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Epidemiology and genetic diversity of *Tobacco streak virus* and related subgroup 1 ilarviruses

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Abstract

A quarter of Australia's sunflower production is from the central highlands region of Queensland and is currently worth six million dollars ($AUD) annually. From the early 2000s a severe necrosis disorder of unknown aetiology was affecting large areas of sunflower crops in central Queensland, leading to annual losses of up to 20%. Other crops such as mung bean and cotton were also affected. This PhD study was undertaken to determine if the causal agent of the necrosis disorder was of viral origin and, if so, to characterise its genetic diversity, biology and disease cycle, and to develop effective control strategies.

The research described in this thesis identified *Tobacco streak virus* (TSV; genus *Ilarvirus*, family *Bromoviridae*) as the causal agent of the previously unidentified necrosis disorder of sunflower in central Queensland. TSV was also the cause of commonly found diseases in a range of other crops in the same region including cotton, chickpea and mung bean. This was the first report from Australia of natural field infections of TSV from these four crops.

TSV strains have previously been reported from other regions of Australia in several hosts based on serological and host range studies. In order to determine the relatedness of previously reported TSV strains with TSV from central Queensland, we characterised the genetic diversity of the known TSV strains from Australia. We identified two genetically distinct TSV strains from central Queensland and named them based on their major alternative hosts, TSV-parthenium from *Parthenium hysterophorus* and TSV-crownbeard from *Verbesina encelioides*. They share only 81 % total-genome nucleotide sequence identity. In addition to TSV-parthenium and TSV-crownbeard from central Queensland, we also described the complete genomes of two other ilarvirus species. This proved that previously reported TSV strains, TSV-S isolated from strawberry and TSV-Ag from *Ageratum houstonianum*, were actually the first record of *Strawberry necrotic shock virus* from Australia, and a new subgroup 1 ilarvirus, Ageratum latent virus. Our results confirmed that the TSV strains found in central Queensland were not related to previously described strains from Australia and may represent new incursions. This is the first report of the genetic diversity within subgroup 1 ilarviruses from Australia.

Based on field observations we hypothesised that parthenium and crownbeard were acting as symptomless hosts of TSV-parthenium and TSV-crownbeard, respectively. We developed strain-specific multiplex PCRs for the three RNA segments to accurately characterise the range of naturally infected hosts across central Queensland. Results described in this thesis show compelling evidence that parthenium and crownbeard are the major (symptomless) alternative hosts of TSV-

parthenium and TSV-crownbeard. While both TSV strains had wide natural host ranges, the geographical distribution of each strain was closely associated with the respective distribution of their major alternative hosts. Both TSV strains were commonly found across large areas of central Queensland, but we only found strong evidence for the TSV-parthenium strain being associated with major disease outbreaks in nearby crops.

The findings from this study demonstrate that both TSV-parthenium and TSV-crownbeard have similar life cycles but some critical differences. We found both TSV strains to be highly seed transmitted from their respective major alternative hosts from naturally infected mother plants and survived in seed for more than 2 years. We conclusively demonstrated that both TSV strains were readily transmitted via virus-infected pollen taken from the major alternative hosts. This transmission was facilitated by the most commonly collected thrips species, *Frankliniella schultzei* and *Microcephalothrips abdominalis*. These results illustrate the importance of seed transmission and efficient thrips vector species for the effective survival of these TSV strains in an often harsh environment and enables the rapid development of TSV disease epidemics in surrounding crops.

Results from field surveys and inoculation tests indicate that parthenium is a poor host of TSV-crownbeard. By contrast, crownbeard was naturally infected by, and an experimental host of TSV-parthenium. However, this infection combination resulted in non-viable crownbeard seed. These differences appear to be an effective biological barrier that largely restricts these two TSV strains to their respective major alternative hosts.

Based on our field observations we hypothesised that there were differences in relative tolerance to TSV infection between different sunflower hybrids and that seasonal variation in disease levels was related to rainfall in the critical early crop stage. Results from our field trials conducted over multiple years conclusively demonstrated significant differences in tolerance to natural infections of TSV-parthenium in a wide range of sunflower hybrids. Glasshouse tests indicate the resistance to TSV-parthenium identified in the sunflower hybrids is also likely to be effective against TSV-crownbeard. We found a significant negative association between TSV disease incidence in sunflowers and accumulated rainfall in the months of March and April with increasing rainfall resulting in reduced levels of disease. Our results indicate that the use of tolerant sunflower germplasm will be a critical strategy to minimise the risk of TSV epidemics in sunflower.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-review papers (published)

Sharman M, Thomas JE, Persley DM (2008) First report of *Tobacco streak virus* in sunflower (*Helianthus annuus*), cotton (*Gossypium hirsutum*), chickpea (*Cicer arietinum*) and mung bean (*Vigna radiata*) in Australia. *Australasian Plant Disease Notes* 3: 27-29. (Included as chapter 2)

Sharman M, Persley DM, Thomas JE (2009) Distribution in Australia and seed transmission of *Tobacco streak virus* in *Parthenium hysterophorus*. *Plant Disease* 93, 708-712. (Included as chapter 3)

Sharman M, Constable F, Perera R, Thomas JE (2011) First report of *Strawberry necrotic shock virus* infecting strawberry (*Fragaria vesca*) from Australia. *Australasian Plant Disease Notes* 6: 54-56. (Included as chapter 4)

Sharman M, Thomas JE (2013) Genetic diversity of subgroup 1 ilarviruses from eastern Australia. *Archives of Virology* 158: 1637-1647. (Included as chapter 5)

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Conference abstracts

Sharman M (2008) Tobacco streak virus in grain and pulse crops in Queensland. Proceedings of the 2008 GRDC Grains Research Update. 20 August, Dalby, Australia.

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Sharman M, Persley DM, Thomas JE (2009) Thrips and seed transmission, and the epidemiology of *Tobacco streak ilarvirus* in Queensland, Australia. Proceedings of the 9th International Symposium on Thysanoptera and Tospoviruses, 31st August – 4th September, Gold Coast, Australia.

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Sharman M, Persley DM, Thomas JE (2010) Epidemiology of *Tobacco streak virus* in Queensland, Australia. Australian Summer Grains Conference. 21-24th June, Gold Coast, Australia.

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Other Industry publications

Sharman M (2014) TSV tolerance in sunflower hybrids and reduced underlying risk in CQ. Better Sunflower e-Newsletter. March 2014.

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Chapter 2

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Contributions by others to the thesis

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Statement of parts of the thesis submitted to qualify for the award of another degree

None.

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Keywords

Seed transmission, thrips, pollen, *Strawberry necrotic shock virus*, Ageratum latent virus, *Parthenium hysterophorus*, control, resistance, ilarvirus

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Abbreviations

bp	base pair
°C	degrees celcius
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
g	gram
ha	hectare
m	metre
min	minute
mM	milli molar
M	molar
MP-PCR	multiplex polymerase chain reaction
nM	nano molar
nt	nucleotide
ORF	open reading frame
pmoles	pico moles
PCR	polymerase chain reaction
RT-PCR	reverse transcription polymerase chain reaction
RNA	ribonucleic acid
s	second
U	units
µl	microliter
µM	micro-molar

Chapter 1

Introduction and Literature Review

Contents

Introduction

Viruses possess some of the properties of living organisms, such as having a genome which is able to evolve and adapt to changing environmental conditions. However, viruses rely on the cellular machinery of their host cells to replicate (Van Regenmortel et al. 2000). Plant viruses have been evolving with endemic plant communities probably since plants have existed and in such situations they generally do not cause damaging virus epidemics (Jones 2009). However, the domestication of plants for food production has resulted in extensive global movement of plant germplasm and the accidental movement of plant viruses. This has resulted in many new interactions between plant viruses and plant species that have not co-evolved, often leading to severe epidemics (Jones 2009; Strange and Scott 2005). While the combined effect of the major plant pathogens (viruses, bacteria, fungi, nematodes and parasitic plants) reduces global food production by more than 10 % (Strange and Scott 2005), it is thought that the relative effect of viruses is greatly underestimated due to their insidious nature and difficulties related to their study (Hull 2014).

Approximately 33,000 ha of sunflowers are grown annually in Australia with almost all production occurring in the States of Queensland and New South Wales (Anonymous 2014). Significant areas of sunflowers have been grown in the central highlands region of central Queensland since the 1970s. In the mid-2000s there were reports of a serious necrosis disorder of sunflowers in this region. Initial testing indicated that *Tobacco streak virus* (TSV) was associated with the disorder (Reppel 2007).

TSV can affect a wide range of grain, vegetable and horticultural crops. The first report of TSV in Australia came from south-east Queensland (Greber 1971). It was later found to be the cause of a disease in tobacco in same region (Finlay 1974) and disease epidemics were found to be closely associated with the major alternative host, *Ageratum houstonianum* (Ageratum or Blue billygoat weed; Greber et al. 1991b) which is common along the eastern seaboard of Australia. Symptoms, serology and thrips transmission were reported for two further strains of TSV from Australia, affecting strawberries and an ornamental plant, *Ajuga reptans* (Greber 1979; Shukla and Gough 1983; Klose et al. 1996; Sdoodee 1989). However, prior to this PhD study, no molecular genetic information was available for TSV strains from Australia and it was unknown if the TSV affecting sunflower crops from inland central Queensland was related to any of the three other strains previously reported from Australia. Given sunflowers have been grown in this region during the same period that TSV has been reported from other regions but no disease was reported from sunflower until the mid-2000s, it was important to determine the genetic diversity of TSV strains

from Australia. This would clarify which TSV strain was affecting sunflowers, and enable investigations into how this strain was surviving in a very different environment to the previously reported TSV strains and which management options may be effective for its control.

Virus properties

Physical and molecular structure of Ilarviruses

Tobacco streak virus (TSV) is the type member of the genus *Ilarvirus* (family: *Bromoviridae*) which have a positive sense single stranded RNA genome with a total length of approximately 8,600 nucleotides (nt), divided into 3 linear segments designated as RNA-1, -2 and -3. The 1a (viral replicase) protein is encoded by RNA-1, the 2a (RNA-dependent RNA polymerase) protein and the 2b protein are encoded by RNA-2. Two proteins are encoded by RNA-3, the 3a cell-to-cell movement protein and the 3b virus coat protein. The coat protein is translated from a sub-genomic RNA-4 which is derived from RNA-3. The first complete nucleotide sequence of RNA 3 for a strain of TSV was reported in 1984 (Cornelissen et al. 1984) with a total length of 2,205 nt. The complete RNA 1 and RNA 2 sequences were later reported for the same strain with total lengths of 3,491 nt and 2,926 nt respectively (Scott et al. 1998). All RNA segments are encapsidated separately in quasi-isometric to bacilliform virions about 30 nm in diameter and 20 to 55 nm in length (Fauquet et al. 2005).

Ilarvirus species characteristics, phylogenetics and demarcation

The demarcation criteria for ilarvirus species are a combination of serological relatedness, host range and genome sequence similarity (King et al. 2012), although there is currently no formal specified level of sequence similarity to distinguish species. Ilarvirus species displaying serological relatedness were originally assigned to subgroups and genome sequence data have generally confirmed these groupings. There are currently 19 recognised species across 4 subgroups, the majority of which infect woody plants (King et al. 2012; Matic et al. 2008).

Within the genus, the putative 2b protein encoded on RNA 2 is unique to species in Subgroups 1 and 2. The homology of this 2b protein to a similar protein in cucumoviruses indicated it may also be involved in viral movement (Lucy et al. 2000) and it has recently been demonstrated that for the ilarvirus *Asparagus virus 2* (AV-2) it acts as an RNA silencing suppressor (Shimura et al. 2013). Recent phylogenetic studies on ilarvirus members have suggested greater emphasis on sequence

homology to RNA 3 coding regions (Codoñer et al. 2005), RNA 1 and RNA 2 sequence (Boulila 2009) or whole proteome (all putative proteins) analysis (Codoñer and Elena 2006) for grouping species.

The genus type member, TSV, was first reported in 1936 (Johnson 1936) and was the only described species within subgroup 1 until the early 2000s when the identification and classification of ilarvirus species rapidly changed with the development of molecular characterisation and genome sequencing. TSV, *Parietaria mottle virus* (PMoV), *Strawberry necrotic shock virus* (SNSV) and *Blackberry chlorotic ringspot virus* (BCRV) are recognised species within Subgroup 1 (ICTV 2013). Two tentative species which are also likely to be Subgroup 1 members are Bacopa chlorosis virus and Tomato necrotic spot virus (King et al. 2012).

Molecular diagnostic tools have also thrown some doubt on the true identity of viruses that were reported in the past. For example, a virus of strawberry causing a necrotic shock reaction when grafted onto susceptible indicator species, was first reported in 1957 (Jorgensen 1957) and the name Strawberry necrotic shock virus was proposed in 1966 (Frazier 1966). However, when later work indicated a serological relationship with TSV, it was assumed SNSV was synonymous with TSV which was then reported as the causal agent of the disease in strawberry (Greber 1979; Johnson et al. 1984; Spiegel and Cohen 1985; Stace-Smith and Frazier 1971; Stenger et al. 1987). It was not until 2004 that sequence data of RNA 3 confirmed that SNSV was a distinct ilarvirus species (Tzanetakis et al. 2004). One reference isolate from Frazier's earlier work and an isolate of "Black raspberry latent virus", BRLV (Converse and Lister 1969; Jones and Mayo 1975) were confirmed as SNSV (Tzanetakis et al. 2004). However, the true identity of other viruses that were reported as TSV from strawberry may remain uncertain without molecular characterisation of the original isolates. This is further complicated by the fact that while SNSV may be the most frequently found ilarvirus in *Rubus* and *Fragaria*, TSV has also been confirmed in strawberry by PCR and partial genome sequence (Tzanetakis et al. 2010).

A recent review of the molecular biology of ilarviruses reported the coat protein sequences for TSV strains to be highly conserved (Pallas et al. 2013) even though TSV has been reported as the causal agent for major disease outbreaks in a diverse range of crops from a number of countries, including oilseed and pulse crops in India (Prasada Rao et al. 2000; Reddy et al. 2002), and in soybean in Brazil (Almeida et al. 2005) and the United States of America (Rabedeaux et al. 2005). In contrast, after confirming that SNSV was distinct from TSV, Tzanetakis et al. (2004) suggested that what

was thought to be a group of highly heterogeneous isolates of TSV may actually represent a number of distinct species.

Ilarvirus diversity in Australia

Several ilarvirus species have been reported from Australia including *Asparagus virus 2* (AV-2; Shimura et al. 2013), *Prune dwarf virus* (PDV; Greber et al. 1992), *Prunus necrotic ringspot virus* (PNRSV; Munro 1987), *Apple mosaic virus* (ApMV; Crowle et al. 2003), Sunflower ringspot virus (SRSV: Dale and Behncken 1981; Thomas et al. 1988) and TSV (Greber 1971). SRSV has only been reported from Australia while the other ilarviruses are common in many other countries. The record of AV-2 by Shimura et al. (2013) is the first report of this virus from Australia and was determined by testing imported asparagus samples collected from markets in Japan. There is partial genome sequence for three of the ilarviruses present in Australia including numerous sequences for isolates of ApMV (Crowle et al. 2003), for one isolate of PNRSV (Sala-Rejczak and Paduch-Cichal 2013) and for one isolate of AV-2 (Shimura et al. 2013). Figure 1.1. illustrates the coat protein relationship between various ilarviruses recorded from Australia and representative members of all subgroup 1 species. Reports of differing strains of TSV (Greber 1979; Klose et al. 1996; Sdoodee 1989) suggest there is significant diversity in TSV strains present in Australia but no molecular information to confirm phylogenetic relatedness was available.

Transmission of multipartite genomes and recombination leading to diversity

Like many other plant viruses with segmented genomes, the multipartite genome of TSV is packaged separately into virions and the successful transmission of the virus into a new host can only be achieved when a set of viral particles containing the entire genome infects the same host cell (Roossinck 2002). This increase in complexity for successful transmission of multipartite genomes compared to the transmission of one single large genome appears to represent a biological cost. However, it has been proposed that the multipartite genome has evolved to favour genetic exchange by reassortment (Chao 1988, 1991; Pressing and Reanney 1984). This increase in genetic exchange would counter the loss of fitness resulting from the accumulation of deleterious mutations which occur due to the high error rates during RNA replication (Drake 1993; Holland et al. 1982; Lin et al. 2004). The relatively high genetic diversity displayed by many RNA viruses, such as the members of the family *Bromoviridae*, is partly as a result of the high error rates during RNA replication.

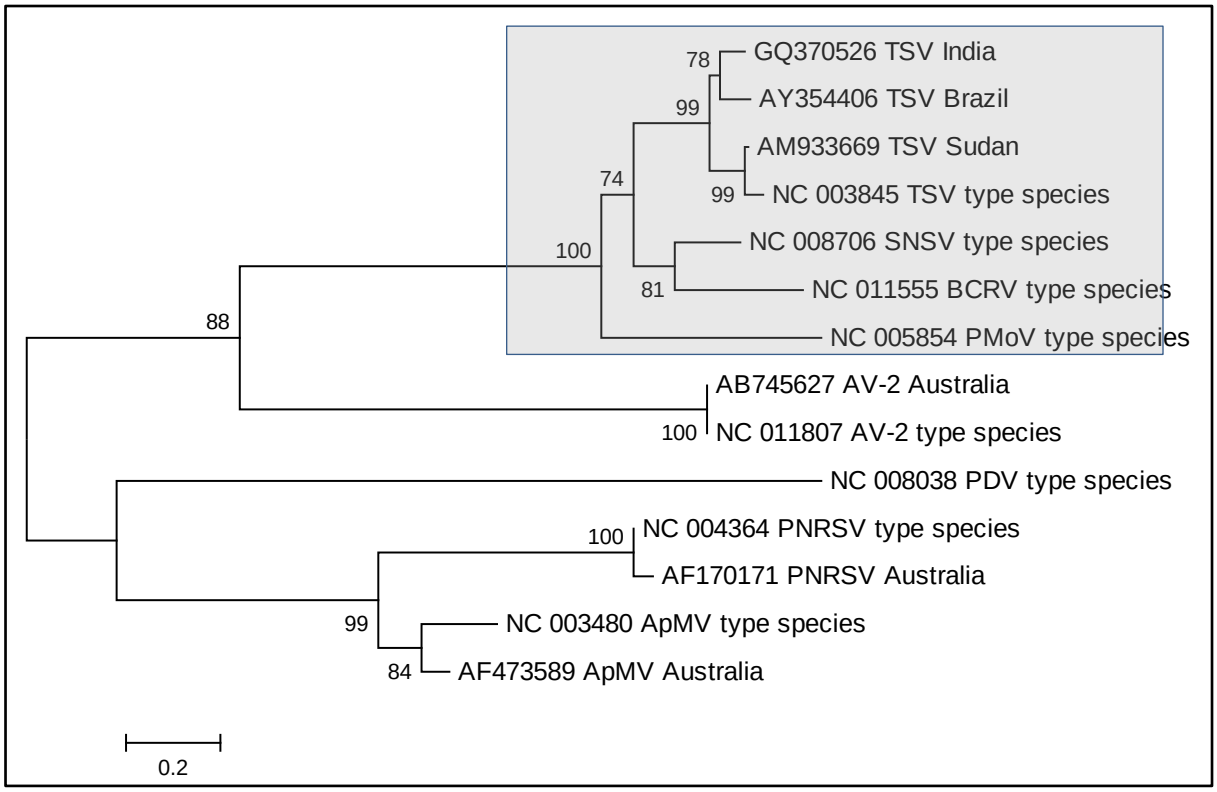

Fig. 1.1. Maximum Likelihood dendrogram, based on WAG parameter model, illustrating coat protein gene amino acid sequence relationships of ilarvirus species recorded in Australia for which sequence is available, and their respective type species, as listed in King et al. (2012). Additionally, representative members of all subgroup 1 species are shown within the grey box (TSV, SNSV, BCRV and PMoV) and TSV isolates from other countries. A discrete Gamma distribution was used to model evolutionary rate differences among sites. Horizontal distances are proportional to amino acid substitutions per site, and the dendrogram was bootstrapped 500 times with branch values greater than 50 % indicated. The scale bar represents the number of amino acid substitutions per site. GenBank accession numbers are shown on the dendrogram and abbreviations are: TSV (*Tobacco streak virus*), SNSV (*Strawberry necrotic shock virus*), BCRV (*Blackberry chlorotic ringspot virus*), AV-2 (*Asparagus virus 2*), PDV (*Prune dwarf virus*), PNRSV (*Prunus necrotic ringspot virus*), and ApMV (*Apple mosaic virus*).

Genetic exchange by recombination and reassortment can lead to an increase in variation within virus species and even evolution of new strains and species. The natural mixing of strains of *Cucumber mosaic virus* (CMV; family *Bromoviridae*) has been reported and can result in reassortants that display some characteristics of both parent strains (Lin et al. 2004; Maoka et al. 2010). This propensity for reassortment of CMV RNA segments is believed to be part of the reason that CMV has had such evolutionary success and such a wide host range (Roossinck 2002). While there have been further studies investigating possible recombination and reassortment between

different virus species of the family *Bromoviridae* (Boulila 2009; Codoñer et al. 2005; Codoner and Elena 2008), the possible genetic exchange between strains of TSV has not been reported.

Diseases caused by TSV

Summary of TSV disease cycle

TSV has an unusual disease cycle which requires at least two critical factors: (a) an abiotic (wind) or biotic (insect) process to move infected pollen from a flowering source to a healthy plant (i.e. non-infected with TSV); and (b) thrips with a feeding habit that creates feeding wounds suitable for pollen to enter and release virions into the healthy plant cells. Some strains of TSV are also seed transmitted in some hosts, which enables the virus to survive between seasons and crops and can also facilitate long distance dispersal. Various aspects of the disease cycle of TSV are discussed in greater detail in the following sections. The use of the term "healthy" in this thesis implies non-infected with TSV.

Geographic distribution

The identification of various plant species as hosts of TSV has been done using one or more diagnostic methods such as serological reactions, differential host range reactions, polymerase chain reaction (PCR, using TSV-specific primers) or partial genome sequencing, or a combination of these methods. Scott (2001) summarised wide natural and experimental host ranges for TSV from many regions of the world. However, the validity of these host lists should now be viewed in light of recent molecular characterisation of ilarviruses as illustrated above with the example of some previously reported strains of TSV now being confirmed to be SNSV or BRLV (Tzanetakis et al. 2004).

TSV has a global distribution with reports from almost all continents. The following are a selection of reports from different regions. In Europe and Western Asia, it has been reported from the United Kingdom (Brunt 1968), The Netherlands (Dijkstra 1983), Yugoslavia (Rana et al. 1987), Israel (Spiegel and Cohen 1985) and Iran (Abtahi and Koohi Habibi 2008). It has spread extensively through the Indian subcontinent (Ahmed et al. 2003; Kumar et al. 2008; Prasada Rao et al. 2000). In Africa it has been reported from Sudan (Ali et al. 2009) and South Africa (Cook et al. 1999). It has been reported in several States within the United States of America (USA; Cornelissen et al. 1984; Fulton 1948; Johnson 1936) and in Brazil (Costa and Carvalho 1961), Argentina (Gracia and

Feldman 1974) and Peru (Salazar et al. 1982) in south America. In the Australasian region it occurs in Australia (Greber 1971) and New Zealand (Pappu et al. 2008).

Host range of TSV strains from different regions of the world

While TSV has been reported to cause crop diseases from many countries since it was first described in the mid-1930s, the most recent and perhaps most severe diseases have been reported from India since the early 2000s where TSV has been identified as the cause of widespread crop losses in many regions. Although disease levels fluctuate from season to season, actual losses of 5-70 % are reported from sunflower (Sujatha 2006) and a single epidemic of TSV in groundnut during 2000 reportedly caused crop losses of greater than $ AUD 60 million (Kumar et al. 2008). Diseases caused by TSV in India have been reported in at least 18 different field crops with some of the most severely affected including; sunflower, peanut, okra and cucurbits (Bhat et al. 2002a; Bhat et al. 2002c; Prasada Rao et al. 2000; Kumar et al. 2008; Vemana and Jain 2010). At least 16 naturally infected alternative weed hosts have also been identified as TSV hosts in India (Kumar et al. 2008; Prasada Rao et al. 2003a; Prasada Rao et al. 2003b). Weeds found near crops with the highest incidence of TSV were *Ageratum conyzoides*, *Corchorus trilocularis* and *Parthenium hysterophorus* (hereafter referred to as parthenium). Parthenium was a non-symptomatic host and was considered the principal source of TSV-infected pollen leading to disease epidemics in nearby crops (Prasada Rao et al. 2003a).

Properties of **Parthenium hysterophorus** *as a host of TSV*

Parthenium is thought to be endemic to the region surrounding the Gulf of Mexico and/or central South America. Outside of its natural range it can be a highly invasive species and due to human activities, it has spread extensively during the 20th century to many tropical and subtropical regions of the world, including across vast regions of Indian and Pakistan (Kohli et al. 2006; Adkins and Shabbir 2014; Shabbir et al. 2012). There may have been multiple incursions of parthenium into India with the first occurring perhaps as early as 1810 and then again in the 1950s as a contaminant of grain imports from the USA. It appears to have remained uncommon in most regions prior to 1955 after which it spread rapidly throughout most agricultural regions of the country (Kohli et al. 2006; Lakshmi and Srinivas 2007). It can form dense and extensive stands and studies in India have indicated the potential for large quantities of airborne pollen to be moved significant distances (Kanchan and Jayachandra 1980; Mishra et al. 2002; Satheeshkumar and Vittal 1998), characteristics that also make it an ideal alternative host of TSV.

In Australia, the geographical range of parthenium currently covers an extensive region of central Queensland (Evans 1997; Navie et al. 1996; Towers et al. 1977). At least two unintentional introductions of parthenium occurred into Australia. The first was near Toogoolawah, south-east Queensland in 1955, although this infestation has not spread significantly (Auld et al. 1983). The second introduction into central Queensland, north of Clermont, occurred in 1958 and has been far more invasive than the first. This introduction is most likely to have originated from Texas, USA, through the importation of contaminated pasture seed (Everist 1976; Picman and Towers 1982; Haseler 1976). The major area of parthenium infestation now covers an extensive region of central Queensland with some isolated infestations also occurring outside this region in southern regions of Queensland and into northern NSW (Navie et al. 1996).

If buried in soil, parthenium seed is very long-lived with a germinable half-life of 4-6 years (Navie et al. 1998; Tamado et al. 2002). It has also been shown to account for greater than 50% of seedbanks at some locations in Queensland (Navie et al. 2004). It is an opportunistic weed and is able to germinate, grow and shed prolific numbers of seeds (achens) in less than two months with adequate soil moisture, but will survive for much longer if sufficient moisture is maintained (Navie et al. 1996; Jayachandra 1971). It has a wide temperature tolerance (Tamado et al. 2002) and can complete its life cycle at any time of year in Queensland (Haseler 1976).

History of TSV in Australia and strains described

In Australia, TSV was first reported in 1971 (Greber 1971) from south-east Queensland from several naturally infected weeds including *Ageratum houstonianum*, *Asclepias physocarpus* (syn. *Gomphocarpus physocarpus*), *Dahlia pinnata*, *Nicandra physalodes*, *Solanum auriculatum* and *S. nigrum*. TSV was subsequently reported as the cause of a sporadic disease of commercial tobacco crops (Finlay 1974; Greber et al. 1991b) and was mostly studied in the south-east Queensland region around, and just north of Brisbane. Three distinct TSV strains have been described from Australia, TSV-Ag, TSV-A and TSV-S. The most commonly reported was collected from *A. physocarpus* (syn. *G. physocarpus*) and was originally referred to as the Asclepias strain (TSV-As; Sdoodee 1989). Sdoodee and Teakle (1993) noted that TSV-As was closely related serologically to the TSV reported by Greber (1991b) from *A. houstonianum* and tobacco. Klose et al. (1996) later assumed that TSV-As was the same as the TSV from *A. houstonianum* and thereafter it was referred to as the Ageratum strain (TSV-Ag) due to the high incidence of this strain in *A. houstonianum* near TSV affected tobacco crops (Greber et al. 1991b).

A TSV strain from strawberry was originally isolated by Greber (1979) from the M9 clone of strawberry cv. Redlands Crimson and was later designated as strain TSV-S by Sdoodee (1989). Klose et al. (1996) suggested that TSV-S may have originated from North America, a major source of germplasm for the Australian strawberry industry.

A third strain, TSV-A, was isolated from the ornamental plant, *Ajuga reptans*, in Victoria by Shukla and Gough (1983) who demonstrated serological differences to strain TSV-Ag. Interestingly, both Shukla and Gough (1983) from Australia and Fisher and Nameth (1997) from the United States of America reported an association between TSV, *Alfalfa mosaic virus* and *Cucumber mosaic virus* infecting *Ajuga reptans*.

The three strains, TSV-Ag, TSV-A and TSV-S were shown to differ in relation to serology, host range and thrips species transmission efficiency (Greber 1971, 1979; Klose et al. 1996; Sdoodee 1989). One notable difference was that while TSV-Ag and TSV-A both produced dentate leaves in systemic infections of *Nicotiana tabacum* cv. Turkish (Greber 1971; Sdoodee 1989), TSV-S failed to do so (Greber 1979). Klose et al. (1996) demonstrated different rates of transmission efficiency between these three strains using different thrips species as vectors as discussed in more detail below.

Recent diseases caused by TSV in central Queensland

A severe necrosis disorder of unknown aetiology has affected sunflower crops in the Central Highlands region of central Queensland since the early 2000s. This region extends over more than 200 km from roughly Springsure in the south to north of Clermont, encompassing the northern section of the Queensland grain belt where a significant proportion of total production occurs for several key grain and oilseed crops. The sunflower necrosis disorder was estimated to have caused annual losses of approximately 20% ($ AUD 4.5 million) across the sunflower industry in Central Queensland for several consecutive years since 2004. Mung bean (*Vigna radiata*) crops were also severely affected in early 2007 across the Central Highlands region. Symptoms typically included severe stem and terminal necrosis in both sunflower and mung bean, often progressing to complete collapse and death of affected plants (Fig. 1.2). Preliminary reports suggested an association with TSV infection (Reppel 2007) and glasshouse experiments indicated that a range of crop plants grown in central Queensland could potentially be affected by TSV including sunflower, mung bean, chickpea (*Cicer arietinum*) and cotton (*Gossypium hirsutum*).

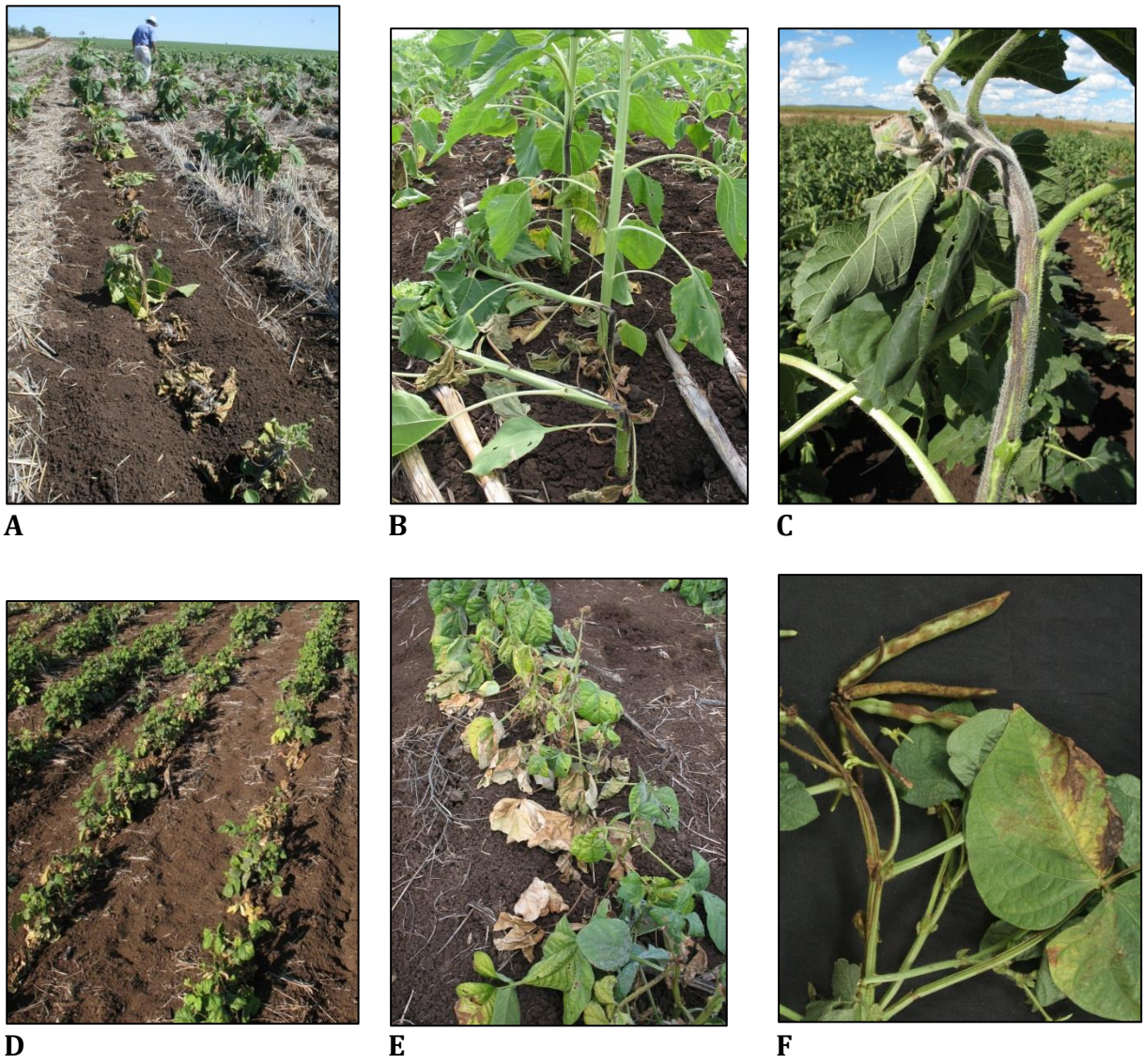

Figure 1.2. Typical necrotic symptoms observed on sunflower (a-c) and mung bean (d-f). Plants affected early often died while plants affected later generally displayed severe stem necrosis and distortion. Sunflowers often lodged (b) due to stem necrosis.

