et al., 2000).

TaqMan real time qPCR technology has several potential advantages over ELISA and over other DNA-based diagnostic technology platforms, including (i) increased sensitivity which enables detection of low pathogen levels in bulked samples, (ii) minimal cross-contamination potential due to close tube assay and lack of post-PCR handling for product detection, (iii) real time quantification, and (iv) reliable high throughput and automation. This technology has been successfully applied for the sensitive detection of many plant and human viruses and other pathogens.

In 1999, the national Horticulture Australia WFT/TSWV project identified several priority areas for further research. These included (i) epidemiological studies in key horticultural crops to link vectors with epidemics and to clarify the role of virus sources, (ii) development of forecasting models for TSWV epidemics, (iii) breeding/selection of TSWV-resistant capsicum varieties, (iv) application of high sensitivity, high throughput diagnostics for routine TSWV detection, (v) determination of the presence of other tospoviruses in Australia, (vi) improved testing procedures for bulk samples, such as of vegetable seedlings, potato tubers and flower bulbs, (vii) study of TSWV strain variability, and (viii) establishment of an Australian Tospovirus Network and reference collection.

Fig.1 *TaqMan*™ real time PCR assay



PROJECT AIMS

1) Implementation and validation of the TSWV *TaqMan* RT-qPCR assay by

- virus detection in a range of field samples from diverse vegetable crops, ornamentals and weeds
- evaluation of RNA extraction methods for template preparation
- comparison with ELISA
- testing of bulked samples

2) Development of a duplex RT-qPCR assay to detect TSWV in thrips vectors

3) Analysis of the genetic variability of TSWV isolates from throughout Australia

4) Investigation of the possibility of developing a broad spectrum RT-qPCR assay to detect other tospoviruses.

MATERIALS & METHODS

Virus isolates

Australian TSWV isolates collected from horticultural crops, ornamentals and weeds (Table 2) were provided by Denis Persley (QDPI), Monica Thomas-Carroll, Donna Atkins, Roger Jones and Brenda Coutts (Dept of Agriculture, WA), and Calum and Annabel Wilson (University of Tasmania). CaCV isolates were provided by Lee McMichael (QDPI).

Enzyme-linked immunosorbent assay (ELISA)

Leaf disks were extracted in phosphate-buffered saline (10 mM potassium phosphate, 150 mM sodium chloride), pH 7.4 containing 0.5% Tween 20 using a leaf press (Pollähne, Hanover, Germany). Extracts were tested in duplicate wells of microtitre plates. Double antibody sandwich ELISA was done as described by Clark and Adams (1977). Antisera and antibody conjugates specific for TSWV were obtained from Sanofi (France) or Loewe Diagnostics (Germany).

Nucleic acid extraction methods

Plants

Phenol-chloroform extraction (Wylie et al., 1993). Single leaf disks (0.5 mm diameter) were ground thoroughly in a microcentrifuge tube with a disposable pestle in 50 μl extraction buffer (50 mM Tris-HCl, pH 8.5, 10 mM EDTA, 200 mM NaCl). Then, 450 μl extraction buffer and 500 μl phenol:chloroform:isoamylalcohol (50:48:2) were added and vortexed for 1 min. The tube was centrifuged for 1 min and the aqueous layer transferred to a fresh tube. An equal volume of chloroform: isoamylalcohol (48:2) was added, mixed by vortexing and centrifuged. The chloroform extraction was repeated once. The aqueous layer was transferred to a fresh tube, an equal volume of cold isopropanol was added, mixed by repeated inverting of the tube and incubated on ice for 15 min. The mixture was centrifuged for 15 min at 4°C, and the supernatant removed. The pellet was vacuum-dried, resuspended in 100 μl TE (10 mM Tris-HCl, pH 7.4, 1 mM

EDTA), 4 *µl* 5 M NaCl, 250 *µl* cold ethanol and the mixture incubated on ice for 30 min. The tube was centrifuged for 30 min at 4°C, the supernatant discarded and the pellet vacuum-dried. 150 *µl* 70% ethanol was added to the pellet and incubated at 56 °C for 3 min, briefly vortexed and centrifuged for 2 min. The pellet was vacuum dried, resuspended in 100 *µl* RNase-free water and centrifuged for 2 min. One *µl* of the supernatant was used as template in PCR.

For bulk extracts, up to 1000 leaf disks were combined and ground in a mortar and pestle using 10 ml extraction buffer. The slurry was transferred to a 50 ml tube, and vortexed for 1 min. An equal volume of phenol:chloroform:isoamylalcohol (50:48:2) was added and vortexed for 1 min. Following centrifugation, 750 *µl* supernatant was transferred into a microcentrifuge tube and extracted twice with chloroform as described above.

RNeasy extraction. Total plant RNA was extracted from 50 mg of freeze-dried or 100 mg of fresh leaf material using RNeasy plant miniprep columns (Qiagen) following the protocol provided by the manufacturer. The concentration was determined from measurement of A_{260nm} in a spectrophotometer.

"Leaf soak" extracts were prepared by adding 30 µl of a Template Preparation Solution (TPS) containing 100 mM Tris-HCl, pH 7.4, 1 M KCl and 10 mM EDTA to a ~ 2 mm^2 piece of leaf and heating of the sample at 95°C for 10 min (Thomson and Dietzgen, 1995). Plant debris was sedimented by centrifugation, the supernatant diluted in sterile DEPC-treated water and 1-5 µl used in RT-PCR.

Combined leaf soak & RNeasy extraction. Leaf disks were submerged in TPS (200 µl/1 disk, 1ml/100 disks, 10 ml/1000 disks) and boiled for 5 min. Of the supernatant 200 µl was transferred to a microcentrifuge tube and an equal volume RNeasy RTL buffer was added. The solution was mixed and incubated at 56°C for 3 min. Ethanol (200 µl) was added and the solution applied to an RNeasy column. The column was washed as described by the manufacturer and RNA eluted in 50 µl RNase-free water.

Trizol® extraction of bulked leaf material. Leaf punches were ground with a mortar and pestle in Trizol reagent (10 ml/1000 punches; 2 ml/100 punches) transferred to a centrifuge tube and 1/5 volume chloroform added. The mixture was vortexed, incubated for 3 min and centrifuged. RNA was precipitated from the supernatant by addition of an equal volume of isopropanol. The nucleic acid pellet was washed with 70% ethanol, dried, resuspended in RNase-free water and used 1:10 diluted in RT-qPCR.

Insects

Trizol® extraction. Individual thrips were homogenised in a microcentrifuge tube in a solution containing 100µl Trizol, 1.25µl glycogen, 1µl 4mg/ml proteinase A and 2µl 10mg/ml RNase-free DNaseI. The extract was incubated for 15 min, mixed vigorously with 20µl chloroform, centrifuged and the aqueous phase transferred to a fresh tube. Following extraction with 50µl isopropanol and centrifugation, RNA was precipitated from the aqueous phase with an equal volume of ethanol, air-dried, dissolved in DEPC-treated water and stored at –70°C.

Chelex resin extraction. Individual thrips were ground with a pestle in a microcentrifuge tube containing 50 µl RNase-free water. Chelex 100 resin (Sigma) was added to a final concentration of 5%, the mixture boiled for 5 min, centrifuged briefly, the pellet discarded and the supernatant and stored at –70°C.

CSL precipitation method. (Boonham et al., 2002). Individual thrips were ground with a pestle in a microcentrifuge tube with 50 µl extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS). Twenty-five µl 3 M sodium acetate, pH 5.2 were added, tubes incubated at –20°C for 10 min and centrifuged for 5 min. The pellet was discarded and 1 volume isopropanol (–20°C) added to the supernatant, mixed well and incubated at room temperature for 30 min. The pellet was collected by centrifugation for 30 min, washed with 70% ethanol, repelleted and dried. Finally, the pellet was resuspended in 25 µl RNase-free water and stored at –20°C.

PCR amplification, purification, direct sequencing and analysis of TSWV N gene

RT-PCR was performed in 0.2 ml tubes in a Perkin Elmer model 2400 or 9700 thermal cycler using the Superscript One-Step RT-PCR system (Life Technologies) in a reaction volume of 25 µl. Primers TSW.1 and TSW.2 (Table 4) were used at 0.4 µM final concentration with 10 ng total RNA extract (RNeasy, Qiagen) as template. Following denaturation at 72°C for 5 min, cDNA was synthesized at 48°C for 45 min and the reaction mix heated to 94°C for 2 min. PCR was carried out for 40 cycles at 94°C for 45 s, 45°C for 45 s, 72°C for 60 s, followed by a final extension step of 72°C for 10 min. PCR products were analysed by 1% agarose gel electrophoresis and purified using Exo SAP-IT (USB) to eliminate dNTPs and primers. Nucleotide sequences were determined through the Australian Genome Research Facility (AGRF, Brisbane) using the PRISM BigDye terminator sequencing kit (PE Biosystems) and primers TSW.1 and TSW.2. PCR

products from at least two independent RT-PCR reactions were sequenced directly without prior cloning to ensure accuracy of sequence data. Consensus sequences were compiled using Sequencher 3.0 software (Gene Codes) and analysed by multiple sequence alignment using ClustalW. Sequence homology and phylogeny were determined through GCG programs networked by the Australian National Genome Information Service (ANGIS).

Preparation of RNA transcripts and standard curves

A recombinant plasmid carrying the 628 bp TSWV N gene insert from isolate #873 was linearised with *SalI* (Roche) and purified using the High Pure PCR purification kit (Roche). RNA transcripts were generated at 37°C for 1 hr using T7 RNA polymerase and the SP6/T7 transcription kit (Roche). Plasmid DNA was subsequently digested with RNase-free DNase I at 37°C for 30 min and the transcripts isolated by two phenol-chloroform extractions and ethanol precipitation. RNA concentration was determined using a GeneQuant spectrophotometer (Pharmacia). Cycle thresholds (Ct) for a 10-fold dilution series of RNA transcripts were plotted to yield a standard curve for each experiment. Ct was initially calculated by the *TaqMan* software to indicate significant fluorescence signals above noise during the early cycles of amplification. When necessary, Ct was adjusted manually to cross an exponential portion of the amplification curves of all samples being compared. Thus, Ct can vary between experiments, and for quantification it is necessary to include common standards in all experiments.

Oligonucleotide primers

Oligonucleotide primers for amplification of a fragment of the TSWV N gene (Roberts et al., 2000) were designed from conserved regions of a consensus sequence obtained by CLUSTAL W alignment of 12 TSWV N gene sequences extracted from GenBank (AF048714-16, AF064469-74, AB010997, X61799, Z36882) and verified using Oligo 4.0 (National Biosciences). Primer Express (PE Biosystems) parameters were used to design the TaqMan primers and the fluorescent dye-labelled probe. The chosen primers and probe were subjected to FastA analysis on GenBank through the Australian National Genome Information Service (ANGIS) to confirm sequence specificity. Other degenerate primers used for detection of tospovirus RNA are listed in Table 4.

