Natural and Engineered Resistance to *Wheat streak mosaic virus* (*Tritimovirus*: *Potyviridae*)

Muhammad Fahim

U4388601

Australian National University

A thesis submitted for the Degree of

Doctor of Philosophy

of The Australian National University

June 2011

Authorship Statement

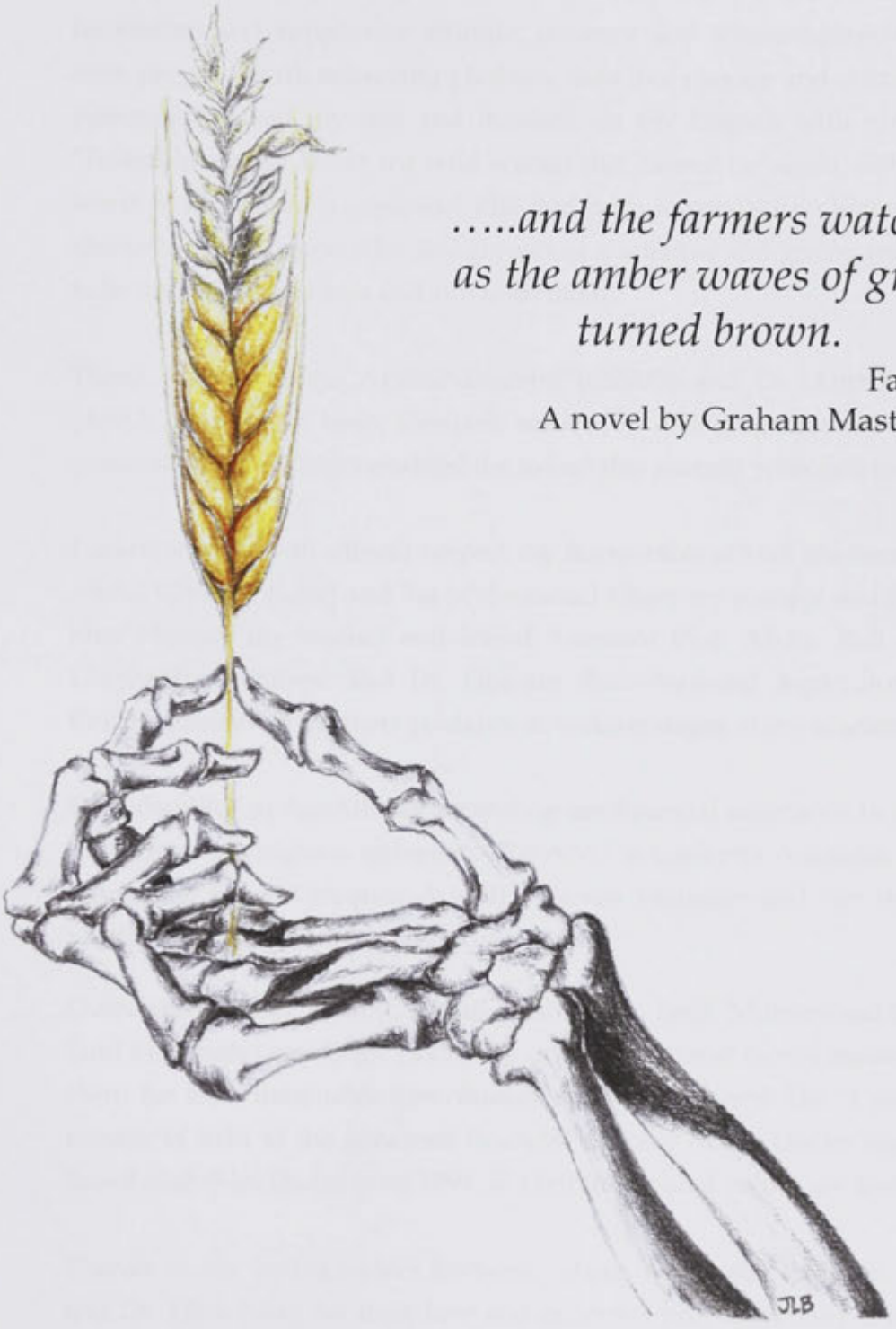
The work contained in this thesis is compilation of research papers, generated from my PhD research,

- Five peer reviewed papers (as first author), framed as individual chapters.
- These papers have been published, are in press, or have been submitted for publication during tenure of my PhD.
- Abstracts of three peer reviewed papers (as secondary author) are framed as Appendices.

I solely conducted the research described in this thesis, except where indicated otherwise. The work presented here has not been submitted elsewhere for any other degree.

Muhammad Fahim

June 2011



.....and the farmers watched as the amber waves of grain turned brown.

Famine
A novel by Graham Masterton

Acknowledgements

I am inspired to extend my gratitude and appreciation to my supervisor Dr. Philip John Larkin (CSIRO) for his excellent supervision, professional guidance, facilitation and supportive attitude; patience and encouragement despite my slow progress with squeezing plethoric data into concise and crispy papers. He patiently checked my text and brushed up my English with a message that "Scientific english is not the wild animal that cannot be tamed; only a lifetime's worth of dedication is required." Phil had been a wonderful mentor and always showed a great concern for my growth as a scientist and giving me a free hand to be creative, take ideas and run with them.

Thank you Dr. Ligia Ayala-Navarette (CSIRO) and Dr. Anthony A. Millar (ANU), you have been constant source of encouragement. Your valuable comments and criticism enabled me to end this journey with success.

I acknowledge with utmost respect my honourable school teachers Syed Salar, Abdul Ghafoor (Late) and Taj Muhammad Khan; my mentor and Professor Dr. Sher Hassan; my teacher and friend Assistant Prof. Abdur Rafi Agricultural University Peshawar and Dr. Hussain Shah National Agricultural Research Centre, Islamabad for their guidance at various stages of my academic journey.

I am thankful to AusAID for providing me financial assistance to study in one of the most prestigious university The ANU in Canberra Australia. I personally thank Ms. Gina Abraquez AusAID Liaison Manager and her staff for their wonderful support throughout this period.

Gulzarullah, Hafiz Rashid, Abdul Nasir, Engr. Latif, Muhammad Sharif Khalil (and his family) and Engr. Fazli Rabi -my inspirational family members- I thank them for their invaluable contribution to my career and life. I would always remain in debt to the generous financial support of my Uncles especially Haji Saeedullah (Gul Dada) from 1994 till I felt the ground below my feet.

Thanks to my loving sisters Romana, Uzma, Kalsoom, Basharat, Asima Jan and Dr. Hina Khan for their love and generous prayers. To my brother Shahid and "Unkle Naeem" for their hardships in managing home and social affairs through the chaotic times in the history of my beloved Swat Valley.

I dedicate this thesis

to my mother for her immense courage and determination that kept her children together, her decisions that changed their lives, her love that gave them a reason to believe in themselves and her prayers that boosted their spirit. May God grant you a long healthy life, full of happiness and may God give us opportunities to serve you to the best of our abilities.

to my sweetest daughter Sabauon (Ayela) and dearest son Talhah for the most precious moments that we missed in each other lives. I have always loved you; now and forever.

to the friendship and love of my father Dr. Muhammad Ismail (1955-1994) – an eminent Dental Surgeon of his time- and my grandfather Husnul Maab (1938-2000) -a great scholar, even greater mathematician and perhaps someone with the greatest patience, strongest nerves and immense courage I ever witnessed in life.

"May your souls rest in peace" Ameen.

Thesis outcome

1. Publications

1. **Fahim, M.**, L. Ayala-Navarrete, A.A. Millar, and P.J. Larkin, 2010: Hairpin RNA derived from viral NIa gene confers immunity to *Wheat streak mosaic virus* infection in transgenic wheat plants. Plant Biotechnology Journal **8**, 821-834.

2. **Fahim, M.**, H. Dove, W.M. Kelman, L. Ayala-Navarrete, and P.J. Larkin, 2010: Does grazing of infected wheat by sheep result in salivary transmission of *Wheat streak mosaic virus*? Crop & Pasture Science **61**, 247-254.

3. **Fahim, M.**, A.A. Millar, C.C. Wood, and P.J. Larkin, (2011) Resistance to *Wheat streak mosaic virus* generated by expression of artificial polycistronic microRNA in Wheat. **Plant Biotechnology Journal**. (early view September 2011) doi: 10.1111/j.1467-7652.2011.00647.x

4. **Fahim, M.**, A. Mechanicos, L. Ayala-Navarrete, S. Haber, and P.J. Larkin, (2011) Resistance to *Wheat streak mosaic virus* in Australia – a Survey of Resources and Development of Markers. **Plant Pathology** (early view October 2011) doi: 10.1111/j.1365-3059.2011.02542.x

5. **Fahim, M.**, P.J. Larkin, S. Haber, S. Shorter, P. Lonergan, and G. Rosewarne, (2011) Effectiveness of three potential sources of resistance against *Wheat streak mosaic virus* under field conditions. **Plant Breeding** (**Accepted**)

6. S. Haber, **M. Fahim**, L. Ayala-Navarrete, P.J. Larkin, and D.L. Seifers (2011) A new source of resistance against *Wheat streak mosaic virus* derived from doubled haploid (DH) spring wheat line C2652. **Plant Disease** (**Submitted**)

7. S. Belide, J.R. Petrie, P. Shrestha, **M. Fahim**, Q. Liu, C.C. Wood, and S. P. Singh (2011) Multi-gene silencing using a single polycistronic artificial miRNA. **Plant Methods** (**Submitted**)

8. **Fahim, M.**, S. Ohms, and P.J. Larkin, (2011) Complete WSMV genome of Australian isolate assembled from deep sequencing of small RNAs from virus infected wheat plants. (**In preparation**)

9. Hayes, R., M. Newell, **M. Fahim**, M. Norton, M. Newberry, L.R. DeHaan, T.S. Cox, S.S. Jones, K.M. Murphy, L.J. Wade, and P.J. Larkin, (2011) Pathways to the development of 'perennial wheat' for Australian environments. (**In preparation**)

2. Patent

- Wheat plants with immunity to *Wheat streak mosaic virus*
  Publication No. : US 2011/01545534 A1, Publication Date: June 23, 2011

Conferences and Symposia

i. As Attendee

- 11th International Wheat Genetics Symposium, Brisbane, Australia 24-29 August 2008

- 1st International Plant Phenomics Symposium. CSIRO Canberra Australia. 21-24 April 2009

ii. As Speaker

- Presented a paper on *Hairpin RNA derived from viral NIa gene confers immunity to Wheat streak mosaic virus infection in transgenic wheat plants* at Australasian Plant Pathology Symposium 2009 Plant Health Management: An Integrated Approach New Castle NSW Australia. 29 September – 1 October 2009

- Presented oral paper *Virus resistance mediated by artificial miRNA against Wheat streak mosaic virus in transgenic wheat plants* in International Association of Plant Biotechnologist 2010 at American Centre St. Louis Missouri USA. June 6-11, 2010

- **Cash Prize and Certificate for 2nd position** on presenting *"Virus resistance mediated by artificial miRNA against Wheat streak mosaic virus in transgenic wheat plants"* in PhD student oral competition held by Society of In vitro Biology 2010 in collaboration with IAPB 2010 at St. Louis Missouri USA. June 4-10, 2010

iii. Poster Presentation

- *Hairpin RNA derived from viral NIa gene confers immunity to wheat streak mosaic virus infection in transgenic wheat plants.* ESF-EMBO Antiviral Applications of RNA interference 2010 St Feliu de Guixols Spain. May 30-June 4, 2010

Table of Contents

Abstract

Wheat streak mosaic virus (WSMV) is a new virus of wheat crop in Australia. Discovered in the Australian Capital Territory (ACT) in 2003, the virus has put Australian commercial bread wheat at a risk of major losses. Although, the virus is naturally transmitted by Wheat curl mites (WCM), some of the Australian farming community expressed concerns that grazing of early sown, dual-purpose wheat for winter forage may have a role in the spread of WSMV. We probed this issue in a series of experiments with housed sheep grazing on WSMV infected wheat plants. However, we find no evidence for the suggestion that grazing sheep spread the WSMV between plants in a grazed wheat crop as a consequence of the grazing process itself.

We tested for natural resistance against WSMV in diverse germplasm including three different known resistance sources in cultivated wheat. Previously reported resistances were effective against the Australian isolate of WSMV. Some accessions of these resistances were ineffective at higher temperatures (all *Wsm1* and most *Wsm2* accessions); some were reported to have linked negative agronomic traits (most accessions of *Wsm1*). Two exceptions were c2652 and *Wsm2* accession CA745 which were very effective at controlled higher temperatures (28°C), in the glasshouse, and also protected plants from symptoms and yield loss following WSMV mechanical inoculation in the field, making these two sources particularly useful in the relatively warm Australian agro-climate. New molecular markers were developed for the various derivatives of *Wsm1* resistance that should help speed up the breeding of resistance into wheat cultivars. These *Wsm1* markers are now being used by CSIRO for breeding *Wsm1*-resistance into elite wheat cultivars.

Furthermore, we developed and tested two independent transgenic strategies based on intron-hairpin RNA (ihpRNAi) and artificial microRNAs (amiRNA). Both strategies were effective in conferring immunity in transgenic wheat to mechanically inoculated WSMV. We classified this resistance as immunity by four criteria: no disease symptoms were produced; Enzyme linked immuno-

sorbent assay (ELISA) readings were as in un-inoculated plants; viral sequences could not be detected by RT-PCR from leaf extracts; and leaf extracts failed to give infections in susceptible plants when used in test-inoculation experiments.

We developed ihpRNA or RNAi based immune transgenic wheat by designing an RNAi construct to target the Nuclear inclusion protein 'a' (NIa) gene of WSMV. The Northern and Southern blot hybridization analysis indicated the ihpRNA transgene integrated into the wheat genome and was processed into typical 21-24 nucleotide long siRNAs and correlated with immunity in transgenic plants. In order to achieve amiRNA immunity, we designed five artificial microRNAs (amiRNA) against different portions of the WSMV genome, utilising published miRNA sequence and folding rules; these amiRNAs were incorporated into five duplex arms of the polycistronic rice primary microRNA (pri-miR395) and transformed into wheat. Southern blot hybridisation showed that the transgene was stably integrated into the wheat genome and processed into small RNAs, both correlating with transgenic resistance against WSMV.

As a consequence of the work described in this thesis, the wheat industry in Australia and abroad has both conventional and transgenic options for the control of this serious viral pathogen.

Chapter 1
Literature Review

1.1 Introduction

The project was designed to confront the potential new threat to the wheat industry resulting from the recent discovery of *Wheat streak mosaic virus* (WSMV) in Australia. The virus has spread across the continent in a relatively short period of time and has been detected in all major wheat growing zones and has put several cereal crops, especially bread wheat, at risk of major losses.

In some parts of Australia, farmers are abandoning cultivation of dual purpose or graze and grain wheat. Moreover, these farmers presented some anecdotal evidence that WSMV could be spread by grazing sheep. Chapter 2 of this thesis deals with testing the hypothesis that sheep can pass the disease from infected to clean wheat via their saliva.

The most effective, economical and environmentally friendly way to combat viruses generally relies on the availability and use of resistant cultivars. Chapter 3 deals with the screening of germplasm to find and confirm sources of resistance to WSMV. Australian wheats have never been bred with WSMV as a selection factor and are generally susceptible to the virus. This chapter also deals with the performance of various resistance sources at elevated temperature, keeping in view the warmer agro-climate of Australia and the temperature sensitivity of resistance in some promising exotic wheat lines. Chapter 3 also reports the development of molecular markers for resistance in some wheat lines that shall facilitate and speed up the breeding of these resistance sources into Australia wheat.

In Chapter 4, the performance of some resistant lines identified in Chapter 3 was tested under natural field conditions and shedding further light on the usefulness of resistance to WSMV in at least the Australian wheat growing environment.

Deployable sources of natural resistance in wheat are quite rare and only a limited number of commercial elite crop cultivars have been released

internationally. Furthermore, the deployment of resistance into crop cultivars is a lengthy process. This becomes of special importance, considering the fact that the evolution of a virus into resistance-breaking mutants can result in the break-down of natural resistances, which are mostly monogenic. This is the motivation for stacking of the available natural resistances and for considering novel sources of resistance including transgenic sources as a complement to conventional breeding. Virus-resistant transgenic crops are becoming an option of choice and sustainable control measures against major virus diseases, where natural resistance is scarce and not readily available. Chapter 5 and Chapter 6 present the result of experiments to use two different approaches to make virus-resistant transgenic plants, both of which resulted in wheat plants with heritable immunity to WSMV. Chapter 7 concludes the major findings of this research and paves the way to further research on the subject.

1.2 Wheat production in Australia

Bread wheat is the second most valuable agricultural commodity in Australia after beef and veal [approx 3.5 billion Australian dollars (AU $) compared to 4.5 billion AU $] and makes up 15% of the gross value of farm production and 56% of grains and oilseed production. Australia exports 80% of its wheat produce that constitutes 4% of world wheat production (Australian Bureau of Agricultural and Resource Economics and Sciences, 2010).

In Australia, wheat is sown in autumn to early winter and harvested from mid spring to early summer. However, a substantial amount of this production is lost due to diseases at a cost of around 913 million AU $ per annum (Murray and Brennan, 2009). Globally, the losses to diseases represent 14.1% of total crop production and are valued at 220 billion US $ (Agrios, 2005). Viral diseases constitute a very small proportion (3-4%) of the total losses to wheat pathogens in Australia, estimated at 28 million AU $ per year (Murray and Brennan, 2009). Among cereal-infecting viruses, *Barley yellow dwarf virus* (BYDV) is the most important followed by the newly discovered *Wheat streak mosaic virus* (WSMV) (Ellis et al., 2003). The recent discovery of *High Plains virus*

(HPV) in Australia (Thomas et al., 2009) may further accentuate the economic importance of WSMV, since WSMV, HPV and *Triticum mosaic virus* (TriMV) are transmitted by the same vector, and the co-infection of a single plant pose greater damage through synergism (Stenger et al., 2007b; Tatineni et al., 2010). This following chapter reviews some of the previous research on the WSMV– a new threat to wheat production in Australia.

1.3 *Wheat streak mosaic virus*

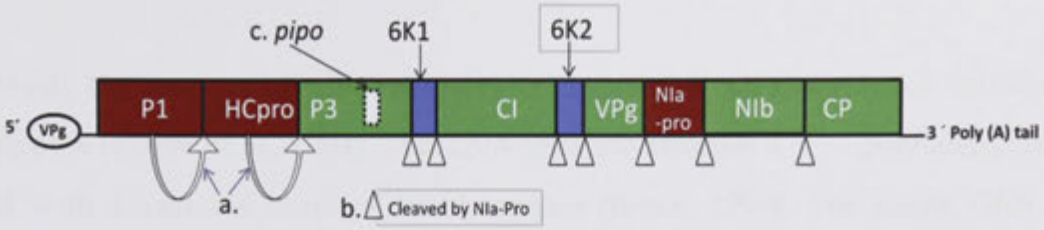
1.3.1 Virus structure, properties and classification

The family *Potyviridae* is the largest family of plant-infecting viruses. Members of this family cause some of the most important diseases in field and horticultural crops and include *Plum pox virus* (PPV) in stone fruits (Cambra et al., 2008), *Bean common mosaic virus* (BCMV) in legumes (Davis and Tsatsia, 2009; Denny and Guy, 2009), *Maize dwarf mosaic virus* (MDMV) in maize (Shukla et al., 1992), *Potato virus Y* (PVY) (Tairo et al., 2006), *Lettuce mosaic virus* (LMV) (Moreno et al., 2007) and *Zucchini yellow mosaic virus* (ZYMV) (Lecoq et al., 2009) in horticultural crops, and WSMV in cereals (Stenger and French, 2009).

WSMV belongs to the Genus *Tritimovirus* in the Family *Potyviridae*. The virus is the type species of the genus *Tritimovirus* which also includes *Brome streak mosaic virus* (BSMV), *Oat necrotic mottle virus* (ONMV), *Wheat eqlid mosaic virus* (WEqMV) and *Yellow oat grass mosaic virus* (YOGMV) (Fauquet and Mayo, 2001; Rastegar et al., 2008; Carstens, 2009; Collins et al., 2010). The virus appears in the Electron Microscope (EM) as rod-like particles that are 700 nm long and 15 nm wide, and the genome consists of a positive sense, single stranded RNA of 9,339 to 9,384 nt in size that encodes a single open reading frame (ORF) of 3,035 amino acids (McNeil et al., 1996; Stenger et al., 1998; Choi et al., 2000a).

1.3.2 Genome description

Like other Potyviruses, the WSMV genomic RNA has a virus-encoded protein covalently attached at the 5′ -end, called the VPg (virus protein genome-



Proteins	Properties
P1 (32–64 KDa)	• Trypsin-like serine proteinase, • C-terminal autocleavage • Symptomatology • Synergism and symptom development • Suppression of gene silencing
HC-Pro (56–58 KDa)	• Mite transmission • Self-interaction • Systemic movement • Papain-like cysteine proteinase • C-terminal autocleavage
P3 (37 KDa)	• Plant pathogenicity
6K1	• ?
CI (70 KDa)	• ATPase/RNA helicase Cell-to-cell movement
6K2	• Anchoring the viral replication complex to membranes
NIa (49 KDa)	• Cellular localization • VPg involved in genome replication • Trypsin-like serine proteinase, acts in cis and in trans • Protein-protein interaction
NIb (58 KDa)	• RNA-dependent RNA polymerase (RdRp) • Involved in genome replication
CP (28–40 KDa)	• Aphid transmission • Cell-to-cell and systemic movement • Virus assembly

Figure 1. Genomic map of *Wheat streak mosaic virus*. Genomic ssRNA is shown as a solid line with the covalently linked VPg protein depicted as a solid circle at the 5′-end and a poly A tail at the 3′-end. ORFs are indicated as boxes, divided into viral products, with their names inside or above, by vertical lines. The *pipo* ORF inside P3, translatable with a frameshift, is indicated by a box inside the P3 region starting downstream of the *N*-terminal part of P3. a. P1 is a trypsin-like serine proteinase located at the amino-terminal end of the polyprotein and has *cis* auto catalytic activity; and helper component (HC-Pro), a conserved carboxy-terminal cysteine proteinase domain that acts in *cis* to cleave the HC-Pro/P3 junction of the viral polyprotein. b. Nuclear inclusion protein "a" (NIa), the major proteinase of potyviruses acts in *cis* and *trans* and cleaves the polyprotein in eight smaller proteins denoted as P3, cylindrical inclusion (CI) protein, NIa, Nuclear inclusion "b" (NIb), CP, and two small proteins known as 6K1, 6K2 and VPg.

linked), the purpose of which appears to bind to the cell's translation initiation complex (Plante et al., 2004). The RNA genome also has a 3′ - polyadenylated tail with a variable number of adenosines (Brunt, 1992). The single ORF of WSMV, like other potyviruses, encodes a single polyprotein of 3,035 amino acids (Stenger et al., 1998). The polyprotein is processed by three virus-encoded proteases P1, Nuclear inclusion "a" (NIa) and Helper component-Protease (HC-Pro) into 10 mature functional proteins (Fig. 1), comprising the following gene products from the *N*- to *C*- termini: P1, HC-Pro (Helper Component Protease), P3 (and *pipo: pretty interesting potyvirus ORF)*, 6K1, CI (Cylindrical Inclusion), 6K2, NIa (Nuclear Inclusion protein "a" comprising of VPg and Protease), NIb (Nuclear Inclusions protein "b") CP (Capsid Protein) (Choi et al., 2001; Hull, 2002) in a fashion similar to other potyviruses such as *Tobacco etch virus* (TEV) (Adams et al., 2005). The whole genome of WSMV has been cloned and transcripts from the clone are infectious and are used as expression vectors in cereals i.e., wheat, maize, barley and oat (Choi et al., 2000b; Tatineni et al., 2011a).

1.3.2.1 P1 protein

Located at the N-terminus, P1 is the first mature protein of all monopartite members of *Potyviridae,* is the least conserved in sequence and is variable in size (Adams et al., 2005). It is a serine proteinase and cleaves itself from HC-Pro (Carrington et al., 1990). The P1 protein of WSMV has a role in virus symptomatology and infectivity (Choi et al., 2000a) and suppression of gene silencing (Stenger et al., 2007a).

1.3.2.2 HC-Pro

HC-Pro is a cysteine proteinase similar in function some of HC-pro of other potyviruses and is required for semi-persistent transmission of WSMV by the WCM (Stenger et al., 2005; Young et al., 2007). HC-Pro is divided into three regions; the amino terminus is necessary for vector transmission (Stenger et al., 2006a; Stenger et al., 2005; Tatineni et al., 2011b), the central region has an

essential role in viral amplification and systemic movement (Choi et al., 2000a), while the carboxy-terminus is a proteinase domain involved in polyprotein maturation (Maia and Haenni, 1994). This protinase acts in *cis* to cleave the HC-Pro/P3 junction of the viral polyprotein (Choi et al., 2000a; Stenger et al., 2005; Stenger et al., 2006b; Tatineni et al., 2010) and releases itself from the polyprotein along with P1 (described above). HC-Pro is a suppressor of gene silencing for the majority of monopartite viruses in the family *Potyviridae* and inhibits the accumulation of the 21nt class small interfering RNA (siRNA) by binding to them and thus either interferes with the methylation of viral RNAs or preventing RISC assembly (Merai et al., 2006). Generally, it mediates synergistic interactions among unrelated viruses in double infections (Pruss et al., 1997; Yang and Ravelonandro, 2002). However, WSMV differs from other potyviruses in the roles of P1 and helper component proteinase (HC-Pro) (Stenger et al., 2007b). When the role of HC-Pro in disease synergism and suppression of post-transcriptional gene silencing (PTGS) was investigated in WSMV-*Maize chlorotic mottle virus* (MCMV) system it was found that HC-Pro is dispensable for disease synergism (Stenger et al., 2007b) Later, the P1-proteinase was identified as the suppressor of RNA silencing in WSMV (Stenger et al., 2007a).

1.3.2.3 P3 protein

P3 has remained for years as one of the least-characterized proteins within the *Potyviridae* family. However, the recent availability of more robust bioinformatics algorithms for the prediction of short overlapping coding sequences (CDSs) (Chung et al., 2008; Firth and Atkins, 2008; Firth and Atkins, 2009; Firth et al., 2008) led to the discovery of an overlapping gene termed *pretty interesting potyviridae ORF* or *pipo* (Chung et al., 2008). This discovery provided insights into its role in plant pathogenicity (see next paragraph). Furthermore, Chung and colleagues further studied *pipo*'s presence within the P3 cistron and found to be well conserved in a similar position in at least 48 viruses from the family *Potyviridae*. They hypothesized that this ORF could yield a different product (~7kDa protein) after a +2 frame-shift relative to the P3 coding

sequence, however, attempts to detect such a product in virus challenged plants failed. Despite this, detection of a ~25kDa protein indicated that the *pipo* protein is not expressed independently but forms a fusion product with the upstream portion of P3 after a +2 frameshift during P3 synthesis (P3N+PIPO) (Chung et al., 2008).

Mutations in *pipo* affect the ability of the virus to replicate in protoplasts (Chung et al., 2008) or restrict *Soybean mosaic virus* (SMV) to only a few cells in inoculated plants (Wen and Hajimorad, 2010). Similar observations were made by Choi et al. (2005) while studying mutations in the P3 cistron of WSMV; however they attributed the loss of viral functions to an internal RNA secondary structure in the P3 cistron and not to a protein coding effect. Identification of such structural and functional conservation of the viral genomes, make them ideal for targeting with amiRNA for developing virus resistant transgenic crop (See Chapter 6).

1.3.2.4 Two smallest proteins 6K1 and 6K2

The two small 6K cistrons flanking the CI of potyviruses are the least characterized genes. The product of 6K1 cistron is sometimes associated with P3 and may have a role in host-range definition and pathogenicity (Saenz et al., 2000). The second small 6K2 peptide appears to be involved in RNA replication and is associated with endoplasmic reticulum and is physically associated with virus movement and symptom induction (Spetz and Valkonen, 2004).

1.3.2.5 Cylindrical Inclusion (CI)

The CI protein is the largest potyviral gene product and forms the very distinctive pinwheel-shaped cylindrical inclusions in the cytoplasm of infected cells, with high taxonomic value because they are unique to members of the family *Potyviridae* (Urcuqui-Inchima et al., 2001). It exhibits adenosine triphosphatase (ATPase) and RNA helicase activity (Fernandez and Garcia, 1996; Fernandez et al., 1997), and is thought to act during RNA replication and perhaps in translation by helping to unwind the viral RNA(Urcuqui-Inchima et al., 2001). The association of CI with other viral proteins, and also with host

factors, suggests a rather central role for this product. It is also implicated in viral movement, a fact that correlates with its presence in structures near plasmodesmata (Carrington et al., 1998).

1.3.2.6 Nuclear Inclusion protein a (NIa)

Genome linked viral protein (VPg)

The NIa of WSMV and other members of the family *Potyviridae*, consists of the C-terminal "NIa" proteinase domain and the N-terminal VPg domain, which are separated by an internal cleavage site, recognized by NIa-proteinase (Dougherty and Parks, 1991). The *N*-proximal part is called the VPg, and is the only other viral protein, in addition to CP, that is present in WSMV virions. VPg is covalently attached to the 5´-end of the genome via a tyrosine (Y) residue (Murphy et al., 1991). The VPg is involved in replication possibly by priming RNA synthesis (Anindya and Savithri, 2004; Chhavi et al., 2008). It was believed that VPg may not have a direct role in potyviral translation because VPg was not required for efficient cap-independent translation of TuMV, *in vitro* (Basso et al., 1994; Niepel and Gallie, 1999). The association of VPg with eukaryotic translation initiation factors [eIF4E and eIF (iso)4E] might have a central role in pathogenicity that might serve to explain some resistance mechanisms (Wittmann et al., 1997; Leonard et al., 2000, 2004).

NIa-protease

The C-terminal portion of NIa is a major trypsin-like protease (NIa-Pro) (Adams et al., 2005a) and is the most studied proteinase of potyviruses (It is the target chosen for our hairpin RNA mediated resistance study reported in chapter 5.) This proteinase acts in *cis* and *trans* to process all cleavage sites other than those processed by P1 and HC-Pro (Fig. 1). The NIa proteinase cleaves the polyprotein into eight smaller proteins: P3, cylindrical inclusion (CI) protein, NIa, Nuclear inclusion "b" (NIb), CP, 6K1, 6K2 and VPg (Riechmann et al., 1992). The NIa-Proteinase from *Pepper vein banding virus* (PVBV) has been also shown to display nonspecific deoxyribonuclease activity (Anindya and Savithri,

2004), suggesting that in the late infection cycle, the NIa protein accumulates in the nucleus and serve to degrade the host deoxyribonucleic acid (DNA).

1.3.2.7 Nuclear inclusion protein b (NIb)

The NIb protein acts as an RNA–dependent RNA polymerase (RdRp) and is the larger than NIa. It is found in the cytoplasm in association with the replication complex during viral genome amplification (Fellers et al., 1998a). NIa interacts with NIb and they co-localize in inclusion bodies in the nucleolus, nucleus or cytoplasm of infected cells, however this localization varies for different potyviruses (Riedel et al., 1998; Urcuqui-Ichima et al., 2001).

NIb is homologous to other RNA-dependent RNA polymerases, displays RNA polymerase activity *in vitro* and presumably is the viral replicase that acts in concert with host-encoded factors (Urcuqui-Ichima et al., 2001). It is directed to the nucleus by specific signals in its sequence. Recruitment to the replication complex is postulated to occur via interaction with NIa (Daròs and Carrington, 1997).

1.3.2.8 Capsid protein (CP)

The coat protein or capsid protein of *Potyviridae* members is divided into three main domains. The C-terminal and the N-terminal domain are exposed on the surface of the virion and are involved in systemic movement, infectivity and transmissibility (López al., 1999), and the conserved central domain is implicated in cell-to-cell movement and virus encapsidation (Dolja et al., 1994; Rojas et al., 1997). In addition, in some members of the *Potyviridae* family the capsid protein is involved in aphid transmission through interaction with HC-Pro (Blanc et al., 1997) and has been shown to display nucleoside triphosphatase (NTPase) activity (Rakitina et al., 2005).

A DAG motif (Asp-Ala-Gly) is conserved within the N-terminal domain of the coat protein of many potyviruses (Harrison and Robinson, 1988) and is a determinant of aphid transmission (Blanc et al., 1997; Dombrovsky et al., 2005). However, the coat protein of WSMV-Sidney 81 and another 50 or more isolates

for which CP sequences are available, only El Batán3 strain encodes a DAG motif (Stenger et al., 2002). As sequences of WSMV isolates and related species from across the globe build up, it is likely that new taxons will arise to clarify peculiarities such as the presence of the DAG motif in the El-Batan isolate of WSMV.

1.4 Geographical distribution

Wheat streak mosaic disease (WSM) was first documented in Nebraska, USA in 1922 as "yellow mosaic disease" and in 1932 the virus was first associated with WSM symptoms (McKinney, 1937). Since its first discovery, WSM disease spread has rapidly across the Great Plains of the United States with sporadic reports of severe endemics (Fellows, 1949). The disease was reported in 1952 in winter wheat fields in Southern Alberta Canada (Slykhuis, 1953b). Since then the virus has become an important pathogen in the Great Plains region of North America and has been reported in several countries across the world including Iran, Brazil, Turkey, Argentin, New Zealand and Australia (Ellis et al., 2003; Lebas et al., 2009; Stenger and French, 2009). In Australia, since its identification in 2003, it has remained a moderate threat to wheat production (Fig. 2) and has rapidly spread to all the wheat producing zones (Dwyer et al., 2007) necessitating an immediate action (addressed in this thesis).

The rapid spread of WSMV in Australia since 2003, was probably due to transmission by both seed and the WCM vector (Jones et al., 2005; Dwyer et al., 2007; Schiffer et al., 2009; Carew et al., 2009). Infected seed from CIMMYT (Mexico) has been implicated as the means of initial introduction of the virus, when grown at a post-entry quarantine facility in Tamworth NSW Australia (Dwyer et al., 2007). However, a sample of cv. Spear in South Australia, stored since 1997 was shown by Enzyme linked immune sorbent assay (ELISA), reverse-trascriptase Polymerase Chain Reaction (RT-PCR), and sequencing to contain the virus (Dwyer et al., 2007). The presence of the virus in Adelaide before the recent discovery in Canberra and other parts of New South Wales (NSW) (Ellis et al., 2003), suggests an earlier time of entry or importation of a

very homogeneous biotype. Although the details remain sketchy, the close phylogenetic relationship to other strains (Turkish and US strains) suggests incursion of a single biotype or a few very closely-related biotypes and their spread through breeders' germplasm (Dwyer et al., 2007).

With the development of DNA sequencing technology and the completion of various sequencing projects across the globe, a total of 13 isolates of WSMV have been completely sequenced (Stenger et al., 1998; Choi et al., 2001; Rabenstein et al., 2002; Stenger and French, 2009). The availability of the genome sequence has facilitated the establishment of thresholds of relatedness between genera and between species (Adams et al., 2005). Based on a phylogenetic analysis of the virus sequences, WSMV is grouped into four distinct clades A, B, C and D. **Clade A** contains only the most divergent strain, El Batán3 from Mexico (Sanchez-Sanchez et al., 2001). It differs in nucleotide sequence from the other three clades by 20% (Choi et al., 2001). WSMV isolates from Central Europe and Russia (Rabenstein et al., 2002) comprise **Clade B** and differ in nucleotide sequence similarity from clade C and D genotypes by 10%. An isolate WSMV-I from Iran (Rabenstein et al., 2002) represents **Clade C**, differing in sequence from clade D genotypes by 8%. Numerous WSMV isolates from temperate North America belong to clade D and share about 96% nucleotide sequence identity. **Clade D** also includes two isolates from Turkey, suggesting intercontinental movement at some point of time in the past (Rabenstein et al., 2002). To our knowledge, no complete sequence of an Australian isolate of WSMV is available in the public domain (NCBI). However, sequence analysis of NIb and CP regions revealed that the 17 Australian isolates are more similar to each other than to isolates from the rest of the world (Dwyer et al., 2007: figure 2). These Australian isolates (and our unpublished data on the full genome sequence of WSMV) are sharing approximately 98.9% homology to the type strain of WSMV (Genebank Accession no. AF285169) and are more similar to USA and Turkish isolates than to Czech or El-Batan isolates (Dwyer et al., 2007). The Australian isolates are grouped into clade D, along with American Pacific Northwest (APNW) isolates and Argentine isolates (Dwyer et al., 2007; Stenger and French, 2009).

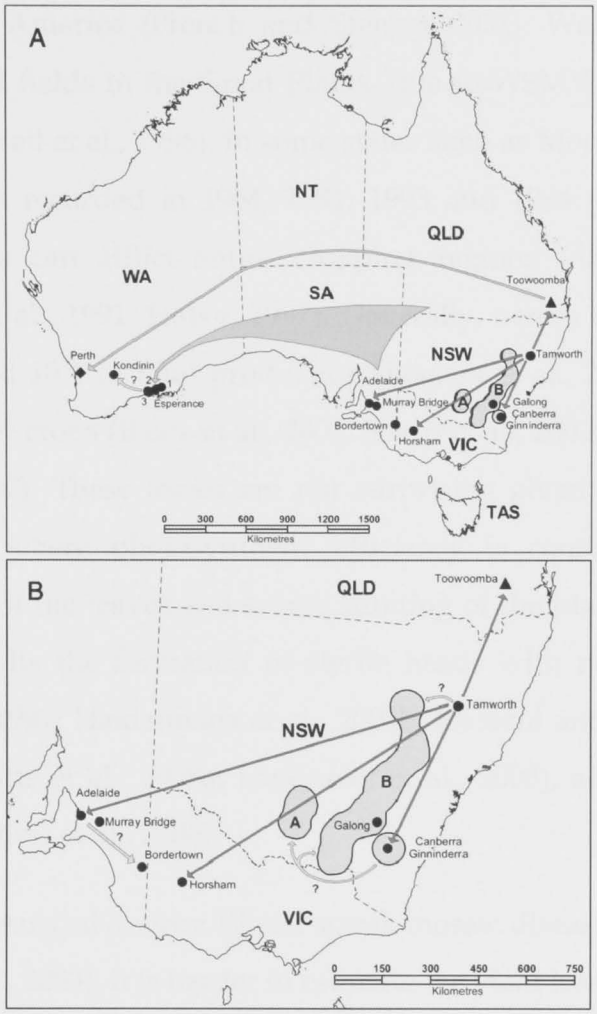


Figure 2. Maps of the Australian continent (A) and southeast Australia (B) showing the locations from which the 17 sequenced *Wheat streak mosaic virus* (WSMV) isolates were obtained, and the probable virus distribution routes in infected wheat seed. Disks (●) show locations where isolates were found; triangle (▲) shows the source of the three seedborne isolates detected in post-entry quarantine; and diamond (♦) shows where seed-infected isolates were identified under post-entry quarantine. Bold arrows indicate probable distribution routes from the incursion site to the primary breeding centers. Shaded arrows indicate suspected onward distribution routes. States: NSW = New South Wales; NT =Northern Territory; QLD = Queensland; SA = South Australia; VIC = Victoria; WA = Western Australia. Areas of NSW affected by major WSMV epidemics in 2005: A, Murrumbidgee irrigation area; B,"slopes" region.

Reference: Dwyer et al 2007 *Plant Disease*

1.5 Economic importance

Wheat streak mosaic disease is of great economic importance in the Great Plains of North America (French and Stenger, 2003; Weise, 1987). Yield losses in individual fields in the Great Plains, due to WSMV can be 100% (Riesselman, 1993; McNeil et al., 1996). In some states such as Montana US, severe epidemics have been recorded in 1964, 1981, 1993 and 1994 (Burrows et al., 2009) and sometimes can afflict entire cropping regions (Atkinson and Grant, 1967; Conner et al., 1991; Haber, 1997). Generally, across the Great Plains the losses can exceed 10% of total production (Bockus et al., 2001) affecting both spring and winter crops (Baley et al., 2001; Sharp et al., 2002; Seifers et al., 2006; Seifers et al., 2007). These losses are not surprising given the impact of early virus infection where photosynthetic efficiency is constrained by streaking and chlorosis of the leaves and severe stunting of the plants. Severe, early infection often results the formation of sterile heads with no seed or shrivelled seed (Nyitrai, 1991; Hakizimana et al., 2004b; Hassani and Assad, 2004; Jones et al., 2005; Coutts et al., 2008a; Lanoiselet et al., 2008), adversly affecting the grain quality (Divis et al., 2006).

In Australia, since Wheat streak mosaic disease is a relatively new threat (Ellis et al., 2003), it is harder to estimate the yield losses that might emerge over time. Resistance is rare in bread wheats and under favourable agro-climatic conditions the virus may cause severe yield losses, especially in early-sown winter wheat and dual purpose "graze and grain" wheat (Andrews and Slykhuis, 1956; Murray and Brennan, 2009). Losses caused by WSMV in Australia during 2003-04 were patchy except in a few irrigated wheat crops in the Murrumbidgee irrigation area in New South Wales, where the losses were significant. However, in 2005 over 5000 hectares (ha) in the High Rainfall Zone of NSW were infected with WSMV, especially among the early sown "graze-and-grain" crops. The yield losses in severely infected wheat fields were approximately 80% in some paddocks (Murray et al., 2007). The area being affected expanded to 20,000 ha in 2006, when the environmental conditions

were favourable for disease establishment (Dwyer et al., 2007). A major shift may occur in the cropping preferences of the farmers in WSMV-affected areas as they may switch away from long-season wheats to lower value grazing cereals such as WSMV-resistant rye and triticales; the lost opportunity cost of such a switch in NSW could be around $ 21 million (Murray and Brennan, 2009).

Although serious outbreaks have been sporadic, it is possible that the drought has minimised the build-up of the WCM population with fewer green bridges being present compared to in a wet summer and autumn period and therefore minimised the impact of the virus to date. However, there is a high risk that the virus could become a major production constraint in Australia. Kansas has similar climate to much of the main Australian wheat belt, including terminal drought, and has had losses to WSMV of 2.6% averaged over many years. If the disease impact in Australia grew to that level it would have an average cost of >$150 m per annum. The highest recorded percent loss in Kansas would translate to >$850 m in Australia. The impact in the High Rainfall Zone (HRZ) may be even greater. The WCM has been shown to be widespread throughout all Australian wheat growing regions (main and HRZ regions) (Schiffer et al., 2009). Under favourable conditions (for mites and volunteer grasses) there is no known reason why WSMV cannot become a major constraint on wheat production in Australia.

1.6 Pathogenesis

The most-common symptoms associated with WSMV include greenish yellow dashes and streaks parallel to the leaf axes that advance to produce more extensive streaks or blotches resulting in chlorosis and eventually necrosis (Martin, 1978). Severely affected plants infected at an early stage, typically exhibit stunted growth, losing up to 80% of plant height with associated reduced tillering. Along with these characteristic symptoms, rolling up of to leaves (in the case of mite infestation) can occur. In the field, initial symptoms are usually recorded on the crop borders. In cases of early infection and with

severely-infected plants, the overall impact is the production of fewer and smaller grains per spike and a drastic reduction in yield (Brakke, 1971; Hunger et al., 1992; Baley et al., 2001; Sharp et al., 2002; Price et al., 2010; Seifers et al., 2011). Several environmental factors play a critical role in symptom development and severity, including soil fertility, temperature and light, as well as the growth stage of the plant at the time of the infection and the genotype of the infected wheat (Haunold, 1958; Stoddard et al., 1987b; Seifers et al., 1995a). Like other members of the *Potyviridae* family, WSMV can interact with other viruses such as *High Plains virus* (HPV) and *Triticum mosaic virus* (TriMV) in co-infection of a host plant. These two viruses are related to WSMV, in terms of transmission by WCM, and co-infections induce more-severe symptoms and severe yield losses (Shahawan and Hill, 1984; Wiese, 1987; Mahmood et al., 1997; Stenger et al., 2007a,b). The recent discovery of *High Plains virus* (HPV) in Australia (Thomas et al., 2009) appears to have major consequences for the wheat industry in Australia and would require further study to prevent major losses resulting from synergism of HPV with WSMV.

1.7 Host Range

The virus is hosted by grasses that are restricted to species in the family *Gramineae*. In addition to wheat, it can also infect corn (*Zea mays*), barley (*Hordeum vulgare* L.), sorghum (*Sorghum vulgare*), oats (*Avena sativa*), rye (*Secale cereale*), and other grasses such as cheat grass (rye brome) (*Bromus secalinus*), millets (*Panicum, Setaria* and *Echinichloa*), sandbur (*Cenchrus pauciflorus* Benth.), Canada wild rye (*Elymus canadensis*), and several wild perennial grasses (Bottacin and Nassuth, 1990; Christian and Willis, 1993; Seifers et al., 1996; Rabenstein et al., 2002; Seifers et al., 2002; Seifers et al., 2005; Haber et al., 2009; Seifers and Martin, 2009; Seifers et al., 2010). Ellis et al. (2004) identified common weeds in Australian pastures as alternative hosts for WSMV including African lovegrass (*Eragrostis curvula*), whorled pigeon grass (*Setaria verticillata*), spike goosegrass (*Eleusine tristachya*) and a panicum (*Panicum sp*). It has been noticed that a high incidence of WSMV is commonly associated with the

presence of alternative hosts and volunteer wheat plants that act as reservoirs or green bridges for the virus and its viruliferous vector (Slykhuis, 1953a; Christian and Willis, 1993). On the other hand various monocots such as some rye varieties are symptomless carriers of WSMV (McKinney, 1949; Sill and Connin, 1953; McMullen, 2002;), and therefore the virus may escape survillance strategies based on the virus symptoms in the field. The host species mentioned here are very unlikely to constitute an exhaustive list of the alternative hosts.

1.8 Transmission

1.8.1 Mite Transmission

Members of the *Tritimovirus* genus, WSMV, HPV and TriMV, are transmitted semi-persistently by the Wheat curl mite (WCM) *Aceria tosichella* Keifer formerly known as *A. tulipae* (Slykhuis, 1955; Keifer 1969). The WCM is a wingless cigar-shape arachnid, which grows up to 0.3 mm long. Mites can be observed near the ligule on the lower end of the leaves of plants, in cases where leaf rolling occurs trapping the spike (Harvey and Martin, 1980; Harvey and Seifers, 1991; Harvey et al., 1997; Harvey et al., 1999). The optimal temperature for rapid mite increase is 24-27° C (Staples and Allington, 1956). Upon population build-up the mites crawl and move to the leaf edges or spike before the wind moves them long distances, from plant to plant (Somsen and Sill, 1970; Liu et al., 2005). In the field symptoms usually appear at margins where viruliferous mites are blown to adjacent plants or more distant mite-infested fields. The likelihood of mite survival between seasons is probably increased by the otherwise desirable increase in conservation tillage (no-till or low-till) farming (Weise, 1987). Movement of the WCM have been reported to be dependent on wheat host senescence and deterioration (Nault et al., 1967; Nault and Briones, 1968), however it is primarily influenced by the size of the source population and not the condition of the host plant (Thomas and Hein, 2003).

The WCM itself impacts directly on the growing wheat plants by damaging the young and succulent tissues of growing seedlings leading to leaf

rolling and frequent trapping of the emerging spikes, whereby the awns are not released from the leaf sheath and the head is bent back over its tiller (Weise, 1987). The indirect and greater damage from WCM comes from its transmission of WSMV. WCM can acquire the virus from a host plant only when at the nymphal-stage, with an acquisition access period (AAP) of 15 minutes or more; subsequently the virus is passed to later developmental stages (transtadial transmission) through moults (Paliwal and Slykhuis, 1967). Though the transmission is transtadial, and is passed from adult mites to offspring, no evidence for transovarial transmission exists (where the virus is passed from an infected female directly to her eggs) (Slykhuis, 1955). Virus has been detected in the alimentary canal, or digestive tract, of the WCM, where it persisted for at least 5 days after feeding, suggesting its *circulative* nature within the vector (Paliwal and Slykhuis, 1967; Sinha and Paliwal, 1977; Paliwal, 1980). However, the virus has not been detected in the hindgut of viruliferous mites (Paliwal, 1980). Mahmood et al. (1997) detected WSMV near the anterior end of viruliferous WCM using virus-specific immuno-fluorescent microscopy. The mites can remain viruliferous for at least 9 days while feeding on virus-immune plants (Staples and Allington, 1956).

Although WCM was reported only recently in Australia (Halliday and Knihinicki, 2004), it has probably been present for many years. Carew et al. (2009) categorized the WCM population in Australia into two genetically-distinct lineages based on the mitochondrial 16S rRNA gene and two nuclear markers (internal transcribed spacer 1 and adenine nucleotide translocase). Both lineages of WCM are equally distributed throughout Australia and are often found in sympatry (Schiffer et al., 2009). However, only one biotype could transmit WSMV efficiently under laboratory conditions, while the other biotype failed to transmit WSMV from infected to healthy wheat plants (Schiffer et al., 2009). It is important to note that at least two other wheat viruses, *Triticum mosaic virus* (TriMV) and *High plains virus* (HPV) (Fellers et al., 2009; Louie et al., 2006; Schiffer et al., 2009; Seifers et al., 2009; Seifers et al., 2004) are transmitted by WCM. It would be interesting to know the relationship between the various

biotypes of WCM and the viruses they transmit in cereal. These mites were also found on alternate host plants, including some plants not previously known to host WCM (Carew et al., 2009). As is evident from the literature, the presence of WCM and its biotypes have serious implications for the management of WCM and WSMV within Australia and elsewhere.

1.8.2 Seed transmission

The seed-borne status of WSMV has been documented in maize at a rate 0.1-0.2% (Hill et al., 1974) and more recently in wheat with transmission ranging from 0.5-2% (Jones et al., 2005; Dwyer et al., 2007; Lanoiselet et al., 2008). The detection of WSMV in a South Australian seed sample of wheat cv. Spear stored since 1997 (Dwyer et al., 2007) implicate the role of seed in initial introduction of the virus long before its initial reports (Ellis et al., 2003).

Other isolates of WSMV from Clade A, B, C, and D have not been evaluated for seed transmission and it remains unclear whether a transmission rate of ~1% (several log unit greater that the previous reports for WSMV in Maize (Hill et al., 1974), is unusual (Stenger and French 2009).

1.9 Epidemiology

The disease severity and incidence is often associated with the presence of volunteer wheat plants and other green bridges that act as reservoirs for both WSMV and WCM. Wide spread epidemics and endemics often occur in the absence of a substantial 'green bridge', suggesting the possible importance of seed transmission in forming primary foci of infection in the field with the mites become agents for secondary spread within crops. The wheat-growing season in Australia is relatively short compared to that of the Great Plain of North America; winters are much warmer, and there is a much longer period between the harvest in late spring and the autumn sowings. Differences, such as the existence of areas with Mediterranean-type climates in the south-western grain belt, with hot and dry summers and wet winters, are likely to have a major effect on the epidemiology of WSMV. In the winter of 2006, WSMV was

detected in a severely stunted wheat crop in the Merredin district in the central low-rainfall zone (250–325mm of rainfall annually) of the south-west Australian grain belt (Coutts et al., 2008a). The follow up studies revealed the occurrence of WSMV in all rainfall zones of the grain belt, with high incidences restricted to the central low-rainfall zone (Dwyer et al., 2007). The region received considerable cyclonic rainfall from the north-west in the preceding summer and autumn, and experienced exceptionally warm conditions during the winter growing period (Coutts et al., 2008a; 2008b). This generated a 'green ramp' of grasses and volunteer cereals before the emergence of the autumn-sown wheat crop. Relatively warm winters also provide ideal conditions for WCM population build-up (Staples and Allington, 1956).

1.10 Disease Management

Control of viral diseases can be difficult; therefore, prophylaxis and management strategies are advocated by The International Plant Protection Convention (IPPC). This includes measures such as the creation of pest-free areas, phytosanitary treatments and limitations to the movement of infected material across international borders. Parallel to this, countries have devised their own quarantine measures to prevent the introduction of new pests and diseases into their agricultural ecosystem, and have imposed inter-state quarantine measures that would prevent the spread of an accidentally-introduced pathogen. However, these measures are not always successful in preventing the entry of exotic pests and diseases. The introductions of WSMV, PVY, and *Tomato yellow leaf curl virus* (TYLCV) into the Australian agricultural system are a few examples of failure (Rodoni, 2009). Extreme measures were taken to eradicate WSMV and prevent its spread in Canberra (Ellis et al., 2003; 2004). However, this second line of defence i.e., destruction of all grasses and wheat by Australian Quarantine and Inspection Services (AQIS), to minimise the escape of the virus into the system seems to have been too late because WSMV was already present elsewhere. Such eradication measures have been successful in containing some non-endemic viruses such as PPV in North

America (Gildow et al., 2004). However, if the early measures fail in eradication of the introduced virus, measures are then directed to preventing the movement of infected propagative material such as seed and through disease indexing of material to minimize the threat of spread by vertical transmission (Fereres et al., 2000; Irwin, 1999; Irwin et al., 2000). In the case of WSMV these containment attempts were also too late as the seed transmission of WSMV was only confirmed 2-3 years after the presence of the virus in the country was accepted (Jones et al., 2005).

Horizontal transmission of viruses is sometimes successfully minimized by the use of pesticides to control the vector along with an effective forecasting system for judicial pesticide applications or by an integrated pest management (IPM) program (Irwin, 1999; Oliveira et al., 2001). However, no effective commercial miticide is available for the control of WCM, as the mites are usually protected in the leaf sheath at the base (ligule area). Furthermore, large-scale use of miticide is not environmentally friendly and would add greatly to the cost of wheat production. Therefore, management strategies are directed towards the elimination of "green bridges", as WCM can only survive for 4 to 6 days without green tissue (Slykhuis, 1955). WCM utilizes wild perennial grasses and volunteer wheat plants (resulting from shattering of seeds before or during harvest) as over-summering hosts (Staples and Allington, 1956). This dependence on the green bridge between growing seasons provides some opportunity to control WCM populations by destroying early volunteer wheat and weeds, and avoiding the overlapping of host plants. These practices can be applied in the immediate vicinity of the farm. Community efforts on a larger scale are required to achieve the benefit from this practice as WCM is disseminated by the wind as far as 3.2 km (Pady and Kapica, 1955). Nevertheless, eliminating "green bridges" close to the farm appears to reduce the severity of losses in a cost effective manner (Christian and Willis, 1993). Efforts are therefore directed at the destruction of volunteers and host plants two or three weeks before the emergence of the wheat crop (Jiang et al., 2005) either through the use of herbicides or tillage.

Management strategies in arid countries like Australia are dependent on the availability of soil moisture. The delaying of planting to late winter does minimise the overlap of wheat growing seasons and the "green bridge" factor, however it may contribute to persistence of WSMV from another factor: younger less established plants in spring may be more prone to severe infections just as the mites become active (Hunger et al., 1992). Interestingly, those who are advocating for the development of perennial wheats because of the environmental benefits they would bring, are conscious of the need for the deployment of WSMV resistance. With perennial wheat there is less of an opportunity to avoid green bridges by crop management. The most promising experimental perennial wheats are in fact amphiploids made by combining wheat genomes with wheatgrass species such as *Thinopyrum intermedium*, which are often themselves very resistant to WSMV and/or WCM (Baley et al., 2001; Chen et al., 2003; Cox et al., 2002; Divis et al., 2006; Fedak and Han, 2005; Fedak et al., 2001; Friebe et al., 2009; Li et al., 2004). The exploration of WSMV resistance and its subsequent incorporation into perennial wheat will be essential for ensuring its successful production over years (Cox et al., 2005). The present author has assessed the WSMV resistance of many of perennial wheat lines being evaluated under field conditions in Australia (see Appendix III, Hayes et al., in preparation).

The elimination of the green bridge alone is helpful in minimizing the sources of WSMV infection, but may not necessarily reduce the chance of WSM reaching epidemic proportions. Furthermore, cultural methods such as destruction of green bridges are a communal activity, requiring coordination among neighbouring farmers with different priorities. Therefore, despite the best individual efforts, alternative host species for both WSMV and WCM might survive. Therefore, genetic resistance against WSMV and WCM is the most economic way of preventing losses in wheat and other cereals.

1.10.1 Natural Resistance

Since the first report associating WSMV with wheat streak mosaic disease, efforts have been made to find sources of resistance in cultivated wheat. The initial survey into wheat germplasm for resistance against WSMV revealed that

commercial bread wheat is susceptible to the virus and its vector. Meanwhile, many wheat-alien hybrids were characterized and documented to have several useful traits, originating from the alien grasses, and not available in wheat (Andrews and Slykhuis, 1956; McKinney and Sando, 1951; Schmidt et al., 1953). Efforts were shifted to exploring resistance in distant wild relatives such as in the genera *Thinopyrum, Aegilops, Elymus* and *Secale*, and their hybrids with wheat. Effective resistance sources to WSMV were identified (Friebe et al., 2009; Graybosch et al., 2009; Haber et al., 2006; Hassani and Assad, 2004; Pfannenstiel and Niblett, 1978; Wang et al.,1980; Stoddard et al., 1987a; Lay et al., 1971; Wang and Liang 1977). Moreover, perennial Triticeae relatives such as *Thinopyrum intermedium* (Host) Barkworth and Dewey ($2n$ = 6x = 42, JJsS) and *Th. ponticum* (Podp.) Barkworth and Dewey ($2n$ = $10x$ = 70, JJJJsJs) are valuable sources of resistance and tolerance to various biotic and abiotic stresses, and are relatively easy to cross with common and durum wheat (Armstrong, 1936; Dewey, 1984). Resistance in *Thinopyrum* was identified against WSMV and its vector WCM (Friebe et al., 1991; Larson and Atkinson, 1972; Sharma et al., 1984).

The resistance described in *Th. intermedium* was of particular interest to plant breeders as it is readily crossable with common wheat. To date, three sources of WSMV-resistance have been reported from *Th. intermedium* but so far only one compensating Robertsonian translocation (where a nonreciprocal translocation involving two homologous (paired) chromosomes or non-homologous chromosomes i.e., two different chromosomes figure 3) has been studied in detail. This translocation carries a temperature-sensitive resistance gene designated *Wsm1* (Friebe et al., 1992; 1996a; 2009) and involved the short arm of one *Th. intermedium* chromosome (4Ai), replacing the short arm of wheat chromosome 4DS. The centric fusion chromosome has been recently re-designated 4Ai#2S.4DL (Friebe et al., 1996b, 2009). The resistance was characterized in germplasm CI 117884 and shown to have probably originated from the Js genome of *Th. intermedium* (Chen et al., 1998a; Friebe et al., 1996b). Lines such as CI 17884 (KS93WGR27) had better agronomic traits (Gill et al., 1995) and were further improved by backcrossing to the hard winter wheat line

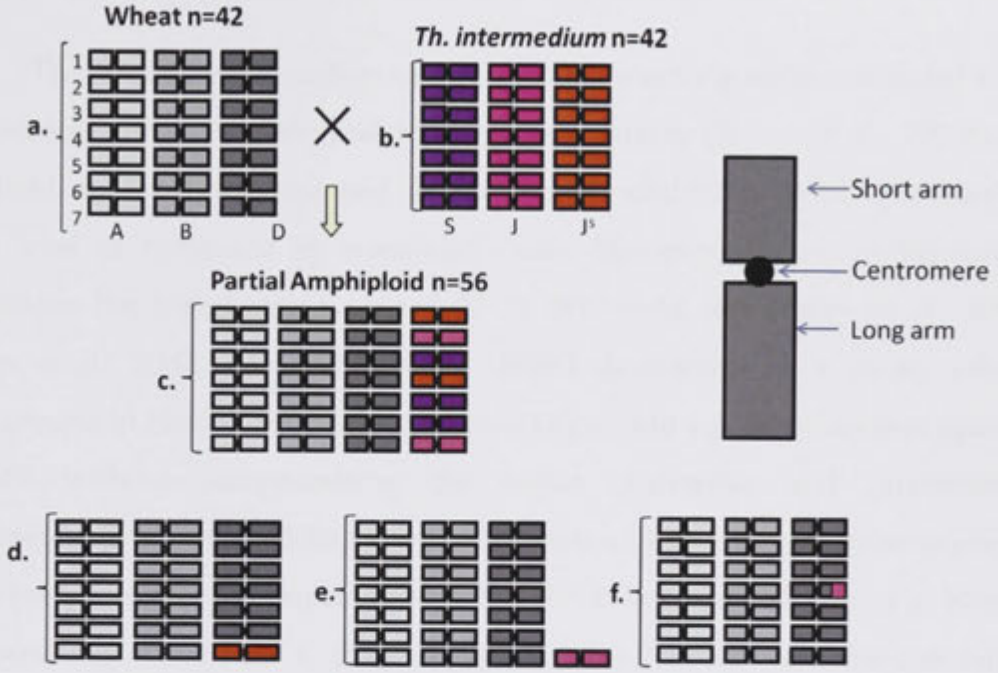


Figure 3. Genome organization of Wheat, *Thinopyrum intermedium* and their derivatives. In this diagram only one of each chromosome pair is represented.
a. Wheat hexaploid (AA BB DD, n=42) consisting of 7 groups.
b. *Th. intermedium* hexaploid (JJ JsJs SS, n=42) genome, just like wheat these chromosomes are organized into 7 groups.
c. Wheat x *Thinopyrum* partial amphiploid is an allopolyploid consisting of the three wheat genomes with another set of chromosomes from *Th. intermedium*. This additional alien set of chromosomes may vary in composition e.g., Zhong1, Zhong2, B84-994 etc. discussed in this thesis.
d. Substitution Line (n=42) where one or more chromosome are replaced by alien chromosome e.g., Yi4212 in chapter 3.
e. Addition line (n=44) where one full genome of wheat has an additional chromosome from *Thinopyrum*. e.g., Z1, Z2, Z6 etc discussed in this thesis.
f. Translocation line (n=42), segment from wheat chromosome is replaced by an alien-chromosomal segment such as *Thinopyrum*. *Wsm1* is one such example discussed at length in this thesis.