The transmission of TSV via pollen and insect vector

While members from at least 15 virus genera are reported to be transmitted via pollen (Card et al. 2007), only a limited number, including ilarviruses such as TSV, appear to be able to infect the leaves of healthy plants via virus-infected pollen with the aid of an insect vector (Mink 1993; Hull 2014). Vertical transmission of viruses via pollen where fertilisation results in infection of the embryo and subsequent seed transmission is more common than horizontal transmission where virus in pollen infects nongametophytic tissues (Card et al. 2007; Hull 2014). A review by Mink

(1993) indicated little evidence that fertilisation by virus-infected pollen can result in infection of the mother plant and generally only results in embryo infection and potential seed transmission. However, a recent study by Isogai et al. (2014) conclusively demonstrated horizontal transmission of *Raspberry bushy dwarf virus* from infected pollen tubes during pollination into maternal tissue leading to systemic infection. Another recent study by Jaspers et al. (2015) indicated that *Asparagus virus 2* was transmitted to healthy plants during fertilization with virus-infected pollen, although they also note the possibility that the method of experimental fertilization may have caused mechanical transmission.

Thrips as vectors of plant viruses

Thrips are small insects, usually only a few millimetres in length, but as a group they display extensive diversity. There are approximately 7700 species of thrips (Order *Thysanoptera*) classified into nine families and about 93% of all species are members of just two families, *Thripidae* and *Phlaeothripidae* (Mound 1997; Mound 2015). In spite of this thrips species diversity, only 19 species have been reported as vectors of plant viruses from the genera *Carmovirus*, *Ilarvirus*, *Machlomovirus*, *Sobemovirus* and *Tospovirus* (Jones 2005; Riley et al. 2011; Prasada Rao et al. 2003; Cabanas et al. 2013; Zhou and Tzanetakis 2013). The majority of virus species reported to be thrips transmitted belong to the tospoviruses (family *Bunyaviridae*) and are transmitted in a persistent circulative manner (Ullman et al. 1997). There are 9 recognised tospovirus species and a further 15 proposed species (ICTV 2013; King et al. 2012; Zhou et al. 2011) and 15 thrips species reported as vectors (Jones 2005; Riley et al. 2011; Rotenberg et al. 2015; Zhou and Tzanetakis 2013).

While Australia has significant diversity of endemic thrips with over 700 species, at least 60 non-native species have also become established (Mound 2004), including western flower thrips (*Frankliniella occidentalis*), melon thrips (*Thrips palmi*) and tomato thrips (*F. schultzei*). These efficient virus vectors are spreading widely in Australia, and concurrently, thrips-transmitted viruses such as *Tomato spotted wilt virus* and Capsicum chlorosis virus (Persley et al. 2006; Sharman and Persley 2006) and TSV (Reppel 2007) are causing significant crop damage.

Thrips as vectors of TSV and other pollen-borne viruses

The majority of work done in relation to associating thrips with the transmission of pollen-borne viruses has been done with ilarviruses following the confirmation by Sdoodee and Teakle (1987) of the critical link between thrips and virus-infected pollen in the transmission of TSV (Mink 1993;

Johansen et al. 1994; Ullman et al. 1997). The ilarviruses transmitted by thrips and pollen are discussed further below and the only other viruses transmitted by this means include *Sowbane mosaic virus* (SoMV; Hardy and Teakle 1992), *Pelargonium flower break virus* (PFBV; Krczal et al. 1995) and *Maize chlorotic mottle virus* (MCMV; Ullman et al. 1992; Cabanas et al. 2013; Mahuku et al. 2015).

The association of thrips (a *Frankliniella* sp.) with the transmission of TSV was first reported from Brazil (Costa and Lima Neto 1976) and then later Kaiser et al. (1982) transmitted TSV with a mixed colony of *F. occidentalis* and *F. schultzei*. Almeida and Corso (1991) also transmitted TSV to about 80 % of test plants by transferring thrips from field infected plants and even noted the high numbers of thrips feeding on pollen of the TSV-infected weeds. In these cases, transmission was achieved when thrips from infected plants were transferred to healthy test plants. However, the critical link between TSV-infected pollen and thrips transmission was not clarified until Sdoodee and Teakle (1987) first demonstrated *Thrips tabaci* could only transmit TSV when virus-infected pollen was also present. It appears likely that TSV transmission was achieved in the earlier reports because thrips taken from TSV-infected plants were contaminated by TSV-infected pollen. Attempts to transmit TSV using a range of other insects have been unsuccessful. Costa and Carvalho (1961) fed insects such as aphids, white flies, leafhoppers and two species of thrips on virus-infected leaves and failed to transmit TSV to healthy test plants. Reddy et al. (2002) also had no transmission when thrips were fed on virus-infected leaves in the absence of pollen. While Kaiser et al. (1982) did transmit TSV using field-collected thrips (from a mixed population of *F. occidentalis* and *T. tabaci*), they did not transmit TSV using pea aphids (*Acyrthosiphon pisum*) or pea leaf weevils (*Sitona lineata*). Interestingly, a recent study on the transmission of another subgroup 1 ilarvirus, PMoV, reported transmission by several insect species in the presence of PMoV-infected pollen (Aramburu et al. 2010). In addition to the thrips *F. occidentalis*, they found transmission was also facilitated by insects such as *Myzus persicae*, *Bemisia tabaci*, *Nesidiocoris tenuis* and *Orius majusculus*. These findings indicate that transmission of PMoV is not exclusively association with thrips.

It seems logical that the most likely thrips species to act as vectors of pollen-borne viruses are those that actively inhabit flowers and feed on pollen. Many thrips species opportunistically feed on the pollen of a wide range of plant species as it provides a rich source of protein and as a result of this activity they can often transport pollen grains on their bodies during flight (Kirk 1984b, 1997).

Several species of thrips have now been reported as vectors of ilarviruses (Jones 2005). In India, TSV has been shown to be transmitted by *F. schultzei* (Prasada Rao et al. 2003a; Reddy et al. 2002), *Megalurothrips usitatus*, and *Scirtothrips dorsalis* (Prasada Rao et al. 2003a). Swamy et al. (2010) reported transmission of TSV in India using *Thrips palmi* but it is not clear if this transmission was done in the presence of TSV-infected pollen.

In Australia, PDV and PNRSV were transmitted using *F. occidentalis* (Greber et al. 1992) from virus-infected pollen. Greber et al. (1992) found that PDV, but not PNRSV, could be thrips transmitted from pollen stored for longer than 2 years at -20°C while both viruses could be readily transmitted by mechanical inoculation from the same stored pollen. This demonstrates a high degree of stability of both these ilarviruses in pollen. PNRSV was also transmitted by either *T. tabaci* alone or in a mixed population of *T. imaginis, T. tabaci* and *T. australis* (Greber et al. 1991a). The three TSV strains reported from Australia appear to be transmitted at different rates depending on the thrips species. TSV-Ag was transmitted by *F. schultzei*, *Microcephalothrips abdominalis*, *T. tabaci* and *T. parvispinus* (Greber et al. 1991b; Klose et al. 1996; Sdoodee and Teakle 1987, 1993). TSV-A was transmitted by *T. tabaci* but not by *F. schultzei* (Klose et al. 1996).

Another ilarvirus, *Asparagus virus* 2 (AV-2), is present on pollen grains (Evans and Stephens 1988) and it has been reported that infected pollen is moved by bees and thrips to female plants (Tomassoli et al. 2012).

Localisation of TSV in pollen

Studies by Sdoodee and Teakle (1993) of pollen from several plant species suggested there is considerable variation in the location of TSV associated with pollen grains, appearing to be either external, internal or both. In studies of several plant species, they found that while considerable amounts of TSV could be washed off the exterior of the pollen grains, high amounts of TSV could still be released from the washed pollen with disruption of the pollen grains. They hypothesised that it was most likely that the externally associated virus was involved in transmission. Externally located virus has also been detected for other ilarvirus species PDV and PNRSV (Cole et al. 1982; Hamilton et al. 1984; Kelley and Cameron 1986). These authors indicated that these viruses were located both externally and internally and they suggested that internally located virus was most likely transmitted to cherry seeds during fertilization but it was possible that externally located virus was involved in tree-to-tree transmission.

In contrast to the results of Sdoodee and Teakle (1993), Klose et al. (1992) used thin section electron microscopy to demonstrate that TSV (presumably TSV-Ag) was only in the cytoplasm of *Ageratum houstonianum* pollen and not in the wall layers of the exine, interbacular spaces or the intine. However, they also showed that TSV particles were at high concentrations in pollen tubes that germinated within minutes of coming in contact with moisture and then lysed after some time, presumably releasing the virus particles. PNRSV was also shown to be at high concentrations in the vegetative cytoplasm of *Prunus persica* pollen by Aparicio et al. (1999) who suggested that virus present in the pollen tube that enters the embryo sac may lead to infection of the seed. The possibility that germ tubes emanating from virus-contaminated pollen may be involved in transmission to the ovule was also hypothesised earlier by Hamilton et al. (1977).

Mode of transmission of TSV via pollen

Using the TSV-Ag strain (syn. TSV-As), Sdoodee and Teakle (1987) demonstrated TSV is pollen-borne and transmission of TSV to healthy plants relies on the virus from infected pollen entering plant cells through the feeding injury caused by thrips. They concluded that it was most likely that TSV located externally on infective pollen was involved in transmission to healthy plants via thrips feeding wounds. A similar mode of transmission was suggested by Hamilton et al. (1977) for viruses that contaminate the exine surface of pollen grains that may be moved by flower-visiting insects to wound sites on leaves, facilitating virus transmission.

Several studies on aspects of this transmission process for TSV have been done. In the absence of thrips, transmission of TSV was also demonstrated when TSV infective pollen was applied to freshly made thrips feeding wounds, indicating these wound sites are the point of infection (Sdoodee and Teakle 1993). The high concentration of TSV-Ag in the germinating pollen tubes (Klose et al. 1992) may be an effective mechanism for internally located TSV to rapidly exit pollen, enter wound sites produced by thrips and then move into the plant cells. Sdoodee and Teakle (1987) also demonstrated that transmission did not occur when thrips that were allowed to feed on TSV-infected pollen were then cleaned to remove pollen grains and then allowed to feed on test plants.

Transmission of TSV via seed

Mechanisms and biological function of seed transmission

Seed transmission of plant viruses can form a critical link between generations of host plants which may be temporally separated by adverse conditions, such as winter or drought. It can also enable

long distance dispersal of a virus to new locations (Maule and Wang 1996). The mechanisms that control seed transmission are complex and have been difficult to study in detail. Approximately 20 % of all plant viruses can be transmitted via seed between generations of plants (Hull 2014; Mink 1993) and while some viruses may be seed-borne as contaminants on seed coats, effective transmission to seedlings almost always requires the infection of the embryo (Maule and Wang 1996). Perhaps the only exception to this is for transmission of the highly stable tobamoviruses, such as *Tobacco mosaic virus*, which contaminates the seed coat and transmission occurs by a mechanical means when seedlings are transplanted, but does not occur if seedlings are untouched (Broadbent 1965; Johansen et al. 1994).

Virus infection of the seed embryo can occur by two pathways, the most common of which is indirect embryo invasion prior to fertilisation where either one or both of the gametes are virus infected. The second pathway is by direct invasion of the embryo after fertilisation and it appears viruses may need to move through the embryonic suspensor to gain access to the embryo before it degenerates and the connection to the embryo is lost (Johansen et al. 1994; Maule and Wang 1996; Wang and Maule 1994). These two pathways are not mutually exclusive and the final rate of seed transmission may be a result of both. Where transmission to the embryo occurs either indirectly or directly from the infected mother plant, the timing of initial infection of the mother plant in relation to flowering has a marked influence on the resulting rate of seed transmission with generally higher rates of seed transmission occurring when the mother plant is infected before the onset of flowering (Hull 2014; Johansen et al. 1994).

The rate of seed transmission of a virus can be significantly affected by genetic variation within the same host species (i.e. between different cultivars) (Wang et al. 1993) or by variation within the same virus species (i.e. different strains or isolates) (Hull 2014; Kaiser et al. 1991; Walter et al. 1995). The rate of seed transmission can also decline markedly with seed maturation and storage (Bowers and Goodman 1991; Wang and Maule 1994). Almost half of the described ilarvirus species have been shown to be seed transmitted (Hull 2014).

Seed transmission of TSV

Transmission of TSV between growing plants (horizontal transmission) occurs via thrips and infected pollen (Klose et al. 1996; Prasada Rao et al. 2003a; Sdoodee and Teakle 1987). However, seed transmission between generations of plants (vertical transmission) has also been reported in a range of plant species (Kaiser et al. 1991; Sdoodee 1989; Sdoodee and Teakle 1988). The rate of

reported transmission from naturally infected hosts is variable, including 3% in *Melilotus alba* (Kaiser et al. 1982), 1.4-6% in *Phaseolus vulgaris* cv. Pinto (Thomas and Graham 1951), 3.8% in *P. vulgaris* cv. Black Turtle Soup (Kaiser et al. 1991) and about 6% in *Raphanus raphanistrum* (Cupertino et al. 1984).

Fertilisation of female megaspores (ovules) with TSV-infected pollen has been demonstrated to lead to infection of seed (i.e. indirect invasion of the embryo) and subsequent transmission to seedlings. When TSV-Ag infected pollen was used to fertilise healthy tomato mother plants, about 11% of the resulting seedlings were TSV-infected (Sdoodee and Teakle 1988). While pollination of TSV-infected or healthy plants with TSV-infected pollen can result in infection of the endosperm, embyro and seedlings, transmission to the leaves of healthy mother plants was not detected (Sdoodee and Teakle 1988). Similar to the suggestion of Aparicio et al. (1999) for seed transmission of PNRSV, it may be the movement of TSV through the pollen tube (Klose et al. 1992) that enables the transmission of TSV to the embryo.

While Sdoodee (1989) did detect TSV (presumably TSV-Ag) in the seed of three naturally infected weed hosts, *A. houstonianum*, *Bidens pilosa* and *Gomphocarpus physocarpus*, she did not detect seed transmission to the seedlings when 50 seeds of each were grown out. Klose (1997) did detect a low rate (less than 1 %) of seed transmission of TSV-Ag to seedlings from naturally infected *A. houstonianum* when 111 seedlings from the seed of six mother plants was tested. It appears that Sdoodee (1989) may not have detected natural seed transmission of TSV-Ag in *A. houstonianum* due to a lower number of seedlings tested. By contrast, experimental inoculation of TSV-Ag to mother plants of other weed species, *Nicandra physalodes* and *Solanum nigrum*, resulted in seed transmission to seedlings at rates of 88 % and 11 % respectively (Sdoodee 1989). Experimental seed transmission to seedlings was also demonstrated for TSV-A at rates of 48 %, 74 % and 100 % respectively for *Chenopodium amaranticolor*, *C. quinoa* and *Vigna unguiculata* (Shukla and Gough 1983).

While Sdoodee and Teakle (1988) did not demonstrate TSV seed transmission in tomato resulting from the combination of TSV-Ag infected mother plants and healthy pollen, only three seedlings were tested in the grow out test. Hence, it remains unclear if fertilisation with TSV-infected pollen was critical to get seed transmission or if TSV can infect the seed embryo by invasion from the mother plant.

Interestingly, four studies of TSV in India have indicated no true seed transmission to seedlings in a range of naturally and experimentally infected hosts. Naturally infected hosts included sunflower, at least three cultivars of peanut, marigold, and parthenium, while experimentally infected hosts included the same hosts (except marigold) and *Phaseolus vulgaris*, *Glycine max*, *Vigna mungo*, *V. radiata*, *Dolichos lablab*, *Trigonella foenum*. *Gomphrena globosa*, *Chenopodium quinoa* and parthenium (Prasada Rao et al. 2009; Prasada Rao et al. 2003a; Reddy et al. 2007; Vemana and Jain 2010). Vemana and Jain (2010) suggest that the non-seed transmission of TSV from India may be a characteristic of this strain, similar to the differences in seed-transmissibility of distinct TSV strains from the USA where Walter et al. (1995) suggested that the presence of a small minor fifth RNA segment may be linked to non-seed transmission. However, the presence or otherwise of this extra, minor fifth RNA has not been determined for the non-seed transmissible TSV from India (Vemana and Jain 2010).

Management of TSV diseases

Effective and appropriate management options for virus control are underpinned by an adequate understanding of the biology and epidemiology of a virus in the affected crop. Control options may target one or more interactions of the plant-host-vector system in an attempt to break the infection cycle. This may involve reducing the virus inoculum pressure on the susceptible host or utilising the natural defence mechanisms of the host plant (Hull 2014). Effective cultural practices can include; altering planting date to avoid the peak influx of inoculum, modifying plant density to disrupt vector movements into crops, or removal of the inoculum source. Reduction of the vector population can be effective to break the link from source to crop. For all control options to be viable, the cost of implementing control measures must be less than the losses incurred without the control. Where available, the most economical and environmentally sustainable control option is often to utilise naturally occurring resistance genes in the host plant in association with other control measures in an integrated approach to minimise damage caused by virus diseases.

Several management options have been reported to control or reduce disease. By delaying soybean planting in Brazil by several weeks, to the end of the normal planting window, and thus avoiding the peak influx of thrips associated with transmission, TSV disease was significantly reduced without compromising the yield potential of the crop (Almeida and Corso 1991). Shirshikar (2003) also reported a large difference in TSV disease incidence when sunflowers were planted monthly at

Latur, India from July to February, with the highest incidence with planting in July-August and the lowest in November-December. Unfortunately, planting dates for most Australian sunflower production are dictated by sporadic rain events, precluding the selection of planting times to avoid peak TSV influxes.

Investigations of TSV in India have reported the benefits of cultural control options such as removal of the major alternative host and virus source, parthenium, close to peanut crops or planting tall, fast growing barrier crops such as sorghum prior to sowing susceptible crops to interrupt the influx of thrips and TSV-infected pollen (Prasada Rao et al. 2003b; Shirshikar 2008). Some benefit from the use of imidacloprid-treated seed and regular in-crop sprays have been suggested for peanut (Prasada Rao et al. 2003b) and demonstrated for sunflower (Shirshikar 2008). However, such treatments may not be economical, can rapidly result in insecticide resistance (Lewis 1997), and can compromise effective integrated pest management where thrips may be effective predators of other pests (Wilson et al. 1996). Almeida and Corso (1991) also noted that insecticide treatment of thrips would only be of benefit if the thrips populations could be controlled at a low level but they consistently observed high numbers of thrips migrating into soybean crops from the surrounding TSV-infected weed, *A. polystachia*. Effective control of the thrips vectors in central Queensland is also unlikely as it would require regular applications of insecticide not only over the susceptible crops that commonly cover thousands of hectares per farm, but also the neighbouring areas where the virus source is likely to be located.

The application of anti-viral compounds such as a mix of a *Bougainvillea spectabilis* extract and goat's milk was shown to reduce TSV infections in cowpea and sunflower presumably by inducing plant defence mechanisms (Lavanya et al. 2009). A study by Srinivasan et al. (2009) also reported reduced virus disease with the use of other biocontrol agents such as species of *Bacillus*, *Pseudomonas* and *Streptomyces*. However, they refer only to Sunflower necrosis virus which is presumably a synonym for TSV.

Control of parthenium as a source

Given the critical role parthenium plays in the epidemiology of TSV in India (Prasada Rao et al. 2003a) and the similarity of its abundance in cropping areas of India and Australia, some consideration should be given to the specific control of this potential TSV host. Widespread control of parthenium is unlikely to be successful in the rangelands of central Queensland where it is widespread and abundant. Parthenium is also a significant pest of grazing lands and while numerous

biological agents have been introduced into Australia for its control (Evans 1997; Wild et al. 1992; Adkins and Shabbir 2014), it continues to infest vast areas across central Queensland. Given the broad acre farming systems of central Queensland, the options for management of diseases caused by TSV appear to be limited to cultural practices to reduce the level of inoculum present in surrounding host plants entering the crop and the use of tolerant germplasm.

Use of genetic resistance

The use of pathogen-derived resistance, whereby a portion of the virus genome is incorporated into the plant genome, has been successfully used for a range of plant viruses (Cillo and Palukaitis 2014). This approach has been recently used to engineer resistance in sunflower against a strain of TSV from India (Pradeep et al. 2012). However, the use of such engineered resistance in food crops such as sunflower is currently unlikely to be widely accepted by the general public in Australia.

Naturally occurring genetic resistance to TSV has been reported from the USA in soybean germplasm (Hobbs et al. 2012), and from India in wild *Arachis* (peanut) germplasm (Kalyani et al. 2007; Kalyani et al. 2005) and sunflower germplasm (Karuna et al. 2008; Lokesh et al. 2005). The sunflower resistance screening by Lokesh et al. (2005) was done in a single trial with relatively low disease pressure and non-replicated plots. The trial by Karuna et al. (2008) was also run with non-replicated plots, TSV disease incidence was much higher and there appeared to be large differences in the tolerance of the tested hybrids. There is also a report of a non-replicated field trial of five sunflower hybrids to investigate TSV tolerance in central Queensland. However, the presented results indicate the disease pressure was too low to detect differences between hybrids, the rated symptoms were not described and no diagnostic confirmation of TSV was reported (Crawford and Philp 2009). There appear to be no other reported studies investigating resistance of sunflower germplasm to field infections of TSV. In addition, any results from resistance screening against the Indian strain of TSV may not be applicable to the TSV strain causing disease epidemics in Australia due to potential genetic differences between the strains. The range of sunflower hybrids currently grown in Australia is also different from those grown in India so any resistance found in Indian germplasm is unlikely to be available in the current germplasm utilised in Australia.

Aims of this study and rationale

Review of the literature has identified several critical gaps in our understanding of the disease cycle of TSV in central Queensland and more broadly the genetic relationship between TSV strains in Australia with strains from other regions of the world. In summary, TSV has caused significant losses in sunflower and mung bean crops in central Queensland. While biologically distinct strains of TSV have been previously described from Australia, there is no reported genomic data for these strains. It is unknown what strain(s) of TSV is the causal agent of disease in central Queensland and how it relates genetically to other TSV strains. A number of other biological characteristics of TSV in central Queensland are unknown, including; what other crops may be affected, what important alternative hosts may exist, what thrips vectors are associated with disease epidemics and what life cycle characteristics enable TSV to survive in this sometimes harsh environment.

Considering the importance of TSV on a global scale and the gaps in our knowledge concerning this pathogen and the diseases it causes in multiple crops, the major aims of this PhD study included:

1. Determining what strain of TSV was the causal agent for the severe disease outbreaks in central Queensland, which crops were affected and if different strains affected different crops (Chapter 2).
2. Characterising the genetic diversity of TSV strains previously reported from Australia and determining their relationships to TSV affecting crops in Central Queensland. This information may enable an hypothesis to be formed as to the origin of this new TSV outbreak (Chapters 4 and 5).
3. Obtaining an understanding of how TSV was surviving and moving into crops in central Queensland. This required determining aspects of its biology including; its natural host range and any key alternative hosts; the thrips vector species associated with disease outbreaks; and if mechanisms such as seed transmission may enable it to survive through the adverse conditions commonly experienced in this region (Chapters 3 and 6).
4. Determining management strategies that could be implemented to minimise the risk of TSV disease in susceptible crops, particularly sunflower and mung bean. A critical part of this was to determine if any useful tolerance to field infections of TSV was available in the sunflower hybrids used by the industry (Chapter 7).

The rationale for undertaking this PhD study was to improve our knowledge about the epidemiology and diversity of TSV strains in Australia. By undertaking detailed studies to address the aims listed above, the data collected will fill the identified knowledge gaps. In doing so this will provide a sound basis for the development of management strategies to minimise the risks of diseases caused by TSV in crops currently affected in central Queensland and ensure the viability of associated cropping industries.

Chapter 2

First report of *Tobacco streak virus* in sunflower (*Helianthus annuus*), cotton (*Gossypium hirsutum*), chickpea (*Cicer arietinum*) and mung bean (*Vigna radiata*) in Australia

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First report of *Tobacco streak virus* in sunflower (*Helianthus annuus*), cotton (*Gossypium hirsutum*), chickpea (*Cicer arietinum*) and mung bean (*Vigna radiata*) in Australia

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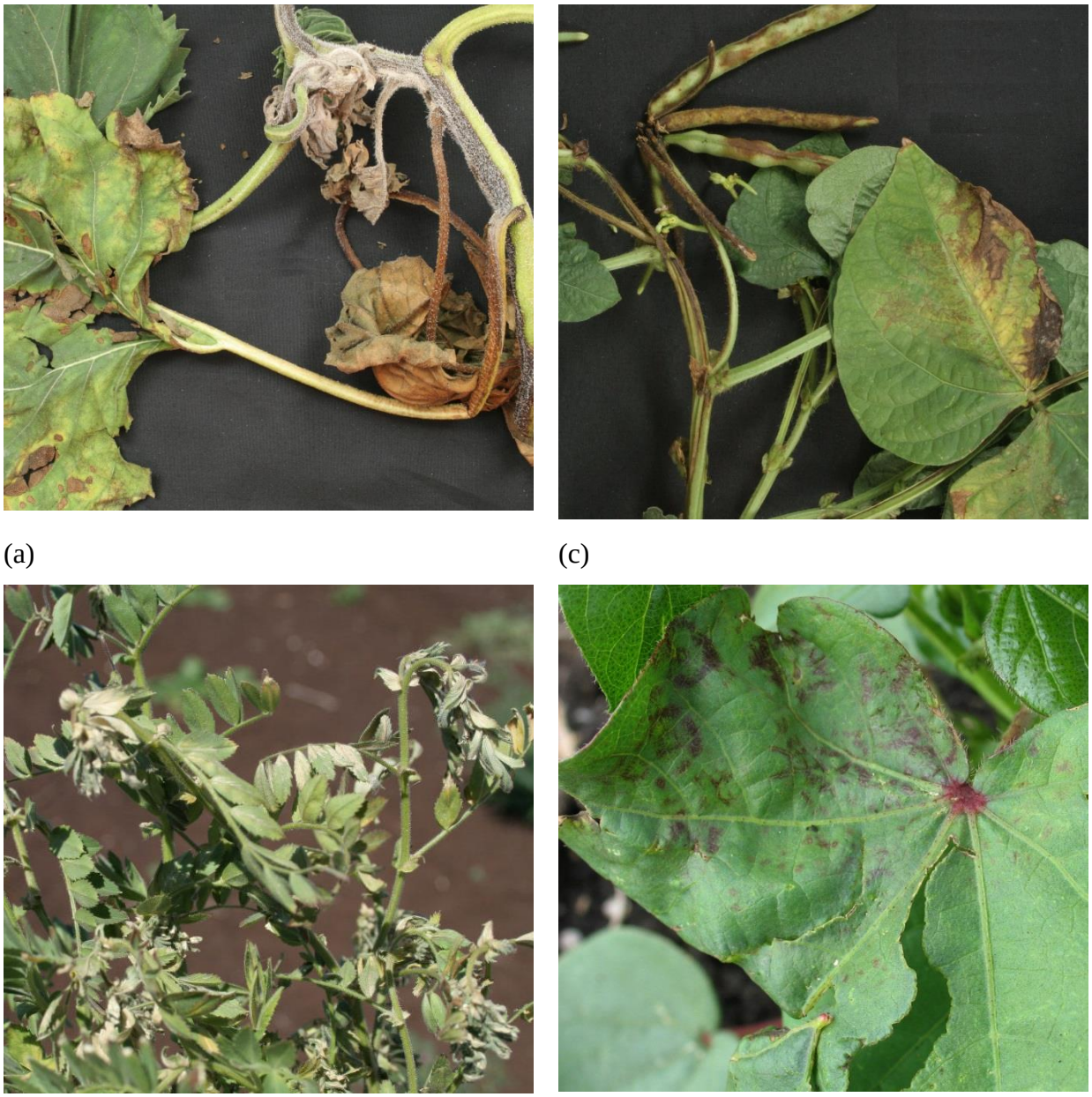
Abstract. *Tobacco streak virus* (genus *Ilarvirus*) is recorded on sunflower (*Helianthus annuus*), cotton (*Gossypium hirsutum*), chickpea (*Cicer arietinum*) and mung bean (*Vigna radiata*) in Australia for the first time.

A significant proportion of the Australian total production of sunflower (*Helianthus annuus*), chickpea (*Cicer arietinum*), mung bean (*Vigna radiata*) and cotton (*Gossypium hirsutum*) occurs in the Queensland grain belt. Sunflower is used primarily for domestic consumption, whilst over 90% of the chickpea, mungbean and cotton production is exported (Douglas 2007; Anon. 2004; Anon. 2008).

Since the summer of 2004 / 2005 an unidentified necrotic disorder of sunflower has caused significant production losses across a large area of the Central Highlands of Queensland, Australia, between the towns of Springsure in the south to Clermont in the north. Subsequently, necrotic symptoms have also been observed in crops of chickpea, mung bean and cotton grown in the same region. While limited field observations in 2007 indicated disease incidence on chickpea and cotton was low, significant losses were reported from numerous mung bean crops across the Central Highlands in early 2007.

Symptoms on sunflower included necrosis of leaf lamina, petiole, stem and floral calyx, often with lodging of taller plants or stunted growth and plant death (Fig 1a). On chickpea, symptoms included stem necrosis, tip wilting and necrotic etching on leaves (Fig 1b). On mung beans there were necrotic line patterns on leaves, severe petiole, stem and tip necrosis, stunted growth and plant death (Fig 1c). On cotton, symptoms ranged from young plants with single diffuse necrotic lesions to older plants with many necrotic lesions, ring spots, chlorotic mottle and leaf deformation (Fig 1d).



(a) (c)

(b) (d)

Fig. 1. Symptoms of TSV on naturally-infected field samples of: (a) sunflower (TSV-1974); (b) chickpea (TSV-1979); (c) mung bean (TSV-2027) and (d) cotton (TSV-2120).

Representative samples from each of the four crops were selected for detailed analysis. Sunflower and chickpea samples were collected in 2006 while mung bean and cotton samples were collected in 2007. Quasi-isometric virions typical of members of the genus *Ilarvirus* were observed in sap extracts of isolate 1974 from sunflower, when negatively contrasted with 1% ammonium molybdate pH 7.0 and examined by electron microscopy. Leaf samples of isolates 1974 (sunflower, from Clermont), 1979 (chickpea, from Clermont), 2027 (mung bean, from Springsure) and 2120 (cotton, from Emerald) tested positive for *Tobacco streak virus* (TSV) by ELISA (AGDIA ELISA reagent set, cat. No. SRA25500/0500) with A_{405nm} values of 15 - 90 times greater than the means of their respective healthy controls.

For confirmation of ELISA results by RT-PCR, RNA was extracted from leaf tissue of the four isolates mentioned above using the Concert RNA Reagent (Invitrogen) prior to preparation of cDNA using SuperScript III reverse transciptase (Invitrogen) as per the manufacturer's instructions. Previously published sequences from GenBank (accessions NC_003845, AY354406 and DQ323518) were used to design TSV-specific PCR primers flanking the coat protein gene. Primers TSVcpR2 (5' CCA CAT CGC ACA CAA GTA TTA C 3') and TSVcpF2 (5' GCT TCT CGG ACT TAC CTG AGA T 3') were used at an annealing temp of 58ºC and primed amplification of an 802 bp fragment from each of the four isolates, containing the entire coat protein gene of 717 nt. The nucleotide sequence obtained for sunflower isolate-1974 (TSV-1974; GenBank accession EU375481) had > 98% identity with a Brazilian TSV isolate reported by Almeida *et al*. (2005) from soybean (GenBank accession AY354406). TSV isolates 1974, 1979, 2027 and 2120 have been lodged in the DPI&F Indooroopilly Plant Virus Collection.

TSV-1974 was isolated from the field sample by manual inoculation to *Nicotiana tabacum* cv. Xanthi nc, which developed systemic necrotic etching and notched leaf margins typical of TSV infection (Greber 1971). When inoculated from tobacco back to sunflower cv. Suncross 53, the range of symptoms observed was similar to that seen in natural field infections, including chlorotic local lesions, and midrib, petiole and stem necrosis (Fig. 2).

TSV was first reported from Australia in 1971 and has subsequently been reported from tobacco, strawberry, dahlia and various weed species, mostly from south-eastern Queensland (Greber 1971, 1979, Greber *et al.* 1991). This is the first report of TSV naturally infecting sunflower, cotton, mung bean and chickpea in Australia. Natural field infections with TSV have previously been reported on sunflower, mung bean and cotton from India (Bhat *et al.* 2002a; Bhat *et al*. 2002c; Prasada Rao *et al.* 2000) and also on cotton from Pakistan and Brazil (Ahmed *et al.* 2003; Costa and

Carvalho 1961). In India, TSV-induced sunflower necrosis disease has been responsible for serious economic losses (Baht 2002b). Kaiser *et al.* (1991) reported TSV naturally infecting chickpea growing adjacent to plots of inoculated plants in the United States of America.