TaqMan RT-qPCR assay

TaqMan® assays were performed in a Model 7700 Sequence Detector (PE Biosystems). One-Step RT-PCR Master Mix (PE Biosystems) was used in Brisbane, while in Perth a two-step *TaqMan* protocol was adopted.

The One-Step RT-PCR mixture (25 µl reaction) contained 1 x *TaqMan* buffer A, 5.5 mM $MgCl_2$, 0.3 mM each of dATP, dCTP and dGTP, 0.6 mM dUTP, 200 nM forward and reverse primers, 100 nM *TaqMan* probe, 0.4 U/µl RNase inhibitor, 0.25 U/µl Multiscribe reverse transcriptase and 0.025 U/µl AmpliTaq Gold DNA polymerase. Thermal cycling conditions were 48°C for 30 min for reverse transcription, 95°C for 10 min for AmpliTaq Gold activation, and 45 cycles of 95°C for 15 s and 60°C for 60 s for PCR.

For the two-step protocol, first strand synthesis was performed in 200µl thin-wall tubes in a 10µL reaction volume. The reaction mix contained 1 X PCR buffer, 4 mM $MgCl_2$, 1 mM each dNTP, 200 nM reverse primer, 5 units RNase inhibitor, and 12.5 units MuLV reverse transcriptase. The reaction was incubated at 42°C for 15 min, then at 99°C for 5 min. In step two, 5µl of the first-strand synthesis reaction was added to optical tubes containing 1x *TaqMan* buffer, 2.5 mM $MgCl_2$, 200 nM forward primer, 100 nM *TaqMan* probe and 0.025 units *Taq* DNA polymerase. Tubes were incubated at 92°C for 3 minutes, followed by 40-45 cycles of 94°C for 60 s, 60°C for 60 s. Finally the tubes were incubated at 60°C for 7 minutes.

TaqMan primer and probe sequences used in this study are listed in Table 5. TSWV and CaCV were detected in uniplex assays in plant specimens or in duplex assays using a WFT actin mRNA-specific internal control in thrips extracts. In assays not utilising a *TaqMan* probe, SYBR® Green PCR Master Mix (PE Biosystems) was used for PCR.

Relative virus load, ie. relative amount of virus in infected extracts was calculated using the formula:

$rvl = 2^{-\Delta Ct}$

where Ct is the cycle threshold. The higher the Ct value, ie. the more cycles of PCR amplification required to reach the threshold level, the lower the virus concentration.

Table 2 TSWV isolates used in this study

Name/number	Original host	Extraction host#	Collected/Notes	TaqMan	Sequence
AhQLD-1	*Arachis hypogaea*	Peanut [f]	Kingaroy, SEQ; 1999	√	√
AhQLD-2	*Arachis hypogaea*	Peanut [f]	Kingaroy, 2001	√	
Ah-QLD-3	*Arachis hypogaea*	Peanut [f]	Kingaroy, 2001	√	
AnWA-1	anemone	Tomato	WA	√	√
AnWA-1di	Derivative from AnWA-1	*C. chinenese* PI152225	*Tsw* resistance breaking from AnWA-1	√	√
AnWA-1eii	Derivative from AnWA-1	*C. chinenese* PI152225	*Tsw* resistance breaking from AnWA-1	√	√
ArWA-1	artichoke	Artichoke	South Perth, WA	√	√
AsWA-1	aster	aster	Yangebup, WA	√	
BaWA-1	basil	Basil [f]	Munster, WA	√	
CalWA-1	*Calendula officinalis* (marigold)	Calendula [f]	Yangebup, WA	√	
CaQLD-867	capsicum	Capsicum	Gumlu, NQ	√	
CaQLD-868	capsicum	Capsicum	Gumlu, NQ	√	
CaQLD-869	capsicum	Capsicum	Rocky Ponds, NQ	√	√
CaQLD-871	capsicum	Capsicum	Gumlu, NQ	√	√
CaQLD-872	capsicum	Capsicum	Bowen, NQ	√	
CaQLD-873	capsicum	Capsicum		√	√
CaQLD-874	capsicum	Capsicum	Ayr, NQ	√	√
CaQLD-876	capsicum	Capsicum	Bowen, NQ	√	
CaQLD-891	capsicum	Capsicum	Bowen, NQ	√	√
CaQLD-1255	capsicum	Capsicum	Gatton, SEQ	√	√
CaQLD-1256	capsicum	Capsicum	Gatton, SEQ	√	√
CaQLD "4"	capsicum	Capsicum [f]	Gumlu, NQ	√	√
CaQLD "34"	capsicum	Capsicum [f]	Mt Kelly, NQ	√	√
CaQLD "36"	capsicum	Capsicum [f]	Giru, NQ	√	√
CaWA-1	capsicum	Tomato	Banjup, WA	√	√
CaWA-3	capsicum	Tomato	Banjup, WA	√	
Cap1	capsicum	Capsicum [f]	Gnangara, WA	√	
CeWA-1	celery	Celery	South Perth, WA	√	√
CiWA-1	chickweed	Chickweed [f]	Munster, WA	√	
Chi1	chilli	Chilli [f]	Gnangara, WA	√	
DaWA-1c	Derivative	*Nicotiana tabacum*	=DaWA-3s; derived from DaWA-1, *Sw-5* resistance breaking	√	√
DaWA-1d	Derivative	Tomato	*Sw-5* resistance breaking from DaWA-1c	√	√
DaTAS1.01	dahlia	Dahlia	TASUni	√	
DaTAS2.01	dahlia	Dahlia	TASUni	√	
DaTAS3.01	dahlia	Dahlia	TASUni	√	
DaTAS4.01	dahlia	Dahlia	TASUni	√	

DaTAS5.01	dahlia	Dahlia	TASUni	√	
Egg1	eggplant	Eggplant [f]	Gnangara, WA (03/01)	√	
IrWA-1	iris	Iris	Perth, WA	√	
LeQLD-1	lettuce	Tomato	Qld	√	√
LeNSW-1	lettuce	Tomato	Griffith, NSW	√	√
LeSA-1	lettuce	Lettuce [f]	Adelaide Hills; 2000	√	√
LeSA-2	lettuce	Lettuce [f]	Adelaide Hills; 2000	√	√
LeSA-3	lettuce	Lettuce [f]	Adelaide Hills; 2000	√	√
LeWA-1	lettuce	Lettuce	AgWA	√	√
Le1	lettuce	Lettuce [f]	Gnangara, WA (03/01)	√	
Le2	lettuce	Lettuce [f]	Gnangara, WA (03/01)	√	
LiWA-1	Lisianthus	Lisianthus	Yangebup, WA	√	
MaWA-1	malva	Malva [f]	Munster, WA	√	
PaWA-1	parsley	Parsley [f]	Munster, WA	√	
PoWA-1	Potato	Capsicum	Ludlow, WA	√	√
RanWA-1	ranunculus	Ranunculus [f]	Yangebup, WA	√	
SoWA-1	sowthistle	Sowthistle [f]	Gnangara, WA (03/01)	√	
SoWA-2	sowthistle	Sowthistle [f]	Munster, WA	√	
ToTAS-1	tomato	Capsicum	Tasmania	√	√
ToTAS-1d	Derivative	*N. tabacum*	*Sw-5* resistance breaking, derived ex ToTAS-1	√	√
ToTAS-1dc	Derivative	Capsicum	*Tsw* resistance breaking ex ToTAS-1d	√	√
To1	tomato	Tomato [f]	Gnangara, WA (03/01)	√	
ZanTAS-6.01	Zantedeschia (Araceae)	zantedeschia	TASUni	√	
ZanTAS-7.01	zantedeschia	zantedeschia	TASUni	√	
ZanTAS08.01	zantedeschia	zantedeschia	TASUni	√	

[f] indicates field-collected samples; other samples were glasshouse-maintained

Table 3 Other tospovirus isolates used in this study

Name/number	Original host	Extraction host	Collected	TaqMan
CaCV "18"	capsicum	Capsicum [f]	Mt. Kelly, NQ	√
CaCV "22"	capsicum	Capsicum [f]	Mt Kelly, NQ	√
CaCV "29"	capsicum	Capsicum [f]	Giru, NQ	√
Tulip tospovirus	tulip	tulip	open quarantine WA	-

Table 4 PCR primers for detection/cloning of tospoviruses

Primer 1	Primer 2	Target	Reference
gL3637 CCTTTAACAGTDGAAACAT	gL4435 CATDGCRCAAGARTGRTARACAGA	L RNA (819bp)	Chu et al., 2001
gL3637 CCTTTAACAGTDGAAACAT	gL4510c TCATCRGARTGBACMATCCATCT	L RNA (895bp)	Chu et al., 2001
3'T12 GWGYTAACGAGAgggggggg	TsMCR2 TCIRDICKIYKRAAIGTCMSRTC	S RNA	Okuda & Hanada, 2001
J13 CCCGGATCCAGAGCAAT	UHP CACTGGATCCTTTTGTTTTTGTTTTTTG	Termini; S RNA IGR	Cortez et al., 2001
S1 UNIV TGTARTGKTCCATWGCA	S2 UNIV AGAGCAATYGTGTCA	S RNA (871bp)	Mumford et al., 1996
Dewey1 AGAGCAATTGTGTCA	Dewey2 TCAAGYCTTCKGAARGTCAT	S RNA (450bp)	Dewey et al., 1996
TSWV.1 TCTGGTAGCATTCAACTTCAA	TSWV.2 GTTTCACTGTAATGTTCCATAG	S RNA (626bp)	Roberts et al., 2000

Table 5 Tospovirus/WFT *TaqMan* primers and probes used in this study

Primer/probe	Sequence (5' to 3')	Target	Reference
TSW.F	GCTTGTTGAGGAAACTGGGAATT		Roberts et al., 2000
TSW.R	AGCCTCACAGACTTTGCATCATC	(117bp)	"
TSWV. probe1	6FAM_AAATCTAAGATTGCTTCCCAC CCTTTGATTCAA_TAMRA	TSWV N gene	"
WFT-25F	GGTATCGTCCTGGACTCTGGTG		Boonham et al., 2002
WFT-93R-C	GGGAAGGGCGTAACCTTCA	(68bp)	"
WFT RNA-48T	VIC_CGGTGTCTCCCACACTGTCCCC A_TAMRA	WFT actin	"
CaCVNF604	GGTGCAAGGCCCTTGATG		this study
CaCVNR691	CTGCTTTGTTTGATTCATGTGCT		"
CaCV.probe1	VIC_AGAGGACCACCAATACACAAC ATCATCCTGA_TAMRA	CaCV N gene	"