"Karl", and released as germplasm KS93WGRC (Gill et al., 1995). However, alien genome segments in wheat sometimes carry undesirable characteristics, resulting in a negative impact on the quality and agronomic traits of the recipient wheat cultivars (Lukaszewski and Gustafson, 1983).

The original translocation carrying *Wsm1*, was frequently associated with undesirable effects on yield and bread-making quality (Seifers et al., 1995b). In the field, under inoculated and un-inoculated conditions, *Wsm1* provided a high level of resistance in inoculated plots. However, under un-inoculated conditions the translocation caused 11 to 28% yield loss (Baley et al., 2001; Sharp et al., 2002). In another study, *Wsm1* derivatives in a winter wheat background in Nebraska USA were shown to provide a good protection against WSMV without compromising the major qualitative and quantitative parameters (Divis et al., 2006). *Wsm1* was shown to be a temperature sensitive resistance, effective at temperatures below 20° C but breaking down at higher temperatures around 24° C (Seifers et al., 1995a). Nevertheless, the resistance has proved useful in the field where the vulnerable growth stages of the plant are at cooler temperatures and where the exposures to higher temperatures are brief and interrupted by cool nights.

Nevertheless, researchers in Kansas continued to develop recombinants with the aim to reduce the translocation size and minimise the linkage drag without compromising resistance to WSMV. Qi et al. (2007) reported an efficient recombination strategy using induced homoeologous recombination, molecular markers and cytology. Lines were produced where there had been recombination between 4Ai#2S and the wheat chromosome arm 4DS. One of these lines is examined in this thesis (chapter 3 and 4). Two more potential sources of resistance were reported to be derived from *Th. intermedium*. The first mapped to a *Th. intermedium* telosome initially believed to belong to long arm of group-4 chromosome but later shown to be homeologous to group-7 long arms (Friebe et al., 1996a). The second mapped to a Js-genome chromosome present in the Zhong series of wheat-*Th. intermedium* partial amphiploids and was

designated as Js2 (Chen et al., 1998b, 2003). The effective utilization of these resistances in wheat breeding will first require compensating translocations in the wheat genome, which are so far not available. In chapter 3 and 4, I report studies on the performance of reported natural resistance, discovery of new sources of natural resistance and of a new *Wsm1* carrying translocation on chromosome 4A rather than 4D. This was developed by Steve Haber (reported briefly in Haber et al. 2007) and has obvious potential advantages for introgression to durum wheat. It will be referred to in the thesis as CA741. This new *Wsm1*-carrying translocation on chromosome 4A, is missing the Talbert J15 molecular marker. We report later on the development of new molecular markers some of which are useful for mapping of the CA741 translocation.

The first substantive resistance to WSMV originating in bread wheat itself, was discovered in germplasm CO960293-2 (Haley et al., 2002) and was deployed to winter wheat cultivar RonL (Martin et al., 2007) as well as in the released germplasm KS03HW12 (Seifers et al., 2007). All derivatives of CO960293-2 were shown to have temperature-sensitive resistance to WSMV, similar to *Wsm1*. The resistance has been recently mapped to chromosome arm 3BS and renamed *Wsm2* (Lu et al., 2011). The second resistance in bread wheat has been found in selections from the Canadian breeding line c2652 (Haber et al., unpublished). The resistance has been given the temporary designation c2652, and its origin is explored in a paper in preparation to which I have contributed (Haber et al., unpublished). The c2652 resistance is readily introgressed into durum wheat and therefore must be on the A or B genome. These three resistances (c2652, *Wsm1* and *Wsm2*) are immediately deployable in wheat and are examined in some detail in chapters 3 and 4 of this thesis.

Molecular markers are very useful in speeding up conventional breeding programs. To facilitate the breeding of *Wsm1* into bread wheat and simultaneously reducing its linkage drage, markers were discussed (Talbert et al., 1996; Friebe et al., 1992; Seifers et al., 1995a). The Talbert marker for the original *Wsm1* translocation was useful in the development of the commercial

US variety Mace. One advantage of resistances introduced on alien translocations is that there is usually no recombination of breeding material along the length of the translocation because the homoeology is insufficient to allow pairing and cross-over with the corresponding wheat arm in this region. As a consequence useful molecular markers are easily found even though they may not be physically close to the gene of interest. However, with efforts to reduce the translocation size new markers become necessary. This is illustrated in the work of Qi et al., (2007) in the generation of a variety of recombinants of the *Th. intermedium* segment using the mutant *ph1b* background to permit homoeologous recombination. The alien chromosome in the recombinant rec213 (T4JsS-4DS.4DL) has only about 20% of the terminal end of the short arm as *Th. intermedium* chromatin as determined by markers and GISH (Qi et al., 2007; Friebe et al., 2009) has the *Wsm1*-temperature sensitive resistance to WSMV as well as the linked resistance to *Triticum mosaic virus* (TriMV) (Friebe et al., 2009) and was introgressed into Kansas hard red winter wheat and released as KS08WGRC50 (Gill et al., 2008). It is hoped that this shortened translocation will prove to have less or no linkage drag leading to poor agronomic performance. Successful recombination strategies resulted in *Wsm1*-carrying lines, having no yield penalties (Friebe et al., 2009), however, the shortening of the *Wsm1* translocation sometimes led to the loss of Talbert's marker. In addition to screening for resistance sources, Chapter 3 also reports the development and testing of new molecular markers to facilitate transfer of the resistance from new recombinants into elite wheat cultivars in Australia and elsewhere.

1.10.2 Engineered Resistance

1.10.2.1 Pathogen Derived Resistance (PDR)

Barton et al. (1983) laid the foundation of plant transgenics and for the first time demonstrated the possibility of achieving foreign gene insertion into a plant genome using the Ti plasmid from *Agrobacterium*. Soon afterwards, it was demonstrated in the lab of Roger Beachy, that expressing a viral coat protein gene from *Tobacco mosaic virus* (TMV) conferred specific resistance to TMV in

transgenic tobacco plants (Abel et al., 1986). Despite extensive studies, the molecular mechanisms that govern CP-mediated resistance (CPMR) are not fully understood, and furthermore, mechanisms of CPMR are different in different viruses (Bendahmane et al., 2007). It was assumed that somehow the expression of the transgenic viral protein disrupted the life cycle of the virus (Abel et al., 1986; Register and Beachy, 1988; Nejidat and Beachy, 1990; Bendahmane et al., 1997). Furthermore, various labs around the world showed that in addition to viral CP genes, the expression of functional and dysfunctional sequences derived from elsewhere in the viral genome i.e., replicase, movement protein or protease genes can also confer various degrees of protection characterized by symptom delay, reduced symptom severity, partial resistance and immunity (Lindbo and Dougherty, 2005; Lomonossoff, 1995). The term ***Pathogen Derived Resistance (PDR)*** was coined for this phenomenon and the technique became widely used for the development of transgenic virus-resistant plants (Baulcombe, 1996; Beachy, 1993; Beachy et al., 1990; Bucher et al., 2006; Dominguez et al., 2002; Fuchs, 2008; Goldbach et al., 2003; Han et al., 1999; Jozsa and Balazs, 2000; Lin et al., 2007; Lius et al., 1997; Maki-Valkama and Valkonen, 1999; Marano and Baulcombe, 1998; Othman, 1996; Palukaitis and Zaitlin, 1997; Sijen et al., 1996). Initial studies suggested the possibility that two types of mechanisms might be responsible for the transgenic resistance phenotypes: the first one disrupting the life cycle of the virus through expression of viral coat protein; and the other one depending on the presence of transgene-derived RNA involving post transcriptional gene silencing i.e., PTGS (Goldbach et al., 2003). However, the picture was far from clear until some fascinating discoveries were made in late 1990s.

Experiments designed to produce over-expression of chalcone synthase (CHS) using a transgene with a strong constitutive promoter in petunia (Napoli et al., 1990; van der Krol et al., 1990) failed to produce the desired or expected results and found that the over-expression of the CHS gene had negative effects on the pigmentation of Petunia flowers. Instead of achieving increased flower pigmentation, they recovered Petunia flowers with patchy or reduced

pigmentation in the presence of a second copy of CHS. It was becoming evident that non-coding viral RNA is sufficient to mediate artificial resistance in transgenic plants and the phenomenon was placed in the same category as "co-suppression" (Lindbo et al., 1993). The expression of antisense RNA to engineer resistance against plant viruses moved the understanding of pathogen derived resistance a step closer to the discovery of RNA interference (RNAi) in plants.

1.10.2.2 Hairpin RNA (hpRNA) mediated Resistance

In late 1990s, a research team in CSIRO led by Peter Waterhouse obtained resistance to PVY in transgenic tobacco by simultaneous expression of sense and antisense virus RNA sequences. Waterhouse et al. (1998) concluded that constructs capable of forming dsRNA transcripts were more effective in protecting the transgenic plant, compared to transgenic plants expressing either sense or antisense RNA alone (Waterhouse et al., 1998). This was the first compelling evidence that a dsRNA is responsible for mediating specific transgenic virus resistance. Furthermore a picture slowly emerged that natural plant recovery from virus infection might also involve similar mechanisms, especially in cases where the virus life cycle was dependent on dsRNA intermediates for replication (Beclin and Vaucheret, 2001).

A pivotal step in the discovery of this mechanism came from a different field of studies and different organism, *Caenorhabditis elegans*, which became famous as RNA interference or RNAi (Fire et al., 1998). Other terms has been applied to this phenomenon such as *Quelling* in fungi (Nakayashiki, 2005) and post-transcriptional gene silencing (PTGS) in plants (Beclin and Vaucheret, 2001; Sijen and Kooter, 2000; Turner and Schuch, 2000; Voinnet et al., 1999).

Later in plants it was revealed that the sense-, antisense- and dsRNA-induced RNA silencing pathways appear to converge at the point of dsRNA formation (Beclin et al., 2002; Di Serio et al., 2002). Although dsRNA is produced by various mechanisms, the steps following the processing of the dsRNA in small interfering RNA (siRNA) are common to all three transgene types (Beclin et al., 2002). It is now understood that siRNAs and microRNAs

(miRNAs) are the two key players in gene silencing [recent review by Carthew and Sontheimer (2009)].

In plant research, the work in the Waterhouse lab continued until the efficiency of RNAi was improved. Smith et al., (2000) showed that transgene constructs encoding a spliceable intron within a hairpin (hp) RNA structure (Fig. 3) can silence the target genes with almost 100% efficiency. They showed that the percentage of PVY-resistant tobacco plants obtained by targeting the nuclear inclusion a (NIa) protease gene of PVY with different constructs (Fig. 3) was 7% for the sense gene, 4% for the antisense gene, 58% for the hpRNA with a non-spliceable loop separating the sense and antisense arms, and 96% for the same hpRNA with a spliceable intron. This method was soon adapted to generate virus-immune transgenic plants against *Cucumber mosaic virus* (CMV) (Kalantidis et al., 2002), PVY (Mitter et al., 2003; Mitter et al., 2006) and a number of other viruses in different plant systems (Duan et al., 2008a; Hily et al., 2007; Li et al., 2008; Ma et al., 2004; Wang et al., 2000). The resistance mechanism relies on small RNAs that either repress or cleave the complementary target RNAs in a sequence-specific manner (Bagasra and Prilliman, 2004; Baulcombe, 1999; Baulcombe, 2004; Buchon and Vaury, 2006; Jana et al., 2004; Ketzinel-Gilad et al., 2006; Stram and Kuzntzova, 2006; Watson et al., 2005). The expressed dsRNA is processed into siRNAs of 21-24 bp by DICER-like enzymes. siRNAs are then incorporated into RISC (RNA-induced silencing complex) which guides recognition of complementary target RNA sequences resulting in their degradation (Fig. 4) and, in the case of a target virus, resistance (Campbell and Choy, 2005). The discovery of RNAi as a eukaryotic surveillance mechanism solved the puzzle underlying the pathogen-derived resistance associated with RNA-mediated resistance and became a useful tool in efficient and effective transgenic protection against viruses in plants (Abbott et al., 2002; Bucher et al., 2006; Smith et al., 2000). dsRNA or hpRNA transgenes successfully induced RNAi against BYDV in both wheat and barley (Wang et al., 2000).

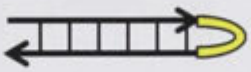
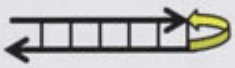
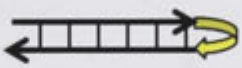
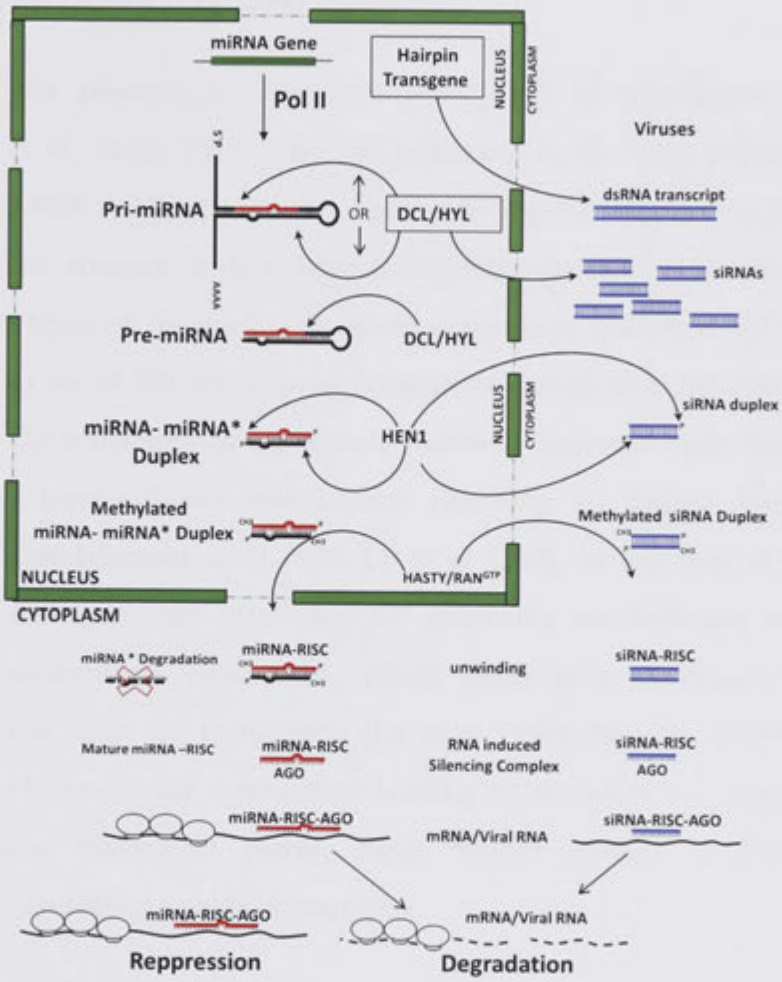
Gene construct	Predicted RNA structure	% immune Lines
Sense PVY		7
Antisense PVY		4
Hairpin PVY (GUS loop)		58
Hairpin PVY (reverse intron)		65
Hairpin PVY (sense intron)		96

Figure 4. Efficiency of induction of RNAi by different gene constructs and the predicted structure of RNA transcribed from the transgenes. In the predicted structures of RNA transcripts, right- and left-pointing arrows represent sense and antisense orientation of sequences, respectively; small vertical arrows represent splice-junction sequences remaining after the intron has been spliced out. Vertical lines in the predicted structures indicate duplex formation

References:

Smith et al. (2000) *Nature.*
Waterhouse et al. (1998) *PNAS.*


miRNA Gene
Pol II
Hairpin Transgene
NUCLEUS
CYTOPLASM
Viruses
dsRNA transcript
Pri-miRNA
OR
DCL/HYL
siRNAs
Pre-miRNA
DCL/HYL
siRNA duplex
miRNA- miRNA* Duplex
HEN1
Methylated miRNA- miRNA* Duplex
Methylated siRNA Duplex
HASTY/RAN^{GTP}
NUCLEUS
CYTOPLASM
miRNA * Degradation
miRNA-RISC
unwinding
siRNA-RISC
Mature miRNA –RISC
miRNA-RISC
AGO
RNA induced Silencing Complex
siRNA-RISC
AGO
miRNA-RISC-AGO
mRNA/Viral RNA
siRNA-RISC-AGO
miRNA-RISC-AGO
mRNA/Viral RNA
Reppression
Degradation

)

The first attempts to achieve transgenic resistance against WSMV were based on PDR strategies (Sivamani et al., 2000; 2002) by expressing NIb or CP genes from the WSMV 'Conrad' isolate in two varieties of wheat, 'Hi-Line' (Lanning et al., 1992) and 'Bobwhite' (Carroll et al., 1982). The transgenic progeny analysis in glasshouse experiments for resistance, exhibited a delayed resistance phenotype (Sivamani et al., 2000, 2002).

However, the phenotypes observed previously in glasshouse experiments (Sivamani et al., 2000; 2002) were not observed in the field where yield losses varied from 46% in NIb-expressing lines to 67% in CP-expressing lines (Sharp et al., 2002). The absence of detectable transgene-transcript in the best transgenic lines which showed delayed glasshouse symptoms, indicated the involvement of RNAi (in case of NIb-expressing transgenic plants). It is believed that in case of CPMR, CP transgene in transgenic plants is mutated such that there is an increase in inter–subunit interactions resulting in higher levels of virus resistance (Bendahmane et al 1997; Lu et al 1998). In the case of resistance to TMV, the transgenically expressed CP sub-units are believed to re-coat the nascent disassembled viral RNA, which leads to a decreased pool of the available viral RNA for translation (Lu et al., 1998; Beachy, 1999) resulting in resistance. However, the mechanism leading to breakdown of resistance in the CP-expressing transgenic plants, need further studies to investigate the mechanism underlying such phenotypes.

The main problem is that the sense transgene approach is an inefficient way to trigger RNAi-type protection (Smith et al., 2000). The overall yield performance of these transgenic lines was poor compared to the parent genotype, both with and without WSMV inoculation; however, this can probably be attributed to the somaclonal variation, transgene silencing and position effect of the integrated transgene in the target genome (Bregitzer et al., 1998; Larkin and Scowcroft, 1981; Matzke and Matzke 1998). Our approach (Discussed in Chapter 5) was to target RNAi against the *NIa* gene of WSMV and we showed that transgenic constructs capable of forming dsRNA transcripts are more effective at

generating immunity against WSMV (Fahim et al., 2010) than were either of the previous two strategies, that involved sense expression of the NIb or CP genes that were followed previously (Sivamani et al., 2000; 2002).

It is known that stacking multiple transgenes for various traits in a single plant poses several challenges, including the need to transform sequentially with different selectable markers and then preferably eliminate the markers to render the plant commercially deployable. Because RNAi does not require the use of large sequences it has been successfully used for multiplexing transgenic resistance in plants against viral disease complexes and also in silencing of multiple endogenous genes (Antony et al., 2005; Bucher et al., 2006; Praveen et al., 2006). However, agricultural scale deployment of transgenic virus resistance has so far been limited to release of *Papaya ringspot virus* (PRSV)- resistant papaya, and the transgenic squash line CZW-3 with resistance to CMV, *Zucchini Yellow mosaic virus* (ZYMV) and *Watermelon mosaic virus* WMV (Fuchs and Gonsalves, 2007). Concerns have been expressed that anti-virus hpRNA expressing-transgenic plants might lead to the evolution of new virus biotypes via heterologous recombination or complementation between the relatively long viral sequences expressed from the transgene and RNA from a non-target virus infecting the same plant (Schnippenkoetter et al., 2001). Because the larger dsRNA, the folded transcript of the transgene, is typically a couple of hundred bp long, it can be processed into a large number of different siRNA species. The resulting diversity of primed RISC complexes will attack the virus at many points along the sequence used to design the hpRNA transgene, resulting in immunity against the target virus (see chapter 5). However the long hpRNA from conventional RNAi vectors increases the probability of "off-target" effects, i.e. silencing of unintended host plant genes (Jackson et al., 2006a; Jackson et al., 2003). Despite this risk a number of transgenic plants with anti-viral RNAi constructs have been produced and perform successfully (Fuchs, 2008). Although the likelihood of events mentioned above seems remote and could be further reduced by judicious selection of smaller sequences for the hpRNA

constructs, attention is given to this possibility in this thesis (Chapter 6) with the adoption of a polycistonic miRNA strategy.

1.10.2.3 Artificial microRNA (amiRNA) Mediated Resistance

Certain limitations in the use of siRNA led to experimentation with a new class of small RNAs called microRNAs (miRNAs), which might be used to achieve transgenic virus resistance. Artificial microRNA (amiRNA)-mediated gene silencing has recently been developed and may specifically address the issues associated with off-target effects and the hypothetical generation of novel recombinant virus biotypes (Schwab et al., 2006).

miRNAs encoded by plant genes are similar to siRNAs in their biogenesis. However they are derived from imperfect hairpin loop structures (pre-miRNAs), which have a characteristic structure from which the DICER-LIKE enzymes processes the mature miRNA, which is then loaded into the RNA-induced silencing complex (RISC). The miRNA-RISC then mediates complementarity-dependent repression or degradation of target mRNA (Brodersen et al., 2008; Meister et al., 2004). It is possible to alter the sequence of the 21-nt mature miRNAs within the natural miRNA precursor backbone, without affecting miRNA biogenesis and maturation, as long as the secondary structure of the pre-miRNA is maintained. This opens up the prospect of creating amiRNAs with new targets by design (Ai et al., 2011; Duan et al., 2008b; Israsena et al., 2009; Khraiwesh et al., 2008; Ossowski et al., 2008; Park et al., 2009; Wagaba et al., 2010; Warthmann et al., 2008; Zhang et al., 2010).

The first successful amiRNA-based virus resistance was achieved in *Arabidopsis thaliana* (Niu et al., 2006) using two plant virus systems. Here, amiRNA amiR-p69-159 was engineered into the backbone of miR159, targeting the p69 gene of *Turnip yellow mosaic virus* (TYMV) amiR-HC-Pro-159, derived from *Turnip mosaic virus* TuMV was targeted the HC-Pro gene of TuMV, which encodes the viral suppressor of RNA silencing. Transgenic lines expressing wither of these two amiRNAs specifically conferred resistance to either TYMV or TuMV (Niu et al., 2006). Since then the strategy has been successfully applied

to other virus-plant systems (Duan et al., 2008; Ai et al., 2011; Qu et al., 2007; Zhang et al., 2010; Wagaba et al., 2010) using a number of different miRNA precursors as backbones for delivery of amiRNAs in plants including miR159a (Niu et al., 2006); miR171a (Qu et al., 2007); miR172a (Schwab et al., 2006); miR30 (Zeng et al., 2002); miR528 (Warthmann et al., 2008); and miR167b (Ai et al., 2011).

However, an obvious disadvantage of amiRNA is that when the virus is only targeted by a single amiRNA sequence, it is more likely to evolve resistance since only minor changes to the target sequence may enable avoidance of miRNA targeting (Lin et al., 2009). Documented examples of this include the selection of HIV escape mutants that avoid RNAi in animals (Boden et al., 2003; Das et al., 2004; Westerhout et al., 2005), and the emergence of PPV mutants in plants (Simon-Mateo and Garcia, 2006). In order to substantially reduce the risk of viruses evolving to avoid miRNA-mediated degradation, a useful strategy would be where an amiRNA precursor gene expressed multiple amiRNAs targeting different conserved structurally and functionally critical portions of the viral genome. Escape from targeting by multiple amiRNAs would require the introduction of simultaneous mutations to avoid all the amiRNA sequences. In 2009, a similar rationale was followed in the work of Israsena and colleagues, where they designed precursor genes encoding three amiRNA against rabies virus and tested them in cell culture. Likewise, multiple siRNA were introduced in the backbone of a multiplex miRNA, directed against HIV in cultured cells (Liu et al., 2008; ter Brake et al., 2007).

We adapted pri-miR395 from *Oryza sativa* (Guddeti et al., 2005) to achieve transgenic resistance to WSMV in wheat (Chapter 6). miR395 targets ATP sulfurylases that are involved in sulphate assimilation (Rotte and Leustek, 2000) and are induced in sulphur starvation to regulate a low-affinity sulphate transporter and two ATP sulphurylases (Allen et al., 2005; Jones-Rhoades and Bartel, 2004). The rice miR395 gene cluster is located within a sequence of about

1 kb, and is transcribed as single polycistronic transcript (Jones-Rhoades and Bartel, 2004).

amiRNA-based virus resistance may have a number of advantages over virus-derived hpRNA or dsRNA-mediated resistance:

i. Depending on the length of the dsRNAs used, multiple species of siRNAs are expected to be expressed in transgenic plants, and these siRNAs may have off-target effects, e.g. on endogenous genes. By contrast, specific small RNA (21nt) are produced by the amiRNA approach, and their sequences can be more easily chosen to reduce the likelihood of off-target effects (Schwab et al., 2006). This solves the issue documented in some reports, suggesting that the long hpRNA from conventional RNAi vectors may cause silencing of unintended genes (Jackson et al., 2003; 2006a; 2006b).

ii. Furthermore, there is concern that the agricultural scale deployment of anti-virus hpRNA-expressing transgenic plants might lead to the evolution of new virus biotypes via heterologous recombination or complementation between the relatively long viral sequences expressed from the transgene and RNA from a non-target virus infecting the same plant. Although the likelihood of such events seems remote and could be further reduced by judicious sequence selection, nevertheless using the very small viral sequences in amiRNAs reduces this risk.

iii. Environmental conditions, such as low temperature, can also affect siRNA-mediated resistance. It has been reported that virus and transgene-mediated RNA-silencing become attenuated at low temperatures, which inhibits siRNA accumulation in insect, plant and mammalian cells (Fortier and Belote, 2000; Kameda et al., 2004; Szittya et al., 2003). This temperature sensitivity explains why siRNA-mediated virus resistance breaks down at 15°C (Szittya et al., 2003). However, miRNA accumulation was hardly affected at low temperatures, and

transgenic lines expressing amiRNAs maintained their specific virus resistance even at low temperatures (Szittya et al., 2003).

1.11 Aims and scope of Thesis

The project was designed to confront the potential new threat to the wheat industry resulting from the recent discovery of *Wheat streak mosaic virus* in Australia. Australian wheats are generally susceptible to WSMV, having never been bred with WSMV-resistance as a selection factor. In assessing and confronting the WSMV threat, the project:

- explored the suspected role of grazing sheep in the spread of WSMV;
- tested the efficacy of reported sources of natural resistance in the glasshouse against the Australian isolate of WSMV
- searched for new sources of natural resistance in wheat, other cereals and wheat-wheatgrass hybrids
- tested the temperature sensitivity of the natural sources of resistance
- tested in field experiments the ability of the various natural resistances to protect yield from repeated heavy inoculation of WSMV
- developed transgenic strategies for synthetic resistance based on RNAi and artificial microRNA technology and tested their efficacy in glasshouse and controlled temperature experiments.

References

Abbott, D., MB. Wang, and PM. Waterhouse, 2002: A single copy of virus-derived transgene-encoding hairpin RNA confers BYDV immunity., In: M. Henry and A. McNab, (eds.) Barley Yellow Dwarf Disease: Recent Advances and Future Strategies., pp 22-26. CIMMYT, Mexico, D.F.

Abel, PP, RS. Nelson, B. De, N. Hoffmann, S.G. Rogers, R.T. Fraley, and R.N. Beachy, 1986: Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232, 738-743.

Adams MJ, Antoniw JF and Beaudoin F (2005) Overview and analysis of the polyprotein cleavage sites in the family Potyviridae. Molecular Plant Pathology 6:471-487.

Agrios, GN, 2005: Plant Pathology. Academic Press, Elsevier

Ai, T., L. Zhang, Z. Gao, C.X. Zhu, and X. Guo, 2011: Highly efficient virus resistance mediated by artificial microRNAs that target the suppressor of PVX and PVY in plants. Plant Biol (Stuttg) 13, 304-16.

Allen, E., ZX Xie, AM. Gustafson, and JC. Carrington, 2005: microRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell 121, 207-221.

Andrews, JE, and JT. Slykhuis, 1956: Reaction of winter wheat varieties and Triticum X Agropyron hybrids when inoculated with streak mosaic virus by the mite vector Aceria tulipae Keifer. Plant Dis Reporter 40, 513-516.

Anindya R and Savithri HS (2004) Potyviral NIa proteinase, a proteinase with novel deoxyribonuclease activity. Journal of Biological Chemistry 279:32159-32169.

Antony, G., AK Mishra, and S. Praveen, 2005: A single chimeric transgene derived from two distinct viruses for multiple virus resistance. Journal of Plant Biochemistry and Biotechnology 14, 101-105.

Armstrong, JM, 1936: Hybridization of Triticum and Agropyron. I. Crossing results and description of the first generation hybrids. . Canadian Journal Research 190-202.

Atkinson, TG, and MN Grant, 1967: An evaluation of streak mosaic losses in winter wheat. Phytopathology 57, 188

Australian Bureau of Agricultural and Resource Economics and Sciences, accessed May10, 2010, <Abare.gov.au>

Bagasra, O, and KR Prilliman, 2004: RNA interference: The molecular immune system. Journal of Molecular Histology 35, 545-553.

Baley, GJ, LE Talbert, JM. Martin, MJ Young, DK Habernicht, GD Kushnak, J.E. Berg, S.P. Lanning, and P.L. Bruckner, 2001: Agronomic and end-use qualities of *Wheat streak mosaic virus* resistant spring wheat. Crop Science 41, 1779-1784.

Barton, K.A., and W.J. Brill, 1983: Prospects in plant genetic engineering. Science 219, 671-675.

Basso J, Dallaire P, Charest PJ, Devantier Y and Laliberte JF (1994) Evidence for an Internal Ribosome Entry Site within the 5' Non-Translated Region of Turnip Mosaic Potyvirus Rna. Journal of General Virology 75:3157-3165.

Baulcombe, D.C., 1996: Mechanisms of pathogen-derived resistance to viruses in transgenic plants. Plant Cell 8, 1833-1844.

Baulcombe, D., 1999: Viruses and gene silencing in plants. Archives of Virology, 189-201.

Baulcombe, D., 2004: RNA silencing in plants. Nature 431, 356-363.

Beachy, RN, S. Loeschfries, and N.E. Tumer, 1990: Coat Protein-Mediated Resistance Against Virus Infection. Annual Review of Phytopathology 28, 451-474.

Beachy, RN, 1993: Transgenic Resistance to Plant-Viruses - Introduction. Seminars in Virology 4, 327-328.

Beachy RN. 1999: Coat-protein-mediated resistance to tobacco mosaic virus: discovery mechanisms and exploitation. Philos Trans R Soc Lond B Biol Sci. ;354(1383):659–64

Beclin, C., and H. Vaucheret, 2001: PTGS in plants, a virus resistance mechanism. M S-Medecine Sciences 17, 845-855.

Beclin, C., S. Boutet, P. Waterhouse, and H. Vaucheret, 2002: A branched pathway for transgene-induced RNA silencing in plants. Current Biology 12, 684-688.

Bendahmane M, Fitchen JH, Zang GH, Beachy RN. 1997: Studies of Coat Protein Mediated Resistance to TMV: Correlation between assembly of mutant coat proteins and resistance. Journal of Virology. 71:7942–7950.

Bendahmane, M., I. Chen, et al. 2007: Coat protein-mediated resistance to TMV infection of *Nicotiana tabacum* involves multiple modes of interference by coat protein. Virology 366(1): 107-116.

Blanc S, Lopez-Moya JJ, Wang R, Garcia-Lampasona S, Thornbury DW and Pirone TP (1997) A specific interaction between coat protein and helper component correlates with aphid transmission of a potyvirus. Virology 231:141-147.

Bockus, W.W., J.A. Appel, R.L. Bowden, A.K. Fritz, B.S. Gill, T.J. Martin, R.G. Sears, D.L. Seifers, G.L. Brown-Guedira, and M.G. Eversmeyer, 2001: Success stories: Breeding for wheat disease resistance in Kansas. Plant Disease 85, 453-461.

Boden, D., O. Pusch, F. Lee, L. Tucker, and B. Ramratnam, 2003: Human immunodeficiency virus type 1 escape from RNA interference. Journal of Virology 77, 11531-11535.

Bottacin, A., and A. Nassuth, 1990: Evaluation of ontario-grown cereals for susceptibility to *Wheat streak mosaic virus*. Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie 12, 267-272.

Brakke, M.K., 1971: *Wheat streak mosaic virus*. Descriptions of Plant Viruses.No. 48. Assoc. Appl. Biol./ Commonw. Mycol. Inst. Kew, Surrey. England., 4pp.

Bregitzer, P., S.E. Halbert, and P.G. Lemaux, 1998: Somaclonal variation in the progeny of transgenic barley. Theoretical and Applied Genetics 96, 421-425.

Brodersen, P., L. Sakvarelidze-Achard, M. Bruun-Rasmussen, P. Dunoyer, Y.Y. Yamamoto, L. Sieburth, and O. Voinnet, 2008: Widespread translational inhibition by plant miRNAs and siRNAs. Science 320, 1185-1190.

Brunt AA (1992) The General-Properties of Potyviruses. Archives of Virology:3-16.

Bucher, E., D. Lohuis, P. van Poppel, C. Geerts-Dimitriadou, R. Goldbach, and M. Prins, 2006: Multiple virus resistance at a high frequency using a single transgene construct. Journal of General Virology 87, 3697-3701.

Buchon, N., and C. Vaury, 2006: RNAi: a defensive RNA-silencing against viruses and transposable elements. Heredity 96, 195-202.

Burrows, M., Ito, D., and Grey, W. 2009. Cereal viruses of importance in Montana. MontGuide MT200911AG, Montana State University Extension Service.

Cambra M, Capote N, Olmos A, Bertolini E, Gorris MT, Africander NL, Levy L, Lenardon SL, Clover G and Wright D (2008) Proposal for a new international protocol for detection and identification of Plum pox virus: Validation of the techniques. Proceedings of the Twentieth International Symposium on Virus and Virus-Like Diseases of Temperate Fruit Crops - Fruit Tree Diseases:181-191

Campbell, T.N., and F.Y.M. Choy, 2005: RNA interference: Past, present and future. Current Issues in Molecular Biology 7, 1-6.

Carew, M., M. Schiffer, P. Umina, A. Weeks, and A. Hoffmann, 2009: Molecular markers indicate that the wheat curl mite, Aceria tosichella Keifer, may represent a species complex in Australia. Bulletin of Entomological Research 99, 479-486.

Carrington JC, Freed DD and Oh CS 1990: Expression of potyviral polyproteins in transgenic plants reveals three proteolytic activities required for complete processing. *EMBO J* 9:1347-1353.

Carrington JC, Jensen PE and Schaad MC 1998: Genetic evidence for an essential role for potyvirus CI protein in cell-to-cell movement. Plant Journal 14:393-400.

Carroll, T.W., S.K. Zaske, and R.H. Brlansky, 1982: Separation of Montana Isolates of Wheat Streak Mosaic-Virus on Michigan Amber Wheat. Plant Disease 66, 916-918.

Carstens EB 2009: Report from the 40th meeting of the Executive Committee of the International Committee of Taxonomy of Viruses. Archives of Virology 154:1571-1574.

Carthew, R.W., and E.J. Sontheimer, 2009: Origins and Mechanisms of miRNAs and siRNAs. Cell 136, 642-655.

Chen, Q., B. Friebe, R.L. Conner, A. Laroche, J.B. Thomas, and B.S. Gill, 1998a: Molecular cytogenetic characterization of Thinopyrum intermedium-derived wheat germplasm specifying resistance to *Wheat streak mosaic virus*. Theoretical and Applied Genetics 96, 1-7.

Chen, Q., R.L. Conner, F. Ahmad, A. Laroche, G. Fedak, and J.B. Thomas, 1998b: Molecular characterization of the genome composition of partial amphiploids derived from Triticum aestivum x Thinopyrum ponticum and T-aestivum x Th-intermedium as sources of resistance to *Wheat streak mosaic virus* and its vector, Aceria tosichella. Theoretical and Applied Genetics 97, 1-8.

Chen, Q., R.L. Conner, H.J. Li, S.C. Sun, F. Ahmad, A. Laroche, and R.J. Graf, 2003: Molecular cytogenetic discrimination and reaction to *Wheat streak mosaic virus* and the wheat curl mite in Zhong series of wheat--Thinopyrum intermedium partial amphiploids. Genome 46, 135-45.

Chhavi M, Anindya R and Savithri HS 2008: Effect of viral genome-linked protein (VPg) on protease function of nuclear inclusion- a (NIa) protein of pepper vein banding virus. Indian Journal of Virology 19:105-106.

Choi IR, Stenger DC and French R 2000a: Multiple interactions among proteins encoded by the mite-transmitted wheat streak mosaic tritimovirus. Virology 267:185-198.

Choi IR, Stenger DC, Morris TJ and French R 2000b: A plant virus vector for systemic expression of foreign genes in cereals. Plant Journal 23:547-555.

Choi, IR, J.S. Hall, M. Henry, L. Zhang, G.L. Hein, R. French, and D.C. Stenger, 2001: Contributions of genetic drift and negative selection on the evolution of three strains of wheat streak mosaic tritimovirus. Arch Virol 146, 619-28.

Choi IR, Horken KM, Stenger DC and French R 2005: An internal RNA element in the P3 cistron of *Wheat streak mosaic virus* revealed by synonymous mutations that affect both movement and replication. J Gen Virol 86:2605-2614.

Christian, M.L., and W.G. Willis, 1993: Survival of Wheat Streak Mosaic-Virus in Grass Hosts in Kansas From Wheat Harvest to Fall Wheat Emergence. Plant Disease 77, 239-242.

Chung BYW, Miller WA, Atkins JF and Firth AE 2008: An overlapping essential gene in the Potyviridae. Proceedings of the National Academy of Sciences of the United States of America 105:5897-5902.

Conner, RL, J.B. Thomas, and E.D.P. Whelan, 1991: Comparison of mite resistance for control of wheat streak mosaic Crop Science 31, 315-318.

Connin, RV, 1956: The host range of the wheat curl mite, vector of wheat streak mosaic. Journal of Economic Entomology 49, 1-4.

Coutts, BA, N.E.B. Hammond, M.A. Kehoe, and R.A.C. Jones, 2008a: Finding *Wheat streak mosaic virus* in south-west Australia. Australian Journal of Agricultural Research 59, 836-843.

Coutts, B.A., G.R. Strickland, M.A. Kehoe, D.L. Severtson, and R.A.C. Jones, 2008b: The epidemiology of *Wheat streak mosaic virus* in Australia: case histories, gradients, mite vectors, and alternative hosts. Australian Journal of Agricultural Research 59, 844-853.

Cox, CM, T.D. Murray, and S.S. Jones, 2002: Perennial wheat germ plasm lines resistant to eyespot, cephalosporium stripe, and wheat streak mosaic. Plant Disease 86, 1043-1048.

Cox, CM, K.A. Garrett, T.S. Cox, W.W. Bockus, and T. Peters, 2005: Reactions of perennial grain accessions to four major cereal pathogens of the great plains. Plant Disease 89, 1235-1240.

Das, AT, T.R. Brummelkamp, E.M. Westerhout, M. Vink, M. Madiredjo, R. Bernards, and B. Berkhout, 2004: Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. Journal of Virology 78, 2601-2605.

Davis RI and Tsatsia H 2009: A survey for plant diseases caused by viruses and virus-like pathogens in the Solomon Islands. Australasian Plant Pathology 38:193-201.

Denny BL and Guy PL 2009: Incidence and spread of viruses in white-clover pastures of the South Island, New Zealand. Australasian Plant Pathology 38:270-276.

Dewey, D.R., 1984: The genomic system of classification as a guide to intergeneric hybridization with the perennial triticeae. Gustafson, J. P. (Ed.). Gene Manipulation in Plant Improvement; 16th Stadler Genetics Symposium, Columbia, Mo., USA, 1984. Xi+668p. Plenum Press: New York, N.Y., USA; London, England. Illus, 209-280.

Di Serio, F., L. Rubino, M. Russo, and G.P. Martelli, 2002: Homology-dependent virus resistance against Cymbidium ringspot virus is inhibited by post-transcriptional gene silencing suppressor viruses. Journal of Plant Pathology 84, 121-124.

Divis, LA, R.A. Graybosch, C.J. Peterson, P.S. Baenziger, G.L. Hein, B.B. Beecher, and T.J. Martin, 2006: Agronomic and quality effects in winter wheat of a gene conditioning resistance to *Wheat streak mosaic virus*. Euphytica 152, 41-49.

Dolja VV, Haldeman R, Robertson NL, Dougherty WG and Carrington JC 1994: Distinct functions of capsid protein in assembly and movement of tobacco etch potyvirus in plants. EMBO Journal 13:1482-1491.

Dombrovsky A, Huet H, Chejanovsky N and Raccah B 2005: Aphid transmission of a potyvirus depends on suitability of the helper component and the N terminus of the coat protein. Archives of Virology 150:287-298.

Dominguez, A., A.H. de Mendoza, J. Guerri, M. Cambra, L. Navarro, P. Moreno, and L. Pena, 2002: Pathogen-derived resistance to Citrus tristeza virus (CTV) in transgenic Mexican lime (Citrus aurantifolia (Christ.) Swing.) plants expressing its p25 coat protein gene. Molecular Breeding 10, 1-10.

Dougherty WG and Parks TD 1991: Posttranslational Processing of the Tobacco Etch Virus 49-Kda Small Nuclear Inclusion Polyprotein - Identification of an Internal Cleavage Site and Delimitation of Vpg and Proteinase Domains. Virology 183:449-456.

Duan, CG, C.H. Wang, and H.S. Guo, 2008a: Delayed resistance to Cucumber mosaic virus mediated by 3'UTR-derived hairpin RNA. Chinese Science Bulletin 53, 3301-3310.

Duan, CG, C.H. Wang, R.X. Fang, and H.S. Guo, 2008b: Artificial MicroRNAs Highly Accessible to Targets Confer Efficient Virus Resistance in Plants. Journal of Virology 82, 11084-11095.

Dwyer, GI, M.J. Gibbs, A.J. Gibbs, and R.A.C. Jones, 2007: *Wheat streak mosaic virus* in Australia: Relationship to isolates from the Pacific Northwest of the USA and its dispersion via seed transmission. Plant Disease 91, 164-170.

Ellis, M.H., G.J. Rebetzke, R. Mago, and P. Chu, 2003: First report of *Wheat streak mosaic virus* in Australia. Australasian Plant Pathology 32, 551-553.

Ellis, M.H., G.J. Rebetzke, W.M. Kelman, C.S. Moore, and J.E. Hyles, 2004: Detection of *Wheat streak mosaic virus* in four pasture grass species in Australia. Plant Pathology 53, 239-239.

Fahim, M., L. Ayala-Navarrete, A.A. Millar, and P.J. Larkin, 2010: Hairpin RNA derived from viral NIa gene confers immunity to *Wheat streak mosaic virus* infection in transgenic wheat plants. Plant Biotechnology Journal 8, 821-834.

Fauquet CM and Mayo MA 2001: The 7(th) ICTV Report. *Archives of Virology* 146:189-194.

Fedak, G., Q. Chen, R.L. Conner, A. Laroche, A. Comeau, and C.A. St-Pierre, 2001: Characterization of wheat-Thinopyrum partial amphiploids for resistance to barley yellow dwarf virus. Euphytica 120, 373-378.

Fedak, G., and F. Han, 2005: Characterization of derivatives from wheat-Thinopyrum wide crosses. Cytogenetic and Genome Research 109, 360-367.

Fellers J, Wan JR, Hong YL, Collins GB and Hunt AG 1998a: In vitro interactions between a potyvirus-encoded, genome-linked protein and RNA-dependent RNA polymerase. Journal of General Virology 79:2043-2049.

Fellers JP, Collins GB and Hunt AG 1998b: The NIa-proteinase of different plant potyviruses provides specific resistance to viral infection. Crop Science 38:1309-1319.

Fellers JP, Seifers D, Ryba-White M and Martin TJ 2009: The complete genome sequence of Triticum mosaic virus, a new wheat-infecting virus of the High Plains. Archives of Virology 154:1511-1515.

Fellows H 1949: A survey of the wheat mosaic disease in western Kansas. Plant Dis Rep 33:356-358.

Fereres, A., M. Thresh, and M. Irwin, 2000: Plant virus epidemiology: Challenges for the twenty-first century - Preface. Virus Research 71, X-Xi.

Fernandez A and Garcia JA 1996: The RNA helicase CI from plum pox potyvirus has two regions involved in binding to RNA. FEBS Letters 388:206-210.

Fernandez A, Guo HS, Saenz P, SimonBuela L, deCedron MG and Garcia JA 1997: The motif V of plum pox potyvirus CI RNA helicase is involved in NTP hydrolysis and is essential for virus RNA replication. Nucleic Acids Research 25:4474-4480.

Fire, A, S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, and C.C. Mello, 1998: Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391, 806-11.

Firth AE and Atkins JF 2008: Bioinformatic analysis suggests that a conserved ORF in the waikaviruses encodes an overlapping gene. Archives of Virology 153:1379-1383.

Firth AE, Chung BYW, Fleeton MN and Atkins JF 2008: Discovery of frameshifting in Alphavirus 6K resolves a 20-year enigma. Virology Journal 5.

Firth AE and Atkins JF 2009: Analysis of the coding potential of the partially overlapping 3 ' ORF in segment 5 of the plant fijiviruses. Virology Journal 6.

Fortier, E., and J.M. Belote, 2000: Temperature-dependent gene silencing by an expressed inverted repeat in Drosophila. Genesis 26, 240-244.

French, R., and D.C. Stenger, 2003: Evolution of *Wheat streak mosaic virus*: dynamics of population growth within plants may explain limited variation. Annu Rev Phytopathol 41, 199-214.

Friebe, B., Y. Mukai, H.S. Dhaliwal, T.J. Martin, and B.S. Gill, 1991: Identification of alien chromatin specifying resistance to wheat streak mosaic and greenbug in wheat-germ plasm by c-banding and in situ hybridization. Theoretical and Applied Genetics 81, 381-389.

Friebe, B., Y. Mukai, B.S. Gill, and Y. Cauderon, 1992a: C-banding and in situ hybridyzation analysis of Agropyron intermedium, a partial wheatXA.intermedium apmphiploid, and 6 Derived chromosome Addition lines. Theoretical and Applied Genetics 84, 899-905.

Friebe, B., K.S. Gill, N.A. Tuleen, and B.S. Gill, 1996a: Transfer of *Wheat streak mosaic virus* resistance from Agropyron intermedium into wheat. Crop Science 36, 857-861.

Friebe, B., J. Jiang, W.J. Raupp, R.A. McIntosh, and B.S. Gill, 1996b: Characterization of wheat-alien translocations conferring resistance to diseases and pests: Current status. Euphytica 91, 59-87.

Friebe, B., L.L. Qi, D.L. Wilson, Z.J. Chang, D.L. Selfers, T.J. Martin, A.K. Fritz, and B.S. Gill, 2009: Wheat-Thinopyrum Intermedium Recombinants Resistant to *Wheat streak mosaic virus* and Triticum Mosaic Virus. Crop Science 49, 1221-1226.

Fuchs, M., and D. Gonsalves, 2007: Safety of virus-resistant transgenic plants two decades after their introduction: lessons from realistic field risk assessment studies. Annu Rev Phytopathol 45, 173-202.

Fuchs, M., 2008: Transgenic plants and control of virus diseases: state of the art and prospects. Virologie 12, 27-37.

Gildow, F., V. Damsteegt, A. Stone, W. Schneider, D. Luster, and L. Levy, 2004: Plum pox in north america: identification of aphid vectors and a potential role for fruit in virus spread. Phytopathology 94, 868-74.

Gill, BS, B. Friebe, D.L. Wilson, T.J. Martin, and T.S. Cox, 1995: Registration of KS93WGRC27 *Wheat streak mosaic virus* resistant T4DL 4AI-number-2S wheat germplasm. Crop Science 35, 1236-1237.

Gill, BS, B. Friebe, L.L. Qi, D.L. Wilson, W.J. Raupp, A.K. Fritz, D.L. Seifers, T.J. Martin, and M.O. Pumphrey. 2008. Notice of release of KS08WGGRC50 *Wheat streak mosaic virus*- and Triticum mosaic virus-resistant hard red winter wheat germ plasm. Agricultural Exp. Station and Cooperative Extension Service Release. Kansas State Univ.

Goldbach, R, E. Bucher, and M. Prins, 2003: Resistance mechanisms to plant viruses: an overview. Virus Research 92, 207-212.

Graybosch, RA, C.J. Peterson, P.S. Baenziger, D.D. Baltensperger, L.A. Nelson, Y. Jin, J. Kolmer, B. Seabourn, R. French, G. Hein, T.J. Martin, B. Beecher, T. Schwarzacher, and P. Heslop-Harrison, 2009: Registration of 'Mace' Hard Red Winter Wheat. Journal of Plant Registrations 3, 51-56.

Guddeti, S, D.C. Zhang, A.L. Li, C.H. Leseberg, H. Kang, X.G. Li, W.X. Zhai, M.A. Johns, and L. Mao, 2005: Molecular evolution of the rice miR395 gene family. Cell Research 15, 631-638.

Haber, S, R. Kurtz, and E. Moats. , 1997: Wheat streak mosaic in southeastern Saskatchewan and southwestern Manitoba in 1996. Can. PI. Dis. Survey. 77, 23.

Haber, S, D.L. Seifers, and T.J. Martin, 2006: Two new sources of resistance to *Wheat streak mosaic virus* (WSMV) in winter wheat. Phytopathology 96, S44-S44.

Haber, S, M. Pradhan, and D. Somers, 2007: Breaking the linkage between the Wsm1 gene for resistance to *Wheat streak mosaic virus* and the alien chromatin of its origin. Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie 29, 215-216.

Haber S, Gilbert J and Golkari S 2008: An evolutionary approach identifies and exploits effective FHB resistance in hitherto susceptible wheat germplasm. Cereal Research Communications **36**:63-69

Haber S, Gilbert J and Golkari S 2009: Using variations in response to infection with Wheat streak mosaic virus to identify resistance to Fusarium head blight in germplasm considered susceptible. Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie **31**:149-149.

Haber, S, A. Singh, and D.L. Seifers, 2009: Evolution of resistance to *Wheat streak mosaic virus* in elite durum wheat germplasm. Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie 31, 487-487.

Haber, S., M. Fahim, LA.Navarrete, PJ Larkin and Dallas L. Seifers 2011: A new source of resistance against *Wheat streak mosaic virus* (WSMV) derived from doubled haploid (DH) spring wheat line C2652. Plant Disease (Submitted)

Haber S, Gilbert J, Seifers DL and Comeau A 2011: Epigenetics Serves Genetics: Fusarium Head Blight (FHB) Resistance in Elite Wheat Germplasm. Americas J Pl Science and Biotechnology **5**:IN PRESS.

Hakizimana, F., A.M.H. Ibrahim, M.A.C. Langham, J.C. Rudd, and S.D. Haley, 2004a: Generation means analysis of *Wheat streak mosaic virus* resistance in winter wheat. Euphytica 139, 133-139.

Hakizimana, F., A.M.H. Ibrahim, M.A.C. Langham, S.D. Haley, and J.C. Rudd, 2004b: Diallel analysis of *Wheat streak mosaic virus* resistance in winter wheat. Crop Science 44, 89-92.

Haley, S.D., T.J. Martin, J.S. Quick, D.L. Seifers, J.A. Stromberger, S.R. Clayshulte, B.L. Clifford, F.B. Peairs, J.B. Rudolph, J.J. Johnson, B.S. Gill, and B. Friebe, 2002: Registration of CO960293-2 wheat germplasm resistant to *Wheat streak mosaic virus* and Russian wheat aphid. Crop Science 42, 1381-1382.

Halliday, RB, and D.K. Knihinicki, 2004: The occurrence of Aceria tulipae (Keifer) and Aceria tosichella Keifer in Australia (Acari : Eriophyidae). International Journal of Acarology 30, 113-118.

Han, SJ, HS. Cho, J.S. You, Y.W. Nam, E.K. Park, J.S. Shin, Y.I. Park, W.M. Park, and K.H. Paek, 1999: Gene silencing-mediated resistance in transgenic tobacco plants carrying potato virus Y coat protein gene. Molecules and Cells 9, 376-383.

Harrison, BD, and D.J. Robinson, 1988: Molecular Variation in Vector-Borne Plant-Viruses - Epidemiological Significance. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 321, 447-462.

Harvey, TL., and T.J. Martin, 1980: Effects of wheat pubescence on infestations of wheat curl mite (acari, eriophyidae) and incidence of wheat streak mosaic. Journal of Economic Entomology 73, 225-227.

Harvey, TL., and D.L. Seifers, 1991: Transmission of Wheat Streak Mosaic-Virus to Sorghum by the Wheat Curl Mite (Acari, Eriophyidae). Journal of the Kansas Entomological Society 64, 18-22.

Harvey, TL., T.J. Martin, D.L. Seifers, and P.E. Sloderbeck, 1997: Change in virulence of wheat curl mite detected on TAM 107 wheat. Crop Science 37, 624-625.

Harvey, TL., D.L. Seifers, T.J. Martin, G. Brown-Guedira, and B.S. Gill, 1999: Survival of wheat curl mites on different sources of resistance in wheat. Crop Science 39, 1887-1889.

Harvey, TL., T.J. Martin, and D.L. Seifers, 2000: Effect of nonviruliferous wheat curl mites on yield of winter wheat. Journal of Agricultural and Urban Entomology 17, 9-13.

Hassani, F., and M.T. Assad, 2004: Inheritance and allelism of *Wheat streak mosaic virus* resistance in two Iranian wheat lines. Euphytica 140, 213-216.

Haunold, E., 1958: Factors affecting the virus content of cheyenne wheat infected by wheat streak mosaic. Phytopathology 48, 411-414.

Hayes R., M. Newell, M. Fahim, M. Norton, M. Newberry, LR DeHaan, T.S Cox, S.S. Jones, K.M. Murphy, L.J Wade, PJ Larkin, 2011: Pathways to the development of 'perennial wheat' for Australian environments. (In preparation)

Hill, JH, Martinso.Ca, and W.A. Russell, 1974: Seed transmission of maize-dwarf mosaic and wheat streak mosaic-viruses in maize and response of inbred lines. Crop Science 14, 232-235.

Hily, JM, M. Ravelonandro, V. Damsteegt, C. Bassett, C. Petri, Z. Liu, and R. Scorza, 2007: Plum pox virus coat protein gene Intron-hairpin-RNA (ihpRNA) constructs provide resistance to plum pox virus in Nicotiana benthamiana and Prunus domestica. Journal of the American Society for Horticultural Science 132, 850-858.

Hull, R., 2002: Matthews' Plant Virology, Academic Press, San Diego.

Hunger, R.M., J.L. Sherwood, C.K. Evans, and J.R. Montana, 1992: Effects of Planting Date and Inoculation Date on Severity of *Wheat streak mosaic virus* in Hard Winter Wheat-Wheat Cultivars. Plant Disease 76, 1056-1060.

Irwin, M.E., 1999: Implications of movement in developing and deploying integrated pest management strategies. Agricultural and Forest Meteorology 97, 235-248.

Irwin, M.E., W.G. Ruesink, S.A. Isard, and G.E. Kampmeier, 2000: Mitigating epidemics caused by non-persistently transmitted aphid-borne viruses: the role of the pliant environment. Virus Research 71, 185-211.

Israsena, N., P. Supavonwong, N. Ratanasetyuth, P. Khawplod, and T. Hemachudha, 2009: Inhibition of rabies virus replication by multiple artificial microRNAs. Antiviral Res 84, 76-83.

Jackson, A.L., S.R. Bartz, J. Schelter, S.V. Kobayashi, J. Burchard, M. Mao, B. Li, G. Cavet, and P.S. Linsley, 2003: Expression profiling reveals off-target gene regulation by RNAi. Nat Biotechnol 21, 635-7.

Jackson, A.L., J. Burchard, J. Schelter, B.N. Chau, M. Cleary, L. Lim, and P.S. Linsley, 2006a: Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. RNA 12, 1179-87.

Jackson, A.L., J. Burchard, D. Leake, A. Reynolds, J. Schelter, J. Guo, J.M. Johnson, L. Lim, J. Karpilow, K. Nichols, W. Marshall, A. Khvorova, and P.S. Linsley, 2006b: Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. RNA 12, 1197-205.

Jana, S, C. Chakraborty, and S. Nandi, 2004: Mechanisms and roles of the RNA-based gene silencing. Electronic Journal of Biotechnology 7.

Jiang, J, B. Friebe, H.S. Dhaliwal, T.J. Martin, and B.S. Gill, 1993: Molecular cytogenetic analysis of agropyron-elongatum chromatic in wheat germplasm specifying resistance to *Wheat streak mosaic virus*. Theoretical and Applied Genetics 86, 41-48.

Jiang, W, K.A. Garrett, D.E. Peterson, T.L. Harvey, R.L. Bowden, and L. Fang, 2005: The window of risk for emigration of *Wheat streak mosaic virus* varies with host eradication method. Plant Disease 89, 853-858.

Jones, RAC., B.A. Coutts, A.E. Mackie, and G.I. Dwyer, 2005: Seed transmission of *Wheat streak mosaic virus* shown unequivocally in wheat. Plant Disease 89, 1048-1050.

Jones-Rhoades, M.W., and D.P. Bartel, 2004: Computational identification of plant MicroRNAs and their targets, including a stress-induced miRNA. Molecular Cell 14, 787-799.

Jozsa, R, and E. Balazs, 2000b: Coat protein gene-mediated cross protection in transgenic plants I. State for the art at the Millenium. Novenytermeles 49, 165-184.

Kalantidis, K, S. Psaradakis, M. Tabler, and M. Tsagris, 2002: The occurrence of CMV-specific short RNAs in transgenic tobacco expressing virus-derived double-stranded RNA is indicative of resistance to the virus. Molecular Plant-Microbe Interactions 15, 826-833.

Kameda, T, K. Ikegami, Y. Liu, K. Terada, and T. Sugiyama, 2004: A hypothermic-temperature-sensitive gene silencing by the mammalian RNAi. Biochemical and Biophysical Research Communications 315, 599-602.