While TSV has been present in south-eastern Queensland since at least the early 1970s (Greber 1971), it remains to be determined why it has only recently become prominent in the Central Highlands region. Research is continuing to determine the relationship between TSV isolates from different regions of Australia. Further studies on the epidemiology of TSV from the Central Highlands of Queensland, including identification of alternative hosts and thrips vectors will be important for development of effective management strategies.

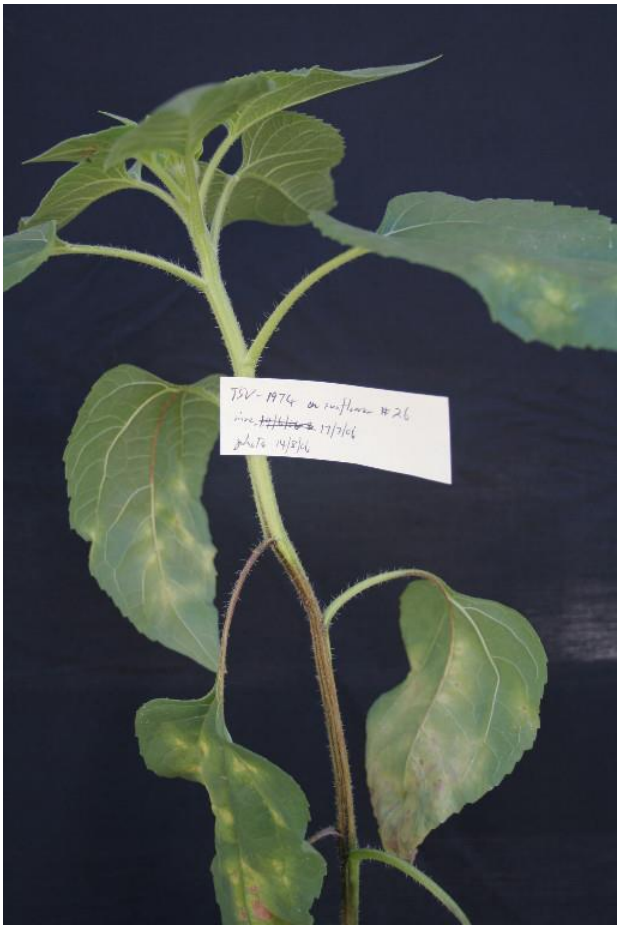


Fig. 2. Sunflower inoculated with TSV-1974, from *Nicotiana tabacum* cv. Xanthi nc, originally isolated from field-infected sunflower. Symptoms shown include chlorotic local lesions and necrosis of leaf veins, petioles and stem.

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Chapter 3

Distribution in Australia and seed transmission of *Tobacco streak virus* in *Parthenium hysterophorus*

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Distribution in Australia and seed transmission of *Tobacco streak virus* in *Parthenium hysterophorus*

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Abstract

Sharman, M, Persley, D. M. and Thomas, J. E. 2008. Distribution in Australia and seed transmission of *Tobacco streak virus* in *Parthenium hysterophorus*. *Plant Dis.*

Tobacco streak virus (TSV) was found to commonly occur in *Parthenium hysterophorus*, as symptomless infections, in central Queensland, Australia across a large area infested with this weed. Several isolates of TSV collected across the geographic range of *P. hysterophorus* were found to share identical coat protein sequence with each other and with TSV from crop plants in the same area. Seed transmission of TSV in *P. hysterophorus* was found to occur at rates of 6.8 to 48%. There was almost no change in the rate of TSV seed transmission when *P. hysterophorus* seed was stored for up to 24 ½ months. Implications of this relationship between TSV and *P. hysterophorus* for the development of virus disease epidemics in surrounding crops are discussed.

Keywords: Ilarvirus, epidemiology, alternative host

Tobacco streak virus (TSV), the type member of the genus *Ilarvirus* (family: *Bromoviridae*), has an extensive host range (7). While the transmission of TSV commonly occurs via thrips and infected pollen (21, 30, 33), seed transmission has also been demonstrated in a range of plant species (18, 32, 34). Seed transmission of plant viruses can form a critical link between generations of host plants which may be temporally separated by adverse seasons, such as winter or drought. It can also enable long distance dispersal of a virus to new locations (23).

In Australia, TSV was first reported in 1971 and has subsequently been reported from tobacco, strawberry, dahlia and various weed species, mostly from south-eastern Queensland (13-15, 37). TSV has recently been reported from several field crops in central Queensland (36) causing a yield loss of approximately 20% across the sunflower industry in central Queensland since 2004 with localised yield losses of greater than 40% observed in many crops. TSV also caused significant losses in mung bean crops throughout central Queensland in early 2007 with up to 70% yield reductions in severely affected crops. Losses have been both direct, from crop damage caused by the virus, and indirect from reduced grower confidence in sunflower (authors, unpublished results). A similar disease caused by TSV, in a range of crops, has recently been reported from India (5, 6, 29). A number of alternative weed host have been identified in India including the symptomless host, *Parthenium hysterophorus* (hereafter referred to as parthenium), which was considered to be the principle source of TSV for the development of disease epidemics in nearby crops (30).

Parthenium is an invasive and prolific weed. Believed to have originated from the area of the Gulf of Mexico or central South America, it has been spread extensively during the 20th century by human activities to many tropical and subtropical regions of the world such as India, Pakistan, Ethiopia, Brazil, Argentina and Taiwan (9, 28, 35, 41). In Australia, parthenium occurs over an extensive region of central Queensland and in isolated pockets in south-east Queensland. Parthenium has also been recorded in small sporadic outbreaks in northern New South Wales (25). Parthenium seed has been shown to account for greater than 50% of seedbanks at some locations in Queensland (26) and buried seed has a germinable half-life of 4-6 years (27, 39).

Given the recent occurrence of serious crop diseases in central Queensland caused by TSV (36) and the extensive distribution of parthenium, this paper aimed to investigate the possible prevalence and diversity of TSV in parthenium populations across its distribution and the possibility of seed transmission.

Materials and Methods

Field surveys of parthenium populations for TSV infection. Parthenium plants were sampled from 31 sites across its range of distribution in Australia (Fig. 1 and Table 1). Sites with a historical record of parthenium were surveyed in northern New South Wales but parthenium plants were not found. Leaf material from between 4 to 100 plants were sampled from each location, stored at 5-10ºC and indexed for TSV within a few days by ELISA, essentially as per the manufacturer's instructions (AGDIA ELISA reagent set, Cat. No. SRA25500/0500). Plants were sometimes bulked in groups of up to 5 plants per extract. Coating antibodies and conjugate were used at 1:500 and conjugate was cross absorbed with a mix of healthy *Helianthus annuus* and *Nicotiana tabacum* cv. Xanthi n.c. in PBS-T. Leaf tissue was extracted at 0.1 g per 1 ml of PBS-T including 2% polyvinylpyrrolidone, with reaction volumes of 50 µl in duplicate. Absorbance values ($A_{410\ nm}$) were measured using a Dynatech MR 7000 ELISA plate reader and values of greater than 3 times the mean of the healthy controls were considered positive.

Test of virus distribution within parthenium plants. Five mature parthenium plants naturally infected with TSV were collected from site 23 north-east of Emerald (Table 1) and plant tissue was tested by ELISA from five different locations on each plant. These included young leaf shoots from two locations: close to the top of the plant and also from a lower branch; flower heads; fully expanded old leaf; and the lower main stem.

Seed transmission tests. Parthenium seeds were collected from four mature plants (seed batches A-D) from site 10 north of Clermont and one plant (batch E) from site 23 north-east of Emerald (Fig. 1). At both locations, seeds were collected from individual plants that were shown to be positive for TSV by ELISA. Another collection of seeds (batch F) from site 10 was bulked from several plants whose virus infection status was unknown but were growing adjacent to a sunflower crop with approximately 50% incidence of TSV-like symptoms. Seed was dried to ambient humidity and stored at room temperature until being grown out in the glasshouse. Individual seedlings were indexed for TSV by ELISA at 3 to 5 weeks post planting, when they were between 10-30 cm tall, with no floral structures present to avoid the possibility of plant to plant contamination from infective pollen. Further controls included growing test seedlings in close proximity to the susceptible hosts mung beans (*Vigna radiata* cv. Emerald), French beans (*Phaseolus vulgaris* cv Bountiful) and tobacco (*Nicotiana tabacum* cv. Xanthi nc) which acted as bait plants to detect any thrips transmission. Glasshouses were routinely spayed with insecticides and no thrips were detected. Where seed transmission of TSV was detected by ELISA, infection was confirmed from a

selection of positive seedlings by mechanical inoculation on to the susceptible indicator hosts mung bean, tobacco and French bean. A further five ELISA positive and six negative seedlings were assayed by TSV-specific PCR as described below.

Test of longevity of TSV in parthenium seed. Individual seedlings were tested from all six batches of seeds (batches A-F above) at between 2½ to 3½ months post collection, seedlings from four of the batches (B, C, D and F above) were grown out again for a second test at between 9½ - 19 months post collection.. Batches C and F were also grown out and tested for a third time at between 20 - 28 months post collection (Table 2). Seed was stored in plastic vials at ambient temperature. Change in seed transmission rates was tested using a Chi-square contingency table for comparison of three sampling times and with Fisher's exact test for one degree of freedom for two sampling times (38).

PCR, sequencing and analysis of TSV isolates. Total RNA was extracted from parthenium leaf tissue using a BioSprint 15 workstation (Qiagen, catalogue number 9000850) with a BioSprint 15 Plant DNA kit (catalogue number 941514) as per the manufacturer's instructions but without the use of RNase A. SuperScript III reverse transcriptase (Invitrogen) was used to prepare cDNA as per the manufacturer's instructions.

Previously published ilarvirus sequences from GenBank (accession NC_003845, X00435, NC_005854 and NC_008706) were used to design a virus sense primer in the movement protein gene of RNA3, TSVmpF1 (5′ CTA TTG AGA AGT CGT GCC TCA A 3′). This was used with a TSV-specific antisense primer, TSVcpR2 (36; 5' CCA CAT CGC ACA CAA GTA TTA C 3'), located 3' of the coat protein gene, to amplify a 1155 bp fragment from each of the six isolates. The fragment contained the entire coat protein gene of 717 nt and partial movement protein gene. PCR was done using 10 pmoles of each primer, 1 unit native *Taq* DNA polymerase (Invitrogen), 1.75 mM $MgCl_2$, 200 mM dNTPs and 2µl of cDNA template in a 25µl reaction volume. Temperature cycling was in a C1000 Thermal Cycler (Bio-Rad) using initial denaturation of 94ºC for 60 s, then 35 cycles of: 94ºC for 15 s, 57ºC for 30 s and 72ºC for 60 s; followed by a final extension of 72ºC for 3 min. PCR products were electrophoresed using an E-gel CloneWell 0.8% SYBR Safe gel (Invitrogen, catalogue number G6618-08) on an iBase (Ethrong Biotechnologies Ltd) as per the manufacturers' instructions and extracted in water. PCR products were directly sequenced with an Applied Biosystems Inc. automated sequencing system. Sequence alignments, dendrograms and genetic distances were produced using the Mega3 software package (22). Nucleotide identity

searches of the GenBank database were done using the Basic Local Alignment Search Tool (BLAST; 3).

Results

Field surveys. TSV was present in parthenium at 25 of the 31 sites tested (Table 1). Within the major area of infestation in central Queensland, TSV was present at 24 out of 26 sites tested (Fig. 1). None of the TSV-infected plants collected displayed virus symptoms. TSV-infected parthenium plants were collected during all seasons of the year and from plants of all ages, from very young seedlings, through to fully mature plants at least several months old. Six field infected parthenium plants, previously shown to be positive for TSV by ELISA were also positive by TSV-specific PCR. These were from locations across the major geographical range of parthenium in Australia. TSV isolate -2012 was collected from site 10, TSV-2077 from site 22, TSV-2084 from site 29, TSV-2087 from site 19, TSV-2103 from site 4 and TSV-2105 from site 1 (Fig. 1 and Table 1).

Collections of TSV-positive seedlings listed in Table 1 suggested possible seed transmission. TSV-infected parthenium plants were also collected from grazing land at several sites that were substantial distances from cropping areas.

Distribution of TSV within parthenium plant. There was no significant difference in ELISA absorbance values from the different plant tissue types tested, though the flowers had the least amount of variation in values and were consistently high. From the five plants tested, $A_{410\ nm}$ absorbance values ranged from 0.260-0.327 for flowers, 0.104-0.298 for young leaves, 0.157-0.388 for old leaves and 0.157-0.274 for the main stem compared to 0.004 for a healthy parthenium control.

Seed transmission and longevity. Seed transmission of TSV was demonstrated from all six batches of seed tested and ranged from 6.8% for batch E to 48% for batch A (Table 2). None of the infected plants displayed any symptoms. ELISA values for infected plants were 4-73 times the averages of the healthy controls with more than 90% of these being higher than 6 times the healthy average. Systemic symptoms typical of TSV infection were observed for all ELISA positive test plants that were mechanically inoculated to the indicator hosts French bean, mung bean and tobacco. All five ELISA positive test plants also tested by TSV-specific PCR produced typical bands of the expected size while all six ELISA negative plants did not produce any bands by PCR.

There was no significant change in the rate of seed transmission over a time period of up to 24 ½ months for the four batches of seed tested on more than one occasion. None of the susceptible bait plants grown next to the parthenium seedlings displayed any symptoms during any of these tests.

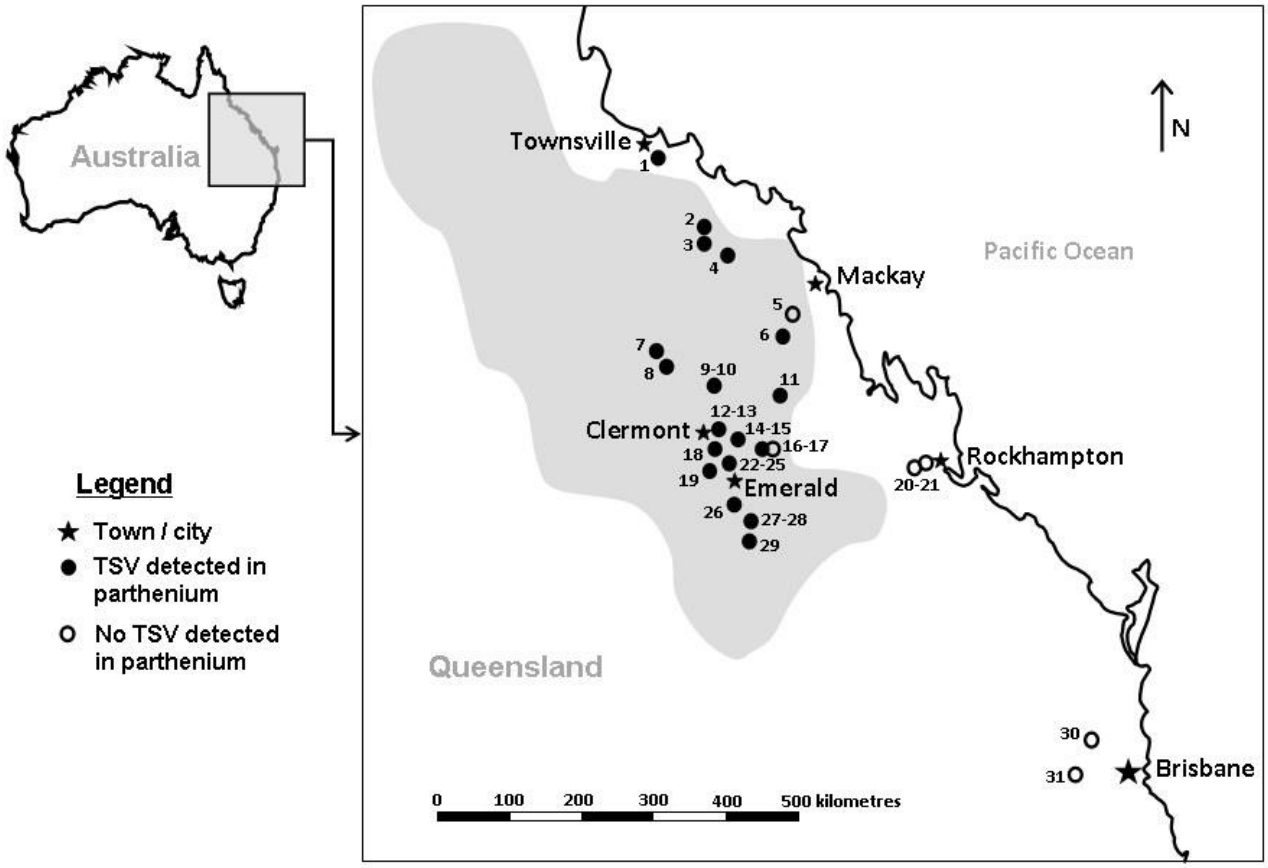

Fig. 1. Distribution of parthenium sampling sites in Queensland, Australia, showing sites where TSV was detected (solid dots) and not detected (open circles). Further details of sampling sites are given in Table 1. Shaded area contains regions with heavy infestations of parthenium, adapted from Navie *et al.* (25).

Table 1. Sample site details and results of TSV testing for *Parthenium hysterophorus* plants.

Site number	Latitude [A]	Longitude	Land use	Age of plants	Collection month	Total individuals	Positive individuals or bulks [B]	Sample bulking [C]
1	-19.39840	146.9484	grazing	mature	October	22	2/22	a
2	-20.46834	147.5928	grazing	mature	October	18	2/18	a
3	-20.51579	147.5925	grazing	mature	October	15	1/15	a
4	-20.65705	147.8613	grazing	mature	October	12	1/12	a
5	-21.52040	148.7789	forest	mature	January	13	0/5	a,b
6	-21.84299	148.6273	grazing	mature	January	24	6/12	c
7	-22.03670	147.1082	grazing	mature	January	39	9/13	b
8	-22.20378	147.2379	cropping	mature	January	33	10/11	b
9	-22.38065	147.6767	cropping	seedlings	August	100	11/20	d
10	-22.40488	147.6951	cropping	seedlings	February	51	16/51	a
10	-22.40488	147.6951	cropping	mature	April	4	4/4	a
11	-22.51065	148.5592	cropping	mature	March	8	1/8	a
12	-22.78142	147.7934	cropping	seedlings	February	40	1/40	a
13	-22.79212	147.7756	cropping	mature	April	56	25/28	c
14	-22.84174	148.0706	grazing	mature	September	10	5/5	c
15	-22.90582	148.0772	cropping	mature	September	11	5/5	b,c
15	-22.90582	148.0772	cropping	seedlings	January	20	0/20	a
15	-22.90582	148.0772	cropping	mature	January	38	2/13	b,c
16	-23.04613	148.3193	cropping	mature	September	21	4/10	b,c
17	-23.05149	148.4679	grazing	mature	September	26	0/13	c
18	-23.05221	147.9269	cropping	mature	September	9	1/9	a
19	-23.34846	147.7053	forest	mature	September	26	2/13	c
20	-23.41017	150.4997	grazing	mature	April	12	0/12	c
21	-23.44035	150.4300	grazing	mature	April	25	0/17	a,c
22	-23.28802	148.0732	cropping	mature	August	9	3/3	b
23	-23.45401	148.3307	cropping	mature	November	30	11/20	a,c
23	-23.45401	148.3307	cropping	mature	February	6	6/6	a

24	-23.47274	148.0807	cropping	mature	January	30	6/10	b
25	-23.48871	148.1449	cropping	mature	February	6	6/6	a
26	-23.76367	148.1063	cropping	various	August	42	4/10	a,d
27	-23.98075	148.3448	cropping	mature	September	19	6/9	a,b,c
27	-23.98075	148.3448	cropping	mature	March	11	11/11	a
28	-24.04109	148.4142	cropping	mature	September	5	2/3	a,c
29	-24.22601	148.3642	grazing	mature	September	17	6/8	b,c
30	-26.94705	152.5000	grazing	mature	May	64	0/32	c
31	-27.55270	152.0969	grazing	mature	May	38	0/19	c

[A] Latitude and longitude are shown as degrees only using the map datum WGS 84.

[B] The number of TSV positive samples and total number of samples tested. A sample may represent one individual plant or a bulk of individual plants.

[C] Samples were either tested as individuals (a), in triples (b), in pairs (c), in bulks of 5 (d) or using combinations of these.

Table 2. Transmission rate of TSV in parthenium seed over time. *P* values are shown to compare rates of transmission over time.

	First test		**Second test**			**Third test**		
Seed batch	**Storage time (months)**	**Proportion of seedlings infected**	**Storage time (months)**	**Proportion of seedlings infected**	**P value [A]**	**Storage time (months)**	**Proportion of seedlings infected**	**P value [B]**
A	2 ½	24/50	n/t [C]	-	-	n/t	-	-
B	2 ½	11/50	11	12/50	0.818	n/t	-	-
C	2 ½	14/50	11	10/32	-	20	19/50	0.558
D	2 ½	19/50	9 ½	11/32	0.728	n/t	-	-
E	2 ½	3/44	n/t	-	-	n/t	-	-
F	3 ½	16/56	19	14/50	-	28	13/50	0.954

[A] *P* value for change in proportion of infected plants over time from the first to second test using Fisher's exact estimate.

[B] *P* value for change in proportion of infected plants over time from the first to third test using standard Chi-square analysis.

[C] n/t = not tested

PCR, sequencing and analysis. The predicted product size of 1155 bp was amplified from all six TSV isolates by PCR. Sequence data for isolate TSV-2012 has been lodged in the GenBank database, accession number EU871659. After removal of primer sequences, the resulting sequence was 1084 bp in length. This includes the 3' terminal 200 nt of the putative movement protein gene, the intergenic region and the complete 717 nt putative coat protein gene. The predicted size of the putative coat protein is 238 amino acids for all six isolates.

The nucleotide sequences of the six TSV isolates from parthenium and the recently published Australian sunflower isolate (TSV-1974; GenBank accession EU375481) were >99.5% identical, and these seven sequences from Australia had >98% identity with a Brazilian TSV isolate reported from soybean (2; AY354406) over a 747 nt overlap. The next closest nucleotide sequence identity by BLAST was approximately 80% between the parthenium isolates and a TSV isolate from white clover, United States of America (USA; NC_003845) over a 1069 nt overlap.

Discussion

At least two accidental introductions of parthenium into Australia have occurred. The first, in 1955, was near Toogoolawah in south-east Queensland, although this infestation has not spread significantly (4). The second, far more invasive introduction occurred in 1958 into central Queensland, north of Clermont, and this introduction is believed to have been from Texas, USA, through the importation of contaminated pasture seed (10, 16, 28). The major area of parthenium infestation now covers an extensive region of central Queensland. Some isolated infestations occur outside this region and its presence has been recorded in southern regions of Queensland and into northern NSW (25; Fig. 1).

Parthenium is an opportunistic weed and given adequate soil moisture is able to germinate, grow and shed prolific numbers of seeds (achens) in less than two months, but will survive for much longer if sufficient moisture is maintained (17, 25). It can complete its life cycle at a wide range of temperatures (39) and will do so at any time of year in Queensland (16).

This is the first record of TSV seed transmission in *P. hysterophorus.* Varying rates of TSV seed transmission in other naturally infected hosts have been reported, including 3% in *Melilotus alba* (19), 1.4-6% in *Phaseolus vulgaris* cv. Pinto (40), 3.8% in *P. vulgaris* cv. Black Turtle Soup (18) and about 6% in *Raphanus raphanistrum* (8). The rates of TSV seed transmission found in naturally infected parthenium in this study are relatively high at between 6.8-48%. The wide variation of

observed rates of transmission in parthenium may be related to the time of infection of the mother plant, as has been observed with other viruses (12),

It is possible that TSV was accidently introduced into Australia via TSV-infected parthenium seed in the mid 20th century from the USA and it has subsequently been spread with the movement of parthenium throughout central Queensland. If TSV did arrive in Australia in parthenium seed, it is unclear why it has only been noticed in crops within the last 10 years when high densities of parthenium have been present in many parts of central Queensland for at least 30 years. There may have been a slow increase in incidence in parthenium over time and only recently has TSV reached high enough incidence to move into, and cause noticeable disease levels in, nearby susceptible crops. Interestingly, TSV was not detected at sites 20, 21, 30 or 31 which are isolated infestations separated from, but believed to have originated from, the major area of infestation in central Queensland (C. McGaw, *personal communication*). This may indicate that these sites became established from a low number of seed from central Queensland which by chance did not contain TSV, or TSV has only recently become widespread in central Queensland parthenium. It is also possible that TSV was accidently introduced into Australia via seed of another host (possibly a crop) recently and has subsequently become established in parthenium. However, the survey results indicated that TSV is randomly distributed throughout the geographical range of parthenium with no obvious association with surrounding land use.

TSV is pollen-borne and transmission of TSV to healthy plants relies on the virus from infected pollen entering plant cells through the feeding injury caused by thrips (33). Surveys of pollen diversity and abundance from several Indian cities indicate the potential for large quantities of parthenium pollen to become airborne and moved significant distances (20, 24, 31). Hence, parthenium is an ideal alternative host for generating TSV disease epidemics as it produces large amounts of pollen which is easily dispersed by wind or thrips. The high rates of seed transmission and long term viability of TSV in parthenium seed demonstrated in this study, along with the ability of parthenium to often dominate seed banks, remain viable for many years (26, 27), and produce large amounts of TSV infected pollen indicates that parthenium plays a critical role in the life cycle of TSV in central Queensland.

The dry tropical climate of Emerald is typical of central Queensland with most of its approximately 600 mm of annual rainfall being received in warm summer months followed by dry, mild winters. Drought periods are not uncommon during which very few alternative host plants of TSV survive and most TSV susceptible crops are only grown in summer. Seed transmission of TSV in

parthenium enables the rapid development of TSV epidemics even after prolonged adverse conditions such as drought and/or harsh winters and provides a link between favourable seasons and cropping cycles. It would also enable the long distance dispersal of TSV into new agricultural regions with the movement of parthenium seed on machinery, livestock or in harvested goods.

Coat protein sequence from six parthenium isolates indicated that only one strain of TSV is present in parthenium across the area of major infestation and this strain is the same as that reported to cause the sunflower necrosis disorder in crops from the same region (36). However, coat protein sequence data for TSV isolates from south-east Queensland indicates a significant difference to the isolates from central Queensland (authors, unpublished results). Thus, while TSV was first reported from south-east Queensland in the early 1970s (13) it is clear there has been a separate introduction and spread of the central Queensland strain of TSV. It is likely that TSV from Brazil and central Queensland share a common origin because strains from central Queensland and Brazil (GenBank AY354406) are very similar to each other but distinct from all other reported TSV strains, including those reported to cause crop diseases from India (5).

Prasada Rao *et al.* (30) suggested that parthenium was a widely distributed alternative host of TSV and that it was crucial for the development of virus epidemics in nearby peanut crops in India. While they found no seed transmission of TSV in sunflower, parthenium seed was not tested. Results presented here warrant the investigation of TSV seed transmission in parthenium from other regions of the world where parthenium and TSV are present, such as India. In the state of Parana, Brazil, *Ambrosia polystachia*, an Asteraceae species, is reportedly an important alternative host associated with soybean bud blight disease caused by TSV (1). The status of TSV seed transmission in *A. polystachia* has not been reported. Interestingly, *Parthenium hysterophorus* also occurs in the state of Parana, Brazil where it is reported to infest soybean crops (11). While no mention of parthenium has been made in the literature in relation to TSV in Brazil, the results of this study suggest parthenium may also be an important host of TSV in that country, particularly considering the Brazilian and central Queensland strains of TSV are very similar by sequence analysis and may share very similar biological characteristics.

The widespread distribution of TSV-infected parthenium and propensity for seed transmission in this host indicates it plays an important role in the epidemiology of TSV in central Queensland. However, several other aspects of the epidemiology of TSV in central Queensland may be important and are under further investigation, including the range of other alternative hosts and the thrips vector species responsible for transmission of TSV into crops. Control strategies may include

the control of parthenium next to cropping areas, crop location based on likely risk, and the use of tolerant or resistant cultivars and barrier crops, some of which are also being investigated in India (30).

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Chapter 4

First report of *Strawberry necrotic shock virus* infecting strawberry (*Fragaria vesca*) from Australia

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First report of *Strawberry necrotic shock virus* infecting strawberry (*Fragaria vesca*) from Australia

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Abstract

The complete coat protein gene sequence is described for three isolates of *Strawberry necrotic shock virus* (SNSV) isolated from strawberry (*Fragaria vesca*) from Australia. Sequences for these isolates were found to have close identity to SNSV isolates from North America. This is the first report of SNSV from Australia.

In Australia, the ilarvirus *Tobacco streak virus* (TSV) was first described from tobacco in the early 1970s (Greber 1971) and has been mostly studied in south-eastern Queensland. An ilarvirus isolated from the M9 clone of strawberry (*Fragaria vesca*) cv. Redlands Crimson, designated TSV-S, was first described by Greber 1979 and Klose *et al.* (1996) suggested that it may have originated from imported North American strawberry clones. Sdoodee (1989) demonstrated serological and host range differences between three strains of "TSV" found in Australia. One notable difference being that while strains TSV-Ag and TSV-A both produced dentate leaves in systemic infections of *Nicotiana tabacum* cv. Turkish (Greber 1971; Sdoodee 1989), TSV-S failed to do so (Greber 1979).

Klose *et al.* (1996) also demonstrated different rates of transmission efficiency between the three strains using different thrips species as vectors.

TSV-S, from Dr Ratana Sdoodee (Sdoodee 1989), was deposited in the Queensland Department of Employment, Economic Development and Innovation plant virus collection as isolate-840, and stored as desiccated leaf tissue in the indicator host *Nicotiana tabacum* cv. Xanthi. Isolates CVIC21 and CVIC22 were sampled from two unknown strawberry cultivars being grown as reference isolates of what was thought to be TSV at the Victoria Department of Primary Industries, Knoxfield laboratories. The origin of these reference plants is unclear but they are likely to have been subsamples of the original M9 clone from Queensland.

For isolate-840, total RNA was extracted from desiccated leaf tissue using a BioSprint 15 workstation (Qiagen) with a BioSprint 15 Plant DNA kit (Qiagen) as per the manufacturer's instructions, but without the use of RNase A. For isolates CVIC21 and CVIC22, total RNA was extracted using using the RNeasy Plant Mini kit (Qiagen, catalogue number 74904) with a modified lysis buffer (MacKenzie *et al.* 1997).

For PCR amplification of the complete coat protein gene from the Victorian isolates CVIC21 and CVIC22, primer SNSV CPbeg F (Tzanetakis *et al.* 2004) was used with primer TSVcpR4 (5' CGG ATG CGY GGY ARC TAT GCA T 3') which was designed to a conserved region of previously published sequences for TSV, *Strawberry necrotic shock virus* (SNSV) and *Parietaria mottle virus* (PMoV) (GenBank accessions NC_003845, AY363228 and NC_005854, respectively). The SuperScript One-Step RT-PCR System (Invitrogen) was used as per the manufacturer's instructions except the total reaction volume was 25 µl. A product of 930 bp was amplified from each isolate. These products were cloned using the pGEM-T Easy Vector system (Promega) as per the manufacturer's instructions. Three clones each of isolates CVIC21 and CVIC22 were sequenced in both directions and after removal of primer sites, the 883 nt consensus sequences were lodged with GenBank (accessions JF781587 and JF781588).

To obtain the complete coat protein gene of Queensland isolate-840, SuperScript III reverse transcriptase (Invitrogen) was used to prepare cDNA with primer TSVcpR4 as per the manufacturer's instructions followed by PCR with primers TSVmpF1 (Sharman *et al.* 2009) and TSVcpR4 at an annealing temperature of 57°C. A product of 1273 bp was amplified. PCR products were either electrophoresed using an E-gel CloneWell 0.8% SYBR Safe gel (Invitrogen) on an iBase (Ethrong Biotechnologies Ltd) as per the manufacturers' instructions and extracted in water, or purified from a 0.5 × TBE agarose gel using a Qiaquick PCR purification kit (Qiagen). PCR

products for isolate-840 were directly sequenced in both directions with an Applied Biosystems Inc. automated sequencing system at the Australian Genome Research Facility, Brisbane, and after removal of primer sites, the 1229 nt sequence was lodged with GenBank (JF781586). Sequence alignments, dendrograms and genetic distances were produced using *MEGA* version 5 (Tamura *et al.* 2011). Nucleotide identity searches of the GenBank database were done using the Basic Local Alignment Search Tool (BLAST; Altschul *et al.* 1990).

The nucleotide sequences of isolate-840, CVIC21 and CVIC22 were >99.5% identical to each other over a 883 nt overlap and >99% identical over the 222 amino acid overlap of the putative coat protein. The next two closest nucleotide sequence identities with Queensland isolate-840, from GenBank by BLAST, were with isolates of SNSV; 99% over a 669 nt overlap with an isolate from Mississippi, United States of America (USA; AY363233), and then 92% over a 1227 nt overlap with an isolate from Maryland, USA (AY363228). When compared with other published putative coat protein gene sequences (Fig. 1) the Australian isolates of SNSV are most closely related to isolates from Mississippi and Louisiana, USA.