RESULTS AND DISCUSSION

1. Genetic variability of Tomato spotted wilt virus isolates in Australia

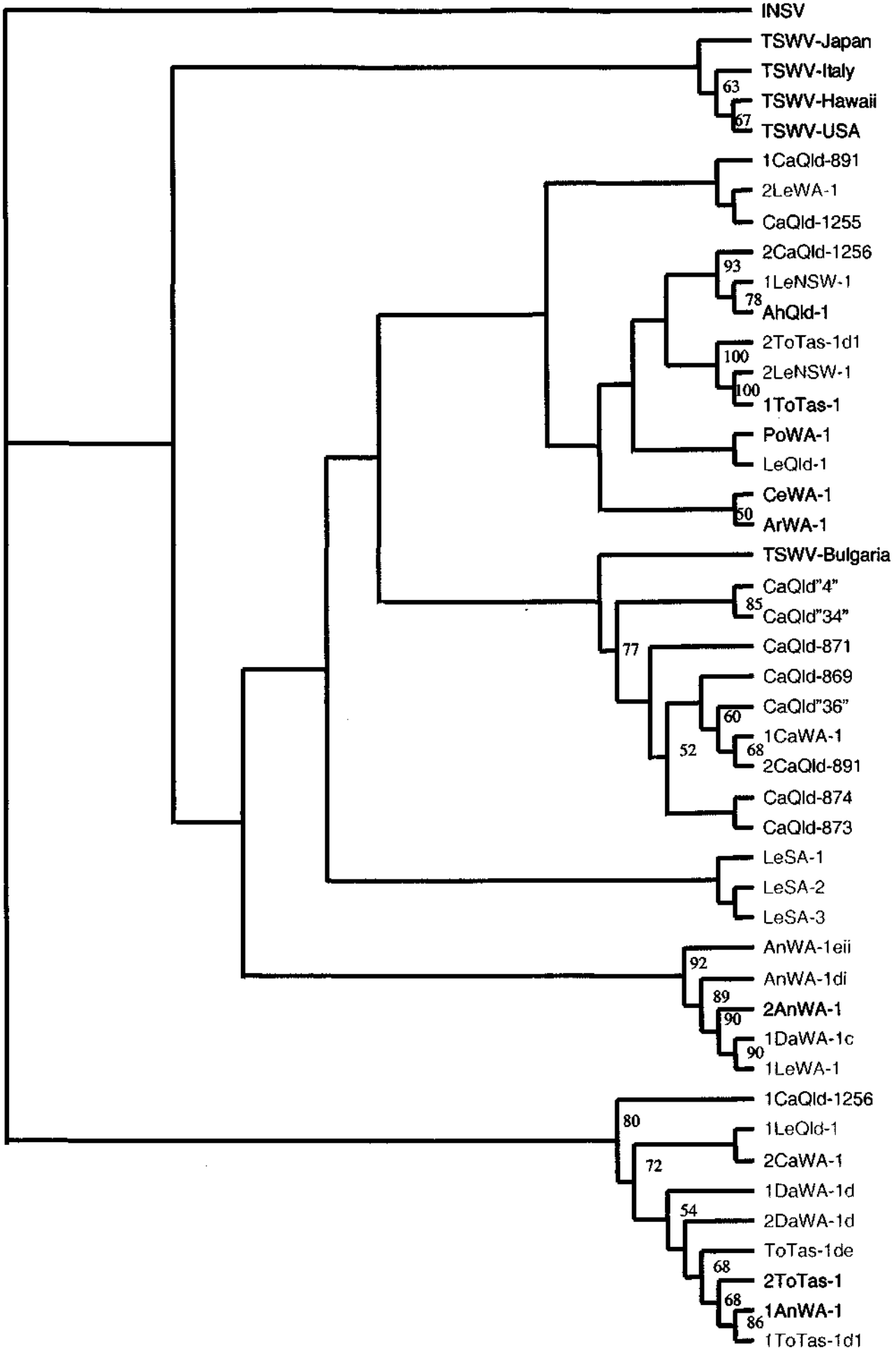
Nucleotide sequences of a 587bp region of the nucleoprotein gene (S RNA) of 29 Australian TSWV isolates were determined and compared among themselves and with selected overseas isolates. Isolates from QLD, WA, SA, NSW and Tasmania from capsicum, lettuce, anemone, dahlia, peanut, potato, celery, artichoke and tomato were amplified by RT-PCR and directly sequenced. The nucleotide sequences of these Australian isolates were 95.7-100% identical in this region of the TSWV genome, but could be differentiated from the majority of analysed overseas isolates (Fig.2). Maximum sequence diversity detected among Australian TSWV isolates was 4.3%.

Resistance-breaking isolates in tomato (*Sw*-5 gene) and *Capsicum chinense* PI152225 (*Tsw* gene) did not differ significantly in this region of the S RNA. They were also very similar to the original field isolates they were derived from, eg. AnWA-1, 1eii and 1di or ToTAS-1, 1d, 1e, 1d1.

Phylogenetic analysis revealed no consistent groupings according to geographic origin or original host plant species (see spread of capsicum and lettuce isolates in Fig.2). However, most Queensland capsicum isolates and the South Australian lettuce isolates collected from the same field at the same time, formed a cluster of closely related sequences (Fig. 2).

Population diversity within single TSWV isolates was detected by sequencing independent RT-PCR reactions (indicated by a number preceding the isolate name). This pseudo-species variability could be low, like in the case of LeNSW-1, or high, like in LeWA-1, where the sequences fall into quite separate groupings (Fig.2).

Figure 2: Neighbour-joining tree of partial TSWV N gene sequences from 29 Australian (39 consensus sequences) and 5 overseas isolates. Impatiens necrotic spot tospovirus (INSV) was used as out-group; isolates are labelled according to host plant species, followed by State collected and isolate number (see Table 2). Capsicum isolates are labelled in 'blue', lettuce isolates in 'green' and resistance breaking isolates in 'red'. Only bootstrap values above 50% are shown.


INSV
TSWV-Japan
TSWV-Italy
63
TSWV-Hawaii
67
TSWV-USA
1CaQld-891
2LeWA-1
CaQld-1255
2CaQld-1256
93
1LeNSW-1
78
AhQld-1
2ToTas-1d1
100
2LeNSW-1
100
1ToTas-1
PoWA-1
LeQld-1
CeWA-1
50
ArWA-1
TSWV-Bulgaria
CaQld"4"
85
CaQld"34"
77
CaQld-871
CaQld-869
CaQld"36"
60
1CaWA-1
52
68
2CaQld-891
CaQld-874
CaQld-873
LeSA-1
LeSA-2
LeSA-3
AnWA-1eii
92
AnWA-1di
89
2AnWA-1
90
1DaWA-1c
90
1LeWA-1
1CaQld-1256
80
1LeQld-1
2CaWA-1
72
1DaWA-1d
54
2DaWA-1d
ToTas-1de
68
2ToTas-1
68
1AnWA-1
86
1ToTas-1d1

2. Validation of TaqMan RT-qPCR assay for detection of TSWV in plants

All ELISA-positive single TSWV-infected samples also proved positive with the TSWV *TaqMan* RT-qPCR assay. When it came to diluted and bulked samples with lower virus concentrations, however, ELISA was somewhat less sensitive than *TaqMan*.

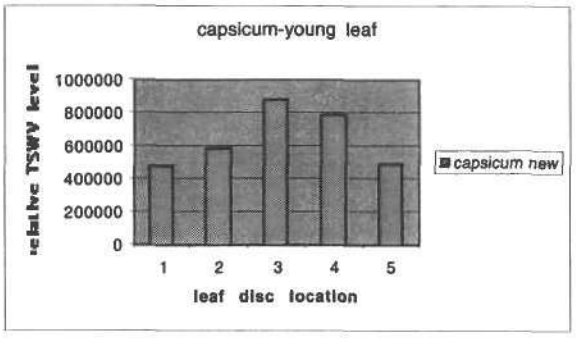
2.1 Distribution of TSWV in leaves of infected plants – impact on sampling

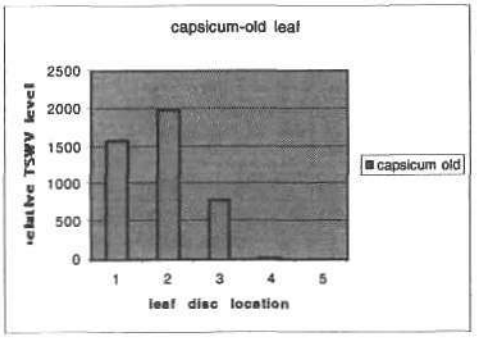
Sampling position and leaf age sometimes effected TSWV detection in capsicum and tomato, since low virus titres were not detectable by ELISA. This was not a problem when using *TaqMan* RT-qPCR due to its high sensitivity and detection range across at least 6 orders of magnitude.

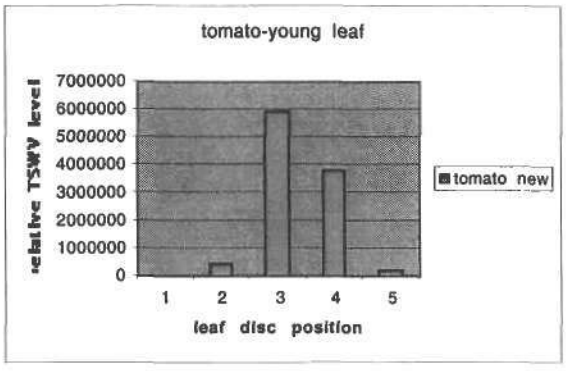
TaqMan RT-q-PCR and ELISA were used to determine relative TSWV levels in tomato and capsicum leaves. Older leaves had lower virus levels than young leaves. TSWV concentration varied by up to 5 orders of magnitude within the same leaf (Fig. 3). For diagnosis of tomato and capsicum leaves, the midrib area between the middle and base of the leaf should be sampled as it contained the highest TSWV titres. Sampling location was most important for testing bulked leaf punch samples (see 2.4).