Keifer, HH, 1969: Eriophyid studies. ARS-USDA, C-3, 1-2

Ketzinel-Gilad, M., Y. Shaul, and E. Galun, 2006: RNA interference for antiviral therapy. Journal of Gene Medicine 8, 933-950.

Khraiwesh, B., S. Ossowski, D. Weigel, R. Reski, and W. Frank, 2008: Specific gene silencing by artificial MicroRNAs in Physcomitrella patens: an alternative to targeted gene knockouts. Plant Physiol 148, 684-93.

Kutter, C. and P. Svoboda 2008: miRNA, siRNA, piRNA: Knowns of the unknown. RNA Biology 5(4): 181-188.

Lanning, SP, L.E. Talbert, F.H. Mcneal, W.L. Alexander, C.F. Mcguire, H. Bowman, G. Carlson, G. Jackson, J. Eckhoff, G. Kushnak, V. Stewart, and G. Stallknecht, 1992: Registration of Hi-Line Wheat. Crop Science 32, 283-284.

Lanoiselet, VM, T.L. Hind-Lanoiselet, and G.M. Murray, 2008: Studies on the seed transmission of *Wheat streak mosaic virus*. Australasian Plant Pathology 37, 584-588.

Larkin, PJ, and W.R. Scowcroft, 1981: Somaclonal variation - a novel source of variability from cell culture for plant improvement. Theor Appl Genet 60, 197-214.

Larson, RI, and T.G. Atkinson, 1972: Isolation of an agropyron-elongatum chromosome conferring resistance to wheat curl mite on a triticum-agropyron hybrid. Canadian Journal of Genetics and Cytology 14, 731.

Lay CL, Wells DG and Gardner WS 1971: Immunity from *Wheat streak mosaic virus* in irradiated Agrotricum progenies. Crop Science 11:431.

Lebas, BSM., F.M. Ochoa-Corona, B.J.R. Alexander, R.A. Lister, J.D.F. Fletcher, S.L. Bithell, and G.M. Burnip, 2009: First Report of *Wheat streak mosaic virus* on Wheat in New Zealand. Plant Disease 93, 430-430.

Lecoq H, Wipf-Scheibel C, Chandeysson C, Le Van A, Fabre F and Desbiez C 2009: Molecular epidemiology of Zucchini yellow mosaic virus in France: An historical overview. Virus Research 141:190-200.

Léonard, S, Plante, D., Wittmann, S., Daigneault, N., Fortin, M. G. & Laliberté, J. F. 2000: Complex formation between potyvirus VPg and translation eukaryotic initiation factor 4E correlates with virus infectivity. Journal of Virology **74**, 7730–7737

Léonard, S, C. Viel, et al. 2004: Interaction of VPg-Pro of Turnip mosaic virus with the translation initiation factor 4E and the poly(A)-binding protein in planta." Journal of General Virology 85(4): 1055-1063.

Li, HJ, RL Conner, Q. Chen, R.J. Graf, A. Laroche, F. Ahmad, and A.D. Kuzyk, 2004: Promising genetic resources for resistance to *Wheat streak mosaic virus* and the wheat curl mite in wheat-Thinopyrum partial amphiploids and their derivatives. Genetic Resources and Crop Evolution 51, 827-835.

Li, Y, Y Song, C. Zhu, and F. Wen, 2008: Effect of stem-loop proportion in hpRNA on the RNA-mediated virus resistance. Acta Phytopathologica Sinica 38, 468-477.

Lin, SS, R. Henriques, H.W. Wu, Q.W. Niu, S.D. Yeh, and N.H. Chua, 2007: Strategies and mechanisms of plant virus resistance. Plant Biotechnology Reports 1, 125-134.

Lin, SS, H.W. Wu, S.F. Elena, K.C. Chen, Q.W. Niu, S.D. Yeh, C.C. Chen, and N.H. Chua, 2009: Molecular Evolution of a Viral Non-Coding Sequence under the Selective Pressure of amiRNA-Mediated Silencing. Plos Pathogens 5, -.

Lindbo, J.A., L. Silvarosales, W.M. Proebsting, and W.G. Dougherty, 1993: Induction of a highly specific antiviral state in transgenic plants - implications for regulation of gene expression and virus resistance. Plant Cell 5, 1749-1759.

Lindbo, JA, and W.G. Dougherty, 2005: Plant pathology and RNAi: A brief history. Annual Review of Phytopathology 43, 191-204.

Liu, J, EA Lee, M.K. Sears, and A.W. Schaafsma, 2005: Wheat curl mite (Acari : Eriophyidae) dispersal and its relationship with kernel red streaking in maize. Journal of Economic Entomology 98, 1580-1586.

Liu, YP, J. Haasnoot, O. ter Brake, B. Berkhout, and P. Konstantinova, 2008: Inhibition of HIV-1 by multiple siRNAs expressed from a single microRNA polycistron. Nucleic Acids Research 36, 2811-2824.

Lius, S, RM. Manshardt, M.M.M. Fitch, J.L. Slightom, J.C. Sanford, and D. Gonsalves, 1997: Pathogen-derived resistance provides papaya with effective protection against papaya ringspot virus. Molecular Breeding 3, 161-168.

Lomonossoff, GP, 1995: Pathogen-Derived Resistance to Plant-Viruses. Annual Review of Phytopathology 33, 323-343.

Lopez-Moya JJ, Wang RY and Pirone TP 1999: Context of the coat protein DAG motif affects potyvirus transmissibility by aphids. J Gen Virol 80 (Pt 12):3281-3288.

Louie, R, DL Seifers, and O.E. Bradfute, 2006: Isolation, transmission and purification of the High Plains virus. Journal of Virological Methods 135, 214-222.

Lu, B, G. Stubbs, and Culver, J. N., 1998: Coat Protein Interactions Involved in Tobacco Mosaic Tobamovirus Cross-Protection. Virology 248(2): 188-198.

Lu, H, J. Price, R. Devkota, C. Rush, and J. Rudd, 2011: A Dominant Gene for Resistance to *Wheat streak mosaic virus* in Winter Wheat Line CO960293-2. Crop Science 51, 5-12.

Lukaszewski, AJ, and J.P. Gustafson, 1983: Translocations and modifications of chromosomes in triticale x wheat hybrids. Theor Appl Genet 64, 239-248.

Ma, ZL, HY. Yang, R. Wang, and P. Tien, 2004: Construct hairpin RNA to fight against rice dwarf virus. Acta Botanica Sinica 46, 332-336.

Mahmood, T., G.L. Hein, and R.C. French, 1997: Development of serological procedures for rapid and reliable detection of *Wheat streak mosaic virus* in a single wheat curl mite. Plant Disease 81, 250-253.

Maia IG and Haenni AL 1994: Early Events in Virus Plant Interactions. Brazilian Journal of Medical and Biological Research 27:2521-2532.

Maki-Valkama, T., and J.P.T. Valkonen, 1999: Pathogen derived resistance to Potato virus Y: mechanisms and risks. Agricultural and Food Science in Finland 8, 493-513.

Marano, MR, and D. Baulcombe, 1998: Pathogen-derived resistance targeted against the negative-strand RNA of tobacco mosaic virus: RNA strand-specific gene silencing? Plant Journal 13, 537-546.

Martin, TJ, 1978: Procedures for evaluating *Wheat streak mosaic virus* resistance. Plant Disease Reporter 62, 1062-1066.

Martin, TJ, A.K., Fritz, D.L., Seifers, and J.P. Shroyer. 2007. RonL hard white wheat. Kansas State Univ., Agricultural Experiment Station and Cooperative Extension Service Release L-926.

Matzke, AJM and M. A. Matzke 1998: "Position effects and epigenetic silencing of plant transgenes." Current Opinion in Plant Biology 1(2): 142-148.

Mayo, MA, 1999: Developments in plant virus taxonomy since the publication of the 6th ICTV Report. International Committee on Taxonomy of Viruses. Arch Virol 144, 1659-66.

McKinney, HH, 1937: Mosaic diseases of wheat and related cereals. U.S. Department of Agriculture Circular 442. .

McKinney, HH, 1949: Virus isolates from mosaic wheat in the hard red winter wheat area. Plant Disease Reporter, 346-349.

McKinney, HH, and W.J. Sando, 1951: Susceptibility and resistance to the wheat streak-mosaic virus in the genera Triticum, Agropyron, Secale, and certain hybrids. Plant Dis Reporter 35, 476-479.

McMullen, MP, 2002: Wheat streak mosaic. NDSU Extension Service Bulletin, pp-646 (revised).

McNeil, JE, R. French, G.L. Hein, P.S. Baenziger, and K.M. Eskridge, 1996: Characterization of genetic variability among natural populations of *Wheat streak mosaic virus.* Phytopathology 86, 1222-1227.

Meister, G., M. Landthaler, Y. Dorsett, and T. Tuschl, 2004: Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. 10, 544-550.

Merai Z, Kerenyi Z, Kertesz S, Magna M, Lakatos L and Silhavy D 2006: Double-stranded RNA binding may be a general plant RNA viral strategy to suppress RNA silencing. Journal of Virology 80:5747-5756.

Mitter, N, E. Sulistyowati, and R.G. Dietzgen, 2003: Cucumber mosaic virus infection transiently breaks dsRNA-Induced Transgenic immunity to Potato virus Y in tobacco. Molecular Plant-Microbe Interactions 16, 936-944.

Mitter, N, R. Mitchell, and R.G. Dietzgen, 2006: Fate of hairpin transcript components during RNA silencing and its suppression in transgenic virus-resistant tobacco. Journal of Biotechnology 126, 115-122.

Moreno A, Nebreda M, Diaz BM, Garcia M, Salas F and Fereres A 2007: Temporal and spatial spread of Lettuce mosaic virus in lettuce crops in central Spain: factors involved in Lettuce mosaic virus epidemics. Annals of Applied Biology 150:351-360.

Murphy JF, Rychlik W, Rhoads RE, Hunt AG and Shaw JG 1991: A Tyrosine Residue in the Small Nuclear Inclusion Protein of Tobacco Vein Mottling Virus Links the Vpg to the Viral-Rna. Journal of Virology 65:511-513.

Murray G, Simpfendorfer S, Hind-Lanioselet T, Lanoiselet V and Wratten K 2007: Wheat streak mosaic: a threat to grazing and main season wheats that can be beaten, in *GRDC Research Update* pp 63-69, Waga Waga: New South Wales Departmen of Primary Industries and EH Graham Center for agriculture Innovation.

Murray, G., and J. Brennan, 2009: Estimating disease losses to the Australian wheat industry. Australasian Plant Pathology 38, 558-570.

Nakayashiki, H., 2005: RNA silencing in fungi: Mechanisms and applications. FEBS Letters 579, 5950-5957.

Napoli, C., C. Lemieux, and R. Jorgensen, 1990: Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. The Plant Cell 2, 279-289.

Nault, LR, M.L. Briones, L.E. Williams, and B.D. Barry, 1967: Relation of wheat curl mite to kernel red streak of corn. Phytopathology 57, 986.

Nault, LR, and M.L. Briones, 1968: Reaction of corn to feeding of wheat curl mite. Journal of Economic Entomology 61, 31

Nejidat, A. and Beachy, R.N. 1990: Transgenic tobacco plants expressing a coat protein gene of tobacco mosaic virus are resistant to some other tobamoviruses. Mol. Plant–Microbe Interact. **3**, 247–251.

Niepel M, Gallie D R. 1999: Identification and characterization of the functional elements within the tobacco etch virus' Ǝeader required for cap -independent translation. Journal of Virology. 73:9080–9088.

Niu, QW, SS. Lin, J.L. Reyes, K.C. Chen, H.W. Wu, S.D. Yeh, and N.H. Chua, 2006: Expression of artificial microRNAs in transgenic Arabidopsis thaliana confers virus resistance. Nature Biotechnology 24, 1420-1428.

Nyitrai, A., 1991: Investigation on the damage caused by *Wheat streak mosaic virus*. Novenytermeles 40, 21-26.

Oliveira, MRV., T.J. Henneberry, and P. Anderson, 2001: History, current status, and collaborative research projects for Bemisia tabaci. Crop Protection 20, 709-723.

Ossowski, S, R. Schwab, and D. Weigel, 2008: Gene silencing in plants using artificial microRNAs and other small RNAs. Plant Journal 53, 674-690.

Othman, Y., 1996: Transgenic virus resistant plants: Prospects for tropical agriculture. Asia-Pacific Journal of Molecular Biology and Biotechnology 4, 145-153.

Pady, SM, and L. Kapica, 1955: Fungi in Air over the Atlantic Ocean. Mycologia 47, 34-50.

Paliwal, YC, and J.T. Slykhuis, 1967: Localization of *Wheat streak mosaic virus* in the alimentary canal of its vector Aceria tulipae Keifer. Virology 32, 344-53.

Paliwal, YC, 1980: Relationship of wheat streak mosaic and barley stripe mosaic viruses to vector and nonvector eriophyid mites. Arch Virol 63, 123-32.

Palukaitis, P., and M. Zaitlin, 1997: Replicase-mediated resistance to plant virus disease. Advances in Virus Research, Vol 48 48, 349-377.

Park, W., J.X. Zhai, and J.Y. Lee, 2009: Highly efficient gene silencing using perfect complementary artificial miRNA targeting AP1 or heteromeric artificial miRNA targeting AP1 and CAL genes. Plant Cell Reports 28, 469-480.

Pfannenstiel, M., and C. Niblett, 1978: The nature of the resistance of agrotricums to *Wheat streak mosaic virus*. Phytopathology 68, p1204-1209.

Plante, D., C. Viel, S. Leonard, H. Tampo, J.F. Laliberte, and M.G. Fortin, 2004: Turnip mosaic virus VPg does not disrupt the translation initiation complex but interferes with cap binding. Physiological and Molecular Plant Pathology 64, 219-226.

Praveen, S., A.K. Mishra, and G. Antony, 2006: Viral suppression in transgenic plants expressing chimeric transgene from tomato leaf curl virus and cucumber mosaic virus. Plant Cell Tissue and Organ Culture 84, 47-53.

Price, JA, F. Workneh, S.R. Evett, D.C. Jones, J. Arthur, and C.M. Rush, 2010: Effects of *Wheat streak mosaic virus* on Root Development and Water-Use Efficiency of Hard Red Winter Wheat. Plant Disease 94, 766-770.

Pruss G, Ge X, Shi XM, Carrington JC and Bowman Vance V 1997: Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. Plant Cell 9:859-868.

Qi, LL, B. Friebe, P. Zhang, and B.S. Gill, 2007: Homoeologous recombination, chromosome engineering and crop improvement. Chromosome Research 15, 3-19.

Qu, J, J. Ye, and R.X. Fang, 2007: Artificial microRNA-mediated virus resistance in plants. Journal of Virology 81, 6690-6699.

Rabenstein, F., D.L. Seifers, J. Schubert, R. French, and D.C. Stenger, 2002: Phylogenetic relationships, strain diversity and biogeography of tritimoviruses. Journal of General Virology 83, 895-906.

Rakitina DV, Kantidze OL, Leshchiner AD, Solovyev AG, Novikov VK, Morozov SY and Kalinina NO 2005: Coat proteins of two filamentous plant viruses display NTPase activity in vitro. FEBS Letters 579:4955-4960.

Rastegar M, Izadpanah K, Masumi M, Siampour M, Zare A and Afsharifar A 2008: Analyses of the complete sequence of the genome of wheat Eqlid mosaic virus, a novel species in the genus Tritimovirus. Virus Genes 37:212-217.

Register, J.C. and Beachy, R.N. 1988: Resistance to TMV in transgenic plants results from interference with an early event in infection. Virology, 166, 524–532.

Riechmann, J.L., S. Lain, and J.A. Garcia, 1992: Highlights and prospects of potyvirus molecular biology. Journal of General Virology 73, 1-16.

Riedel D, Lesemann DE and Maiss E 1998: Ultrastructural localization of nonstructural and coat proteins of 19 potyviruses using antisera to bacterially expressed proteins of plum pox potyvirus. *Archives of Virology* 143:2133-2158.

Riesselman, J. 1993. Wheat streak identified. Montana Crop Health Report. 3.

Rodoni, B, 2009: The role of plant biosecurity in preventing and controlling emerging plant virus disease epidemics. Virus Research 141, 150-157.

Rojas MR, Zerbini FM, Allison RF, Gilbertson RL and Lucas WJ 1997: Capsid protein and helper component proteinase function as potyvirus cell-to-cell movement proteins. Virology 237:283-295.

Rotte, C, and T. Leustek, 2000: Differential subcellular localization and expression of ATP sulfurylase and 5 '-adenylylsulfate reductase during ontogenesis of arabidopsis leaves indicates that cytosolic and plastid forms of ATP sulfurylase may have specialized functions. Plant Physiology 124, 715-724.

Saenz P, Cervera MT, Dallot S, Quiot L, Quiot JB, Riechmann JL and Garcia JA 2000: Identification of a pathogenicity determinant of Plum pox virus in the sequence encoding the C-terminal region of protein P3+6K(1). Journal of General Virology 81:557-566.

Sanchez-Sanchez, H., M. Henry, E. Cardenas-Soriano, and H.F. Alvizo-Villasana, 2001: Identification of *Wheat streak mosaic virus* and its vector Aceria tosichella in Mexico. Plant Disease 85, 13-17.

Schiffer, M, P Umina, M. Carew, A. Hoffmann, B. Rodoni, and A. Miller, 2009: The distribution of wheat curl mite (Aceria tosichella) lineages in Australia and their potential to transmit *Wheat streak mosaic virus*. Annals of Applied Biology 155, 371-379.

Schmidt, JW, E.G. Heyne, C.O. Johnston, and E.D. Hansing, 1953: Progress of Agrotricum breeding in Kansas. Trans Kansas Acad Sci 56, 29-45.

Schnippenkoetter, W.H., D.P. Martin, J.A. Willment, and E.P. Rybicki, 2001: Forced recombination between distinct strains of Maize streak virus. Journal of General Virology 82, 3081-3090.

Schwab, R, S. Ossowski, M. Riester, N. Warthmann, and D. Weigel, 2006: Highly specific gene silencing by artificial microRNAs in Arabidopsis. Plant Cell 18, 1121-1133.

Seifers, DL and T. Martin, 1988: Correlation of low level *Wheat streak mosaic virus* resistance in Triumph 64 wheat with low virus titer. Phytopathology 78, 703-707.

Seifers, DL, T.J. Martin, T.L. Harvey, and B.S. Gill, 1995a: Temperature Sensitivity and Efficacy of Wheat Streak Mosaic-Virus Resistance Derived from Agropyron Intermedium. Plant Disease 79, 1104-1106.

Seifers, DL, T.L. Harvey, and R.L. Bowden, 1995b: Occurrence and Symptom Expression Of American Wheat Striate Mosaic-Virus In Wheat In Kansas. Plant Disease 79, 853-858.

Seifers, DL, T.L. Harvey, K.D. Kofoid, and W.D. Stegmeier, 1996: Natural infection of pearl millet and sorghum by *Wheat streak mosaic virus* in Kansas. Plant Disease 80, 179-185.

Seifers, DL, T.L. Harvey, R. Louie, D.T. Gordon, and T.J. Martin, 2002: Differential transmission of isolates of the High Plains virus by different sources of wheat curl mites. Plant Disease 86, 138-142.

Seifers, DL, Y.M. She, T.L. Harvey, T.J. Martin, S. Haber, W. Ens, K.G. Standing, R. Louie, and D.T. Gordon, 2004: Biological and molecular variability among High Plains virus isolates. Plant Disease 88, 824-829.

Seifers, DL, S. Haber, W. Ens, Y.M. She, K.G. Standing, and R. Salomon, 2005: Characterization of a distinct Johnsongrass mosaic virus strain isolated from sorghum in Nigeria. Archives of Virology 150, 557-576.

Seifers, DL, T.J. Martin, T.L. Harvey, S. Haber, and S.D. Haley, 2006: Temperature sensitivity and efficacy of *Wheat streak mosaic virus* resistance derived from CO960293 wheat. Plant Disease 90, 623-628.

Seifers, DL, T.J. Martin, T.L. Harvey, and S. Haber, 2007: Temperature-sensitive *Wheat streak mosaic virus* resistance identified in KS03HW12 wheat. Plant Disease 91, 1029-1033.

Seifers, DL, and J. Martin, 2009: Differential hosts for *Triticum mosaic virus* and *Wheat streak mosaic virus*. Phytopathology 99, S117-S117.

Seifers, DL, T.J. Martin, T.L. Harvey, J.P. Fellers, and J.P. Michaud, 2009: Identification of the Wheat Curl Mite as the Vector of Triticum mosaic virus. Plant Disease 93, 25-29.

Seifers, DL, T.J. Martin, and J.P. Fellers, 2010: An Experimental Host Range for Triticum mosaic virus. Plant Disease 94, 1125-1131.

Seifers, DL, T.J. Martin, and J.P. Fellers, 2011: Occurrence and Yield Effects of Wheat Infected with Triticum mosaic virus in Kansas. Plant Disease 95, 183-188.

Shahwan, IM, and Hill, J.P. 1984. Identification and occurrence of *Wheat streak mosaic virus* in winter wheat in Colorado and its effects on several wheat cultivars. Plant Disease. 68:579-581.

Sharma, HC, B.S. Gill, and J.K. Uyemoto, 1984: High-levels of resistance in Agropyron species to *Barley yellow dwarf* and *Wheat streak mosaic viruses*. Phytopathologische Zeitschrift-Journal of Phytopathology 110, 143-147.

Sharp, GL, J.M. Martin, S.P. Lanning, N.K. Blake, C.W. Brey, E. Sivamani, R. Qu, and L.E. Talbert, 2002: Field Evaluation of Transgenic and Classical Sources of *Wheat streak mosaic virus* Resistance. Crop Sci 42, 105-110.

Shukla DD, Lauricella R and Ward CW 1992: Serology of potyviruses - current problems and some solutions. Archives of Virology:57-69.

Sijen, T, J Wellink, J.B. Hiriart, and A. Vankammen, 1996: RNA-mediated virus resistance - role of repeated transgenes and delineation of targeted regions. Plant Cell 8, 2277-2294.

Sijen, T., and J.M. Kooter, 2000: Post-transcriptional gene-silencing: RNAs on the attack or on the defense? Bioessays 22, 520-531.

Sill, WH, and JRV. Connin, 1953 Summary of the Known Host Range of the Wheat Streak-Mosaic Virus Transactions of the Kansas Academy of Science (1903-) 56, 411-417.

Simon-Mateo, C., and J.A. Garcia, 2006: MicroRNA-Guided processing impairs Plum pox virus replication, but the virus readily evolves to escape this silencing mechanism. Journal of Virology 80, 2429-2436.

Sinha, R.C., and Y.C. Paliwal, 1977: Detection of wheat streak mosaic-virus antigens in vector mites with fluorescent-antibodies. Phytopathology 67, 570-572.

Sivamani, E., C.W. Brey, W.E. Dyer, L.E. Talbert, and R. Qu, 2000: Resistance to *Wheat streak mosaic virus* in Transgenic Wheat Expressing the Viral Replicase (NIb) Gene. Molecular Breeding 6, 469-477.

Sivamani, E., C.W. Brey, L.E. Talbert, M.A. Young, W.E. Dyer, W.K. Kaniewski, and R. Qu, 2002: Resistance to *Wheat streak mosaic virus* in transgenic wheat engineered with the viral coat protein gene. Transgenic Res 11, 31-41.

Slykhuis, JT, 1953a: The relation of Aceria tulipae Keifer to streak mosaic and other chlorotic symptoms on wheat. Phytopathology 43, 484-485.

Slykhuis, JT, 1953b: Striate mosaic, a new disease of wheat in South-Dakota. Phytopathology 43, 537-540.

Slykhuis, JT, 1955: *Aceria tulipae* Keifer (Acarina, Eriophyidae) in relation to the spread of wheat streak mosaic. Phytopathology 45, 116-128.

Smith, NA, S.P. Singh, M.B. Wang, P.A. Stoutjesdijk, A.G. Green, and P.M. Waterhouse, 2000: Gene expression - Total silencing by intron-spliced hairpin RNAs. Nature 407, 319-320.

Somsen, HW, and W.H. Sill, 1970: The wheat curl mite, Aceria tulipae Keifer, in relation to epidemiology and control of wheat streak mosaic. . Agriculture Experiment Station Research Publication

Spetz C and Valkonen JPT 2004: Potyviral 6K2 protein long-distance movement and symptom-induction functions are independent and host-specific. Molecular Plant-Microbe Interactions 17:502-510.

Staples, R., and W.B. Allington, 1956: Streak mosaic of wheat in Nebraska and its control. University of Nebraska, Lincoln. Agriculture Experiment. Station. Research Bulliton.

Stenger, DC, J.S. Hall, I.R. Choi, and R. French, 1998: Phylogenetic relationships within the family Potyviridae: *Wheat streak mosaic virus* and brome streak mosaic virus are not members of the genus Rymovirus. Phytopathology 88, 782-787.

Stenger, DC, D.L. Seifers, and R. French, 2002: Patterns of polymorphism in *Wheat streak mosaic virus*: sequence space explored by a clade of closely related viral genotypes rivals that between the most divergent strains. Virology 302, 58-70.

Stenger DC, Hein GL, Gildow FE, Horken KM and French R 2005: Plant virus HC-Pro is a determinant of eriophyid mite transmission. Journal of Virology 79:9054-9061.

Stenger DC, Hein GL and French R 2006a: Nested deletion analysis of *Wheat streak mosaic virus* HC-Pro: Mapping of domains affecting polyprotein processing and eriophyid mite transmission. Virology 350:465-474.

Stenger DC, Young BA and French R 2006b: Random mutagenesis of *Wheat streak mosaic virus* HC-Pro: non-infectious interfering mutations in a gene dispensable for systemic infection of plants. J Gen Virol 87:2741-2747.

Stenger D, Young B, Qu F, Morris T and French R 2007a: *Wheat streak mosaic virus* P1, not HC-Pro, facilitates disease synergism and suppression of post-transcriptional gene silencing. Phytopathology 97:S111-S111.

Stenger DC, Young BA, Qu F, Morris TJ and French R 2007b: *Wheat streak mosaic virus* lacking helper component-proteinase is competent to produce disease synergism in double infections with Maize chlorotic mottle virus. Phytopathology 97:1213-1221.

Stenger, D.C., and R. French, 2009: *Wheat streak mosaic virus* genotypes introduced to Argentina are closely related to isolates from the American Pacific Northwest and Australia. Archives of Virology 154, 331-336.

Stoddard, S., B. Gill, and Lommel. SA., 1987b: Genetic expression of *Wheat streak mosaic virus* resistance in two wheat-wheatgrass hybrids. Crop Science 27, 514-519.

Stoddard, S., S. Lommel, and B. Gill, 1987a: Evaluation of wheat germplasm for resistance to *Wheat streak mosaic virus* by symptomatology, ELISA, and slot-blot hybridization. Plant Disease 71, 714-719.

Stram, Y., and L. Kuzntzova, 2006: Inhibition of viruses by RNA interference. Virus Genes 32, 299-306.

Szittya, G., D. Silhavy, A. Molnar, Z. Havelda, A. Lovas, L. Lakatos, Z. Banfalvi, and J. Burgyan, 2003: Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. EMBO Journal 22, 633-640.

Tairo F, Jones RAC and Valkonen JPT 2006: Potyvirus complexes in sweet potato: Occurrence in Australia, serological and molecular resolution, and analysis of the sweet potato virus 2 (SPV2) component. Plant Disease 90:1120-1128.

Talbert, L.E., P.L. Bruckner, L.Y. Smith, R. Sears, and T.J. Martin, 1996: Development of PCR markers linked to resistance to *Wheat streak mosaic virus* in wheat. Theoretical and Applied Genetics 93, 463-467.

Tatineni S, Graybosch RA, Hein GL, Wegulo SN and French R 2010: Wheat Cultivar-Specific Disease Synergism and Alteration of Virus Accumulation During Co-Infection with *Wheat streak mosaic virus* and Triticum mosaic virus. Phytopathology 100:230-238.

Tatineni S, McMechan AJ, Hein GL and French R 2011a: Efficient and stable expression of GFP through *Wheat streak mosaic virus*-based vectors in cereal hosts using a range of cleavage sites: Formation of dense fluorescent aggregates for sensitive virus tracking. Virology 410:268-281.

Tatineni S, Van Winkle DH and French R 2011b: The N-Terminal Region of *Wheat streak mosaic virus* Coat Protein Is a Host- and Strain-Specific Long-Distance Transport Factor. Journal of Virology 85:1718-1731.

ter Brake, O., K.J. von Eije, Y.P. Liu, and B. Berkhout, 2007: Silencing of HIV-1 with RNA interference: A multiple shRNA approach. Human Gene Therapy 18, 1068-1068.

Thomas, JA, and G.L. Hein, 2003: Influence of volunteer wheat plant condition on movement of the wheat curl mite, Aceria tosichella, in winter wheat. Experimental and Applied Acarology 31, 253-268.

Thomas, JE, V. Steele, A.D.W. Geering, D.M. Persley, C.F. Gambley and B.H., Hall. 2009. Recent plant incursions into Australia. Poster presentation at Australasian Plant Pathology Society Conference, Plant Health Management: An integrated approach September 29, 2009- October 1, 2009.

Turner, M., and W. Schuch, 2000: Post-transcriptional gene-silencing and RNA interference: genetic immunity, mechanisms and applications. Journal of Chemical Technology and Biotechnology 75, 869-882.

Urcuqui-Inchima S, Haenni AL and Bernardi F 2001: Potyvirus proteins: a wealth of functions. Virus Research 74:157-175.

Urcuqui-Inchima, S., I.G. Maia, P. Arruda, A.L. Haenni, and F. Bernardi, 2000: Deletion mapping of the potyviral helper component-proteinase reveals two regions involved in RNA binding. Virology 268, 104-111.

van der Krol, A.R., L.A. Mur, M. Beld, J.N.M. Mol, and A.R. Stuitje, 1990: Flavanoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expession. The Plant Cell 2, 291-299.

Vaucheret, H. 2006. Post-transcriptional small RNA pathways in plants: mechanisms and regulations. Genes & Dev. 2006. 20: 759-771

Voinnet, O, YM Pinto, and D.C. Baulcombe, 1999: Suppression of gene silencing: A general strategy used by diverse DNA and RNA viruses of plants. Proceedings of the National Academy of Sciences of the United States of America 96, 14147-14152.

Wagaba, H., Basavaprabhu, L. Patil, Y. Jitender, S., T. Nigel, J., T. Alicai, Y. Baguma, B. Settumba Mukasa, and C.M. Fauquet, 2010: Testing the efficacy of artificial microRNAs to control cassava brown streak disease. Second RUFORUM Biennial Meeting 20 - 24 September 2010, Entebbe, Uganda, 287-291.

Wang RC and Liang GH 1977: Cytogenetic location of genes for resistance to Wheat streak mosaic in an Agropyron substitution line. Journal of Heredity 68:375-378.

Wang RC, Barnes EE and Cook LL 1980: Transfer of *Wheat streak mosaic virus* resistance from *Agropyron intermedium* to homoeologous chromosome of wheat *Triticum aestivum*. Cereal Research Communications 8:335-340.

Wang, M-B, DC. Abbott, and PM. Waterhouse, 2000: A single copy of a virus-derived transgene encoding hairpin RNA gives immunity to barley yellow dwarf virus. Molecular Plant Pathology 1, 347-356.

Warthmann, N., H. Chen, S. Ossowski, D. Weigel, and P. Herve, 2008: Highly specific gene silencing by artificial miRNAs in rice. PLoS One 3, e1829.

Waterhouse, PM, H.W. Graham, and M.B. Wang, 1998: Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proceedings of the National Academy of Sciences of the United States of America 95, 13959-13964.

Watson, JM, AF Fusaro, M.B. Wang, and P.M. Waterhouse, 2005: RNA silencing platforms in plants. FEBS Letters 579, 5982-5987.

Wittmann S, Chatel H, Fortin M G, Laliberte J F. 1997: Interaction of the viral protein genome linked of turnip mosaic potyvirus with the translational eukaryotic initiation factor (iso) 4E of *Arabidopsis thaliana* using the yeast two-hybrid system. Virology. 234:84–92

Weise, MV, 1987: Wheat streak mosaic. Compendium of Wheat Diseases. 2nd edition. American Phytopathological Society, St. Paul. MN. , 80-81.

Wen RH and Hajimorad MR 2010: Mutational analysis of the putative *pipo* of soybean mosaic virus suggests disruption of PIPO protein impedes movement. Virology 400:1-7.

Westerhout, E.M., M. Ooms, M. Vink, A.T. Das, and B. Berkhout, 2005: HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. Nucleic Acids Research 33, 796-804.

Yang S and Ravelonandro M 2002: Molecular studies of the synergistic interactions between plum pox virus HC-Pro protein and potato virus X. Archives of Virology 147:2301-2312.

Young BA, Hein GL, French R and Stenger DC 2007: Substitution of conserved cysteine residues in *Wheat streak mosaic virus* HC-Pro abolishes virus transmission by the wheat curl mite. *Arch Virol* 152:2107-2111.

Zeng, Y, EJ. Wagner, and B.R. Cullen, 2002: Both natural and designed micro RNAs technique can inhibit the expression of cognate mRNAs when expressed in human cells. Molecular Cell 9, 1327-1333.

Zhang, X, H Li, J. Zhang, C. Zhang, P. Gong, K. Ziaf, F. Xiao, and Z. Ye, 2010: Expression of artificial microRNAs in tomato confers efficient and stable virus resistance in a cell-autonomous manner. Transgenic Research. 20(3):569-81

Chapter 2
Grazing sheep and transmission of WSMV

Authors' contribution

MF and HD designed the experiment
MF carried out viral assays and glasshouse work
MF and HD wrote the paper
PL reviewed and edited the paper

CSIRO PUBLISHING
www.publish.csiro.au/journals/cp
Crop & Pasture Science, 2010, **61**, 247–254

Does grazing of infected wheat by sheep result in salivary transmission of *Wheat streak mosaic virus*?

M. Fahim[A], *H. Dove*[A], *W. M. Kelman*[A], *L. Ayala-Navarrete*[A], *and P. J. Larkin*[A,B]

[A]CSIRO Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia.
[B]Corresponding author. Email: philip.larkin@csiro.au

Abstract. Research is reported probing the concern of some wheat producers that grazing of early sown, dual-purpose wheat for winter forage may accentuate the spread of *Wheat streak mosaic virus* (WSMV). In experiments with housed sheep, we investigated whether there were any grounds for this concern. In the first experiment, sheep were allowed to graze heavily virus-infected wheat in trays, followed over a period of 24 h by a series of test trays of healthy wheat. The grazed plants were allowed to recover and new leaves were tested for symptoms and the presence of virus. In total, 2352 test plants were negative for WSMV, assessed through symptoms, ELISA, and RT-PCR. In the second experiment, no WSMV particles (assayed with ELISA) or RNA (assayed by RT-PCR) were detected in any saliva samples collected from sheep 0.5, 7.5, and 24.5 h after being fed heavily virus-infected wheat. Furthermore, these saliva samples, when inoculated onto test wheat seedlings under optimal conditions, failed to transmit the virus. In a third experiment we showed that the urea concentration in sheep saliva is at least two orders of magnitude lower than that required to render WSMV non-infective, and therefore is not responsible for the failure of sheep to transmit the virus. Our data provide no support for the suggestion that grazing sheep spread the WSMV between plants in a grazed wheat crop as a consequence of the grazing process itself.

Introduction

Longer-season, dual-purpose wheats have been a profitable addition to mixed farming systems in southern Australia, because they allow an income stream from improved winter grazing, without major sacrifice of income from grain production (Virgona *et al.* 2006; Kelman and Dove 2007). The advent of *Wheat streak mosaic virus* (WSMV) in crops in southern Australia led to some apprehension about the use of dual-purpose wheats, because for optimum use as a grazing resource, this crop has to be sown earlier than 'grain-only' wheat, and some survey data in New South Wales (NSW) (Murray *et al.* 2005) suggested that earlier sowing was associated with greater incidence of WSMV. For example, grain yield losses of up to 100% occurred in some early-sown and grazed fields in 2005. Total losses were valued at more than A$2 million (Murray *et al.* 2005) and this caused some producers to reconsider their use of dual-purpose wheats, which are otherwise valued for their provision of forage of high nutritive value and their high grain-yield potential after grazing. There are precedents for some pasture viruses being spread by grazing and trampling of livestock. McKirdy *et al.* (1998) simulated grazing and trampling on clovers and demonstrated the spread of white clover mosaic and subterranean clover mottle viruses. The same authors also found that leaf extracts with these two viruses were still infective after 4 weeks of storage mixed with bovine saliva. The suggestion that WSMV is worse in grazed crops has not been adequately supported by objective data and in fact runs counter to the results of early experiments that found no increase in field transmission of this virus in grazed crops (Sill *et al.* 1954). These authors found that, for there to be any spread of the virus, the presence of the primary vector, the wheat curl mite (WCM; *Aceria tosichella*), was essential.

Reduced use of dual-purpose wheat because of the fear of WSMV indicates a need for fuller investigation of the role of grazing livestock as potential vectors of the disease. The grazing animal, if it is functioning as a secondary vector of the virus, would presumably do so by: transmitting the virus in saliva or on hooves that have trampled infected plants; assisting the spread of the mite within the crop; or causing physical damage to the crop (grazing, trampling), which renders it more susceptible to the mite or the virus. A recent summary of preliminary findings by Murray *et al.* (2007) suggested that mites might be carried throughout a crop on the mouth and/or hooves of sheep but that WSMV infectivity was greatly reduced in ovine saliva. Infectivity of virus in saliva was much lower (18%) than WSMV in either water or buffer (90+%) (Murray *et al.* 2006, 2007; and pers. comm.). However, even this reduced infectivity could potentially be of practical importance, especially at high stocking rates or for extended grazing periods, if grazing sheep are effective in mechanical inoculation of infective virus particles in the mouth or in saliva, to newly grazed, uninfected plants.

The possible role of such transmission is tested in this study, in which we report 3 experiments investigating the salivary transmission of WSMV, using viral detection techniques not available when early field studies (Sill *et al.* 1954) were conducted. In the first experiment, housed sheep were allowed to consume a meal of WSMV-infected wheat followed by a series

10.1071/CP09301 1836-0947/10/030247

of small meals of uninfected wheat. Our hypothesis was that this would permit the salivary transmission of virus to the uninfected wheat. In a second, similar experiment, housed sheep consumed a single meal of infected wheat and the infectivity of their saliva was monitored over the subsequent 24 h. Our hypothesis here was that there would be a time-related decline in infectivity of WSMV in saliva. The third experiment evaluated the possible role of urea, a normal component of ovine saliva, as a possible cause of reduced infectivity of WSMV in saliva. Extracts from wheat were mixed with urea solutions, using urea concentrations ranging from those known to denature viruses (Tremaine *et al.* 1982, 1983; Tremaine and Ronald 1985; Da Poian *et al.* 1995) to concentrations similar to ovine saliva.

Materials and methods

Our first 2 experiments involved housed sheep fed WSMV-infected or uninfected wheat plants. The third experiment was a laboratory study examining the response of WSMV to serial dilutions of urea in buffer. All housing, feeding, and experimental procedures to which the housed sheep were subjected received the prior written approval of the Animal Experimentation Committee of the CSIRO Divisions of Plant Industry and Entomology (Approval Certificate #106).

Preparation of WSMV inoculum and of WSMV-infected wheat

A WSMV inoculum was prepared by grinding infected tissue in a mortar and pestle at a 1 : 10 w/v ratio in 0.02 M potassium phosphate buffer (pH 7). The homogenate was filtered through 4 layers of Miracloth, and abrasive Celite (Johns-Manville, Analytical filter grade) was added at 2% w/v to the final volume of inoculum.

Seedlings of wheat cv. Sunbrook, growing in rows in seedling trays (30 by 60 cm) at 70 plants per tray, were inoculated at the 2–3 leaf stage with WSMV inoculum, using a spray-gun procedure previously shown to result in uniform and 100% infection. WSMV-infected trays were regularly monitored for characteristic WSMV symptoms, and at 21 days post-inoculation, the trays were shifted to the animal house for the sheep feeding.

Leaf samples were randomly collected from WSMV-inoculated trays and were assayed for WSMV through RT-PCR and ELISA.

Experiment 1

Experimental animals and their management

Ten days before the start of Expt 1, 8 Merino sheep aged 15 months and with a mean weight of 35 kg were taken from pasture and housed in individual pens with slatted floors, in an animal house with continuous fluorescent lighting. The sheep had never previously grazed wheat forage. During the pre-experimental phase, animals were offered a daily ration of 800 g (air-dry) lucerne chaff and allowed to become accustomed to the animal house conditions. Water was freely available at all times. Two days before the start of the experiment, the sheep were also offered small quantities of freshly cut wheat forage (virus free), so that they were accustomed to this feed.

On the morning on which Expt 1 started, saliva samples were taken from all sheep, commencing at 0900 hours, using sterile, domestic cotton swabs. Swabs were immediately transferred to individual plastic vials and stored on ice before being transferred to the laboratory for further analysis. At 0930 hours, 6 of the sheep were offered a meal consisting of 1 seedling tray of fresh wheat infected with WSMV as described above (70 plants per tray; plants 30-days old).

Two sheep, regarded as controls, were offered seedling trays of virus-free wheat of the same cultivar. All sheep readily consumed the fresh wheat, and seedling trays were removed from the pens as soon as the wheat had been 'grazed' to soil level. For some sheep this 'grazing' step took ~30 min.

Further seedling trays of virus-free wheat were then offered to both 'virus' and 'control' sheep at 0.5, 1, 2, 4, 8, 12, and 24 h after the initial feed was first offered (i.e. 1000, 1030, 1130 hours,. . .etc.). Each feeding was preceded by saliva sampling as described above.

Processing of plant samples

After being 'grazed' by sheep, the seedling trays of wheat were transferred to a glasshouse to allow recovery and re-growth and were regularly monitored for WSMV-specific symptoms. Twenty-one days post grazing, the youngest re-grown fully expanded leaves were collected within each tray; border rows were excluded from this sampling. Eight composite samples, representing 56 plants, were produced per tray, to allow us to analyse a large number of samples more efficiently. The full analysis involving 8 sheep, each fed 7 trays over the different time intervals, with each tray consisting of 8 tested rows and each row a composite of 7 plants, resulted in a total of 3136 tested plants, 2352 of which had been grazed by sheep exposed to infected wheat.

Crude virus extracts from WSMV-infected plants were prepared by grinding leaf at 1 : 1 w/v with phosphate-buffered saline pH 7.0 (PBS) and were heated to 95, 85, 75, 65, or 55°C for 10 min, to choose the best temperature for inactivation of PCR inhibitors and disruption of WSMV coat protein, without perturbing the integrity of WMSV RNA. Extracts from WSMV-infected wheat plants were centrifuged at 13000 rpm for 30 s and the supernatant serially diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} of their original concentration. Ten µL of each dilution was then treated at the optimum temperature (85°C) for 10 min before subjecting it to RT-PCR.

Processing of saliva samples

To test for the presence of virus, saliva samples on cotton buds were transferred to 600-µL tubes in the base of which a small hole had been made using a hot needle. The tubes were placed in turn into 1.5-mL centrifuge tubes and 20 µL of sterile deionised, diethylpyrocarbonate-treated water (DEPC-H_2O) was added before spinning the tube assembly at 13 000 rpm for 2 min, so that the liquid was collected at the bottom of the centrifuge tube. The mixture was divided into 2 aliquots: 10 µL of mixture was removed and stored at −20°C to check for the presence of WSMV using RT-PCR, while the rest (approx. 90 µL) was stored at −20°C for further analysis by ELISA (Clark and Adams 1977).

To prepare viral RNA for the molecular assay, the samples were heated at 85°C for 10 min to disrupt the virus capsid and release RNA before the reverse transcription step.

RT-PCR reaction

One-step reverse transcription (RT) reactions were performed using the Qiagen onestep RT PCR kit. Primers *Nla*F1 (5554) 5′-CTGGACCGATCGGATTAAGA-3′ (forward) and *Nla*R1 (5895) 5′-ACTTGGTTTGGCTTGTGAGG-3′ (reverse) were designed to amplify 341 bp of the *Nla* region of WSMV. The PCR conditions were: 50°C for 30 min, 95°C for 15 min, 40 cycles of 94°C (for 30 s, 58°C for 30 s, 72°C for 60 s) with a final extension at 72°C for 10 min. The amplified DNA was separated on 1% agarose and visualised with ethidium bromide and UV illumination.

Experiment 2

In the second experiment, housed sheep consumed a single meal of infected wheat and the infectivity of their saliva was monitored over the subsequent 24 h, to check whether there was a time-related decline in infectivity of WSMV in saliva. The presence of virus in saliva was monitored as RNA using RT-PCR, as intact particles using ELISA, and as infectious particles by immediately hand-inoculating saliva samples onto virus-free wheat test plants. Given the high sensitivity of the RT-PCR assay, particular care was taken to avoid cross-contamination; sterile gloves were worn when handling sheep and samples, and gloves were replaced between sheep and between time points.

Experimental animals and their management

Five young sheep of similar age and liveweight to those in Expt 1 were transferred to individual pens in the animal house, and were fed lucerne chaff (800 g/day air-dry) until they became accustomed to the conditions and to being handled.

At 0930 hours on the morning of the experiment, and before sheep were fed, 3 saliva samples were taken from each sheep using cotton swabs and processed as described below. Four of the animals were then offered WSMV-infected wheat seedlings growing in seedling trays, as described for Expt 1. The fifth sheep was offered uninfected wheat. When wheat seedlings had been 'grazed' to nearly level with the potting mix, seedling trays were removed and saliva samples (3 per sheep) were taken again (called the 0.5 h time point) and processed as described below. At 1000 hours, all sheep were given 800 g (air-dry) of lucerne chaff. Saliva samples (3 per sheep) were then taken at 1700 hours (7.5 h time point) and at 1000 hours the next morning (24.5 h time point).

Collection and analysis of saliva samples

Three saliva swabs were taken per sheep per time point.

(*a*) One swab was sprinkled with Celite and used directly to inoculate 3 test wheat seedlings at the 2–3 leaf stage (susceptible cv. BobWhite 26); after 1 h the leaves were rinsed with water. Cultivars Sunstar (Expts 1 and 3) and Bob White 26 (Expt 2) are equally susceptible to WSMV. Wheat plants which had been inoculated using the saliva samples were transferred to a glasshouse as in Expt 1. Positive controls consisted of wheat plants inoculated with 1/10 diluted crude extract from infected leaves, using cotton buds and Celite as above.

(*b*) The saliva from the second cotton bud was collected for RT-PCR as described previously by adding 20 µL of DEPC-treated water and centrifuging through a pinhole from a 600-µL tube into a 1.5-mL tube.

(*c*) The saliva from the third cotton bud was collected by the same centrifugation method but using 100 µL of ELISA extraction buffer and was used for DAS-ELISA assay (Clark and Adams 1977) following the manufacturer's directions (Agdia Inc.). The plates were coated with capture/coating antibody mixed at 1 : 200 with carbonate coating buffer (pH 9.6) and the plates were incubated at room temperature for 2 h. After incubation the plates were washed with PBS-Tween washing buffer (pH 7.4) 3 times at ~3-min intervals. Saliva samples, extracted from cotton buds with 100 µL of extraction buffer (pH 7.4), were added to the plates and incubated as described previously, followed by washing with PBS-Tween. Conjugate anti-WSMVAntibody (Agdia Inc.), coupled to alkaline phosphatase, was diluted 1 : 200 in Conjugate/ECL Buffer (pH 7.4) and added to the plate, followed by incubation and washing as described previously. Substrate *p*-nitrophenylphosphate (0.5 mg/mL in 10% diethanolamine, pH 9.8) was added to the wells and absorbance (A405 nm) measured 30 min and 60 min later with an ELISA plate reader. A sample was considered positive if its absorbance (A405) was more than double that of the healthy control sample.

The plants inoculated with saliva swab (*a*) (above), were grown for another 2 weeks before they were examined for symptoms and leaves extracted for ELISA. The extracts were 0.1 (w/v) in ELISA extraction buffer; 200 µL was placed in the coated ELISA plates and processed as described above.

Experiment 3

Urea solutions have been used to denature viruses (Stanley 1935; Bawden and Pirie 1940; Bawden 1954; McCarthy *et al.* 1980; Lakshmanan *et al.* 1985; Tremaine and Ronald 1985; Da Poian *et al.* 1995; Rao *et al.* 1995). Urea is also a normal constituent of ovine saliva, so Expt 3 was conducted to ascertain whether urea, at the concentrations normally found in sheep saliva, was sufficient to deactivate virus particles if they were present in saliva. Primary virus extracts were prepared by homogenising 1 g fresh weight of wheat leaf tissue in 10 mL of 0.02 M phosphate buffer, pH 7.0. A 10 M urea solution was then prepared by dissolving 6 g of urea in 10 mL of the same buffer. By serial dilution, further urea solutions of 5 M, 2 M, 1 M, 500 mM, 200 mM, 50 mM, 20 mM, 10 mM, 5 mM, and 2 mM were prepared in the same phosphate buffer.

From each of these solutions, 100-µL samples were taken and mixed with the same volume of primary virus extract, to give final urea concentrations in the mixtures of 5 M, 2.5 M, 1 M, 500 mM, 250 mM, 100 mM, 25 mM, 10 mM, 5 mM, 2.5 mM, and 1 mM. Solutions of 100 µL of virus extract and 100 µL of 0.02 M phosphate buffer were also prepared (+virus, –urea control), plus mixtures of phosphate buffer and urea solution to give final concentrations of 5 M, 1 M, and 100 µM urea in buffer (+urea, –virus controls). The samples were kept at room temperature for 2 h before being applied to plants.

Samples of all the above mixtures were then mechanically inoculated onto leaves of wheat plants (cv. Sunbrook) growing in potting mix in a glasshouse; 8 plants were inoculated per treatment, using cotton buds dipped in Celite. Plants were then allowed to grow for a further 2 weeks to allow virus replication

and symptom development. At that time, symptoms, if present, were scored on a scale of 0–4: 0, healthy; 1, mild with very few streaks; 2, moderate with streaks that coalesce; 3, severe with ~50 percent of leaf area showing streaks; and 4, the most severe or lethal symptoms where the streaks become chlorotic and cover more than 70% of leaf area. At 14 days post inoculation, leaf samples were collected (2 plants/treatment) from new leaves and the presence of virus determined using ELISA as described above.

Results

Experiment 1

In this experiment, both saliva samples and the extracts of wheat plants 'grazed' by the sheep were assayed by RT-PCR. The extreme sensitivity of this assay is evident in Fig. 1, which demonstrates that in a 10^{-8} dilution of extract from an infected plant, viral RNA could still be detected. The relatively poor amplification at the highest concentration (10^{-1}) was attributed to inhibition by components of the leaf extract. The level of sensitivity demonstrated in Fig. 1 is such that RT-PCR could readily have detected a single plant with WSMV infection at a level 1000 times less than our positive control plants.

Saliva samples collected before each meal from the 6 sheep consuming infected wheat, were negative for WSMV when assayed using DAS-ELISA; however, when assayed with RT-PCR, saliva samples were positive only at 24 h and only from 3 sheep. These few positive RT-PCR results may have resulted from residual infected wheat tissue in the mouth, either from the original meal of infected wheat or from regurgitation and rumination by the sheep. However, given the extreme sensitivity of the RT-PCR assay, it is also possible that some of the positive saliva samples could have been false positives. Experiment 2 specifically investigates the question of virus in saliva. Most importantly for Expt 1, of the 2352 virus-free wheat plants 'grazed' by the sheep over the 24 h after they had consumed heavily infected wheat, none became infected with WSMV or displayed any symptoms of infection. Figure 2 shows the RT-PCR results at one time point. The result at all time points was the same. It must be emphasised that although the first saliva sampling and the first 'meal' of uninfected wheat are described as 0.5 h, this 30-min interval was in fact the time between first offering the tray of infected wheat and offering the first tray of uninfected wheat. In reality, most sheep had only just finished eating the infected wheat when their saliva was sampled and they were offered uninfected wheat. The experiment thus mimics the field situation where a sheep can move directly from an infected to an uninfected plant.

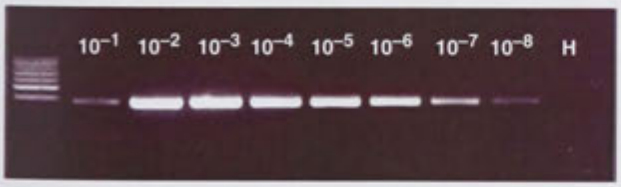


Fig. 1. RT-PCR of serial dilution of infected leaf extracts (Expt 1). RT-PCR reactions to detect WSMV RNA in a series of 10-fold serial dilutions of leaf extracts from a WSMV infected plant. The 10-fold serial dilution is shown over each track; H is the healthy extract control at 10^{-2}. A 341-bp product corresponding to a portion of the *Nla* region of the WSMV genome is evident even at 10^{-8}. Amplification at the highest concentration (10^{-1}) was relatively poor due to inhibition of the PCR reaction by components of the leaf extracts (unpubl. data).

Experiment 2

Experiment 2 was designed specifically to test the infectivity of saliva after sheep were fed infected wheat followed by different forage, with collection and monitoring of saliva over the subsequent 24 h. Saliva samples were assayed by both RT-PCR and ELISA. The infectivity of saliva was also checked through virus bioassay where saliva samples were inoculated directly onto healthy wheat test plants.

No WSMV coat protein (assayed by ELISA) or RNA (assayed by RT-PCR) was detected in any of the sheep saliva samples from Expt 2. Regardless of the sampling time before or after the consumption of a meal of WSMV-infected wheat, the ELISA results for the saliva of sheep which consumed infected wheat did not differ significantly from those for the sheep which consumed uninfected wheat (Fig. 3); results for positive and negative controls were as expected. Likewise, no viral RNA could be detected in any of the saliva samples by RT-PCR (Fig. 4); results for positive and negative controls were as expected.

When the extracts from the infected plants used in Expt 2 were used to inoculate healthy wheat plants, the presence of

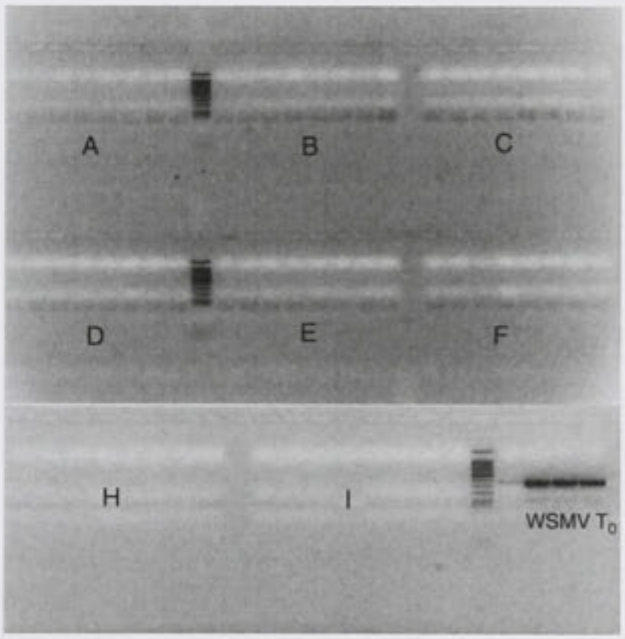


Fig. 2. RT-PCR of pooled leaf samples in Expt 1. The figure shows the RT-PCR products (inverted colour image) for the healthy plants grazed at one time point, namely 1 h after the sheep fed on WSMV infected plants. Sheep A–F were fed WSMV infected plants. Sheep H and I were fed healthy plants. Each track represents the pooling of a young leaf of 7 test plants after the recovery period. The 8 tracks per sheep represent the 56 plants analysed per sheep per time point. Molecular markers are shown between the 8 tracks for sheep A and B, and for sheep D and E, and after sheep I. The positive WSMV 341-bp amplicon is shown from random pooled samples from the WSMV infected tray at the zero time point, labelled WSMV T_0.

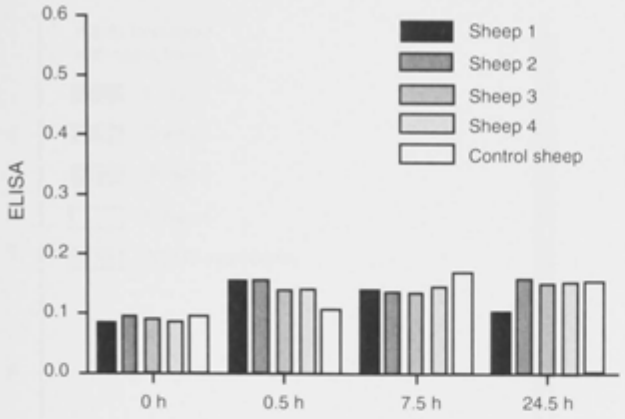


Fig. 3. Results of ELISA assay for WSMV in sheep saliva either before consuming the WSMV infected meal in Expt 2, or at 3 specific time periods afterwards (0.5, 7.5, and 24.5 h). Sheep 1–4 consumed an initial meal of WSMV-infected wheat; the control sheep was initially fed with healthy wheat. Shown are means of duplicate ELISA readings (A405). At each of the time points, differences between sheep 1–4 and the control sheep were not significant (one-way ANOVA, Friedman test).

WSMV was readily detected in extracts collected 2 weeks later (positive control in Fig. 5). By contrast, ELISA readings from the extracts of plants inoculated with saliva from sheep which consumed infected wheat, never differed significantly from those infected with saliva collected before sheep consumed the infected wheat, or from those infected with saliva from the sheep which consumed uninfected wheat (Fig. 5). These test plants were also devoid of virus symptoms up to 6 weeks after inoculation. These results indicate that saliva collected at 0.5, 7.5, and 24.5 h from sheep fed infected wheat, was not infective even under the optimal inoculation conditions attained using Cellite abrasive and careful manual inoculation of individual test plants.

Experiment 3

Experiment 3 was designed to test the effect of urea on the infectivity of WSMV, given that urea has been used to denature viruses and is a normal constituent of ovine saliva, and thus might explain the lack of infection noted in Expts 1 and 2. Leaf extracts from infected leaves were incubated in urea solutions at final concentrations of 1 mM to 5 M. The treated extracts were then manually inoculated onto young wheat plants. Urea-free controls confirmed the infectivity of the extracts and virus-free controls established that the urea concentrations were not deleterious to the leaves.

Mean ELISA readings for the extracts of plants inoculated with virus exposed to urea concentrations between 1 mM and 1 M did not differ significantly from each other, nor from the urea-free control (0 mM urea; Table 1). However, they all differed significantly from the mean of the virus-free controls. By contrast, ELISA readings for the 2.5 M and 5 M treatments were significantly different (reduced) from the 0 mM urea treatment ($P<0.001$) but not significantly different from the mean of the virus-free controls. The ELISA results thus show that virus was only deactivated by urea concentrations of 2.5 M and above. At concentrations typical of sheep saliva (1–5 mM) there was no deactivation.

The data for symptom development, assessed 2 weeks after inoculation with the treated extracts, suggest a more gradual effect of urea treatment (Fig. 6). Nevertheless, compared with the virus-free control, symptom development was significantly greater ($P<0.05$ to $P<0.001$) in all treatments except for the two highest urea treatments, which did not differ from the virus-free controls.

Discussion

In parts of south-eastern Australia in 2005, WSMV caused major yield losses in some wheat crops and some farmers perceived that the disease was more severe in crops which had been sown early and then grazed. One conceivable way in which grazing could exacerbate WSMV disease in a crop would be if the sheep functioned as a 'secondary vector', by spreading the virus from infected to uninfected wheat through the grazing process itself, particularly *via* the salivary route. Our data provide evidence that this is not a mechanism of WSMV spread.