This is the first report of SNSV from Australia and confirms that Queensland isolate-840, which was previously referred to as a strain of Tobacco streak virus, TSV-S (Greber 1979; Klose *et al.* 1996; Sdoodee 1989), is SNSV. The close nucleotide identity of the Australian SNSV isolates with isolates from Mississippi, USA, is in agreement with the suggestion by Klose *et al.* (1996) that strain TSV-S may have originated from North American strawberry plants introduced into Australia. It appears likely that the Victorian isolates of SNSV described here represent sub-samples of the original Queensland isolate-840. However, SNSV has not been found to naturally infect other host plants in Australia.

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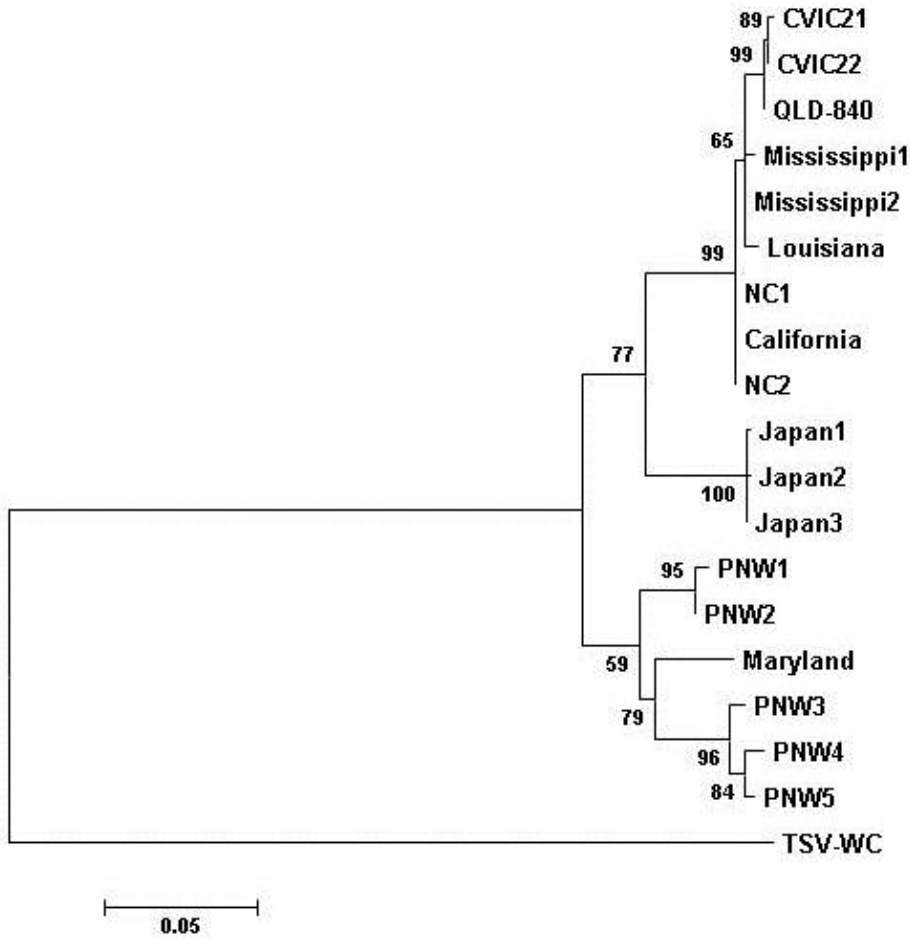

Fig. 1. Maximum Likelihood dendrogram, based on the Kimura 2-parameter model, illustrating coat protein gene nucleotide sequence relationships of SNSV isolates. A discrete Gamma distribution was used to model evolutionary rate differences among sites. Horizontal distances are proportional to nucleotide substitutions per site, and the dendrogram was bootstrapped 500 times with branch values greater than 50% indicated. The scale bar represents the number of nucleotide substitutions per site. TSV was used as an out-group to root the phylogenetic tree. Abbreviations and GenBank accession numbers used are: CVIC21 (JF781587), CVIC22 (JF781588), QLD-840 (JF781586), Mississippi-1-2 (AY363232, AY363233), Louisiana (AY363234), NC1-2 (North Carolina; AY363235, AY363237), California (AY363236), Japan1-3 (AY363229, AY363230, AY363231), PNW-1-5 (Pacific North West, USA; AY363238, AY363239, AY363240, AY363241, AY363242), Maryland (AY363228) and TSV-WC (NC_003845).

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Chapter 5

Genetic diversity of subgroup 1 ilarviruses from eastern Australia

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Genetic diversity of subgroup 1 ilarviruses from eastern Australia

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Abstract

This is the first report of the genetic diversity within subgroup 1 ilarviruses from eastern Australia. It supports the separation of *Tobacco streak virus* (TSV) strains from parthenium (*Parthenium hysterophorus*) and crownbeard (*Verbesina encelioides*) based on serology and host specificity, has confirmed one previously described strain of TSV as an isolate of *Strawberry necrotic shock virus* and has identified a new subgroup 1 ilarvirus, Ageratum latent virus (AgLV) from *Ageratum houstonianum*. All had been previously identified as strains of TSV. A multiplex RT-PCR showed that the genetically distinct strains of TSV and AgLV were commonly found as symptomless infections in virus-specific alternative weed hosts growing over a wide geographical range in eastern Australia. TSV has been one of the most damaging viruses in Australian oilseed and pulse crops in recent years and this study has provided the taxonomic knowledge essential for the development of control programs for these viruses.

Introduction

Ilarviruses (family: *Bromoviridae*) have a positive sense single-stranded RNA genome consisting of three linear segments (RNA-1 to -3). The 1a (viral replicase) protein is encoded by RNA-1 and the 2a (RNA dependent RNA polymerase) and 2b proteins are encoded by RNA-2. The 3a (cell-to-cell movement) protein (MP) and 3b coat protein (CP) are encoded by RNA-3, although the CP is translated from the sub-genomic RNA-4, which is derived from RNA-3 [1].

The demarcation criteria for ilarvirus species combine serological relatedness, host range and genome sequence similarity [1], although there is currently no specified level of sequence similarity to distinguish species, strains or subgroups. Ilarvirus species have been assigned to subgroups based on serological relatedness and strains of ilarvirus species have also been reported based on serological differences, host specificity or geographical location. Members of subgroups 1 and 2 have a unique second open reading frame (ORF) on RNA-2 for the putative 2b protein which is absent from other ilarvirus subgroups. Recent phylogenetic studies on ilarvirus members have suggested greater emphasis on sequence identity in RNA-3 coding regions [2], RNA-1 and RNA-2 sequence [3] or whole proteome (all putative proteins) analysis [4] for defining species.

Tobacco streak virus (TSV), the type species for the genus *Ilarvirus*, was identified in 1936 [5] and was the only described species within subgroup 1 until the mid 1990s when the development of genome sequencing techniques resulted in the recognition of several additional species. The type isolate of TSV was originally isolated from white clover (TSV-WC; [6]). Species within subgroup 1 include TSV, *Strawberry necrotic shock virus* (SNSV), *Parietaria mottle virus* (PMoV), *Blackberry chlorotic ringspot virus* (BCRV) [7] and the proposed possible member Bacopa chlorosis virus [8]. Two subgroup 1 species have been previously reported from Australia: TSV [9] and more recently SNSV [10].

In Australia, TSV was first described in the early 1970s as the cause of a sporadically occurring disease of commercial tobacco crops in south-eastern Queensland [9]. Three distinct strains of TSV have been described from Australia and were shown to differ in relation to serology, host range and thrips species transmission efficiency [9,11-13]. The most commonly reported strain was originally referred to as the Asclepias strain (TSV-As; [13]) and later as the Ageratum strain (TSV-Ag; [12]) due to its high incidence in *Ageratum houstonianum* (ageratum) near TSV affected tobacco crops [14]. A strain originally isolated from strawberry, TSV-S [11,13], has recently been demonstrated to actually be SNSV [10]. A third strain, TSV-A, was isolated from *Ajuga reptans* in Melbourne,

Victoria in the late 1970s and was shown to be serologically distinct from strain TSV-Ag [15]. However, no further reports of this TSV-A strain have been made and it is unclear if it is still present in Australia. Similarly, TSV-S (SNSV) has recently been reported from reference collections but has not been reported in natural infections since the late 1970s [10].

We recently reported a TSV strain from central Queensland causing significant economic losses in sunflower and mung bean crops over several seasons [16]. This TSV strain (hereafter referred to as TSV-parthenium) was commonly found in asymptomatic *Parthenium hysterophorus* and this prolific weed is considered to be a major source of inoculum for disease epidemics in surrounding crops [17]. Partial RNA-3 sequence of the TSV-parthenium strain indicated it was most closely related to a soybean TSV isolate from Brazil [18] but preliminary results demonstrated TSV-parthenium was genetically divergent from TSV-Ag from south-east Queensland. In the course of the present work, we found a further distinct TSV strain commonly infecting *Verbesina encelioides* (crownbeard), hereafter referred to as TSV-crownbeard. These results suggested there was significant diversity in TSV strains present in eastern Australia. With the recent discovery of the TSV-parthenium and TSV-crownbeard strains, there were a total of five distinct ilarvirus subgroup 1 members, which could constitute TSV strains or other ilarvirus species. Partial RNA-3 sequence was published for only SNSV (TSV-S) [10] and TSV-parthenium [17].

The main focus of this paper is to describe the genetic diversity of subgroup 1 ilarviruses from eastern Australia and in doing so clarify the taxonomy of a range of strains previously reported as TSV. Serological and host data are used to support conclusions made from the phylogenetic analyses.

Materials and Methods

Virus isolates. Leaf material was collected between 2006 and 2010 from many locations in central and south-east Queensland and northern New South Wales (Table 1). Samples were either collected from locations isolated from cultivated areas or close to crops affected by TSV. The isolates -1973 from parthenium, -1998 from ageratum, and -2334 from crownbeard were selected for further detailed investigation as typical representatives of distinct ilarvirus strains from the three commonly found weed hosts. They were propagated and maintained in *N. tabacum* cv. Xanthi plants in the glasshouse for use as diagnostic controls and for additional characterization experiments. All isolate

numbers refer to samples lyophilised and stored at -20ºC in the Queensland Department of Agriculture, Fisheries and Forestry plant virus collection.

ELISA. Fresh or lyophilised samples were tested for TSV by ELISA (AGDIA ELISA reagent set, Cat. No. SRA25500/0500) as per the manufacturer's instructions with minor modifications. Coating antibodies and conjugate were used at 1:500 and conjugate was cross absorbed with a mix of healthy *Helianthus annuus* and *Nicotiana tabacum* cv. Xanthi, each diluted 0.1 g per 10 ml of PBS-T. Leaf tissue was extracted at 0.1 g per 1 ml of PBS-T containing 2% polyvinylpyrrolidone, with reaction volumes of 50 or 100 µl in duplicate. Absorbance values ($A_{410\ nm}$) were measured using a Thermo Electron Corporation Multiskan EX plate reader and values of greater than three times the mean of the healthy controls were considered positive.

Polymerase chain reaction (PCR). To design PCR primers (Table 2), previously published RNA-1, -2, and -3 sequences for TSV-WC, SNSV-MD and PMoV (Table 3) were aligned using the MUSCLE algorithm [19] and conserved regions selected by eye. Various combinations of primer pairs were used to amplify overlapping fragments from the four reference isolates characterised (see below).

Total Nucleic Acid Extracts (TNAEs) were prepared from lyophilised leaf tissue using the BioSprint 15 workstation (Qiagen, Cat. No. 9000850) with a BioSprint 15 Plant DNA kit (Cat. No. 941514) as per the manufacturer's instructions but without the use of RNase A in the RLT buffer. SuperScript III reverse transcriptase (Invitrogen) was used to prepare cDNA as per the manufacturer's instructions. PCR was done using 10 pmoles of each primer, 1 unit native *Taq* DNA polymerase (Invitrogen), 1.75 mM $MgCl_2$, 200 mM dNTPs and 2µl of cDNA template in a 25µl reaction volume. Due to the large number of combinations of different primer pairs and templates, generic ramped annealing temperature cycling parameters [20] were used for as many primer pairs as possible, consisting of an initial denaturation of 95ºC for 60 s, then 35 cycles of: 95ºC for 20 s, 62ºC for 20 s, 56ºC for 10 s and 72ºC for 45 s; followed by a final extension of 72ºC for 3 min on a C1000 Thermal Cycler (Bio-Rad).

Table 1. Details for virus isolates used in this study.

Isolate number [A]	Host species	Collection year	Nearest town / locality	Collection location (Lat' Long')	Ilarvirus strain / species by multiplex RT-PCR
835	*Xanthium occidentale*	1975	Airdmillan	19.528° S 147.472° E	TSV-crownbeard
837	*Gomphocarpus physocarpus* [E]	1987 [C]	Brookfield	27.493° S 152.910° E	AgLV
840 [B]	*Fragaria ananassa*	1979 [C]	Redlands	27.525° S 153.250° E	n/a [D]
1025	*Ajuga reptans* [F]	1978	Melbourne	37.79° S 144.95° E	n/a
1973 [B]	*Helianthus annuus*	2006	Clermont	22.77200° S, 147.78888° E	TSV-parthenium
1998 [B]	*Ageratum houstonianum*	2006	Mt Glorious	27.331820° S, 152.767317° E	AgLV
2012	*P. hysterophorus*	2007	Clermont	22.40816° S, 147.68428° E	TSV-parthenium
2084	*P. hysterophorus*	2007	Springsure	24.22601° S, 148.36423° E	TSV-parthenium
2086	*P. hysterophorus*	2007	Tieri	23.04613° S, 148.31934° E	TSV-parthenium
2087	*P. hysterophorus*	2007	Rubyvale	23.34846° S, 147.70531° E	TSV-parthenium
2103	*P. hysterophorus*	2007	Colinsville	20.46834° S, 147.59282° E	TSV-parthenium
2105	*P. hysterophorus*	2007	Alligator Creek	19.39840° S, 146.94844° E	TSV-parthenium
2139	*P. hysterophorus*	2008	Nebo	21.84299° S, 148.62732° E	TSV-parthenium
2140	*P. hysterophorus*	2008	Mt Coolon	22.03670° S, 147.10818° E	TSV-parthenium
2282	*V. encelioides*	2008	Theodore	24.93518° S, 150.01425° E	TSV-crownbeard
2291	*A. houstonianum*	2008	Woodburn	29.098083° S, 153.340302° E	AgLV
2295	*V. encelioides*	2008	Emerald	23.48702° S, 148.06104° E	TSV-crownbeard
2333	*P. hysterophorus*	2009	Injune	Unknown	TSV-parthenium
2334 [B]	*Verbesina encelioides*	2008	Emerald	23.51936° S, 148.29465° E	TSV-crownbeard
2338	*V. encelioides*	2009	Springsure	24.16183° S, 148.43636° E	TSV-crownbeard

2339	*V. encelioides*	2009	Gindie	23.85828° S, 148.23199° E	TSV-crownbeard
2396	*V. encelioides*	2009	Springsure	24.02136° S, 148.51714° E	TSV-crownbeard
2400	*V. encelioides*	2009	Duaringa	23.63383° S, 149.92381° E	TSV-crownbeard
2403	*A. houstonianum*	2009	Yeppoon	23.09433° S, 150.73456° E	AgLV
2514	*P. hysterophorus*	2009	Moura	24.63083° S, 149.50124° E	TSV-parthenium
2558	*A. houstonianum*	2010	Woolgoolga	30.0839° S, 153.1844° E	AgLV

[A] Isolate number from the Queensland Department of Agriculture, Fisheries and Forestry plant virus collection.

[B] Isolate used for full genome characterisation in this study. See Table 4 for further details.

[C] The collection year is unknown so the first record of publication is shown

[D] Not available or not tested by MP-RT-PCR.

[E] Isolate-837 was originally referred to as strain TSV-As [13].

[F] Isolate-1025 was originally referred to as strain TSV-A [15].

Reference isolates selected for whole genome characterisation. Although isolate TSV-1973 was originally collected from sunflower displaying typical TSV symptoms (Table 1), preliminary partial RNA-3 sequence demonstrated it was a member of the strain found commonly in parthenium isolates [17] and as such TSV-1973 was retained as a reference isolate for the TSV-parthenium strain. Isolates -2334 (TSV-crownbeard) and -840 (SNSV; TSV-S) were selected for characterisation. Isolate-1998 was selected as the reference isolate for the proposed new subgroup 1 ilarvirus species, "Ageratum latent virus" (AgLV) previously reported as strain TSV-Ag.

Table 2. PCR primers for identification of TSV strains, AgLV and SNSV and primers used in strain-specific multiplex RT-PCRs

Target region	Primer name	Sequence (5' to 3')
RNA-1	TSVRNA1_471R	GGGCAGCARGAATGMACATT
	TSVrepF2	CAGKAATGGTGATTTYCARAA
	TSVrepR1	GTAACRGCTAAWGGAACATATT
	TSVrep1080F	GTRCCGARTGGTWTACBGAA
	TSVrep2769R	GGAACTTGCTCKGTRTCACCAA
	TSVrep2756F	ACVGAGCARGTTCCYTTCAT
	TSVrep3000F	CACACACGMGAGCWGATAA
RNA-2	TSVpol795R	CMACCCAYTCYTGGAARAAT
	TSVpolF1	CACCTTGAAGCCWGTSGAAGA
	TSV2b2451R	CCAGCACARTCAATGCAHTT
	TSVpol2432F	CAAAWTGCATTGAYTGTGC
	TSV2b2478F	CTCHGTSAAGGTDCCGAAGT
RNA-3	TSVRNA3_506R	GCRAYRGCRTGDGTGGTRTT
	TSVRNA3_1F	GCGCCGWATTCTCYGAGC
	TSVRNA3_1251R	GCCAGACATGRCGTTGGAT
	TSVmpF1	CTATTGAGAAGTCGTGCCTCAA
	TSVRNA3.1982R	CCRCATCKCACACARGWATT
	TSVcp1789F	GCWRTYGTBTGGTGYCTCGA
TSV-parthenium (RNA-3)	CQTSVF	CCTACTCCAACCCTGATTA
TSV-crownbeard (RNA-3)	CrbTSVF	GCCCGTTTACCAGTACCAAT
AgLV (RNA-3)	SEQTSVF	CGCCATGTCTACTTCTAGGA
5' and 3' ends	AdaptR1	CTGTCTCGGCAATATATGGGAT
5' and 3' ends	AdaptR2	GCAATATATGGGATTTCCGTT

Multiplex RT-PCR for segment RNA-3. To differentiate TSV-parthenium, TSV-crownbeard and AgLV and to enable identification of mixed infections, a multiplex RT-PCR was developed. A degenerate reverse primer (TSVRNA3.1982R) was used for cDNA synthesis for all strains and then combined with strain-specific forward primers for PCR. To test if the multiplex RT-PCR could detect mixed infections of these ilarvirus strains, artificial mixes were made by combining TNAEs in roughly equal proportions prior to cDNA synthesis. PCR mix and temperature cycling were as described above but with the following optimised mix of primers: 200 nM of TSVRNA3.1982R, 300 nM of CQTSVF, 100 nM of SEQTSVF and 80 nM of CrbTSVF (Table 2).

5' and 3' terminal sequencing. The 5' and 3' termini of each RNA segment for each of the reference isolates, -1973, -1998, -2334 and -840 were determined utilising a single oligo, Adaptor2 (5'-PO_4-CGACATAACCTTGCACATGACTCGAACGGAAATCCCATATATTGCCGAGACAG-3') with a 3' C-3 spacer to stop self ligation. Adaptor2 was either ligated directly to the 3' terminus of RNA molecules, including viral genomic RNA or to the 3' end of cDNA made to the 5' end of genomic RNA. Ligation reactions were modified from previously described methods [21,22].

For the 5' terminus, cDNA was prepared using primers located about binding 500 nt from the predicted 5' terminus and with 7 µl of TNAE. To remove excess ssRNA and RNA from the RNA/cDNA duplex, 1 U of RNase A (Qiagen) and 2 U of RNase H (Epicentre) was added to the cDNA reaction for 30 min at 37ºC followed by 10 min at 65ºC to inactivate the enzymes. To remove unincorporated primer, the reaction was cleaned using a PCR purification kit (Qiagen) as per the manufacturer's instructions and eluted in 30 µl of 10 mM Tris-Cl pH 8.5. Ligations were done at 24ºC for 18 h in 1 × T4 DNA ligase buffer containing a final concentration of 1 mM ATP (New England Biolabs; Cat. No. B0202S), 25% (wt/vol) PEG 8000, 10 µg/ml of bovine serum albumin, 10 pmoles of Adaptor2 oligo, 10 U T4 RNA ligase (Epicentre), 1 mM Hexammine cobalt(III) chloride (Sigma-Aldrich) and made up to a reaction volume of 35 µl with cleaned cDNA. Ligations were terminated at 65ºC for 10 min. The preparation was then used in PCR with a primer complementary to the Adaptor2 oligo, AdaptR1 (Table 2), and a virus specific downstream primer, preferably internal to the primer used for cDNA synthesis. Nested PCR using a second virus-specific primer and AdaptR2 was sometimes required to obtain a distinct PCR band.

For the 3' terminus, ligations were carried out as described above but with 20 pmoles of Adaptor2 oligo, 15 U T4 RNA ligase and reaction made up to volume with TNAE. Following ligation termination, unincorporated Adaptor2 oligo and enzyme were removed by passage through a

Sephadex G15 column with elutions made in H_2O and 1 µl then used in cDNA synthesis reactions with primer AdaptR1. Resulting cDNA was used in PCR as described above.

In the case of RNA-3 for SNSV-840 the above method did not work for obtaining the 3' terminal sequence and polyadenylation of the 3' end was carried out using *E. coli* Poly(A) Polymerase (Ambion, Cat. No. AM1350) as per the manufacturer's instructions, followed by cDNA synthesis with primer Poty 1 [23].

Sequencing and analysis. PCR products were analysed by electrophoresis on 0.5 × TBE agarose or using an E-Gel® CloneWell™ 0.8% SYBR Safe™ gel on an E-Gel® iBase™ Power System (Invitrogen, Cat. No. G6618-08 and G6400) as per the manufacturers' instructions and extracted in water. PCR products were directly sequenced using an Applied Biosystems Inc. automated sequencing system (Australian Genome Research Facility, Brisbane), to provide at least two-fold coverage of the target regions.

A representative selection of complete genome sequences of currently recognised ilarvirus subgroup 1 members [7] including TSV, SNSV, BCRV and PMoV, along with isolates characterised in the present work, were used for phylogenetic analyses (Table 3). The tentative subgroup 1 member, Bacopa chlorosis virus (BaCV) was also included in the analysis for the coat protein. Nucleotide and putative amino acid sequences were aligned using the MUSCLE algorithm [19] included in the MEGA5 software package [24]. MEGA5 was also used to calculate pairwise uncorrected genetic distances between sequences in the alignments. For nucleotide alignments, putative ORFs were translated into proteins, aligned by MUSCLE and returned to the original nucleotide sequence. Non-coding regions were aligned by MUSCLE and resulting nucleotide alignments were used for phylogenetic analysis. *Spinach latent virus* (SpLV) and *Prunus necrotic ringspot virus* (PNRSV) were included as out groups from ilarvirus subgroups 2 and 3 respectively (Table 3).

Phylogenetic relationships were inferred using the Maximum Likelihood method as implemented in RaxML [25] and PhyML 3.0 [26]. GTR-GAMMA was specified as the model of evolution in both programs. The RaxML analyses were run with a rapid bootstrap analysis using a random starting tree and 1000 Maximum Likelihood bootstrap replicates. The PhyML analyses were implemented using the ATGC bioinformatics platform (available at: http://www.atgc-montpellier.fr/phyml/), with SPR and NNI tree improvement, and support obtained from an approximate likelihood ratio test [27]. For analysis of the complete genome nucleotide sequence, the three complete RNA segments were included as separate partitions in a Maximum Likelihood analysis so each locus could be run

under different optimal model parameters. A similar method was used for protein analysis (whole proteome) with the five putative proteins (loci) included as separate partitions in a Maximum Likelihood analysis. Resulting trees were observed with FigTree (available at http://www.tree.bio.ed.ac.uk/software/figtree/). Nucleotide identity searches of the GenBank database were done using the Basic Local Alignment Search Tool (BLAST; [28]). Recombination analysis was carried out using the program RDP3 with the default parameters [29].

Table 3. Ilarvirus members included in the study and their accession numbers for RNA segments.

Virus-isolate	RNA-1	RNA-2	RNA-3
TSV-WC	NC_003844	NC_003842	NC_003845
TSV-2012	- [A]	-	EU871659 [B]
TSV-1974	-	-	EU375481 [B]
TSV-1973	JX463334	JX463335	JX463336
TSV-2334	JX463337	JX463338	JX463339
TSV-1025	JX463345	JX463346	JX463347[B]
TSV-835	-	-	JX463349[B]
TSV-Brazil	-	-	AY354406 [B]
AgLV-1998	JX463340	JX463341	JX463342
AgLV-837	-	-	JX463348[B]
SNSV-840	JX463343	JX463344	JF781586
SNSV-MD	NC_008708	NC_008707	NC_008706
BCRV	DQ091193	DQ091194	NC_011555
PMoV	NC_005848	NC_005849	NC_005854
SpLV	NC_003808	NC_003809	NC_003810
PNRSV	NC_004362	NC_004363	NC_004364

[A] There is no sequence data published for this RNA segment.

[B] These GenBank accessions are partial for segment RNA-3.

Results

Field surveys and identification by ELISA and multiplex RT-PCR. All isolates listed in Table 1 were positive by TSV ELISA and all ELISA-positive samples of *P. hysterophorus*, *A. houstonianum* and *V. encelioides* were symptomless. When the reference isolates -1973 (TSV-parthenium), -2334 (TSV-crownbeard) and -1998 (AgLV) were maintained in tobacco, consistent

differences in relative A_{410nm} values by TSV ELISA were observed, suggesting serological variability. From three independent tests, A_{410nm} values were 17 to 21 times the average of the healthy controls for isolate-1998 (AgLV), 329 to 726 times for isolate-1973 (TSV-parthenium) and 740 to 1113 times for isolate-2334 (TSV-crownbeard). The relatively low A_{410nm} values for isolate-1998 were similar to that of SNSV-840 which was nine times the average healthy control in a single test.

Using the multiplex RT-PCR, products of unique size were amplified (Fig. 1a) from the reference samples of TSV-parthenium (isolate-1973, 921 bp), AgLV (isolate-1998, 743 bp) and TSV-crownbeard (isolate-2334, 571 bp). The three strains were found in many samples across a wide geographical area, each spanning several hundred kilometres from north to south, apparently coinciding with, and possibly endemic throughout, the geographical range of their respective alternative host (Table 1). The TSV-parthenium strain was never found in crownbeard nor visa versa even though both strains were found in central Queensland, often occurring in the same locations. The distribution of AgLV had no overlap with the TSV strains, and was found in ageratum which occurs in higher rainfall areas mostly east of the Great Dividing Range along the eastern seaboard of Australia. While no natural mixed infections of the three strains were found, all were detected by multiplex RT-PCR in artificial mixes although there was a moderate decrease in amplification of AgLV when all three strains were combined (Fig 1b). A summary of the multiplex RT-PCR results are shown in Table 1 and gel electrophoresis results for a selection of the parthenium, ageratum and crownbeard samples are shown in Fig 1a.

Characterisation of complete genomes for representative isolates. The PCR primers (Table 2) for RNA-1, -2 and -3 worked for all four reference isolates characterised: isolate-1973, -1998, -2334 and -840. The use of the single oligo, Adaptor2, to attach to both 5' and 3' ends was an effective, low cost means for determining the terminal sequences as described. The option of A-tailing the 3' end by polyadenylation was also effective for templates that were problematic with the first method such as the 3' end of RNA-3 for isolate-840. Complete nucleotide (nt) sequence of RNA-1, -2 and -3 was determined for isolates -1973, -1998, -2334 and -840, and partial RNA-1, -2 and -3 for isolate-1025. Partial RNA-3 sequences were also determined for archived isolates -837 and -835 with GenBank accessions shown in Table 3. For the four isolates for which complete genomes were determined, a summary of genome features and a comparison of amino acid (aa) identity with the type ilarvirus species, TSV-WC, are shown in Table 4.

Table 4. Organisation of complete genome information for RNA-1, RNA-2 and RNA-3 of TSV strains, AgLV and SNSV characterised in this study and comparison of aa identity with type ilarvirus species TSV-WC.

	TSV-1973	**TSV-2334**	**AgLV-1998**	**SNSV-840**
RNA-1				
Total length (nt)	3512	3481	3481	3474
1a gene position (nt)	38-3322	38-3322	35-3328	38-3319
1a comparative identity [A]	87%	92%	70%	76%
1a protein Mr (kDa) [B]	123.1	123.4	123.4	123.9
RNA-2				
Total length	2922	2901	2872	2876
2a gene position	39-2462	40-2415	35-2407	36-2447
2a comparative identity	84%	91%	73%	75%
2a protein Mr	92.5	90.1	90.9	92.1
2b gene position	2152-2805	2150-2758	2157-2759	2155-2790
2b comparative identity	79%	85%	65%	61%
2b protein Mr	24.0	22.1	22.1	23.6
RNA-3				
Total length	2215	2208	2224	2249
3a gene position	212-1087	212-1084	214-1098	216-1112
3a comparative identity	81%	92%	79%	80%
3a protein Mr	32.0	31.7	32.0	32.0
3b gene position	1212-1928	1208-1921	1225-1932	1289-1957
3b comparative identity	86%	96%	75%	66%
3b protein Mr	26.2	26.3	26.0	24.6

[A] Comparison of amino acid identity for the complete putative protein to the type ilarvirus species TSV-WC.
[B] Molecular mass predicted from ExPASy website http://web.expasy.org/compute_pi/

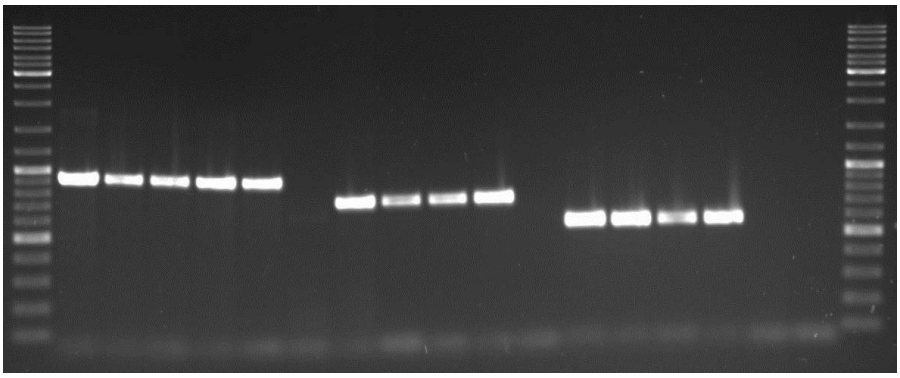

Fig. 1a. Multiplex RT-PCR for segment RNA-3 of TSV-parthenium, TSV-crownbeard and AgLV. Lanes 1 to 6 are TSV-parthenium isolates-1973, -2084, -2087, -2103, -2105 and healthy parthenium. Lanes 7 to 11 are AgLV isolates-1998, -2291, -2403, -2558 and healthy ageratum. Lanes 12 to 17 are TSV-crownbeard isolates-2334, -2282, -2338, -2400, healthy crownbeard and water control. Marker lanes are 100bp ladder (Fermentas).

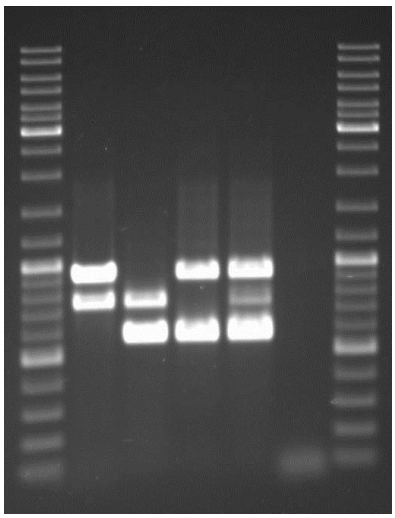

Fig. 1b. Multiplex RT-PCR for segment RNA-3 of mixed TSV-parthenium (isolate-1973), TSV-crownbeard (isolate-2334) and AgLV-1998. Lane 1, TSV-parthenium and AgLV; lane 2, AgLV and TSV-crownbeard; lane 3, TSV-parthenium and -crownbeard; lane 4, TSV-parthenium and -crownbeard and AgLV; and lane 5, water control. Marker lanes are 100bp ladder (Fermentas).

RNA-1 of the four completely sequenced isolates contained the putative ORF for the 1a (replicase) protein within which the methyl transferase-like and helicase-like signatures were found [30,31]. RNA-2 contained the putative 2a (RdRp) protein, with the conserved polymerase signature [30], including the region ASGDDSLI, highly conserved among ilarviruses. RNA-2 also contained the putative 2b protein. RNA-3 contained the putative ORFs for the 3a MP and 3b CP. The conserved folded stem-loop structures and AUGC-like motifs at the 3' end of each RNA segment [32] were identified for the characterised isolates. Using the motif numbering proposed by Bol [32], motif 2 was highly conserved as AUGC for every RNA segment characterised while motif 1 was found to be AUGC or AAGC. Motif 3 was most variable but with conserved U and C at the third and first nucleotide as (A/G/U)U(A/G/U)C. Isolate-1998 was unique in having UUUC for motif 3 of each RNA segment.