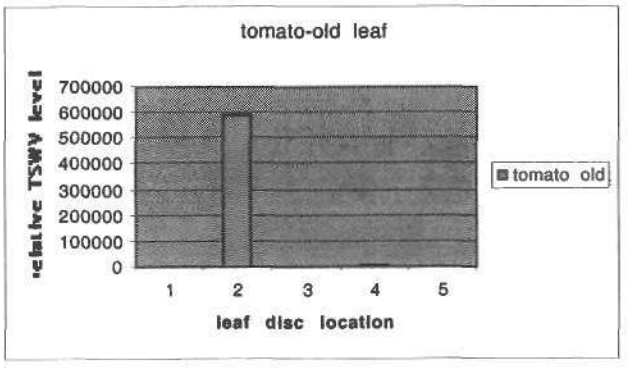
Figure 3. Relative TSWV levels determined by *TaqMan* qRT-PCR vary strongly within infected tomato and capsicum leaves.

leaf position	*tomato new*	*tomato old*	*capsicum old*	*capsicum new*
1	498	690	1584	479110
2	405685	593957	1965	593957
3	5931641	2210	781	881743
4	3754007	7643	19	783732
5	231395	873	10	489178









2.2 Comparison of sample extraction methods

A commercial extraction method, previously developed for *TaqMan* assays at SABC for detection of *Cucumber mosaic virus* in lupin seeds (Wylie et al., 1993), worked well for field samples from vegetable and ornamental crops, except that dilution of RNA was needed to overcome PCR inhibitors. This method involves the use of phenol & chloroform and, though effective, it is costly, somewhat time-consuming and labour-intensive, and also involves use of toxic chemicals and is therefore not ideal. However, all TSWV-infected samples found infected by ELISA were reliably detected using it.

To test potentially more user-friendly, cheaper and faster methods of sample extraction for *TaqMan*, RNeasy (Qiagen, Germany) and "leaf soak" extracts (Thomson and Dietzgen, 1995) of TSWV-infected samples were prepared and tested using *TaqMan*. The former yields relatively pure RNA, but is more costly and time consuming, whereas the latter provides an inexpensive and quick, but more crude nucleic acid preparation. The "leaf soak" extraction method enabled reliable detection of TSWV in all individual infected samples assayed of tomato, capsicum, lettuce, dahlia, zantedeschia (Fig. 5), basil, chickweed, malva, parsley and sowthistle at extract dilutions of 1/100 and 1/500. However, at the lower dilution of 1/20, detection was sometimes affected by inhibitor activity intrinsic to extracts of some plant species. This need to dilute extracts further poses a drawback to its broader scale application when bulked samples are to be tested rather than individual samples. RNeasy extracts were generally superior in terms of reliability and signal intensity for most plant samples tested throughout the project.

In one experiment, samples of anemone, capsicum, lettuce and tomato, which had been shown previously by ELISA to be infected with TSWV, were extracted. *TaqMan* detected all isolates when 1 ng of RNeasy-purified RNA was used as template. TSWV was also reliably detected in leaf soak extracts diluted 1/100, but in some instances when virus concentration was low, the 1/500 dilution was negative (Table 6). This was also the case when extract was diluted less, as PCR was inhibited. We therefore recommend that 'leaf soak' TPS extracts should be used at 1/100 dilution for tomato, lettuce and capsicum leaf samples. *TaqMan* qRT-PCR using 1 ng RNeasy or 1/100 diluted leaf soak extracts worked reliably for both glasshouse- grown and field-collected leaf samples.

Table 6: Ct values indicating relative concentration of TSWV*

Sample	Isolated from	Extracted from	RNeasy	TPS 1/100	TPS 1/500
AnWA-1	anemone	tomato	30	36	37
CaQ1255	capsicum	capsicum	40	39	>39
CaQ1256	capsicum	capsicum	33	>36	negative
CaWA-1	capsicum	tomato	35	>40	>41
LeQ-1	lettuce	tomato	>38	36	38
LeSA-1	lettuce	lettuce	31	34	36
LeSA-2	lettuce	lettuce	29	36	39
LeSA-3	lettuce	lettuce	27	32	34
LeWA-1	lettuce	lettuce	28	37	negative
ToTas-1	tomato	capsicum	33	34	negative

* the higher the Ct value, the lower the TSWV concentration

TaqMan can also be used to estimate virus concentration in infected plants. For this purpose, a dilution series of TSWV RNA transcripts containing the target sequence was included in the set-up of every *TaqMan* experiment. A standard curve was generated and the unknown samples placed on it for quantification as illustrated in the example shown in Fig.6. Using this system, symptomatic field samples of lettuce, tomato, capsicum and chillies collected in WA in March 2001 were shown to have high virus titres of ~10^9 TSWV particles/ng total RNA.

2.3 Assay of single samples

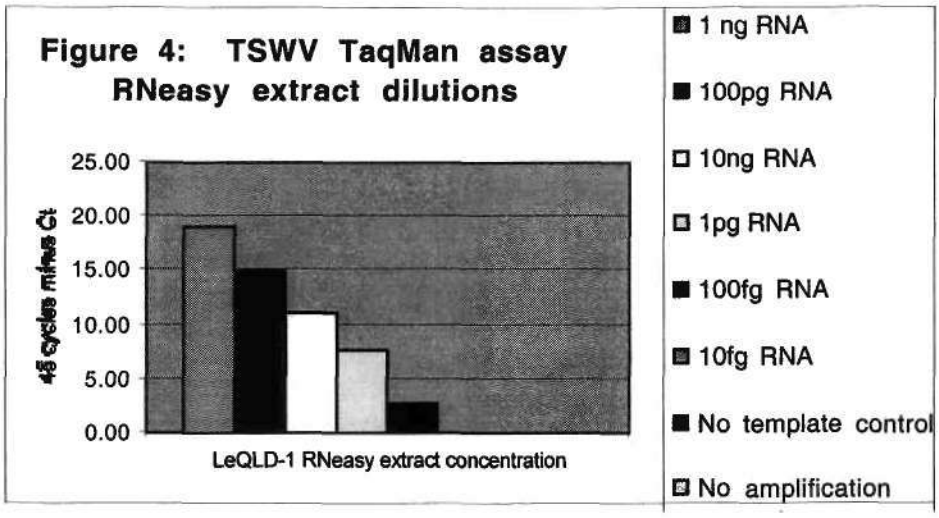
In order to develop an understanding of the epidemiology of TSWV and provide information necessary to improve control strategies, it is vital to be able to detect the virus reliably in all naturally-infected host crop species as well as in a representative range of important weed virus reservoir host species. *TaqMan* successfully detected TSWV in all of the >60 infected field-collected and glasshouse-maintained samples tested (Table 2). A range of horticultural and ornamental crops and some key weeds were successfully analysed by our TSWV *TaqMan* assay. Overall during this project, the *TaqMan* assay detected TSWV in samples (previously shown to contain TSWV by ELISA serological tests) of: artichoke, basil, capsicum, celery, chilli, eggplant, lettuce, parsley, potato, peanut, tomato (crops), anemone, aster, dahlia, iris, calendula, *Lisanthus*, ranunculus, zantedeschia (ornamentals), and malva, sowthistle, chickweed and paddy melon. Thus all samples positive for the virus by ELISA were also positive by *TaqMan* RT-qPCR confirming its reliability.

To test the reproducibility of the assay, RNeasy extracts from five samples containing varying amounts of TSWV, were analysed in duplicate in three independent *TaqMan* assays. Standard deviation of Ct values between replicates in the same test and across the three tests was less than 1 (Table 7).

Table 7: Reproducibility of *TaqMan* RT-PCR Ct values assaying 2ng total RNA from RNeasy extracts

	Test 1 [8/3/02]		**Test 2** [14/3/02]		**Test 3** [21/3/02]		
Isolate	*Rep1*	*Rep2*	*Rep1*	*Rep2*	*Rep1*	*Rep2*	**Mean**
SoWA-2	15.6	15.5	15.75	15.5	15.1	14.8	15.4±0.35
PaWA-1	32.5	31.8	31.6	32.2	33.3	33.3	32.5±0.66
BaWA-1	18.0	18.1	17.6	17.4	17.3	16.6	17.5±0.54
MaWA-1	22.4	22.8	24.0	24.3	22.8	23.1	23.2±0.75
CiWA-1	15.3	15.3	15.0	15.1	14.5	14.5	15.0±0.37

The assay detected TSWV across four to five orders of magnitude and TSWV was reproducibly detected in as little as 1 pg RNeasy-extracted total plant RNA (Fig. 4 and 7).



Note: Three replicates were tested for each dilution and controls; standard deviations were below 0.5 for all means, except 100fg (+/- 1.32) and do not show on the scale of this graphic representation.

Figure 5. TSWV *TaqMan* amplification plot of ZanTAS-7 – Comparison of RNeasy extracts and "leaf soak" extract dilutions

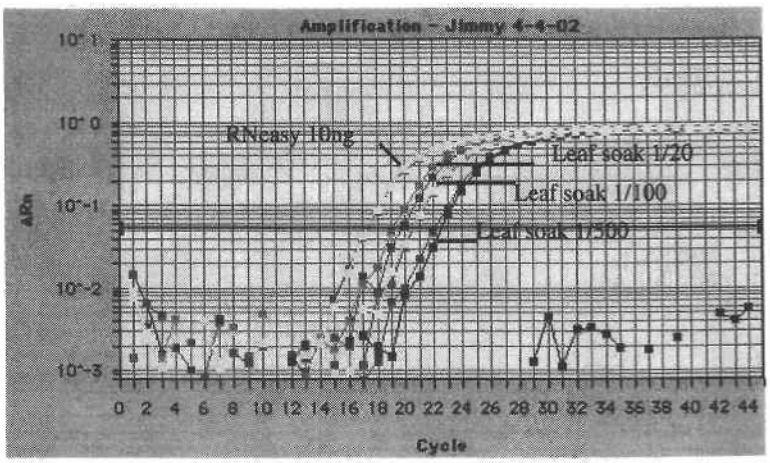


Figure 6. TSWV *TaqMan* amplification plot and standard curve of TSWV N gene RNA transcripts of known concentration