In a small-scale trial, Murray *et al.* (2007) added WSMV to water, phosphate buffer solution, or ovine saliva and then inoculated uninfected plants with these mixtures. When plants were inoculated with WSMV in water or phosphate buffer, 95–100% of plants became infected and went on to display symptoms. However, the 'infectivity' of WSMV in ovine saliva was greatly reduced to only 18% (Murray *et al.* 2006, 2007). This is evidence suggesting that some component of saliva is inhibiting viral infectivity, but even this seemingly low level of infectivity could assume practical importance. From our estimates of daily herbage intake and the average

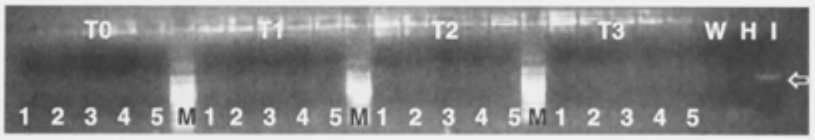


Fig. 4. RT-PCR detection of WSMV RNA in sheep saliva at various time points after feeding with WSMV infected wheat. Controls include: W, water; H, extract from healthy wheat; I, extract from infected wheat; M, molecular weight markers. The expected WSMV amplicon appears in track I (and marked with arrow). Shown are the reactions for saliva of 5 sheep per time point. T0 is sample before feeding; T1, T2, and T3 are samples at 0.5, 7.5, and 24.5 h after feeding. Each lane represents the saliva sample from one sheep, with the first 4 lanes in each time point (1–4) from sheep that fed on WSMV infected plants; and the fifth lane (5) for each time point is from the control sheep that had no exposure to WSMV infected wheat.

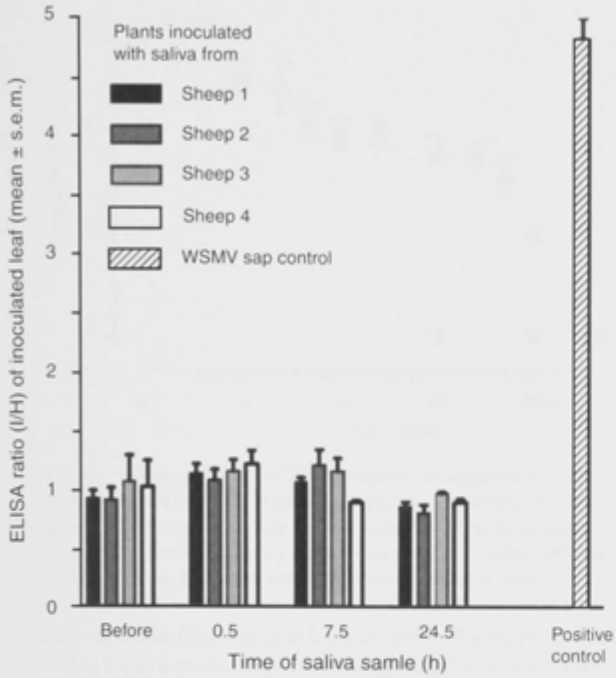


Fig. 5. Plants inoculated with saliva from sheep fed WSMV infected wheat in Expt 2. ELISA was conducted on 0.1 (w/v) extracts of the test plant leaves after 2 weeks from inoculation. The results are expressed as a ratio of ELISA readings at 14 days post inoculation from 3 inoculated plants per time point per sheep divided by mean ELISA readings of healthy plants. Plants were inoculated with saliva samples of sheep 1, 2, 3, and 4, which had been fed infected wheat. Control plants were inoculated with saliva from the control sheep 5, which had been fed healthy wheat. Saliva samples were collected at 4 time points as shown, before and after feeding with WSMV infected wheat plants. None of the control plants or test plants showed any symptoms, even after 28 days. The positive control is the mean of 4 plants inoculated with known infected extract. s.e.m. is standard error of the mean.

weight of a wheat plant (data not shown), we have calculated that even with an infectivity of only 18%, and in the absence of the WCM, the number of wheat plants bitten in a day by sheep grazing wheat at a typical grazing pressure of 25–35 animals/ha would be more than enough to result in 30–50% of the plants in the crop becoming infected over a typical grazing period of 30–40 days.

We therefore approached the assessment of possible salivary transmission in a different way, by allowing housed sheep to graze infected wheat followed by a series of meals of uninfected wheat (Expt 1), or by closely monitoring WSMV presence in sheep saliva, and its infectivity, over a 24-h period following a single meal of infected wheat (Expt 2). While RT-PCR assays on sheep saliva in Expt 1 were occasionally positive for WSMV, equivalent assays on extracts from previously healthy plants 'grazed' by the sheep showed no trace of WSMV (Fig. 2) and, during their regrowth under glasshouse conditions, none of these plants developed any WSMV symptoms. This was true even at the shortest time point, which was offering the test tray immediately after removal of the infected tray. In Expt 2, saliva from sheep fed a single meal of WSMV-infected wheat was assayed by both RT-PCR and ELISA, with WSMV not being detected from any of the sheep at any of the time points. When these same saliva samples were inoculated onto healthy wheat plants, ELISA also failed to detect WSMV in plant extracts 2 weeks after inoculation, and none of the inoculated plants displayed symptoms. In summary: virus coat protein could not be detected in saliva by ELISA in either Expt 1 or 2; viral RNA could only rarely be detected in saliva in only Expt 1; and virus infection could not be transferred from heavily infected plants to test plants either by the mouth parts of grazing sheep (Expt 1) or by manual inoculation using collected saliva (Expt 2).

Our results thus contrast with those obtained by McKirdy *et al.* (1998) with white clover mosaic and subterranean clover mottle viruses in cattle, by suggesting that WSMV is inactive in ovine saliva and that the grazing process itself does not result in cross-infection from infected to uninfected wheat plants. Our data, together with the earlier results of Murray *et al.* (2006, 2007), suggest the presence in saliva of one or more components that eliminate or greatly reduce the infectivity of WSMV, and Expt 3 was conducted to see if urea might be this component. Several studies with other plant viruses have demonstrated a decline in virus integrity and infectivity after incubation of virus in

Table 1. Results of ELISA assays of the extracts of test plants 2 weeks after they were inoculated with virus containing leaf extracts treated with urea at varying concentrations

Negative controls were virus-free urea solutions (Expt 3). $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; n.s., not significant

Final urea conc.	ELISA reading (s.e.m.)	*t*-test that value differs from 0 mM urea	*t*-test that value differs from mean of virus-free control
0 mM	0.482 (0.0401)	n.s.	10.10**
1 mM	0.438 (0.0830)	n.s.	4.36*
2.5 mM	0.398 (0.0391)	n.s.	8.22*
5 mM	0.444 (0.0522)	n.s.	7.05*
10 mM	0.552 (0.0901)	n.s.	5.27*
25 mM	0.586 (0.0626)	n.s.	8.14*
100 mM	0.512 (0.0771)	n.s.	5.65*
250 mM	0.377 (0.0655)	n.s.	4.59*
500 mM	0.451 (0.0772)	n.s.	4.85*
1 M	0.368 (0.0739)	n.s.	3.95*
2.5 M	0.090 (0.0092)	42.79***	n.s.
5 M	0.070 (0.0051)	80.05***	n.s.

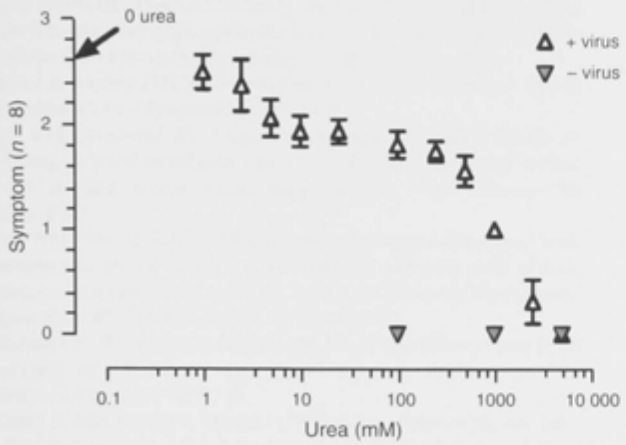


Fig. 6. Mean scores for WSMV symptom development at 14 days post inoculation (scale of 0–4) in susceptible plants inoculated with virus-infected extracts following incubation in urea solutions of varying concentrations (triangles), or buffer ('0 urea'), or virus-free urea solutions (inverted triangles) in Expt 3. Error bars show standard errors of means (8 plants).

urea solution (Stanley and Lauffer 1939; Tremaine and Ronald 1985; Da Poian *et al.* 1995), although usually only with solutions in the molar range of concentrations. Urea is a normal constituent of ovine and bovine saliva, functioning as a means of recycling nitrogen back to the rumen. However, normal salivary urea concentrations are much lower (<10 mM) than those typically used to denature plant viruses. Our results in Expt 3 clearly demonstrate that urea concentration had to be 2.5 M or above before it had any significant effect on WSMV infectivity, and at concentrations typical of ovine saliva, effects of urea did not differ significantly from the 'no urea' control. This suggests that, whatever the mechanism whereby WSMV is reduced or eliminated in sheep saliva, it is not mediated through any effect of urea.

Overall, our results have used the much greater sensitivity of modern assays such as RT-PCR and ELISA to confirm the results in an earlier report (Sill *et al.* 1954) that, in the absence of the primary WCM vector, grazing ruminants do not transmit WSMV by the oral route between grazed wheat plants. Our experiments did not address the issue of whether the mechanical damage to plants made them more susceptible to WSMV infection; however, Sill *et al.* (1954) conducted a large field experiment to address this question. They used large plots of infected and healthy plants and attempted to spread virus by leading cattle repeatedly between infected and healthy plots, or by driving a tractor 25 times between infected and healthy plots. They concluded that 'all data obtained indicate that grazing and trampling of livestock and moving of machinery are not involved in the transmission of this virus in the field'. The results of McKirdy *et al.* (1998) indicate that some pasture viruses can be spread through livestock trampling (McKirdy *et al.* 1998) and there is scope to revisit this 'trampling' question with WSMV. There is also the possibility that grazing animals might serve to spread the mite around the field and thereby indirectly increase the spread of WSMV. While it is conceivable that the WCM could be carried between plants on grazing animals, we suggest that this means of spread would be insignificant compared with the very effective and rapid spread of the mites by means of the wind (Thomas and Hein 2003).

In conclusion, our data provide no support for the suggestion that WSMV is spread between plants in a grazed wheat crop as a consequence of the grazing process by sheep. Despite this, anecdotal data remain that the disease is worse in grazed crops, but this may be because in order to be grazed, such crops have to be sown early in the season and thus experience greater exposure to the WCM as it 'over-summers' on alternate host plants (Hunger *et al.* 1992; Coutts *et al.* 2008). If this is indeed the mechanism, it in turn suggests that WSMV incidence in grazed crops could be better managed by the use of virus-resistant cultivars, by ensuring better removal of WCM host plants between harvest and the next sowing, and/or by better control of the mite itself. WSMV resistance is available by both natural (Haber *et al.* 2006*a*, 2006*b*, 2007; Harrison and Murray 2007; Seifers *et al.* 2007) and transgenic (Fahim *et al.* 2010) strategies, making the development of resistant cultivars achievable.

Acknowledgments

The work we report was financially supported by Australian grain growers, through a grant to HD and WMK by the Grains Research and Development Corporation. MF was supported by a postgraduate scholarship from AusAID. The animal house studies were designed and supervised by HD and WMK with assistance from the other co-authors, while viral assays and glasshouse work were conducted principally by MF. The manuscript was prepared by all authors after being initially drafted by MF and HD. The technical support of Mr Scott McDonald and the assistance of Dr Andrew Moore in the theoretical calculations of disease incidence in relation to level of infectivity are also gratefully acknowledged.

References

Bawden FC (1954) Inhibitors and plant viruses. *Advances in Virus Research* **2**, 31–57. doi:10.1016/S0065-3527(08)60528-X

Bawden FC, Pirie NW (1940) The inactivation of some plant viruses by urea. *Biochemical Journal* **34**, 1258–1277.

Clark MF, Adams AN (1977) Characteristics of microplate method of enzyme-linked immunosorbent assay for detection of plant-viruses. *Journal of General Virology* **34**, 475–483. doi:10.1099/0022-1317-34-3-475

Coutts BA, Strickland GR, Kehoe MA, Severtson DL, Jones RAC (2008) The epidemiology of *Wheat streak mosaic virus* in Australia: case histories, gradients, mite vectors, and alternative hosts. *Australian Journal of Agricultural Research* **59**, 844–853. doi:10.1071/AR07475

Da Poian AT, Oliveira AC, Silva JL (1995) Cold denaturation of an icosahedral virus – the role of entropy in virus assembly. *Biochemistry* **34**, 2672–2677. doi:10.1021/bi00008a034

Fahim M, Ayala-Navarrete L, Millar AA, Larkin PJ (2010) Hairpin RNA derived from viral *NIa* gene confers immunity to *Wheat streak mosaic virus* infection in transgenic wheat plants. *Plant Biotechnology Journal*, (in press).

Haber S, Gilbert J, Steinberg JG, Clarke J, Thomas J (2006*a*) Resistance to *Wheat streak mosaic virus* in durum wheat. *Canadian Journal of Plant Pathology – Revue Canadienne De Phytopathologie* **28**, 351–352.

Haber S, Pradhan M, Somers D (2007) Breaking the linkage between the *Wsm1* gene for resistance to *Wheat streak mosaic virus* and the alien chromatin of its origin. *Canadian Journal of Plant Pathology – Revue Canadienne De Phytopathologie* **29**, 215–216.

Haber S, Seifers DL, Thomas J (2006*b*) A new source of resistance to *Wheat streak mosaic virus* in spring wheat. *Canadian Journal of Plant Pathology – Revue Canadienne De Phytopathologie* **28**, 324–324.

Harrison LA, Murray TD (2007) Resistance to *Wheat streak mosaic virus* in perennial wheat. *Phytopathology* **97**, S168.

Hunger RM, Sherwood JL, Evans CK, Montana JR (1992) Effects of planting date and inoculation date on severity of *Wheat streak mosaic virus* in hard winter wheat-wheat cultivars. *Plant Disease* **76**, 1056–1060.

Kelman WM, Dove H (2007) Effects of a spring-sown brassica crop on lamb performance and on subsequent establishment and grain yield of dual-purpose winter wheat and oat crops. *Australian Journal of Experimental Agriculture* **47**, 815–824. doi:10.1071/EA06152

Lakshmanan P, Manoharan T, Jagannathan NT (1985) Effect of urea foliar spraying on rice tungro virus (RTV) infection. *International Rice Research Newsletter* **10**(2), 10.

McCarthy D, Bleichmann S, Thorne J (1980) Some effects of ph, salt, urea, ethanediol and sodium dodecyl-sulfate on tobacco necrosis virus. *Journal of General Virology* **46**, 391–404. doi:10.1099/0022-1317-46-2-391

McKirdy SJ, Jones RAC, Sivasithamparam K (1998) Determining the effectiveness of grazing and trampling by livestock in transmitting white clover mosaic and subterranean clover mottle viruses. *Annals of Applied Biology* **132**, 91–105. doi:10.1111/j.1744-7348.1998.tb05187.x

Murray GM, Hind-Lanoiselet T, Lanoiselet V, Wratten K (2006) Sap transmission of *Wheat streak mosaic virus*: Experiment by High School Students 2006. Technical Report from E.H. Graham Centre for Agricultural Innovation, Charles Sturt University Wagga Wagga and NSW Dept of Primary Industry.

Murray GM, Knihinicki D, Wratten K, Edwards J (2005) *Wheat streak mosaic virus* and the Leaf Curl Mite. NSW Department of Primary Industries *Primefact 99*. www.dpi.nsw.gov.au

Murray G, Simpfendorfer S, Hind-Lanioiselet T, Lanoiselet V, Wratten K (2007) Wheat streak mosaic: a threat to grazing and main season wheats that can be beaten. In 'GRDC Research Update Feburary 2007'. (Eds D Kaminskas, S Rawlings) pp. 63–69. (Grains Research and Development Corporation: Wagga Wagga, NSW)

Rao TN, Raja R, Zaidi AA (1995) Effect of foliar spray of urea and inoculation schedule on quantitative bioassay of carnation vein mottle virus. *New Agriculturist* **6**, 15–18.

Seifers DL, Martin TJ, Harvey TL, Haber S (2007) Temperature-sensitive *Wheat streak mosaic virus* resistance identified in KS03HW12 wheat. *Plant Disease* **91**, 1029–1033. doi:10.1094/PDIS-91-8-1029

Sill WH, Lowe AE, Bellingham RC, Fellows H (1954) Transmission of *Wheat streak mosaic virus* by abrasive leaf contacts during strong winds. *Plant Disease Reporter* **38**, 445–447.

Stanley WM (1935) Chemical studies on the virus of tobacco mosaic. IV. Some effects of different chemical agents on infectivity. *Phytopathology* **25**, 899–921.

Stanley WM, Lauffer MA (1939) Disintegration of *Tobacco mosaic virus* in urea solutions. *Science* **89**, 345–347. doi:10.1126/science.89.2311.345

Thomas JA, Hein GL (2003) Influence of volunteer wheat plant condition on movement of the wheat curl mite, *Aceria tosichella*, in winter wheat. *Experimental & Applied Acarology* **31**, 253–268. doi:10.1023/B:APPA.0000010384.12678.46

Tremaine JH, Ronald WP (1985) The effect of pH and some selected chemicals on the temperature-reversible aggregation of Carnation ringspot virus. *Phytopathology* **75**, 467–471. doi:10.1094/Phyto-75-467

Tremaine JH, Ronald WP, Mcgauley EM (1982) The effect of sodium dextran sulfate on some spherical plant-viruses. *Phytopathology* **72**(7), 954.

Tremaine JH, Ronald WP, Mcgauley EM (1983) Effect of sodium dextran sulfate on some isometric plant-viruses. *Phytopathology* **73**, 1241–1246. doi:10.1094/Phyto-73-1241

Virgona JM, Gummer FAJ, Angus JF (2006) Effects of grazing on wheat growth, yield, development, water use, and nitrogen use. *Australian Journal of Agricultural Research* **57**, 1307–1319. doi:10.1071/AR06085

Manuscript received 20 October 2009, accepted 23 December 2009

Chapter 3
Natural Resistance to WSMV
-Resources and Molecular Markers-

Authors' contribution

MF designed the experiments with assistance from PL and LAN
MF carried out all experimental work
MF wrote the paper
PL reviewed and edited the paper

 Doi: 10.1111/j.1365-3059.2011.02542.x

Resistance to *Wheat streak mosaic virus* – a survey of resources and development of molecular markers

M. Fahim[a,b,c], A. Mechanicos[a], L. Ayala-Navarrete[a], S. Haber[d] and P. J. Larkin[a]*

[a]*CSIRO Plant Industry, P.O. Box 1600 Canberra, ACT 2601, Australia;* [b]*Department of Microbiology, Hazara University, Mansehra, KPK, Pakistan;* [c]*Division of Plant Sciences, Research School of Biology, Australian National University, Canberra, ACT, Australia; and* [d]*Cereal Research Centre, Agriculture & Agri-Food Canada, Winnipeg, MB R3T 2M9, Canada*

Wheat streak mosaic virus (WSMV) has been newly documented in Australia. The vulnerability of contemporary Australian elite wheat germplasm prompted a survey for effective resistance against an Australian isolate, WSMV-ACT. This study confirms the effectiveness of previously reported sources of resistance and shows that new sources of resistance also confer protection. The resistance derived from *Thinopyrum intermedium* (*Wsm1*) as a 4D translocation and a new 4A translocation, and two bread wheat resistances, *Wsm2* and the new source c2652, were effective against WSMV-ACT in glasshouse experiments. *Wsm1* was effective at lower temperatures but ineffective above 20°C, a temperature sensitivity shared with many of the derivatives of *Wsm2* except for one new selection which was effective at 26°C. True wheats c2652 and *Wsm2* selection CA745, and amphiploids Zhong1, Zhong2, Zhong4, Zhong5, TAF46, Summer1, Ot38 and OK7211542 were uniformly resistant at 20, 25 and 28°C. New sources of resistance were identified in a *Th. scirpeum*-wheat amphiploid, B84-994, and in chromosome addition lines Z2, Z6 and TAi27, derived from wheat-*Th. intermedium* partial amphiploids. Several new, tightly linked SSR, RAPD and EST-ILP PCR markers were developed for tracking the various *Th. intermedium* translocations associated with *Wsm1*, including the smaller translocations on wheat chromosome 4AS and 4DS. Three markers for the 4A-*Wsm1* translocation were validated on a segregating breeding population.

Keywords: disease resistance, molecular markers, temperature sensitive resistance, *Tritimovirus*, wheat diseases, *Wheat streak mosaic virus*

Introduction

Wheat streak mosaic virus (WSMV: *Tritimovirus, Potyviridae*) is transmitted by the wheat curl mite (WCM) *Aceria tosichella* (Slykhuis, 1955). The presence of the virus was first confirmed in Australia in 2002. Evidence from molecular characterization and demonstration of seed transmission suggest WSMV was accidentally introduced to Australia in the 1980s (Ellis *et al.*, 2003; Dwyer *et al.*, 2007). The virus causes sporadic epidemics in the Great Plains of North America and is of great economic importance, causing severe losses in some years. In Australia, the virus has to-date remained a moderate threat to wheat production, although it has spread rapidly to all the wheat producing states. Both the virus and the leaf curl mite vector are present throughout the Australian wheatbelt. Where environmental conditions have favoured survival over summer and early infestation of crops by viruliferous mites, substantial losses have been observed. Should the combination of management practices and environmental conditions that favour the vector's transmission of the virus become widespread, major industry losses are possible.

Since wheat streak mosaic (WSM) disease was first reported (Mckinney, 1937), efforts have been made to find sources of resistance in cultivated wheat. Sources of resistance or tolerance in true wheat were slow to emerge, prompting efforts to explore resistance in perennial *Triticeae* relatives such as *Thinopyrum intermedium* ($2n = 6x = 42$, JJsS) and *Th. ponticum* ($2n = 10x = 70$, JJJJsJs) (Friebe *et al.*, 1993; Chen *et al.*, 2003). These two species have been valuable sources of resistance and tolerance to various biotic (both against WSMV and its vector WCM) and abiotic stresses and are relatively easy to cross with common and durum wheat (Larson & Atkinson, 1970).

The resistance called *Wsm1* was transferred to wheat initially as Robertsonian translocations 4Ai#2S.4AL and 4Ai#2S.4DL (Chen *et al.*, 1998a,b) and attempts were made to breed with it and deploy it as WSMV resistant varieties (Friebe *et al.*, 1996a; Baley *et al.*, 2001; Sharp *et al.*, 2002; Divis *et al.*, 2006). However, the initial linkage drag associated with *Wsm1* in the absence of WSMV infection affected the deployment of this resistance into the field (Friebe *et al.*, 1996b; Sharp *et al.*, 2002). To assist

*E-mail: philip.larkin@csiro.au

the development of useful recombinants and crossing of *Wsm1* into elite wheat germplasm, Talbert *et al.* (1996) developed a sequence-tagged site (STS) marker (STS-J15). Backcrossing and selection eventually succeeded in producing lines with *Wsm1* that did not appear to suffer yield penalties. This success, possibly due to recombination between 4DS and the alien chromatin (Divis *et al.*, 2006; Friebe *et al.*, 2009), has led to the release of the cultivar Mace (Graybosch *et al.*, 2009). The development of recombinant shortened *Wsm1* translocations and *Wsm1* translocations on 4A (Haber *et al.*, 2007; Qi *et al.*, 2007) has required new molecular markers to be developed.

More recently, there have been a small number of reports of resistance in conventional wheat lines, not involving alien translocations, such as Hume derived from the cultivar McKenzie (Haber *et al.*, 2011), c2652 (our unpublished data) and CO960293-2 (Haley *et al.*, 2002; Seifers *et al.*, 2006; Lu *et al.*, 2011b). The resistance in CO960293-2 (now called *Wsm2*) has been introgressed into two cultivars, RonL (Seifers *et al.*, 2007) and Snowmass (Haley *et al.*, 2011). With their deployment in cultivars, the few available resistances may be compromised or broken by the selective pressure on the virus, hence the need to find new resistances and deploy them in stacks.

This paper reports the effectiveness of known and new genetic resistances against the Australian isolate of WSMV and the stability of the resistances to increased growth temperatures. It also reports the discovery of new sources of genetic resistance in amphiploids and addition lines and the development of new, more closely-linked, molecular markers for *Wsm1* that should be useful in further refinement of *Wsm1* translocations, marker-assisted breeding, and resistance gene stacking.

Materials and methods

Plant material

Wheat and other cereals

Screening for resistance to WSMV-ACT isolate was carried out on the following: a collection of 53 wheats without reported resistance to WSMV; a collection of wheats with reported resistance including a number of accessions of these sources in different backgrounds from Canada, Nebraska and Kansas (Table 1, Fig. 2); and 42 wheat land races collected in the 1920s (Table 1, Fig. S1), kindly made available by Dr Harbans Bariana, University of Sydney. Bob White selection 26 (BW26) in particular was used as a susceptible control throughout this study.

Amphiploids and chromosome addition and substitution lines

The tertiary gene pool for wheat was also sampled for resistance by including a range of wheat-alien amphiploid hybrids, substitution and chromosome addition lines derived from some of them (Table 1).

Screening for resistance

Virus inoculum was prepared by grinding WSMV infected tissue (stored at −80°C) in 1:10 w/v ratio in 0·02 M potassium phosphate buffer (pH 7) in a Sorvall Omni Mixer (at 10 000 rpm). The virus inoculum used in all experiments was prepared from a WSMV infected wheat collected in Canberra (the Australian Capital Territory) and hereafter called the WSMV-ACT isolate. The homogenate was filtered through four layers of Miracloth® (Calbiochem), abrasive diatomaceous earth (celite; Johns-Manville) was added at 2% w/v to the final volume of inoculum, and the mixture was left on ice for 1 h. Germplasm was inoculated at the 2–3 leaf stage, with the prepared sap extracts from WSMV-infected leaf material. The sap plus celite abrasive was applied with an air-powered spray gun. A side mount gravity type Mini Spray Gun (Star S2F; Rich Star Precision Industrial) with adjustable 0·5–0·8 mm nozzle was used with a portable tank of compressed air (Fig. 1). Following spraying at 270 kPa, the leaves were also gently rubbed with gloved fingers.

In all experiments eight plants per genotype were used; six were inoculated and two were left uninoculated. The plants were scored for symptoms at 14 and 28 days post-inoculation (dpi) as described previously (Fahim *et al.*, 2010). Newly emerged leaf samples were collected at either 14 or 28 dpi for WSMV-specific ELISA using Agdia® reagents following manufacturer's instructions and extracted at a standard weight to extract ratio as detailed in Fahim *et al.* (2010). Plates were read at $A_{405\ nm}$ in ELISA Reader Spectra Max 340 PC (Molecular Devices) 60 min after addition of substrate. Every sample, inoculated and uninoculated healthy controls, had duplicate wells on the ELISA plate, and means were used in calculating the ELISA value ratio between inoculated and healthy controls.

Temperature sensitivity of resistance to the Australian isolate of WSMV

Lines that expressed resistance to WSMV in glasshouse experiments were evaluated for resistance against WSMV in controlled temperature growth cabinets. When screening for temperature sensitivity of the resistance, two sets of eight plants per genotype were used; one set in a cabinet at 18°C night, 20°C day temperature and the other set in a cabinet with 18°C night, 25 or 28°C day temperature. Each set of eight plants per genotype was grown in 10 cm pots and six of these were inoculated with WSMV at the three leaf stage; the remaining two plants were kept as uninoculated controls. Light conditions varied from 190 to 375 $\mu E\ m^{-2}\ s^{-1}$ depending upon experiment (Table 2) and the day length was set at 16 h, with 8 h dark. After inoculation plants were rated for symptoms at 7, 14, 21 and 28 dpi, and the youngest fully expanded leaf was harvested from each plant for ELISA analysis as described above.

Table 1 Germplasm screened for resistance against WSMV-ACT. Eight wheat plants per genotype were grown in a glasshouse, six inoculated at the three-leaf stage with *Wheat streak mosaic virus* (WSMV) and two left as healthy controls. Each experiment included susceptible BW26 controls. Germplasm with resistance was subsequently assessed at controlled temperatures. Resistance (R) is recorded only if resistance was confirmed in subsequent testing at 20°C

Germplasm	Source	Identifiers/accession	Description	WSMV	References/source
Wheat	Wheat cultivars and breeding lines		Annuello, Barian, Bolac, Brennan, Bob White26, Camm, Chara, Corella, CSP44, Currowong, Declic, Diamond Bird, Drysdale, EGA Gregory, Frame, GBA Ruby, Giles, H45, HD2009, WC03.87, WC03.008, WC03.1010.3, Janz, Jinan 177, Karl, Kellalac, Kennedy, Lalbahadur, Mackellar, Marombi, Myna, Pugsley, Romany, Rosella, Rudd, Silver Star, Sunvale, Sunbri, Sunstate, Super Seri, Tennant, Ventura, Vilmorin, Vl404, Wedgetail, WL711, Wylah, Xiaoyan 54, Xiaoyan 6, Yitpi, Young, Z1-sib	S	
	Wheat land races	Figure S1	42 wheat land races collected during 1920s	S	Harbans Bariana (Sydney University)
	c2652	CA716, CPI146018	Spring wheat selected from breeding line C2652	R	Steve Haber (AAFC, Winnipeg)
		CA742, Haber9379, CPI146896	Advanced resistant derivative of CA716	R	
Wheat (contd)	CO960293-2 (*Wsm2*) various	CA744 Original	PI222668/TAM 107//(NOVI SAD 14/NOVI SAD 603//NEWTON/3/PROBRAND 835_ CO850034). PI222668 is ex- Azerbaijan	R	Haley *et al.* (2002) Seifers *et al.* (2006, 2007) Lu *et al.* (2011b)
		CA717, AUS34288, PI615160	As above but sourced ex- AWCC Tamworth, Australia	R	
		CA743, Haber7760, CPI146635	Winter wheat '4th cycle' WSMV selection seed, CO960293-1-1-3gen	R	
		CA745, Haber9104, CPI146894	Spring wheat, Superb2*/CO960293-2 (BC1F9)	R	
		CA833, RonL, CPI147321	Hard white winter wheat (awned) KS03HW158, selected from the cross Trego/CO960293-2	R	
	Wsm1 various	CA739, KS93WGRC27	Pedigree CI17884/4* KARL, *Wsm1* translocation (4Ai#-2S.4DL) from intermedium 4Ai-2, CI17884 also contained *Ae. speltoides* 7S substituting for 7A	R	Gill *et al.* (1995)
		CA740, KS95H10-3	*Wsm1* translocation (4Ai#2S.4DL); an improved selection of KS93WGRC27	R	Dallas Seifers, Kansas State University
		CA741, Haber9376, CPI146895	Pai Toborochi/CI15091 F_{12} derivative, where CI15092 is a disomic substitution line 4Ai#2 (4A). Selection for resistance and against an J15 marker. Likely *Wsm1* translocation on 4AS (T4Ai#2S-4AS.4AL)	R	Haber *et al.* (2007)
		CA 837, Rec213, KS09WRGC51	Shorter *Wsm1* recombinant translocation, derived from KS93WGRC27. 4Ai#2S-4DS.4DL (where only 18% of S arm is alien)	R	Friebe *et al.* (2009)
		CA832, Mace, CPI147322	Winter wheat with *Wsm1*, bred from CI17884	R	Graybosch *et al.* (2009)

Table 1 (Continued)

Germplasm	Source	Identifiers/accession	Description	WSMV	References/source
Wheat-alien amphiploids and partial amphiploids	*Th. intermedium* derived	Zhong1	2*n* = 56 wheat/*Th. intermedium*	R	Qi *et al.* (1979) Sun (1981) Chen *et al.* (2003)
		Zhong2	2*n* = 56 wheat/*Th. intermedium*	R	
		Zhong4	2*n* = 56 wheat/*Th. intermedium*	R	
		Zhong5	2*n* = 56 wheat/*Th. intermedium*	R	
		TAF46	2*n* = 56 wheat/*Th. intermedium*	R	Cauderon (1966)
		Summer 1, CPI119107, CA830	2*n* = 56 wheat/*Th. intermedium*	R	NE Normal University, Changchun, China
		Otrastajuscaja 38, Ot38, CPI114085, CA596,	2*n* = 56, wheat/*Th. intermedium*. Produced in 1920–30s. Released in Russia in 1978 as a grain and fodder plant	R	Berezhnoi (1987)
	Th. ponticum derived	OK7211542, CPI114084, CA595	2*n* = 56 wheat/*Th. ponticum*	R	
	Th. scirpeum derived	B84-994, CPI109887, CA574	2*n* = 56 *T. turgidum*/*Th. scirpeum* (J1J1J2J2)	R	Mujeeb-Kazi & Miranda (1984)
	L. elongatum derived	CS-LE, CA731	2*n* = 56 Wheat/*Lophopyrum elongatum* amphiploid	R	Rana Munns, CSIRO
	Haynaldia villosa derived	CA657, CPI115741	2*n* = 42 T. durum/*Haynaldia villosa* amphiploid, from Nanjing Agricultural University	S	
Addition lines (2*n* = 44)	TAi Series	TAi11	TAI11: 2*n* = 44, ex Zhong2	S	He *et al.* (1989)
		TAi12	2*n* = 44, ex Zhong2	S	
		TAi14	2*n* = 42 + 2t, ex Zhong2	S	
		TAi15	2*n* = 44, ex Zhong2	S	
		TAi21	2*n* = 44, ex Zhong4. Pedigree Ken149 *2/Zhong4	S	
		TAi22	2*n* = 44, ex Zhong4. Pedigree Ken149 *2/Zhong4	S	
		TAi23	2*n* = 44, ex Zhong5. Pedigree Zhuocheng1 *2/Zhong5	S	
		TAi24	2*n* = 44, ex Zhong5. Pedigree Zhuocheng1 *2/Zhong5	S	
		TAi26	2*n* = 44, ex Zhong3. Pedigree 3B2 *2/Zhong3	S	
		TAi27	2*n* = 44, ex Zhong3. Pedigree 3B2 *2/Zhong3 (see Discussion)	R	Dong *et al.* (2004); Jiang *et al.* (2005); Gao *et al.* (1994)
	Z-Series	Z2	2*n* = 44, ex Zhong5	R	Larkin *et al.* (1995)
		Z3	2*n* = 44, ex Zhong5	S	
		Z4	2*n* = 44, ex Zhong5	S	
		Z5	2*n* = 44, ex Zhong5	S	
		Z6	2*n* = 44, ex Zhong5	R	
	L-Series	L1	2*n* = 44 ex-TAF46 group 7 addition	S	Cauderon (1966)
		L2	2*n* = 44 ex-TAF46 group 3 addition	S	
		L3	2*n* = 44 ex-TAF46 group 1 addition	S	
		L4	2*n* = 44 ex-TAF46 group 4 addition	S	
		L5	2*n* = 44 ex-TAF46 group 5 addition	R	
		L7	2*n* = 44 ex-TAF46 group 6 addition	S	
Substitution lines (2*n* = 42)		L1 (7A)	L1 substituting for 7A	S	Bob McIntosh, University of Sydney

Table 1 (Continued)

Germplasm	Source	Identifiers/accession	Description	WSMV	References/source
L1 (7B)	L1 substituting for 7B	S			
L1 (7D)	L1 substituting for 7D	S			
6E (6A)	6E (ex-*L. elongatum*) substituting for 6A	S			
6E (6B)	6E (ex-*L. elongatum*) substituting for 6B	S			
6E (6D)	6E (ex-*L. elongatum*) substituting for 6D	S			

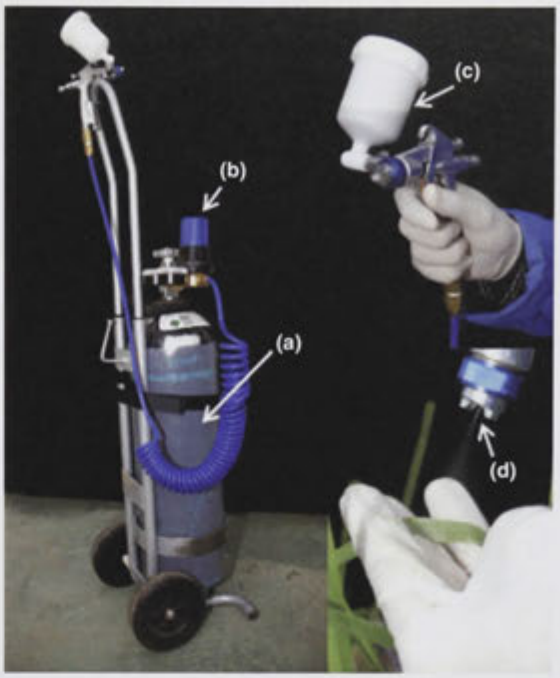


Figure 1 Mechanical inoculation system: spray gun with 0·8 mm nozzle. (a) air cylinder, (b) air pressure regulator at 40 psi, (c) container filled with infected sap and suspended celite shaken regularly to keep them suspended, (d) the nozzle and sap–celite spray is directed at leaf lamina. Celite acts as abrasive to assist in the infection process.

Molecular markers

New molecular markers were developed for the *Wsm1*-carrying translocations. Initial screening of potential markers was carried out on DNA obtained from *Th. intermedium* and Chinese Spring wheat. The polymorphic markers between wheat and *Th. intermedium* were tested on the following material: *Wsm1*-CA739 (4Ai#2S·4DL); *Wsm1*-CA740 (4Ai#2S·4DL); *Wsm1*-CA837 (Rec213, the shortest translocation on 4D; 4Ai#2S-4DS·4DL; Friebe *et al.*, 2009); *Wsm1*-Mace (Original 4Ai#2S·4DL with no yield penalty); and *Wsm1*-CA741 (4Ai#2S-4AS·4AL; Haber *et al.*, 2007). The controls included: *Th. intermedium*; wheats (Chinese Spring and Karl); Yi4212, a substitution of a group 2 *Th. intermedium* chromosome for 2D (Xin *et al.*, 2001); and Mackellar, a wheat carrying a group 7 *Th. intermedium* translocation on 7DL (Larkin *et al.*, 2002). A total of 120 primer pairs were designed and tested using the following approaches; all primers were ordered either from Sigma or Invitrogen.

Wild grasses derived ESTs and SSRs

The NCBI database was searched for expressed sequence tags (ESTs) and simple sequence repeats (SSRs) from the following *Triticeae* species: *Th. intermedium*, *Th. ponticum*, *Th. bessarabicum*, *Th. junceiforme*, *Th. caespitosum*, *Th. junceum*, *Agropyron cristatum*, *Lophopyrum elongatum*, *Pseudoroegneria*

Table 2 Temperature sensitivity of resistance in germplasm against *Wheat streak mosaic virus* (WSMV)

Resistance source/ germplasm	Accessions	Resistance at the following growth temperatures (°C) 20°C day, 18°C night 190 $\mu E\ m^{-2}\ s^{-1}$	25°C day, 18°C night 250 $\mu E\ m^{-2}\ s^{-1}$	28°C day, 18°C night 242 $\mu E\ m^{-2}\ s^{-1}$
c2652, $n = 42$	CA716, CA742	R	R	R
CO960293-2 (*Wsm2*), $n = 42$	CA717, CA744, CA743, RonL	R	S	S
	CA745	R	R	R
Translocation-*Wsm1*, $n = 42$	CA739, CA740, CA741, Rec213, Mace	R	S	S
Wheat/*Th. intermedium* partial amphiploids, $n = 56$	Zhong (1,2, 4, 5), TAF46, Summer 1, OT38	R	R	R
Wheat/*Th. ponticum* partial amphiploid, $n = 56$	OK7211542	R	R	R
T. turgidum/*Th. scirpeum* partial amphiploid, $n = 56$	B84-994	R	S	S
Wheat/*L. elongatum* partial amphiploid, $n = 56$	CS-LE	R	S	S
Th. intermedium addition lines to wheat	Z2, Z6, L5[a], TAi27	R	S	S

[a]L5 showed necrosis at 21 dpi, and at 28 dpi all plants were dead.

stipifolia, *Ps. spicata*, *Ps. strigosa*, *Elymus hispidus*, *Secale cereale* and *Avena vaviloviana*.

Wheat ESTs

As the original source of the *Th. intermedium* derived gene *Wsm1* is a compensating translocation 4Ai#2S·4DL, and *Wsm1* has since been associated with the telomeric region (Friebe *et al.*, 2009), wheat ESTs associated with the deletion bin 4DS2-0.82-1.00 were sought (Grain Gene 2·0; http://wheat.pw.usda.gov). A total of 49 primer pairs were designed based on ESTs mapped to deletion bin 4DS2-0.82-1.00. The ESTs were CoGe Blasted against rice and *Brachypodium* genomic sequences. The sequences were exported as FASTA files and aligned using AlignX (Vector NTI 10). Where alignment revealed the likely position of an intron, primers were designed to interspecific conserved exonic regions spanning at least one intron. This approach has previously been used for isolating polymorphism in genes and is known as intron-length polymorphism (ILP) or exon-primed intron-crossing PCR (EPIC-PCR) (Wang *et al.*, 2006).

Markers from literature

Markers reported in the literature to amplify sequences from the alien genomes were also tested as potential markers. These included: 3P3/3P4 (Wang & Wei, 1995); STSJ-15 (Talbert *et al.*, 1996); BG263898 (without restriction digestion in this study; Qi *et al.*, 2007); wheat resistance gene analogue (RGA)-ILPs (Shang *et al.*, 2010); and ILP markers to *Thinopyrum* TiERF1 (Liang *et al.*, 2008).

DNA extraction and marker assessment

DNA was extracted using a QIAGEN kit, according to manufacturer's instructions. Four microlitres of 10 ng μL^{-1} genomic DNA was amplified using the HotStartTaq® DNA polymerase and Master Mix buffer from QIAGEN following the manufacturer's guidelines. Amplification was performed in a ThermoHybaid PX2 or Corbette thermacycler as follows: one cycle of 15 min at 95°C; 35 cycles of 30 s at 94°C, 30 s at 52–65°C (depending on the individual pair of primers' temperature melting point, T_m) and 30 s at 72°C; and a final 5 min extension step at 72°C. Amplification products were separated on 1%, 2% or 3% agarose gels and visualized with ethidium bromide under UV light; where higher resolution was required products were separated by electrophoretic microchip (MultiNA; Shimadzu). Primers amplifying polymorphic sequences from *Thinopyrum* and wheat were then tested on lines derived from *Wsm1* carrying translocations. When a pair of primers amplified a *Thinopyrum* band from the translocation lines but not from the corresponding wheat line, the band was considered a dominant marker for the translocation. Likewise, if a pair of primers amplified a band in wheat but not in *Thinopyrum* it was

considered dominant for wheat. Co-dominant markers were those that gave a different size product on wheat and *Thinopyrum*.

Results

Resistance in common wheat

The inoculation method adopted achieved almost 100% of susceptible controls being infected and showing symptoms by 14 dpi. Almost all the common wheat accessions, including Australian and international cultivars and land races, were susceptible and developed characteristic wheat streak symptoms with attendant accumulation of virus as detected by ELISA. The results of many experiments are summarized in Table 1 (land race results in Fig. S1). The differences in early virus accumulation between susceptible cultivars and land races in one experiment were not reproducible and all were considered susceptible. The assessment is largely confined to substantial and reproducible resistance shown by greatly reduced symptoms and ELISA readings that do not differ significantly from background. All the true wheats examined, other than those with previously reported resistance (see below), were susceptible to the WSMV-ACT isolate and accumulated virus to a significant level by 14 dpi.

All lines previously reported to resist infection with North American isolates of the virus also succeeded in resisting the Australian WSMV-ACT isolate (Table 1, Fig. 2). Wheats with c2652 resistance and all five wheats carrying the CO960293-2 (*Wsm2*) resistance prevented systemic infection, as shown by the absence of symptoms and failure to accumulate virus in the youngest fully expanded leaf at 14 and 28 dpi (Table 1).

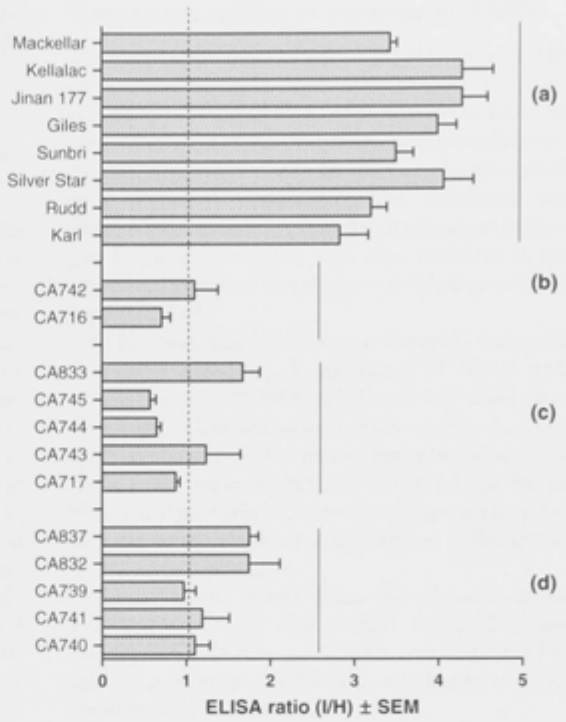


Figure 2 Resistance in wheat against WSMV-ACT. Eight plants per genotype were grown in the glasshouse. At the three leaf stage, six plants per genotype were inoculated and two plants were left as healthy controls. At 14 dpi, a newly emerged, fully expanded leaf was sampled for ELISA. $OD_{450\ nm}$ values for infected plants were divided by $OD_{450\ nm}$ values from healthy controls and presented as ELISA ratio ± SEM. (a) Selection of susceptible Australian wheat lines, (b) two accessions of c2652, (c) five accessions derived from CO960293-2, (d) five accessions derived from *Wsm1* translocation lines. The raw ELISA values show the group of varieties in (a) are significantly different to all the other genotypes at $P \leq 0.05$.

Resistance in wheat carrying the *Wsm1*-alien translocation

The *Wsm1* gene is derived originally as a Robertsonian translocation from *Th. intermedium* to wheat, 4Ai#2S.4DL. Subsequently some derivatives have had the alien portion shortened by recombination and can be symbolized as 4Ai#2S-4DS.4DL, where *Wsm1* appears to reside in the terminal 18% of the short arm (Friebe *et al.*, 2009). All lines carrying the *Wsm1* were also resistant to WSMV-ACT (Table 1, Fig. 2). This includes the shortened translocation rec213 (CA837) and Mace, an advanced *Wsm1* line recently released as a cultivar.

CA741-*Wsm1* is derived from the same *Th. intermedium* group 4 chromosome (4Ai#2) contributing *Wsm1*, but is an independently derived translocation to 4AS developed from the 4Ai#2(4A) substitution, CI15092 (Seifers *et al.*, 1995; Haber *et al.*, 2007). Evidence that it is the same group-4 chromosome from the hexaploid *Th. intermedium* comes from the amplification of the same 341-bp STS marker sequence from the 4A substitution line CI15092 and the 4D translocation (Talbert *et al.*, 1996). In accession CA741 this translocation appears to be smaller than the 4DS translocations and is no longer linked to the STS marker (Haber *et al.*, 2007 and later under molecular markers), but appears also to contain *Wsm1* as it protects against WSMV-ACT (Fig. 2). Being on 4A makes it a suitable source of resistance for introgression into durum wheat (AABB) (Haber *et al.*, 2007).

Resistance in amphiploids and partial amphiploids

The responses of various alien-wheat hybrids to inoculation with WSMV are given in Table 1. Wheat hybrids derived from *Th. intermedium* were highly resistant to WSMV-ACT, including $2n = 56$ partial amphiploids Zhong1, Zhong2, Zhong4, Zhong5, Summer1, TAF46 and Otrastajuscaja 38. The wheat/*Th. ponticum* partial amphiploid OK7211542, the wheat/*Agropyron scirpeum* (B84-994) and wheat/*Lophopyrum elongatum* amphiploids were also resistant. While some of these had been shown previously to be resistant to North American isolates (Chen *et al.*, 2003; Li *et al.*, 2004), this is the first report of the resistance of Summer1, the *scirpeum* and

elongatum amphiploids. The wheat/*Haynaldia villosa* amphiploid was not resistant when challenged with WSMV.

Resistance in chromosome addition and substitution lines

A total of 28 single chromosome alien addition lines ($2n = 44$) and substitution lines ($2n = 42$) were assessed for resistance. Several addition lines derived from *Th. intermedium* partial amphiploids ($2n = 56$) were resistant to the WSMV-ACT (Table 1). Addition line L5 (ex-TAF46) was shown previously to resist North American isolates of WSMV (Stoddard *et al.*, 1987) and it likewise resisted the Australian isolate, at least at low growth temperatures. However, the other addition lines in the L-series were uniformly susceptible to WSMV-ACT, including L2 which was previously reported to be resistant (Table 1, Fig. 3). Other addition lines Z2, Z6 (ex-Zhong5) and TAi27 (ex-Zhong3) are newly reported sources of resistance to WSMV. Z2 and Z6 have a group 2 *Th. intermedium* chromosome (Larkin *et al.*, 1995). TAi27 is a novel and complex $2n = 44$ addition line which appears to involve introgressions from a number of *Th. intermedium* chromosomes including from groups 2, 4, 7 and possibly others (Liu *et al.*, 2001; Dong *et al.*, 2004; our unpublished data), perhaps as recombinant chromosomes, perhaps as both an addition and a substitution (Han *et al.*, 1998). Line L5 has a 5J chromosome (Forster *et al.*, 1987; Chen *et al.*, 1999b). Therefore, it can be concluded that *Th. intermedium* has various resistances to WSMV at least on chromosomes of groups 2, 4 and 5.

Temperature stability of resistance to WSMV

The germplasm which showed WSMV resistance in glasshouse experiments in cool but not strictly controlled conditions was retested in growth chambers with different controlled temperature regimes. In all experiments where the day temperature was 18–20°C, only susceptible controls developed symptoms when inoculated with the virus. All the genotypes found to be resistant in the earlier glasshouse experiments were also resistant in the controlled temperature chambers at this low growth temperature (Table 2).

ELISA readings were taken at 4-weekly time points. As previously reported, all accessions of *Wsm1* and most accessions of CO960293-2 (*Wsm2*) lost resistance at the higher growth temperatures (Table 2, Fig. 4). The plants showed characteristic virus symptoms and accumulated virus, as shown by ELISA, at least by 14 dpi. In susceptible control germplasm, virus symptoms appeared as early as 5 dpi at the elevated temperatures, reflecting a faster rate of virus replication.

Similar to the *Wsm1* lines, the wheat/*L. elongatum* amphiploid CS-LE and durum wheat/*Th. scirpeum* amphiploid B84-994 were both resistant at 20°C, had delayed symptoms at 25°C (Fig. 4d), but were totally susceptible at 28°C (Table 2).

The resistance in addition lines Z2, Z6, L5 and TAi27 behaved in a similar fashion as *Wsm1* translocation lines, ceasing to be effective at 25 and 28°C (Table 2, Fig. 4d). The resistances in Z2, Z6 and L5 were already overcome at 25°C at 14 dpi, whereas TAi-27 was only overcome after 21 dpi. Despite the breakdown of resistance in the addition lines, they did delay symptoms and virus accu-

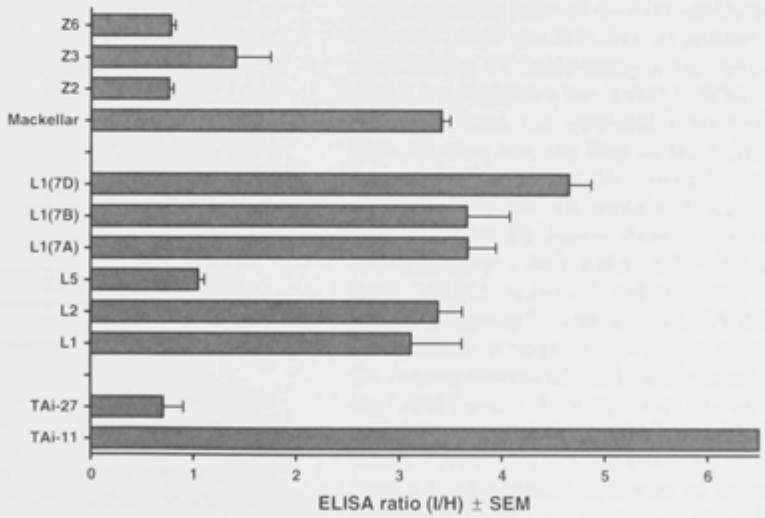


Figure 3 Resistance in Addition lines. Eight plants per genotype were grown in the glasshouse. At the three leaf stage, six plants per genotype were inoculated with WSMV and two plants left as healthy controls. At 14 dpi, a newly emerged, fully expanded leaf was sampled and ELISA performed. The apparent resistance by ELISA ratio of the lines Z6, Z2, L1 and TAi27 was confirmed by the total absence of symptoms in these lines and the development of symptoms in all the other lines.

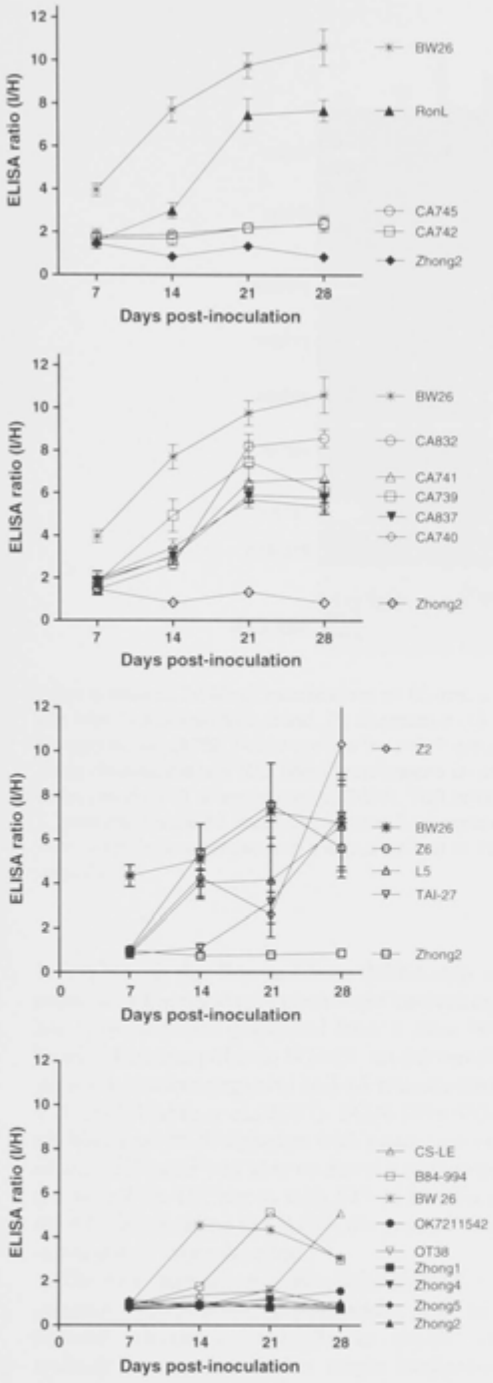


Figure 4 Temperature sensitive resistance in wheat. Germplasm with resistance against WMSV in the glasshouse was grown at controlled elevated temperature in a growth cabinet at 18°C night, 25°C day with 250 μE m^{-2} s^{-1} of light. Plants (n = 6) were inoculated at the three leaf stage. New expanded leaf samples were collected at 7, 14, 21 and 28 dpi and stored at −80°C until assayed together. Presented are the ELISA ratios (inoculated/healthy) at each time point. The first two plots are data from genotypes in one experiment; the third and fourth plots are from two further independent experiments.

mulation at the higher temperatures compared to the fully susceptible wheat control BW26.

The wheat lines c2652, CO960293-CA745 and amphiploids Zhong1, Zhong2, Zhong4, Zhong5, TAF46, Summer1, Ot38 and OK7211542 were uniformly resistant at all time points in all three temperature regimes: 20, 25 and 28°C (Table 2). One accession derived from CO960293-2 (*Wsm2*), namely CA745, was stably resistant at higher growth temperatures in a number of experiments; this interesting trait, distinguishing it from the other *Wsm2* derivatives, may reflect the *de novo* alteration of a trait's expression that can be identified and subsequently fixed with the iterative exposure to cycles of virus inoculation and selection over several generations (Seifers *et al.*, 2006; Haber *et al.*, 2011).

Development of molecular markers for *Wsm1* translocation

A total of 130 pairs of primers from various origins were initially tested using DNA from *Th. intermedium*, wheat and *Wsm1*–CA740. Included in this were 28 resistance gene analogue (RGA)-ILP markers developed by Shang *et al.* (2010), selected based on their location on 4A or 4D; none of these RGA primers detected any useful polymorphisms for *Wsm1* translocations. Nine markers in total were useful in differentiating the presence of some or all of the *Wsm1* carrying lines, the published STS-J15 (Talbert *et al.*, 1996), and BG263898-STS (Qi *et al.*, 2007) markers, and six new ones (Fig. 5, Table 3).

WSR2 amplified only from *Th. intermedium* and the 4A translocation CA741, but not from any of the wheats or 4D translocations, CA739, CA740, CA837 (rec213) or CA832 (Mace). WSR9 amplified *Th. intermedium* but not the wheats. The product from the 4A translocation (CA741) was of similar size to the *Th. intermedium* band. It also amplified from all the 4D translocations except the reduced CA837 (rec213), but the product size was much smaller (Fig. 5). Surprisingly it also amplified from the group 2 substitution line Yi4212. WSR11 amplified no bands in wheat, but amplified a band of the same size from all alien carrying lines including Yi4212 (group 2 substitution), and Mackellar (group 7 translocation); the exception was the 4A translocation, CA741. WSR17 amplified from *Th. intermedium*, the 4D *Wsm1* translocations, (except CA837), the 4A *Wsm1* translocation and from Yi4212 (group 2 substitution), but not from Mackellar (group 7 translocation). WSR65 is an EST-ILP co-dominant marker, amplifying a wheat band and a *Th. intermedium* band in all the 4D translocations including CA837 (rec213), but not from the 4A *Wsm1* translocation CA741. SCM4 amplified from all the *Wsm1* lines except CA837 (rec213). It also amplified from Yi4212 (group 2 substitution), but none of the wheats and not Mackellar. CL167 amplified only from the large *Wsm1* translocations and Yi4212 (group 2 substitution).

The previously reported marker STS-J15 (Talbert *et al.*, 1996) identified *Wsm1* translocations in various backgrounds with the exception of the 4A translocation

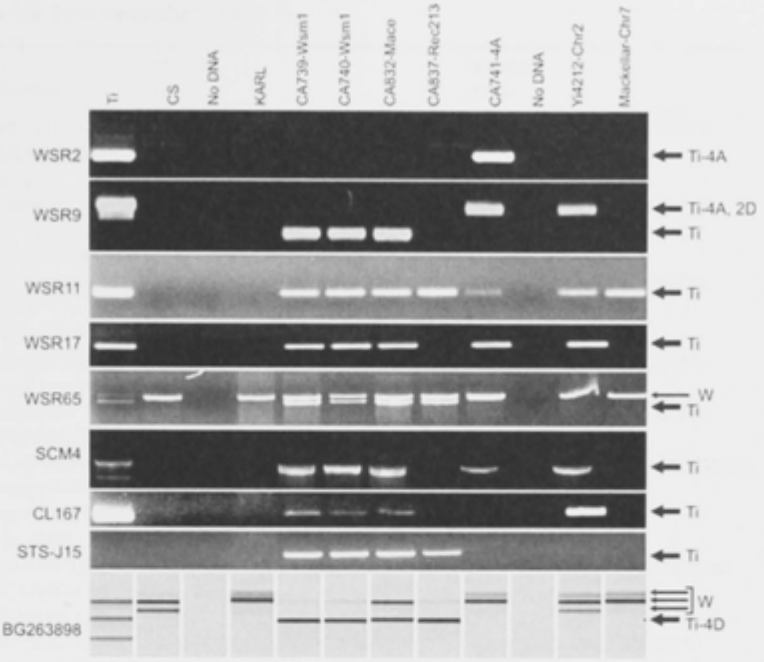


Figure 5 Markers for *Wsm1* translocations on 4D and/or 4A. Markers from various sources were used to detect polymorphisms associated with *Wsm1* translocations in wheat. *Th. intermedium* (Ti) and wheats Chinese Spring, Karl and Mackellar were used as controls. Karl was the background for CA739. Yi4212 is wheat line with Ti group 2 chromosome substituting for 2D. Mackellar is carrying a group 7 Ti fragment on wheat chromosome arm 7DL. *Wsm1* translocations on wheat 4D are represented by CA739, CA740, CA832, CA837, while the *Wsm1* translocation on 4A is represented by CA741. PCR products associated with some or all the tested Ti fragments are shown with an arrow and Ti; products associated specifically with the Ti 4A translocation or the 2D substitution, are notated as Ti-T4A or Ti-sub2D, respectively. Wheat specific products are shown with a light arrow and W. PCR product for marker BG263898 (last row) was resolved with MultiNA® for better resolution.

in CA741, as this line had been deliberately selected as expressing the resistance phenotype but lacking the marker in populations descended from a cross between Pai Toborichi (susceptible to WSMV, no *intermedium* translocation, marker-negative) and 4A translocation-bearing CI15092 (Haber *et al.*, 2007). BG263898-STS was used without post-PCR digestion with restriction enzyme (Qi *et al.*, 2007) and was able to detect a polymorphic band for *Wsm1* on 4D, but as with STS-J15, it did not detect the 4A translocation in CA741, the group 2 substitution, or the group 7 translocation.