Phylogenetic analysis. The phylogram produced using Maximum Likelihood analysis with RaxML for the complete nt genome sequence of the newly characterised TSV isolates-1973 and -2334, AgLV-1998 and SNSV-840, and other ilarvirus species showed a number of strong relationships (Fig 2). The topology of this phylogram was strongly supported by Maximum Likelihood analysis with RaxML for the complete proteome sequence of the same samples and also by Maximum Likelihood analyses with PhyML for the separate complete RNA-1 and RNA-2 nt sequences. The recognised subgroup 1 species formed a well supported clade that was separate from the subgroup 2 and 3 out group representatives, SpLV and PNRSV. AgLV-1998 was sister to PMoV within the subgroup 1 clade but only has 69-71% total genome nt identity with PMoV, SNSV or TSV-WC. By comparison, recognised subgroup 1 species BCRV and SNSV share 81% total genome nt identity. The phylogram produced for the complete RNA-3 nt sequence using Maximum Likelihood analysis with PhyML showed a slightly different topology with the BCRV / SNSV clade grouping with the AgLV / PMoV clade instead of the TSV clade (data not shown).

Of the complete genome sequences presented in this study, TSV-2334 (TSV-crownbeard) was the most closely related to the type species TSV-WC (Fig 2), with a total genome nt identity of 88%. TSV-2334 had 81% total genome nt identity with TSV-1973. Partial RNA-3 sequence for isolate-835 had 98% nt identity with TSV-2334 over a 1141 nt overlap demonstrating that the TSV-crownbeard strain has been present in Australia since at least 1975 when isolate-835 was collected.

TSV-1973 (TSV-parthenium) along with isolates-1974 and -2012 from related studies [17,16] and a Brazilian isolate, TSV-BR [18] share very high identity (99-100%) for partial RNA-3 sequence over a 762 bp overlap and appear to all be isolates of a genetically divergent strain of TSV. The complete

RNA-1, -2 and -3 sequences of TSV-1973 had relatively low nt identity (closest matches 77%-81%) to all other published complete RNA-3 TSV sequences (data not shown) including the type isolate TSV-WC. All phylogenetic analyses (Fig 2) and putative aa identities (Table 4) indicated TSV-1973 is distinct from the type species TSV-WC (Fig 2).

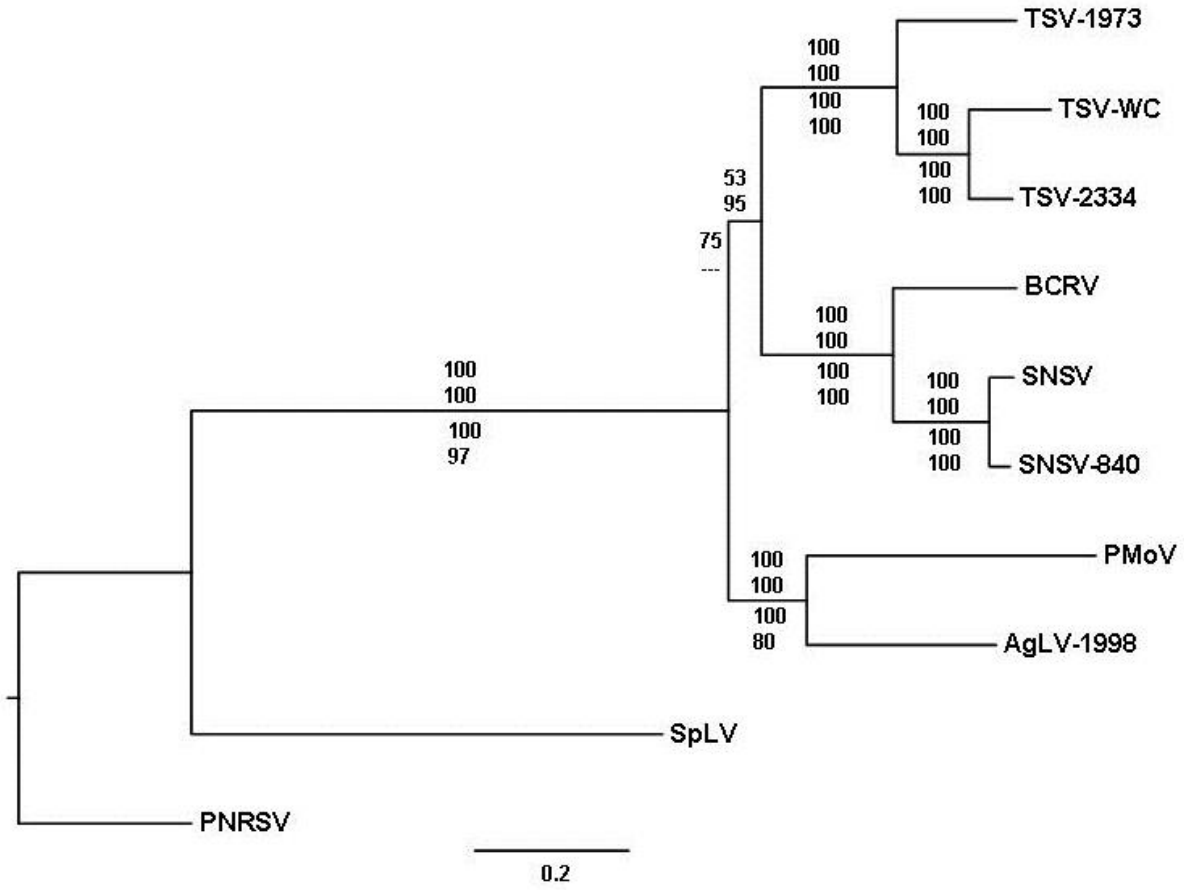

Fig. 2. Phylogram obtained from a Maximum Likelihood analysis in RAxML for the complete genome nucleotide sequence of the ilarviruses characterised in this study and other previously published ilarviruses. The complete sequence of each RNA segment was included as separate partitions in a Maximum Likelihood analysis. The scale bar represents the number of nucleotide substitutions per site. Maximum Likelihood support values (> 50%) from RAxML 1000 bootstrap replicates shown above the nodes as the upper values. The lower values (above the nodes) are from the RAxML analysis of the separate partitioned putative protein sequences (whole proteome) for which the phylogram topology was the same as that shown. There is no corresponding scale bar for the protein analysis. The same phylogram topology was obtained from Maximum Likelihood analyses in PhyML for the complete RNA-1 and RNA-2 nucleotide sequences. Maximum Likelihood support values > 50% from PhyML are shown below the nodes for RNA-1 (closest to the node) and RNA-2. SpLV and PNRSV were used as out-groups to root the phylograms. Abbreviations and GenBank accession numbers used are listed in Table 3.

The six separate phylogenetic analyses demonstrated that AgLV-1998 should be considered as a distinct ilarvirus subgroup 1 species. Closest BLASTN searches for AgLV-1998 were 76% nt identity with BCRV for RNA-1 and RNA-2, and 72% with TSV-WC for RNA-3. Partial RNA-3 sequence for isolate-837 had 99% nt identity with AgLV-1998 over a 584 bp overlap, indicating that isolate-837 should be considered an isolate of AgLV. The closest aa identities for each of the putative proteins of AgLV-1998 were 81% (replicase, PMoV), 76% (RdRp, PMoV), 68% (2b, BCRV), 79% (MP, TSV-WC), 75% (CP, TSV-WC).

Isolate-840, a reference sample referred to as strain TSV-S [13] was shown to be an isolate of SNSV in all phylogenetic analyses, supporting its previous identification based on partial RNA-3 sequence data [10]. Isolate SNSV-MD from Maryland, USA is the only other completely sequenced isolate of SNSV with which SNSV-840 shares 96%, 95% and 93% nt identity for complete RNA-1, -2 and -3 respectively. However, SNSV-840 was more closely related to a Mississippi, USA SNSV isolate (AY363233) with 99% nt identity for partial RNA-3 sequence [10] over a 669 bp overlap.

Isolate-1025, a reference sample from *Ajuga reptans* referred to as TSV-A [15], most closely grouped with the type species TSV-WC in the putative 3b CP ORF with 99% aa identity. However the putative 3a MP of isolate-1025 shared only 85% aa identity with TSV-WC. Partial RNA-1 and RNA-2 nt sequence of isolate-1025 were also divergent from TSV-WC with 90% and 87% identity over 642 bp and 1100 bp overlaps respectively. Interestingly, partial RNA-2 sequence for a Rhubarb TSV isolate from the USA (HQ130450) had 99% nt identity with isolate-1025 over a 419 bp overlap but the relationship between these two isolates is unclear for other regions of the genome. For isolate-1025, a likely recombination event on RNA-3 of an unknown major parent with TSV-WC as minor parent was strongly supported by the RDP [29], GENECONV [33], BootScan [34], MAXCHI [35] and SiScan [36] methods, all with p-values less than $1x10^{-12}$ and also by the CHIMAERA [37] method with a p-value of $6x10^{-5}$. No other putative recombination events were strongly supported for any of the other strains of TSV, AgLV or SNSV in this study.

Partial RNA-1, -2 and -3 sequences have been reported for an isolate of TSV from South Africa [38] but were not available on the GenBank database. No close relationship was found between the South African TSV isolate and the characterised isolates presented here. For example, for a 691 bp overlap of the partial RNA-3, the South African TSV isolate shared 96%, 89% and 80% nt identity with TSV-WC, isolate-2334 and isolate-1973 respectively.

Discussion

This study has clarified the genetic identity of several ilarvirus isolates from eastern Australia, some of which had previously been identified as strains of TSV. In the process, one of these strains (TSV-Ag) was shown to be a member of a new ilarvirus species, AgLV, while another (TSV-S) was shown to be SNSV. A further two strains of TSV were also described from central Queensland, TSV-parthenium and TSV-crownbeard. TSV-parthenium, TSV-crownbeard and AgLV display serological differences and are common and widespread in eastern Australia in symptomless weed species which appear to be specific to each. This new knowledge of the taxonomy and biology of these strains and species is an essential prerequisite for the development of control programs for these viruses. These results also underline the value of retaining reference and voucher specimens, as previously published biological data could be linked to genetic analyses through these specimens.

The complete genomes of the reference isolates -1973 (TSV-parthenium), -2334 (TSV-crownbeard), AgLV-1998 and SNSV-840, and the partial genome of isolate-1025 (TSV-A), revealed that significant genetic diversity exists in eastern Australia and adds greatly to the known diversity of ilarvirus subgroup 1 members. The degree of genetic diversity found in this study supports the proposition by Tzanetakis et al. [39] that the cluster of TSV-like strains may be several distinct species.

Sequence data demonstrated that isolate-1998 from ageratum is closely related to reference isolate-837 which was also referred to as the Ageratum strain (TSV-Ag) by Klose et al. [12] and both are members of a new subgroup 1 ilarvirus species. With consideration of its previous naming as Ageratum TSV along with our observations and the previous reports of this strain being commonly found as symptomless infections in *Ageratum houstonianum* [14,12] we propose the name Ageratum latent virus (AgLV). The phylogenetic results of this study and previous work showing serological and host range differences between AgLV and other TSV strains [13] along with the apparent geographical isolation of AgLV from the TSV-parthenium and -crownbeard strains also support AgLV being considered a distinct species and satisfy the requirements for the demarcation of new ilarvirus species [1].

The differential reactions observed for the TSV strains, AgLV and SNSV in TSV ELISA correlate with the relative identities of their respective putative coat proteins to that of TSV-WC, but complicate definitive diagnosis. The use of the multiplex RT-PCR eliminates this ambiguity in diagnosis and is being used in a related investigation to assist with screening of sunflower germplasm for tolerance to the TSV-parthenium strain.

The TSV-parthenium and TSV-crownbeard strains and AgLV were commonly found across wide geographical ranges in strain-specific alternative weed hosts as symptomless infections. This was also the case for many more field samples of parthenium that we tested from central Queensland [17]. These virus strains have no apparent impact on their respective strain-specific weed hosts suggesting a stable interaction. The presence of the viruses is only apparent when they induce disease symptoms in nearby susceptible plant species including crops. The possible reasons why TSV-parthenium has not been found in natural infections of crownbeard or TSV-crownbeard in parthenium are not well understood. Both plant host species, with their respective TSV strains, often grow in direct contact in the same locations throughout large areas of central Queensland and the same common thrips vector species have been found on both hosts (data not shown).

The use of alternative host names, parthenium and crownbeard, to describe the TSV strains from this study has been adopted as it appears that these are the major hosts for these TSV strains over the geographical distribution of these hosts in Australia. However, given the diversity of TSV strains now published, care needs to be taken in how these are referred to. For example, parthenium in India is also considered to be the principle source of TSV for the development of disease epidemics in nearby crops [40] but the TSV strains reported from India are genetically distinct from the TSV-parthenium strain reported here from Australia.

The current wide geographical distribution of TSV-crownbeard in *V. encelioides* in central Queensland and the identification of a closely related isolate collected in 1975 from coastal north Queensland would indicate the TSV-crownbeard strain has been present in this region for several decades. However, severe TSV disease epidemics in sunflower and mungbeans have only been reported since the early 2000s and these epidemics have only been associated with the TSV-parthenium strain [16] so it seems unlikely that the TSV-crownbeard strain has caused severe disease epidemics in the past.

While substantial previous work has been done to characterise the biology of AgLV, further investigation of the TSV-parthenium and -crownbeard strains are being conducted to further characterise their natural host range, biological characteristics including seed and thrips transmission, and their respective roles in the development of disease epidemics in central Queensland crops. We are also conducting further studies to investigate possible mechanisms that may be restricting these two strains to their respective weed hosts in nature and if reassortments of RNA segments can be found in natural infections or induced by artificial mixed infections.

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Chapter 6

Natural host range, thrips and seed transmission of distinct *Tobacco streak virus* strains in Queensland, Australia

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Natural host range, thrips and seed transmission of distinct *Tobacco streak virus* strains in Queensland, Australia

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Abstract

Diseases caused by *Tobacco streak virus* (TSV) have resulted in significant crop losses in sunflower and mung bean crops in Australia. Two genetically distinct strains from central Queensland, TSV-parthenium and TSV-crownbeard, have been previously described. They share only 81 % total-genome nucleotide sequence identity and have distinct major alternative hosts, *Parthenium hysterophorus* (parthenium) and *Verbesina encelioides* (crownbeard). We developed and used strain-specific multiplex PCRs for the three RNA segments of TSV-parthenium and TSV-crownbeard to accurately characterise the strains naturally infecting 41 hosts species. Hosts included species from 11 plant families, including 12 species native to Australia. Results from field surveys and inoculation tests indicate that parthenium is a poor host of TSV-crownbeard. Crownbeard was a natural and experimental host of TSV-parthenium but this infection combination resulted in non-viable seed. These differences appear to be an effective biological barrier that largely restricts these two TSV strains to their respective major alternative hosts. TSV-crownbeard was seed transmitted from naturally infected crownbeard at a rate of between 5 % and 50 % and was closely associated with the geographical distribution of crownbeard in central Queensland. TSV-parthenium and TSV-crownbeard were also seed transmitted in experimentally infected ageratum (*Ageratum houstonianum*) at rates of up to 40 % and 27 % respectively. The related subgroup 1 ilarvirus, Ageratum latent virus, was also seed transmitted at a rate of 18 % in ageratum which is its major alternative host. Thrips species *Frankliniella schultzei* and *Microcephalothrips*

abdominalis were commonly found in flowers of TSV-affected crops and nearby weed hosts. Both species readily transmitted TSV-parthenium and TSV-crownbeard. The results are discussed in terms of how two genetically and biologically distinct TSV strains have similar life cycle strategies in the same environment.

Keywords

Epidemiology; sunflower; *Parthenium hysterophorus*; *Helianthus annuus*; *Ilarvirus*

Introduction

Tobacco streak virus (TSV), the type member of the plant infecting Ilarviruses (Family: *Bromoviridae*), has a wide host range (Brunt et al. 1996), is pollen-borne and transmitted by thrips (Sdoodee and Teakle 1987; Prasada Rao et al. 2003a). Some strains of TSV have also been shown to be seed transmitted (Kaiser et al. 1991; Sharman et al. 2009). TSV has a single-stranded RNA genome, separated into three linear segments designated RNA-1 to -3 (King et al. 2012) which are encapsidated separately in quasi-isometric to bacilliform virions.

TSV has been reported as the causal agent for major disease outbreaks in sunflower and mung bean in Australia (Sharman et al. 2008), in oilseed and pulse crops in India (Prasada Rao et al. 2000; Reddy et al. 2002) and in soybean in Brazil (Almeida et al. 2005) and the United States of America (Rabedeaux et al. 2005). In Australia and India, parthenium weed (*Parthenium hysterophorus*) is the major alternative host of TSV and is closely associated with disease outbreaks in nearby crops (Prasada Rao et al. 2003a; Sharman et al. 2009). However, the TSV strains from the two countries are genetically distinct (Sharman and Thomas 2013).

The subgroup 1 ilarviruses reported to date from Australia are three genetically distinct TSV strains, Ageratum latent virus (AgLV) and *Strawberry necrotic shock virus* (SNSV; Sharman et al. 2011; Sharman and Thomas 2013). AgLV and SNSV were originally described as strains of TSV in earlier work (Greber 1979; Sdoodee 1989), but we have shown these to be distinct viruses (Sharman and Thomas 2013). The two most commonly found TSV strains in Australia that have been associated with disease outbreaks are referred to as TSV-parthenium and TSV-crownbeard. They have symptomless major alternative hosts of parthenium (*P. hysterophorus*) and crownbeard (*Verbesina encelioides*) respectively, they share only 81 % total-genome nucleotide sequence identity and TSV-crownbeard reacts more strongly in a commercially available TSV ELISA (Sharman and Thomas 2013). Seed transmission of the TSV-parthenium strain occurs at rates of up

to 48 % in naturally infected parthenium and is likely to be a critical survival mechanism for the virus to survive drought conditions (Sharman et al. 2009). While TSV-parthenium appears to be more important than TSV-crownbeard in disease outbreaks in sunflower crops (Sharman and Thomas 2013) several aspects of the biology of these two TSV strains have not been reported.

In this paper, we aim to fill the current knowledge gaps for aspects of the biology of the distinct TSV strains, TSV-parthenium and TSV-crownbeard. This includes describing their respective natural host ranges, the thrips species that transmit them and seed transmission. We also monitored for AgLV in central Queensland, a region previously unsurveyed for this virus. These results are discussed in terms of how these biological characteristics enable these distinct strains to persist in the same environment and lead to disease epidemics in nearby susceptible crops.

Materials and methods

Virus isolates

We collected leaf material from a variety of plant species from many locations in central Queensland between 2006 and 2014, spanning a distance of about 750 km from Injune in the south to Alligator Creek in the north (Table 1). Samples were selected for indexing based on the presence of virus-like symptoms or randomly from locations close to high levels of typical TSV infection in susceptible crops. Observed symptoms varied depending on the host (Table 1) but often included chlorotic or necrotic line patterns, stem or terminal necrosis, stunting and leaf deformation.

We tested samples by TSV ELISA as previously described (Sharman et al. 2009) and positive samples were tested by PCR as described below. The reference isolates previously used for complete genome characterisation (Sharman and Thomas 2013), TSV-parthenium isolate-1973, TSV-crownbeard isolate-2334 and AgLV isolate-1998 were maintained in *Nicotiana tabacum* cv. Xanthi for further use as diagnostic controls and for additional biological studies. All isolate numbers refer to samples lyophilised and stored at -20 ºC in the Queensland Department of Agriculture and Fisheries plant-virus collection.

RNA segment-specific multiplex RT-PCRs for TSV strains

To design PCR primers (Table 2) we aligned previously published RNA-1 and -2 sequences (GenBank accessions listed in Sharman and Thomas (2013)) for TSV-WC, TSV-parthenium, TSV-

crownbeard, AgLV, SNSV-MD and *Parietaria mottle virus* (PMoV) using the MUSCLE algorithm (Edgar 2004). Regions that were either in common to both, or specific to TSV-parthenium or TSV-crownbeard were selected by eye. In doing so, primers for cDNA synthesis were designed to work for both TSV-parthenium and TSV-crownbeard (and other ilarvirus species) while other strain-specific primers were for use in PCRs.

Total nucleic acid extracts were prepared as previously described (Sharman and Thomas 2013). To differentiate TSV-parthenium and TSV-crownbeard and to identify mixed infections and possible reassortments of RNA segments, we developed separate multiplex (MP) PCRs for RNA-1 and -2 to produce size specific products for these two TSV strains. A MP-PCR for RNA-3 which detected the TSV strains and AgLV was used as previously described (Sharman and Thomas 2013) except with the modified cDNA synthesis described here. SuperScript III reverse transcriptase (Invitrogen) was used to prepare cDNA essentially as per the manufacturer's instructions with the following modifications; a mix of 1 µM of each reverse primer TSVrep2769R (RNA-1), TSV2b2451R (RNA-2) and TSVRNA3.1982R (RNA-3) was used in a 10 µl reaction with the inclusion of 150 ng of bovine serum albumin (BSA; Life Techologies).

We used the resulting cDNA with TSV strain-specific forward primers for PCR (Table 1) with 1 unit native *Taq* DNA polymerase (Invitrogen), 1.75 mM $MgCl_2$, 200 mM dNTPs and 2 µl of cDNA template in a 25 µl reaction volume. Generic ramped annealing temperature cycling parameters were used for all PCRs, consisting of an initial denaturation of 95 ºC for 60 s, then 35 cycles of 95 ºC for 15 s, 62 ºC for 20 s, 56 ºC for 10 s and 72 ºC for 40 s, followed by a final extension of 72 ºC for 3 min.

Cross-infection studies of TSV strains and AgLV into major alternative hosts

The reference isolates maintained in *N. tabacum* cv. Xanthi, TSV-parthenium (-1973), TSV-crownbeard (-2334) and AgLV-1998 were manually inoculated using 0.1 M phosphate buffer and carborundum onto healthy seedlings of parthenium, crownbeard and ageratum. Test plants were grown for 2-3 weeks before the newly emerging terminal growth was tested by TSV ELISA.

Table 1 Natural host range of TSV-parthenium and TSV-crownbeard from surveys in central Queensland

Plant family	Species	Symptoms on each host species [A]	Isolate number and TSV-strain; parthenium (P) or crownbeard (C) [B]	Month / year of collection	Nearest locality
Amaranthaceae	*Amaranthus mitchellii* [C]	tn, sn, ld	2343 (P)	Mar 2009	Emerald
Apocynaceae	*Parsonsia sp.* [C]	crs, cll	2198 (P)	Apr 2008	Mt McLaren
Asteraceae	*Bidens pilosa*	small ld, reddening	2201 (P)	Apr 2008	Clermont
	Carthamus tinctorius	cm, tn	2591 (P)	Apr 2010	Mt McLaren
	Conyza bonariensis	ld, ln, stunted	2520 (P)	Dec 2009	Arcturus
			2513 (P)	Nov 2009	Emerald
	Eclipta prostrata [C]	Small ld	2521 (C)	Dec 2009	Arcturus
	Helianthus annuus	tn, sn, cm, cll, nll	1973 (P) [D]	Jun 2006	Clermont
			1974 (P) [D]	Jun 2006	Clermont
			2337 (C)	Apr 2009	Orion
			2341 (C)	Apr 2009	Arcturus
			2344 (P)	Apr 2009	Mt McLaren
			2580 (P)	Mar 2010	Clermont
			5127 (P)	Apr 2014	Clermont
			5128 (P)	Apr 2014	Clermont
			5129 (P)	Apr 2014	Capella
			5140 (P)	May 2012	MtMcLaren
			5139 (P)	Apr 2013	Clermont
	Lactuca serriola	chl	2610 (C)	May 2010	Arcturus
	Parthenium hysterophorus	none	2012 (P) [D]	Feb 2007	Mt McLaren
			2084 (P) [D]	Sep 2007	Orion
			2086 (P)	Sep 2007	Tieri
			2087 (P) [D]	Sep 2007	Rubyvale
			2103 (P) [D]	Oct 2007	Collinsville
			2105 (P) [D]	Oct 2007	Alligator Ck
			2139 (P)	Jan 2008	Nebo
			2140 (P)	Jan 2008	Frankfield
			2514 (P)	Nov 2009	Bauhinia
			2589 (P)	Mar 2010	Mt McLaren

			2590 (P)	Mar 2010	Fernlees
			5131 (P)	Apr 2014	Capella
			5132 (P)	Apr 2014	Capella
			5137 (P)	Apr 2014	Clermont
			5138 (P)	Apr 2014	Clermont
	Senecio madagascariensis	none	2508 (P)	Nov 2009	Arcturus
	Sonchus oleraceus	ln, cm	2037 (P)	Apr 2007	Mt McLaren
			2511 (C)	Nov 2009	Arcturus
	Tridax procumbens	none	3065 (C)	Jan 2012	Emerald
	Verbesina encelioides	none	2334 (C) [D]	Apr 2008	Emerald
			2282 (C)	Nov 2008	Theodore
			2338 (C)	Apr 2009	Orion
			2400 (C)	Jun 2009	Gogango
			5141 (C)	Apr 2010	Fernlees
			5142 (P)	Apr 2009	Emerald
			5143 (P+C)	Apr 2009	Emerald
			5130 (P+C)	Apr 2014	Arcturus
			5133 (C)	Apr 2014	Comet
			5134 (C)	Apr 2014	Comet
			5135 (C)	Apr 2014	Arcturus
			5136 (C)	Apr 2014	Arcturus
	Xanthium occidentale	ln, ld, nrs, nll, cm	2032 (P)	Apr 2007	Orion
			2102 (P)	Oct 2007	Collinsville
			2512 (P)	Nov 2009	Arcturus
			2524 (P)	Nov 2009	Arcturus
			834 (C)	Aug 1975	Airdmillan
			835 (C)	Aug 1975	Airdmillan
Boraginaceae	*Trichodesma zeylanicum* [C]	none	2322 (P)	Feb 2009	Clermont
Commelinaceae	*Commelina benghalensis*	cm, tn	2615 (P)	May 2010	Arcturus
Fabaceae	*Alysicarpus muelleri* [C]	cm, clp	2323 (P)	Feb 2009	Clermont
	Arachis hypogaea	tn, cm, ld, crs	2165 (C)	Feb 2008	Emerald
			2401 (C)	May 2009	Gogango

			3098 (C)	Feb 2012	Emerald
			2594 (P)	Apr 2010	Mt McLaren
	Cajanus cajan	cll, nll, sn, crs	2143 (P)	Jan 2008	Clermont
	Cicer arietinum	tn, sn, wilting	1979 (P) [D]	Aug 2006	Clermont
			2074 (P)	Aug 2007	Gindie
			2075 (P)	Aug 2007	Gindie
	Crotalaria mitchellii subsp. *Mitchellii* [C]	cm, crs	2199 (C)	Apr 2008	Emerald
	Glycine max	tn, mild cm	2200 (P)	Apr 2008	Emerald
			2348 (P)		
	Lupinus sp.	ld, tn	2592 (P)	Apr 2010	Mt McLaren
	Macroptilium lathyroides	cm, clp, cll	2163 (C)	Feb 2008	Emerald
	Phaseolus vulgaris	severe ld, cm	2346 (P)	Apr 2009	Mt McLaren
	Tephrosia sp. [C]	cll, crs	5126 (P)	Feb 2014	Mt McLaren
	Vicia faba	tn, cm	2347 (P)	Apr 2009	Mt McLaren
	Vigna radiata	tn, sn, nrs	2027 (P) [D]	Mar 2007	Orion
			2028 (P)	Mar 2007	Dysart
			2342 (P)	Mar 2009	Emerald
			2025 (P)	Mar 2007	Arcturus
	Vigna radiata var. sublobata [C]	vn	2197 (P)	Apr 2008	Clermont
	Vigna unguiculata	nll, nrs	2345 (P)	Apr 2009	Mt McLaren
Malvaceae	*Abelmoschus ficulneus* [C]	chl.patches, clp	2036 (P)	Apr 2007	Mt McLaren
			2164 (P)	Feb 2008	Emerald
	Gossypium hirsutum	purple rs, spreading purple nll	2120 (P) [D]	Nov 2007	Emerald
			2285 (P)	Nov 2008	Moura
			2399 (C)	June 2009	Springsure
			2510 (C)	Nov 2009	Theodore
			2735 (P)	Jan 2011	Emerald
Phyllanthaceae	*Notoleptopus decaisnei* [C]	mild clp, crs	2324 (P)	Feb 2009	Clermont
	Phyllanthus sp.	Mild clp, crs	2325 (P)	Feb 2009	Clermont
Solanaceae	*Capsicum annuum*	Mild cll	3107 (P)	Apr 2012	Clermont
	Datura ferox	ns, nrs	2326 (P)	Feb 2009	Clermont
	Datura leichhardtii	ld, ns, nrs	2035 (P)	Apr 2007	Mt McLaren

	Nicotiana megalosiphon subsp. megalosiphon [C]	ln, nlp	2593 (P)	Apr 2010	Capella
	Physalis lanceifolia	tn, sn, nrs	2034 (P)	Apr 2007	Mt McLaren
Tiliaceae	*Corchorus trilocularis* [C]	chl patches	2038 [E]	Apr 2007	Mt McLaren
Verbenaceae	*Verbena bonariensis*	none	3063 (P)	Jan 2012	Arcturus

[A] Abbreviations for observed symptoms: chl - chlorosis; clp - chlorotic line patterns; cm - chlorotic mottle; tn - tip necrosis; sn - stem necrosis; ld - leaf deformation; crs - chlorotic ringspots; cll - chlorotic local lesions; nll - necrotic local lesions; ns - necrotic spots; nrs - necrotic ringspots; nr - necrotic rings; rs - ring spots; nlp - necrotic line patterns; ln - leaf narrowing; vn - vein necrosis.

[B] TSV strain determined by strain specific MP-PCR for each RNA segment. TSV strain was confirmed as either TSV-parthenium (P) or TSV-crownbeard (C) for all three RNA segments.

[C] Plant species endemic to Australia.

[D] Partial or complete genome sequence has been derived for these isolates (Sharman *et al.*, 2009; Sharman and Thomas, 2013; Sharman *et al.*, 2008).

[E] Isolate-2038 failed in the MP-PCRs but had a TSV ELISA absorbance value of greater than 200 times the healthy control.

Table 2 PCR primers used in strain-specific MP-PCRs for RNA-1, -2 and -3 for identification of TSV-parthenium and TSV-crownbeard

Target RNA	**Target TSV strain**	**Primer name**	**Sequence (5' to 3')**	**Final concentration in PCR (nM)**	**Approximate PCR product size (bp)**
RNA-1	TSV-parthenium	ParTSVrep2228F	CCCTCTGCACCCACTTCCGAA	200	540
	TSV-crownbeard	CrbTSVrep2420F	CTAGTCCCAACCTTCAAAATC	200	350
	Both strains	TSVrep2769R	GGAACTTGCTCKGTRTCACCAA	200	
RNA-2	TSV-parthenium	ParTSVpol1722F	GATAGTTTGATTGGATCGTTAAG	280	760
	TSV-crownbeard	CrbTSVpol2144F	GAGTTCCAAGGTTTGTATTCGT	200	300
	Both strains	TSV2b2451R	CCAGCACARTCAATGCAHTT	200	
RNA-3 [A]	TSV-parthenium	CQTSVF	CCTACTCCAACCCTGATTA	300	920
	TSV-crownbeard	CrbTSVF	GCCCGTTTACCAGTACCAAT	80	570
	AgLV	SEQTSVF	CGCCATGTCTACTTCTAGGA	100	740
	TSV and AgLV	TSVRNA3.1982R	CCRCATCKCACACARGWATT	200	

[A] Primers and conditions for RNA-3 MP-PCR described by Sharman and Thomas (2013).

Seed transmission of TSV strains and AgLV in major alternative hosts and crop plants

In order to test for seed transmission of the TSV strains and AgLV from different hosts, we collected seed from either naturally infected plants or from plants inoculated with reference isolates. TSV-crownbeard transmission was tested from three naturally infected crownbeard plants (one of which was isolate-2334). TSV-parthenium transmission was tested from one plant each of naturally infected *Bidens pilosa* (isolate-2201) and *Conyza bonariensis* (isolate-2520), and multiple plants of infected sunflower and mung bean. All mother plants were tested by strain-specific PCR except for sunflower and mung bean mother plants which were tested by TSV ELISA prior to the PCR being available. The sunflower and mung bean mother plants were collected from the Clermont region where all other samples tested by PCR over several years have been TSV-parthenium with no TSV-crownbeard detected.

In order to determine whether seed transmission could occur with other virus-host combinations, we inoculated healthy plants of parthenium, crownbeard or ageratum with reference cultures as part of the cross-infection studies described above and collected seed from ELISA positive plants. Test seed was grown in isolation of virus sources, glasshouses were routinely treated with insecticide and no thrips were detected. Seedlings were tested by ELISA prior to flowering, generally within 3-4 weeks of germination.

Thrips surveys and transmission tests

The aim of this study was to determine which are the major thrips species associated with disease outbreaks caused by TSV-parthenium and TSV-crownbeard and to test if these are capable of transmitting the two TSV strains. Between 2006 and 2011, we made a total of 35 collections of thrips from flowers of TSV-affected crops and nearby weed hosts from locations across central Queensland from Theodore in the south to Mt McLaren about 400 km to the north-west. Identifications were confirmed by Queensland Department of Agriculture, Fisheries and Forestry senior entomologist Desley Tree and the species commonly collected from many locations were used to test their ability to transmit the TSV strains. *Frankliniella schultzei* and *Thrips tabaci* were established as live colonies in cages constructed with 106 µm thrips proof mesh. Due to difficulties in establishing a culture, *Microcephalothrips abdominalis* was used as direct field collections.