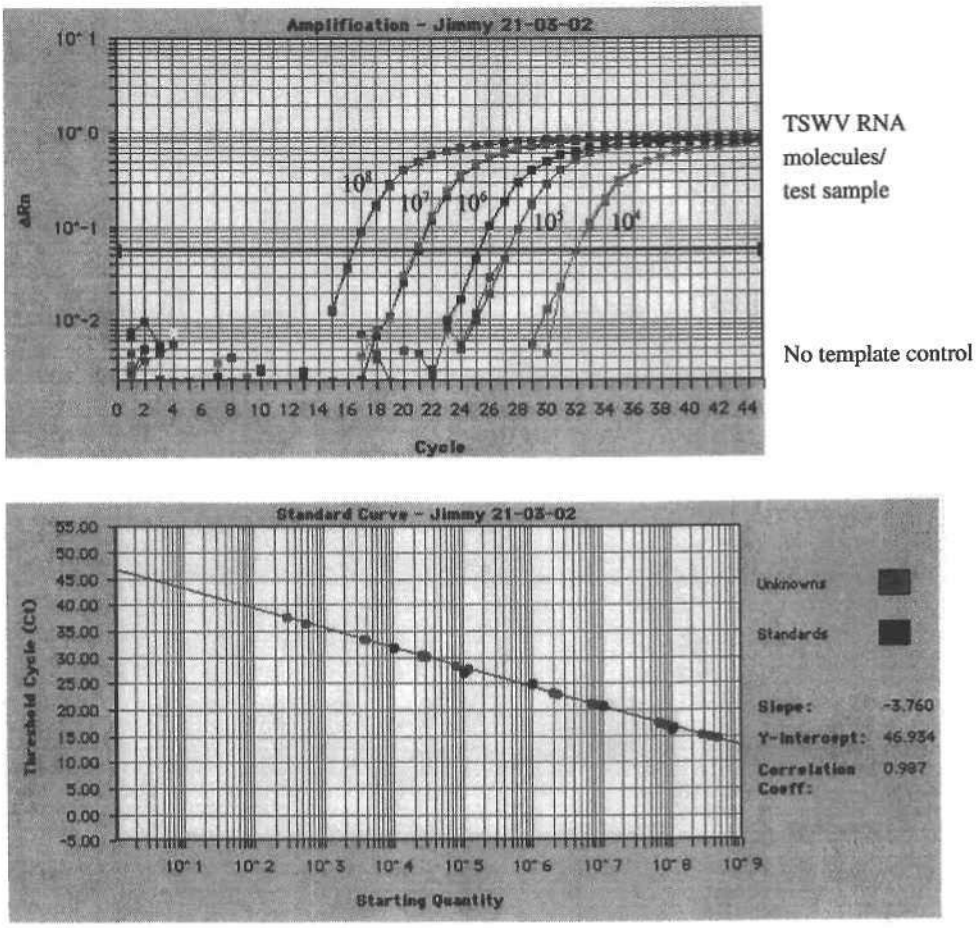
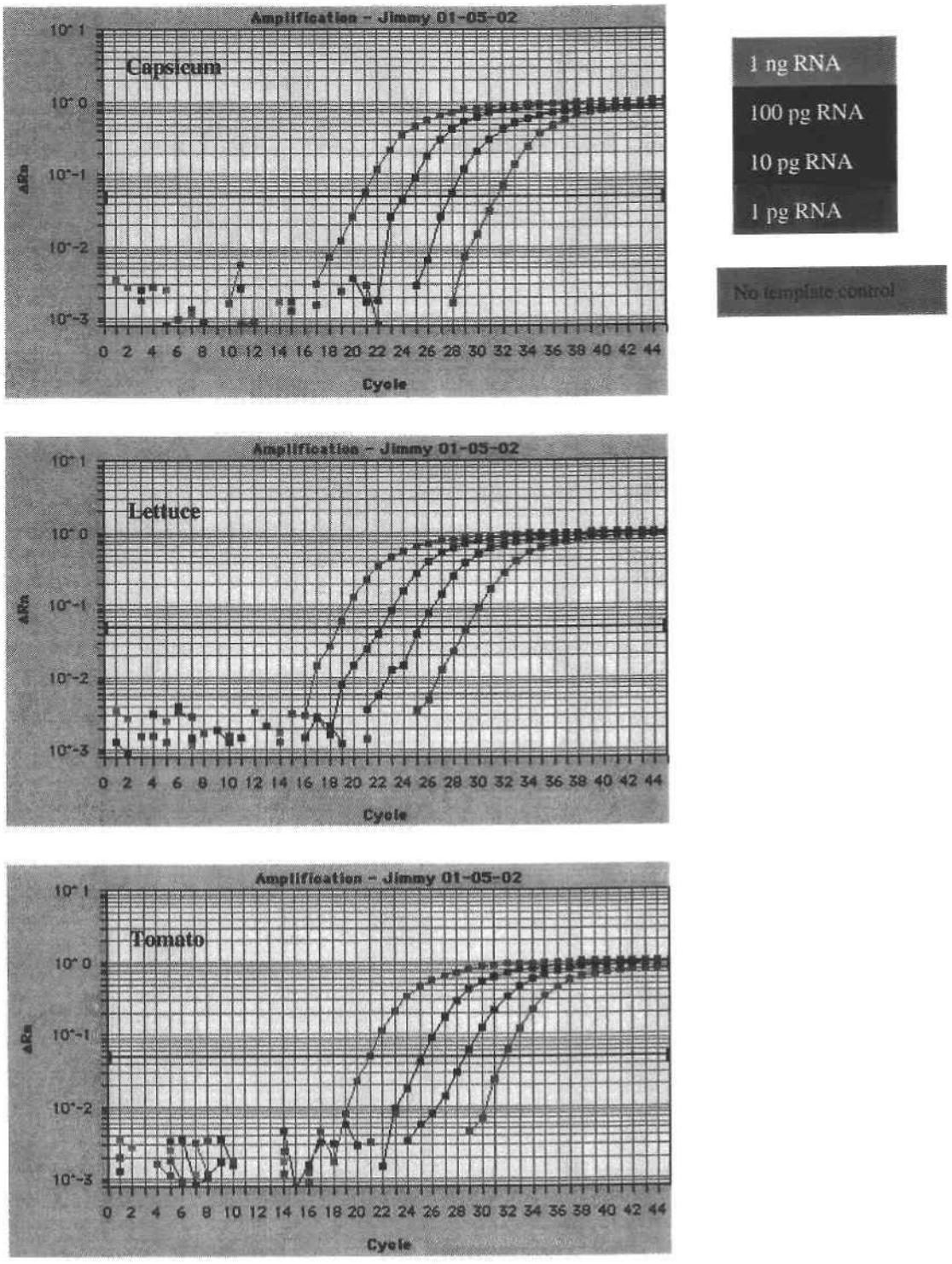


Figure 7. TSWV *TaqMan* amplification plots for three field samples
10-fold dilution series of RNeasy extracts

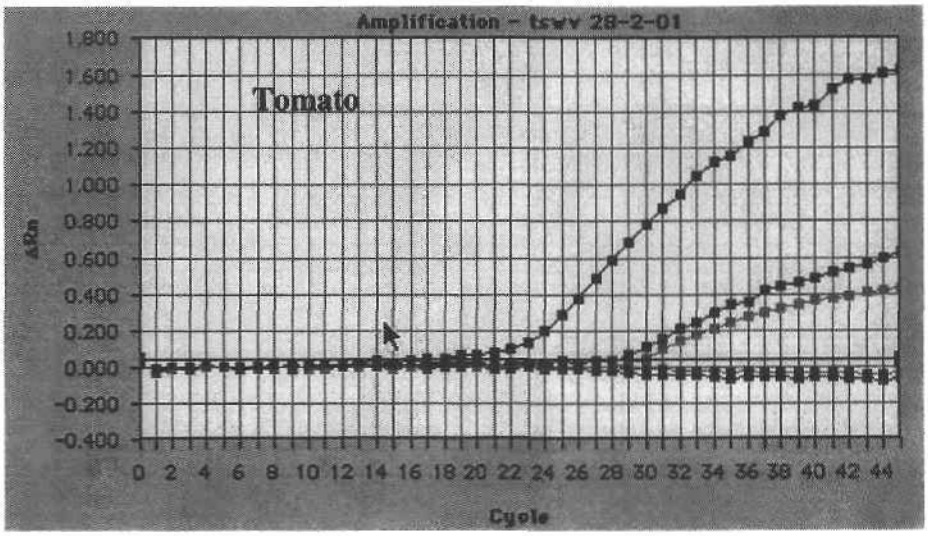


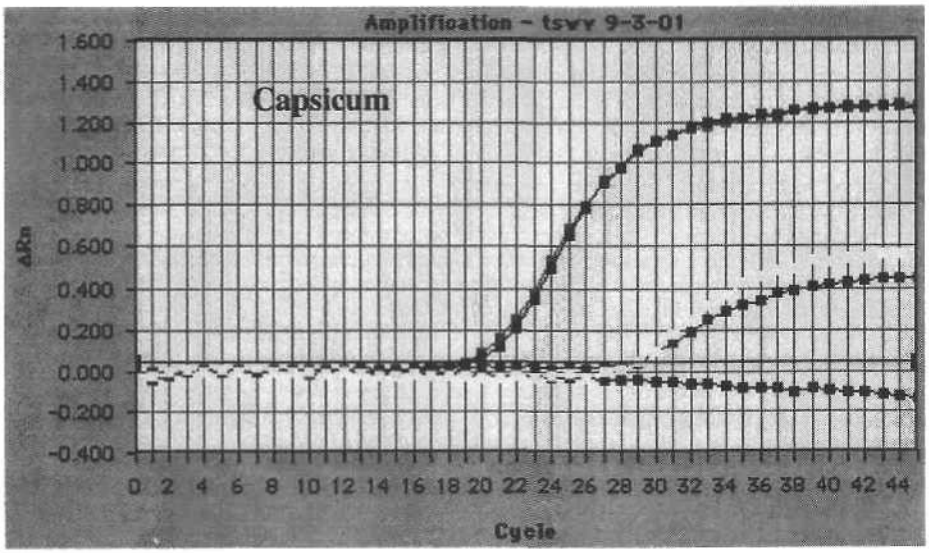
2.4 Assay of bulked samples

In order to establish the sensitivity of TSWV detection by *TaqMan* and compare this sensitivity with that of ELISA, leaves with TSWV symptoms and very large numbers (up to 2000 at a time) of asymptomatic leaves were collected on different properties at different times. Cork bore punch discs from single symptom-affected leaves were grouped with different proportions of cork bore punch discs from asymptomatic leaves so as to establish dilutions of 1/100 (both), 1/200 (ELISA only), 1/500 (both), 1/800 (ELISA only) and 1/1000 (both). These dilutions were each tested separately for TSWV presence by *TaqMan* (phenol-chloroform extraction of RNA) and ELISA. Thorough grinding and use of fresh samples were both essential for effective detection at high dilution. Presence of symptomless TSWV-infected leaves sometimes interfered with the early dilution assays as initially it was assumed that infected leaves would always show visible symptoms but this proved not always to be the case with some crops.