The new markers may be useful in further efforts to produce recombinant translocations and for marker-assisted selections, especially to enable pyramiding multiple resistances into a single background. Most immediately pertinent is the fact that there are now markers that can follow the 4A translocation in CA741, which STS-J15 is unable to do.

Validation of new molecular markers for *Wsm1* translocation on wheat arm 4A in a breeding population

Some of the promising markers were tested on a population of 65 BC_1F_1 individuals from the breeding pedigree CA741/2* CW3.87, where CW3.87 is an elite breeder's genotype. A total of 65 plants were assessed for WSMV resistance and with markers WSR2, WSR9 and SCM4. Figure 6 shows that 24 segregants were positive for the 4A *Wsm1* molecular markers and all 24 were also resistant or moderately resistant to WSMV as indicated by lack of symptoms and low ELISA values. Of the 41 segregants without the markers, 40 were fully susceptible and one moderately susceptible. All tested individuals of parent CA741 were resistant, while the individuals of CW3.87 were susceptible (Fig. 6).

This confirmed the usefulness of the markers in following the apparently shortened 4A translocation in CA741 and that *Wsm1* expresses dominant resistance, effective in the heterozygotes. However, this experiment also indicated that the 4A translocation segregates in a manner distorted from the expected 1:1 ratio. In three other populations (two BC_1 populations in different backgrounds, and an F_2 population) assayed in the glasshouse at a different time of year, the presence of the alien marker corresponded almost perfectly (after virus inoculation at around the three leaf stage) with plant death at about the tillering or early elongation stage. The leaves from plants carrying molecular markers for CA741-4A translocation did not develop normal virus symptoms; instead the leaf

Table 3 Potential markers for some or all *Wsm1* translocations (see Fig. 5)

Marker	Source	Description	Forward primer	Reverse primer	Size (bp)	T_m
WSR2	EF174397.1	*R. fastigiata* isolate LMCB1 mRNA sequence	TAGCTCATACACGACAGCGG	GAGAGTGTGCAACTACCGCA	220 Ti	60
WSR9	U58022	*Ps. stipifolia* RAPD fragment Primer OPB08	GTTTCATGCAGATTGGCCTT	TGTTAGGTCGTCCGATAGGG	~320 Ti4A ~250 Ti4D	60
WSR11	U43516	*Th. bessarabicum* RAPD marker DNA	TCCCGGTACTTATCGAGGTG	CCGCAAGTCTTACTGCAACA	200 Ti	60
WSR17	AY618664	*Th. intermedium* repeat sequence	TACCAATGTCTTCAGCTGCG	ACTGCTCCTCCGTCTCAAAA	220 Ti	60
WSR65	BE443500_RC	Deletion Bin 4DS2-0.821.00	TGTTGTGACCAGTAGTGCTGC	CCTCAAAAGCTGCTACGACA	1300 Ti	60
SCM4	EF566899	*Pseudoroegneria spicata* RAPD sequence (Zhang *et al.* 2002)	GCCCTGCCATTGATCCCAAGCTG	TGGGCCAGGTCTTTCAGGTGACG	1300 Ti	60
CL167	BQ172287	2DL9-0.76-1.00; 2AL1-0.85-1.00; 2BL6-0.89-1.00	CGGAAGGACTTCATCATCATTTGT	CCTCTGCTGCTTCTCCTTCTCAG	300 Ti	66
STS-J15	RFLP Probe WG232/4L	*Wsm1* STS marker Talbert *et al.* (1996)	GTAGCAGGGGAAGCTGAAGA	CCGAGCTCACACGCTAATTT	341 Ti	50
BG263898-STS	BG263898	Ex- Qi *et al.* (2007) (without RE digestion)	TGCTCAATAAGAACTGGCAGAACG	GGAATCACAACTCAGGGGAAACAG	310 Ti	56

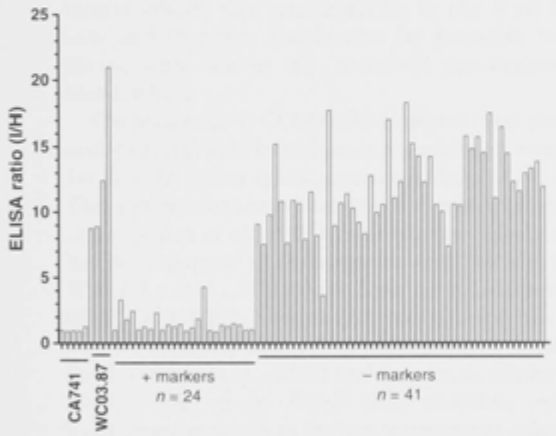


Figure 6 Segregation of resistance with molecular markers in *Wsm1*-4A population. Wheat lines segregating for *Wsm1* translocation on 4A (in accession CA741) were tested for resistance and the resistant phenotype was correlated with markers for the *Wsm1* translocation (WSR2, WSR9, SCM4) (see Fig. 5). The segregating population derived from CA741 was grown in the glasshouse along with resistant parent CA741 and susceptible parent WC03.087. At the three leaf stage plants were inoculated with WSMV-ACT isolate. At 14 dpi, a newly emerged, fully expanded leaf was sampled for ELISA. ELISA values for infected plants were divided by ELISA values from healthy controls (CA741 parental line in this case) and presented as an ELISA ratio.

lamina narrowed, followed by rapid and spreading leaf senescence, and plant death at 21–28 dpi. The phenomenon is suggestive of virus-induced plant hypersensitivity or programmed cell death, rather than the usual symptoms of WSMV. Circumstantial evidence suggests periods of elevated temperature can trigger the phenotype. In all these experiments the homozygous CA741 parent, with and without inoculation, was not dying.

Similar observations were made in Canada in F_6–F_9 generations during development of CA741; the hypersensitivity was selected out of homozygotes at F_{10} and seed made available for the Canberra experiments at F_{12}. During that development, resistance was selected by virus assay and the absence of the STS-J15 marker, and many of those individuals in each generation displayed necrotic leaves that led to plant death (Haber *et al.*, 2007; S. Haber, Agriculture and Agrifood Canada, unpublished data). The preliminary observations of this phenomenon can be summarized as follows: it is a senescence phenotype associated with heterozygosity of the 4A alien translocation, is triggered by the presence of virus, and may be accentuated by short periods of high temperatures.

Discussion

In susceptible common wheat and land race genotypes WSMV symptoms generally appeared at 4–7 dpi in summer and 8–10 dpi in winter in glasshouse experiments. Symptoms usually appear as light green and yellow streaking of the leaves, and as the disease progresses, the streaks coalesce, leading to general chlorosis of the leaf lamina and reduced photosynthetic efficiency. Susceptible plants accumulated moderate to high level of virus titre, displayed foliar symptoms and, as is characteristic

of severe WSMV infections, were stunted with shortened tillers lying prostrate. In the most severe cases, heads were shrivelled or failed to emerge and produced little or no seed.

Although all were susceptible to virus inoculation, some Australian wheats and international land races appeared to have a degree of tolerance as shown by plants growing to near-normal height. There has been no attempt to define this further in this present work. These lines would need to be studied in controlled yield trials involving WSMV inoculation to determine what contribution, if any, tolerance and/or partial resistance made in protecting yield under disease pressure.

This survey has confirmed that the genetic sources previously shown to resist North American isolates also resist the Australian WSMV-ACT isolate. Amongst the true wheats the most stable resistance was the c2652 source, which proved effective at higher growth temperatures. This resistance (Haber *et al.*, 2006) now appears to be an example of a *de novo*-evolved trait that became genetically fixed with repeated selection (Haber *et al.*, 2011; our unpublished data). It has been possible to introgress c2652 into both hexaploid and tetraploid germplasm by simple backcrossing and it was inherited in a manner consistent with that of a single dominant gene. Because c2652 resistance is easily introgressed into durum wheat, this gene must be in the A or B genome and therefore should also be amenable to combining with one of the *Wsm1*-4D translocations in bread wheat.

The resistance in CO960293-2 (*Wsm2*) was identified under natural field infections in a population being bred for Russian wheat aphid resistance (Haley *et al.*, 2002). The resistance has been characterized as temperature sensitive (Seifers *et al.*, 2006). Recently the resistance gene has been mapped to chromosome arm 3BS and named *Wsm2* (Lu *et al.*, 2011b) with flanking SSR markers identified. CO960293-2 was readily introgressed into wheat with no apparent yield penalty and released as cultivars RonL (Seifers *et al.*, 2007) and Snowmass (Haley *et al.*, 2011). Most of the *Wsm2* lines available, including RonL, were resistant in the low temperature cabinet but failed at higher temperatures. However, the CA745 derivative of CO960293-2, which had been iteratively selected in a regime that included exposure to elevated temperatures, continued to be effective even at 28°C. Since both CA745-*Wsm2* and c2652 were effective at elevated temperature, combining them could confer protection that should be particularly valuable for wheat in warmer zones. The location of *Wsm2* on 3BS ensures that it can be combined with *Wsm1* (either on 4A or 4D), but it remains to be determined whether it can be combined with c2652.

This study has also confirmed the effectiveness of the *Wsm1* translocations against the Australian isolate of WSMV, and confirmed that this resistance is temperature sensitive. The temperature sensitivity of *Wsm1* to North American isolates has been well documented (Friebe *et al.*, 1996a, 2009; Sharp *et al.*, 2002; Divis *et al.*, 2006). Friebe *et al.* (2009) have recently substantially reduced the size of the alien translocation in a recombinant called rec213. The original *Wsm1* translocation has been released in cultivar Mace in Nebraska (Graybosch *et al.*, 2009).

It is perhaps worth noting that even the temperature sensitive *Wsm1* and *Wsm2* lines appear to be effective in the field in Canberra (our unpublished data). The reason may be that during the critical times of the growing season, day temperatures only reach permissive levels for certain times of the day and are followed by long periods of non-permissive cool night temperatures (Seifers *et al.*, 1995).

Wild relatives of wheat, the so called tertiary gene pool, have been important sources for traits of agronomic importance, especially resistance to biotic and abiotic stresses (Jauhar & Peterson, 1996; Qi *et al.*, 2007). In order to become deployable in wheat improvement, a series of introgression steps are often required as follows: wheat-alien hybrids, full or partial amphiploids, chromosome additions, chromosome substitutions, and finally compensating translocation lines (Friebe *et al.*, 1991; Banks *et al.*, 1995; Luan *et al.*, 2010; Niu *et al.*, 2011).

Two species among these wild relatives, *Th. intermedium* and *Th. ponticum*, have previously displayed very high levels of resistance to WSMV and WCM (Fedak & Han, 2005). There was some evidence in *Th. ponticum* that the genes controlling resistance to WCM and WSMV are closely linked (Martin *et al.*, 1976) and therefore might be introgressed together, however even the WSMV resistance from *Th. ponticum* has proved to be complex (Hakizimana *et al.*, 2004).

All seven wheat-*Th. intermedium* amphiploids tested in the present study were highly resistant to WSMV-ACT at all tested temperatures. The resistance of this type of amphiploid has been noted previously (Chen *et al.*, 1998a). Zhong1 and Zhong2, produced by Qi *et al.* (1979) and characterized by Han *et al.* (2003), were reported resistant to both WSMV and its vector WCM (Chen *et al.*, 2003) and also highly resistant to *Barley yellow dwarf virus* (BYDV), leaf rust and stem rust (Banks *et al.*, 1993; Zhang *et al.*, 1996; Fedak *et al.*, 2001; Xin *et al.*, 2001). The diversity of resistances in the Zhong series adds to the justification for attempting to generate translocations to wheat.

All of the four Zhong lines tested in the present study showed high levels of resistance to WSMV-ACT (Table 1). The amphiploids or partial amphiploids with *Th. ponticum* (OK7211542), *Th. scirpeum* (B84-994) and *L. elongatum* (CS-LE) were all resistant to WSMV in glasshouse and lower controlled temperatures. B84-994 and CS-LE showed various degrees of instability at controlled elevated temperatures.

Screening chromosome addition lines ($2n = 44$) revealed new sources of WSMV resistance in Z2, Z6 and

TAi27 (this study), as well as confirming the already reported L5 (Stoddard *et al.*, 1987). The resistance in these addition lines was effective at low temperature and in the glasshouse experiments. Unlike the amphiploids from which they were derived, all these addition lines become susceptible at elevated temperature.

The alien chromosome present in Z2 and Z6 belongs to homoeologous group 2 (2Ai#2) and carries *Barley yellow dwarf virus* (BYDV) resistance (Larkin *et al.*, 1995; Tang *et al.*, 2000; Zhang *et al.*, 2000). At least some derivatives of Z2 have become substitution lines where wheat chromosome 2D was replaced by 2Ai#2 and was resistant to *Fusarium graminearum* (Han *et al.*, 2003). At least some accessions of Z6 ($2n = 44$) appear to have one pair of chromosomes derived from *Th. intermedium* plus another pair of translocated chromosomes involving B-genome chromosomes of wheat (Han *et al.*, 2003). In the present study resistance to WSMV-ACT was present in Z2 and Z6.

TAi27 is the third new addition line ($2n = 44$) identified with resistance to WSMV (this study). It is one of 14 in the TAi series involving *Th. intermedium* chromosomes added to wheat (He *et al.*, 1989). The literature suggests uncertainty whether TAi27 was derived from Zhong3 (Dong *et al.*, 2004), Zhong4 (Jiang *et al.*, 2005, 2009), or Zhong5 (Liu *et al.*, 2002). However, TAi27 is complex and appears to involve introgressions from a number of *Th. intermedium* chromosomes including from groups 2, 4, 7 and possibly others (Liu *et al.*, 2001; Dong *et al.*, 2004; our unpublished data), perhaps as recombinant chromosomes, or perhaps as both an addition and a substitution (Han *et al.*, 1998). Isozyme data implicated a *Th. intermedium* group 4 chromosome (Gao *et al.*, 1994). TAi27 has other useful traits such as BYDV resistance (Jiang *et al.*, 2009) and stem rust resistance (our unpublished data) which would help justify the effort required to generate translocations.

Stoddard *et al.* (1987) previously evaluated resistance in the L-series addition lines against WSMV and concluded that L2 carries intermediate resistance while L5 conferred stronger resistance at least at low temperature. In the experiments here L2 became readily infected with WSMV-ACT in the glasshouse, while L5 was resistant. L5 possesses a 5J chromosome (Forster *et al.*, 1987; Chen *et al.*, 1999a); Z2 and Z6 have a group 2 *Th. intermedium* chromosome (Larkin *et al.*, 1995); TAi27 has chromatin from at least groups 2 and 4, possibly others (Liu *et al.*, 2001; Dong *et al.*, 2004); *Wsm1* derives from a group 4 *Th. intermedium* chromosome. Therefore, it can be concluded that *Th. intermedium* has various resistances to WSMV at least on chromosomes of groups 2, 4 and 5. The cumulative effect of multiple genes in *Th. intermedium* and the derived amphiploids would explain their high level of resistance. This would also encourage ongoing efforts to render further wheatgrass WSMV resistance genes deployable and to pyramid multiple genes in cultivars.

The stability and strength of the WSMV resistance in the wheat/*Th. intermedium* partial amphiploids, and the observation that multiple genes on multiple chromosomes are involved, demonstrates the value of gene stacking in breeding for resistance to this serious disease. The general wisdom of resistance gene pyramids to increase the longevity of protection to agricultural diseases is well accepted (Ayliffe *et al.*, 2008).

Even before new alien sources of WSMV resistance become available, there are opportunities to pyramid resistance genes. Using molecular markers makes this much easier to accomplish. *Wsm1* is available on 4D or 4A, and CO960293-2 (*Wsm2*) is on 3B (Lu *et al.*, 2011b), allowing for their combination. This study has developed a number of new markers for the various *Wsm1* translocations; importantly this includes three simple markers for the previously unmarked CA741 version of *Wsm1* on chromosome 4A. Although the flanking markers to *Wsm2* in Lu *et al.* (2011b) are not close enough to be safely used in marker assisted selection, more closely linked markers are now available (Lu *et al.*, 2011a). Furthermore this study has shown that the CA745 derivative of CO960293-2 consistently performed well at elevated temperatures, as did c2652, making them attractive for warmer agroclimatic wheat zones. Even without markers for the heat stable resistances, it might be possible to combine them with *Wsm1*; segregants with both might be distinguished by the presence of a *Wsm1* marker and the presence of heat stable resistance. Despite that theoretical possibility, geneticists and breeders concerned with this disease will welcome the development of tightly linked molecular markers for both c2652 and *Wsm2*.

Acknowledgements

We are thankful to Dr Bob Furbank (CSIRO) for providing controlled climate growth cabinet for the temperature sensitive experiment, to Dr Garry Rosewarne (CSIRO) for providing seed of various Australia wheats, Dr Harbans Bariana (University of Sydney) for generously sharing the land races; and Jenny Gibson (CSIRO) for excellent technical assistance. The first author thankfully acknowledges AusAID for financial assistance as a PhD Studentship.

References

Ayliffe M, Singh R, Lagudah E, 2008. Durable resistance to wheat stem rust needed. *Current Opinion in Plant Biology* **11**, 187–92.

Baley GJ, Talbert LE, Martin JM *et al.*, 2001. Agronomic and end-use qualities of *Wheat streak mosaic virus* resistant spring wheat. *Crop Science* **41**, 1779–84.

Banks PM, Xu SJ, Wang RRC, Larkin PJ, 1993. Varying chromosome composition of 56-chromosome

wheat × *Thinopyrum intermedium* partial amphiploids. *Genome* **36**, 207–15.

Banks PM, Larkin PJ, Bariana HS *et al.*, 1995. The use of cell culture for sub-chromosomal introgressions of *Barley yellow dwarf virus* resistance from *Thinopyrum intermedium* to wheat. *Genome* **38**, 395–405.

Berezhnoi P, 1987. Development of the ideas of N. I. Vavilov on distant hybridization in wheat breeding. *Selektsiya i Semenovodstvo, Moscow* **6**, 49–52.

Cauderon Y, 1966. Cytogenic study of material resulting from a cross between *Triticum aestivum* and *Agropyron intermedium*. I. Creation of stable addition lines. *Annales de L'Amélioration des Plantes* **16**, 43–70.

Chen Q, Conner RL, Ahmad F, Laroche A, Fedak G, Thomas JB, 1998a. Molecular characterization of the genome composition of partial amphiploids derived from *Triticum aestivum* × *Thinopyrum ponticum* and *T. aestivum* × *Th. intermedium* as sources of resistance to *Wheat streak mosaic virus* and its vector, *Aceria tosichella*. *Theoretical and Applied Genetics* **97**, 1–8.

Chen Q, Friebe B, Conner RL, Laroche A, Thomas JB, Gill B, 1998b. Molecular cytogenetic characterization of *Thinopyrum intermedium*-derived wheat germplasm specifying resistance to *Wheat streak mosaic virus* and its vector, *Aceria tosichella*. *Theoretical and Applied Genetics* **96**, 1–7.

Chen Q, Conner RL, Laroche A, Fedak G, Thomas JB, 1999a. Genomic origins of *Thinopyrum* chromosomes specifying resistance to *Wheat streak mosaic virus* and its vector, *Aceria tosichella*. *Genome* **42**, 289–95.

Chen Q, Conner RL, Laroche A, Ji WQ, Armstrong KC, Fedak G, 1999b. Genomic in situ hybridization analysis of *Thinopyrum* chromatin in a wheat – *Th. intermedium* partial amphiploid and six derived chromosome addition lines. *Genome* **42**, 1217–23.

Chen Q, Conner RL, Li HJ *et al.*, 2003. Molecular cytogenetic discrimination and reaction to *Wheat streak mosaic virus* and the wheat curl mite in Zhong series of wheat – *Thinopyrum intermedium* partial amphiploids. *Genome* **46**, 135–45.

Divis LA, Graybosch RA, Peterson CJ *et al.*, 2006. Agronomic and quality effects in winter wheat of a gene conditioning resistance to *Wheat streak mosaic virus*. *Euphytica* **152**, 41–9.

Dong YS, Bu XL, Luan YS, He MY, Liu B, 2004. Molecular characterization of a cryptic wheat-*Thinopyrum intermedium* translocation line: evidence for genomic instability in nascent allopolyploid and aneuploid lines. *Genetics and Molecular Biology* **27**, 237–41.

Dwyer GI, Gibbs MJ, Gibbs AJ, Jones RAC, 2007. *Wheat streak mosaic virus* in Australia: relationship to isolates from the Pacific Northwest of the USA and its dispersion via seed transmission. *Plant Disease* **91**, 164–70.

Ellis MH, Rebetzke GJ, Mago R, Chu P, 2003. First report of *Wheat streak mosaic virus* in Australia. *Australasian Plant Pathology* **32**, 551–3.

Fahim M, Ayala-Navarrete L, Millar AA, Larkin PJ, 2010. Hairpin RNA derived from viral NIa gene confers immunity to *Wheat streak mosaic virus* infection in transgenic wheat plants. *Plant Biotechnology Journal* **8**, 821–34.

Fedak G, Han F, 2005. Characterization of derivatives from wheat-*Thinopyrum* wide crosses. *Cytogenetic and Genome Research* **109**, 360–7.

Fedak G, Chen Q, Conner RL, Laroche A, Comeau A, St-Pierre CA, 2001. Characterization of wheat-*Thinopyrum* partial amphiploids for resistance to barley yellow dwarf virus. *Euphytica* **120**, 373–8.

Forster BP, Reader SM, Forsyth SA *et al.*, 1987. An assessment of the homoeology of six *Agropyron intermedium* chromosomes added to wheat. *Genetical Research* **50**, 91–7.

Friebe B, Hatchett JH, Gill BS, Sebesta EE, 1991. Transfer of hessian fly resistance from rye to wheat via radiation-induced terminal and intercalary chromosomal translocations. *Theoretical and Applied Genetics* **83**, 33–40.

Friebe B, Jiang J, Gill BS, Dyck PL, 1993. Radiation-induced nonhomologous wheat *Agropyron intermedium* chromosomal translocations conferring resistance to leaf rust. *Theoretical and Applied Genetics* **86**, 141–9.

Friebe B, Gill KS, Tuleen NA, Gill BS, 1996a. Transfer of *Wheat streak mosaic virus* resistance from *Agropyron intermedium* into wheat. *Crop Science* **36**, 857–61.

Friebe B, Jiang J, Raupp WJ, Mcintosh RA, Gill BS, 1996b. Characterization of wheat-alien translocations conferring resistance to diseases and pests: current status. *Euphytica* **91**, 59–87.

Friebe B, Qi LL, Wilson DL *et al.*, 2009. Wheat-*Thinopyrum intermedium* recombinants resistant to *Wheat streak mosaic virus* and Triticum mosaic virus. *Crop Science* **49**, 1221–6.

Gao MJ, Bu XL, He MY, Hao S, 1994. Biochemical evidence of homoeology between wheat and wheatgrass chromosomes in Set II wheat-wheatgrass alien addition lines. *Acta Botanica Sinica* **36**, 79–85.

Gill BS, Friebe B, Wilson DL, Martin TJ, Cox TS, 1995. Registration of KS93WGRC27 wheat streak mosaic virus resistant T4DL.4Ai#2S wheat germplasm. *Crop Science* **35**, 1236–7.

Graybosch RA, Peterson CJ, Baenziger PS *et al.*, 2009. Registration of 'Mace' hard red winter wheat. *Journal of Plant Registrations* **3**, 51–6.

Haber S, Seifers DL, Thomas J, 2006. A new source of resistance to *Wheat streak mosaic virus* (WSMV) in spring wheat. *Canadian Journal of Plant Pathology* **28**, 324.

Haber S, Pradhan M, Somers D, 2007. Breaking the linkage between the *Wsm1* gene for resistance to *Wheat streak mosaic virus* and the alien chromatin of its origin. *Canadian Journal of Plant Pathology* **29**, 215–6.

Haber S, Gilbert J, Seifers DL, Comeau A, 2011. Epigenetics serves genetics: fusarium head blight (FHB) resistance in elite wheat germplasm. *The Americas Journal of Plant Science and Biotechnology* **5**, in press.

Hakizimana F, Ibrahim AMH, Langham MAC, Rudd JC, Haley SD, 2004. Generation means analysis of wheat streak mosaic virus resistance in winter wheat. *Euphytica* **139**, 133–9.

Haley SD, Martin TJ, Quick JS *et al.*, 2002. Registration of CO960293-2 wheat germplasm resistant to *Wheat streak mosaic virus* and Russian wheat aphid. *Crop Science* **42**, 1381–2.

Haley SD, Johnson JJ, Peairs FB *et al.*, 2011. Registration of 'Snowmass' wheat. *Journal of Plant Registrations* **5**, 87–90.

Han FP, Zhang XQ, Bu XL *et al.*, 1998. Variation of wheatgrass chromosomes in wheat-wheatgrass alien addition line "TAI-27" revealed by fluorescence in situ hybridization (FISH). *Science in China Series C – Life Sciences* **41**, 367–71.

Han FP, Fedak G, Benabdelmouna A, Armstrong K, Ouellet T, 2003. Characterization of six wheat × *Thinopyrum intermedium*

derivatives by GISH, RFLP, and multicolor GISH. *Genome* **46**, 490–5.

He MY, Xu ZY, Zou MQ *et al.*, 1989. The establishment of the two sets of alien addition lines of wheat-wheatgrass. *Scientia Sinica (Series B)* **32**, 695–705.

Jauhar PP, Peterson TS, 1996. *Thinopyrum* and *Lophopyrum* as sources of genes for wheat improvement. *Cereal Research Communications* **24**, 15–21.

Jiang SM, Hu J, Yin WB, Chen YH, Wang RRC, Hu ZM, 2005. Cloning of resistance gene analogs located on the alien chromosome in an addition line of wheat-*Thinopyrum intermedium*. *Theoretical and Applied Genetics* **111**, 923–31.

Jiang SM, Yin WB, Hu J *et al.*, 2009. Isolation of expressed sequences from a specific chromosome of *Thinopyrum intermedium* infected by BYDV. *Genome* **52**, 68–76.

Larkin PJ, Banks PM, Lagudah ES *et al.*, 1995. Disomic *Thinopyrum intermedium* addition lines in wheat with *Barley yellow dwarf virus* resistance and with rust resistances. *Genome* **38**, 385–94.

Larkin PJ, Kleven S, Banks P, 2002. Utilizing *Bdv2*, the *Thinopyrum intermedium* source of BYDV resistance, to develop wheat cultivars. In: Henry M, McNab A, eds. *Barley Yellow Dwarf Disease: Recent Advances and Future Strategies*. Mexico: CIMMYT, 60–3.

Larson R, Atkinson T, 1970. Identity of the wheat chromosome replaced by *Agropyron* chromosomes in a triple alien chromosome substitution line immune to wheat streak mosaic. *Canadian Journal of Genetics and Cytology* **12**, 145–50.

Li HJ, Conner RL, Chen Q *et al.*, 2004. Promising genetic resources for resistance to *Wheat streak mosaic virus* and the wheat curl mite in wheat-*Thinopyrum* partial amphiploids and their derivatives. *Genetic Resources and Crop Evolution* **51**, 827–35.

Liang HX, Lu Y, Liu HX, Wang FD, Xin ZY, Zhang ZY, 2008. A novel activator-type ERF of *Thinopyrum intermedium*, TiERF1, positively regulates defence responses. *Journal of Experimental Botany* **59**, 3111–20.

Liu B, Luan YS, Han FP, Ji WQ, He MY, 2001. Cell culture induced introgression of *Thinopyrum intermedium* chromatin into common wheat. *Plant Cell Tissue and Organ Culture* **65**, 9–13.

Liu WG, Zheng MY, Polle EA, Konzak CF, 2002. Highly efficient doubled-haploid production in wheat (*Triticum aestivum* L.) via induced microspore embryogenesis. *Crop Science* **42**, 686–92.

Lu HJ, Kottke R, Martin J, Bai G, Haley S, Rudd J, 2011a. Identification and validation of molecular markers for marker assisted selection of *Wsm2* in wheat. Plant and Animal Genomes XIX Conference, Abstract W433. [http://www.intl-pag.org/19/abstracts/W68_PAGXIX_433.html].

Lu HJ, Price J, Devkota R, Rush C, Rudd J, 2011b. A dominant gene for resistance to *Wheat streak mosaic virus* in winter wheat line CO960293-2. *Crop Science* **51**, 5–12.

Luan Y, Wang X, Liu W *et al.*, 2010. Production and identification of wheat-*Agropyron cristatum* 6P translocation lines. *Planta* **232**, 501–10.

Martin TJ, Harvey TL, Livers RW, 1976. Resistance to *Wheat streak mosaic virus* and its vector *Aceria tulipae*. *Phytopathology* **66**, 346–9.

Mckinney HH, 1937. Mosaic diseases of wheat and related cereals. *U.S. Department of Agriculture* Circular number **442**, 1–23.

Mujeeb-Kazi A, Miranda JL, 1984. High frequency of synthetic genome formation potentialities in backcross I selfed derivatives from some intergeneric hybrids involving *Triticum aestivum* and *Agropyron*. *Agronomy Abstracts* **76**, 80.

Niu Z, Klindworth DL, Friesen TL *et al.*, 2011. Targeted introgression of a wheat stem rust resistance gene by DNA marker-assisted chromosome engineering. *Genetics* **187**, 1011–21.

Qi SY, Yu SX, Zhang YH, Yu GH, Song FY, 1979. Studies on distant hybridization between spring wheat and *Agropyron glaucum*. *Scientia Agricultura Sinica* **2**, 1–11.

Qi LL, Friebe B, Zhang P, Gill BS, 2007. Homoeologous recombination, chromosome engineering and crop improvement. *Chromosome Research* **15**, 3–19.

Seifers DL, Martin TJ, Harvey TL, Gill BS, 1995. Temperature sensitivity and efficacy of *Wheat streak mosaic virus* resistance derived from *Agropyron intermedium*. *Plant Disease* **79**, 1104–6.

Seifers DL, Martin TJ, Harvey TL, Haber S, Haley SD, 2006. Temperature sensitivity and efficacy of *Wheat streak mosaic virus* resistance derived from CO960293 wheat. *Plant Disease* **90**, 623–8.

Seifers DL, Martin TJ, Harvey TL, Haber S, 2007. Temperature-sensitive *Wheat streak mosaic virus* resistance identified in KS03HW12 wheat. *Plant Disease* **91**, 1029–33.

Shang W, Zhou R, Jia J, Gao L, 2010. RGA-ILP, a new type of functional molecular markers in bread wheat. *Euphytica* **172**, 263–73.

Sharp GL, Martin JM, Lanning SP *et al.*, 2002. Field evaluation of transgenic and classical sources of *Wheat streak mosaic virus* resistance. *Crop Science* **42**, 105–10.

Slykhuis JT, 1955. *Aceria tulipae* Keifer (Acarina, Eriophyidae) in relation to the spread of wheat streak mosaic. *Phytopathology* **45**, 116–28.

Stoddard SL, Gill BS, Lommel SA, 1987. Genetic expression of *Wheat streak mosaic virus* resistance in two wheat-wheatgrass hybrids. *Crop Science* **27**, 514–9.

Sun S, 1981. The approach and methods of breeding new varieties and new species from *Agrotriticum* hybrids. *Acta Agronomica Sinica* **7**, 51–7.

Talbert LE, Bruckner PL, Smith LY, Sears R, Martin TJ, 1996. Development of PCR markers linked to resistance to *Wheat streak mosaic virus* in wheat. *Theoretical and Applied Genetics* **93**, 463–7.

Tang S, Li Z, Jia X, Larkin PJ, 2000. Genomic in situ hybridization (GISH) analyses of *Thinopyrum intermedium*, its partial amphiploid Zhong 5, and disease-resistant derivatives in wheat. *Theoretical and Applied Genetics* **100**, 344–52.

Wang RRC, Wei JZ, 1995. Variations of two repetitive DNA sequences in several Triticeae genomes revealed by polymerase chain reaction and sequencing. *Genome* **38**, 1221–9.

Wang X, Zhao X, Zhu J, Wu W, 2006. Genome-wide investigation of intron length polymorphisms and their potential as molecular markers in rice (*Oryza sativa* L.). *DNA Research* **12**, 417–27.

Xin ZY, Zhang ZY, Chen X *et al.*, 2001. Development and characterization of common wheat-*Thinopyrum intermedium* translocation lines with resistance to barley yellow dwarf virus. *Euphytica* **119**, 161–5.

Zhang XY, Dong YS, Wang RRC, 1996. Characterization of genomes and chromosomes in partial amphiploids of the hybrid

Triticum aestivum × *Thinopyrum ponticum* by *in situ* hybridization, isozyme analysis, and RAPD. *Genome* **39**, 1062–71.

Zhang ZY, Xin ZY, Lin ZS, Chen X, Wang XP, 2000. Identification of molecular markers for the *Thinopyrum intermedium* chromosome 2Ai-2 with resistance to barley yellow dwarf virus. *Acta Botanica Sinica* **42**, 1051–6.

Zhang ZY, Wang LL, Xin ZY, Lin ZS, 2002. Development of new PCR markers specific to a *Thinopyrum intermedium* chromosome 2Ai-2 and cloning of the St-specific sequences. *Journal of Genetics and Genomics* **29**, 627–33.

Supporting Information

Additional Supporting Information can be found in the online version of this article:

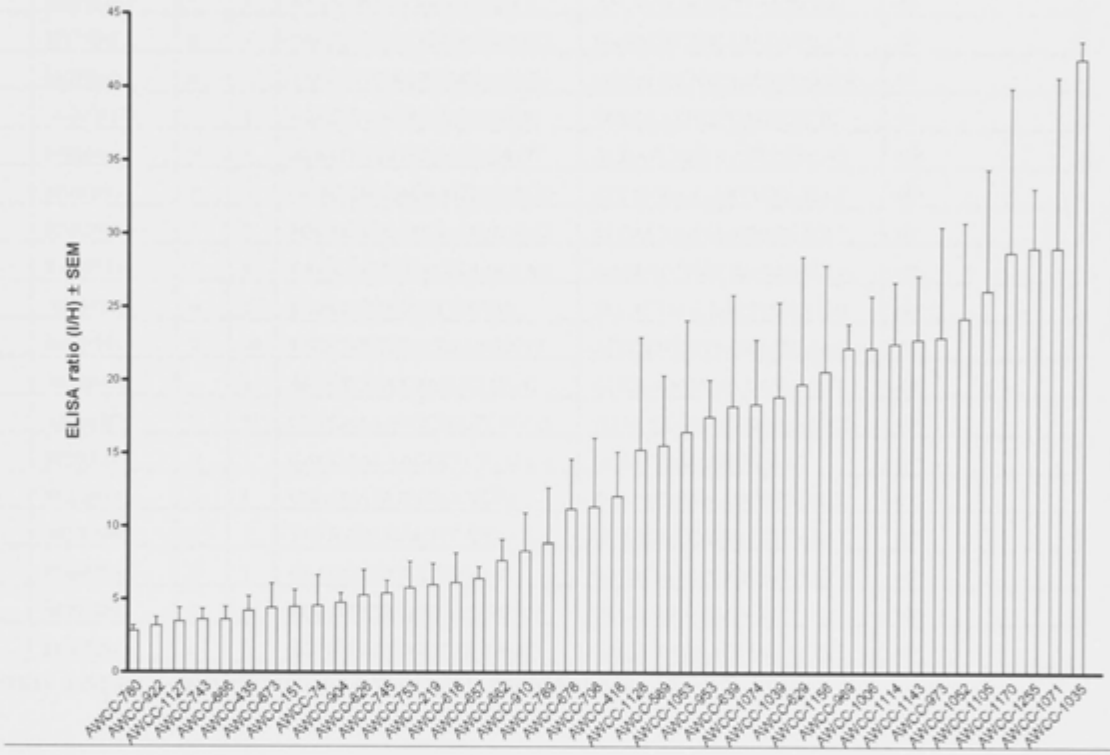
Figure S1. Reaction of land races to infection by WSMV-ACT isolate. Eight plants per genotype were grown in glasshouse. At three leaf stage, six plants per genotype were inoculated and two plants were left as healthy controls. At 14 dpi, newly emerged, fully expanded leaf was sampled for ELISA. ELISA values for infected plants were divided by ELISA values from healthy controls and presented as ELISA ratio ± SEM.

Table S1 Molecular markers amplifying diagnostic bands between *Thinopyrum intermedium* and wheat.

Table S2 Potential markers to detect polymorphism between wheat and *Thinopyrum intermedium*.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding authors for the article.

Suppl-Figure 1. Reaction of Land Races to infection by WSMV-ACT isolate. Eight plants per genotype were grown in glasshouse. At three leaf stage, six plants per genotype were inoculated and two plants were left as healthy controls. At 14dpi, newly emerged, fully expanded leaf was sampled for ELISA. ELISA values for infected plants were divided by ELISA values from healthy controls and presented as ELISA ratio ±SEM. Genotypes with ELISA ratio above 2 are considered as susceptible.



Supplementary Data (Chapter 3)

Supplementary Table : 1. Molecular markers amplifying diagnostic bands between *Th. intermedium* and wheat

WSR	Gene Bank	CS	Ai	Forward Primer (5'-------3')	Reverse Primer (3'-------5')	Melting Temperature
1	L02368	-	1	GTCTTCTGAATCTCCAGCGG	GTCAAGCAGACAGGCAAGC	60
19	DQ992032.1	-	1?	CCGACGAGAGTGTGCAATTA	TAGCTCATACACGACTGCGG	60
20	DQ992033	-	1	CCGCAGTCCTGTGTGAAGTA	CTTCTGAGGAGGCGAATCAC	60
21	EF014218.1	-	1	ATATATCGTCTTTGGCCCCC	ATGTCTCACGCTCACATTGC	60
22	EF174397.1	0	1	TAGCTCATACACGACAGCGG	GAGAGTGTGCAACTACCGCA	60
23	EF174405.1	0	1	TAGCTCATACACGACAGCGG	GAGAGTGTGCAACTACCGCA	60
26	ACU31172	-	1	GGCCGACCCGTCTTTAGTAT	CGCCCATTCTTGACTCTCTC	60
29	BE406462	0	1	TCACGTCGCACAAGAGATTC	ACGATCATGTCGTCCCAGAT	60*
33	BF482950	?	1	GGAGCTGGACAAGGTCTACG	CCCACAAATAATTGGCTGCT	60*
34	BF482950-aln	?	1	TGGACAAGGTCTACGACACG	CCCACAAATAATTGGCTGCT	60*
38	BE489555	0	1	CAAAATTCCCACAGCACCAG	GAACTTCTTGCAGAAGGCCA	60*
40	BF484144	0	1	CCAGGGTACGCTTCGTTG	TCCACTAGCAACTTCTGGCA	60*
43	BE627594	0	10	TGCTTCGTTTGCTAAATGCTT	ATCCTTGTCTGGGCATGAAG	60*
50	BG604678	-	1	ACCCTCCTCCACTGGTCAAT	GTCTCAAGCACCCGTCATCT	60*
52	BE404977	0	1?	GATCAAAAGGCTCGTGAGGA	TGAGTGGATATGGAAACCCC	60*
59	BF200816	0	1	GAGGAAGAAGGCTCTCACCA	GCACGCCAGGTTGAGC	60*
62	BF428613	0	1	CGGCGATATCGTGCTGTA	TCACTGATGCAGCGTAGACC	60*
63	BG262940	0	1	TTGTGTACGACTTCATGCCC	GCAAGGCCATTGTCTGAAAC	60*
64	BE405350	0	1	CAAGGCCATCGTGGAGG	GACAAGAGAGGAATTTCGCA	60*
67	BF292180	0	1	ACCTCTCTCGCCGTCTTCTC	GCCGCGGGTGAAGTT	60*
76	BE405592	0	1	TGTTGCATTTGTTGCCTAGC	CACCCCTGGAGTAGAGCTTG	60*

? may require confirmation on higher percentage 3%-5% gels.

Supplementary Data (Chapter 3)

Supplementary Table 2. : Potential markers to detect polymorphism between Wheat and *Thinopyrum intermedium*.

WSR	ACCESSION	Forward Primer (5'-------3')	Reverse Primer (3'-------5')	Melting Temperature
3	AY242388	TATCAATGGCCGGAGAAAAG	TGCATTTCTGCCAAAACATC	60
4	AY249525	ATCGCTGACCATTTTGAAGC	TTCGTTCAACACCGTGACAT	60
5	AY249524	AGTTGGCAAGAGGAGCTTCA	AGGCCAGAAGCATAAGCAAC	60
6	AF525279	TTGGGAAAACCACACTGTCC	ACCAGTGGTGCTGCTTTCTT	60
12	AY217117	GTGGGGAAGACCGAGAAGTT	AGGAGAAGGGGAATTTCAGC	60
14	EU520257	CTGACCTTTTTAGCAACGCC	AGGAGTGCTGCTACGTCCAT	60
24	Z12616	GGACCTTCTGGTTCGACAAC	GTACAACGACGACAAACGCA	60
25	AJ780952	ACTGGGTTGATAAAGCAGGC	TTTGCTCATATGGTTGAAAGGA	60
27	BV679213.1	GACCTAGTGG	GACCTAGTGG	36
28	EF570121.1	ACGGCGAGGATGCTGCTGAAC	GGTTGCTTTGCCGGATTGCTC	60
30	BE445831	GAATGGAGGGACACCATTTG	CCCACAATGCTGTGTTTGTC	60
36	BE489890	GTGATGCTGAGAGACGCCTT	ATTCGCTAGCCTCGGGTATG	60
37	BE489890	GGCTCAGCCTCCAGGTAAAC	TGCACCAGATTTAAGAGGCA	60
41	BF484144	CGAAGCATGGTCTGCTGATAAGGC	GCCTTATCAGCAGACCATGCTTCG	60
44	BE627594	GCCCTTGTCAAACTCGGACATG	CATGTCCGAGTTTGACAAGGGC	60
46	BF292015	TGGCCTTCGCGTGGGAGCACGAG	CTCGTGCTCCCACGCGAAGGCCA	60
47	BF482849	TTGAATCTGTCCTTTGTGAGGA	CCGGACTATTACGAGAAGTGTG	60
48	BF482849	AGCAAATGTGAGGGTGGAAA	AGGCTAGGCCAGTCACTGAA	60
49	BG604678	ACCCTCCTCCACTGGTCAAT	TTCAAGTGCAAGCCACAATC	60
51	BG262757	GGGATGCCAGGCAATTTAT	TAAGAGCATGATCACAGCCG	60
54	BE490281	ATTCTGGCGATGGTGTCAG	TAGGAGCCAAGGCAGTGATT	60
55	BE490281	GTCGTTGCTCCTCCTGAAAG	TGTGTCCAAAATCACAAGTAGCA	60
56	BE490281	TACAACTCCATCATGAAGTGCGACG	CGTCGCACTTCATGATGGAGTTGTA	60
60	BF428613	TTCCTCTCGCAGATTCTGGT	TATCTGCGCTTAGAGGCACC	60
61	BF428613	ACAGACGGATTGCTCAGGTT	CCCTCTTCAGGAAATAGCCA	60
68	BF292180_RC	TACAACCTCTCTCGCCGTCT	TAAGACATCGATGCACCAGC	60
73	BF478437	GCCCTTTGTCTCTCTCGTGT	AAGATACCGAAGCTGCCCTT	60
74	BF478437	CCCTTGAAATCTTGTTGAGTGA	TTGCACATTCTCTGGCAGTC	60
75	BF478437	CATGGTTCTGAATTACATCGACA	CAACGAATGTCTTCTGGCCT	60
77	BE405592	AGTACCCACCGGTGAACTTG	AGTCCACACCGTTCATCGAC	60

_RC indicate the Reverse complement of ETS from wheat deletion bin was used to design the primers.

Chapter 4
Yield protection against WSMV

Authors' contribution

MF designed the experiments with assistance from GR
MF carried out all experimental work
MF wrote the paper
PL and GR reviewed and edited the paper

Effectiveness of three potential sources of resistance in wheat against *Wheat streak mosaic virus* under field conditions

Journal:	*Plant Breeding*
Manuscript ID:	PLBR-11-OA-257.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Fahim, Muhammad; CSIRO, Plant Industry Larkin, Philip; CSIRO, Plant Industry Haber, Steve; Agriculture and Agri-Food Canada, Cereal Research Centre Shorter, Steve; Plant and Food Research Lonergan, Paul; CSIRO, Plant Industry Rosewarne, Garry; CSIRO, Plant Industry
Keywords:	Wheat streak mosaic virus, resistance, Wsm1, Wsm2, CO960293-2

Effectiveness of three potential sources of resistance in wheat against *Wheat streak mosaic virus* under field conditions

Muhammad Fahim[1,2,3], Philip Larkin[1], Steve Haber[4], Steve Shorter[5], Paul Lonergan[1], Garry Rosewarne[1, 6]

[1]CSIRO Plant Industry, Canberra, ACT, Australia
[2]Division of Plant Sciences, Research School of Biology, Australian National University Canberra ACT Australia
[3]Department of Microbiology, Hazara University Mansehra, KPK, Pakistan
[4]CRC, Agriculture & Agri-Food Canada, Winnipeg, MB R3T 2M9 Canada
[5]New Zealand Crop and Food, Lincoln, New Zealand
[6]CIMMYT, Chengdu, Sichuan Province, China

Abstract

Wheat streak mosaic virus is an established major threat to wheat in North America and is newly identified in Australia. Three genetic sources of resistance were examined, *Wsm1* (an alien translocation), *Wsm2* (from CO960293-2), and c2652 (selected in Canada). We report their effectiveness in the field when inoculated with an Australian WSMV isolate. Also included were advanced breeding lines with and without *Wsm2* and a number of elite Australian cultivars.

We achieved 85-100% infection with WSMV in susceptible lines following mechanical inoculation which reduced their yield by 22 to 44% and height by 19 to 51%. Thousand kernel weight was significantly affected in some of the susceptible lines.

All three sources of resistance (*Wsm1*, *Wsm2*, c2652) and *Wsm2* derivatives protected wheat against infection despite repeated inoculation. Inoculated resistant plots were virtually disease free and suffered neither significant yield loss nor height reduction. National yield trials of the breeding derivatives showed no difference in yields between those with and without *Wsm2* under non-WSMV conditions.

Introduction

Wheat streak mosaic virus (WSMV) (Genus *Tritimovirus*: Family *Potyviridae*), naturally transmitted by the wheat curl mite (WMC), causes one of the most destructive viral diseases of wheat worldwide in both spring and winter crops

(Baley et al. 2001, Sharp et al. 2002; Seifers et al. 2006; Coutts et al. 2008a, Murray and Brennan 2009). Infected plants display symptoms of streaking and chlorosis of the leaves, may be severely stunted, and produce less grain which is almost always of lower quality (Finney and Sill 1963). The greatest losses occur when early infection so weakens plants that they suffer from poor head fertility and grain fill or fail to produce fertile heads altogether (Staples and Allington 1956; Atkinson and Grant 1967, Lanoiselet et al. 2008).

Until WSMV was recently reported in Australia (Ellis et al. 2003) and New Zealand (Lebas et al. 2009), wheat production in temperate Oceania was not thought to be at risk from losses due to this virus. With outbreaks of wheat streak mosaic (WSM) disease having been confirmed in areas of wheat production (Ellis et al. 2003, Coutts et al. 2008a, 2008b), the absence of genetic resistance in Australian wheat cultivars has put the wheat industry at risk of major losses. Moreover, as wheat in Australia is mostly grown in rain-fed areas with frequent terminal drought, the finding that WSMV infection shortens roots and reduces water use efficiency (Price et al. 2009, 2010) adds urgency to the need to apply effective control measures. With its attendant foliar chlorosis and necrosis, WSM also reduces the quantity and quality of wheat grown for forage (Price et al. 2010). This effect is also of concern in Australia where, to an increasing extent in recent years, dual-purpose wheat is sown early in zones of higher rainfall to provide forage for sheep or cattle without loss of grain yield at harvest. With the arrival of WSMV and the double threat it poses, farmers in these areas of higher rainfall are specifically advised to delay sowing in autumn until temperatures drop (GRDC Fact Sheet, Wheat Curl Mite, 2009). This severely limited the usefulness of dual purpose wheat sowings and profitable grain and graze options for farmers. In such a cropping regime the WCM vector is more likely to find the "green bridge" it needs to continue its life cycle and spread the virus. The early-sown crop is thus predisposed to a greater likelihood of inoculation from an increased mite activity with the warmer conditions during early growth stages, and once inoculated the virulence of the infection is enhanced by the higher temperatures (Pfannenstiel and Niblett 1978, Chen 1985, Seifers et al. 1995, 2006, 2007). In sum the stage is set for severe

endemics which affect both forage and grain production. In the absence of natural resistance in cultivated wheat, the Australian wheat industry is clearly at risk of major losses to WSMV.

There are two broad general strategies available to control WSM. One is to control the mite vector, the second is to enhance the wheat host's ability to tolerate or resist the virus infection itself. Mites cannot be controlled through chemical means as these are too toxic, therefore control can only be achieved through cultural practices. In the Great Plains region of the U.S.A., where winter wheat is sown in autumn, late sowing and the removal of volunteer grass help prevent WCM from finding a green bridge and completing their life cycle (Velandia et al. 2010). The emergence of volunteers that provide the green bridge can never be perfectly controlled, so cultural practice will, at best, be only partially effective. Similar issues occur in certain regions of Australia where winter-habit wheat crops are sown in early autumn where higher temperatures dramatically increase the risk of WSMV infection. The vector can be controlled by introgressing one or more sources of genetic resistance to WCM feeding from an array of species related to wheat (Harvey et al. 1999). This approach has been shown to offer effective control for a time but suffers from the ability of mite vector populations to evolve rapidly from avirulence to virulence (Harvey et al. 1999).

The second strategy, to control losses from WSM by host genetic resistance to the virus disease agent itself, has been pursued since natural resistance to WSMV infection was first discovered in wild relatives of wheat. Resistance from *Thinopyrum intermedium*, first identified by McKinney and Sando (1951), was later translocated to bread wheat on the short arm of chromosome 4D and designated as *Wsm1* (Friebe et al. 1992, 1996a). However, limitations arising from factors such as linkage drag (Friebe et al. 1996a, 1996b) and temperature sensitivity (Pfannenstiel and Niblett 1978, Seifers et al. 1995, 2006, 2007) have hindered the deployment of such resistance in commercial cultivars. Recently, recombination of the translocation has reduced the linkage drag and the attendant yield decrement in the absence of virus infection (Friebe et al. 2009). One U.S. cultivar, Mace (unrelated to the cultivar Mace released by Australian

Grains Technologies), has been released with *Wsm1* resistance and improved agronomic performance (Graybosch et al. 2009). A second source of resistance (*Wsm2*), derived from the experimental wheat selection CO960293-2 (Haley et al. 2002), has been identified and mapped to chromosome 3B (Lu et al., 2011). The *Wsm2* resistance was temperature-sensitive in its original source (Seifers et al. 2006) and in the first North American commercial cultivar, RonL, in which it was deployed (Seifers et al. 2011). A second North American cultivar with *Wsm2* resistance, Snowmass (Haley et al. 2011, Seifers et al. 2011), has also been released. Deploying *Wsm2* resistance may not necessarily be limited by temperature sensitivity as lines derived by repeated cycles of selection have been shown to sustain virus resistance at higher temperature (Fahim et al. in press). A third source of resistance, effective at elevated temperature and designated "c2652", has been selected from an experimental doubled haploid spring wheat line C2652, following repeated cycles of selection (Haber et al. unpublished).

We report here the impact in the field of the Australian (ACT) isolate of WSMV on a number of wheats cultivars and how effectively these three sources of genetic resistance protect yield when challenged by repeated rigorous artificial inoculation.

Methodology

Plant material

The germplasm examined in this study fell into three categories: a) the specific lines used as sources of resistance; b) advanced breeding lines derived by crossing from one of the sources of resistance (CO960293-2, *Wsm2*); and c) an array of contemporary Australian cultivars now commonly grown in areas at risk from WSMV infection.

The specific lines tested as examples of three different sources of resistance were: i) a *Wsm1*-carrying 4Ai#2S.4DL translocation line designated *Wsm1*-CA740 (Wells et al., 1982); ii) two lines (CA743 and CA745) carrying *Wsm2* resistance (Haley et al., 2002) that had been derived by iterative selection from

sublines descended by selfing from CO960293-2; and iii) c2652 resistance in line CA742, developed following repeated cycles of WSMV selection in doubled haploid spring wheat line C2652 (Haber et al. unpublished).

Advanced breeding lines were derived from CO960293-2 (*Wsm2*) as follows: CO960293-2 was backcrossed to Superb (a high yield spring milling wheat of pedigree Grandin2*/AC Domain) or straight crossed to HY644 (a Fusarium Head Blight resistant high yield spring feed wheat). Two homozygous resistant derivatives were selected from each cross and used as the pollen donor to three Australian wheats, Sunstate, EGA Hume, or Yitpi. A number of lines were advanced without selection for resistance and F_6 lines selected for yield traits. A suite of these lines were then screened in the glasshouse to identify resistant and susceptible lines. A number of elite Australian lines were used that are commonly grown in WSMV prone areas. Table 1 describes all lines.

Preparation of virus inoculum

Virus infected wheat leaves were weighed and blended with 0.02 M potassium phosphate buffer pH 7 (1 /10 w/v) in a blender. The homogenate was filtered through four layers of Miracloth® (Calbiochem, La Jolla, California, USA), abrasive Celite (Spectrum Chemicals, Gardena, California, USA) was added at 2% w/v to the final volume of inoculum, and the mixture was left on ice for 1 h and then sprayed at 270 kPa on leaves using a spray gun (Fahim et al. 2010).

Glasshouse screening of germplasm

In the glasshouse experiment, six wheat plants of each accession were grown in 10cm pots. The plants were doubly inoculated at the 2–3 leaf stage, with the prepared sap extracts from WSMV-infected leaf material. The plants were scored for symptoms at 14 dpi (days post inoculation) on a scale of 0–4 with 0 as healthy, 1 as mild with very few streaks, 2 as moderate with streaks that coalesce, 3 as severe with approximately 50% leaf area with streaks, 4 as the most severe or lethal symptoms where the streaks develop into chlorosis of more than 70% of leaf area. Leaf samples were collected from individual plants and assayed for the virus using ELISA (as detailed in Fahim et al., 2010). Two uninoculated plants per line were included as healthy controls.

WSMV field trial

A field trial was conducted in the winter growing season of 2009 at Ginninderra Experimental Station, Canberra (35°12′2.59″S, 149°5′22.74″E). The experiment was designed in a complete randomised block design with two replicates of each line and treatment arranged in two blocks. A total of 19 genotypes were grown in 4.9m long by 0.8m wide plots with each consisting of 3 rows. There was a 54cm gap between plots and a 1m gap at the end of each plot. The field trial was established on 10th of July 2009 and harvested on December 21, 2009.

In the field experiment, half of the plots were inoculated with WSMV the at 2-3 leaf stage on the morning of 23rd of August using a spray gun, again on the 1st of September and finally on the 21st of September. The youngest fully expanded leaf with virus like symptoms was collected on 6th of November (6 weeks after the last inoculation). Relative incidence of WSMV was calculated on all plots by sampling five plants at random and assaying using ELISA (Fahim et al. 2010).

The trial was sprayed with fungicide (Tilt250E) at late tillering according to the manufacturer's instructions (Syngenta Crop Protection, Guelph, Canada). The trial was grown under rainfed conditions, however due to a dry spell in October; four allocations of irrigation of approximately 3mm each were added at weekly intervals.

A maturity score (Zadoks 1974) was taken when most of the lines were at anthesis. Prior to harvest, height measurements (cm) were taken as was a harvest index (HI) cut. This cut measured 0.5m × 0.8m and was taken randomly across the width of each plot by cutting at ground level. The tillers were bundled together, dried and weighed for total biomass. Heads and grain yield per m^2 were recorded and HI calculated. The remainder of each plot was harvested with a single row harvester and total grain yield recorded along with grain weight from the HI cut. Thousand grain weight (from 250 grains) and hectolitre weight were calculated on a subsample from each plot.

Uninoculated yield trials

A total of 29 advanced derivatives of the *Wsm2* crosses into Australian cultivars were included in breeding trials in 2008. Briefly, these lines were included as unreplicated entries -percentage replicated designed trials (Cullis et al. 2006) conducted in nine high rainfall sites, covering four States of Australia. The earliest maturing lines were grown in SW Western Australia, the mid-maturing lines in trial in SE New South Wales and the latest maturing lines were grown in trial in SE Victoria and SE South Australia. All trials were run by commercial enterprises that base their agronomy on "best farmer practice" for the specific region. Kalyx Agriculture (Carlisle WA) ran three trials in Western Australia (Kojonup, Mount Barker and Gibson). Agritech Crop Research Pty Ltd (Young NSW) ran trials in New South Wales (Cowra, Monteagles and Wallendbeen). Southern Farming Systems (Newtown Vic) ran trials in Mininerra and Inverleigh and Mackillop Farm Management Group Inc. (Naracoorte SA) ran a trial at Conmurra.

Statistical analysis

Genstat (v13) was used for spatial analysis of the high rainfall zone (HRZ) yield trials and also to conduct two-way ANOVA with and without interactions on all measured parameters in the WSMV trial. Significant differences between WSMV treatment of lines were identified at $P<0.05$ when the differences between means of the measured parameter were greater than the least significant difference (LSD).

Results

Resistance in glasshouse

The presence of the *Wsm1* resistance gene was inferred using molecular markers. (Talbert et al. 1996, Fahim et al. in press). However no suitable markers are available for *Wsm2* and c2652, therefore these genotypes were inferred using the established glasshouse bioassay to identify the resistance phenotype. All original sources of resistance held up to WSMV infection in the glasshouse. Testing of sets of F_6 Australian derived sibling lines showed that 13 were evidently either homozygous for resistance (*Wsm2*) and 13 were

homozygous for susceptibility (*wsm2*), with three having an intermediate average ELISA level because both resistant and susceptible plants were amongst the six tested individuals of those lines (Fig. 1). From these glasshouse studies, uniformly resistant and susceptible sibling lines were chosen for the inoculated field trial. Lines were chosen that had a uniform disease reaction whilst also ensuring all backgrounds were represented.

Resistance in the field

Spray inoculation of field plots resulted in excellent uniform infection and efficiency of inoculation was almost as good as in the glasshouse. The ELISA performed on leaf samples showed 85 to 100% infection in all inoculated susceptible genotypes. All of the inoculated plots of resistant lines had very low or zero infection (Table 2). Neither visual inspection of plots nor ELISA detected virus infections in any un-inoculated plots, regardless of whether they contained resistance genes or not. This indicated the successful establishment of infection in the treated plots, lack of migration into untreated plots, and lack of natural presence of viruliferous mites during the experiment.