Transmission test methods were similar to those described by Klose *et al.* (1996). Pollen was harvested from TSV-parthenium infected parthenium or TSV-crownbeard infected crownbeard and stored at 5 ºC for up to six months before being used in thrips transmission tests. The same batch of TSV-infected pollen was stored for more than 6 years at 5 ºC and used in manual inoculation to test

longevity of the virus in pollen. The TSV-strains present in each source of pollen was confirmed by PCR as described above. Thrips were mixed with TSV-infected pollen to cover the thrips bodies and 6-10 thrips per plant were placed onto test plants. After 1-2 days of feeding access, thrips were killed with insecticide spray and test plants were grown for 1-2 weeks before being assessed for symptoms and tested by TSV-ELISA. Control plants included plants dusted with TSV infected pollen but no thrips added, thrips added without pollen and neither treatment.

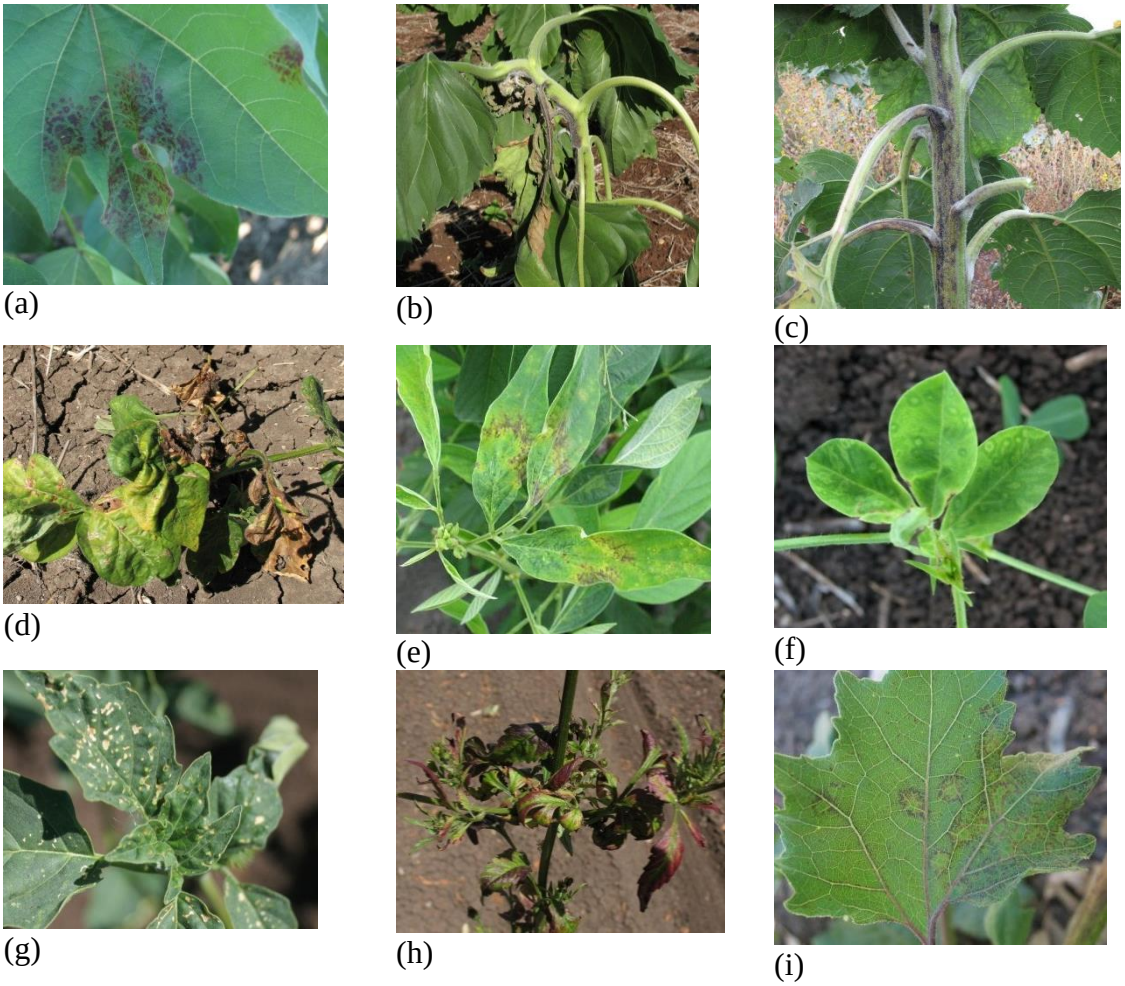

Figure 1 TSV symptoms on; (a) *G. hirsutum*, isolate-2285; (b) *H. annuus*, -5127 and (c) -2398; (d); *V. radiata*, -2342; (e) *C. cajan*, -2143; (f) *A. hypogaea*, -2594; (g) *D. leichhardtii*, -2035; (h) *B. pilosa*, -2201; and (i) *X. occidentale*, -2512. Isolate details and descriptions of host symptoms are given in Table 1.

Results

Multiplex RT-PCRs for RNA segments and host range studies

A diverse range of symptoms are described for the hosts listed in Table 1. We have previously illustrated symptoms on sunflower (isolate-1973), mung bean (isolate-2027), cotton (isolate-2120)

and chickpea (isolate-1979) (Sharman et al. 2008) and further images of TSV symptoms on a range of crop and weed hosts are shown in Fig. 1a – 1i.

The strain-specific MP-PCRs for each RNA segment worked very well for identification of TSV strains from a wide range of hosts (Table 1). Size-specific PCR products were produced for each TSV strain for the three RNA segments (Fig. 2). All samples tested had at least one complete set of RNA segments for either TSV-parthenium or TSV-crownbeard (Table 1).

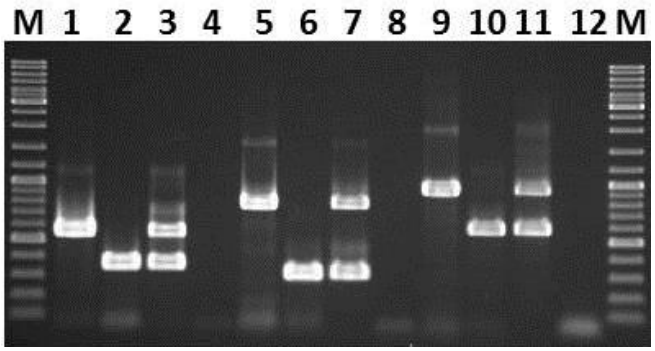

Figure 2 Electrophoresis gel (1.2 % agarose in 0.5 x TBE) of MP-PCRs for RNA-1, -2 and -3. Lane 1 is TSV-parthenium isolate-1973; lane 2 is TSV-crownbeard isolate-2334; lane 3 is mixed isolate-5130; and lane 4 is PCR negative control (no-template). These samples are repeated for RNA-2 (lanes 5 to 8) and RNA-3 (lanes 9 to 12) MP-PCRs. Marker lanes (M) are GeneRuler DNA ladder mix (Catalogue # SM0332, Life Technologies). See table 1 for isolate details.

From locations where both parthenium and crownbeard were growing, some samples had both complete sets of RNA segments or one complete set and one incomplete. From locations where parthenium or crownbeard (but not both) were growing, then only the respective TSV strain was found in surrounding host species. At these locations, testing for only one of the three RNA segments by strain-specific PCR would be adequate for identification of the strain present.

There were 41 naturally infected host species from 11 plant families, including 12 species native to Australia. Of the 41 species, 29 were infected with TSV-parthenium only, five were infected with TSV-crownbeard only and six host species had individual plants with each TSV strain as separate infections. Crownbeard was the only species to have mixed infections of both TSV strains in some individual plants. One species, *Corchorus trilocularis* (isolate-2038), failed in the PCRs mostly likely due to high levels of polysaccharides, a known inhibitor of PCR reactions. However, this sample displayed chlorotic mottle symptoms and produced TSV ELISA absorbance values greater than 200 times those of the healthy controls. The RNA-3 MP-PCR detected the positive control used for AgLV(AgLV-1998; Sharman and Thomas 2013) but AgLV was not detected in any samples tested in this study from central Queensland.

While results for a selection of parthenium and crownbeard samples are shown in Table 1, a total of 30 TSV ELISA-positive crownbeard samples were tested by PCR and gave positive results for all three RNA segments of one or both TSV strains. From locations where crownbeard was the dominant weed with very low numbers of parthenium, 11 crownbeard plants were TSV-crownbeard-only, one had both strains but none were TSV-parthenium-only. From locations where there were many of both weeds growing together, seven crownbeard plants were TSV-crownbeard-only, nine had both strains and two were TSV-parthenium-only. All 17 parthenium samples with complete PCR results for RNA segments were TSV-parthenium-only. However, there were detections of one or two TSV-crownbeard RNA segments from four additional parthenium samples collected from locations were both weeds occurred.

Cross-infection studies of TSV strains and AgLV into major alternative hosts

The test hosts crownbeard and ageratum were readily infected by TSV-parthenium or TSV-crownbeard by manual inoculation (Table 3). However, parthenium was only readily infected by TSV-parthenium. PCR testing of 15 field samples of parthenium (Table 1) and inoculation results (Table 3) indicate parthenium is a poor host of TSV-crownbeard. AgLV did not infect parthenium but did infect crownbeard and ageratum via inoculation.

Some significant differences in symptoms where observed when tobacco (*Nicotiana tabacum* cv. Xanthi) was infected with the TSV strains or AgLV. Both TSV-parthenium and AgLV caused systemic symptoms essentially as illustrated by Costa (1961) with deeply notched leaves and flower petals with a filament-like appendage not present in healthy flowers. TSV-crownbeard did not induce systemic notched leaves or affected flowers but slightly reduced and distorted leaves only.

Table 3 Experimental cross-infection of TSV strains and AgLV into major alternative hosts

	Test host and number of inoculated test plants positive by TSV ELISA from total tested		
Virus strain	**Parthenium**	**Crownbeard**	**Ageratum**
TSV-1973 (TSV-parthenium)	7/8, 3/6 [A]	8/8	9/10, 3/4, 8/11
TSV-2334 (TSV-crownbeard)	1/18	8/8	8/12
AgLV-1998	0/11, 0/7	2/6	1/11, 3/12

[A] Numerator is number of plants positive by TSV ELISA and denominator is total tested. Results shown are from either single or multiple tests.

Seed transmission of TSV strains and AgLV in major alternative hosts and crops

TSV-crownbeard was seed transmitted at relatively high rates from naturally infected crownbeard and from experimentally infected ageratum (Table 4) after up to 23 months storage at ambient room temperature and humidity. TSV-parthenium was seed transmitted from experimentally infected ageratum after up to 11 months storage. TSV-parthenium can readily infect crownbeard (Table 3) but all infected crownbeard plants had greatly reduced (shrivelled) seeds that were not viable. The weight of 100 crownbeard seeds was 196 mg from plants infected with TSV-crownbeard isolate-2334, and 44 mg from plants infected with TSV-parthenium isolate-1973. AgLV-1998 was seed transmitted from experimentally infected ageratum at a rate of 18 % after more than 6 months storage.

TSV-parthenium was also seed transmitted at high rates from naturally infected *B. pilosa* and *C. bonariensis* (Table 4). The TSV-infected seedlings of *B. pilosa* and *C. bonariensis* were significantly stunted with narrowed leaves compared with the non-infected seedlings. TSV was not seed transmitted from naturally infected mother plants of sunflower or mung bean when 678 and 930 seedlings respectively were tested by ELISA.

Table 4 Test of seed transmission of TSV strains and AgLV in different hosts

	Number of seedling test plants positive by TSV ELISA from total tested				
Virus strain	**Parthenium**	**Crownbeard**	**Ageratum [A]**	**Bidens pilosa**	**Conyza**
TSV-parthenium	24/50, 3/44 [B]	No viable seed	1/13, 2/10, 0/4, 4/10	31/47 [C]	8/30 [C]
TSV-crownbeard	n/t [D]	6/12, 2/39, 6/21 [E]	5/27, 3/11, 3/22	n/t	n/t
AgLV	n/t	n/t	5/27	n/t	n/t

[A] TSV-parthenium isolate-1973, TSV-crownbeard isolate-2334 or AgLV-1998 were used to infect ageratum plants (Table 3) from which seeds were collected and used in grow out tests of seed transmission. Results shown are of seedlings tested from either single or multiple mother plants.

[B] TSV-parthenium was previously shown to be seed transmitted in parthenium (Sharman *et al.*, 2009). The highest and lowest rates from 6 mother plants are shown.

[C] TSV isolates-2201 (*Bidens pilosa*) and -2520 (*Conyza bonariensis*) were the naturally infected mother plants for the seedlings tested and were shown to be positive for TSV-parthenium by PCR (Table 1).

[D] Not tested (n/t).

[E] Three naturally infected mother plants of crownbeard were confirmed as TSV-crownbeard by PCR and seedlings were tested for seed transmission. The reference isolate-2334 was one of the progeny from the third mother plant.

Thrips surveys and transmission tests

We made 35 collections of thrips from sunflower (12), mung bean (2), parthenium (14) and crownbeard (7). From the 726 individuals collected, 44 % were *M. abdominalis* and 40 % were *F. schultzei.* These two species were dominant from almost all locations and hosts (Table 5). However,

Megalurothrips usitatus accounted for 44 % of the thrips collected from mung bean and *Tusothrips sp*. for 10 % of thrips from sunflower. *M. usitatus* has been reported as an efficient TSV vector (Prasada Rao et al. 2003a), so it is possible that this species is involved in TSV transmission in mung beans.

We selected *F. schultzei* and *M. abdominalis* for transmission tests because they were the most numerous and commonly found species on the range of field hosts surveyed and they have been previously shown to be vector species of other TSV strains and AgLV (Klose et al. 1996). We also tested *T. tabaci* as a vector of the TSV strains because it was found in some field collections and it has been shown to be an efficient TSV vector species (Klose et al. 1996).

Table 5 The major thrips species collected from weeds and crop plants as a percentage of the total thrips collected from each

	Proportion of thrips collected from different hosts			
Thrips species	**Parthenium (269)[A]**	**Crownbeard (132)**	**Sunflower (243)**	**Mung bean (82)**
F. schultzei	41 %	17 %	45 %	54 %
M. abdominalis	49 %	76 %	37 %	0 %
others	10 %	7 %	18 %	46 %

[A] Total number of individuals collected from each host shown in parentheses.

F. schultzei, *M. abdominalis* and *T. tabaci* were efficient vectors of the TSV-parthenium strain (Table 6). TSV-crownbeard was also efficiently transmitted from crownbeard pollen to mung bean by *F. schultzei* in six of six test plants, by *M. abdominalis* in 11 of 11 plants, and by *T. tabaci* in seven of eight plants.

There was one positive plant for the thrips-only control treatment for TSV-parthenium transmission using *M. abdominalis* (Table 6). This is likely to be a false positive due to the use of *M. abdominalis* individuals collected directly from field samples of parthenium flowers where thrips may have been contaminated with TSV-infected pollen. *M. abdominalis* was unable to be cultured as was done for the other test species, leaving open the risk of collecting TSV-contaminated individuals.

Transmission was also attempted using TSV-parthenium pollen and an infestation of two-spotted mites (*Tetranychus urticae*) on mungbeans with no transmission to six test plants. TSV-parthenium infected pollen stored for more than 6 years at 5 ºC was still infective when manually inoculated to *Vigna unguiculata* (cowpea) with three of three test plants displaying typical local and systemic symptoms of TSV infection.

Table 6 Test of TSV-parthenium transmission using different thrips species

		Number of infected plants from total tested using different vector species		
Treatment	**Test host**	***F. schultzei***	***M. abdominalis***	***T. tabaci***
Thrips + TSV-pollen	Sunflower	17/24	n/t	2/5, 4/5
	Mung bean	24/24	5/6, 10/10, 11/11	4/5, 5/9
TSV-pollen only	Sunflower	0/12	n/t	0/5
	Mung bean	0/12	n/t	0/5
Thrips only	Sunflower	0/12	n/t	0/5
	Mung bean	0/12	1/6, 0/12	0/5
Nil	Sunflower	0/6	n/t	n/t
	Mung bean	0/6	n/t	n/t

Discussion

We report previously unknown biological characteristics such as host range, seed transmission and thrips transmission for two TSV strains from central Queensland, TSV-parthenium and TSV-crownbeard. A diverse natural host range was identified for both TSV strains. TSV-parthenium had a wider natural host range over a larger geographical area in central Queensland compared to TSV-crownbeard. TSV-parthenium was very common in parthenium across most of its geographical range (Sharman et al. 2009) but only infected other host species in locations where infected parthenium was growing. Similarly, TSV-crownbeard only infected hosts other than crownbeard in locations where infected crownbeard was growing. These results demonstrate the close association these two distinct TSV strains have with their respective major alternative hosts, parthenium and crownbeard. The exception to this were the TSV-crownbeard infected archived isolates of *Xanthium occidentale* (isolates 834 and 835) collected in 1975 from Ayr in north Queensland. It is unknown if crownbeard was in the Ayr region at that time.

In a previous study of fewer samples we did not find TSV-parthenium in crownbeard nor TSV-crownbeard in parthenium (Sharman and Thomas 2013). Now we have found that parthenium was a poor host of TSV-crownbeard in nature and by experimental inoculation while crownbeard was both a natural and experimental host of TSV-parthenium. However, inoculations of crownbeard with one isolate of TSV-parthenium resulted in no viable seed. This could act as a biological barrier stopping TSV-parthenium persisting in crownbeard populations and provide parthenium with a biological advantage over crownbeard in locations where they are found together. A similar synergistic plant-virus interaction was also described by Malmstrom *et al.* (2005) who described a plant community shift in favour of virus-tolerant grass species over susceptible native grasses.

There may be variation in the reaction of TSV-parthenium isolates infecting crownbeard and testing with further isolates would help to clarify if the effect on crownbeard seed is consistently observed.

Ageratum has been shown to be a natural host of TSV in India (Prasada Rao et al. 2003a) and was also indicated as a critical host of AgLV and thrips vectors that caused disease in tobacco crops in south east Australia (Greber et al. 1991b). We did not detect AgLV from any samples from central Queensland and ageratum is rarely recorded in this region. AgLV is most likely restricted to eastern coastal areas of Queensland and northern New South Wales where ageratum is often abundant (Klose 1997; Sharman and Thomas 2013).

There are similarities between the Indian TSV strain and TSV-parthenium from Australia in the disease epidemics they cause in sunflower and mung beans. Both have parthenium as their major alternative host. However, they are genetically distinct (Sharman and Thomas 2013) and appear to have differences in host range and seed transmissibility. No seed transmission of TSV from India has been reported from several studies of crop plants and weeds including sunflower, groundnut (peanut), mung bean, soybean and parthenium (Prasada Rao et al. 2009; Prasada Rao et al. 2003a; Reddy et al. 2007; Vemana and Jain 2010). In contrast, we have demonstrated high rates of seed transmission from several Asteraceae species for TSV-parthenium, TSV-crownbeard and AgLV. Along with our previous record of TSV-parthenium transmission in parthenium seed (Sharman et al. 2009), and to the best of our knowledge, these are the first records of TSV seed transmission in Asteraceae species. While TSV-parthenium is genetically closely related to a Brazilian strain of TSV (Sharman and Thomas 2013; Almeida et al. 2005), limited tests have been reported for the Brazilian strain and no seed transmission was found (Costa and Carvalho 1961).

Similar to our observations for TSV in parthenium and crownbeard, several other disease outbreaks caused by TSV or AgLV have also been linked to TSV-infected Asteraceae species. They produce large amounts of pollen and can sustain high thrips populations. These include sunflower and parthenium in India (Prasada Rao et al. 2003a), *Ambrosia polystachia* in Brazil (Almeida and Corso 1991), and *Ageratum houstonianum* in Australia (Greber et al. 1991).

The genetically distinct TSV-parthenium and TSV-crownbeard share similar life cycle strategies that enable them to survive and persist in an environment that is often unpredictable and harsh. Inland regions of central Queensland typically have a dry tropical climate that often reduces alternative host populations to isolated patches. The high rates of seed transmission of these two TSV strains in their respective major alternative hosts enables them to remain dormant for up to several years (Sharman et al. 2009). This enables them to rapidly re-establish and spread when conditions improve and is critical to the rapid development of TSV epidemics in this region.

TSV-parthenium and TSV-crownbeard were readily transmitted via infected pollen and three thrips species: *F. schultzei, M. abdominalis and T. tabaci*. *F. schultzei* and *M. abdominalis* play a critical role in facilitating the movement and transmission of TSV into susceptible crops via infected parthenium pollen. It is likely these thrips carry virus-infected pollen on their bodies in a similar manner to previously described for another pollen feeding species, *T. imaginis* (Kirk 1984a).

While all samples tested had a complete set of genome segments from either strain (TSV-parthenium or TSV-crownbeard), one or two additional segments from the other strain were also detected in some samples. Mixed infections provide an opportunity for recombination and reassortment of genetic material from multipartite viruses (Pressing and Reanney 1984; Roossinck 1997). Genetic exchange occurs with other Bromoviridae members (Codoner and Elena 2008) to potentially result in new species which may have quite different host range and pathogenicity characteristics. It is unclear if our observations of mixed RNA segments from TSV-parthenium and TSV-crownbeard indicate these strains are capable of complementing each other or if a new hybrid strain could arise from a reassortment of RNA segments. This could be clarified with further investigation of naturally occurring or experimentally induced mixed infections of these two strains.

Acknowledgments

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Chapter 7

Field evaluation of tolerance to *Tobacco streak virus* in sunflower hybrids, and observations of seasonal disease spread

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Field evaluation of tolerance to *Tobacco streak virus* in sunflower hybrids, and observations of seasonal disease spread

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Abstract

Significant differences in tolerance to natural infections of *Tobacco streak virus* (TSV) in sunflower hybrids were identified. Data from 470 plots involving 23 different sunflower hybrids tested in multiple trials over 5 years in Australia were analysed. Using a Bayesian Hierarchical Logistic Regression model for analysis provided: (i) a rigorous method for investigating the relative effects of hybrid, seasonal rainfall and proximity to inoculum source on the incidence of severe TSV disease; (ii) a natural method for estimating the probability distributions of disease incidence in different hybrids under historical rainfall conditions; and (iii) a method for undertaking all pairwise comparisons of disease incidence between hybrids whilst controlling the familywise error rate without any drastic reduction in statistical power. The tolerance identified in field trials was effective against the main TSV strain associated with disease outbreaks, TSV-parthenium. Glasshouse tests indicate this tolerance to also be effective against the other TSV strain found in central Queensland, TSV-crownbeard. The use of tolerant germplasm is critical to minimise the risk of TSV epidemics in sunflower in this region. We found that rainfall during the early growing

months of March and April had a significant negative affect on the incidence of severe infection with greatly reduced disease incidence in years that had high rainfall during this period.

Keywords: epidemiology; Stan; Hamiltonian Monte Carlo; *Helianthus annuus.*

Introduction

Sunflower (*Helianthus annuus*) is an important oilseed crop grown in many countries in Europe, the Indian subcontinent, South and North America and Australia. The majority of the approximately 33,000 ha of production in Australia occurs in Queensland and New South Wales (Anonymous 2014). Significant losses due to disease caused by *Tobacco streak virus* (TSV) occurred during the mid-2000s in sunflower and mung bean crops in the central highlands region of Queensland (Sharman *et al.* 2008; Sharman *et al.* 2009). The region affected by TSV spans more than 200 km from south of the town of Springsure to north of Clermont.

TSV is a pollen-borne virus and transmission to the leaves of susceptible hosts requires both virus-infected pollen and thrips feeding damage (Sdoodee and Teakle 1987). There are two genetically and biologically distinct TSV strains reported from central Queensland, TSV-parthenium and TSV-crownbeard (Sharman and Thomas 2013; Sharman *et al.* 2015). These strains were named after their respective major alternative hosts, *Parthenium hysterophorus* (parthenium) and *Verbesina encelioides* (crownbeard). While both strains naturally infect sunflower, TSV-parthenium has been the causal agent in all recent major disease outbreaks (Sharman *et al.* 2009). Parthenium is an opportunistic, invasive weed that is established across an extensive region of central Queensland (Navie *et al.* 1996; Adkins and Shabbir 2014) and is an ideal host for generating TSV epidemics (Sharman *et al.* 2009; Sharman *et al.* 2015).

Control options for TSV in sunflower crops in central Queensland appear to be limited to cultural practices such as reducing the source of the virus, limiting vector populations, or the identification and use of tolerant germplasm. While some measures can be taken to control parthenium in the immediate area around crops, adequate control of parthenium across central Queensland is unlikely to be successful. At least 10 biological control agents have been released in Australia to target parthenium (Adkins and Shabbir 2014) but it continues to infest vast regions. Effective control of

the thrips vectors is also unlikely to be feasible in broad-acre farming systems as it would be uneconomical and impractical to apply the insecticides required on both the crops and surrounding parthenium-infested areas. The most effective long-term control option is likely to be the use plant host resistance.

There are limited reports of screening for TSV resistance in sunflower germplasm. In India, resistance screening by Lokesh *et al.* (2005) was done in a single trial with non-replicated plots and relatively low disease pressure. A similar trial by Karuna *et al.* (2008) was also run with non-replicated plots. However, TSV disease incidence was much higher and there appeared to be large differences in the tolerance of the tested hybrids.

In this paper, we tested the hypothesis that sunflower hybrids differ in their relative tolerance to natural field infections of TSV. Another objective was to characterise some aspects of disease spread such as the distance that it can spread into a crop, the association to seasonal rainfall, and if any likely benefit may be achieved with the use of barrier crops. This knowledge will be important for the development of management strategies to minimise the risk of TSV in sunflower crops.

Methods and materials

Field trial design

We conducted field trials over five consecutive seasons from 2008 to 2012 at two sites near Clermont, Queensland. Long term weather data from Clermont (Australian bureau of meteorology site number 035019) show the prevailing wind direction throughout the day during the most common cropping period for rain-fed sunflower (February to May) is from the south-east with winds from between south to east for greater than 70 % of the day. Trial sites were selected to be downwind of infestations of TSV-infected parthenium at locations where high TSV disease levels had recently been observed in commercial sunflower crops.

The first site, hereafter referred to as Kenlogan, was approximately 49 km north of the town of Clermont and the second site, hereafter referred to as Langton Cottage, was approximately 16 km east-north-east of Clermont. We planted the field trials in late February to early March each year depending on suitable rain events for planting. In 2008, only the Kenlogan trial site was used while both sites were used in subsequent years.

Field observations in commercial crops indicated an edge effect which resulted in a higher incidence of TSV affected plants close to the paddock boundary and downwind of the areas harbouring TSV-infected parthenium. To minimise any effect, trial sites had a long narrow, randomised block design parallel to the edge of the cropping area, downwind of areas infested with parthenium and no plot further than 20 m from the edge. A planting density of 35,000 plants per hectare was used as is recommended for commercial sunflower production in the rain fed area of central Queensland.

The design of the trials was altered over the 5 year period to reflect the needs of the industry and to test new hybrids. In the 2008 and 2009 trials, the plot size was two rows at 1 m apart by 8 m long, containing about 55 plants and replicated four times in a randomised block design. The same design was used in 2010 with additional larger plots for three hybrids; two hybrids with good tolerance to TSV (Hysun 304 and NH2201) and one with poor tolerance (Ausigold 61). These larger plots were six rows by 16 m, containing about 340 plants and replicated three times. In 2011 and 2012, hybrids were tested in plot sizes of four rows by 16 m, containing about 225 plants and replicated six times. The hybrids tested in each trial are shown in Figure 1.

Rating for TSV disease

We rated plants for severe TSV symptoms that would prevent harvesting. For the field trials, we assessed plants at 2 months post planting, rating for severe TSV symptoms including death, stem necrosis leading to lodging and heads severely reduced in size (Fig. 2). Disease incidence was considered to be the proportion of total plants that were severely infected from each trial plot or disease count from commercial crops. To confirm TSV infections and the strain present from representative samples, TSV ELISA and RNA 3 strain-specific PCR were done as previously described (Sharman *et al.* 2009; Sharman and Thomas 2013).

Statistical analysis

The data we collected consisted of counts of disease incidence in plots to which different sunflower hybrids had been randomly assigned. Such data is most naturally modelled as arising as samples from a binomially distribution with parameters p (the probability of disease) and n (the total number of plants counted in each plot). The probability of disease in each plot is modelled as a function of a number of contributing factors, such as the sunflower hybrid, the amount of rainfall, and the proximity to parthenium weed. An appropriate statistical model for modelling these probabilities (that take values between 0 and 1) is a logistic regression model.

Such a logistic regression could have been performed as a standard Generalised Linear Model or Generalised Linear Mixed Model, however, we sought to test if a Bayesian Hierarchical Logistic Regression (BHLR) model offered a more robust means of analysis. In order to address our research questions we sought to determine: (i) what the expected probability distributions of disease for different hybrids under historical rainfall conditions were; and (ii) how does disease resistance compared between each of the 23 hybrids.

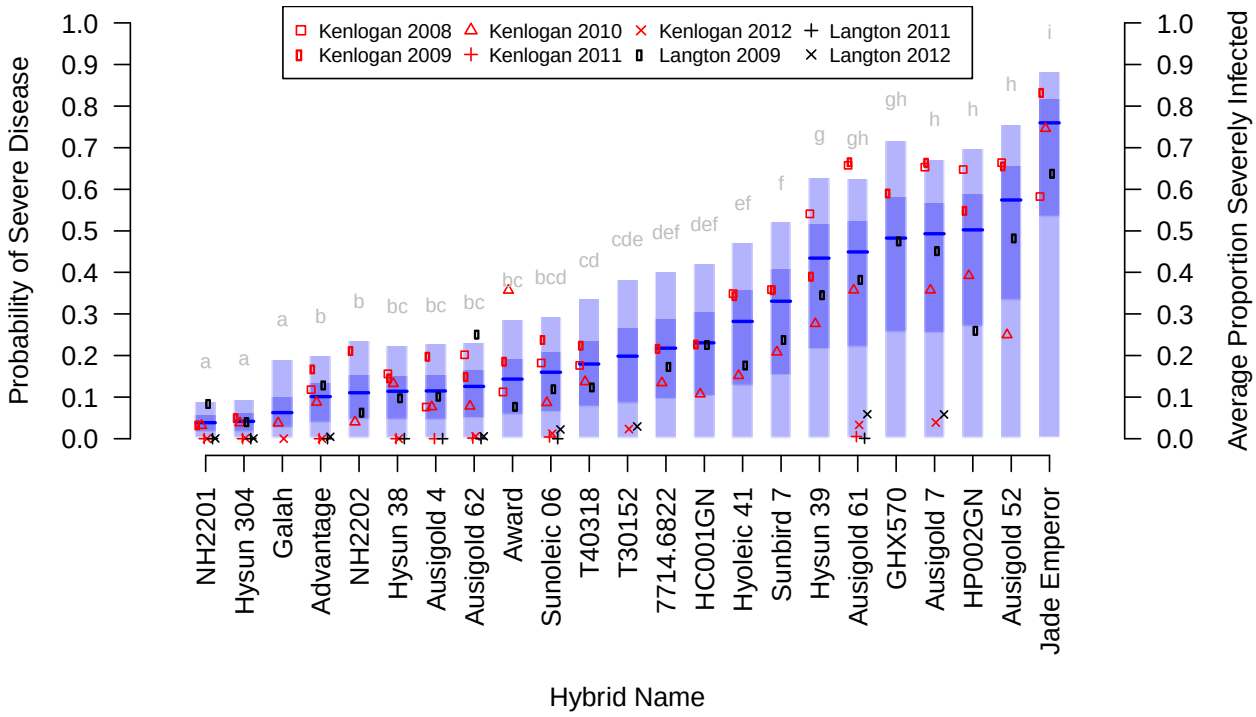

Figure 1 Modelled and observed probabilities of disease incidence for different hybrids. Horizontal dark blue lines show the modelled median, dark coloured, inner rectangles span the 25th-75th percentiles, and outer rectangles span the 95% predictive interval. The position of symbols show the average proportion of severely diseased plants for each hybrid for each site/year combination. Red symbols represent data for the Kenlogan site and black symbols for Langton Cottage. Grey letters above the blue rectangles show the groupings from Table 2.