- In capsicum and tomato *TaqMan* detected TSWV at 1/1000, provided that leaves were fresh and leaf punch discs thoroughly ground, but only at 1/500 when they were not fresh. Inadequately ground leaf punch disc samples sometimes only gave positive results at up to 1/100. By ELISA, detection was up to a sensitivity of 1/800 with 2 capsicum samples, but only up to 1/100 with another sample apparently due to inadequate grinding. In tomatoes, by ELISA the maximum positive dilution result was 1/200 with samples that were fresh and well ground.
- In lettuce and potato samples from 1 site each, *TaqMan* detected TSWV at 1/1000 dilution. ELISA detected it at a dilution of up to 1/200 in lettuce (potato dilutions not tested by ELISA).
- In aster and *Lisianthus* samples, *TaqMan* gave positive detection results at 1/1000 dilution but these tests were compromised by symptomless infection in some asymptomatic leaves. In the same material, ELISA also gave positive detection up to 1/1000 with aster and *Lisianthus*. Thus, here the true limit of sensitivity could not be established with the material tested because of symptomless infection in field collected asymptomatic samples.

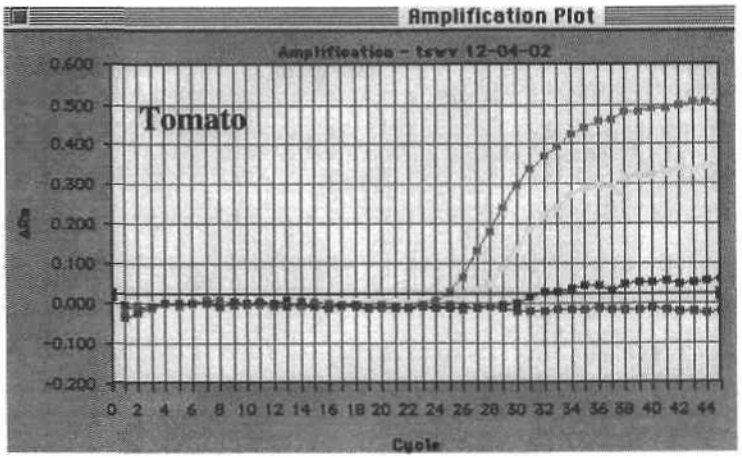
Figure 8. TSWV *TaqMan* amplification plots for bulked tomato and capsicum leaf samples collected on grower's properties in Western Australia.





Artificial bulk samples composed of 1 symptomatic leaf in 1000 asymptomatic leaves were extracted using the phenol-chloroform method and assayed for TSWV by qRT-PCR. Infection levels of 0.01% (ie. 1/1000) were detected when TSWV concentration in the infected tomato and capsicum leaf sample was high; optimal leaf punch position was found to be important (see Fig. 3). Bulked samples with lower TSWV amounts were only positive at 1/500 or 1/100 (not shown).

Figure 9. *TaqMan* amplification plots for bulked tomato, lettuce and capsicum leaf samples, extracted with Trizol® or combined leaf soak/RNeasy (LSR) protocols.

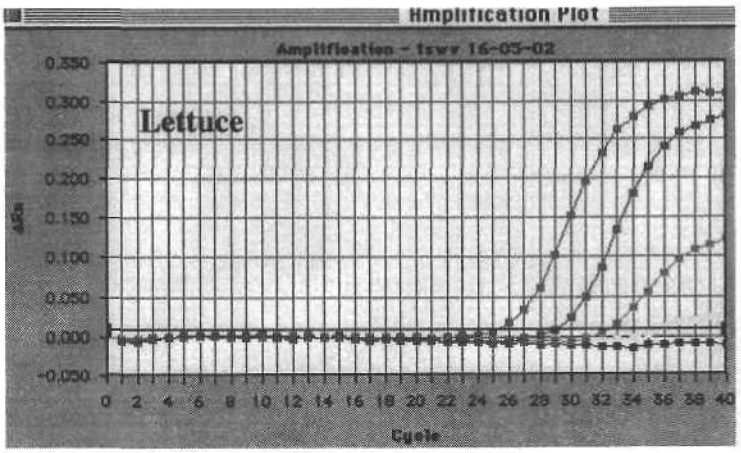


Trizol 1/1000 (1:10)

Trizol 1/1000 (1:100)

LSR 1/1000 (undiluted)

Trizol 1/1000 (undiluted)



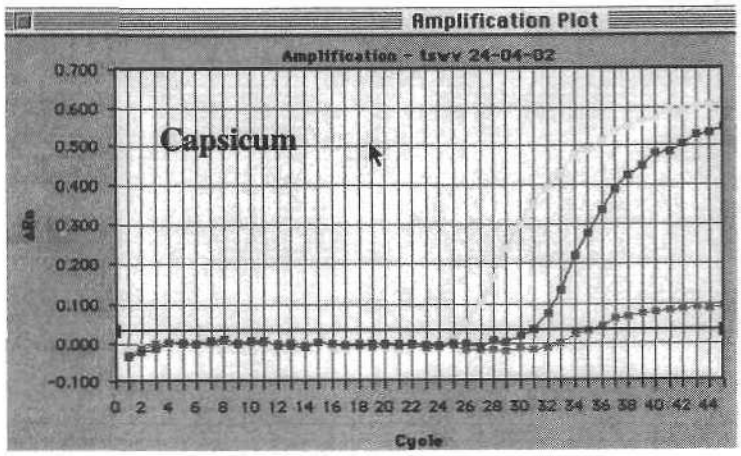
Trizol 1/1000 (1:10)

LSR 1/100 (undiluted)

LSR 1/500(undiluted)

LSR 1/1000 (undiluted)

LSR healthy (undiluted)



Trizol single leaf punch (undiluted)

Trizol 1/1000 (undiluted)

Trizol 1/1000 (1:100)

Extraction method – infected/uninfected leaves – (extract dilution for RT-PCR);
Note: Ct values of Trizol and LSR extracts not directly comparable, because different volumes of extract was processed for each protocol and independent assays for each crop.

Additional disks from TSWV-infected capsicum, lettuce and tomato leaves were tested in different sized bulks of healthy leaves mixed with single disks from known infected leaf samples. *TaqMan* with phenol-chloroform extraction reliably detected TSWV infections in leaf samples to a sensitivity of 1 infected leaf in 1000 uninfected leaves (higher dilutions were not tested). Instead of the phenol-chloroform extraction method used previously, two other extraction methods were also compared, the Trizol® method (which we used successfully to extract RNA from thrips) and a method combining "leaf soak" extracts followed by a commercial method using RNeasy RNA-binding columns.

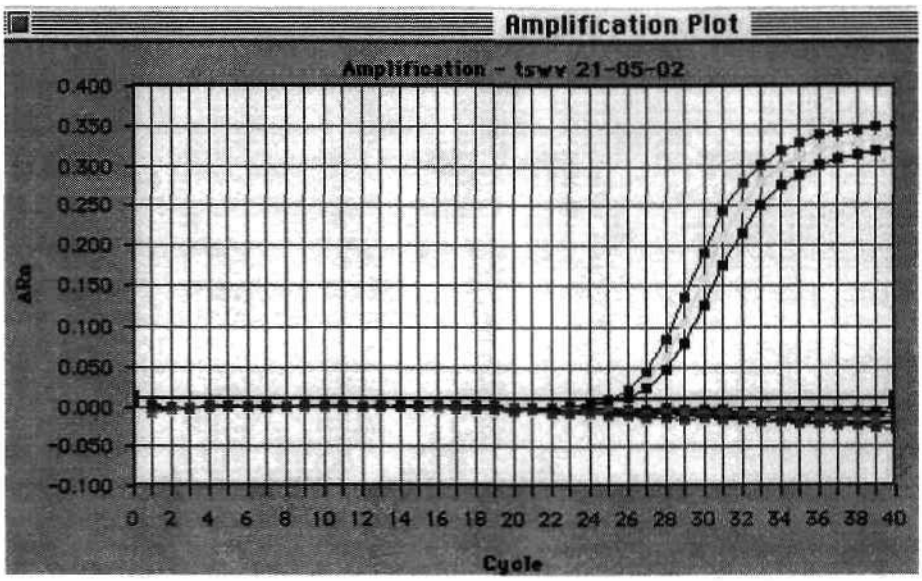
The combined leaf soak/RNeasy (LSR) method successfully detected TSWV infections in 1:1000 bulked lettuce and tomato leaf samples (Fig. 9), where infection levels (ie virus concentrations) in individual leaves were high. However, where the infection level within individual leaves was low, this extraction method had a maximum sensitivity of less than 1:100. This method, therefore, cannot be used to reliably detect TSWV in bulked leaf samples of capsicum. Thus, with capsicum, where PCR inhibitors appeared to build up, even where the infection level within individual leaves was high, the leaf soak/RNeasy method still only had a maximum sensitivity of approximately 1:100. Therefore, we cannot recommend this combined extraction method for routine diagnosis of TSWV in diverse samples.

Using the Trizol® extraction method *TaqMan* was able to detect TSWV infection reliably in bulked samples of leaf discs up to a level of 1:1000 in all samples tested, regardless of crop species (lettuce, tomato and capsicum) or the infection level within individual leaves (Fig. 9). A 1:10 dilution of Trizol extracts worked consistently best. However, this method has the drawback that it requires grinding of leaf disks in the phenol-based Trizol® reagent. As phenol is a hazardous reagent, it is not well suited to routine implementation in a diagnostic laboratory. It may be possible to grind the leaf punches in another aqueous buffer prior to treatment with the Trizol® method in an attempt to eliminate the more hazardous grinding in Trizol® reagent.

Seven commercial vegetable crops growing on properties in Western Australia with previous histories of TSWV infection were sampled by taking 100 shoots at random within the crop, and tested for TSWV by ELISA following grouping in batches of 10x10 leaves per crop. There were three lettuce, one capsicum and three tomato crops. TSWV was detected in one lettuce and the capsicum crop by ELISA at an estimated infection rate of 2% (capsicum) and 9% (lettuce). Twenty batches of 5 leaves were required for ELISA to detect the one infected tomato crop at 1% infection (Gibbs and Gower). *TaqMan* detected all 3 infected crops when 10 groups of 10 leaves were combined per

crop, extracted with Trizol® and assayed by *TaqMan* for the presence of TSWV(Fig 10). The other crops sampled tested negative by both ELISA and *TaqMan*.

Figure 10. *TaqMan* amplification plot of farmer's fields bulked leaf samples (10x10) extracted using the Trizol method.



Crop D: Capsicum (1:10)
Crop B: Lettuce (1:100)
Crop F: Tomato (1:10)

Crops A,C: Lettuce (1:10);
Crops E, G: Tomato (1:10)

2.5 Replacement of the 'probe' by SYBR Green

The most expensive component of the *TaqMan* diagnostic assay is the fluorescently labelled probe, which provides a second level of specificity, filtering out any potential non-specific PCR amplification products. A less expensive alternative is provided by the use of the SYBR Green reagent, which emits a fluorescent signal when intercalated into PCR amplicons (ie. dsDNA). Because of its non-specific action SYBR Green can potentially be used broadly for all qPCR reactions, independent of the target sequence.