A two-way ANOVA was used to analyse the growth and yield components by genotype and WSMV treatment (Table 3). There were significant genotype differences in all yield and growth components, while WSMV inoculation had significant effects on five of the components. Table 3 summarises the yield components which were affected by inoculation, and Table 4 showed which lines contributed to the significant results.

The yield trial suffered from a mid-late season drought (October) and despite attempts at irrigation, the trial average was approximately 1 tonne ha^{-1}. However, we still saw significant yield effects of WSMV in many of the susceptible lines (Fig. 2). All lines carrying resistance genes *Wsm1*, *Wsm2* or c2652 had yields that were unaffected by the disease, despite the highly effective inoculation procedure.

The disease reactions of the Australian derived sibling lines observed in the glasshouse were also very evident in the field trial. There was no loss of yield

on average for the five *Wsm2* resistant lines (range 6% loss to 5% increase for individual genotypes), however, total grain yields of all lines not carrying resistance were reduced in the presence of disease, and for most of these lines the reduction was significant ($P<0.05$). All five Australian wheat cultivars showed a reduction of yield ranging from 29 to 44%. The susceptible sibling lines also had reduced yields, ranging from 22 to 44 %.

The harvest index and most of the parameters that contribute to this measure were significantly affected by WSMV inoculation. Total biomass was affected by inoculation, whereas the number of heads per m^2 was not. The lines that were significantly different for HI parameters were generally susceptible although one resistant selection (HRZ07.0433) had a higher harvest index and another (HRZ07.0485) had higher total biomass in the uninoculated plots.

WSMV had no significant effect on the height of resistant lines whereas it significantly ($P<0.05$) stunted Sunbrook, Preston and Wedgetail. Wedgetail was most severely affected (51% reduction), followed by Preston (31%), Sunbrook (30%), Chara (20%) and Yitpi (19%). Grain size was affected by WSMV infection in Chara and two of the HRZ susceptible lines; thousand-kernel weight was significantly reduced in inoculated plots. WSMV had no effect on maturity score, establishment, or hectolitre weight.

To assess whether linkage drag might be a problem for the *Wsm2* resistance, we grouped the germplasm into four pools for comparisons: 1) the unadapted resistant sources, 2) the lines from elite Australian backgrounds with introgressed *Wsm2*, selected for resistance; iii) the susceptible sibling lines of pool 2; and 4) the adapted Australian parents. Each pool was analysed with and without the virus treatment (Fig. 3). The un-inoculated resistant sources (pool 1) which were not in adapted backgrounds yielded significantly less than any of the other un-inoculated pools. The other three un-inoculated pools did not differ significantly from each other. When crossed into adapted germplasm, *Wsm2* protects against yield losses from inoculated WSMV, as seen in the comparison of the resistant pool against its susceptible pool counterpart and

also has comparable yield in the absence of virus to the cultivars and the susceptible sibling lines (Fig. 2, Fig. 3).

This was further explored by yield trialing of the 26 homozygous Australian derived sibling sets of lines segregating for *Wsm2*. This showed that *Wsm2* carrying derivatives had no yield penalty compared to their siblings (Fig. 4) in the absence of wheat streak mosaic disease. With only one cross into the Australian backgrounds they were achieving on average only about 80% of the site mean yields, compared to standard varieties and breeding lines. However the average yields of the 13 resistant lines was very similar to the 13 susceptible lines in the absence of disease.

Discussion

We had previously documented in glasshouse trials that inoculating susceptible wheat lines with WSMV adversely affected their growth, while resistant lines derived from *Wsm1*, *Wsm2* and c2652 remained unaffected (Fahim et al. in press). The current report extends the analysis and confirms that *Wsm1*, *Wsm2* and c2652 are also effective in the field to protect against yield losses.

Earlier studies conducted in controlled temperature cabinets had also shown that there were differences in the temperature sensitivity of the resistances expressed in derivatives from the three different sources (Fahim et al. in press). All were similarly effective up to 20°C against the ACT isolate of WSMV. At temperatures above 20°C, the resistance expressed by line CA740 (= KS95H10-3), which carried the *Wsm1* gene on a 4Ai#2S.4DL translocation, was ineffective. This response against the ACT isolate was similar to that reported earlier against the Sidney81 isolate from Kansas (Seifers et al. 1995). The resistance derived from CO960293-2 (now designated *Wsm2*) was similarly temperature-sensitive in most lines, but one selection expressed effective resistance at 26°C. Finally, the tested lines that derived their resistance from c2652 expressed resistance that was effective at temperatures as high as 28°C.

With the current field study we established that lines that were susceptible in the glasshouse also suffered major yield losses to WSMV in the field, and lines

that expressed resistance to WSMV were protected from yield losses in the field. *Wsm1* was shown by Sharp et al. (2002) to confer significant yield protection in the field over two years; the average yield was 80% compared to uninoculated plots, whereas sibling lines without *Wsm1* when inoculated yielded on average only 26% of the uninoculated plots. Divis et al. (2006) also reported the effectiveness of *Wsm1* against infection and yield loss in the field. Seifers et al. (2006, 2007) likewise showed that both *Wsm1* and *Wsm2* were effective in the field at protecting yield against North American isolates of the virus. This current study extends these findings to the warmer field conditions of Australia and shows *Wsm1* and *Wsm2* conferring total protection against infection and yield loss using an Australian isolate, WSMV-ACT. This is also the first demonstration that resistance derived from a new high temperature effective resistance, c2652, is completely effective under field conditions.

The five cultivars we tested were all susceptible to WSMV and along with the five susceptible siblings from Australian derived material, all experienced substantial losses (Table 4, Fig. 2). Among the tested cultivars, Wedgetail illustrates most clearly the potential for losses and, correspondingly, the benefits of conferring effective genetic resistance. As a winter wheat with high grain quality which is grown in environments where it is also sown early and used as winter forage, it is vulnerable to becoming infected at early plant growth stages if large populations of WCM are present, for example following a mild summer with moisture and appropriate green bridges. This heightened risk of WSMV infection causing losses of both forage and grain yield has undermined confidence in the valuable dual-purpose application of this cultivar.

Infection with WSMV induces an array of changes that interact to reduce biomass, grain yield and quality. The manner and extent of these interactions vary with the earliness of infection, intensity of inoculation, host genotype and environmental conditions. In this study we observed, for example, that virus infection, stunted plants of the cultivar Sunbrook but did not reduce plot yield significantly. In the cultivar Wedgetail, by contrast, infection reduced height (a proxy for biomass) and yield dramatically, but in sufficiently similar measure

that the HI parameter was not changed significantly. The cultivars Preston and Yitpi experienced proportionally greater losses of grain yield than biomass, reducing their HI. Foliar symptoms that range from light chlorotic streaking and mosaic to severe chlorosis and necrosis affect the volume and quality of wheat as forage. Severe infection stunts growth and retards development, producing fewer tillers and delayed or abolished anthesis and seed set (Martin and Harvey 1992, Tatineni et al. 2010), followed by poor seed filling (Weise 1987). When a high proportion of plants in a field are infected at an early growth stage, these effects combine to reduce yields substantially. For example, in a two-year field study for seven cultivars in Oklahoma, Hunger et al. (1992) estimated that WSMV infection reduced fertile tillers and grain yield by as much as 75% and 87%, respectively.

The expression of a resistance gene may protect against losses from virus infection but be associated with other genes that are refractory to producing high yields in the absence of infection. This effect of linkage drag stymied the deployment of the *Wsm1* resistance gene in its original form (Sharp et al. 2002); though studies that have examined more recently developed *Wsm1* lines suggest the linkage drag may be overcome (Baley et al. 2001, Divis et al. 2006). In the present study *Wsm2* derivatives in adapted backgrounds showed no signs of linkage drag in the absence of virus (Figs. 3 and 4) which augurs well for the deployment of this resistance in new cultivars. That assessment for c2652 will have to wait for backcross derivatives in adapted backgrounds to be developed.

Resistance conferred by *Wsm1*, *Wsm2* and c2652 were effective in protecting against WSMV under field condition. However, these WSMV resistant wheat lines are still susceptible to other viruses such as BYDV, HPV and TriMV (Gieck et al. 2007, Seifers et al. 2009, 2011). Therefore, it would be of great value to stack resistance genes against these viruses into high yielding cultivars. Continuing evolution of virus populations may also bring about shifts from avirulence to virulence in interactions with any single resistance gene. This highlights the importance of pyramiding genes for resisting WSMV along with those that protect against other important diseases.

Acknowledgements

The authors thankfully acknowledge Megan Hemming and Maryse Bourgault CSIRO, for critically reviewing the manuscript. The first author thankfully acknowledges AusAID for financial assistance as PhD studentship.

References

Atkinson, T.G., and M.N. Grant, 1967: An evaluation of streak mosaic losses in winter wheat. Phytopathology **57**, 188-192.

Baley, G.J., L.E. Talbert, J.M. Martin, M.J. Young, D.K. Habernicht, G.D. Kushnak, J.E. Berg, S.P. Lanning, and P.L. Bruckner, 2001: Agronomic and end-use qualities of *Wheat streak mosaic virus* resistant spring wheat. Crop Science **41**, 1779-1784.

Carew, M., M. Schiffer, P. Umina, A. Weeks, and A. Hoffmann, 2009: Molecular markers indicate that the wheat curl mite, *Aceria tosichella* Keifer, may represent a species complex in Australia. Bulletin of Entomological Research **99**, 479-486.

Chen, S.T., 1985: Environmental factors affecting the resistance of certain Agrotricums and their derivatives against wheat streak mosaic. Dissertation Abstracts International, B (Sciences and Engineering) **45**, 3680B.

Coutts, B.A., G.R. Strickland, M.A. Kehoe, D.L. Severtson, and R.A.C. Jones, 2008b: The epidemiology of *Wheat streak mosaic virus* in Australia: case histories, gradients, mite vectors, and alternative hosts. Australian Journal of Agricultural Research **59**, 844-853.

Coutts, B.A., N.E.B. Hammond, M.A. Kehoe, and R.A.C. Jones, 2008a: Finding *Wheat streak mosaic virus* in south-west Australia. Australian Journal of Agricultural Research **59**, 836-843.

Divis, L.A., R.A. Graybosch, C.J. Peterson, P.S. Baenziger, G.L. Hein, B.B. Beecher, and T.J. Martin, 2006: Agronomic and quality effects in winter wheat of a gene conditioning resistance to *Wheat streak mosaic virus*. Euphytica **152**, 41-49.

Ellis, M.H., G.J. Rebetzke, and P. Chu, 2003: First report of *Wheat streak mosaic virus* in Australia. Plant Pathology **52**, 808-808.

Fahim, M., A. Mechanicos, L. Ayala-Navarette, S. Haber, and P.J. Larkin (2011) Resistance to Wheat Streak Mosaic Virus in Australia – a Survey of Resources and Development of Markers. Plant Pathology (in press)

Fahim, M., H. Dove, W.M. Kelman, L. Ayala-Navarrete, and P.J. Larkin, 2010: Does grazing of infected wheat by sheep result in salivary transmission of *Wheat streak mosaic virus*? Crop & Pasture Science **61**, 247-254.

Finney, K.F., and W.H. Sill, 1963: Effects of 2 virus diseases on milling and baking properties of wheat grain and flour and on probable nutritive value of forage wheat. Agronomy Journal **55**, 476-&.

Friebe, B., J. Jiang, W.J. Raupp, R.A. McIntosh, and B.S. Gill, 1996b: Characterization of wheat-alien translocations conferring resistance to diseases and pests: Current status. Euphytica **91**, 59-87.

Friebe, B., K.S. Gill, N.A. Tuleen, and B.S. Gill, 1996a: Transfer of *Wheat streak mosaic virus* resistance from *Agropyron intermedium* into wheat. Crop Science **36**, 857-861.

Friebe, B., L.L. Qi, D.L. Wilson, Z.J. Chang, D.L. Selfers, T.J. Martin, A.K. Fritz, and B.S. Gill, 2009: Wheat-*Thinopyrum intermedium* Recombinants Resistant to *Wheat streak mosaic virus* and *Triticum mosaic virus*. Crop Science **49**, 1221-1226.

Friebe, B., Y. Mukai, B.S. Gill, and Y. Cauderon, 1992: C-banding and in situ hybridization analyses of *Agropyron intermedium*, a partial wheat x *Ag intermedium* amphiploid, and 6 derived chromosome addition lines. Theoretical and Applied Genetics **84**, 899-905.

Gieck, S.L., P.B. Hamm, G.H. Clough, and N.L. David, 2007: *High plains virus*: An emerging disease of sweet corn in the Columbia basin of Oregon and Washington. Phytopathology **97**, S167-S168.

Graybosch, R.A., C.J. Peterson, P.S. Baenziger, D.D. Baltensperger, L.A. Nelson, Y. Jin, J. Kolmer, B. Seabourn, R. French, G. Hein, T.J. Martin, B. Beecher, T. Schwarzacher, and P. Heslop-Harrison, 2009: Registration of 'Mace' Hard Red Winter Wheat. Journal of Plant Registrations **3**, 51-56.

GRDC Fact Sheet: Wheat Curl Mite, 2009 <http://www.grdc.com.au/uploads/documents/ GRDC_ WheatCurlMite_4pp.pdf>

Guddeti, S., D.C. Zhang, A.L. Li, C.H. Leseberg, H. Kang, X.G. Li, W.X. Zhai, M.A. Johns, and L. Mao, 2005: Molecular evolution of the rice miR395 gene family. Cell Research **15**, 631-638.

Haley, S.D., J.J. Johnson, F.B. Peairs, J.A. Stromberger, E.E. Heaton, S.A. Seifert, R.A. Kottke, J.B. Rudolph, T.J. Martin, G.H. Bai, X.M. Chen, R.L. Bowden, Y. Jin, J.A. Kolmer, D.L. Seifers, M.S. Chen, and B.W. Seabourn, 2011: Registration of 'Snowmass' Wheat. Journal of Plant Registrations **5**, 87-90.

Haley, S.D., T.J. Martin, J.S. Quick, D.L. Seifers, J.A. Stromberger, S.R. Clayshulte, B.L. Clifford, F.B. Peairs, J.B. Rudolph, J.J. Johnson, B.S. Gill, and B. Friebe, 2002: Registration of CO960293-2 wheat germplasm resistant to *Wheat streak mosaic virus* and Russian wheat aphid. Crop Science **42**, 1381-1382.

Harvey, T.L., D.L. Seifers, T.J. Martin, G. Brown-Guedira, and B. S. Gill, 1999: Survival of wheat curl mites on different sources of resistance in wheat. Crop Science **39**, 1887-1889.

Hunger, R.M., J.L. Sherwood, C.K. Evans, and J.R. Montana, 1992: Effects of Planting Date and Inoculation Date on Severity of Wheat streak mosaic virus in Hard Winter Wheat-Wheat Cultivars. Plant Disease **76**, 1056-1060.

Lanoiselet, V.M., T.L. Hind-Lanoiselet, and G.M. Murray, 2008: Studies on the seed transmission of *Wheat streak mosaic virus*. Australasian Plant Pathology **37**, 584-588.

Lebas, B.S.M., F.M. Ochoa-Corona, B.J.R. Alexander, R.A. Lister, J.D.F. Fletcher, S.L. Bithell, and G.M. Burnip, 2009: First Report of *Wheat streak mosaic virus* on Wheat in New Zealand. Plant Disease **93**, 430-430.

Lu, H.J., J. Price, R. Devkota, C. Rush, and J. Rudd, 2011: A Dominant Gene for Resistance to *Wheat streak mosaic virus* in Winter Wheat Line CO960293-2. Crop Science **51**, 5-12.

Martin, T., and T. Harvey, 1992: Field screening procedure for resistance to wheat streak mosaic virus. Cereal Research Communications 20, p213-215.

McKinney, H.H., and W.J. Sando, 1951: Susceptibility and resistance to the wheat streak-mosaic virus in the genera *Triticum*, *Agropyron*, *Secale*, and certain hybrids. Plant Dis Reporter **35**, 476-479.

Murray, G., and J. Brennan, 2009: Estimating disease losses to the Australian wheat industry. Australasian Plant Pathology **38**, 558-570.

Pfannenstiel, M., and C. Niblett, 1978: The nature of the resistance of agrotricums to *Wheat streak mosaic virus*. Phytopathology **68**, p1204-1209.

Price, J.A., F. Workneh, S.R. Evett, D.C. Jones, J. Arthur, and C.M. Rush, 2010: Effects of Wheat streak mosaic virus on Root Development and Water-Use Efficiency of Hard Red Winter Wheat. Plant Disease **94**, 766-770.

Seifers, D.L., T.J. Martin, and J.P. Fellers, 2011: Occurrence and Yield Effects of Wheat Infected with *Triticum mosaic virus* in Kansas. Plant Disease **95**, 183-188.

Seifers, D.L., T.J. Martin, T.L. Harvey, and B.S. Gill, 1995: Temperature Sensitivity and Efficacy of *Wheat streak mosaic virus* Resistance Derived from *Agropyron Intermedium*. Plant Disease **79**, 1104-1106.

Seifers, D.L., T.J. Martin, T.L. Harvey, and S. Haber, 2007: Temperature-sensitive *Wheat streak mosaic virus* resistance identified in KS03HW12 wheat. Plant Disease **91**, 1029-1033.

Seifers, D.L., T.J. Martin, T.L. Harvey, J.P. Fellers, and J.P. Michaud, 2009: Identification of the Wheat Curl Mite as the Vector of *Triticum mosaic virus*. Plant Disease **93**, 25-29.

Seifers, D.L., T.J. Martin, T.L. Harvey, S. Haber, and S.D. Haley, 2006: Temperature sensitivity and efficacy of *Wheat streak mosaic virus* resistance derived from CO960293 wheat. Plant Disease **90**, 623-628.

Sharp, G.L., J.M. Martin, S.P. Lanning, N.K. Blake, C.W. Brey, E. Sivamani, R. Qu, and L.E. Talbert, 2002: Field evaluation of transgenic and classical sources of *Wheat streak mosaic virus* Resistance. Crop Science **42**, 105-110.

Staples, R., and W.B. Allington, 1956: Streak mosaic of wheat in Nebraska and its control. University of Nebraska, Lincoln. Agriculture Experiment. Station. Research Bulletin.

Talbert, L.E., P.L. Bruckner, L.Y. Smith, R. Sears, and T.J. Martin, 1996: Development of PCR markers linked to resistance to wheat streak mosaic virus in wheat. Theoretical and Applied Genetics **93**, 463-467.

Tatineni S, Graybosch RA, Hein GL, Wegulo SN and French R (2010) Wheat Cultivar-Specific Disease Synergism and Alteration of Virus Accumulation During Co-Infection with Wheat streak mosaic virus and Triticum mosaic virus. *Phytopathology* **100**:230-238.

Velandia, M., R.M. Rejesus, D.C. Jones, J.A. Price, F. Workneh, and C.M. Rush, 2010: Economic impact of *Wheat streak mosaic virus* in the Texas High Plains. Crop Protection **29**, 699-703.

Weise, M.V., 1987: Wheat streak mosaic. . Compendium of Wheat Diseases. 2nd edition. American Phytopathological Society, St. Paul. MN. , 80-81.

Wells D, Kota R, Sandhu H, Gardner W, Finney K (1982) Registration of one disomic substitution line and five translocation lines of winter wheat germplasm resistant to wheat streak mosaic virus (Reg. No. GP 199 to GP 204). Crop Science **22**, 1277-1278.

Zadoks, J.C., T.T.Chang, and C.F. Konzak, 1974. A decimal code for the growth stages of cereals. Weeds Research **14,** 415-421.

Table 1. Germplasm and glasshouse WSMV test. Eight plants per genotype were grown in a glasshouse, six inoculated at the three-leaf stage with WSMV and two left as healthy controls. Resistance (R) is recorded only if resistance was confirmed in subsequent testing at 20°C
Superb is a high yield spring milling wheat Grandin*2/AC Domain, HY644 is FHB resistant high yield spring feed wheat and HY644/CO960293 was homozygous for resistance.

Identifier	Other accession names	Resistance gene	Description	WSMV
CA740	KS95H10-3	*Wsm1*	*Wsm1* translocation (4Ai#2S .4DL); an improved selection	R
CA743	Haber7760, CPI146635	*Wsm2*	Winter wheat '4th cycle' WSMV selection	R
CA745	Haber9104, CPI146894	*Wsm2*	Spring wheat, Superb 2*/CO960293 (BC1F9)	R
CA742	Haber9379, CPI146896	c2652	Selection from repeated rounds of WSMV selection in doubled haploid line C2652	R
HRZ07.0422		*Wsm2*	EGA Hume// HY644/CO960293	R
HRZ07.0425		*Wsm2*	EGA Hume//CA745	R
HRZ07.0433		*Wsm2*	Sunstate// HY644/CO960293	R
HRZ07.0485		*Wsm2*	Yitpi// HY644/CO960293	R
HRZ07.0486		*Wsm2*	Sunstate// HY644/CO960293	R
HRZ07.0423		-	EGA Hume// HY644/CO960293	S
HRZ07.0428		-	EGA Hume// CA745	S
HRZ07.0431		-	Yitpi// HY644/CO960293	S
HRZ07.0436		-	Sunstate// HY644/CO960293	S
HRZ07.0437		-	Sunstate// CA745	S
Chara		-	Australian wheat cultivar	S
Sunbrook		-	Australian wheat cultivar	S
Preston		-	Australian wheat cultivar	S
Wedgetail		-	Australian wheat cultivar	S
Yitpi		-	Australian wheat cultivar	S

Table 2. WSMV status of inoculated plots. Five plants were sampled at random from each plot and assayed by ELISA for presence of WSMV. Percentage incidence was calculated for each plot and the average from two replicate plots presented. All uninoculated plots had zero infection (data not shown).

Germplasm	**Resistance** Locus/Allele	**% Incidence (WSMV)**
CA-740	*Wsm1*	0
CA-742	c2652	0
CA-743	*Wsm2*	0
CA-745	*Wsm2*	0
HRZ07.0422	*Wsm2*	0
HRZ07.0425	*Wsm2*	0
HRZ07.0433	*Wsm2*	5
HRZ07.0485	*Wsm2*	0
HRZ07.0486	*Wsm2*	0
HRZ07.0423	Null	85
HRZ07.0428	Null	85
HRZ07.0431	Null	85
HRZ07.0436	Null	82.5
HRZ07.0437	Null	97.5
Chara	Null	100
Sunbrook	Null	100
Preston	Null	100
Wedgetail	Null	100
Yitpi	Null	100

Table 3. Two-way analysis of variance of 19 wheat genotypes treated with and without WSMV showing F statistic. * represents significant difference in at least one of the genotypes tested at $P<0.05$

Growth and Yield Components	Significance Difference Between Genotypes	Significant Difference between WSMV Treatments
Grain Yield	<0.001*	<0.001*
Heads/M^2	<0.001*	0.254
Total Biomass	<0.001*	<0.001*
HI	<0.001*	<0.001*
Thousand Kernel Wt	<0.001*	0.011*
Hectolitre Weight	<0.001*	0.094
Height	<0.001*	<0.001*
Zadoks Score	<0.001*	0.074
Establishment	<0.001*	0.304

Table 4. Percentage change caused by inoculation for significant growth/yield components (from table 1) and associated Means and Least Significant Difference (L.S.D.). * represents genotypes that were significantly affected for the associated yield/growth component at $P<0.05$.

		Percentage Reduction (Inoculated- Uninoculated*100/Uninoculated)				
Genotype	Resistance Locus/Allele	Grain Yield (t/Ha)	Total Biomass	HI (%)	Height	TKW (g)
CA-740	*Wsm1*	37	-8	-7	6	-4
CA-742	c2652	5	-10	-14	-5	0
CA-743	*Wsm2*	27	85*	-13	4	2
CA-745	*Wsm2*	8	-15	18	-14	1
HRZ07.0422	*Wsm2*	13	15	-3	8	-3
HRZ07.0425	*Wsm2*	-10	12	-5	8	-2
HRZ07.0433	*Wsm2*	3	1	-35*	-20	7
HRZ07.0485	*Wsm2*	5	-34*	-16	-12	-1
HRZ07.0486	*Wsm2*	3	-29	-14	-15	1
HRZ07.0423	Null	-22	13	-3	-28	-13*
HRZ07.0428	Null	-31*	-12	-22	-18	-7
HRZ07.0431	Null	-33*	-33*	-26	-19	-1
HRZ07.0436	Null	-31	13	-1	3	-9*
HRZ07.0437	Null	-44*	-38*	-13	-19	-6
Chara	Null	-34	-37	-30*	-20	-13*
Sunbrook	Null	-29	-41	-5	-30*	3
Preston	Null	-46*	-46*	-25*	-31*	-7
Wedgetail	Null	-49*	-33	-12	-51*	3
Yitpi	Null	-49*	-21	-33*	-19	-3
Mean		**0.95**	**100.68**	**30.37**	**57.51**	**30.14**
L.S.D.		**0.38**	**46.74**	**9.53**	**14.31**	**2.8**

Figure 1. Glasshouse study of WSMV reaction of F_6 Australian cultivars x *Wsm2* sibling sets (blocked into pedigrees). Mean ELISA ratios (inoculated/healthy) of a range of lines derived from F_6 lines developed without WSMV selection. Scores above two are considered susceptible.

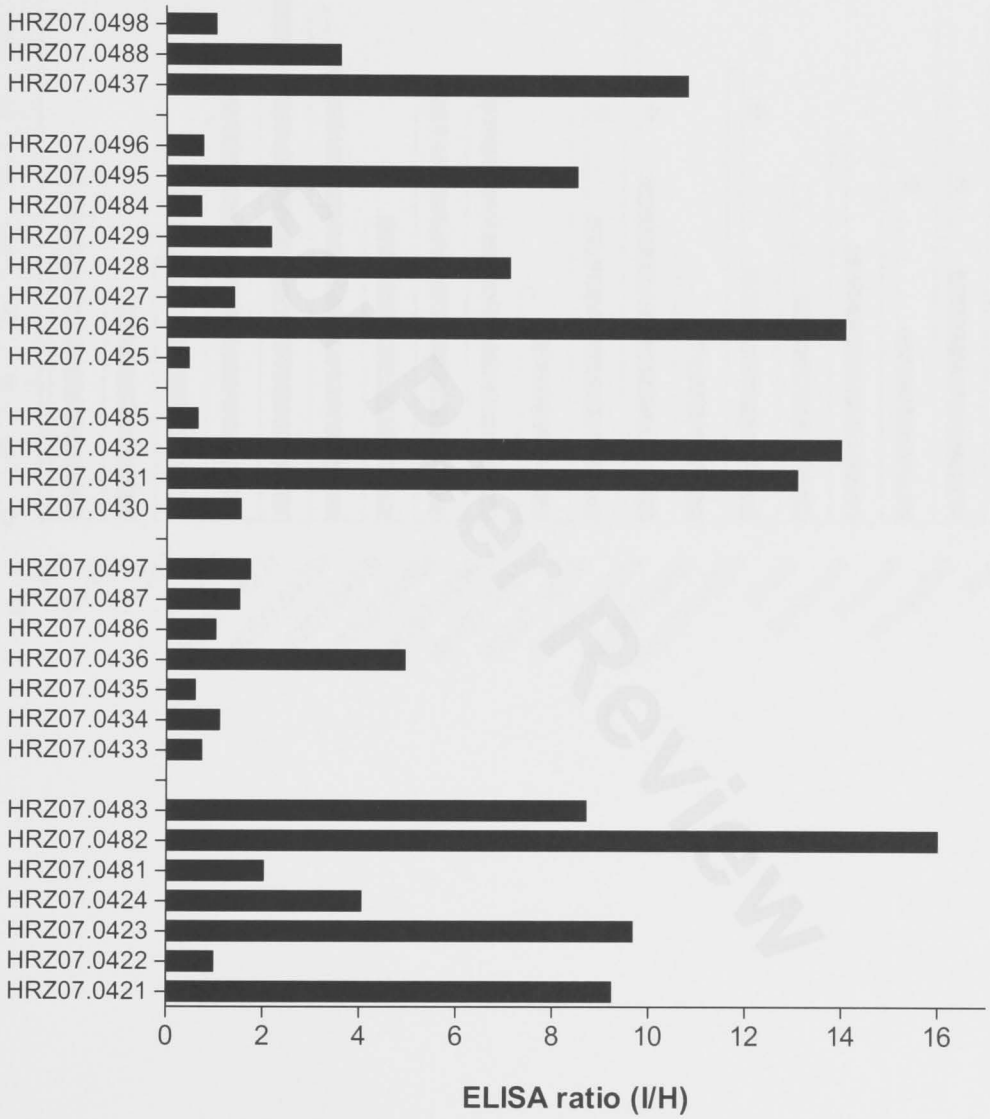


Figure 2. Effect of resistance on yield: Yield comparison (t/ha). * indicated lines that were significantly affect by WSMV inoculation (P<0.05)

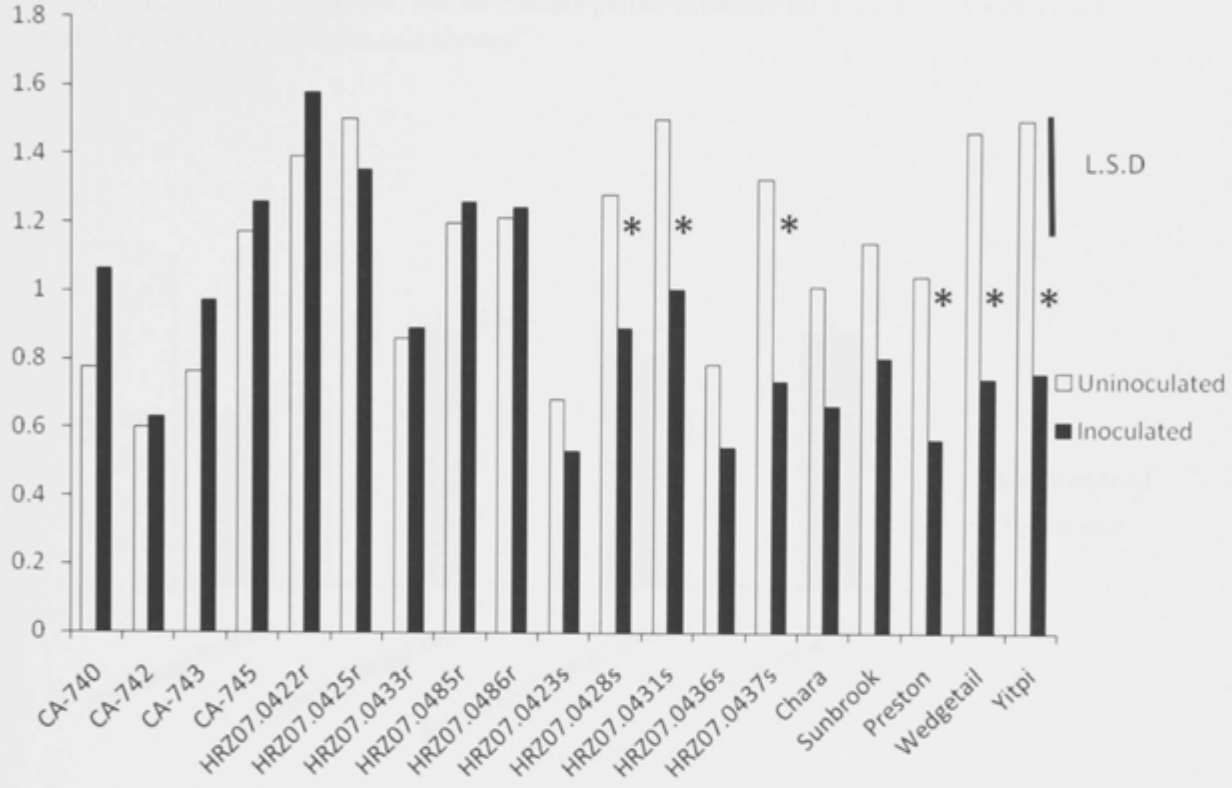


Figure 3. Average yield (t/Ha) of lines grouped as follows: Resistance sources, the four sources of resistant germplasm; Resistant Breeder Lines, the five derived lines carrying *Wsm2*; Susceptible Breeder Lines, the five derived lines without *Wsm2*; Australian varieties, the five susceptible Australian varieties. Means and standard errors of means are shown.

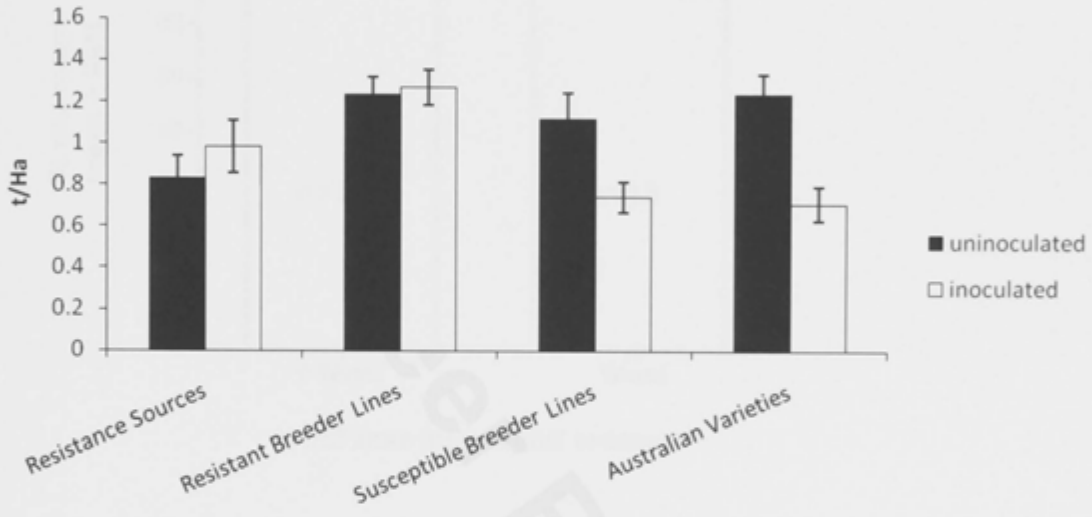
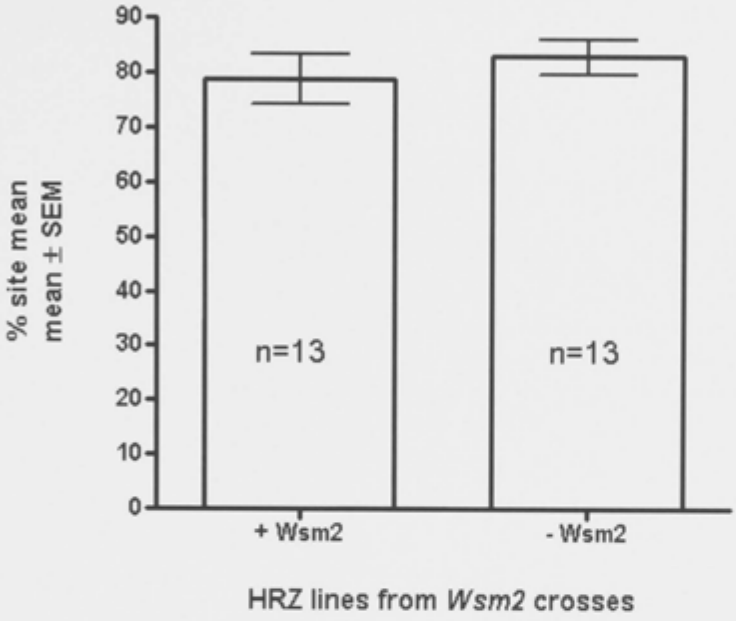


Figure 4. Yield Data of 26 Australian derived sets of sibling lines with either *Wsm2* or *wsm2*. Data was normalised to yield as a percentage of site means from 13 trials conducted in 2008. WSMV was not observed at any of the sites.



Chapter 5
Intron hairpinRNA-mediated immunity against WSMV

Authors' contribution

MF designed the experiments
MF carried out all experimental work
MF wrote the paper
PL edited the paper
AAM reviewed the paper

Plant Biotechnology Journal

Plant Biotechnology Journal (2010) 8, pp. 821–834 doi: 10.1111/j.1467-7652.2010.00513.x

Hairpin RNA derived from viral *Nla* gene confers immunity to wheat streak mosaic virus infection in transgenic wheat plants

Muhammad Fahim[1,2,3], Ligia Ayala-Navarrete[1], Anthony A. Millar[2] and Philip J. Larkin[1,*]

[1]CSIRO Plant Industry, Canberra, ACT, Australia

[2]Research School of Biology, Division of Plant Sciences, Australian National University, Canberra, ACT, Australia

[3]Department of Microbiology, Hazara University Mansehra, Pakistan

Received 28 January 2010;
revised 28 January 2010;
accepted 30 January 2010.
*Correspondence (fax +61 2 6246 5000;
email Philip.larkin@csiro.au)

Summary

Wheat streak mosaic virus (WSMV), vectored by Wheat curl mite, has been of great economic importance in the Great Plains of the United States and Canada. Recently, the virus has been identified in Australia, where it has spread quickly to all major wheat growing areas. The difficulties in finding adequate natural resistance in wheat prompted us to develop transgenic resistance based on RNA interference (RNAi). An RNAi construct was designed to target the nuclear inclusion protein 'a' (*Nla*) gene of WSMV. Wheat was stably cotransformed with two plasmids: pStargate-*Nla* expressing hairpin RNA (hpRNA) including WSMV sequence and pCMneoSTLS2 with the *nptII* selectable marker. When T_1 progeny were assayed against WSMV, ten of sixteen families showed complete resistance in transgenic segregants. The resistance was classified as immunity by four criteria: no disease symptoms were produced; ELISA readings were as in uninoculated plants; viral sequences could not be detected by RT-PCR from leaf extracts; and leaf extracts failed to give infections in susceptible plants when used in test-inoculation experiments. Southern blot hybridization analysis indicated hpRNA transgene integrated into the wheat genome. Moreover, accumulation of small RNAs derived from the hpRNA transgene sequence positively correlated with immunity. We also showed that the selectable marker gene *nptII* segregated independently of the hpRNA transgene in some transgenics, and therefore demonstrated that it is possible using these techniques, to produce marker-free WSMV immune transgenic plants. This is the first report of immunity in wheat to WSMV using a spliceable intron hpRNA strategy.

Keywords: WSMV, Potyviridae, RNAi, resistance.

Introduction

Wheat streak mosaic tritimovirus (WSMV) of the Family *Potyviridae* has a monopartite genome of single-stranded RNA (ssRNA) with messenger polarity and a genome size that varies from 9 339 to 9 384 nucleotides depending upon the isolate (Stenger *et al.*, 1998; Choi *et al.*, 2001; Rabenstein *et al.*, 2002). The host range of WSMV is restricted to species in the family Gramineae, and it is naturally transmitted by the wheat curl mite *Aceria tosichella* Keifer (Slykhuis, 1955; Harvey and Seifers, 1991; Seifers *et al.*, 1998).

Wheat streak mosaic virus is one of the most destructive viral diseases of wheat (Conner *et al.*, 1991; Nyitrai, 1991; Jiang *et al.*, 1993; Makkouk and Kumari, 1997); for instance, in the Great Plains of North America endemics may cause yield losses up to 100% (Stenger *et al.*, 2002; French and Stenger, 2003). In Australia, WSMV was first identified in 2003 in South Australia, Victoria, New South Wales and Queensland, followed by Western Australia in 2006 and more recently in Tasmania (Ellis *et al.*, 2003, 2004; Dwyer *et al.*, 2007), with losses reaching 80% in some instances (Dwyer *et al.*, 2007; Murray *et al.*, 2007).

The two sources of natural resistance that have been described to date in wheat and in its wild relatives, have proved to be temperature sensitive (Seifers *et al.*, 1995, 2006). However, one of these sources, a *Thinopyrum intermedium* translocation to wheat, can incur a significant yield penalty in the absence of the virus (Baley *et al.*, 2001; Sharp *et al.*, 2002; Divis *et al.*, 2006). The other resistance has been released once in the wheat cultivar RonL (Seifers *et al.*, 2007). As a consequence, there has been interest to create transgenic resistance to minimize losses, especially for environments with higher early season temperatures where the existing resistances break down.

Earlier pathogen-derived resistance strategies (Abel *et al.*, 1986) have evolved into more efficient and effective transgenic protection against viruses in plants utilizing induced RNA interference (RNAi) (Waterhouse *et al.*, 1998). Since then RNAi has been employed in development of virus-resistant transgenic plants through expression of transgenes that form double-stranded RNAs (dsRNA) or hairpin RNAs (hpRNA) that are based on virus sequence (Kalantidis *et al.*, 2002; Di Nicola-Negri *et al.*, 2005; Fuentes *et al.*, 2006; Tougou *et al.*, 2006). These RNAs are processed into small interfering RNAs (siRNAs) by DICER-like enzymes. siRNAs are then incorporated into RISC (RNA-induced silencing complex) which guides recognition of complementary target RNA sequence resulting in degradation and resistance (Hammond *et al.*, 2001; Campbell and Choy, 2005).

Previously, resistance (but not immunity) to WSMV in transgenic wheat has been reported using viral NIb and CP sense transgenes (Sivamani *et al.*, 2000, 2002). Transgenic constructs capable of forming dsRNA transcripts proved to be more effective in yielding high-level virus-resistant plants than constructs producing either sense or antisense RNA alone (Waterhouse *et al.*, 1998). It is now well established that sense-, antisense- and dsRNA-induced RNA silencing pathways converge at the point of dsRNA formation, and that the steps following the processing of the dsRNA are common to all three strategies (Beclin *et al.*, 2002). Smith *et al.* (2000) demonstrated that constructs encoding a spliceable intron within an hpRNA structure can induce RNAi with almost 100% efficiency. Genes transcribing hpRNA have proven effective in inducing RNAi against Barley yellow dwarf virus in both wheat and barley (Wang *et al.*, 2000).

Our approach was to target RNAi against the nuclear inclusion protein 'a' (*Nla*) gene of WSMV. The Nla is one of three virus-encoded proteinases and its NH_2-terminal half is also the VPg, a viral protein covalently bound to the 5′-terminus of genomic RNA (Shahabuddin *et al.*, 1988; Murphy *et al.*, 1990). Nla is responsible for proteolytic cleavage of viral proteins at conserved heptapeptide sequences located in the viral polyprotein, at sites other than the COOH-termini of P1 and HC-Pro (Carrington and Dougherty, 1987, 1988; Carrington *et al.*, 1988; Dougherty *et al.*, 1988).

We describe the development of the first successful transgenic strategy using a spliceable intron hpRNA approach to achieve immunity in wheat to WSMV.

Results

Generation of wheat transformants with an RNAi construct against WSMV

A highly conserved portion of the *Nla* gene of WSMV (Figure 1a, and defined in Experimental Procedures) was amplified and cloned into the vector pStargate, to generate the pStargate-*Nla* (Figure 1b) that would transcribe an hpRNA. The construct consisted of a sense and antisense copy of the *Nla* sequence, separated by spliceable pyruvate dehydrogenase kinase (pdk) intron and was driven by the maize polyubiquitin promoter. Although a hygromycin selectable marker was present on pStargate-*Nla*, transformation selection was based on cobombardment with pCMneoSTLS2 which contain an *nptII* marker and transformants were selected with 50 mg/L geneticin. A total of 16 independent T_0 transgenic wheat plants were generated with a transformation efficiency of 3.5%. All the primary transgenic lines presumed to have the hairpin transgene were designated as *hpws*. Where multiple T_0 plants were recovered from a single bombarded embryo, they were distinguished with letters, e.g. *hpws2a* and *hpws2b*. All T_1 progeny had no obvious visible difference in morphological phenotype from the parental cultivar, Bob White selection 26 (BW26).

Molecular and serological characterization of transgenic resistance to WSMV

An initial assessment of 6–8 T_1 individuals of all sixteen transgenic families indicated the presence of the selectable marker *nptII* via genomic PCR, verifying that these plants were transgenic. Further analysis involved inoculating each individual plant with WSMV and assaying with a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) at 14 days post inoculation (dpi). WSMV

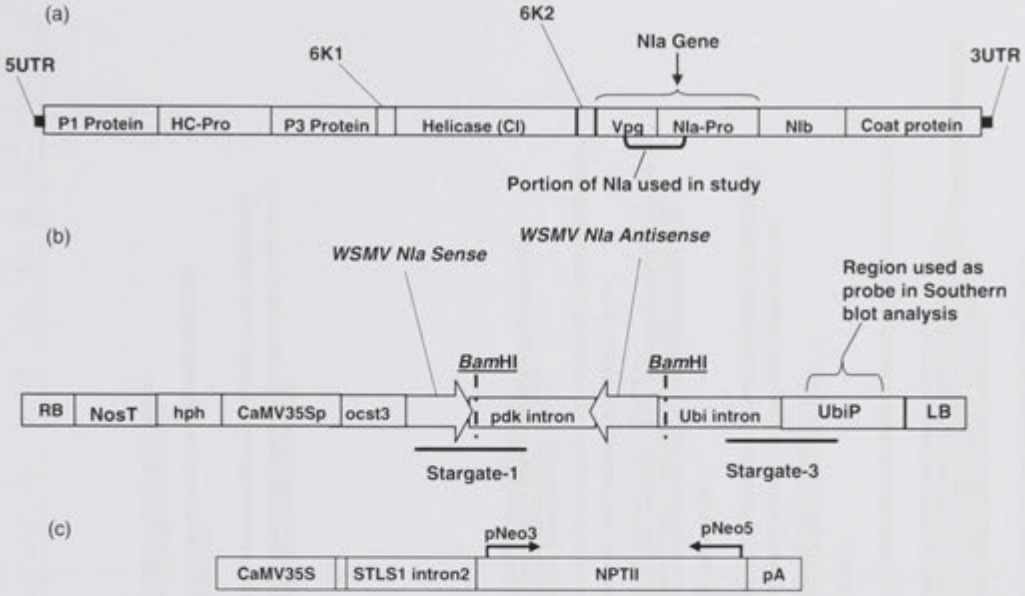


Figure 1 Structure of the *Wheat streak mosaic virus* (WSMV) genome and the pStargate-*Nla* transgene. (a) Genome map of WSMV showing the region used to generate pStargate-*Nla*, (b) Design of pStargate-*Nla* construct used to transform wheat using biolistics, and the two Stargate amplicons (1 & 3) used to characterize the putative transgenics. (c) Diagram of pCMneoSTLS2 containing the *nptII* gene for Geneticin resistance, used in the cotransformation experiments, and showing the position of the primers pNeo3 and pNeo5.

typically causes light-green to faint yellow streaks in wheat leaves parallel to the veins. As the disease progresses, affected plants appear retarded and show a general yellow mottling. Diseased plants are also moderately to severely stunted (Figure 2) with prostrated tillers often with empty spikes or spikes with shrivelled kernels.

Virus accumulation in leaves was determined using ELISA and expressed as a ratio of inoculated plants to noninoculated controls. Ten families had at least one highly resistant individual (ELISA ratio approximately 1), while all Bobwhite 26 (BW26) nontransformed control plants were highly susceptible (ELISA ratio >9) (Figure 3). All T_1 individuals of three families *hpws2a, 17* and *18* were completely immune to WSMV when challenged, suggesting they possess multiple loci of the Stargate-*Nla* transgene. Seven families were segregating for both

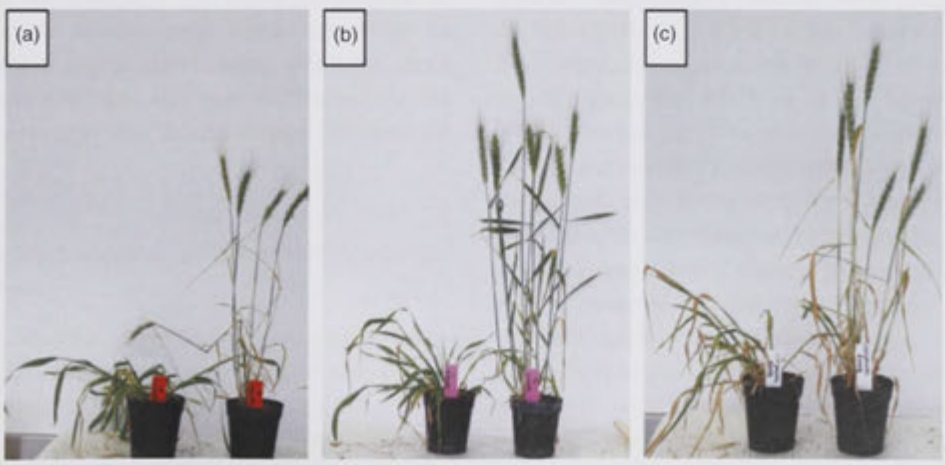


Figure 2 Phenotypes of wheat lines infected by Wheat streak mosaic virus. Wheat plants were inoculated at three leaf stages (a) family *hpws24b* where the presence of the transgene was responsible for an intermediate resistance or recovery phenotype evident in the inoculated right plant compared to an inoculated susceptible plant (left). (b) Fully resistant inoculated transgenic segregant (right) and inoculated susceptible nontransgenic segregant (left) of family *hpws2b*. (c) Bobwhite26 controls, infected (left) and uninoculated (right).

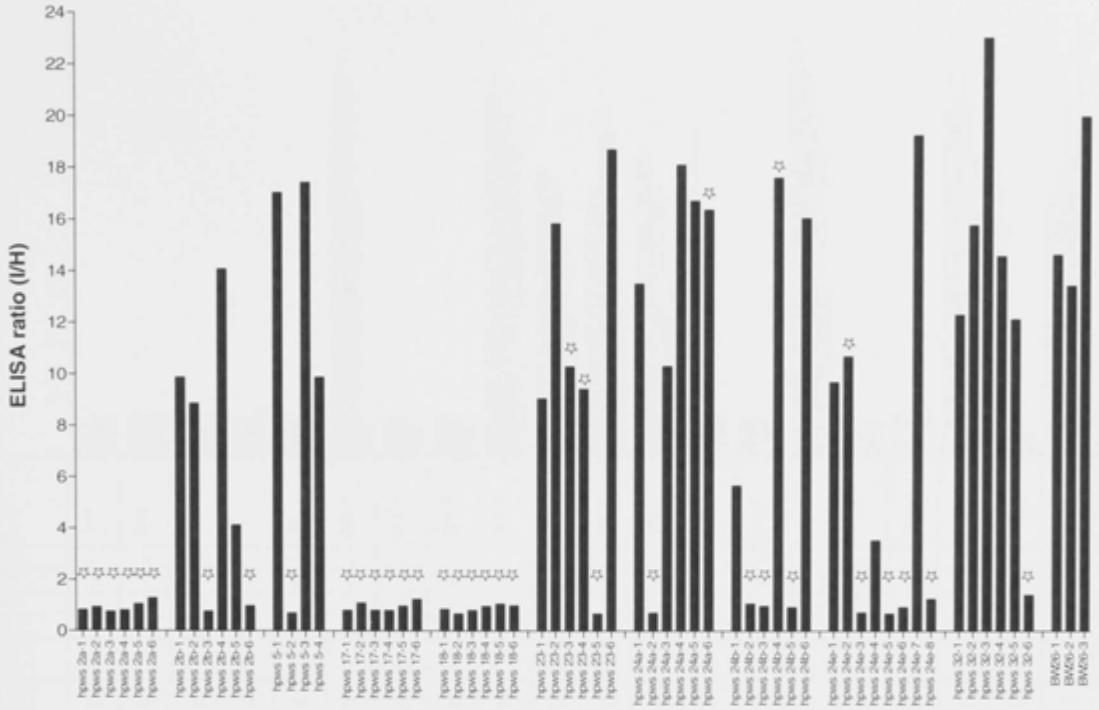


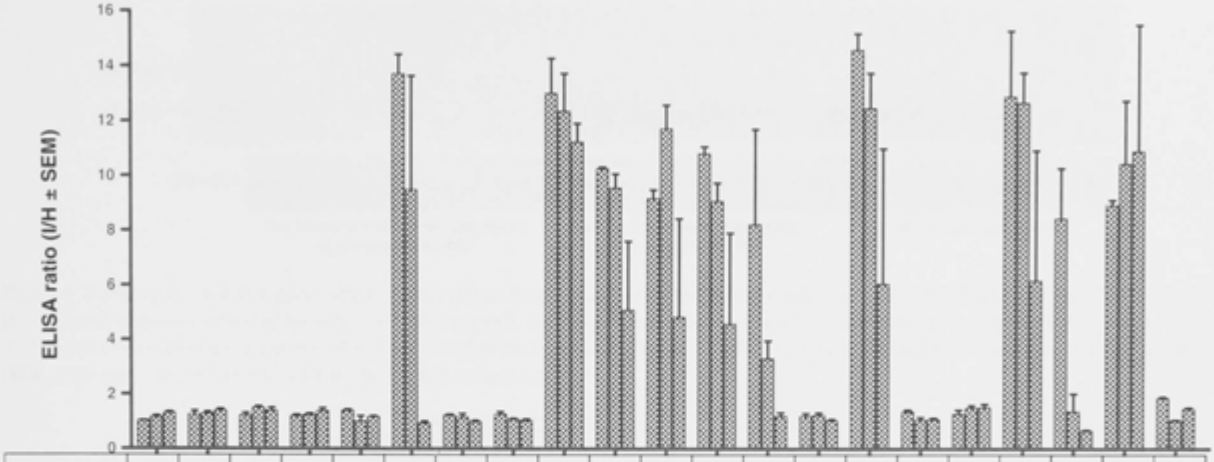
Figure 3 The T_1 segregating transgenic families showing at least one resistant individual. Virus levels were detected by ELISA 14 days post inoculation. The ELISA ratio plotted is the ELISA reading of the inoculated plant divided by the average ELISA reading of all healthy controls. The star (☆) represents the presence of both Stargate1 and Stargate3 amplicons in a transgenic plant, whereas the absence of the star indicates that at least one of the amplicons is missing.

resistant and susceptible T_1 individuals. The immune individuals in all families were indistinguishable from the healthy uninoculated controls. Both Stargate 1 and 3 amplicons (Figure 1b) could be amplified from the 10 transgenic lines, suggesting that they contained the complete Stargate-*Nla* transgene. All tested T_1 progeny of the remaining six families were highly susceptible to WSMV and lacked one or both Stargate amplicons; these families had the selectable *nptII* gene but lacked the full Stargate-*Nla* transgene and therefore were not analysed further.

The Stargate-*Nla* transgene confers immunity against WSMV in wheat

The complete absence of symptoms in inoculated transgenic individuals from some transgenic events over a number of experiments led us to hypothesize that they were immune. Experiments were conducted to see if infectious virus or viral RNA could be recovered from the resistant inoculated transgenic plants. Leaf sap from plants in four transgenic inoculated families was extracted and inoculated onto test plants of control BW26 at various dilutions to investigate the presence of any infectious WSMV particles.

Results from these dilution experiments revealed that all plants from the T_1 families of *hpws2a, hpws17* and *hpws18* were immune to WSMV as no virus was recovered and carried over to control wheat through mechanical inoculation, even at the highest sap concentration. Sap from segregants with transgenes failed to transmit infection to susceptible BW26 as judged by symptoms and ELISA, whereas sap from segregants with no transgene and nontransformed controls (BW26) did transmit infection in every case and at all dilutions (Figure 4). Transgenic lines *hpws24b* and *hpws24e*, which may be clones of the same transgenic event, showed some deviation from the pattern observed for the other transgenic families. As expected, segregants with neither Stargate amplicon had fully infectious sap and some segregants amplifying both amplicons were immune. However, some segregants (e.g. *hpws24e-2*, Figure 4) amplifying both amplicons were themselves intermediate in susceptibility and their sap was infectious, at least at the highest concentration. Also some segregants amplified only one of the Stargate amplicons (*hpws24b-1* and *hpws24e-8*, Figure 4) and



Hpws-line	2a-1	2a-2	2a-3	17	18	24b-1	24b-2	24b-3	24b-4	24b-5	24b-6	24e-1	24e-2	24e-3	24e-4	24e-5	24e-6	24e-7	24e-8	Bw26i	Bw26h
nptII (T0)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
nptII (T1)	–	+	–	+	+	+	–	+	+	–	–	–	–	+	+	–	+	+	+	–	–
Stargate 1	+	+	+	+	+	–	+	+	+	+	–	–	+	+	–	+	+	–	+	–	–
Stargate 3	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	+	+	–	–	–	–
ELISA ratio (sap donor)	1.1	1.3	1.1	1.2	1.0	4.7	0.9	0.8	14.7	0.8	13.4	9.2	10.2	0.7	3.4	0.7	0.9	18.2	1.2	15.7	1.2
Plant height (sap donor)	43	42	35	44	39	34	30	30	28	30	10	8	42	50	12	42	53	13	46	12	47

Figure 4 Virus transmission from the inoculated transgenic plants onto Bobwhite26. Sap was extracted from inoculated transgenic plants at three dilutions, 1/10, 1/250 and 1/500 from left to right in each cluster of three bars. Each dilution was inoculated onto three nontransgenic Bobwhite26 plants. At 14 dpi samples were collected and processed for *Wheat streak mosaic virus* ELISA. Plotted is the average ELISA ratio (inoculated divided by healthy) for the three test-inoculated plants for each sap dilution. Also tabulated are the molecular analysis of the T_0 parent (*nptII*), the molecular analysis of the inoculated T_1 individual serving as sap donor (*nptII*, Stargate1 and Stargate3 PCR), and the ELISA ratio and plant height (cm) at booting stage of the inoculated sap donor T_1 individual.

were intermediate in their susceptibility and yielded infectious saps.

Furthermore, even the highly sensitive RT-PCR was unable to detect viral RNA in resistant transgenic individuals. RT-PCR was carried out on all members of *2b, 17* and *24a* transgenic events representing both susceptible and resistant transgenics, as determined using ELISA reaction and the presence of both Stargate amplicons. No virus RNA was amplified from the transgenic plants that were negative for WSMV through ELISA, indicating RNA interference was fully effective and was conferring immunity. The sensitivity of the assay, and the extent of resistance, can be appreciated from the observation that WSMV sequence could not be amplified from resistant transgenics using as high as 500 ng of total RNA in the RT-PCR reaction; on the other hand, WSMV was amplified from as little as 5 pg of total RNA from infected susceptible controls (Figure 5). In other words, the suppression of viral RNA replication appears to be complete and can be quantified at more than five orders of magnitude.

Segregation of pStargate-*Nla* and pCMneoSTLS2 plasmids in transgenic wheat

The aforementioned preliminary studies indicated that resistance was cosegregating with the transgene in all *hpws* transgenic families. The inheritance of transgenic resistance was more extensively examined in larger T_1 populations of four events, *hpws2b, hpws17, hpws18* and *hpws24b*, through PCR amplification of two regions of the pStargate-*Nla* construct (Stargate1 and Stargate3) and also by WSMV bioassay. These four events were representative of the different preliminary segregation patterns observed in the smaller T_1 populations of the ten transgenic lines showing some resistance. In three of four analysed transgenic families, the resistance perfectly cosegregated with the hairpin transgene, and the immune or highly resistant plants remained symptom-less throughout the experiment (Figure 2). The plants, where one or both fragments (Stargate 1 and 3) were absent, developed characteristic symptoms of WSMV and were markedly shorter when compared to resistant transgenic plants. In *hpws24b*, the

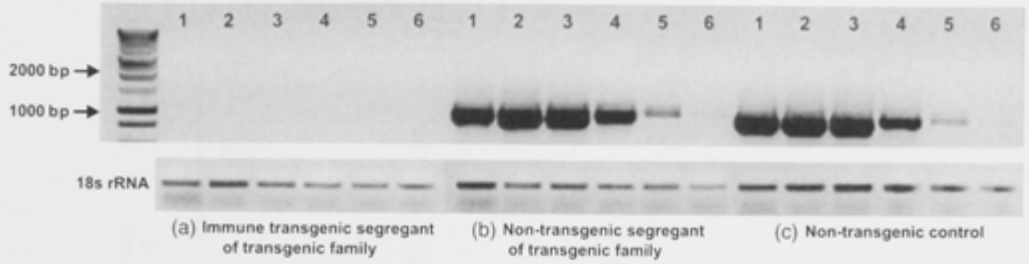


Figure 5 Immunity in *hpws* transgenic wheat against *Wheat streak mosaic virus* (WSMV) as evident from RT-PCR for WSMV in inoculated plants: (a) transgenic segregant wheat of *hpws2b-5*; (b) nontransgenic segregant wheat *hpws2b-6*; and (c) susceptible nontransgenic BW26. Lanes 1, 2, 3, 4, 5 and 6 have decreasing amounts of leaf RNA used in the RT-PR reaction: 500, 50, 5, 0.5, 0.05 and 0.005 ng, respectively. Primers for 18S rRNA (18S) were used for an internal loading standard in lower panel.

plants developed severe symptoms where one or both the amplicons of the transgene were missing. However, all transgenic wheat plants of *hpws24b* containing both amplicons developed mild to moderate symptoms.