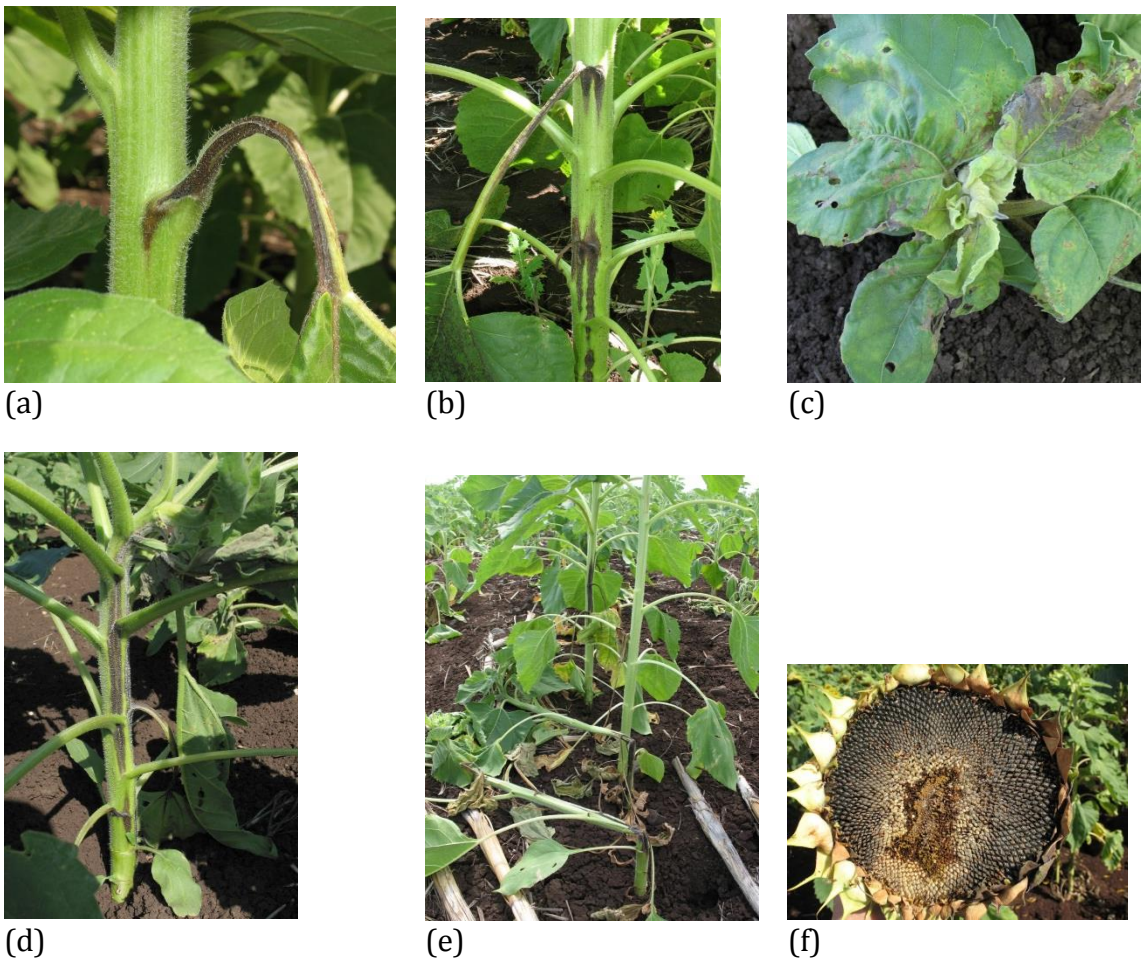

Figure 2 The range of typical TSV symptoms observed in sunflower from the field trials. Mild symptoms commonly seen but not rated as "severe" in the field trials; (a) mild symptoms of isolated necrotic lesions on petioles; (b) necrotic lesions on petioles with spreading necrosis on the stem but not causing plant death or collapse. Severe symptoms that prevented harvesting of marketable seed included; (c) severe chlorosis and necrosis on young plants leading to death; (d) severe stem and terminal necrosis and death; (e) severe stem necrosis resulting in lodging and; (f) severe distortion of mature flower head and seeds.

We aimed to model the probability of sunflower hybrids having severe TSV disease and the effect on this probability from variables including the hybrid, rainfall in March or April and distance from the source of inoculum (parthenium weed). Total rainfalls for the months of March and April were included in the analysis because this is the time period that most commercial crops in central Queensland are germinating and are most susceptible to TSV infection. Field observations indicated that variation in rainfall during this early crop stage had a marked effect on TSV disease. The BHLR model development is described in the BHLR Development document, Supporting Information (see Chapter 7 appendix below), or from the corresponding author.

Movement of TSV into crops and effect of barrier crops

To assess potential edge effects and the distance that TSV disease can typically move into commercial crops, we visually estimated severe TSV disease incidence from at least 300 plants at different distances from the edge of crops. Disease counts were done along transect lines perpendicular to an edge of the crop which was downwind of a parthenium-infested areas.

As part of the 2009 and 2010 trials, we included additional treatments to test the effect of a fast growing barrier crop. Test plots of a TSV-susceptible hybrid (Ausigold 61) were grown within a block of forage sorghum which was planted at the same time as the sunflower and with 8 m of sorghum between the sunflower test plots and the edge of the crop. Severe TSV disease incidence was estimated visually as described above.

Comparison of sunflower hybrid reactions to TSV-parthenium and TSV-crownbeard strains

Reference cultures of TSV-parthenium isolate-1973 and TSV-crownbeard isolate-2334 were maintained as previously described (Sharman and Thomas 2013). To compare the reactions of these distinct TSV strains on sunflower hybrids, they were manually inoculated onto test plants of hybrids with good and poor tolerance to TSV-parthenium as determined in the field trials. Manual inoculations were done using 0.1 M phosphate buffer with sodium sulphite added and a mix of diatomaceous earth and carborundum as abrasives. Test plants were rated visually for severe disease at 8 days post inoculation.

Results

A range of TSV symptoms were observed in naturally infected sunflowers from field trials and commercial crops. While only severe symptoms that would prevent harvesting were considered for the assessment of tolerance, symptoms also included small necrotic lesions on petioles, through to severe stem and terminal necrosis, complete plant collapse and death (Fig. 2).

Field trials for TSV tolerance in sunflower hybrids

Over the course of the 5 year study, the proportion of total plants that were severely infected from each plot was determined from a total of 470 plots within the randomized block designs. The overall observed incidences of severe disease for each hybrid in each year/site combination along

with the predicted probability distributions of severe disease under historical rainfall distributions using the BHLR model are shown in Figure 1.

The BHLR analysis of the field trial results demonstrated that all hybrids, with the exception of Jade Emperor were significant components in the model (i.e. 95% credible intervals for the hybrid specific intercepts did not enclose zero). One way to think about the hybrid specific intercept term in our model is that a value of zero (in the absence of other factors) corresponds to a 50% probability of a plant showing severe disease symptoms. Therefore, all hybrids except for Jade Emperor significantly reduced disease incidence to below this 50% benchmark. Table 1 provides the summary of results from the 253 pairwise comparisons of each hybrid. In total, there were nine groups of hybrid-specific intercepts (labelled with letters a - i) that were not significant (in terms of the credible intervals of their differences) from each other.

Disease incidence varied greatly between seasons and our observations indicate this was closely associated with rainfall. As a comparison of relative disease incidence over the five year period, severe TSV disease incidence in the susceptible hybrid Ausigold 61 at the Kenlogan site was 90 % in 2007, 66 % in 2008, 67 % in 2009, 41 % in 2010, 0.5 % in 2011 and 4.8 % in 2012 (Fig. 3).

The effects for rainfall in March, April and their interaction were all significant (95% credible intervals did not contain zero) and negative, indicating that when rainfall was high in March or April, the probability of observing severe disease decreased. This supports our observations that the worst TSV epidemics were observed when plantings that occurred between late February to early March were preceded by summer rains and followed by dry conditions throughout March-April. This period is when plants were young and most susceptible to infection and conditions were favorable for high thrips populations which enabled significant TSV epidemics to develop. For completeness, Table 2 also includes the means and credible intervals for the hierarchical parameters (μ and σ_0) over hybrid effects and the residual error variance.

Figure 1 shows medians along with 2.5th, 25th, 75th and 97.5th percentiles of the posterior predictive distributions in the absence of residual variation within plots. These intervals can be interpreted as the expected distributions of disease under historic rainfall conditions. The median and the widths of the intervals in Figure 1 are largely controlled by different sunflower hybrids.

Table 1 Means and 95% credible intervals for parameters in the BHLR model.

Hybrid Specific Intercept ($\beta_{0,k}$)	**Mean (2.5th percentile, 97.5th percentile)**	**[A]Grouping**
NH2201	-4.08 (-4.61, -3.57)	a
Hysum 304	-4.00 (-4.51, -3.50)	a
Galah	-3.46 (-4.39, -2.59)	a
Advantage	-3.07 (-3.57, -2.57)	b
NH2202	-2.94 (-3.55, -2.36)	b
Hysun 38	-2.94 (-3.43, -2.44)	bc
Ausigold 4	-2.92 (-3.43, -2.40)	bc
Ausigold 62	-2.84 (-3.26, -2.40)	bc
Award	-2.65 (-3.22, -2.07)	bc
Sunoleic 06	-2.55 (-2.98, -2.09)	bcd
T40318	-2.39 (-2.91, -1.86)	cd
T30152	-2.25 (-2.86, -1.64)	cde
7714.6822	-2.13 (-2.73, -1.56)	def
HC001GN	-2.05 (-2.64, -1.47)	def
Hyoleic 41	-1.80 (-2.30, -1.29)	ef
Sunbird 7	-1.578 (-2.09, -1.06)	f
Hysun 39	-1.14 (-1.64, -0.69)	g
Ausigold 61	-1.11 (-1.48, -0.75)	gh
GHX570	-0.897 (-1.58, -0.223)	gh
Ausigold 7	-0.931 (-1.36, -0.479)	h
HP002GN	-0.859 (-1.37, -0.351)	h
Ausigold 52	-0.58 (-1.10, -4.74E-2)	h
Jade Emperor	0.28 (-0.261, 0.780)	i

[A] Varieties with the same letter for column "Grouping" are not significantly different based on 95% credible intervals for posterior distributions of their differences. Credible intervals that contain the value zero are judged not to be significantly different.

Table 2 Means and 95% credible intervals for parameters other than hybrid specific intercepts in the BHLR model.

[A] **Parameter (descriptor)**	[B] **Mean (2.5th percentile, 97.5th percentile)**
μ (mean for hybrid effects)	-2.13 (-2.71, -1.55)
σ_0 (std. dev. for hybrid effects)	1.20(0.896, 1.71)
β_1 (coef. for March rainfall)	-1.10 (-1.21, -0.996)
β_2 (coef. for April rainfall)	-1.47 (-1.95, -1.02)
β_3(coef. for rainfall interaction)	-1.32 (-1.78, -0.889)
β_4(coef. for proximity)	-2.13E-2 (8.77E-2, 4.22E-2)
σ_ε (std. dev. for residual variation in plots)	0.888 (0.808, 0.975)

[A] The parameters shown are those appearing in equation (2) described in BHLR Development, Supporting Information.
[B] The estimated mean and 95% credible intervals for the parameters.

The wetter than average summers of 2009-10 and 2010-11 resulted in grasses competing effectively with parthenium across much of central Queensland. Figure 4 illustrates a dramatic change from an almost pure stand of parthenium in 2008 after several years of El Niño drought conditions, to an almost pure stand of perennial grasses in 2014 after several favorable years of rain. This resulted in reduced inoculum source (TSV-infected parthenium) and thrips populations across most of central Queensland and in areas adjacent to the field trial sites.

Historical observations from the nearest weather station for the period of 1962 to 2014 were used to predict the expected frequency of severe TSV outbreaks. It could be expected that conditions with equal or lower rainfall than those observed in 2008 and 2009, for both March and April, have a probability of occurrence of 11% and would therefore occur on average roughly one year in ten. However, these conditions are most likely to occur during El Niño conditions resulting in several consecutive years of high TSV pressure as was observed from years 2007-2009.

Movement of TSV into crops and effect of barrier crops

The incidence of TSV disease appeared to be reduced with the use of an 8 m wide barrier crop of fast growing forage sorghum. In the 2009 Kenlogan trial, the plot of Ausigold 61 sunflower within the sorghum barrier crop had an incidence of 33 % severe TSV infection compared to 77 % for a comparable plot the same distance from the crop edge but surrounded by sunflower. The effect was less pronounced in the 2010 Kenlogan trial for the same sunflower hybrid with 27 % severe TSV incidence within the barrier crop compared to 35 % outside the barrier.

A strong edge effect was clearly demonstrated by the significantly higher incidence of severely infecting sunflower plants closer to the edge of a commercial crop of hybrid Sunbird 7 located downwind of a weedy area infested with TSV-infected parthenium (Table 3). This effect was more pronounced at 5 weeks post planting but was still significant at 8 weeks post planting. The effect of prevailing wind direction was further demonstrated from a second paddock of the same hybrid, planted at the same time and located upwind of the parthenium-infested area. At the 5 week disease count, TSV disease incidence was 3 % at 5 m upwind of the weedy area, compared to 25 % at 5 m downwind of the same area.

At another site, well within the boundary of a crop of sunflower hybrid Sunbird 7 and approximately 1.2 km downwind of the nearest source of TSV-infected parthenium, severe disease incidence ranged from 16 – 22 % from four counts. This crop was not flowering so all TSV inoculum would have been from outside the crop.

These field observations are supported by evidence from the BHLR analysis of the field trial data with the coefficient for distance from parthenium having an 82% probability of taking a negative value, providing some evidence that increasing distance from parthenium reduced disease incidence across the relatively narrow width (less than 20 m) of the field trials. However, we did not consider this term to be significant, as the 95% credible interval enveloped the value zero (Table 2).

Table 3 TSV disease incidence in commercial sunflower crop to assess edge effect and distance of movement into crop

5 weeks [A]	**8 weeks**
5 m, 109 / 321 (25 %) [B]	5 m, 168 / 286 (37 %)
50 m, 36 / 421 (8 %)	60 m, 135 / 250 (35 %)
150 m, 23 / 286 (7 %)	150 m, 90 / 257 (26 %)
350 m, 22 / 395 (6 %)	300 m, 67 / 266 (20 %)
400 m, 30 / 622 (5 %)	
χ^2=139.4; p<0.001 [C]	χ^2=33.3; p<0.001

[A] Age post planting of sunflower hybrid Sunbird 7. Transects ran in the direction of prevailing wind, from SE to NW, perpendicular to crop edge.

[B] Distance from edge of crop, number of plants with severe TSV symptoms / number of symptomless plants and percent incidence of severe TSV infection.

[C] Chi-square (χ^2) value for comparison frequency of symptomatic and symptomless plants with significance level indicated.

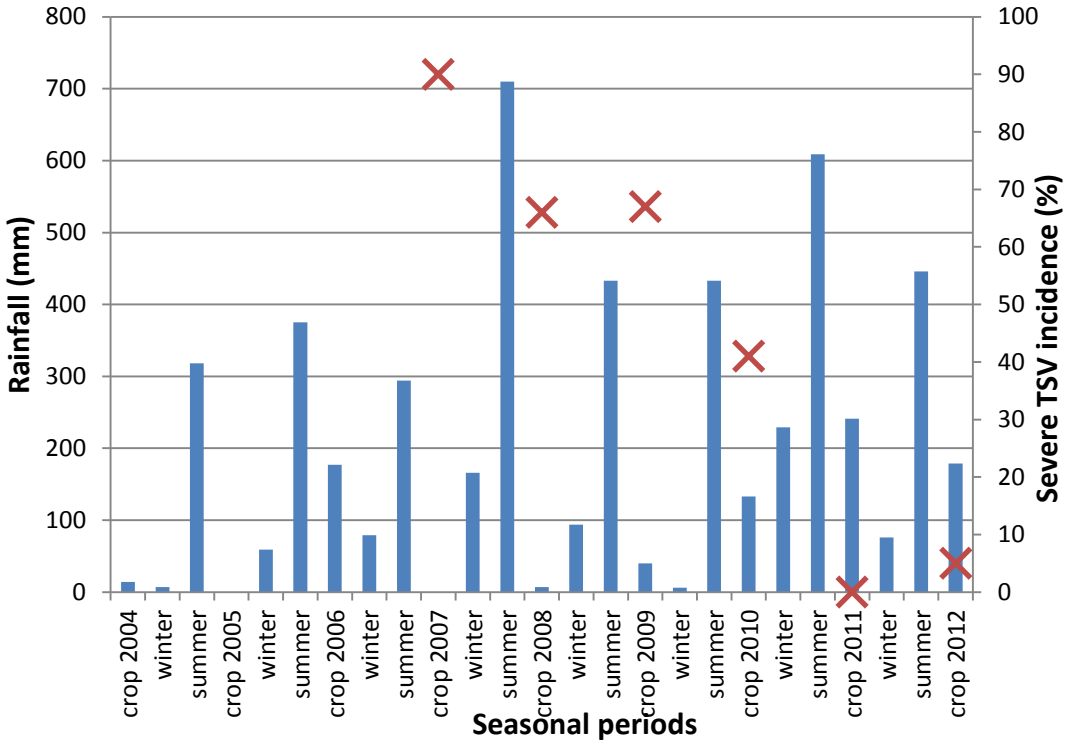

Figure 3 The relationship between changes in seasonal rainfall and incidence of severe TSV disease in sunflower hybrid Ausigold 61 at Kenlogan. Accumulated rainfall data (vertical bars) and severe TSV disease incidence (crosses) are shown. "Winter" is months of June-September, "summer" is October-February and "crop" is March-April.

(a) (b)

Figure 4 Photos of the same site north of Clermont, taken in April 2008 (a) and Feb 2014 (b) illustrating the change in the parthenium population over the six year period.

Comparison of sunflower hybrid reactions to TSV-parthenium and TSV-crownbeard strains

There were only minor differences between the observed symptoms of TSV-parthenium and TSV-crownbeard on the sunflower hybrids tested in the glasshouse (Table 4). TSV-parthenium induced a more severe reaction than TSV-crownbeard but the later still induced a severe disease on all but one of the susceptible Ausigold 61 test plants. In the field trials, hybrids NH2201 and Hysun 304 displayed good tolerance against natural infections of TSV-parthenium while Ausigold 61 had significantly higher incidence of TSV disease. Although the differences were less pronounced compared to the field trials, this pattern of good and poor tolerance was also observed for both TSV strains in the glasshouse testing. The susceptible control plants, mung beans (*Vigna radiata*) and French beans (*Phaseolus vulgaris*), were 100 % infected.

Glasshouse observations indicated that manual inoculation was more severe than field testing and masked some differences that were apparent with field screening. During field surveys over several years TSV-crownbeard was only observed in a few locations infecting commercial sunflower, and like the glasshouse testing, the disease appeared to be less severe than was commonly the case with TSV-parthenium.

Table 4 Glasshouse comparison of reaction of TSV-parthenium and TSV-crownbeard strains on sunflower hybrids and susceptible hosts.

Test host	**TSV-parthenium (isolate-1973)**	**TSV-crownbeard (isolate-2334)**
Sunflower, Ausigold 61	15/15 [A]	7/8
Sunflower, NH2201	8/15	4/10
Sunflower, Hysun 304	10/15	2/9
Vigna radiata	6/6	6/6
Phaseolus vulgaris	5/5	5/5

[A] Number of severely diseased plants out of total tested.

Discussion

This is the first detailed report of the relative tolerance to TSV for a range of sunflower hybrids in Australia. Several hybrids displayed significantly better tolerance to field infections of TSV compared to susceptible hybrids in multiple trials over several years. Notably, those hybrids listed in groupings a and b (Table 1) performed very well even in years with high TSV disease pressure. We did not attempt to identify the genetic basis of the observed tolerance to TSV in sunflower hybrids. However, the high levels of tolerance in a few hybrids suggests that this may be a fruitful area for further research.

We previously tested 33 individual plants from 23 species from the Clermont area from within, or close to the trial sites by strain-specific PCRs and found all were TSV-parthenium (Sharman *et al.* 2015). While TSV-crownbeard has a wide host range, we only ever found TSV-crownbeard in locations where crownbeard grew. Our direct testing of representative samples from the trial sites and the fact that both trial sites were at least 50 km from the nearest known locations of crownbeard, provided strong evidence that TSV-parthenium was the only TSV strain present at the field trials during our study.

Our glasshouse screening results indicate that the observed field tolerance to TSV-parthenium is likely to also confer tolerance to the distinct strain, TSV-crownbeard, and the latter strain appears unlikely to cause the same level of damage in field infections as observed for TSV-parthenium. Given these two TSV strains are genetically and biologically distinct (Sharman *et al.* 2015), the tolerances we have identified in sunflower hybrids may also be effective in other regions affected by TSV such as India.

We acknowledge that the Bayesian statistical analysis employed in this study may seem foreign to some researchers and industry members who are more familiar with traditional frequentist approaches based on the analysis of variance. However, modern computing hardware and computational statistics has led to more sophisticated approaches for data analysis that can enhance our abilities to answer certain types of questions. In this study, the use of a Bayesian Hierarchical Logistic Regression (BHLR) model has provided an elegant method by which we could: (i) answer the same questions that can be answered using traditional frequentist approaches; (ii) quantify the probability distributions of severe disease incidence under historical rainfall conditions using samples from the posterior distribution; and (iii) undertake a large number of pairwise comparisons (253 in total) without being overly concerned about controlling familywise error rates. We should

see greater uptake of these methods in the future with advances in the availability of statistical software for fitting complex BHLR models, like *Stan*, the recently developed probabilistic programming language by the Stan Development Team (2014) used in this study. To aid this, we have made the data and Stan programming code used in this analysis available from the corresponding author.

Bayesian statistical methods can provide an advantage over traditional frequentist methods in providing a simple means of addressing questions such as those in this study. Firstly, the output of a Bayesian analysis is a set of samples from the joint posterior probability distribution (the distribution of the parameters given the observed data). Sampling from the posterior distribution and from the historical rainfall distribution is very simple and allows us to easily combine the knowledge gained from the field experiment with new data (i.e. the historical data; external to the experiment) to make some predictions about future levels of disease incidence that might be expected. Secondly, in the Bayesian hierarchical model outlined below, we model each of the hybrid effects (intercepts in a linear model) as arising from some probability distribution of hybrid effects. Consequently, there is a natural "shrinkage" of the effects of hybrids towards each other that implicitly makes all pairwise comparisons conservative. This addresses the multiple comparison problem and has recently been argued as yielding more efficient estimates than under the traditional frequentist approach of applying corrections to post-hoc analyses (Gelman *et al.* 2012).

For the reasons outlined above, we chose to use the Bayesian statistical procedure, a BHLR rather than a frequentist method such as a Generalised Linear Model. This approach is similar to that presented by Zeger and Karim (1991), but makes use of the Hamiltonian Monte Carlo (Betancourt and Girolami 2013) rather than the more traditional Gibbs sampler to sample from the posterior distribution.

The high level of variation in disease incidence between years illustrates the importance of conducting these replicated trials over several seasons and different sites to ensure a rigorous assessment of field tolerance to TSV. Given the widespread occurrence of TSV in the parthenium population in central Queensland (Sharman *et al.* 2009), we expect TSV inoculum from surrounding parthenium will pose a risk to sunflower crops for the foreseeable future. It will therefore be important to monitor the relative tolerance to TSV for any new hybrids and we hope that the new information presented in this study may be utilised by breeders to predict the tolerance of related hybrids.

Screening hybrid tolerance to TSV by manual inoculation in glasshouse conditions appeared to be too severe and was not a reliable method to identify differences in tolerance observed in the field trials. While glasshouse testing may be useful for comparisons of very different levels of tolerance, testing by field trials was a better method to accurately assess the true tolerance of hybrids under natural conditions. Other techniques have been reported for inoculation of very young sunflower seedlings by injuring the growing point (Sundaresha *et al.* 2012) which may warrant further comparison to standard manual inoculation.

The severity of a TSV disease epidemic is determined by a complex interaction of factors including the growth stages of the virus source (parthenium) and the susceptible crop, the size and dispersal behaviour of the thrips population feeding on the virus source, and the orientation of the crop in relation to prevailing winds. We observed significantly lower incidences of TSV during periods of regular rainfall in the critical growing months of March and April, most likely as a result of reduced total inoculum and thrips populations. Almeida and Corso (1991) also observed a correlation between increased accumulated rainfall and a marked decrease in thrips populations and correspondingly lower levels of TSV disease incidence in soybean in Brazil. Lokesh *et al.* (2005) observed very low TSV disease incidence related to high rainfall which was unfavourable for vector populations.

Rainfall in central Queensland is often sporadic, making it difficult to predict the dry periods which increase the risk of TSV disease. The most common time of year for planting rain-fed sunflowers is late summer which also coincides with the main growing periods of parthenium. Where irrigation is available, the risk of TSV can be greatly reduced when crops are planted in Spring (September – November) when there is generally much less flowering parthenium. Similar strategies of changing planting times to avoid peak influx of TSV inoculum have also been recommended for soybean in Brazil (Almeida and Corso 1991) and sunflower in India (Shirshikar 2003). Unfortunately, irrigated areas are limited in central Queensland and this strategy would be unavailable to most growers.

To minimise the risk of TSV disease in sunflower crops in central Queensland several approaches are recommended. Avoid planting downwind of large areas of flowering parthenium which is the major source of TSV that moves into crops (Sharman *et al.* 2009). The use of a barrier crop, or some means of spatial separation of the crop from flowering parthenium may help to reduce the risk of severe damage near the edge of crops but is unlikely to restrict the long distance dispersal of thrips carrying TSV-infected pollen into crops. Slashing or herbicide control of surrounding parthenium prior to planting susceptible crops is also advisable to reduce TSV inoculum during the

most susceptible early crop stage. The use of tolerant hybrids identified in this study will greatly reduce the risk of significant losses due to TSV in central Queensland and potentially other regions around the world where TSV affects sunflowers.

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Supporting Information

A detailed description of the development of the BHLR model is provided in file BHLR_Development_SuppInfo.docx. See Chapter 7 appendix below.

Chapter 7 Appendix

BHLR Development

The following Bayesian Hierarchical Logistic Regression (BHLR) model was developed and utilised for the study described in Chapter 7.

Let n_{ijkl} denote the number of plants examined and Y_{ijkl} denote the number of those plants that were severely infected, for the *i*th site, *j*th year, *k*th sunflower hybrid, with the *l*th plot. Here $i \in \{1,2\}$ (corresponding to the Kenlogan and Langton Cottage sites), $j \in \{1,2,3,4,5\}$ (corresponding to data collected in years 2008 – 2012 inclusive), and $k \in \{1,2,\dots,23\}$ indexes the 23 hybrid varieties in the experiment. For each of the observations, a number of predictors for the incidence of severe disease were identified. Two of these predictors were centred and scaled rainfall variables: $r_{ij}^{March} =$ (R_{ij}^{March}- 59.29) / 58.65, where R_{ij}^{March} is the total rainfall in mm for the month of March at the Australian bureau of meteorology station number 035094 in each year with historical monthly mean and standard deviation of 59.29 and 58.65 respectively; and $r_{ij}^{April} =$ (R_{ij}^{March}- 33.99) / 44.20, where R_{ij}^{March} is the total rainfall in mm for the month of April, and 33.99 and 44.20 are the historical mean and standard deviation for the month. An interaction between these two rainfall terms was also included in the model. Another predictor in the model was d_{ijkl}, a variable describing the distance of each plot to the inoculum source (parthenium weed upwind of the trial edge). Finally, a number of additional effects were also included in the model to account for: natural variability between sites, $\epsilon_i \sim N(0, \sigma_\epsilon^2)$; between years, $\gamma_j \sim N(0, \sigma_\gamma^2)$; and between each of the plots, $\eta_l \sim N(0, \sigma_\eta^2)$.

Initially, the probability that an individual plant in each plot would exhibit severe disease symptoms was considered using a logistic regression model of the form:

$$log\left(\frac{p_{ijkl}}{1-p_{ijkl}}\right) = \beta_{0,k} + \beta_1 \ r_{ij}^{March} + \beta_2 r_{ij}^{April} + \beta_3 \ r_{ij}^{March} r_{ij}^{April} + \beta_4 d_{ijkl} + \epsilon_i + \gamma_j + \eta_l \quad (1)$$

with $\beta_{0,k} \sim N(\mu, \sigma_0^2)$ being random intercept terms modelled as realisations from a common probability distribution and reflecting the contribution from the sunflower hybrid grown. The data were modelled conditionally on the probability of severe disease as binomial random variables:

$$Y_{ijkl} \mid p_{ijkl} \sim Binomial\left(n_{ijkl}, p_{ijkl}\right).$$

The model was then fit using Bayesian inference using Hamiltonian Monte Carlo (HMC) with uninformative priors on the remaining model parameters, namely: $\beta. \sim N(0, 10^3)$; $\mu \sim N(0, 10^3)$; $\sigma_\varepsilon \sim U(0, 10^3)$; $\sigma_\gamma \sim U(0, 10^3)$; $\sigma_0 \sim U(0, 10^3)$; and $\sigma_\eta \sim U(0, 10^3)$.

HMC was run using 5 independent chains each of which used 1000 burn-in samples, followed by a further 1000 samples that were treated as representative samples from the posterior distribution (used for making inferences). Samples from the posterior distribution were used to assess whether each of the effects in the model made a statistically significant contribution to the probability of severe disease symptoms, by examining whether the 95% credible intervals for the corresponding parameters contained the value zero.

Following this process, the hierarchical terms for years and sites were found to: (i) be insignificant (95% credible intervals for all γ_j and η_l contained the value zero); (ii) contributed an unrealistic amount of additional noise to the model; (iii) and reduced the overall fit to the observed data. Consequently, these components were removed from the final model and equation (1) was replaced with the simpler structure:

$$log\left(\frac{p_{ijkl}}{1-p_{ijkl}}\right) = \beta_{0,k} + \beta_1 r_{ij}^{March} + \beta_2 r_{ij}^{April} + \beta_3 r_{ij}^{March} r_{ij}^{April} + \beta_4 d_{ijkl} + \eta_l. \quad (2)$$

The model was run again under this formulation using HMC, with 5 Markov chains each of which had 1000 burn-in samples followed by 1000 posterior samples.

Subsequently, the posterior samples of the model parameters were used to construct predictive intervals over the probabilities of severe infection for each of the varieties. These predictive intervals were modelled by including variability arising from rainfall in March and April (distance to parthenium was not found to be significant and excluded from this analysis), by sampling with replacement from the distributions over these variables. Pairs of monthly rainfall variables r_{ij}^{March} and r_{ij}^{April} were sampled with replacement from the bivariate empirical distribution of rainfall measured at a nearby Bureau of Meteorology weather station between years 1963-2013.

For comparing the resistance of each of the hybrids to each other, we performed all 253 pairwise comparisons between the 23 hybrids. For the comparison of hybrid i and j ($i \neq j$), the 5000 HMC samples from the posterior distribution were used to compute the differences $\omega_{i,j} = \beta_{0,i} - \beta_{0,j}$. For each pair of hybrids, 95% credible intervals were then computed from the posterior distribution of $\omega_{i,j}$ and used to determine whether this difference was significantly different from zero. Pairs of hybrids that did not differ from one another were assigned letters to signify groups that did not show evidence of being significantly different from one another.

Chapter 8

General discussion

Summary of key findings

In this study, we determined the critical factors affecting the disease cycle of TSV in central Queensland and how this leads to disease epidemics. We determined that while there were two distinct TSV strains present in the central Queensland region (TSV-parthenium and TSV-crownbeard), we only found evidence for the TSV-parthenium strain being associated with the major disease outbreaks in nearby crops of sunflower and mung beans (Chapters 5-7). TSV-parthenium had a wide natural host range in crops, weeds and native plants but its major alternative host was the prolific weed *Parthenium hysterophorus* (Chapters 3 and 6). We determined that TSV-parthenium was endemic and commonly found in parthenium as symptomless infections across the major geographical range of this weed (Chapter 3). We identified the thrips *Frankliniella schultzei* and *Microcephalothrips abdominalis* as the two most important vector species for TSV-parthenium. Both thrips species were the most commonly collected from parthenium and TSV-infected sunflower and were both shown to be efficient vectors (Chapter 6). We demonstrated that the high rate of seed transmission of TSV-parthenium in parthenium is a critical survival mechanism for the virus. It enables it to survive between seasons separated by harsh environmental conditions, allowing it to rapidly re-establish in parthenium populations which can then result in TSV disease epidemics in nearby crops (Chapter 3). Tolerant sunflower hybrids were identified and are now being used to effectively manage TSV (Chapter 7). Based on our findings we can now describe a detailed disease cycle for TSV that affects grain crops in Australia (Fig 8.2).

Identification of causal agent

Our first significant finding was to demonstrate that TSV was the causal agent of the severe necrosis disorder affecting sunflower and mung beans in the central highlands region of central Queensland. We used a combination of diagnostic assays to confirm TSV infection including ELISA, TSV-specific PCR and sequence identity comparisons to type species listed on GenBank. We also found TSV causing disease symptoms in crop plants of mung bean, chickpea and cotton (Chapter 2; Sharman et al. 2008). TSV was causing significant losses in sunflower and mung bean but was a minor issue in chickpea and cotton. We rarely found natural infections of chickpea. This may have been because the crop timing is from late Autumn to early Spring when parthenium and thrips populations are generally at their lowest and hence inoculum pressure is correspondingly low. TSV symptoms were often seen on scattered plants near the edge of cotton crops but almost always as isolated local lesions on one or two leaves and there was rarely evidence of systemic spread. There appeared to be no significant impact on cotton due to TSV infection.

This study provided the first published reports from Australia of TSV infecting these four crop plants. We obtained partial RNA 3 sequence from a sample of infected sunflower which indicated a close nucleotide identity to a TSV strain reported from soybean in Brazil (Almeida et al. 2005).

Relationship of central Queensland TSV strains to other reported strains

In order to determine the relationship between TSV isolates obtained from different hosts, we characterised the complete genome sequences for TSV strains from central Queensland and also for members of two previously reported TSV strains from Australia, TSV-S and TSV-Ag (Table 8.1). We found two genetically distinct TSV strains from central Queensland, TSV-parthenium and TSV-crownbeard (Chapter 5; Sharman and Thomas 2013). Our results also clarified the true identity of strain TSV-S as an isolate of SNSV which was the first report of this virus from Australia (Chapter 4; Sharman et al. 2011). We also demonstrated that TSV-Ag represented a new distinct ilarvirus subgroup 1 species that we called Ageratum latent virus (AgLV; Sharman and Thomas 2013).