We attempted to replace the TSWV, CaCV or WFT probes by SYBR Green in the *TaqMan* RT-qPCR assays we had developed. However, this only proved successful when a high concentration of viral RNA template was present. At lower concentrations, we observed considerable background fluorescence, which was due to SYBR Green detecting primer-dimer amplification products, rather than the target amplicons. This was confirmed by gel-based detection. Due to this fact, water or healthy controls, which were negative using the *TaqMan* probe, showed false-positive signals using SYBR Green. It appears that for successful use of SYBR Green in qPCR, new primers would need to be designed which will not give rise to any spurious primer-dimer products.

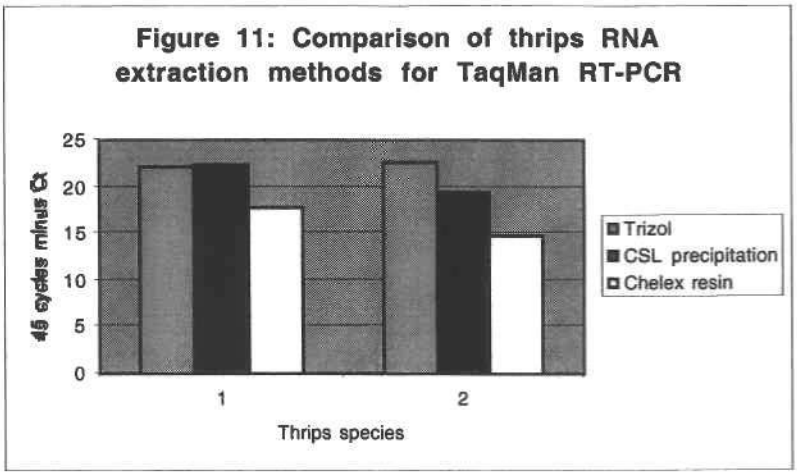
3. Development of a duplex RT-qPCR assay for detection of TSWV in thrips

In order to develop an understanding of the epidemiology of TSWV, it is important to be able to detect the virus in individual trapped thrips vectors as well as in infected plants. Reliable detection of TSWV in individual thrips is also important for forecasting TSWV epidemics. Standard RT-PCR lacks the sensitivity to detect TSWV in total RNA extracted from infected thrips. Using the real-time RT-qPCR assay, TSWV has been consistently detected in the two thrips species tested, Western Flower Thrips (*Frankliniella occidentalis*) and Tomato thrips (*F. schultzei*) collected in Western Australia and in Queensland.

Three RNA extraction methods were evaluated – Trizol® solution; a method adapted from one used for whiteflies; and a rapid but crude Chelex resin-based method with the potential for high throughput. Of these methods, Trizol® solution reproducibly yielded RNA free of PCR inhibitors from both thrips species, which was readily amplified by both *TaqMan* TSWV and WFT simplex and duplex assays (Fig. 11).

RNA extraction from single thrips was possible and yielded sufficient template for RT-PCR analysis. A duplex assay was developed using an internal control primer/probe set for thrips RNA (Fig. 12). This is particularly important with single thrips extractions to verify template presence and eliminate false negatives. *TaqMan* primer and probe sequences for detection of thrips RNA were provided to us, before publication, by Dr. Neil Boonham from the CSL. This thrips-specific *TaqMan* assay using primers and VIC-labelled probe designed from WFT sequences (Table 5) worked not only for the *Frankliniella* species *F. occidentalis* (WFT) and *F. schultzei* (tomato thrips), but also for the *Thrips* species *T. palmi* (melon thrips) and *T. tabaci* (onion thrips).

RNA was extracted from pools of five individuals of *F. schultzei* using the Trizol® method. Varying levels of TSWV were detected in such pooled extracts of dry specimens caught in sticky traps in WA (Fig. 13), indicating that 1) *TaqMan* is sufficiently sensitive to detect TSWV in pools of five individuals in which at least one individual per group carried the virus, and 2) that live thrips specimens are not a prerequisite to successful detection. Thus when trapping takes place in epidemiological studies, the trapped insects can be left for some time before virus testing to see if they are infective. The alternative of live trapping is much more labour intensive requiring many more visits to trapping sites.



Notes: Thrips species WFT (1) and *T. schultzei* (2); 100 pg RNA tested in duplicate using WFT primers and probe; extraction methods detailed in Material & Methods section.

Figure 12: Duplex *TaqMan* RT-PCR for detection of (A) WFT and (B) TSWV in Trizol-extracted RNA from virus-free and TSWV-carrying WFT using FAM and VIC reporter dyes

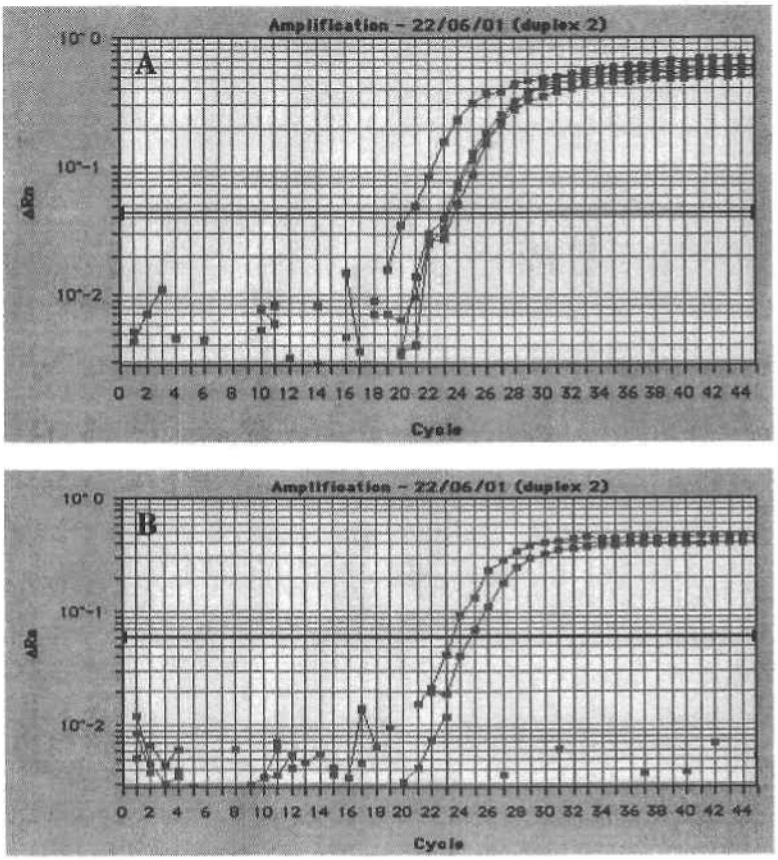
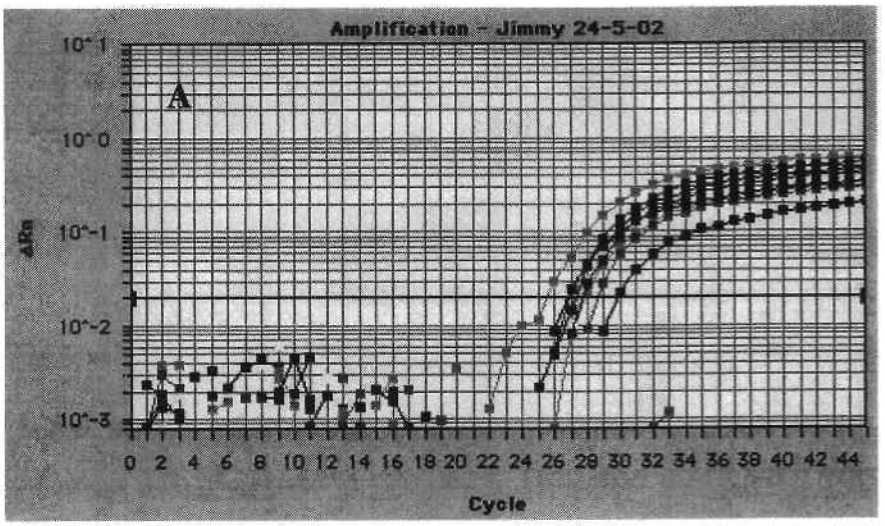
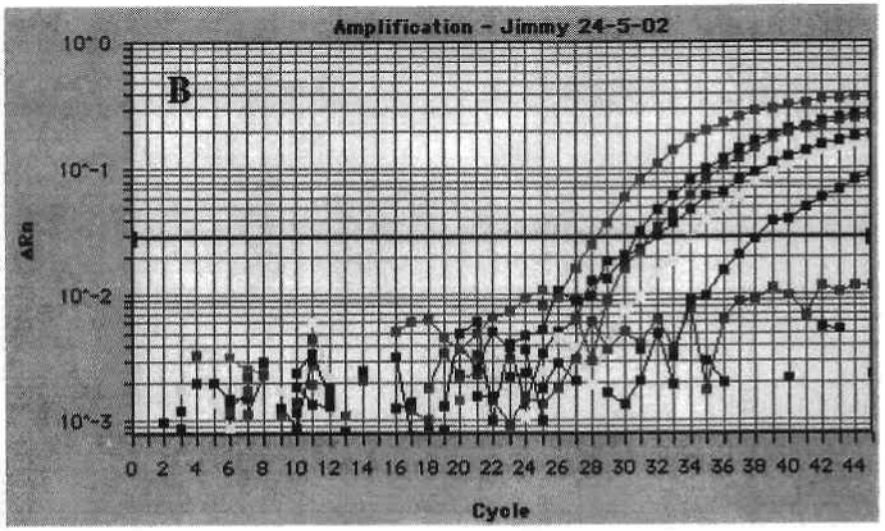


Figure 13: *TaqMan* duplex thrips (A) and TSWV (B) RT-PCR amplification plots of Trizol extracts (1ng RNA) from pools of 5 dry trapped *F. schultzei*



9/9 thrips pools positive;
Ct ~ 26-30

No template control



6/9 thrips pools carried TSWV
Ct ~ 28-38

3/9 thrips pools were virus-free
Ct ~ 45

4. Development of *TaqMan* RT-qPCR assays for detection of other tospoviruses

4.1 Design and use of degenerate primers in RT-qPCR

The best-known tospovirus causing losses to horticultural crops in Australia is TSWV. As mentioned above, worldwide there are at least 16 other biologically and genetically distinct tospoviruses, which belong to at least four different serogroups. These tospoviruses pose a threat to Australian horticultural industries if they become established here. Tests to detect them reliably are urgently needed. We have developed and applied to horticultural and ornamental crops and key weed species a *TaqMan* assay, which detects all Australian TSWV isolates tested. To detect more than one of the non-TSWV tospoviruses simultaneously in infected plant samples, a special general-purpose probe and primers are needed for the qPCR assay. We designed such "degenerate" primers and probes by targeting genome sequences, which are conserved across several tospovirus species.