In the T_1 family of *hpws2b*, there was perfect cosegregation of Stargate1 and Stargate3 amplicons with virus resistance (Figure 6). The segregation of resistance and transgene amplicons conformed to a 3:1 Mendelian ratio (27 resistant: 7 susceptible; $P = 0.779$ Fisher's exact test). Interestingly, the *nptII* selectable marker was segregating independently of the *hpws* insertion. This independence was confirmed by a segregation pattern consistent with a 9 : 3 : 3 : 1 ratio ($P = 0.6$, Fisher's exact test), where of 27 resistant plants, 17 carried the marker gene while 10 did not; and where of seven susceptible plants, four plants carried the marker gene while three plants did not. It therefore appears that this transgenic event had an *hpws* and *nptII* gene, integrated at independent loci. Southern blot hybridization analysis of *hpws2b*, using maize polyubiquitin promoter as a probe, indicated two copies of the transgene (Figure 8); however, the segregation data transgene suggest the two copies are integrated at a single

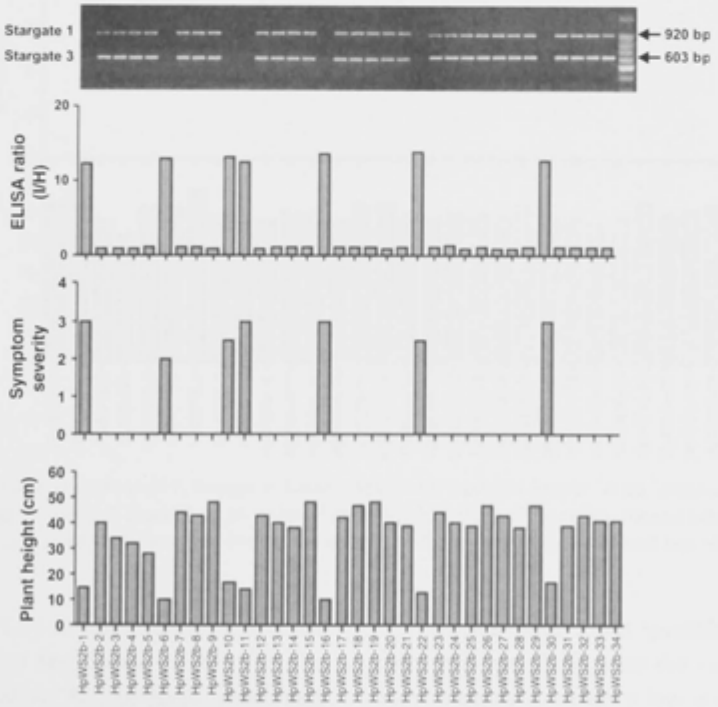


Figure 6 *Wheat streak mosaic virus* inoculation of 34 T_1 individuals of the *hpws2b* transgenic family. Shown are the PCR amplification of both Stargate fragments, ELISA ratio at 14 dpi, symptom severity (0–4 scale) and plant height (cm) at boot stage.

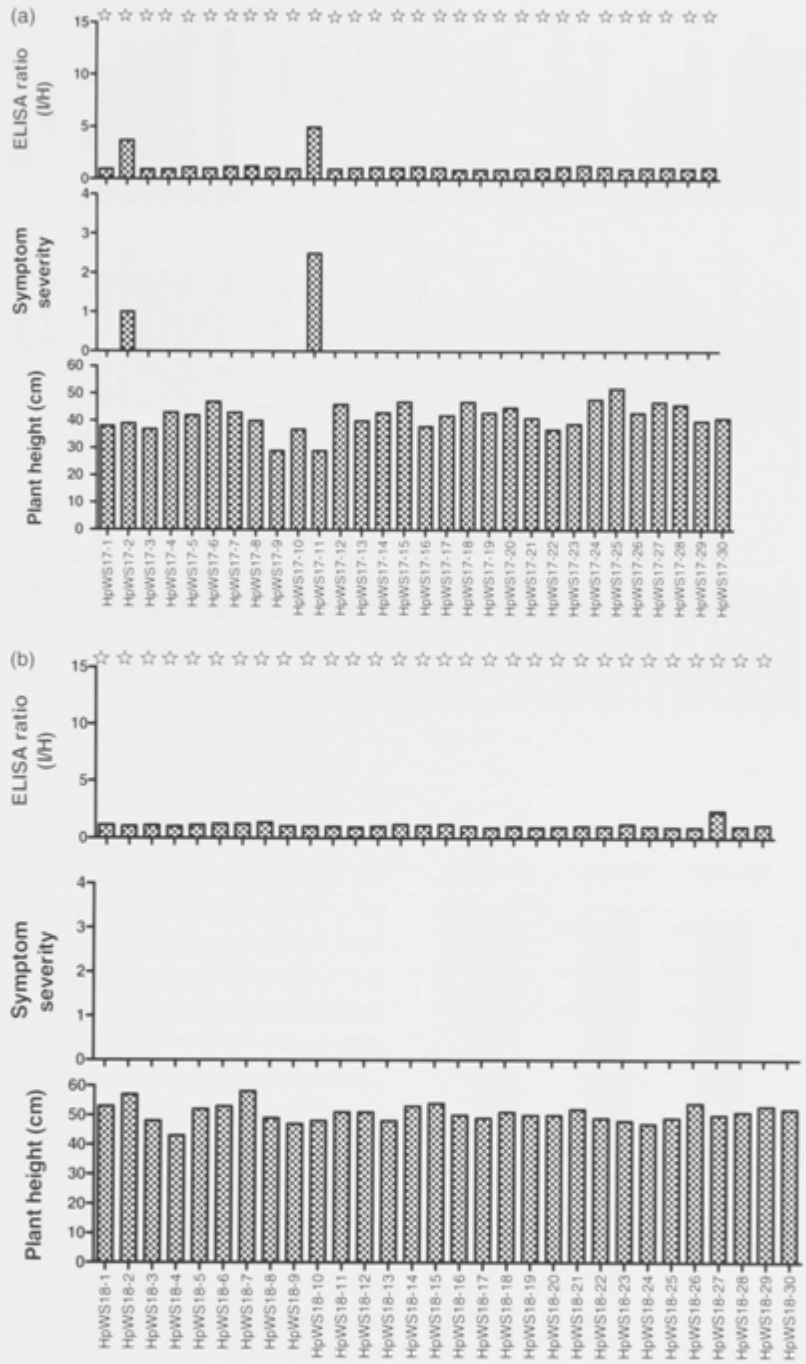


Figure 7 *Wheat streak mosaic virus* inoculation of T_1 transgenic families. Shown are the ELISA ratio at 14 dpi, symptom severity (0–4) and plant height (cm) at booting stage. (a) 30 plants of *hpws17*; (b) 30 plants of *hpws18*; (c) 36 plants of *hpws24b*. The star represents the presence of both Stargate1 and Stargate3 amplicons in a transgenic plant, whereas the absence of the star indicates that at least one of the amplicons is missing (on the next page).

locus. The small RNA Northern blot revealed that the transgene is processed into two classes of siRNA of sizes approximately 21 and 24 nt. T_1 individuals were easily recovered which had the Stargate-*Nla* transgene but were missing the selectable marker gene.

In families *hpws17* and *hpws18*, the resistance was uniform in all test plants, with the exception of two plants of family *hpws17* in which a low level of virus accumulation was evident causing mild symptoms. The ELISA ratio for *hpws17-2* and *hpws17-11* was 3.6 and 4.9, respectively,

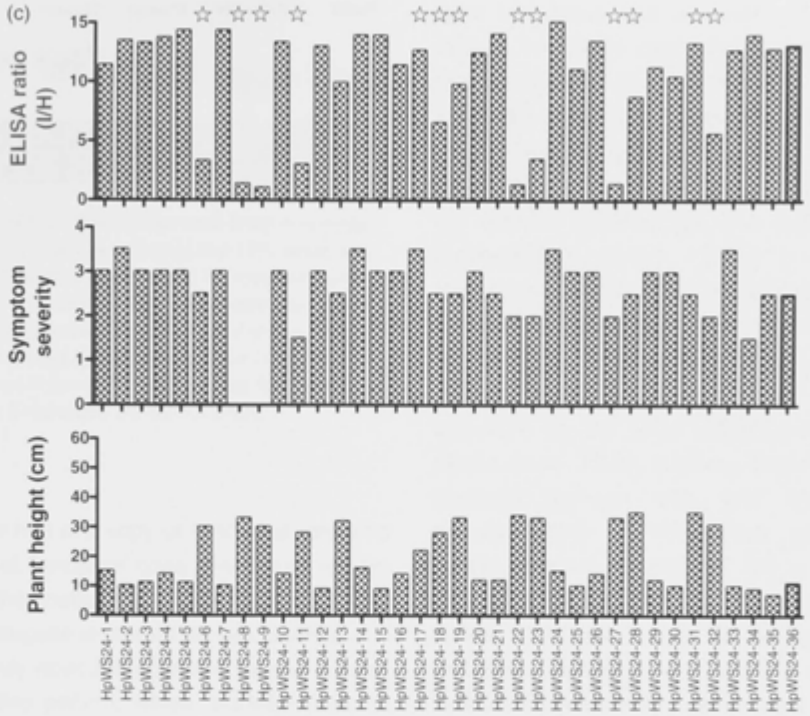


Figure 7c Continued.

indicating a slight accumulation of virus (Figure 7). However, the plants recovered by 21 dpi, and at boot stage the plant height was not significantly different from the immune inoculated plants or healthy controls. Both Stargate amplicons were successfully amplified in these two plants. These moderately resistant plants had upright tillers in contrast to the prostrate tillers of inoculated susceptible nontransgenic controls or susceptible segregants of transgenic family *hpws2b* (Figure 2). The selectable marker in these two families segregated independently of the *hpws* transgene. The segregation for resistance and the selectable marker gene in family *hpws17* was 23 R, *nptII*+ : 5 R, *nptII*– : 2 r, *nptII*+ : 0 r, *nptII*– (where R is resistant, r is susceptible). Southern blot hybridization analysis of DNA from the T_0 *hpws17* and *hpws18* plants using the maize polyubiquitin promoter as probe showed identical banding pattern with five hybridizing fragments (Figure 8). Based on this and the similar behaviour in virus bioassay, we conclude that *hpws17* and *hpws18* are clones of a single transgenic event possibly as consequence of mislabelling of callus pieces. Both *hpws17* and *hpws18* processed the transgene message to siRNA (Figure 9). There were no susceptibles in a total of 36 *hpws18* T1 individuals (Figures 3 and 7). Family *hpws18* in T1 generation segregated for *nptII* 23 + : 7–. The working hypothesis for *hpws17* and

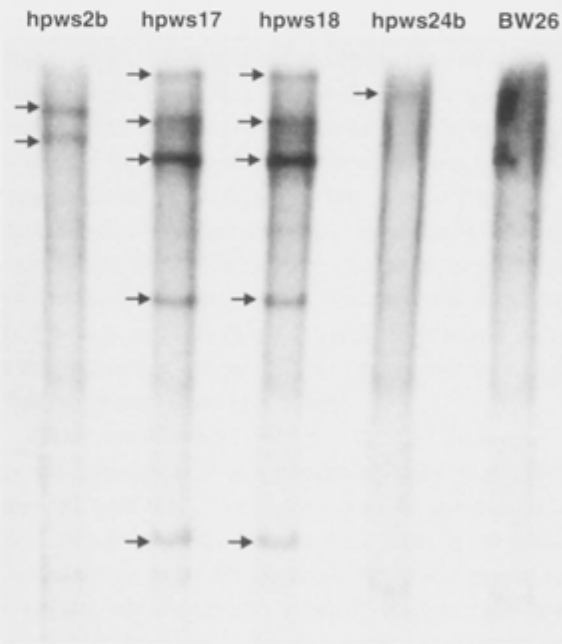


Figure 8 Southern Blot Hybridization Analysis: 15 μg of genomic DNA was digested with *Bam*HI Hybridized to the Maize Ubi promoter probe. Lanes: *1-4 transgenic plants, lane 5:* wild-type/nontransgenic control. Arrow points at the transgene insertion. The similar banding pattern in hpws17 and hpws18 indicate they were the clones of the same transgenic event.

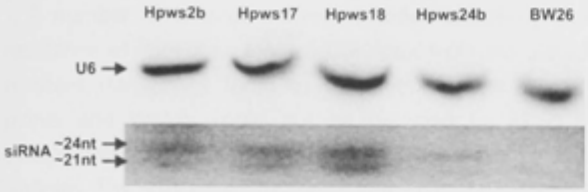


Figure 9 Northern Blot of transgene derived small RNAs in noninfected transgenic plants: Small RNA was separated in a 17% denaturing polyacrylamide gel, electroblotted onto Hybond N+ membrane and hybridized with ^{32}P-labelled antisense RNA corresponding to the viral sequence used in the pStargate-Nla. The upper panel shows the small nuclear RNA, U6, transcript as a loading control. Four of the total small RNA samples shown in (lanes 1–4) were derived from transgenic hpws wheat, while lane 5 represents the control BW26).

hpws18 is that they had one copy of functional *nptII* and at least five copies of functional *hpws*. The higher relative intensity of one of the Southern bands also suggests multiple copies of the transgene at one site as concatamers.

The plants in family *hpws24b* appeared to have a non-Mendelian segregation pattern, where fourteen of 36 T_1 plants amplified both Stargate amplicons. Initially only two plants, *hpws24b-8* and *hpws24b-9*, were resistant to WSMV inoculation at 14 dpi (Figure 7) and 21 dpi, but the resistance in *hpWS24b-8* started breaking down at 28 dpi. The *hpws24b* plants, with both amplicons, had erect stems and achieved reasonable height when compared to susceptible controls. In some plants only one Stargate amplicon was amplified and these were completely susceptible; some with Stargate 1 only, and some with Stargate 3 only. The segregation pattern for *nptII* was independent of the transgene. Only one plant, *hpws24b-9*, was fully resistant by ELISA at 28 dpi, and had no *nptII* selectable marker. Thirteen plants displayed intermediate resistance phenotypes where the virus accumulated but the plant developed normally. Of the 23 susceptible transgenics, 17 did not have the *nptII* selectable marker, while 6 had an *nptII* insert. The Southern blot analysis using the maize polyubiquitin promoter as probe, suggested a single insert of the *hpws* transgene (Figure 8). The accumulation of siRNA in *hpws24b* appears to be less than that in *hpws 2a*, *17* or *18* which might suggest the single insert is not fully of functional copy of the transgene in the transgenic line (Figure 9).

Discussion

It was notable that in a relatively small set of transgenics, events were obtained with a single insertion of the transgene of interest and that were completely immune to WSMV. In selected lines, the immunity cosegregated with the transgene in simple Mendelian ratios. It is assumed that RNA silencing, induced by the expression of the intron-separated hpRNA from the WSMV-*Nla* gene, was responsible for the observed immunity to WSMV. Targeting the *Nla* sequence has also been effective in other potyvirus-plant systems. The percentage of PVY-resistant transgenic tobacco plants obtained by targeting the PVY *Nla* protease gene was 7% for the sense gene, 4% for the antisense gene, 58% for the hpRNA with a nonspliceable loop separating the sense and antisense arms, and 96% for the same hpRNA with a spliceable intron (Smith *et al.*, 2000). Similarly, Mitter *et al.* (2003, 2006) observed between 40% and 50% highly resistant tobacco plants in independent transformations using hpRNA directed against PVY *Nla* in which the inverted repeats were separated by an unrelated loop sequence. Others have also described the greater efficiency of protection associated with the use of a splicable intron hpRNA (Sivamani *et al.*, 2000, 2002; Smith *et al.*, 2000; Wesley *et al.*, 2001).

Cotransformation with different transgenes carried on two separate plasmids has been shown to facilitate the segregation of transgenes of interest from selectable marker genes (Komari *et al.*, 1996; Matthews *et al.*, 2001; Huang *et al.*, 2004; Vidal *et al.*, 2006; Zhao *et al.*, 2007; Jayaraj *et al.*, 2008). It is evident also in this study that a number of our transgenic events had the transgene and selectable marker inserted in unlinked loci and segregating independently of each other. This was very evident in *hpws2b*. This technique therefore will permit the development of marker-free WSMV immune plants. Modifications of the transformation protocol, especially the molar ratio of the two plasmids may be useful in achieving desired cotransformation efficiencies and opportunity for marker-free segregants (Chen *et al.*, 1998).

Wheat streak mosaic virus may be seed transmitted in wheat although at a low frequency (Jones *et al.*, 2005), infected seed may serve as initial foci for infection in the field. *Acaria tosichella*, the wheat curl mite, is the primary natural vector of WSMV (Slykhuis, 1955) and plays a very important role in secondary spread of the virus, and development of epidemics under favourable agro-climatic conditions. It is therefore of particular epidemiological importance that the *hpws* construct could achieve immunity, with no prospect of infectious particles being recovered for secondary infection, especially for non- and semi-persistently transmitted viruses.

A number of lines of evidence justified classifying the resistance as immunity. In particular, saps from inoculated resistant transgenics failed to transmit infection to test plants and WSMV could not be detected by RT PCR analysis even from 500 ng of leaf RNA from the same leaf samples. The resistance suppressed viral RNA accumulation more than 10^5 fold in the immune transgenic *hpws* events when compared to susceptible controls. Our working hypothesis is that the transgenic plants were preprimed by the *hpws* transgene so that corresponding RISC RNA–protein complexes were ready to degrade invading viral RNA and thus effective at preventing any viral replication.

T1 progeny of *hpws17* and *hpws18*, with multiple inserts, were almost all immune and nonsegregating for the transgene. The evidence suggests in these cases that the multiple inserts continue to be functional and are not inactivated through mechanisms such as methylation. Two plants, *hpws17-2* and *hpws17-11*, were not immune but did display a recovery type of resistance; the initial virus accumulation at 14 dpi did not arrest normal plant growth and at the booting stage the plant height was not significantly different from that of immune transgenics or uninoculated controls. Transgenic line *hpws24b* produced a number of segregants with moderate resistance that changed over time to susceptible phenotypes. Transgenic individuals were symptom free up to at least 14 dpi when nontransgenic controls had evident symptoms, but later developed symptoms and higher ELISA ratios.

This article reports for the first time engineered RNAi-mediated immunity in wheat against WSMV using an hpRNA derived from nuclear inclusion protein a (*Nla*) protease gene. WSMV is arguably the third most important virus of wheat behind BYDV and CYDV (barley and cereal yellow dwarf viruses). Marker-free transgenic plants have advantages with respect to regulatory approval and public acceptance (Miki and McHugh, 2004; Miki *et al.*, 2009). Our study indicates that marker-free WSMV immune plants can be readily produced using biolistics and cobombardment.

Experimental procedures

Generation of RNAi Construct

Wheat streak mosaic virus sequences were retrieved using VirusFASTAs® Rothamsted Research Institution (Harpenden, UK) Version 2005.05.20. Sequences of *Nla* region from five full genome sequences (Type strain AF2851169, Turkey Isolate AF454455, Czech Isolate AF454454, El-Batan strain AF285170 and Sydney Isolate AF057533) were retrieved and aligned using AlignX (Vector NTI Advance® 10.3.0; Invitrogen, Carlsbad, California, USA). Primers were designed against the *Nla* gene (Figure 1a) based on the most conserved regions identified from the alignment.

Samples were collected from infectious wheat plants in Canberra in June 2006 and were preserved at −80 °C. RNA was extracted from leaf tissues with Qiagen miniprep kit, following manufacturer's instructions. One step reverse transcription (RT) reactions were performed using Qiagen onestep RT PCRKIT. Primers *Nla*F1(5554) 5′-CTGGACCGATCGGATTAAGA-3′ (forward) and *Nla*R2(6249) 5′-GGCAAGGTTAATGCTACCAGATCC-3′ (reverse) were used for *Nla* amplification using 50 °C for 30 min, 95 °C for 15 min, followed by (94 °C for 30 s, 60 °C for 45 s, 72 °C for 60 s) for 40 cycles with a final extension at 72 °C for 10 min. The amplified fragment was cloned into pGEM-T-Easy vector (Promega), sequenced and aligned with reported WSMV sequences to confirm the identity of the virus.

To generate the RNAi constructs, a 696-bp Topo fragment, covering 451 bp of the genome-linked protein (Vpg) and 245 bp of nuclear inclusion protein (*Nla*) genes including the cleavage site between the two genes, was generated using primers, *Nla*F1-Topo1, 5′-CACCtcctcacaatactggcacttcta-3′ and *Nla*R2(6249) 5′-GGCAAGGTTAATGCTACCAGATCC-3′ using PCR amplification from the *Nla* clone. The resulting PCR fragment was cloned into pENTR/D-TOPO® Entry vector (Invitrogen, Carlsbad, California, USA), which was subsequently transferred into pStargate (monocot version of pHellsgate, Wesley *et al.*, 2001) destination vector by a single LR Clonase reaction (Invitrogen). In these reactions, the PCR-derived fragments are inserted into two regions flanked by two recombination sites (*attB1* and *attB2*) in opposite directions, and the spliceable intron is flanked by the two inverted repeats. The resulting plasmid was called pStargate-*Nla* (Figure 1b).

pStargate, a binary vector, is a modification of pHellsgate for use in *Agrobacterium*-mediated transformation in monocot plants and uses the same Gateway™ (Invitrogen, Carlsbad, California, USA) recombination system. It contains a maize ubiquitin promoter and its intron for the hairpin construct, along with a 35S: *hph* for plant selection using hygromycin, and spectinomycin resistance gene for bacterial selection. (http://www.pi.csiro.au/RNAi/vectors.htm). Biolistics rather than *Agrobacterium*-mediated transformation was employed in these studies.

For confirmation of insertion in the binary vector, pStargate, with and without RNAi fragment VPg-*Nla*, was digested with *NotI* at 37 °C for 3 h and compared with simulated restriction analysis carried out with Vector NTI Advanced (10.03.0). The constructs were also validated via sequencing.

Selected colonies of *Escherichia coli* with pStargate-*Nla* were grown in 400 mL of LB media with spectinomycin (100 μg/μL) and the plasmid DNA was extracted using Nucleobond Machery Nagel® (Duren, Germany) Maxi kit following manufacturer's instructions, for use in wheat transformation with biolistics.

Transformation of wheat embryos and regeneration of transgenic plants

The scutella of 450 freshly isolated embryos from BobWhite 26 spring wheat, at about 14 days post anthesis were cotransformed

by biolistics bombardment with DNA of pStargate-*Nla* and pCMneoSTLS2 (Maas *et al.*, 1997) carrying *nptII* as plant selectable marker; three fold greater weight of pCMneoSTLS2 was used to give approximately equal moles of the two plasmids. The biolistics and tissue culture protocols were large as previously described (Pellegrineschi *et al.*, 2002). The only modification was the use of Phytagel instead of Bacto-Agar (Terese Richardson, unpublished data). At the end of the tissue culture phase, T_0 plants were transferred to a glasshouse and seed collected individually from each spike separately as a precaution against transplanted plants containing unseparated shoots from different events.

Analysis of T_0 transgenic plants - genomic PCR

DNA extraction was carried out using ''DNAeasy Plant Mini Kit'' following manufacturer's instructions (Qiagen Inc., Valencia, CA USA), and PCR was carried out for selectable marker *nptII* using PCR. Approximately 700-bp *nptII* fragment was amplified using the forward primer Neo3 5'- TACGGTATCGCCGCTCCCGAT-3' and reverse primer Neo5 5'-GGCTATTCGGCTATGACTG -3', both sequences being in the *nptII* coding region using the following thermocycler conditions: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s for 40 cycles with a final extension at 72 ° C for 10 min.

Analysis of Transgenic plants – Southern hybridization

Because limited DNA was available from T_0 plants, the primary DNA insertion profile was determined by Southern hybridization of DNA collected and pooled from 30 T_1 plants. Wheat genomic DNA was extracted from 30 T_1 transgenic wheat plants and used for Southern hybridization analysis as previously described by Lagudah *et al.* (1991). Approximately 15 μg of DNA was digested with *Bam*HI for 3 h and separated by 1% agarose gel electrophoresis and blotted onto Hybond™ (GE Healthcare, Rydalmere, Australia) N^+ membrane. *BamHI* cuts outside the probe region (Fig 1b). Maize polyubiquitin promoter was radio-labelled as probe, with [α-^{32} P] dCTP (NEN) using the multiprime system (Amersham, IL USA). DNA hybridization was carried out in 50% formamide, 6X SSPE (1x SSPE: 0.15 M NaCl, 10 mM NaH_2PO_4 H_2O, and 1 mM EDTA, pH 7.4), 5X Denhardt's reagent, 6% dextran sulphate, and denatured salmon sperm DNA (100 μg/ml) at 42 °C. The hybridized membranes were washed at 20- to 30-minutes intervals at 65 °C with three changes of 2X SSC, 0.1% SDS and a final wash with 1X SSC, 0.1% SDS(1X SSC; 0.15 M NaCl, 0.015 M sodium citrate and analysed using a Fujifilm FLA-5000 phosphorimager.

Analysis of T_1 transgenic plants – genomic PCR

T_1 seeds of each transgenic event were kept on moist filter paper in petri plates for 3–4 days, and the germinated seeds were transferred to pots. Approximately a three-centimetre young leaf was collected from each plant and freeze dried. Genomic DNA was extracted in DNA extraction buffer containing 0.1 M Tris-HCL, pH 8.0, 0.05 M EDTA pH 8.0, 1.25% SDS.

Primers were designed against the two extremes of the hairpin construct. Primers Stargate 1F 5'-ATATCATGCGATCATAGGCG-TCTCG-3' and Stargate 1R 5'-ATGATGATAACTGCAGCGCAAG-CTT-3' covering regions from pdk intron through *Nla* sense fragment to OcsT 3' terminator and amplifying 920-bp PCR product. Primers Stargate-3F 5'-CCCAAAGAGAAACACTGGCA-3' and Stargate-3R 5'-TAAACGCCGTCGACGAGTCTAA-3', covering Ubi Intron and Ubi Promotor, were used to amplify a PCR product of 603 bp.

PCR reaction was performed in Thermalcycler PC-960C (Corbett Research, Mortlake, N.S.W., Australia) with the following protocol: 95 °C for 15 min; (94 for 1 min; 63 °C (stargate1)/65 °C (stargate3) for 45 s; 72 °C for 1 min) × 35 cycles and final extension of 10 min at 72 °C. Two amplicons were used in the study to assay for both ends of the hpRNA transgene including a large portion of the promoter (Figure 1b).

Analysis of T_1 transgenic plants – virus bioassay

Virus inoculum was prepared by grinding WSMV infected tissue in a mortar and pestle at a 1 : 10 w/v ratio in 0.02 M potassium phosphate buffer (pH 7). The homogenate was filtered through four layers of Miracloth® (Calbiochem, La Jolla, California, USA), abrasive Celite (Spectrum Chemicals, Gardena, California, USA) was added at 2% w/v to the final volume of inoculum, and the mixture was left on ice for 1 h. Putative transgenic BW26 plants were doubly inoculated at the 2–3 leaf stage, with the prepared sap extracts from WSMV-infected leaf material. The sap plus Celite abrasive was first applied with an air-powered spray gun and then leaves were gently rubbed with gloved fingers to ensure the infection of plant by the virus. The plants were scored for symptoms at 14 dpi on a scale of 0–4 with 0 as healthy, 1 as mild with very few streaks, 2 as moderate with streaks that coalesce, 3 as severe with approximately 50% leaf area with streaks, 4 as the most severe or lethal symptoms where the streaks develop into chlorosis of more than 70% of leaf area. Samples were collected for WSMV-specific ELISA using Agdia reagents (Elkhart, IN) following manufacturer's instructions. Plates were read at A_{405nm} in ELISA Reader Spectra Max 340 PC (Molecular Devices, CA USA) 60 min after addition of substrates. Healthy controls were included on every plate, every sample was duplicated, and means were used in calculating the ELISA value ratio between inoculated and healthy controls. Data were also recorded on the fertility and height of plants.

Analysis of T_0 transgenic plants- Northern blot of small RNAs

For Northern blot hybridization analysis, total RNA was prepared from leaf of plants at the four-leaf stage by using the TRIzol reagent (Invitrogen), separated in 17% polyacrylamide gels, the RNA was electroblotted and UV-cross-linked onto a Hybond N Plus membrane (Amersham Bioscience, Piscataway, NJ, USA). Using PerfectHyb (Sigma, St Louis, Missouri, USA), the membrane was prehybridized for 2 h at 42 °C. The membrane was hybridized with SP6 polymerase-synthesized, α-^{32}P-labelled riboprobes, obtained by *in vitro* transcription from the viral sequence used in

the pStargate-Nla and cloned into pGEM-T Easy plasmids (Promega, Madison, WI, USA). Hybridization was performed overnight at 42 °C, and then membranes were washed four times using low-stringency washing buffer (2× SSC, 0.1% SDS) at 50 °C and imaged by using a Fujifilm FLA-5000 phosphorimager.

Detection of WSMV RNA from inoculated transgenic lines

Total RNA was extracted from WSMV inoculated transgenic plants using Qiagen RNAeasy mini kit following manufacturer's instructions. Concentration of 500 ng total RNA was serially diluted in 1 : 10 steps to 5 pg (final 10^{-5}). In order to amplify viral RNA but avoid amplifying transcripts from the transgene, primers were designed to hybridize the sequences just outside the cloned *Nla* sequence used in the transgene. The primers used were *Nla*-1F 5′-CTGGACCGATCGGATTAAGA-3′ and *Nla*-3R 5′-CTGAGAACTT CCATGGCACA-3′ and amplified a 1045-bp viral product. Reverse transcription (RT) reaction was carried out at 50 °C for 30 min, following by 95 °C for 15 min; (94 for 1 min; 60 °C for 45 s; 72 °C for 1 min) × 35 cycles and final extension of 10 min at 72 °C.

Test-inoculation to detect infectious virus in leaf sap

Sap was extracted from inoculated transgenic plants at 28 dpi using 0.02 M potassium phosphate buffer; the initial concentration was 1 : 10 (w leaf/v buffer). This was further diluted to 1 : 250 and 1 : 500 concentrations. Each dilution was mixed with Celite abrasive and then inoculated onto three plants each. This method was used to evaluate the effectiveness of the hpRNA construct in eliminating viral replication and preventing the formation of infectious particles. Symptoms were scored and leaf samples collected 14 dpi for ELISA as described previously.

Segregation analysis of *Nla* transgene and resistance in selected T_1 families

Twenty five to 35 seeds from four selected transgenic line were germinated in pots. Leaf samples were collected and DNA was extracted as described previously and Genomic PCR carried to detect both Stargate 1 and Stargate 3 amplicons (Figure 1b), to ensure the presence of the transgene promoter and hairpin construct. In order to observe if resistance cosegregated with the transgene, the plants were inoculated with WSMV as described previously, ELISA was performed 14 dpi on inoculated plants, plant heights and symptoms were recorded. Segregation of selectable marker *nptII* was also determined using PCR as described previously.

Acknowledgements

We acknowledge AusAID for the studentship support of MF; Peter Waterhouse for invaluable advice on hpRNA design; Terese Richardson, Anna Mechanicos and Jenny Gibson for technical support and Craig Wood, Neil Smith and Sam Kuppusamy for assistance with Northern blot of small RNAs and Southern blot.

References

Abel, P.P., Nelson, R.S., De, B., Hoffmann, N., Rogers, S.G., Fraley, R.T. and Beachy, R.N. (1986) Delay of disease development in transgenic plants that express the Tobacco mosaic virus coat protein gene. *Science*, **232**, 738–743.

Baley, G.J., Talbert, L.E., Martin, J.M., Young, M.J., Habernicht, D.K., Kushnak, G.D., Berg, J.E., Lanning, S.P. and Bruckner, P.L. (2001) Agronomic and end-use qualities of Wheat streak mosaic virus resistant spring wheat. *Crop Sci.* **41**, 1779–1784.

Beclin, C., Boutet, S., Waterhouse, P. and Vaucheret, H. (2002) A branched pathway for transgene-induced RNA silencing in plants. *Curr. Biol.* **12**, 684–688.

Campbell, T.N. and Choy, F.Y.M. (2005) RNA Interference: Past, Present and Future. *Curr. Issues Mol. Biol.* **7**, 1–6.

Carrington, J.C. and Dougherty, W.G. (1987) Processing of the Tobacco etch virus 49k protease requires autoproteolysis. *Virology*, **160**, 355–362.

Carrington, J.C. and Dougherty, W.G. (1988) A viral cleavage site cassette - identification of amino-acid sequences required for Tobacco etch virus polyprotein processing. *Proc. Natl Acad. Sci. USA*, **85**, 3391–3395.

Carrington, J.C., Cary, S.M. and Dougherty, W.G. (1988) Mutational Analysis of Tobacco Etch virus polyprotein processing - Cis and Trans proteolytic activities of polyproteins containing the 49-kilodalton proteinase. *J. Virol.* **62**, 2313–2320.

Chen, W.P., Gu, X., Liang, G.H., Muthukrishnan, S., Chen, P.D., Liu, D.J. and Gill, B.S. (1998) Introduction and constitutive expression of a rice chitinase gene in bread wheat using biolistic bombardment and the bar gene as a selectable marker. *Theor. Appl. Genet.* **97**, 1296–1306.

Choi, I.R., Hall, J.S., Henry, M., Zhang, L., Hein, G.L., French, R. and Stenger, D.C. (2001) Contributions of genetic drift and negative selection on the evolution of three strains of wheat streak mosaic tritimovirus. *Arch. Virol.* **146**, 619–628.

Conner, R.L., Thomas, J.B. and Whelan, E.D.P. (1991) Comparison of mite resistance for control of wheat streak mosaic Crop Science. *Crop Science*, **31**, 315–318.

Di Nicola-Negri, E., Brunetti, A., Tavazza, M. and Ilardi, V. (2005) Hairpin RNA-mediated silencing of Plum pox virus P1 and HC-Pro genes for efficient and predictable resistance to the virus. *Transgenic Res.* **14**, 989–994.

Divis, L.A., Graybosch, R.A., Peterson, C.J., Baenziger, P.S., Hein, G.L., Beecher, B.B. and Martin, T.J. (2006) Agronomic and quality effects in winter wheat of a gene conditioning resistance to wheat streak mosaic virus. *Euphytica*, **152**, 41–49.

Dougherty, W.G., Carrington, J.C., Cary, S.M. and Parks, T.D. (1988) Biochemical and mutational analysis of a plant-virus polyprotein cleavage site. *EMBO J.* **7**, 1281–1287.

Dwyer, G.I., Gibbs, M.J., Gibbs, A.J. and Jones, R.A.C. (2007) Wheat streak mosaic virus in Australia: relationship to isolates from the Pacific Northwest of the USA and its dispersion via seed transmission. *Plant Dis.* **91**, 164–170.

Ellis, M.H., Rebetzke, G.J. and Chu, P. (2003) First report of Wheat streak mosaic virus in Australia. *Plant Pathol.* **52**, 808.

Ellis, M.H., Rebetzke, G.J., Kelman, W.M., Moore, C.S. and Hyles, J.E. (2004) Detection of Wheat streak mosaic virus in four pasture grass species in Australia. *Plant Pathol.* **53**, 239.

French, R. and Stenger, D.C. (2003) Evolution of Wheat streak mosaic virus: dynamics of population growth within plants may explain limited variation. *Annu. Rev. Phytopathol.* **41**, 199–214.

Fuentes, A., Ramos, P.L., Fiallo, E., Callard, D., Sanchez, Y., Peral, R., Rodriguez, R. and Pujol, M. (2006) Intron-hairpin RNA derived from replication associated protein C1 gene confers immunity to Tomato Yellow Leaf Curl Virus infection in transgenic tomato plants. *Transgenic Res.* **15**, 291–304.

Hammond, S.M., Caudy, A.A. and Hannon, G.J. (2001) Post-Transcriptional Gene Silencing by Double-Stranded RNA. *Nat. Rev. Gen.* **2**, 110–119.

Harvey, T.L. and Seifers, D.L. (1991) Transmission of wheat streak mosaic virus to sorghum by the wheat curl mite (Acari, Eriophyidae). *J. Kans. Entomol. Soc.* **64**, 18–22.

Huang, S.S., Gilbertson, L.A., Adams, T.H., Malloy, K.P., Reisenbigler, E.K., Birr, D.H., Snyder, M.W., Zhang, Q. and Luethy, M.H. (2004) Generation of marker-free transgenic maize by regular two-border Agrobacterium transformation vectors. *Transgenic Res.* **13**, 451–461.

Jayaraj, J., Liang, G.H., Muthukrishnan, S. and Punja, Z.K. (2008) Generation of low copy number and stably expressing transgenic creeping bentgrass plants using minimal gene cassette bombardment. *Biol. Plant.* **52**, 215–221.

Jiang, J., Friebe, B., Dhaliwal, H.S., Martin, T.J. and Gill, B.S. (1993) Molecular cytogenetic analysis of agropyron-elongatum chromatic in wheat germplasm specifying resistance to wheat streak mosaic virus. *Theor. Appl. Genet.* **86**, 41–48.

Jones, R.A.C., Coutts, B.A., Mackie, A.E. and Dwyer, G.I. (2005) Seed transmission of Wheat streak mosaic virus shown unequivocally in wheat. *Plant Dis.* **89**, 1048–1050.

Kalantidis, K., Psaradakis, S., Tabler, M. and Tsagris, M. (2002) The occurrence of CMV-specific short RNAs in transgenic tobacco expressing virus-derived double-stranded RNA is indicative of resistance to the virus. *Mol. Plant Microbe Interact.* **15**, 826–833.

Komari, T., Hiei, Y., Saito, Y., Murai, N. and Kumashiro, T. (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. *Plant J.* **10**, 165–174.

Lagudah, E.S., Appels, R. and McNeil, D. (1991) The nor-D3 locus of Triticum-Tauschii – natural variation and genetic-linkage to markers in chromosome-5. *Genome* **34**, 387–395.

Maas, C., Simpson, C.G., Eckes, P., Schickler, H., Brown, J.W.S., Reiss, B., Salchert, K., Chet, I., Schell, J. and Reichel, C. (1997) Expression of intron modified *Nptll* genes in monocotyledonous and dicotyledonous plant cells. *Mol. Breeding*, **3**, 15–28.

Makkouk, K. and Kumari, S. (1997) Natural occurrence of wheat streak mosaic virus on wheat in Syria. *Rachis*, **16**, 74–76.

Matthews, P.R., Wang, M.B., Waterhouse, P.M., Thornton, S., Fieg, S.J., Gubler, F. and Jacobsen, J.V. (2001) Marker gene elimination from transgenic barley, using co-transformation with adjacent 'twin T-DNAs' on a standard Agrobacterium transformation vector. *Mol. Breeding*, **7**, 195–202.

Miki, B. and McHugh, S. (2004) Selectable marker genes in transgenic plants: applications, alternatives and biosafety. *J. Biotechnol.* **107**, 193–232.

Miki, B., Abdeen, A., Manabe, Y. and MacDonald, P. (2009) Selectable marker genes and unintended changes to the plant transcriptome. *Plant Biotechnol. J.* **7**, 211–218.

Mitter, N., Sulistyowati, E. and Dietzgen, R.G. (2003) Cucumber mosaic virus infection transiently breaks dsRNA-Induced Transgenic immunity to Potato virus Y in tobacco. *Mol. Plant Microbe Interact.* **16**, 936–944.

Mitter, N., Mitchell, R. and Dietzgen, R.G. (2006) Fate of hairpin transcript components during RNA silencing and its suppression in transgenic virus-resistant tobacco. *J. Biotechnol.* **126**, 115–122.

Murphy, J.F., Rhoads, R.E., Hunt, A.G. and Shaw, J.G. (1990) The vpg of Tobacco etch virus-RNA is the 49-kda proteinase or the n-terminal 24-kda part of the proteinase. *Virology*, **178**, 285–288.

Murray, G., Simpfendorfer, S., Hind-Lanioiselet, T., Lanoiselet, V. and Wratten, K. (2007) *Wheat Streak Mosaic: A Threat to Grazing and Main Season Wheats that Can Be Beaten, in GRDC Research Update*. Waga Waga: New South Wales Department of Primary Industries and EH Graham Center for agriculture Innovation, pp. 63–69,

Nyitrai, A. (1991) Investigation on the damage caused by wheat streak mosaic virus. *Novenytermeles*, **40**, 21–26.

Pellegrineschi, A., Noguera, L.M., Skovmand, B., Brito, R.M., Velazquez, L., Salgado, M.M., Hernandez, R., Warburton, M. and Hoisington, D. (2002) Identification of highly transformable wheat genotypes for mass production of fertile transgenic plants. *Genome*, **45**, 421–430.

Rabenstein, F., Seifers, D.L., Schubert, J., French, R. and Stenger, D.C. (2002) Phylogenetic relationships, strain diversity and biogeography of tritimoviruses. *J. Gen. Virol.* **83**, 895–906.

Seifers, D.L., Martin, T.J., Harvey, T.L. and Gill, B.S. (1995) Temperature sensitivity and efficacy of Wheat streak mosaic-virus resistance derived from *Agropyron intermedium*. *Plant Dis.* **79**, 1104–1106.

Seifers, D.L., Harvey, T.L., Martin, T.J. and Jensen, S.G. (1998) Partial host range of the high plains virus of corn and wheat. *Plant Dis.* **82**, 875–879.

Seifers, D.L., Martin, T.J., Harvey, T.L., Haber, S. and Haley, S.D. (2006) Temperature sensitivity and efficacy of Wheat streak mosaic virus resistance derived from CO960293 wheat. *Plant Dis.* **90**, 623–628.

Seifers, D.L., Martin, T.J., Harvey, T.L. and Haber, S. (2007) Temperature-sensitive Wheat streak mosaic virus resistance identified in KS03HW12 wheat. *Plant Dis.* **91**, 1029–1033.

Shahabuddin, M., Shaw, J.G. and Rhoads, R.E. (1988) Mapping of the Tobacco vein mottling virus vpg cistron. *Virology*, **163**, 635–637.

Sharp, G.L., Martin, J.M., Lanning, S.P., Blake, N.K., Brey, C.W., Sivamani, E., Qu, R. and Talbert, L.E. (2002) Field evaluation of transgenic and classical sources of Wheat streak mosaic virus resistance. *Crop Sci.* **42**, 105–110.

Sivamani, E., Brey, C.W., Dyer, W.E., Talbert, L.E. and Qu, R.D. (2000) Resistance to wheat streak mosaic virus in transgenic

wheat expressing the viral replicase (NIb) gene. *Mol. Breeding*, **6**, 469–477.

Sivamani, E., Brey, C.W., Talbert, L.E., Young, M.A., Dyer, W.E., Kaniewski, W.K. and Qu, R.D. (2002) Resistance to wheat streak mosaic virus in transgenic wheat engineered with the viral coat protein gene. *Transgenic Res.* **11**, 31–41.

Slykhuis, J.T. (1955) *Aceria tulipae* Keifer (Acarina, Eriophyidae) in relation to the spread of wheat streak mosaic. *Phytopathology*, **45**, 116–128.

Smith, N.A., Singh, S.P., Wang, M.B., Stoutjesdijk, P.A., Green, A.G. and Waterhouse, P.M. (2000) Gene expression – Total silencing by intron-spliced hairpin RNAs. *Nature*, **407**, 319–320.

Stenger, D.C., Hall, J.S., Choi, I.R. and French, R. (1998) Phylogenetic relationships within the family Potyviridae: wheat streak mosaic virus and brome streak mosaic virus are not members of the genus Rymovirus. *Phytopathology*, **88**, 782–787.

Stenger, D.C., Seifers, D.L. and French, R. (2002) Patterns of polymorphism in wheat streak mosaic virus: sequence space explored by a clade of closely related viral genotypes rivals that between the most divergent strains. *Virology*, **302**, 58–70.

Tougou, M., Furutani, N., Yamagishi, N., Shizukawa, Y., Takahata, Y. and Hidaka, S. (2006) Development of resistant transgenic soybeans with inverted repeat-coat protein genes of Soybean dwarf virus. *Plant Cell Rep.* **25**, 1213–1218.

Vidal, J.R., Kikkert, J.R., Donzelli, B.D., Wallace, P.G. and Reisch, B.I. (2006) Biolistic transformation of grapevine using minimal gene cassette technology. *Plant Cell Rep.* **25**, 807–814.

Wang, M.-B., Abbott, D.C. and Waterhouse, P.M. (2000) A single copy of a virus-derived transgene encoding hairpin RNA gives immunity to Barley yellow dwarf virus. *Mol. Plant Pathol.* **1**, 347–356.

Waterhouse, P.M., Graham, H.W. and Wang, M.B. (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc. Natl Acad. Sci. USA*, **95**, 13959–13964.

Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M.B., Rouse, D.T., Liu, Q., Gooding, P.S., Singh, S.P., Abbott, D., Stoutjesdijk, P.A., Robinson, S.P., Gleave, A.P., Green, A.G. and Waterhouse, P.M. (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J.* **27**, 581–590.

Zhao, Y., Qian, Q., Wang, H.-Z., Huang, D.-N. and Dutis, D. (2007) Co-transformation of gene expression cassettes via particle bombardment to generate safe transgenic plant without any unwanted DNA. *In Vitro Cell. Dev. Biol., Plant*, **43**, 328–334.

Chapter 6
Artificial microRNA-mediated immunity against WSMV

Authors' contribution

MF designed the experiments
MF carried out all experimental work
MF wrote the paper
PL edited the paper
AAM and CCW reviewed the paper

Plant Biotechnology Journal

Plant Biotechnology Journal (2011), pp. 1–14 doi: 10.1111/j.1467-7652.2011.00647.x

Research article

Resistance to *Wheat streak mosaic virus* generated by expression of an artificial polycistronic microRNA in wheat

Muhammad Fahim[1,2,†], Anthony A. Millar[2], Craig C. Wood[1] and Philip J. Larkin[1,*]

[1]CSIRO Plant Industry, Canberra, ACT, Australia

[2]Division of Plant Sciences, Research School of Biology, Australian National University, Canberra, ACT, Australia

Received 16 April 2011;
revised 26 May 2011;
accepted 7 July 2011.
*Correspondence (Tel 61 2 6246 5060;
fax 61 2 6246 5000;
email Philip.larkin@csiro.au)
†Present address: Lecturer, Department of Microbiology, Hazara University Mansehra, KPK, Pakistan. Tel +92 997 414130;
email mfahim@hu.edu.pk

Keywords: *wheat streak mosaic virus*, artificial microRNA, multiplex amiRNA, transgenic resistance, *Triticum aestivum*, *Tritimovirus*, Potyviridae.

Summary

Wheat streak mosaic virus (WSMV) is a persistent threat to wheat production, necessitating novel approaches for protection. We developed an artificial miRNA strategy against WSMV, incorporating five amiRNAs within one polycistronic amiRNA precursor. Using miRNA sequence and folding rules, we chose five amiRNAs targeting conserved regions of WSMV but avoiding off-targets in wheat. These replaced the natural miRNA in each of five arms of the polycistronic rice miR395, producing amiRNA precursor, *FanGuard* (FGmiR395), which was transformed into wheat behind a constitutive promoter. Splinted ligation detected all five amiRNAs being processed in transgenic leaves. Resistance was assessed over two generations. Three types of response were observed in T_1 plants of different transgenic families: completely immune; initially resistant with resistance breaking down over time; and initially susceptible followed by plant recovery. Deep sequencing of small RNAs from inoculated leaves allowed the virus sequence to be assembled from an immune transgenic, susceptible transgenic, and susceptible non-transgenic plant; the amiRNA targets were fully conserved in all three isolates, indicating virus replication on some transgenics was not a result of mutational escape by the virus. For resistant families, the resistance segregated with the transgene. Analysis in the T_2 generation confirmed the inheritance of immunity and gave further insights into the other phenotypes. Stable resistant lines developed no symptoms and no virus by ELISA; this resistance was classified as immunity when extracts failed to transmit from inoculated leaves to test plants. This study demonstrates the utility of a polycistronic amiRNA strategy in wheat against WSMV.

Introduction

Wheat streak mosaic virus (WSMV; Genus *Tritimovirus*; Family Potyviridae) has remained a threat to wheat production wherever it occurs, and its distribution is expanded as evidenced by its confirmed presence in Australia in 2003. Although no data are available for seed transmission of other isolates of WSMV, the Australian isolate is transmitted both through its natural vector wheat curl mite *Aceria tosichella* (Slykhuis, 1955; Harvey and Seifers, 1991; Seifers *et al.*, 1998) and through seed (Jones *et al.*, 2005). The virus spread rapidly across the Australian continent between 2003 and 2007 (Dwyer *et al.*, 2007). The virus poses a new threat to the wheat production and required development of new bio-security practices. The widespread occurrence of the virus and its vector, the potential major impacts on yield, and the impracticality of managing the mites add to the priority of breeding virus-resistant varieties and developing alternative methods of virus control through development of virus-resistant transgenic wheat.

Pathogen-derived resistance was pioneered with the expression of viral coat protein in transgenic tobacco plants (Abel *et al.*, 1986) and developed into more efficient and effective transgenic protection against viruses in plants utilizing double-stranded RNA (dsRNA)-induced RNA interference (RNAi) (Abbott *et al.*, 2002; Smith *et al.*, 2000; Waterhouse *et al.*, 1998). It is now established that RNAi is a natural surveillance mechanism conserved across eukaryotic organisms, where small RNAs either repress or cleave the complementary mRNAs in sequence-specific manner (Baulcombe, 2004). Since then, the strategy has been successfully employed to confer resistance in various plants against invading pathogens.

Previously, we have shown that transgene constructs capable of forming dsRNA transcripts are more likely to result in immunity against WSMV (Fahim *et al.*, 2010) than either of the previous two strategies that involved sense expression of the nuclear inclusion b or coat protein genes (Sivamani *et al.*, 2000, 2002). However, the use of long hairpin RNA (hpRNA) from conventional RNAi vectors as in Fahim *et al.*'s (2010) study theoretically entails an increased risk of 'off-target' effects, i.e. silencing of unintended genes (Jackson *et al.*, 2003). Furthermore, some express concern that agricultural-scale deployment of antivirus hpRNA-expressing transgenic plants might lead to evolution of new virus biotypes via heterologous recombination or complementation between the relatively long viral sequences expressed from the transgene and RNA from a non-target virus infecting the same plant. Although the likelihood of such events seems remote and could be further reduced by judicious selection of smaller sequences for the hpRNA constructs, nevertheless the

utility of other approaches is worth exploring. One such approach, amiRNA-mediated gene silencing, has recently been developed specifically to address the risk of off-target effects and transgene–virus recombination to form new biotypes (Schwab *et al.*, 2006).

The amiRNA approach utilizes a naturally occurring miRNA precursor as a backbone, with the mature miRNA sequence being replaced to gain new targeting ability (Ossowski *et al.*, 2008; Vaucheret *et al.*, 2004). In plants, amiRNAs have been successfully used to down-regulate endogenous genes (Alvarez *et al.*, 2006; Khraiwesh *et al.*, 2008; Molnar *et al.*, 2009; Schwab *et al.*, 2006; Warthmann *et al.*, 2008) and also for developing transgenic virus resistance against *Turnip yellow mosaic virus* (TYMV), *Turnip mosaic virus* (TuMV) (Niu *et al.*, 2006), *Cucumber mosaic virus* (CMV) (Duan *et al.*, 2008), *Potato virus X* (PVX), and *Potato virus Y* (PVY) (Ai *et al.*, 2011) in *Arabidopsis*; against CMV (Qu *et al.*, 2007) in tobacco; against CMV (Zhang *et al.*, 2011) in tomato; and against *Cassava brown streak virus* (CBSV) and *Cassava brown streak Uganda virus* (CBSUV) in cassava (Wagaba *et al.*, 2010).

It has been argued that the use of short viral sequences in this amiRNA approach is less likely to enable the emergence of novel viral entities through recombination and trans-encapsidation (Schnippenkoetter *et al.*, 2001). However, when only a small viral sequence is used, the virus is more likely to evolve in the amiRNA target sequence via transition mutation and enable avoidance of amiRNA complementarity and defence (Simon-Mateo and Garcia, 2006; Lin *et al.*, 2009). Other examples include HIV escape mutants to avoid RNAi (Boden *et al.*, 2003; Das *et al.*, 2004; Westerhout *et al.*, 2005). To substantially reduce the risk of viruses evolving to avoid degradation, a strategy would be very useful where an amiRNA precursor gene expressed multiple amiRNAs targeting different conserved structural and functional portions of the viral genome. Resistance to this protection would require simultaneous mutations to avoid all the amiRNA sequences. A similar rationale was invoked in the work of Israsena *et al.* (2009) where they designed precursor genes encoding three amiRNA against rabies virus and tested them in cell culture. Likewise, multiple siRNA were developed from a multiplex miRNA directed against HIV in cultured cells (Liu *et al.*, 2008; ter Brake *et al.*, 2006).

miRNA precursors that have been used for the delivery of amiRNAs in plants include miR159a (Niu *et al.*, 2006); miR171a (Qu *et al.*, 2007), miR172a (Schwab *et al.*, 2006), miR30 (Zeng *et al.*, 2002), miR528 (Warthmann *et al.*, 2008), and miR167b (Ai *et al.*, 2011). In plants, Niu *et al.* (2006) demonstrated that a dimeric amiRNA precursor in *Arabidopsis* could be effective against two different viruses. Others have successfully targeted two endogenous transcripts with dimeric amiRNA precursors in *Arabidopsis* (Park *et al.*, 2009) and *Chlamydomonas* (Zhao *et al.*, 2009) using different miRNA precursors. Here, in our studies, we used the multiplex precursor of rice miR395 family of miRNAs that was identified in both *Arabidopsis thaliana* and *Oryza sativa* computationally and was later experimentally verified (Guddeti *et al.*, 2005; Kawashima *et al.*, 2009). OsmiR395 targets ATP sulphurylases that are involved in sulphate assimilation (Rotte and Leustek, 2000) and is induced in sulphur starvation to regulate a low-affinity sulphate transporter and two ATP sulphurylases (Allen *et al.*, 2005; Jones-Rhoades and Bartel, 2004). The rice miR395 is a single ~1-kb transcript that generates a convoluted RNA structure that generates seven fully processed miRNA (Jones-Rhoades and Bartel, 2004; S. Belide, J. R. Petrie, P. Shrestha, M. Fahim, Q. Liu, C. C. Wood and S. P. Singh, unpublished).

Here, we expressed five pre-amiRNA, potentially generating ten amiRNA species, to different conserved regions of the WSMV genome from a modified version of rice miRNA precursor miR395. The polycistronic amiRNA strategy is able to produce marker-free transgenic wheat plants immune to WSMV, demonstrating that this is a viable strategy for a major crop species. Moreover, it alleviates the concerns of recombinant novel viral entities forming and also produces plants predicted to avoid the loss of resistance caused by virus mutation. The resolution of technological problems and concerns implies this strategy has a strong biotechnological potential for agriculture.

Results

Design of polycistronic amiRNA construct

We chose pre-miR395 as the backbone for simultaneous expression of multiple amiRNAs targeting various conserved regions in the WSMV genome. We combined published amiRNA selection criterion (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) into a software application we call 'miR Mate'. Applying this to the available full WSMV genome sequences (five at the time) identified approximately 120 potential target sites for amiRNA. As a final selection filter, we searched Wheat TIGR mRNA databases for potential off-targets of these potential WSMV amiRNAs using the online miRU program (http://plantgrn.noble.org/psRNATarget/). No expressed sequences were found as potential targets when three mismatches were allowed. Through this process, five amiRNA were chosen and designated amiRNA-1, amiRNA-2, amiRNA-3, amiRNA-4, and amiRNA-5; their WSMV genome targets and their target coordinates are given in Table 1 and Figure 1. The target of amiRNA-1 lies in 5′ UTR region, amiRNA-2 targets the newly described open reading frame (ORF) *pipo* region of P3 cistron (Chung *et al.*, 2008), amiRNA-3 targets P1 gene, amiRNA-4 targets P3 cistron (upstream of *pipo*), and amiRNA-5 targets the HCpro gene on WSMV genome (Figure 1). We deliberately chose a mix of targets on the genomic and replicative strands of the virus in case one strand was more available for the amiRNA surveillance than the other. While further good targets could be identified in the 3′ genes, the bias to the 5′ genes is simply a result of beginning the screening for potential off-targets from that end. Surprisingly, no 21-nt sequence in the extreme 3′ region of the virus could be identified, which was both conserved and met the design rules. The endogenous miRNAs and miRNA* that are derived from the miR395 precursor were replaced with these amiRNA and amiRNA* sequences to conserve the secondary structure of the transcript. The predicted secondary structure of the polycistronic miR395 and the modified artificial miR395 were almost identical (Figure 2), presumably enhancing the prospect of the predicted biogenesis of mature amiRNAs. This artificial polycistronic precursor was named FanGuard395 (*FGmiR395*).

Generation of wheat carrying the FanGuard395 transgene

FGmiR395 was synthesized by Geneart and cloned into pWubi vector, to generate *FGWS*-pWubi, where FGmiR395 was behind a constitutive maize polyubiquitin promoter separated from the transgene by a spliceable ubiquitin intron. FGWS-pWubi was cobombarded into wheat immature embryos along with

Table 1 Conservation of amiRNA targets in WSMV Genome. The alignment used to design amiRNA against conserved targets in WSMV genome. AlignX was used with default settings using Vector NTI 10. (a) Alignment of the five chosen target regions in the five published WSMV genomes at the time of the design of the amiRNA. (b) Alignment in the five target regions in the ten new WSMV genomes that became available subsequent to amiRNA design. (c) Alignment of the amiRNA target regions in the WSMV-ACT isolate (unpublished and obtained subsequent to the design of FGmiR395 and production of the transgenic plants). The mismatched nucleotides with other isolates are highlighted. amiRNA-1 and amiRNA-2 target the replicating strand, signified by numbering 1–21 from left to right; amiRNA-3, amiRNA-4 and amiRNA-5 target the genomic strand of the WSMV, signified by numbering 1–21 from right to left. The chosen targets remain absolutely conserved in all five target regions of the WSMV-ACT isolate, when virus population RNA was resequenced from inoculated plants in the study including −S, +R, and the transgenic breakdown plants +S

			amiRNA-1 Target sequence	amiRNA-2 Target sequence	amiRNA-3 Target sequence	amiRNA-4 Target sequence	amiRNA-5 Target sequence
			AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATTCCATTATGTGCCGA	CCAGGAAGCATTTTCTGGTCA	CCGCGAACGTCTTGCAAGTTA
Accession	Description	Origin	1 21	1 21	21 1	21 1	21 1
(a)							
AF057533	Sydney 81	Nebraska	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATTCCATTATGTGCCGA	CCAGGAAGCATTTTCTGGTCA	CCGCGAACGTCTTGCAAGTTA
1AF285169	Type Strain	Kansas	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATTCCATTATGTGCCGA	CCAGGAAGCATTTTCTGGTCA	CCGCGAACGTCTTGCAAGTTA
AF285170	El Batan	Mexico	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATACCACTATGTACCGA	CCAGGAAGCATTTTCTGGTCA	CCGCGAACGTCTTGCAAGTTA
AF454454	Czech	Czech	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATTTCATTATGTGCCGA	CCAGGAAGCATTTTCTGGTCA	CCGCGAACGTCTTGCAAGTTA
AF454455	Turkish	Turkey	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATTCCATTATGTGCCGA	CCAGGAAGCATTTTCTGGTCA	CCGCGAACGTCTTGCAAGTTA
(b)							
AF511614	H95S	Kansas	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGGTTCCATTATGTGCCGA	CCAGGAAGCATTTTCTGGTCA	CCGCGAACGTCTTGCAAGTTA
AF511615	H98	Kansas	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATTCCATTATGTGCCGA	CCAGGAAGCATTTTCTGGTCA	CTGCGAACGTCTTGCAAGTTA
AF511618	ID96	Idaho	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATTCCATTATGTGCCGA	CCAGGAAGCATTTTCTGGTCA	CCGCGAACGTCTTGCAAGTTA
AF511619	ID99	Idaho	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATTCCATTATGTGCCGA	CCAGGAAGCATTTTCTGGTCA	CCGCGAACGTCTTGCAAGTTA
AF511630	Mon96	Montana	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATTCCATTATGTGCCGA	CCAGGAAGCATTTTCTGGTCA	CCGCGAACGTCTTGCAAGTTA
EU914917	Naghadeh	Iran	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATTTCATTATGTACCAA	CCAGGAAGCATTTTCTGGTCA	CCGCGAACGTCTTGCAAGTTA
EU914918	Sadat-Saher	Iran	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATTCCATTATGTGCCGA	CCAGGAAGCATTTTCTGGTCA	CCGCGAACGTCTTGCAAGTTA
F511643	WA99	WA, USA	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATTTCATTATGTGCCGA	CCAGGAAGCATTTTCTGGTCA	CCGCGAACGTCTTGCAAGTTA
FJ348358	WA94	WA, USA	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATTTCATTATGTGCCGA	CCAGGAAGCATTTTCTGGTCA	CCGCAAACGTCTTGCAAGTTA
FJ348359	ARG2	Argentina	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATTCCATTATGTGCCGA	CCAGGAAGCATTTTCTGGTCA	CCGCGAACGTCTTGCAAGTTA
(c)							
Unpublished	ACT	Australia	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATTCCATTATGTGCCGA	CCAGGAAGCATTTTCTGGTCA	CCGCGAACGTCTTGCAAGTTA

WSMV, *Wheat streak mosaic virus*.