We characterised the partial genome of strain TSV-A (isolate-1025 from *Ajuga reptans*) which had a strongly supported recombination event on RNA-3 involving a strain closely related to the type species (TSV-WC) as a minor parent (Chapter 5; Sharman and Thomas 2013). Interestingly, TSV has also been reported from *Ajuga reptans* from the United States of America (Fisher and Nameth 1997) but no associated genome data has been reported.

This study provides the first genetic information about subgroup 1 ilarviruses from Australia and illustrates significant genetic diversity of TSV strains, and other subgroup 1 members previously reported to be TSV strains (Table 8.1). In the process, we confirmed that the TSV strain associated with sunflower necrosis disease in central Queensland, TSV-parthenium, was not closely related to any of the other previously reported TSV strains from Australia or overseas. In fact, two of the previously reported TSV strains from Australia, TSV-S and TSV-Ag, were not TSV but were actually members of distinct ilarvirus species. As such, we could not assume that the extensive biological studies conducted by previous workers would be relevant to TSV-parthenium or TSV-crownbeard. Hence, it was important to then characterise various aspects of the biology of both the TSV strains present in central Queensland.

Table 8.1. Comparison of molecular identity of new and archived ilarviruses characterised in this PhD study with samples referred to as TSV strains from previous studies.

Confirmed identity from this study	**Previously called (references)** [1]	**(Our publications)** [1] **/ (Chapters)**
TSV-parthenium	Described in this study	(1-4) / (2, 3, 5, 6, 7)
TSV-crownbeard	Described in this study	(3-4) / (5-7)
TSV-A	TSV-A (5, 6, 7), Ajuga-TSV (8)	(3) / (5)
SNSV	TSV-S (5, 6, 7)	(3, 9) / (4, 5)
AgLV	TSV-Ag (5, 7), TSV-As (6)	(3, 4) / (5, 6)

[1] References cited in Table: (1) Sharman et al. (2008); (2) Sharman et al. (2009); (3) Sharman and Thomas (2013); (4) Sharman et al. (2015); (5) Klose et al. (1996); (6) Sdoodee (1989); (7) Klose (1997); (8) Shukla and Gough (1983); (9) Sharman et al. (2011).

Extensive field surveys conclusively showed that TSV-parthenium and TSV-crownbeard were both geographically widespread and common across a large area of the Central Highlands region (Fig 8.1). In contrast to TSV-crownbeard, our data clearly showed that TSV-parthenium was always associated with major disease outbreaks in crops. They both had wide host ranges, but their geographical distributions were closely associated with that of their respective (and symptomless) major alternative hosts, parthenium (*Parthenium hysterophorus*, Chapter 3; Sharman et al. 2009) and crownbeard (Verbesina encelioides, Chapters 3, 5 and 6; Sharman et al. 2009; Sharman and Thomas 2013; Sharman et al. 2015). These two TSV strains appear to be common and persistent in their respective major alternative hosts.

Parthenium was shown to be a poor host of TSV-crownbeard but crownbeard was found to be naturally infected by TSV-parthenium and was also readily infected by manual inoculation. We demonstrated conclusively TSV seed transmission in Asteraceae species with relatively high rates of seed transmission of TSV-parthenium and TSV-crownbeard identified in their respective major alternative hosts. TSV-parthenium was also seed transmitted at high rates in naturally infected *Bidens pilosa* and *Conyza bonariensis* (Chapters 3 and 6; Sharman et al. 2015; Sharman et al. 2009).

The relatively high rates of seed transmission of TSV-parthenium and TSV-crownbeard enable these two TSV strains to survive in the often harsh and unpredictable climate of central Queensland. They can survive for long periods of time in the soil seed bank and then rapidly re-establish and spread when conditions are favourable for the hosts and vectors.

Our results showed that parthenium is the key host for TSV-parthenium in central Queensland and the interaction between this host and pathogen was shown to be critical for the rapid development of

disease epidemics in nearby crops such as sunflower. This is because of the vast areas of parthenium in central Queensland, the large amounts of TSV-infected pollen it produces, the large populations of thrips that it supports and the high rates of TSV seed transmission in parthenium.

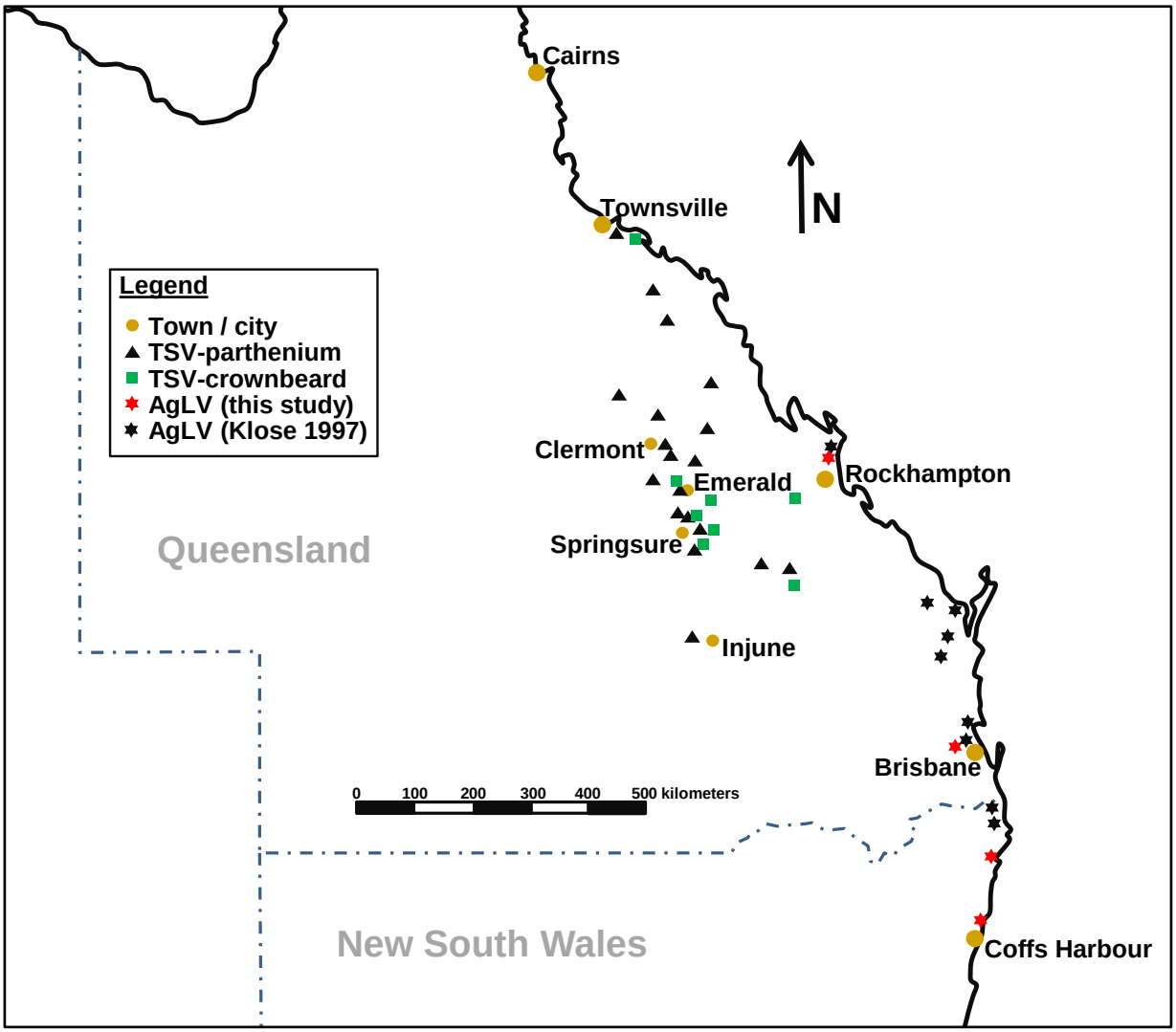

Fig. 8.1. Distribution of sampling sites of TSV-parthenium, TSV-crownbeard and AgLV characterised in this study by TSV-strain and AgLV specific PCRs. The sampling sites of AgLV (TSV-Ag) in *Ageratum sp.* collected by Klose (1997) and identified by ELISA are also shown (black stars).

Thrips transmission of TSV strains

We hypothesised that the transmission mechanism for TSV was similar to that of AgLV (TSV-Ag) demonstrated by Sdoodee and Teakle (1987). We have now conclusively demonstrated this for two distinct strains of TSV. Our study identified the thrips species *F. schultzei* and *M. abdominalis* to be the most important vector species for both TSV-parthenium and TSV-crownbeard in relation to moving these virus strains into susceptible crops. Both were the most commonly collected species

during our surveys from parthenium, crownbeard and sunflower over several seasons in central Queensland. Our experiments showed conclusively that both thrips species readily transmitted both TSV strains in controlled experiments. Although not commonly collected during field surveys, *Thrips tabaci* also readily transmitted both TSV strains (Chapter 6; Sharman et al. 2015).

Seasonal spread of TSV

Our field observations indicated that the disease incidence caused by TSV fluctuated markedly between seasons. There appears to be a complex and dynamic interaction between the major alternative host (parthenium), the pathogen (TSV) and the vector (thrips). Our field observations and results showed that disease incidence in crops was affected by the level of available inoculum (TSV-infected parthenium pollen), the population size of thrips vectors and the proximity and orientation of the susceptible crops to the inoculum. We also demonstrated that some of these factors were also influenced significantly by prevailing weather conditions (Chapter 7).

We found a significant negative association between TSV disease incidence in sunflowers planted in early March and accumulated rainfall in the months of March and April with increasing rainfall resulting in reduced disease incidence. Our field observations indicated that increased rainfall had a negative effect on parthenium and thrips populations, thereby reducing the available inoculum and spread. Parthenium is an opportunistic weed but increased rainfall makes it less competitive against other species. Our observations over several years illustrate the marked change in parthenium population densities across much of central Queensland as the seasons changed from drought conditions in the early to mid-2000s to several seasons of higher rainfall, notably from 2010-12 (Chapter 7).

Our observations of TSV disease incidence within sunflower crops indicated a pronounced edge effect sometimes occurs, resulting in significantly higher incidences of disease close to and downwind from the virus source; TSV-infected parthenium. While TSV disease incidence was usually higher close to the crop edge, we also observed disease incidence of up to 22 % at over one kilometre from the crop edge in moderately susceptible sunflower hybrids. This indicated that while spatial separation of the crop from TSV-infected parthenium is likely to reduce an edge effect, TSV can still be dispersed considerable distances into crops (Chapter 7).

Tolerance in sunflower hybrids and control options for TSV

Based on our initial field observations, we hypothesised that there were differences between sunflower hybrids in their tolerance to TSV infection. Our field trial results collected over five consecutive years, clearly demonstrate significant differences between many sunflower hybrids in

their respective tolerance to field infections of TSV-parthenium (Chapter 7). This is the first report of TSV tolerant hybrids from Australia. Limited glasshouse testing and field observations indicate that the tolerance to TSV-parthenium identified from the field trials is likely to also be effective against TSV-crownbeard. However, TSV-crownbeard infections of sunflower crops were rarely observed during disease surveys and the disease appeared to be less severe than that observed for TSV-parthenium infections. Our results indicate that the use of tolerant sunflower germplasm is an effective means to reduce the risk of TSV disease (Chapter 7).

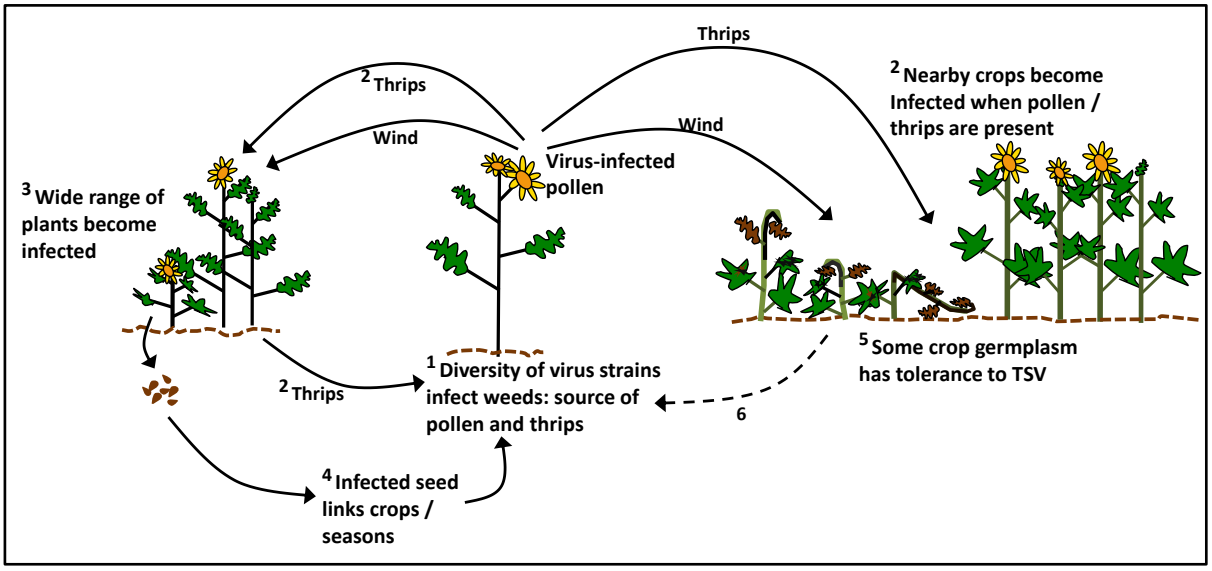

Fig 8.2. Disease cycle of TSV showing points at which we have investigated. **1.** We found significant diversity of ilarvirus subgroup 1 species and strains in Australia but the newly characterised strains, TSV-parthenium and TSV-crownbeard were both common in central Queensland (Chapters 3-6). **2.** A range of thrips were identified from weeds and crops but *F. schultzei* and *M. abdominalis* were the most prevalent. Both TSV strains were readily transmitted in virus-infected pollen in the presence of thrips species *F. schultzei*, *M. abdominalis* and *T. tabaci* (Chapter 6). **3.** Both TSV strains had wide host ranges in weeds and crops but their respective major alternative hosts were parthenium and crownbeard (Chapters 2, 3, 5 and 6). **4.** Both strains were highly seed transmitted in several plant species including in their major alternative hosts, parthenium and crownbeard, providing critical links between seasons and cropping cycles (Chapters 3 and 6). **5.** We identified significant differences between many sunflower hybrids for their tolerance to natural field infections of TSV which will be critically important for effective disease management strategies (Chapter 7). **6.** Severely affected crops such as sunflower were often dead end hosts but other crops may have produced TSV-infected pollen that could act as a source of inoculum. Various aspects of this disease cycle are discussed further in the summary of key findings above.

General discussion

The findings from this PhD study have greatly increased our understanding about the diversity of TSV and ilarvirus subgroup 1 members and some of the fundamental interactions these virus species have with their plant hosts and the thrips vectors that transmit them.

One of the major outcomes of this study was the characterisation of the true molecular identity of archived and new TSV strains and related subgroup 1 species from Australia. The high degree of diversity identified in this study has implications for our global understanding of TSV taxonomy. A recent review of ilarvirus genetic diversity indicated that while TSV has wide geographical and host ranges, there is relatively little diversity in the coat protein with greater than 95 % aa identity between all published sequences (Pallas et al. 2013). However, our published coat protein sequence for the TSV-parthenium strain (Sharman et al. 2009) was not considered in the study by Pallas et al. (2013) and indicates only an 86 % aa identity with the type species TSV-WC.

A recent study reported another divergent strain of TSV infecting soybean in Oklahoma, the United States of America (Dutta et al. 2015). Their analysis included our representative sequences for TSV-parthenium (TSV-1973) and TSV-crownbeard (TSV-2334) and clearly demonstrated that TSV-parthenium was most divergent from other reported strains. Interestingly, Dutta et al. (2015) also reported evidence for recombination events involving both TSV-1973 and TSV-2334 as either major or minor parents. These findings may provide some indication to the origin of the TSV-parthenium and TSV-crownbeard strains.

In a phylogenetic analysis, Tzanetakis et al. (2004) observed that the coat protein nucleotide identity between the reported subgroup 2 species, being less than 84 %, was similar to the difference between TSV and SNSV, hence supporting the proposal to consider them distinct species. Our results demonstrate the coat protein nucleotide identity of TSV-1973 (TSV-parthenium) compared to the type species TSV-WC, to be less than 81 % while the corresponding amino acid identity was 86 % (Chapter 5; Sharman and Thomas 2013). As also illustrated by Dutta et al. (2015), this clearly shows the TSV-parthenium strain is the most divergent TSV strain reported to date. The criteria for species demarcation within the genus *Ilarvirus* include a combination of serology, host range and sequence similarity, although no specific levels of sequence similarity have been defined (King et al. 2012). In addition to the clear sequence differences, we have also demonstrated some serological and host range differences between TSV-parthenium and TSV-crownbeard (Chapters 5 and 6; Sharman and Thomas 2013; Sharman et al. 2015). However, it is not clear if these differences demonstrate that TSV-parthenium should be considered a distinct species based on the current

criteria for species demarcation. If a more specific level of sequence similarity is defined for ilarvirus demarcation, it may help to clarify the status of TSV-parthenium.

The development and use of sequencing methods has enabled archived samples from some previous studies to be accurately identified. For example, several studies reported what was thought to be a distinct TSV strain (Johnson et al. 1984; Spiegel and Cohen 1985; Stace-Smith et al. 1987; Stenger et al. 1987) but no supporting genome information was available. A later molecular study found that reference material from some of the earlier work, including the proposed Black raspberry latent virus (Converse and Lister 1969; Jones and Mayo 1975) were in fact SNSV (Tzanetakis et al. 2004). Unfortunately, the true molecular identity of the viruses from many of the other earlier studies may remain unknown unless reference material is available for sequence analysis. We used similar archived reference material from in our study to confirm the first record of SNSV from Australia and to characterise AgLV, a new ilarvirus species (Sharman et al. 2011; Sharman and Thomas 2013), both of which were previously reported as TSV strains (Table 8.1).

Most host range studies previously published for TSV have not had any supporting virus sequence data to confirm the true identity of the studied virus. To the best of our knowledge, this PhD study is the first of its kind to investigate the natural host range of TSV strains using RNA segment-specific PCRs. This was necessary because our study in central Queensland was complicated by the presence of the two distinct TSV strains. To overcome this, we developed and used strain-specific PCRs for all three RNA segments to ensure we developed an accurate picture of the true composition of RNA segments in each host. This approach allowed us to check if reassortments commonly occurred between the two TSV strains but it may not detect recombination events as we were not attempting to sequence each fragment or characterise the RNA sequence outside of the PCR amplicons. For the purposes of this study, we have assumed that when we identified a sample as a particular strain, its genome sequence was most likely to match the complete genome of either of the reference isolates -1973 (TSV-parthenium) or -2334 (TSV-crownbeard).

Within Australia, TSV and ilarvirus subgroup 1 members have only been reported from eastern States. However, these regions are historically where most of the related research has been conducted so it may be possible that some of the subgroup 1 species are present in other regions of Australia.

It is not clear why the disease caused by TSV-parthenium has only become apparent in sunflower crops in central Queensland since the early 2000s considering the major alternative host, parthenium has been at high densities in the same region for about 30 years longer. There are

several possible scenarios for how TSV has become so widespread and important. Perhaps the most likely is that TSV-parthenium was accidently introduced into central Queensland via infected seed of either a weed or crop species sometime in the 1990s and then moved into parthenium and rapidly spread. Alternatively, it is possible that TSV arrived with the accidental introduction of parthenium seed that occurred in the late 1950s into central Queensland (Everist 1976; Haseler 1976; Picman and Towers 1982). However, this appears less likely because sunflowers have been grown in this region since at least the 1970s with no TSV disease reported in the earlier years. It is also possible that TSV-parthenium arrived and established in a non-cropping region, possibly close to a port of entry and then subsequently moved into central Queensland.

One of the more interesting findings from our study was that the TSV-parthenium strain from Australia appears to be the same, or closely related to the strain described from Soybean in Brazil (Chapter 5; Almeida et al. 2005; Sharman and Thomas 2013). Based on their sequence identity, it seems likely that both have a common ancestor and there may have been movement of this strain between these continents.

We identified a wide host range for each of the two TSV strains from central Queensland, including numerous native plants. Perhaps these viruses survived at relatively low incidences in a number of native plant species and were transmitted by native thrips adapted to those plants. The introduction of pest thrips species may have facilitated transmission of these TSV strains from the native plants into prolific weeds such as parthenium and crownbeard. Pest thrips, such as those identified as efficient vectors in our study, have the characteristics of being polyphagous and able to rapidly multiply and disperse. Even if this hypothesis is not supported by strong evidence, the polyphagous nature and high fecundity of the pest thrips appear to be critical factors in the rapid development of epidemics in combination with the key alternative hosts, parthenium and crownbeard. To test some of these hypotheses about the origin of TSV-parthenium, it would be very useful to have the complete genome sequence of the Brazilian strain so that an evolutionary analysis could be done to estimate the time of divergence from a common ancestor.

By studying the recent TSV disease epidemics in Australia, we can now make some interesting comparisons with recent disease epidemics in India. In both countries, parthenium and pest thrips species have played a critical role in the build up of inoculum and rapid development in disease in nearby susceptible crops such as sunflower and mung bean. In both locations, parthenium has been present at high population levels for at least several decades prior to the first recognition of TSV disease epidemics in both countries, which occurred within several years of each other. However,

the TSV strains responsible for disease epidemics in both countries are genetically quite distinct (Chapter 5; Sharman and Thomas 2013). This suggests that at roughly the same time, both countries had incursions of, or the spread of, endemic TSV strains, different to each other but with the same major alternative host (parthenium) and causing essentially the same diseases in crops. It is interesting to note that while there is a strong synergistic relationship between TSV and parthenium in both Australia and India, parthenium is a poor host of TSV-crownbeard, even though it is common in the same environment. This suggests that there may be something unique and in common between the Indian TSV strain and TSV-parthenium that make parthenium a major host for these strains. This in turn enables these two distinct TSV strains to cause similar severe disease epidemics on two continents.

Another difference between the TSV pathosystems in India and Australia is the seed transmissibility of the different strains. Both TSV-parthenium and TSV-crownbeard from Australia are highly seed transmitted in their respective major alternative hosts (Chapters 3 and 6; Sharman et al. 2009; Sharman et al. 2015). By contrast, TSV from India is not seed transmitted in parthenium, nor in several other plant species tested (Prasada Rao et al. 2009; Reddy et al. 2007; Vemana and Jain 2010). It is unknown if there is an underlying genetic factor controlling seed transmissibility of these TSV strains. Seed transmission studies of different TSV strains from the United States of America indicated the presence of an additional, small RNA segment may be associated with non-seed transmission (Walter et al. 1995). The presence of a similar additional RNA segment has not yet been investigated in either Australia or India (Vemana and Jain 2010).

Seed transmission in the central Queensland environment appears to be a critical factor that enables TSV to survive across adverse climatic conditions, rapidly re-establish in major alternative hosts when conditions are favourable, leading to epidemics in susceptible crops. It is not clear how TSV persists in certain areas in India without seed transmission. Perhaps the climatic conditions are not as harsh as in central Queensland and there are always host refuges in which the virus can survive between cropping cycles; or perhaps there is another alternative host that the virus survives in to bridge between populations of parthenium.

The high rate of seed transmission of TSV-parthenium that we identified in naturally infected *Conyza bonariensis* may enhance the potential for this TSV strain to be moved and become established in regions outside of central Queensland. *C. bonariensis* is a small seeded weed that is widely distributed across much of the cropping regions in north-east Australia and has greatly increased in prevalence over the last couple of decades (Walker and Robinson 2008). Many

populations of this weed across this region have also developed significant resistance to glyphosate (Walker et al. 2011), the dominant herbicide used for fallow weed control in minimum tillage farming systems across this region (Osten et al. 2007). It appears quite feasible that the very small, TSV-infected seeds of this weed could be easily moved on machinery or livestock into new cropping regions. However, it is unknown if the pollen and thrips-hosting capacity of *C. bonariensis* would enable TSV to survive solely in this species across generations or if TSV will only become established in new regions if it can move from *C. bonariensis* into another alternative host more suitable for TSV. Being endemic to South America (Wu 2007), *C. bonariensis* may represent the sort of small seeded species that could have easily been inadvertently moved to Australia with TSV-infected seed, if South America was the origin of TSV-parthenium.

Ageratum conyzoides was reported as a commonly infected natural host of TSV in India (Prasada Rao et al. 2003a), although it is unclear if it can play a significant role in the development of TSV epidemics in the absence of parthenium. We found that TSV-parthenium and TSV–crownbeard readily infect, and are seed transmitted in *A. houstonianum*. This suggests that if these TSV strains become established in *A. houstonianum* or *A. conyzoides*, which are very common weeds in coastal regions of eastern Australia, they could potentially affect several important, susceptible crop plants, such as French beans (*P. vulgaris*), peanuts, soybean and capsicum (Sharman et al. 2015).

AgLV was shown to be transmitted by three thrips species (Klose et al. 1996) and seed transmitted at high rates in some inoculated hosts but not from ageratum, its major alternative host (Greber et al. 1991b; Sdoodee 1989; Sdoodee and Teakle 1988). However, Klose (1997) did later detect seed transmission of TSV-Ag at a low rate (less than 1 %) in seedlings from naturally infected *A. houstonianum*. In this PhD study, we did not detect seed transmission of AgLV from naturally infected ageratum (data not shown) but we did observe seed transmission of AgLV from experimentally infected ageratum at a rate of 18 %. Klose (1997) suggested that seed transmission was of lesser importance for disease spread compared to thrips mediated transmission for TSV-Ag (AgLV). Given the more persistent nature of the ageratum population along tropical and sub-tropical coastal areas of Australia, it may be true that AgLV does not need to have efficient seed transmission in ageratum in order to persist. However, seed transmission, even at a low rate, may still be a useful back up survival strategy for AgLV in more marginal environments where ageratum populations may be fragmented or die out seasonally.

An interesting finding from our study was evidence of synergistic relationships where plant viruses potentially increase the ecological fitness of their hosts. Most notably, the negative effect that TSV-

parthenium infection had on crownbeard seed production, may enable parthenium to compete more effectively for space in locations where both weeds grow together in central Queensland. Also, both TSV-parthenium and TSV-crownbeard were symptomless in parthenium and crownbeard respectively, but caused disease in a number of plant species growing in the same environment, including many species endemic to Australia (Chapter 6; Sharman et al. 2015). Hence, the infection of parthenium and crownbeard with their respective TSV strains is hypothesised to be detrimental to a number of other plant species that would otherwise compete for space, resulting in localised plant community changes favouring TSV-tolerant and non-susceptible species. Malmstrom et al. (2005) also observed a similar synergistic plant-virus interaction when a newly introduced grass species to the grasslands of California, which had tolerance to two cereal viruses, acted as a significant reservoir for these viruses which greatly reduced the population of a susceptible native species, resulting in a plant community shift in favour of virus-tolerant, introduced species. A controlled study of the potential effects of several plant viruses on native plant biodiversity also found that generalist viruses, similar to TSV, which are able to cause disease in plant species across numerous families, pose a risk to the composition of native plant communities (Vincent et al. 2014). The effects on plant population composition when these ecosystems are exposed to new plant viruses can be significant even when the effect on plant fitness is mild (Jones 2009).

Based on our findings concerning several aspects of the disease cycle of TSV, several management options can be implemented to minimise the risk of serious TSV disease in susceptible crops in central Queensland:

- Grow TSV tolerant sunflower hybrids to greatly reduce risk if cropping in regions infested with parthenium. No useful tolerance to TSV has been identified in mung beans, so the use of the cultural practices described below are recommended.
- Avoid planting downwind of large areas of flowering parthenium and aim to be at least several hundred meters from such areas.
- Maintain farm hygiene and control flowering parthenium, particularly during the early stages of a susceptible crop. Chemical control or slashing of flowering parthenium may be effective prior to planting to reduce inoculum.
- The use of a barrier crop, or some form of separation of the crop from parthenium may reduce severe damage on a crop edge but is unlikely to reduce long distance dispersal into crops.

- It is most likely that thrips are carrying TSV-infected pollen into crops from the outside, so there is unlikely to be any reduction in TSV incidence with applications of insecticides to the crop.

The major aims of this PhD study have been addressed, greatly increasing our understanding of the diversity of TSV and related subgroup 1 ilarviruses, how they survive in the environment and how to control the diseases they cause. A detailed TSV disease cycle has now been established and a number of effective disease control methods are now available to growers.

Topics for further investigation

While this PhD study has clarified many aspects related to the diversity and biology of TSV strains and related subgroup 1 ilarviruses, it has also highlighted some interesting questions outside the scope of this current study but worthy of further investigation.

Walter et al. (1995) suggested an association between TSV strains having an additional RNA segment and the absence of seed transmission. It could be a fruitful area for further research to investigate if the presence of such an additional RNA segment, or some other genetic basis, is controlling the apparent difference in seed transmissibility of TSV strains from India and Australia.

We determined that both TSV-parthenium and TSV-crownbeard are highly seed transmitted in their respective major alternative hosts. However, it would be interesting to investigate whether seed transmission results from direct or indirect infection of the embryo. If there is direct infection of the embryo, is it from the mother plant or pollen, or both? It may be possible to determine whether indirect transmission occurs to the embryo via the pollen by pollinating a healthy mother plant with TSV-infected pollen in the absence of thrips. Then check the mother plant to confirm no transmission has occurred and then check whether seed transmission has occurred. It may be difficult to determine whether indirect transmission is occurring from a TSV-infected mother plant to the embryo as this would require emasculation of the flowers to remove the TSV-infected pollen and pollination with healthy pollen. The small size of parthenium and crownbeard flower clusters would make this a difficult task. It may be better to use TSV-infected pollen to pollenate both healthy and TSV-infected mother plants to then compare the resulting rates of TSV seed transmission which may suggest if there is an accumulative effect on the rate of transmission when

both mother plant and pollen are TSV-infected. This may give some indication of the relative importance of the mode of transmission into the embryo.

We hypothesised that there were synergistic effects of TSV-parthenium and TSV-crownbeard strains that provided some biological advantages to their respective major alternative hosts, parthenium and crownbeard respectively (Chapters 6 and 8). Further observations may be required of natural infections of TSV-parthenium-infected crownbeard to confirm if the small deformed seeds observed from experimental inoculations confers a biological advantage for TSV-parthenium and its major host, parthenium in locations where weed species occur. Similar observations could determine if TSV-parthenium provides a biological advantage to parthenium over other TSV-susceptible plant species, resulting in plant community changes. The interaction between TSV-parthenium and crownbeard populations may be a good case study to investigate further.

We found that some field samples had RNA segments present from both TSV-parthenium and TSV-crownbeard, suggesting that such mixed infections could provide the opportunity for recombination and/or reassortment events (Chapter 6; Sharman et al. 2015). Further investigation into the potential for genetic exchange to occur between TSV-parthenium and TSV-crownbeard may provide a good model system to determine how reassortment and recombination between TSV strains may result in hybrid strains, some of which may be able to overcome crop resistance. Conversely, if no genetic exchange occurs between these two TSV strains, even though they are often found as mixed infections, then it may provide some evidence for how they remain genetically distinct.

To better understand how genomic recombinations occur in ilarviruses, it would be useful to determine the full genome sequence of TSV-A (isolate-1025 from *Ajuga reptans*) which had a strongly supported recombination event on RNA-3 involving a strain closely related to the type species (TSV-WC) as a minor parent (Chapter 5; Sharman and Thomas 2013). It would be most useful to do this in collaboration with workers from the USA who lodged a partial RNA-2 sequence from an isolate of TSV from rhubarb (Genbank accession HQ130450) that had 99 % nt identity with RNA-2 of TSV-1025. Comparison of complete genomes of both TSV isolates could clarify their relationship. It would also be useful to determine the genetic relationship of TSV isolated from *Ajuga reptans* from the USA (Fisher and Nameth 1997), for which no sequence data was available, compared to the TSV strain isolated from *Ajuga reptans* in Australia.

We clearly demonstrated the thrips-facilitated transmission of TSV-parthenium and TSV-crownbeard (Chapter 6; Sharman et al. 2015). However, the exact mechanism that enables TSV to

be released from pollen grains and infect healthy plant cells remains poorly understood. Observations of high concentration of AgLV (TSV-Ag) in the germinating pollen tubes by Klose et al. (1992) may provide some clues to an effective mechanism for internally located TSV to enter wound sites produced by thrips and move into the plant cells. This fundamental part of the disease cycle for TSV and other related ilarviruses would be another interesting area to investigate further.

We hypothesise that thrips can carry TSV-infected parthenium pollen during flight, often considerable distances, and subsequently facilitate infection of susceptible plants. This may represent the most likely mechanism for TSV-infected pollen moving from parthenium into crops. This could be tested in caged experiments by mixing thrips and TSV-infected pollen, separating them from test plants using a barrier of water over which they must fly to get to the test plants.

We hypothesise that based on our field trial observations; there is an underlying genetic basis to the observed tolerance to TSV in sunflower germplasm. Determining this genetic basis could be used to breed for tolerance and/or resistance to TSV in Australia and India.

We collected data during from field trials that demonstrated an association between TSV disease incidence in sunflower crops and accumulated rainfall in the months of March and April. We believe that further observations of seasonal changes in thrips and parthenium populations would enable the development of a predictive risk model based on: recent and forecast rain events, soil moisture (related to weed growth and crop stress), predicted and observed thrips and parthenium populations, stage of flowering for parthenium, crop orientation to prevailing winds and proximity to known areas of parthenium.

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