The large (L) RNA of tospoviruses encodes an RNA-dependent RNA polymerase. The amino acid sequences of viral polymerases generally contain small conserved regions due to their common function in RNA replication. Therefore, a primer pair was designed from conserved motifs in the L RNA using published sequences of the type member of each of four tospovirus serogroups. Only limited regions of sequence conservation were found to exist between the different published sequences, so 'redundant' nucleotides were incorporated to design primers that fitted within the assay parameters for the *TaqMan* chemistry. Multiple sequence alignments of other tospovirus sequences in the small (S) and medium (M) RNAs were also done, but no suitably conserved regions for *TaqMan* primer design were identified. Several sets of degenerate primers for more or less broad spectrum detection of tospoviruses by standard RT-PCR have recently been described in the scientific literature.

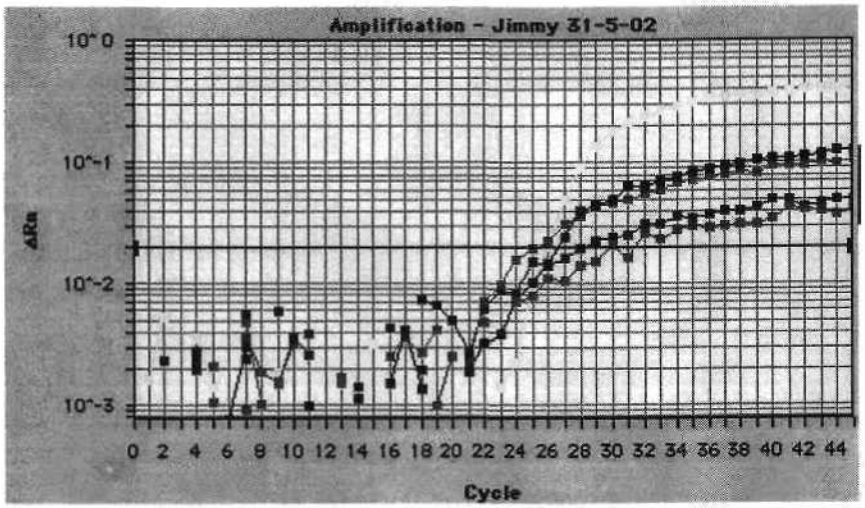
The degenerate primer pair readily detected several isolates of TSWV and capsicum chlorosis virus in standard RT-PCR with gel-based detection. However, in real-time RT-PCR, amplification using these degenerate primers was not successful in detecting any TSWV or CaCV isolate, due to high non-specific background fluorescence. It appears that *TaqMan* chemistry does not support such degenerate primers well. This is supported by information obtained from PE Applied Biosystems, the supplier of the *TaqMan* real-time PCR system, who subsequently advised us that degenerate primers are not recommended in this assay. Work towards establishing a general tospovirus *TaqMan*

assay has not been successful due to extensive variability between the known tospoviruses and the lack of highly conserved "signature sequences".

4.2 RT-qPCR for the detection of capsicum chlorosis virus

A *TaqMan* RT-qPCR assay was newly developed and applied successfully to tospovirus-infected capsicum leaf samples (Fig. 14). CaCV-specific primer and probe sequences are shown in Table 5. This assay detects the recently described capsicum chlorosis virus from Queensland, which belongs to tospovirus serogroup IV, and was designed to potentially also detect other confirmed serogroup IV tospoviruses. This aspect has not been assessed due to the lack of other serogroup IV tospoviruses in Australia and would best be tested overseas. The CaCV assay was quite sensitive, as CaCV could be detected in < 5pg of total RNA extracted from infected field-collected capsicum leaves.

Figure 14: ***TaqMan*** **amplification plot for detection of CaCV in capsicum RNeasy extracts**



4.3 Other tospoviruses in Australia

A so far undescribed tospovirus isolate was identified from a single tulip sample collected growing under "open quarantine" conditions in WA and received in September 2001. The sample tested negative for TSWV but positive for general tospovirus antibody by ELISA. TSWV-specific *TaqMan* assay of total RNA (RNeasy) of this sample also proved negative.

Two sets of degenerate tospovirus primers yielded RT-PCR products (Superscript One - step RT-PCR, BRL) from this sample using total RNA as template:

a) primers 1&2	(S RNA)	Dewey et al. (1996)
b) primers GL3637 & GL4510c	(L RNA)	Chu et al. (2001)

The DNA fragments were cloned into the vector pCR2.1Topo and sequenced.

The evidence for a novel tulip tospovirus is based on the following observations:

1) The specific primer sequences of 20-30 nucleotides at either end of the amplified fragments matched the conserved sequence of tospoviruses fully;
2) Two independent sets of degenerate primers in the S RNA and L RNA, respectively, led to a PCR product;
3) These PCR products were of the size expected from using these primers on a tospovirus sequence and the size was similar to the product obtained when using TSWV as positive control, but not from healthy plant controls;
4) Nucleotide sequence alignment of N gene sequences shows only 30-40% sequence identity between tospovirus species, the same as observed for the tulip N gene sequence.

No close similarity to IYSV or any other recognized tospovirus was apparent for this particular sample, although other tulip samples have given positive reactions with IYSV specific antiserum in ELISA (R.A.C. Jones, pers. communication).

TECHNOLOGY TRANSFER

The outputs of this project include diagnostic real time polymerase chain reaction (PCR) assays for the detection of TSWV and CaCV. The former assay was developed by QDPI before VG00025 and details have been published in the scientific literature. The latter will need to be tested further and validated to determine if it has a broader specificity for other serogroup IV tospoviruses (which would make it more useful); this could not be done within the project because no other viruses of this serogroup are known to occur in Australia.

The project aimed to determine if the advantages of real time PCR assays such as high sensitivity and high throughput could be applied to the detection of tospoviruses in horticultural and ornamental crops. It appears that even though real time PCR provides a more sensitive alternative to the currently used ELISA for single samples, PCR inhibitor problems preclude its use for bulk samples when a simple extraction method is used. More extensive RNA/DNA extraction methods for bulk samples are not user friendly due to the time involved and use of hazardous chemicals, but they do work very well for bulked samples in *TaqMan*. The development of a better RNA/DNA extraction method would significantly improve the large-scale applications of our *TaqMan* assay. For single samples, ELISA appears to be adequate and diagnostic laboratories would be unlikely to switch to the more expensive *TaqMan* assay for these.

During this project, the TSWV assay has been used by Saturn Biotech Ltd. in WA who already test commercially lupin seeds for the presence of CMV using *TaqMan*. They already use more extensive extraction methods successfully for large-scale routine assays and should be in a good position to include TSWV testing in their program. Saturn has indicated that they would be prepared to offer the *TaqMan* TSWV assay service commercially to industry. The assay protocols can also be made available to other diagnostic centres in Australia, such as the Northern Australian Diagnostic Network in Brisbane, Queensland. The South Australian R&D Institute and the Institute for Horticultural Development in Knoxfield, Victoria have indicated that they would not be able to provide such a service at this point.

As we understand the situation from discussions with PE Applied Biosystems, commercial applications of *Taqman* assays will be subject to the PCR patent, managed for agricultural applications by Applied Biosystems for Roche Pharmaceuticals. *TaqMan*™ chemistry is commercialised and patented by Applied Biosystems. Licensing costs for commercial applications of PCR (without any additional IP-protected sample

processing or detection technologies) are currently a one-off licence fee of US$10,000 plus 15% of the charge to customers for conducting each test, with the 15% charges in the first year deducted from the licence fee. A 5% additional charge for the use of *TaqMan* is likely to be added, bringing the *TaqMan* assay royalty fee to 20%. Research institutions and private companies, that want to use such PCR-based assays on a commercial basis, will need to negotiate appropriate licence agreements with Applied Biosystems.

Given the above, the tests will not be appropriate for commercial royalty-bearing licensing. Instead we will transfer the TSWV RT-qPCR assay i.e. provide information and know how to Saturn Biotech Ltd. and the Northern Australian Diagnostic Network and reach agreement with these organisations for them to include the assay as part of their normal portfolio. A simple press article aimed at growers and other users will be prepared highlighting the test and where it is available. This will be published in *Good Fruit and Vegetables* and two state magazines appropriate to horticultural/agricultural industries, which would benefit from being able to detect TSWV.

RECOMMENDATIONS

This project aimed to determine, if the advantages of real time PCR assays such as high sensitivity and high throughput could be applied to the routine detection of tospoviruses in horticultural and ornamental crops. It appears that even though real time PCR provides a more sensitive alternative to the currently used ELISA for single samples, PCR inhibitor problems preclude its use for bulk samples when a simple extraction method is used. More extensive RNA/DNA extraction methods for bulk samples are not user friendly due to the time involved and use of hazardous chemicals, but they do work very well for bulked samples in *TaqMan*. The development of a better RNA/DNA extraction method would significantly improve the large-scale applications of our *TaqMan* assay. For single samples, ELISA appears to be adequate and diagnostic laboratories would be unlikely to switch to the more expensive *TaqMan* assay for these. However, the *TaqMan* assay is an ideal tool for detection of TSWV in thrips vectors, non-symptomatic alternative plant hosts or quarantine import/exports, where highest sensitivity is essential to enable detection.

ACKNOWLEDGMENTS

We thank Lee McMichael (QDPI Indooroopilly) for making available the nucleotide sequence of CaCV S RNA prior to publication, Denis Persley (QDPI Indooroopilly) and Des McGrath (QDPI Bowen) for provision of plant specimens; Bronwyn Walsh (QDPI, Gatton) and David Cousins (AgWA) for thrips specimens; Calum and Annabel Wilson (University of Tasmania) for TSWV isolates. We also thank Don Maclean (UQ School of Molecular and Microbial Sciences) for insightful discussions and advice in real time PCR and his enduring friendship, and Bob Simpson for expert Real time PCR facility staff training. Neil Boonham generous provision of WFT internal control primer/probe sequences and advice prior to publication is gratefully acknowledged.

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PUBLICATIONS FROM THE PROJECT

Manuscript in preparation

Genetic variability of Australian *Tomato spotted wilt virus* isolates and validation of a real time PCR assay for their detection in plants and thrips vectors. (target journal: Annals of Applied Biology)

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Grower magazine item

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