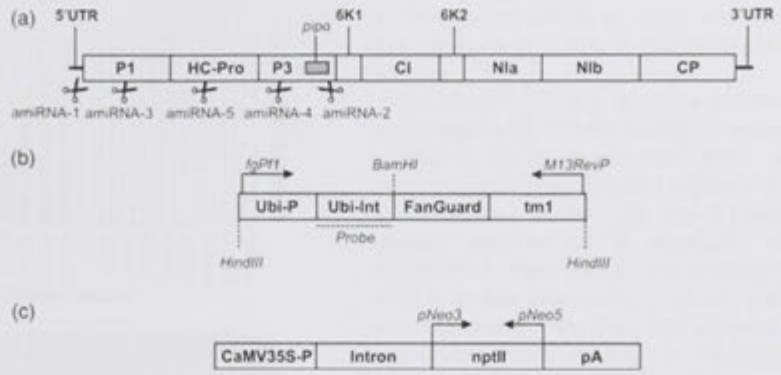


Figure 1 Structure of the *Wheat streak mosaic virus* (WSMV) genome (approximately 9400 nt), the target sites for amiRNAs and the *FGmiR395* transgene. (a) Genome map of WSMV showing the five conserved regions (indicated by scissors) targeted by amiRNAs, amiRNA-1 to amiRNA-5. (b) Design of *FGmiR395* construct (1400 nt) used to transform wheat using biolistics; shown are the probe region for Southern blot and primer sequences FgPf1 and M13RevP used in PCR. (c) Diagram of pCMneoSTLS2 containing the *nptII* gene for geneticin resistance, used in the cotransformation of immature wheat embryos and showing the position of the PCR primers pNeo3 and pNeo5.

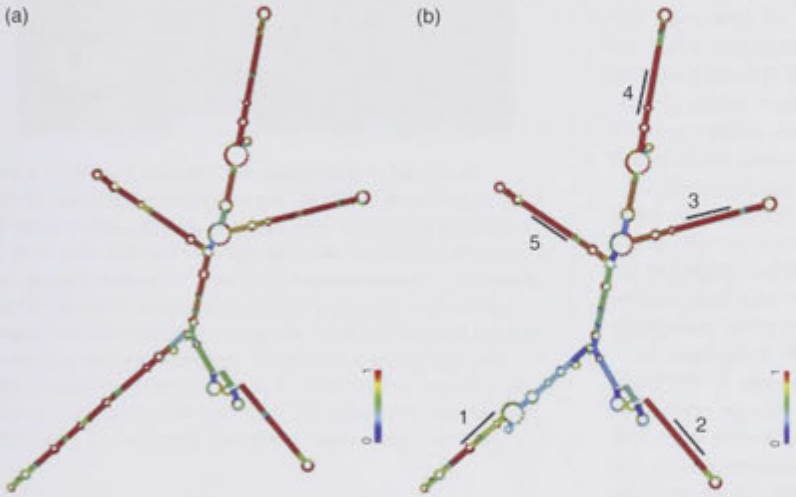


Figure 2 amiRNA Secondary Structure. A comparison of truncated *Osa-miR395* and *FGmiR395* secondary structures. (a) Predicted secondary structure of *miR395* truncated to include only the first five native miRNAs. (b) Predicted secondary structure of *FGmiR395* replacing the first five natural miRNA sequences with amiRNAs design against *Wheat streak mosaic virus*, numbered 1–5. These are the predicted fold structures of transcripts using *RNAfold*. Bars showing regions corresponding to amiRNA guide strand. Secondary structure probabilities are indicated by heat map (Blue, weak; Red, strong).

plasmid pCMneoSTLS2 (Maas *et al.*, 1997) that contained neomycin phosphotransferase gene (*nptII*), conferring resistance to geneticin (G418). From a wheat transformation of 379 embryos, a total of 23 T_0 transgenic wheat plants were generated from 16 different embryos; therefore, there were at least 16 independent events.

The transgenic lines were designated FanGuard plasmid (FGP) plus a number corresponding to the bombarded embryo. Where multiple T_0 plants were obtained from a single embryo, they were distinguished with lower-case letters, e.g. *FGP1a* and *FGP1b*. All T_0, T_1, and T_2 transgenic plants were morphologically indistinguishable from the wild-type parental cultivar, Bob White selection 26 (BW26), implying that the FGmiR395 does not influence growth or development (Figure 3b).

Plants recovered from the transformation selection cultures were screened through PCR for the *nptII* selectable marker from the pCMneoSTLS2 cotransformation vector (Figure 1c). This confirmed that all 23 plants coming through the antibiotic selection were true transgenics carrying the selectable marker. Genomic PCR screening for the presence of *FGmiR395* was carried out using primers, fgPf1 and M13RevP, that span from within the promoter region, full ubiquitin intron and all of the *FGmiR395* transgene including the *nos* terminator (Figure 1b). PCR analysis of T_1 families confirmed that 14 of the 23 families had *FGmiR395* as well as *nptII* (Figure 3a, PCR data not shown) and 10 of these were from different embryos and were therefore independent transgenic events. Southern blot analysis of T_2 plants from a subset of these lines confirmed the presence of transgene (Figure 4). Nine families were negative for *FGmiR395* and positive for *nptII*. The latter lines were discarded after the preliminary assessment for resistance (next section).

Preliminary assessment of *FGmiR395* transgenic wheat in T_1

The T_1 generation was subsequently challenged with WSMV through mechanical inoculation at the three-leaf stage (4–17 plants per family) using the spray gun. Wheat streak mosaic disease is characterized by light-green-to-faint-yellow streaks in wheat leaves parallel to the veins. The virus arrests growth, and plants show moderate-to-severe stunting with prostrated tillers often with empty spikes or spikes with shrivelled kernels. Serological characterization of the transgenic families involved

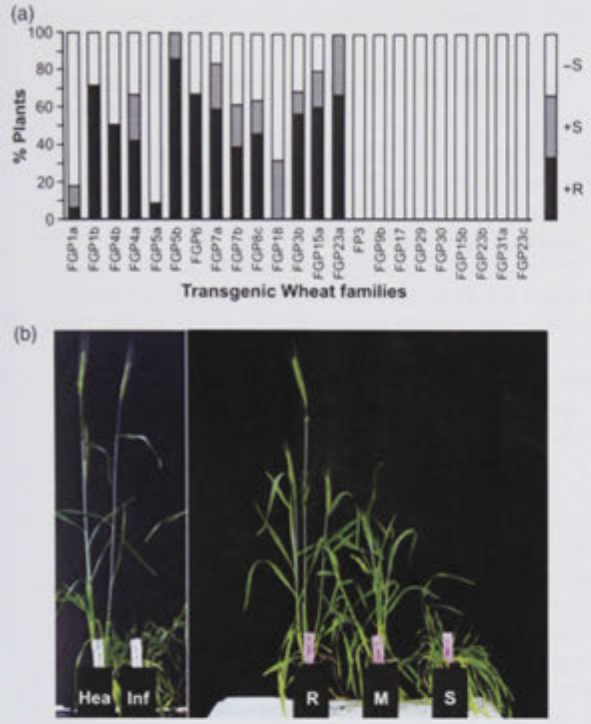


Figure 3 Preliminary assessment of resistance in T_1 families of FGmiR395 expressing transgenic wheat. (a) *Wheat streak mosaic virus* ELISA-based bioassay analysis of resistance in segregating populations. Virus levels were detected by double-antibody sandwich enzyme-linked immunosorbent assay at 14 d p.i. (days postinoculation). –S indicated *FGmiR395* transgene–negative susceptible segregants, +S indicated transgene-carrying susceptible segregants, while +R indicated the transgene-carrying resistant segregants. (b) Resistance phenotypes: Hea., healthy control; Inf., infected control; R., FGP15a2.10, an inoculated transgenic immune; M., FGP15a2.7, an inoculated moderate resistant; S., FGP15a2.2, an inoculated susceptible negative segregant (–S).

inoculating each individual plant with WSMV and assaying with a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) at 14 days postinoculation (d p.i.) and 28 d p.i. Virus accumulation in leaves was determined using ELISA and expressed as a ratio of inoculated plants to non-inoculated controls.

The progeny of nine geneticin-resistant *nptII* positive lines were negative by PCR for *FGmiR395* and were completely susceptible to the challenged virus. The T_1 families from all the other transgenic lines included plants inheriting *FGmiR395* and plants resistant to the virus. The transgenic (signified by +), resistant (signified by R) segregants were designated as *FGmiR395*+R. These plants were completely free of virus symptoms at all four data points 7, 14, 21, and 28 d p.i. They were indistinguishable from uninfected wild-type BW26 plants. Such phenotypes were observed in segregating families of all *FGmiR395*-carrying events except FGP18.

In several transgenic families, some *FGmiR395*-carrying individuals were incompletely resistant, characterized as either: (i) intermediate phenotype between susceptible and resistant arising from either resistance breakdown or plant recovery from virus infection, or (ii) fully susceptible phenotype identical to infected wild-type BW26. The intermediate phenotype was characterized by intermediate plant height and a lower virus titre compared to susceptible control BW26; these types of transgenic segregants were designated as moderately resistant or *FGmiR395*+*MR* (Figure 3b). The fully susceptible *FGmiR395*-carrying plants were designated as *FGmiR395*+S; these were indistinguishable from infected BW26 or the null segregant *FGmiR395*–*S* phenotype.

In this preliminary analysis, most *FGmiR395* families segregated for all three phenotypes (Figure 3a); however, in four events, FGP1b, FGP4b, FPG5a, and FGP6, all *FGmiR395*-inheriting segregants were fully resistant. The numbers of plants were variable and low (4–17), so little attention was paid to the segregation ratios at this stage.

All segregants in FGP18 displayed either *FGmiR395*+S or *FGmiR395*–S phenotype in this preliminary assessment and showed no resistance. These susceptible transgenic plants along with other *FGmiR395*-S from other families exhibited characteristic virus symptoms and were comparable with virus-infected wild-type control BW26 plants.

The initial analysis revealed a variety of phenotypes in response to WSMV inoculation. Transgenic families FGP4a, 6, 8c, 15a, and 18 were selected as representative of the range of

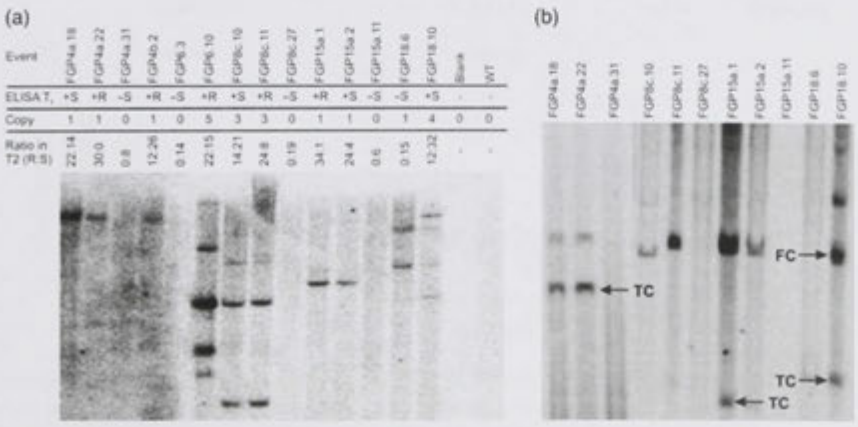


Figure 4 Southern blot analysis of families in T_2 generation. (a) *Bam*HI digested wheat DNA for transgene copy number. (b) *Hin*dIII-digested wheat DNA for transgene copy size. FC is full copy insert size 3 KB; TC is truncated copy inserts. Plant FGP18.6 is labelled –S by PCR and bioassay; it has FG sequence present, but that sequence is truncated so that it is not detected by the PCR.

phenotypes to be studied in greater numbers and detail in T_1 and T_2 generations.

Analysis of transgenic family FGP6 for resistance to WSMV

The preliminary analysis indicated that all *FGmiR395*-positive segregants in FGP1b, FGP4b, FGP5a, and FGP6 were resistant (Figure 3a). Subsequently, a bioassay on 26 T_1 individuals of FGP6 showed that all 13 *FGmiR395*-carrying segregants were symptom free at all four time points (7, 14, 21, and 28 d p.i.) and had background ELISA ratio at 14 and 28 d p.i. One of the major effects of virus infection on plant physiology is the severe stunting and extreme reduction in plant height. When we plotted the virus concentration (ELISA ratio of virus inoculated plant) in segregating FGP6 progeny, we found that they clearly grouped into two clusters (Figure 5b), showing that the plant height is inversely related to the virus concentration.

PCR analysis of selectable marker *nptII* in this family revealed that 17 of 26 plants carried the selectable marker (Table 2). One immune *FGmiR395* transgenic plant FGP6.22 was negative for selectable marker *nptII*, while five *FGmiR395*-negative segregants were carrying *nptII*, and eight segregants were negative for both transgenes. It is worth noting that the approach and technique utilized in this work can yield marker-free immune transgenics.

Southern blot analysis carried out on T_2 segregants confirmed stable integration of *FGmiR395* transgene(s) into the wheat genome (Table 2) and apparently multiple copies of the transgene (*Bam*HI digest: Figure 4a).

Efficacy of viral suppression in transgenic families

The analyses reported thus far for the resistant transgenic families showed the complete absence of symptoms in inoculated transgenic individuals and ELISA readings very similar to the ELISA readings of uninoculated controls, suggesting the complete absence of virus from the inoculated transgenic plants. Experiments were conducted to see whether infectious virus or viral RNA could be recovered from the resistant inoculated transgenic plants. Leaf sap from inoculated plants in three transgenic families (FGP8c, FGP13b, and 15a) was extracted and inoculated onto test plants of control BW26 at 1/10 (w/v) dilution to investigate the presence of any infectious WSMV particles. Results from these test inoculation experiments revealed that all *FGmiR395*+R phenotypes were immune to WSMV, as no infectious virus could be recovered and carried over to control wheat through mechanical inoculation with the most concentrated leaf extract inoculum. Sap from inoculated segregants with *FGmiR395* transgenes failed to transmit infection to susceptible BW26 as judged by symptoms and ELISA, whereas sap from segregants with no transgene and non-transformed controls (BW26) did transmit infection in every case. Examples of these tests are shown in Figure 6.

The formation of amiRNAs from the *FGmiR395* transcript

The expression of the amiRNAs was analysed in virus-free transgenic wheat leaves and detected by splinted ligation using miRtect IT (Maroney *et al.*, 2007). Potentially, from the five duplex arms of the precursor *FGmiR395*, five guide strands (amiRNA) could be produced; moreover, if one also considers the loading of passenger strand (amiRNA*) into RNA Induced Silencing Complex (RISC), then a total of ten amiRNA could potentially be produced against the virus. To detect both amiRNA and amiRNA* sequences using splinted ligation, we

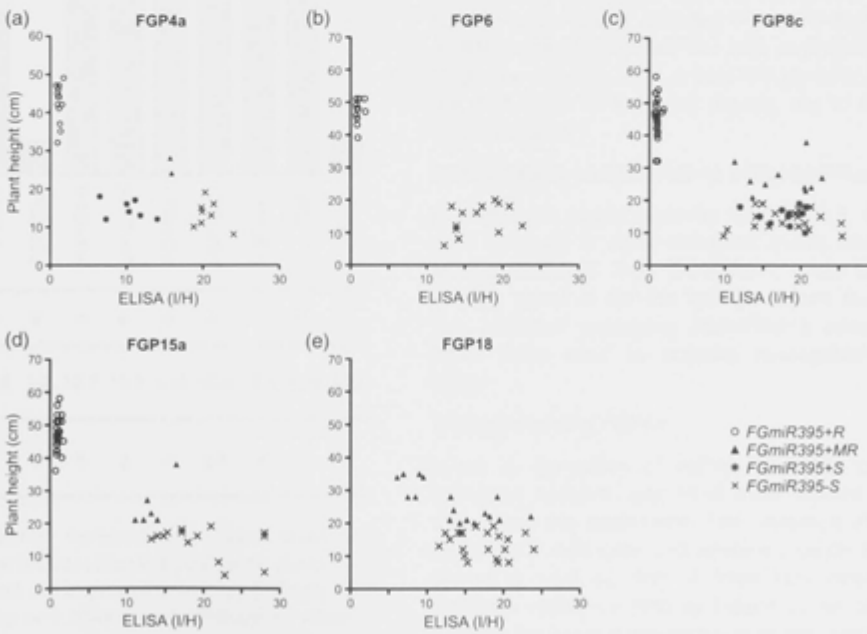


Figure 5 Segregation of resistance in FGP families expressing FGmiR395 transgene. Virus levels were detected by double-antibody sandwich enzyme-linked immunosorbent assay at 28 d p.i. postinoculation and expressed as ratio of inoculated and healthy control. The plant height (in cm) was measured at the heading stage and plotted against the corresponding ELISA ratio. –S indicated the *FGmiR395* transgene–negative susceptible segregant, +S indicated transgene-carrying susceptible segregant, +MR indicated segregants that carried the transgene but accumulated virus titre owing to resistance breakdown, while +R indicated the transgene-carrying resistant segregants. Transgenic families are shown as (a) FGP4a, (b) FGP6, (c) FGP8c, (d) FGP15a, and (e) FGP18. MR, moderate resistance.

Table 2 Segregation of transgene and resistance in T_1 and T_2. Analysis of six selected FGmiR395 families in T_1 and T_2. Recorded for T_1 are the results of Southern hybridisation, segregation for genomic PCR, and resistance. Segregation for resistance in T_2 is also shown for a series of selected derivative families. Selected T_2 families were derived as shown from T_1 individuals that were transgene carrying and resistant (+R); transgene-carrying susceptible (+S); and transgene-negative susceptible (−S). Southern blots are shown in Figure 4

T0 (NT)	T_1					T_2		
T0 Parent	*FGmiR395* Copy no. Southern blot (examples Figure 4a)	*FGmiR395* no. of loci (segregation)	PCR *ptII* + : −	PCR *GmiR395* + : −	ELISA WSMV R : S	T_1 parent	ELISA-based phenotype in T_1	ELISA phenotype in T_2 segregants R : S
FGP4a	2	1	21 : 11	24 : 8	15 : 9	4a.18	+S	20 : 15
						4a.22	+R	29 : 0
						4a.31	−S	0 : 8
FGP6	4	–	17 : 9	13 :173	13 : 13	6.3	−S	0 : 14
						6.10	+R	22 : 15
FGP8c	3	1	49 : 21	51 : 19	30 : 21	8c.10	+S	14 : 21
						8c.11	+R	24 : 8
						8c.27	−S	0 : 19
FGP15a	1	1	39 : 13	39 : 13	32 : 7	15a.1	+R	34 : 1
						15a.2	+S	24 : 4
						15a.11	−S	0 : 6
FGP18	3	–	26 : 12	17 : 21	0 : 38	18.6	−S	0 : 15
						18.10	+S	12 : 32

WSMV, *Wheat streak mosaic virus*.

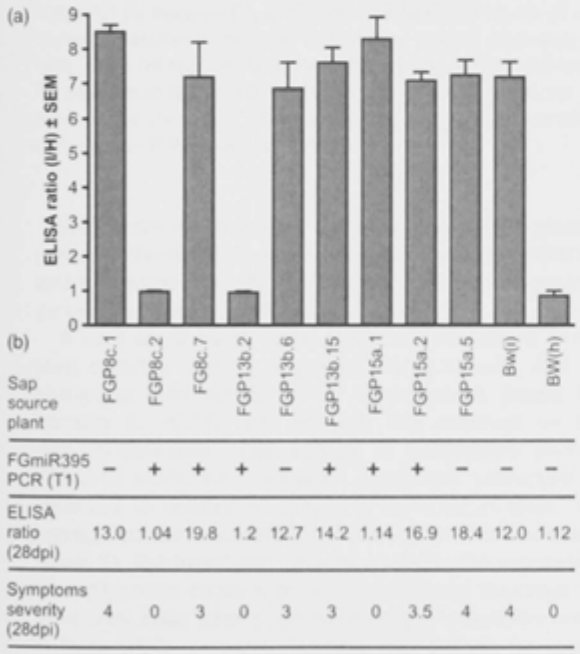


Figure 6 Virus transmission from the inoculated transgenic plants onto Bobwhite26. Sap was extracted from inoculated transgenic plants at 1/10 (w/v). Each sap extract was inoculated onto three wild-type BW26 plants. At 28 d p.i., samples were collected and processed for *Wheat streak mosaic virus* ELISA. (a) Plotted is the average ELISA ratio (inoculated divided by healthy) for the three test-inoculated plants. (b) Also tabulated are the molecular analyses of the inoculated T_1 individuals whose sap extracts were being tested. Shown for each sap donor is the *FGmiR395* PCR result, the bioassay result for that donor plant (as 28 d p.i. ELISA ratio), and the symptom score at 28 d p.i. (0–4 scale). Control BW(i) is inoculated BW26. BW(h) is the uninoculated BW26.

designed the ten bridging oligos accordingly (Table S1). All five designed anti-WSMV miRNAs, amiRNA-1, amiRNA-2, amiRNA-3, amiRNA-4, and amiRNA5, were expressed in the plant. Using an immune plant in T_2 generation, seven of the potential ten amiRNA were generated from *FGmiR395* and accumulated sufficiently to be readily detected (Figure 7). At least these seven amiRNAs would be loaded into RISC and would be expected to target the invading virus at both the genomic RNA and replicative RNA levels in four viral regions, the 5′ UTR, P1, P3, and HCpro regions.

Investigating the resistance breakdown phenotype

In addition to stable immunity *FGmiR395*+R, other phenotypes were observed in some transgenic events described previously as *FGmiR395*+MR and *FGmiR395*+S. These also were studied in more detail in the five families chosen for closer examination. Negative segregants *FGmiR395*−S along with wild-type BW26 were used as negative (susceptible) controls in all assays.

Transgenic event FGP4a

In the T_1 generation of FGP4a, 24 of 32 plants carried the transgene; however, only 15 of these showed strong resistance throughout the experiment. The resistance in nine individuals broke down with time, and symptoms began to appear as faint streaks at 14 d p.i. Two of these nine individuals showed a moderate resistance (MR) as judged by no symptoms at 14 d p.i., but the gradual development of leaf symptoms, growth to only intermediate height, and accumulated virus titre at 28 d p.i.; these segregants were characterized as *FGmiR395*+MR (Figure 5a). PCR, ELISA ratio, and plant height plot suggested four groups in this family. The third cluster (*FGmiR395*+S) accumulated less virus than the null segregants but was equally affected in height.

Figure 7 Expression analysis of *Wheat streak mosaic virus*-specific amiRNAs: Levels of the amiRNAs in immune transgenic segregant FGP4a.22 were examined using the MiRtect IT splinted ligation assay. Assays with bridge oligonucleotides specific for (a) amiRNA, (b) amiRNA*, and (c) internal controls. The numbers in the bottom panel correspond to amiRNA-1 to -5, respectively.

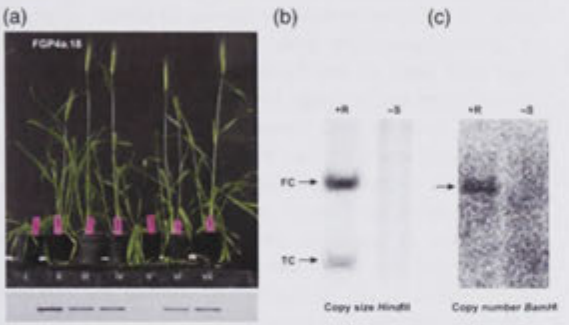


Figure 8 Segregation of resistance in T_2 progeny of T_1 individual FGP4a.18: (a) Inoculated T_2 plants and FGmiR395 PCR results. (i) and (v) transgene-negative susceptibles; (ii) transgene carrying moderately resistant; (iii) (iv) (vi) and (vii) transgene-carrying immunes. (b) Southern blot for copy size of FGP4a (+R) and FGP4a (−S), where FC shows the expected full size, and TC shows a truncated copy. (c) Southern blot for copy number FGP4a (+R) and FGP4a (−S).

Seeds collected from three FGP4a T_1 individuals, representing immune (*FGmiR395*+R), resistance breakdown (*FGmiR395*+MR), and susceptible phenotypes (*FGmiR395*−S) were analysed in T_2 generation (Table 2 and Table S2).

A total of 29 plants were grown and bioassayed from the seed collected from T_1 segregant FGP4a.22 (+R), and when challenged with WSMV, all 29 T_2 individuals proved to be immune to WSMV and no virus was retrieved on BW26 through back-assay. The progeny of intermediate phenotype FGP4a.18 (+MR) with resistance breakdown phenotype in T_1 produced 15 resistant progeny in T_2 plants (of 35 total). This T_2 segregation for resistance in FGP4a.18 is also illustrated in Figure 8a. We hypothesize this may indicate the segregation of an antagonistic factor such as an interfering truncated transgene copy away from a functional copy of *FGmiR395* resulting in more stable resistance. Southern blot analysis for copy size did reveal the presence of a truncated copy (Figure 8b). Furthermore, it is also possible that the zygosity of *FGmiR395* might be involved in the amiRNA production where the homozygous level of expression may result in more stable resistance. The T_2 progeny propagated from a negative segregant parent FGP4a.31 (*FGmiR395*−S) were fully susceptible and indistinguishable from the wild-type susceptible BW26 controls.

Transgenic event FGP15a

In FGP15a, a total of 52 individuals were analysed in T_1 generation. There appears to be one copy of *FGmiR395* and one copy of *nptII*, and both segregate in simple Mendelian proportions (Table 2). Most of 32 tested *FGmiR395* + segregants were strongly resistant (+R), but six had moderate resistance (+MR) (Figure 5d). These six segregants had lower virus accumulation and were less stunted compared to the *FGmiR395* null segregants (Figure 5d).

Three different FGP15a T_1 segregants were selected for analysis in T_2, and once again, resistant phenotypes were recovered from a susceptible transgene-positive T_1 parent (FGP15a.2). The family had one site of insertion of the transgene; however, a strong signal might suggest concatamerization at this site; there was also evidence of a truncated copy (Figure 4). From the +R T_1 individual FGP15a.1, only one of 35 T_2 was not immune.

Transgenic event FGP8c

In FGP8c, four classes of phenotype were observed. A total of 70 individuals were analysed for the transgenes, and 51 were assayed in T_1 for WSMV resistance (Table 2). Of T_1 plants tested (Figure 5c), 30 were +R with average plant height of 45.56 cm; seven were +MR where resistance held up at 14 d p.i. but gradually broke down with the virus titre ratio above ten at 28 d p.i. and average plant height of 28.5 cm; 14 segregants were +S despite carrying a full copy of *FGmiR395*, with average plant height of 16.92 cm and indistinguishable from the 19 −S segregants with average plant height 14.26 cm.

Seeds were collected from three T_1 individuals representing +R, +S, and −S classes (Table 2). From the immune +R individual FGP8c.11, resistance segregated normally (24 : 8) in T_2. Again, the T_2 progeny of a +S plant, FGP8c.10, segregated some strongly resistant plants (14 : 21). The Southern blot analysis revealed the presence of three copies (Table 2, Figure 4) that appeared to be of equal size but segregated together at one insertion locus.

Leaf saps from the strongly resistant segregants of event FGP 8c were back-inoculated to susceptible controls and found to be immune; this is illustrated in Figure 6 by T_1 individual FGP8c.2. However, individuals such as FGP8c.7 showed some breakdown of resistance after 14 d p.i., and sap from this plant was infective to the same extent as the null segregant FGP 8c.1 (Figure 6).

Transgenic event FGP18

Event FGP18 was examined further because the apparent full-length insertions of the *FGmiR395* gene failed to confer any plants with a high level of resistance in T_1 (Figure 3a, Figure 5e). In 38 T_1 segregants, the *nptII* gene segregated simply, but segregation was significantly distorted away from the *FGmiR395* transgene (17 : 21, Table 2). While none of the T_1 individuals were strongly resistant, Figure 5e shows that the *FGmiR395*-carrying plants clustered distinctly from the null segregants, with average plant height of +S being 26.1 cm, compared to 14.6 cm in −S segregants (Figure 5e, Table S2). Southern blot analysis revealed the presence of four copies (Figure 4a) including truncated copies (Figure 4b). The T_2 progeny from a +S and −S T_1 parent were analysed for virus resistance. Resistance was evident in 12 T_2 individuals from FGP18.10 of 44 tested (Table 2). It is noteworthy that even in this multi-insertion transgenic event, which appeared in T_1 generation to be ineffective, by T_2, it was possible to identify fully resistant transgenic segregants. Plant FGP18.6 is noteworthy. Using PCR and bioassay, it was classified as −S; it did have transgenic sequence present (Figure 4a), but the sequence is truncated

(Figure 4b). Presumably, the truncation has eliminated one or both of the primer sites so that the PCR gave no product. When only the truncated copy is present, the plants are susceptible in both T_2 and T_3 generations (Table 2).

The possibility that the virus was evolving to avoid all five amiRNA species can be dismissed based on two lines of evidence. T_2 transgenics were challenged with the infected sap derived from both susceptible and transgene-carrying susceptible segregants in T_1. Leaf sap collected from *FGmiR395*+S T_1 plants was used to inoculate T_2 progeny. These potentially evolved virus populations continued to be controlled by resistant transgenics. Furthermore, in a number of families (Table 2), +R and +MR phenotypes emerged from +S T_1 parents. Therefore, the original +S susceptibility was not the result of virus mutation; otherwise, resistance would not have emerged in T_2 when challenged with the putatively mutant virus preparations. In addition, deep sequencing was carried out of small RNA fractions extracted from virus-inoculated plants of the three phenotypes, +R, +S, and −S. This allowed the full virus genomic sequence to be reconstructed from each of these virus populations. As reported in Table 1, the virus sequence of WSMV-ACT recovered from the non-transgenic plant (−S), in each of the five target regions, was exactly the sequence as used in the construction of the amiRNA. Furthermore, the sequence in these regions was entirely unchanged in the virus population from both the transgenic resistant plant (+R) and the transgenic susceptible plant (+S).

The recovery phenotype

Individuals with a *recovery* phenotype were observed in transgenic family FGP13b. These plants had virus symptoms at 14 d p.i. and a high virus titre; however, when assayed at 28 d p.i., virus was not detected through ELISA nor were there any symptoms observed on the newly emerged leaves. An example of this phenomenon is displayed in transgenic segregant FGP13b.2 (Figure 9), where the new leaf displayed characteristic virus symptoms at 14 d p.i. (still evident at 28 d p.i., Figure 9b) and had an ELISA ratio of 2.5. However, as the plant developed further, no virus symptoms were observed on the newly emerged leaves at 28 d p.i. (Figure 9c). Moreover, no WSMV-specific PCR product could be amplified from reverse-transcribed RNA from the newly emerged leaves. This suggests that the amiRNA was ineffective in the young plant but expressed better and became effective in overcoming virus multiplication as the plant developed further.

Sap was extracted from the newly emerged leaves and inoculated onto susceptible BW26 plants to test whether the virus has been completely eliminated from the newly emerged leaves. No infectious virus could be recovered in this back-assay (Figure 6, FGP13b.2). This lead to the conclusion that in some resistant transgenic events, the virus was able to get away to initial establishment but that the amiRNA expression subsequently was able to completely eliminate the virus from the recovering plant.

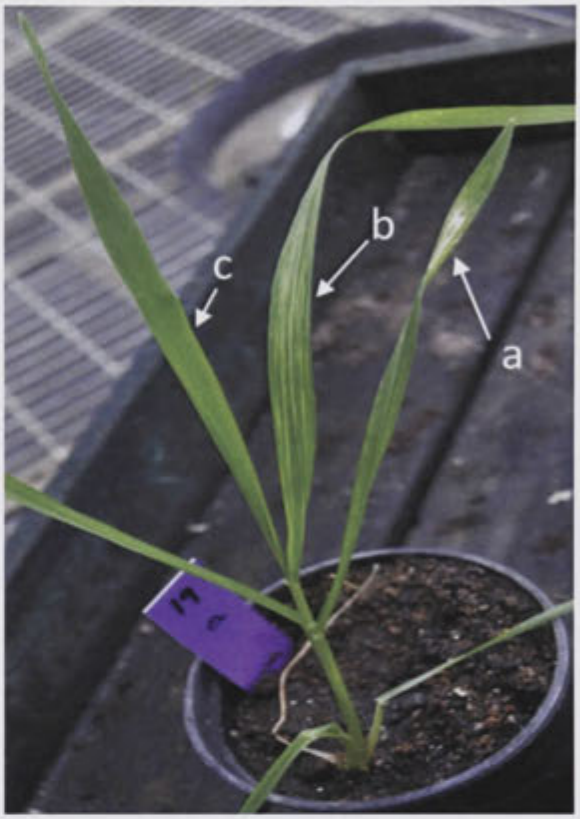


Figure 9 Recovery phenotype. (a) Inoculated leaf (b) leaf showing symptoms (c) newly emerged healthy leaf. In this transgenic FGP13b.2 individual, we inoculated the leaf with spray gun (necrotic spots shows mechanical injury resulting from spray inoculation); at 14 d p.i., the plants showed symptoms and accumulated virus by ELISA ratio. However, at 28 d p.i., newly emerged leaves were completely symptom-free with no virus detected by ELISA. When sap from new leaves of this plant was test-inoculated onto BW26, no infectious virus could be recovered (Figure 5).

Discussion

We engineered a complex rice-derived miR395 with five artificial miRNA precursors designed to target WSMV genome, to achieve amiRNA-mediated resistance in wheat. We retained the predicted secondary structure of miR395 in the synthetic *FGmiR395* transgene and hypothesized that the FanGuard transcript would be processed upon expression to produce five 21-bp amiRNA duplexes and then up to ten species of amiRNA, because secondary structure of the plant precursor miRNAs appears to be more important for processing by DCL1 than the sequence of the mature miRNA itself (Schwab *et al.*, 2006; Ossowski *et al.*, 2008). WSMV has a +ssRNA single-stranded (monopartite) genome that replicates through a dsRNA intermediate to generate a negative (−) strand during this replication process. This monopartite genome provides the opportunity to target the virus at any accessible position that would result in homology-dependent degradation of the viral genome or the replicative strand. amiRNA-1 and amiRNA-2 were designed to be complementary to and target the replicating strand, while amiRNA-3, amiRNA-4, and amiRNA-5 were designed to be complementary to and target the genomic strand of the virus. It has been shown that both positive and negative strands of hepatitis C virus may be targeted by siRNAs (Wilson and Richardson, 2005). The amiRNA* (passenger strand) might also get loaded into RISC and thus mediate the degradation of the opposite strand of the virus (either genomic or replicative strand) than the predicted one. In fact, our results showed that seven of the potential ten types of amiRNA could be readily detected.

Resistance cosegregated with the transgene in most of the transgenic events of this study. However, analysis of T_1 families revealed a range of phenotypes. (i) Immune individuals were obtained, which remained symptomless and with no viral coat protein accumulation all the way through to maturity. Sap prepared from these inoculated plants failed to transmit infection to susceptible controls. Such immunity was evident across two generations, (ii) In other cases, the plants were resistant without symptoms or ELISA detected coat protein at 14 d p.i., but subsequently, the resistance broke down, allowing virus to accumulate by 28 d p.i., (iii) A third transgenic phenotype may be called *plant recovery*, in which early susceptibility is followed by full recovery and resistance, (iv) The forth phenotype is where the presence of multiple copies of the transgene confers only moderate resistance. We challenged the transgenic families with high-titre virus inoculum at two time points 0 and 10 d p.i. and therefore suggest that the breakdown of resistance in some events might result from an excessive virus pressure that overwhelms the amiRNA-mediated resistance. Such a phenomenon has been observed in transgenic barley against *Barley yellow dwarf virus* (BYDV) where an increase in viruliferous aphid infestation resulted in breakdown of RNAi resistance to BYDV (M.-B. Wang, pers. commun.). Ai *et al.* (2011) showed that although amiRNAs were detectable and resistance to PVX or PVY evident in their transgenic plants, the resistance was overcome by reinoculation at 35 d p.i., resulting in increased viral pressure. The pressure of inoculation in our experiments was very high and may be responsible for some of the more complex resistance phenotypes. It would be interesting to investigate the efficiency of the *FGmiR395*-expressing wheat plants in the field under the milder pressures expected from natural wheat curl mite infestations.

It could also be informative to quantify the level of amiRNA expression in the various transgenic phenotypes to observe whether there is a correlation with the degree or stability of resistance. Such an analysis would need to follow all ten of the potential amiRNA species and would ideally include the various phases of resistance breakdown and various phases of recovery. Previous studies have shown some degree of correlation between amiRNA expression and virus resistance (PVX and PVY in *Arabidopsis*, Ai *et al.*, 2011; CMV in tobacco, Qu *et al.*, 2007). When there are at least five and potentially ten species of amiRNA attacking the virus, as with *FGmi395*, the analysis of correlation with resistance will be complex.

One potential risk with amiRNA-mediated resistance is the generation of virus mutants that escape the amiRNA surveillance (Simon-Mateo and Garcia, 2006; Lin *et al.*, 2009). We addressed this issue in our studies by selecting amiRNA targets based upon conserved regions in five full WSMV genome sequences available. We observed no evidence that the virus was evolving (mutating) during the course of the experiment. This was evident because virus populations collected from *FGmiR395*+S T_1 plants was used to inoculate T_2 progeny. Not only did the resistant transgenic continue to be resistant to these inoculations, but progeny from some +S T_1 parents included +R and +MR phenotypes. We take this as evidence that the original +S susceptibility was not the result of virus mutation; otherwise, resistance would not have emerged in T_2 when challenged with the putatively mutant virus preparation. Furthermore, deep sequencing of virus populations from infected transgenic plants confirmed no mutations in the target sequences. By the end of this study, the number of full-length WSMV genomes available online (NCBI) had grown from five to 13. An alignment of all 13 revealed that three of five chosen amiRNA targets are still completely conserved in the WSMV genome (Table 1). This highlights the importance of having multiple targets in polycistronic amiRNA and the importance of aligning as many virus genomes as possible to select highly conserved regions.

One of the targets in *FGmiR395* was the functional region called *pipo* (pretty interesting Potyviridae ORF) within the gene encoding the P3 protein. *Pipo* was initially identified as a small ORF embedded in the P3 cistron of *Turnip mosaic virus* (TuMV; genus *Potyvirus*; family Potyviridae) (Chung *et al.*, 2008), and its presence was confirmed in 48 viruses representing all genera in the family Potyviridae, including WSMV. Mutation in *pipo* hinders various important functions; in the case of WSMV that includes effects on replication and movement in the plant. When mutations are introduced into the *pipo* region of P3, without affecting the amino acid sequence of the translated protein, the virus loses the ability to replicate in protoplasts (Chung *et al.*, 2008) or it is restricted to only a few cells in inoculated plants (Wen and Hajimorad, 2010).

As one would expect, the presence of one full copy of the transgene can be enough to confer resistance (immunity) against WSMV. However, we saw evidence in some transgenic events of additional truncated *FGmiR395* insertions and behaviour in T_1 and T_2 generations suggestive that the truncated copy may be interfering with the expression of the full-length copy; subsequent loss of the truncated copy restores effective resistance. This interpretation will require further experimentation to confirm. We sometimes observed resistance segregation ratios inconsistent with Mendelian expectation based on the inferred insert locus number. Usually, this involved lower than expected numbers of resistant individuals and is likely associated with some inserts of the transgene being ineffective or conditionally effective (Matzke *et al.*, 2009).

Previously, we have reported the use of long hairpin dsRNA-mediated WSMV immunity in wheat (Fahim *et al.*, 2010). The comparison to the present study is a rare opportunity to contrast the two approaches in the same genetic background against the same virus. Compared to amiRNA, long hairpin RNA had the advantage of a very high frequency of insertion events with stable heritable immunity. In this respect, long hairpin RNAi is very attractive from a biotechnology application perspective. However, long hpRNA would have a higher probability of unintended silencing of off-target genes in the host (Xu *et al.*, 2006; Duan *et al.*, 2008; Khraiwesh *et al.*, 2008). Furthermore, long hpRNA approaches are seen by some as posing a risk in the field of heterologous recombination with other virus genomes and resulting in new virus biotypes. Low temperatures can also compromise the efficacy of RNAi silencing strategies (Szittya *et al.*, 2003). On the other hand, miRNAs appear to be completely temperature independent, and the transgenic lines expressing virus-derived amiRNA retain their resistance at low temperatures (Niu *et al.*, 2006; Szittya *et al.*, 2003). We were able to achieve immunity in wheat to WSMV from both the long hairpin dsRNA and the amiRNA strategies. We conclude that amiRNA-based viral resistance, especially polycistronic amiRNA as advocated here, deserves and needs further in-depth studies to improve the amiRNA efficiency. An even better comparison would be achieved if the long hpRNA was designed to cover the same regions as the amiRNA used in this study.

The work described here exemplifies the utility of miR395 and similar miRNA clusters as a carrier of multiple amiRNAs.

They can be used to target multiple regions of the one virus (as here), multiple viruses, or multiple endogenous mRNA species (S. Belide, J. R. Petrie, P. Shrestha, M. Fahim, Q. Liu, C. C. Wood and S. P. Singh, unpublished). Mixed viral infections are common in the field especially in fruits and vegetables. Using the polycistronic amiRNA, it will be possible to target highly conserved regions of multiple viruses. Similarly, polycistronic pre-amiRNA genes will be effective in targeting multiple plant endogenous genes for functional genomics and in applications such as redirecting plant metabolism into novel products.

We conclude that polycistronic amiRNAs can be utilized to induce virus resistance in commercially valuable plants, where there are limited options of natural resistance. We anticipate ongoing improvements in the understanding of miRNA biogenesis and design of amiRNA to further enhance the utility for virus resistance and engineering other agronomically important traits. Furthermore, the expression of multiple amiRNAs from a single precursor transgene will minimize the difficulties of repeated transformations, need for multiple selectable markers, and the constraint of breeding with multiple independent loci.

Experimental procedures

Designing WSMV-specific amiRNA

To select conserved regions in WSMV genome as targets for artificial miRNAs, full-genome sequences of WSMV were retrieved from NCBI (Table 1). The sequences were aligned with Clustal W/AlignX (a component of Vector NTI Advance® 10.3.0) to screen for highly conserved regions in the viral genome. The possible amiRNA sequences were generated from the highly conserved regions (20 nt or more in length) using the basic criteria defined at WMD3 (http://wmd3.weigelworld.org/, a web microRNA design tool) and incorporated into a software algorism called *miR Mate* developed specifically for this study. The algorithm was developed using Microsoft. NET Framework and also incorporates the Vienna RNA Package 1.7 algorithm RNAfold.exe (Hofacker *et al.*, 1994; McCaskill, 1990; Zuker and Stiegler, 1981). The miRNA design criteria used include A/U at position 1 (Mi *et al.*, 2008; Eamens *et al.*, 2009; Takeda *et al.*, 2008), A at position 10 (Reynolds *et al.*, 2004; Mallory *et al.*, 2004), and G/C at position 21 (P. Waterhouse, pers. commun.). *miR Mate* utilizes the *RNAfold* algorithm to calculate minimal free energy (mfe) values for the formation of the candidate miRNA's folded structure; values of ≤−30 kcal/mol represent optimal stability. The negative values reflect the fact that stored energy is released during the formation of the structure; the more negative the value, the more energy is released and the more favourable is the formation of the structure. Candidate amiRNAs with the lowest mfe value (the highest stability) were then assessed for potential off-targets in wheat and barley.

The set of potential virus target sequences were used to search for genes that may be potential off-targets in wheat or barley, using miRU: Plant microRNA Potential Target Finder http://bioinfo3.noble.org/miRNA/miRU.htm (a recent version *psRNATarget: A Plant Small RNA Regulator Target Analysis Server* is available at http://bioinfo3.noble.org/psRNATarget/) (Brennecke *et al.*, 2005; Jones-Rhoades and Bartel, 2004; Lim *et al.*, 2005; Mallory *et al.*, 2004). WSMV-derived amiRNAs were selected having the least probability of targeting any sequence in the gene or EST databases of wheat or barley.

The stemloop backbone

For the delivery of the final five amiRNAs as a polycistronic transgene, we selected the precursor of rice miR395 that is expressed under sulphur stress conditions (Guddeti *et al.*, 2005; Jones-Rhoades and Bartel, 2004; Kawashima *et al.*, 2009). A synthetic gene called *FanGuard* (FG) was designed by replacing the five native miRNA sequences in the first five duplex arms of native miR395 with five amiRNA designed to target WSMV. In a parallel study, a similar construct was used to simultaneously silence five endogenous genes in Arabidopsis (Belide *et al.* submitted). The designed *FGmiR395* was synthesized through GENEART® GmbH (http://www.geneart.com) flanked by restriction sites for *Bam*HI and *Kpn*I in the carrier plasmid. The FG gene was excised from the carrier plasmid using appropriate restriction enzymes and ligated between the Ubiquitin promoter and tm1 terminator of vector pWubi-tm1 vector (Wang and Waterhouse, 2000) generating cereal transformation plasmid FG-pWubi.

Wheat transformation

Transgenic wheat plants were generated following microparticle bombardment of 186 immature cv. Bob White 26 (BW26) wheat embryos. The embryos were cobombarded with two plasmids, FG-pWubi and a selectable marker plasmid pCMneoSTLS2, as described previously (Fahim *et al.*, 2010; Pellegrineschi *et al.*, 2002).

Analysis of T_0 transgenic plants—PCR

DNA extraction was carried out using DNAeasy Plant Mini Kit following manufacturer's instructions (Qiagen Inc, Valencia, CA). For PCR-based genotyping of the *FGmiR395* transgene, DNA was amplified (Figure 1b) using FgPf1 5′-TGCAGCATCTATTCATATGC-3′ and M13RevP 5′-CATGGTCATAGCTGTT-3′, that generated approximately 1.4 kb of FG-pWubi amplicon covering promoter, transgene, and terminator regions, under the following thermocycler conditions 94 °C for 30 s, 60 °C for 45 s, 72 °C for 60 s for 35 cycles with a final extension at 72 °C for 10 min. For the selectable marker *nptII* (Figure 1c), a 700-bp *nptII* fragment was amplified using the forward primer Neo3 5′-TACGGTATCGCCGCTCCCGAT-3′ and reverse primer Neo5 5′-GGCTATTCGGCTATGACTG-3′, both sequences being in the *nptII* coding region, using the following thermocycler conditions: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s for 40 cycles with a final extension at 72 °C for 10 min.

Analysis of T_1 and T_2 transgenic plants—Virus bioassay

Virus inoculum was prepared by grinding WSMV-infected tissue in a mortar and pestle at a 1 : 10 w/v ratio in 0.02 M potassium phosphate buffer (pH 7). After filtering through four layers of Miracloth® (Calbiochem, La Jolla, CA), abrasive celite (Johns-Manville, Denver, CO) was added at 2% w/v to serve as an abrasive. For the analysis in T_1 plants, the inoculum was prepared from virus-infected non-transgenic BW26; for the analysis of T_2 plants, sap from a mixture of *FGmiR395* carrying susceptible and *FGmiR395*-negative segregant susceptible plants was used. The sap–celite mixture was first applied with an air-powered spray gun, and then, leaves were gently rubbed with fingers to ensure the infection of plant by the virus. At 10 d p.i., plants were reinoculated with the virus-infected sap to ensure high inoculum pressure.

The plants were scored for symptoms at 7, 14, 21, and 28 d p.i. on a scale of 0–4 with 0 as healthy, 1 as mild with very few streaks, 2 as moderate with streaks that coalesce, 3 as severe with approximately 50% leaf area with streaks, and 4 as the most severe or lethal symptoms where the streaks develop into chlorosis of more than 70% of leaf area based on visual observation. WSMV-specific ELISA was performed on leaf samples collected at 14 d p.i. and 28 d p.i., using Agdia reagents (Agdia, Elkhart, IN) following the manufacturer's instructions. Plates were read at A_{405nm} in ELISA Reader Spectra Max 340 PC (Molecular Devices, Sunnyvale, CA) 60 min after the addition of substrates. Healthy controls were included on every plate, every sample was duplicated, and duplicate value means were used in calculating the ELISA value ratio between inoculated and healthy controls.

Segregation analysis of FanGuard transgene and resistance in T_1 and T_2 generations

For detailed segregation analysis of selected events in T_1 generation, 25–35 seeds were germinated in pots. Leaf samples were collected, and DNA was extracted as described previously, and genomic PCR was conducted to detect both *FGmiR395* and *npt*II amplicons. The cosegregation of resistance with the transgene was assessed by challenging with WSMV as described earlier. ELISA was performed 14 and 28 d p.i. in T_1 and only 28 d p.i. for T_2 generations on inoculated plants. Plant heights and symptoms were recorded at 7, 14, 21, and 28 d p.i.

Test inoculation to detect infectious virus in leaf sap

Sap was extracted at 28 d p.i. from each inoculated transgenic plant to be tested, using 0.02 M potassium phosphate buffer at 1 : 10 (1 g leaf per 10 mL buffer) concentration and mixed with celite abrasive and then inoculated onto three control BW26 plants. This test inoculation (or back-inoculation) method was used to evaluate the effectiveness of the FanGuard (*FGmiR395*) transgene in eliminating viral replication and preventing the formation of infectious particles. Symptoms were scored on the test-inoculated plants and leaf samples collected 28 d p.i. for ELISA as described previously.

Analysis of transgenic plants—Southern hybridization

Southern hybridization was carried out as described previously (Fahim *et al.*, 2010; Lagudah *et al.*, 1991). Instead of T_0 plants, a pool of 8–44 T_1 individuals per family were used for the analysis of transgene copy number and copy size. This method was used so as not to compromise the initial transgenic T_0 plant yet to capture all the insertion events likely to have been present in the T_0 plant. DNA was digested with *Bam*HI to determine the number of independent insertions; there is only one site for *Bam*H1 in *FGmi395* (Figures 1b and 4). DNA was digested with *Hin*dIII to assess whether the inserted copies were full length or truncated.

amiRNA analysis in immune transgenic plants

The small RNA fraction was enriched from a fraction of total RNA extracted from 100 mg of transgene-carrying immune FGP4a.22 T_2 plants, using miRvana Kit (Ambion, Austin, TX) following manufacturer instructions. The extracted total RNA was run on denaturing 17% PAGE and stained with EtBr. Using 100-bp RNA ladder as reference, the region corresponding to 15–50 bp was dissected and small RNA was extracted from excised gel overnight in 4 M NaCl. The RNA concentration was measured in 1 μL of solution using Nanodrop (Thermo Scientific, Wilmington). The splinted ligation was performed on the purified fraction with miRtect-IT™ miRNA Labeling and Detection Kit (USB, Cleveland, OH) (Maroney *et al.*, 2007). Specific bridge oligonucleotides (Table S1) were designed according to the manufacturer's directions. Using 50 ng of enriched smRNA per reaction, amiRNAs were captured by a specific bridge oligonucleotide and ligated to the P^{32}-labelled detection oligonucleotide with T4 DNA ligase. Ligated products were separated on 17% urea–polyacrylamide gel and visualized using Fujifilm FLA-5000 phosphor imager.

Small RNA library preparation and deep sequencing

Small RNAs were enriched using the mirVana miRNA Isolation Kit (Invitrogen) following manufacturer instructions. Small RNA-Seq libraries were prepared based on Illumina's alternative v1.5 protocol and a published method (Lu *et al.*, 2007) and run on the Illumina's GAIIx platform at the Genome Discovery Unit of Australian National University. WSMV sequences were assembled with assistance from Dr Stephen Ohms, JCSMR, The Australian National University.

Acknowledgements

We are thankful to Dr Peter Waterhouse and Dr Ming-Bo Wang for their technical advice at various stages of these studies, Mr. Zarman Mazhar Rizvi for his assistance with putting together various algorithms under one miR Mate, Drs. James Petrie and Qing Liu for technical supervision in amiRNA detection, and Dr Jun Fan, The John Curtin School of Medical Research, The Australian National University, for providing excellent training and technical supervision in small RNA library preparation. Dr Stephen Ohms is gratefully acknowledged for his assistance with bioinformatic analysis of small RNAs. The first author thankfully acknowledges AusAID for financial assistance as PhD studentship.

References

Abbott, D., Wang, M.-B. and Waterhouse, P.M. (2002) A single copy of virus-derived transgene-encoding hairpin RNA confers BYDV immunity. In *Barley Yellow Dwarf Disease: Recent Advances and Future Strategies* (Henry, M. and McNab, A., eds), pp. 22–26. Mexico, DF: CIMMYT.

Abel, P.P., Nelson, R.S., De, B., Hoffmann, N., Rogers, S.G., Fraley, R.T. and Beachy, R.N. (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science*, **232**, 738–743.

Ai, T., Zhang, L., Gao, Z., Zhu, C.X. and Guo, X. (2011) Highly efficient virus resistance mediated by artificial microRNAs that target the suppressor of PVX and PVY in plants. *Plant Biol. (Stuttg)*, **13**, 304–316.

Allen, E., Xie, Z.X., Gustafson, A.M. and Carrington, J.C. (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell*, **121**, 207–221.

Alvarez, J.P., Pekker, I., Goldshmidt, A., Blum, E., Amsellem, Z. and Eshed, Y. (2006) Endogenous and synthetic microRNAs stimulate simultaneous, efficient, and localized regulation of multiple targets in diverse species. *Plant Cell*, **18**, 1134–1151.

Baulcombe, D. (2004) RNA silencing in plants. *Nature*, **431**, 356–363.

Boden, D., Pusch, O., Lee, F., Tucker, L. and Ramratnam, B. (2003) Human immunodeficiency virus type 1 escape from RNA interference. *J. Virol.* **77**, 11531–11535.

ter Brake, O., Konstantinova, P., Ceylan, M. and Berkhout, B. (2006) Silencing of HIV-1 with RNA Interference: A Multiple shRNA Approach. *Molecular Therapy*, **14**, 883–892.

Brennecke, J., Stark, A., Russell, R.B. and Cohen, S.M. (2005) Principles of microRNA-target recognition. *PLoS Biol.* **3**, 404–418.

Chung, B.Y.W., Miller, W.A., Atkins, J.F. and Firth, A.E. (2008) An overlapping essential gene in the Potyviridae. *Proc. Natl Acad. Sci. USA*, **105**, 5897–5902.

Das, A.T., Brummelkamp, T.R., Westerhout, E.M., Vink, M., Madiredjo, M., Bernards, R. and Berkhout, B. (2004) Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. *J. Virol.* **78**, 2601–2605.

Duan, C.G., Wang, C.H., Fang, R.X. and Guo, H.S. (2008) Artificial microRNAs highly accessible to targets confer efficient virus resistance in plants. *J. Virol.* **82**, 11084–11095.

Dwyer, G.I., Gibbs, M.J., Gibbs, A.J. and Jones, R.A.C. (2007) Wheat streak mosaic virus in Australia: relationship to isolates from the Pacific Northwest of the USA and its dispersion via seed transmission. *Plant Dis.* **91**, 164–170.

Eamens, A.L., Smith, N.A., Curtin, S.J., Wang, M.B. and Waterhouse, P.M. (2009) The *Arabidopsis thaliana* double-stranded RNA binding protein DRB1 directs guide strand selection from microRNA duplexes. *RNA*, **15**, 2219–2235.

Fahim, M., Ayala-Navarrete, L., Millar, A.A. and Larkin, P.J. (2010) Hairpin RNA derived from viral Nla gene confers immunity to *Wheat streak mosaic virus* infection in transgenic wheat plants. *Plant Biotechnol. J.* **8**, 821–834.

Guddeti, S., Zhang, D.C., Li, A.L., Leseberg, C.H., Kang, H., Li, X.G., Zhai, W.X., Johns, M.A. and Mao, L. (2005) Molecular evolution of the rice miR395 gene family. *Cell Res.* **15**, 631–638.

Harvey, T.L. and Seifers, D.L. (1991) Transmission of wheat streak mosaic virus to sorghum by the wheat curl mite (Acari, Eriophyidae). *J. Kans. Entomol. Soc.* **64**, 18–22.

Hofacker, I.L., Fontana, W., Stadler, P.F., Bonhoeffer, L.S., Tacker, M. and Schuster, P. (1994) Fast folding and comparison of RNA secondary structures. *Monatshefte Für Chemie*, **125**, 167–188.

Israsena, N., Supavonwong, P., Ratanasetyuth, N., Khawplod, P. and Hemachudha, T. (2009) Inhibition of rabies virus replication by multiple artificial microRNAs. *Antiviral Res.* **84**, 76–83.

Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G. and Linsley, P.S. (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* **21**, 635–637.

Jones, R.A.C., Coutts, B.A., Mackie, A.E. and Dwyer, G.I. (2005) Seed transmission of *Wheat streak mosaic virus* shown unequivocally in wheat. *Plant Dis.* **89**, 1048–1050.

Jones-Rhoades, M.W. and Bartel, D.P. (2004) Computational identification of plant MicroRNAs and their targets, including a stress-induced miRNA. *Mol. Cell*, **14**, 787–799.

Kawashima, C.G., Yoshimoto, N., Maruyama-Nakashita, A., Tsuchiya, Y.N., Saito, K., Takahashi, H. and Dalmay, T. (2009) Sulphur starvation induces the expression of microRNA-395 and one of its target genes but in different cell types. *Plant J.* **57**, 313–321.

Khraiwesh, B., Ossowski, S., Weigel, D., Reski, R. and Frank, W. (2008) Specific gene silencing by artificial MicroRNAs in *Physcomitrella patens*: an alternative to targeted gene knockouts. *Plant Physiol.* **148**, 684–693.

Lagudah, E.S., Appels, R. and Mcneil, D. (1991) The nor-D3 locus of *Triticum tauschii* – natural variation and genetic-linkage to markers in chromosome-5. *Genome*, **34**, 387–395.

Lim, L.P., Lau, N.C., Garrett-Engele, P., Grimson, A., Schelter, J.M., Castle, J., Bartel, D.P., Linsley, P.S. and Johnson, J.M. (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*, **433**, 769–773.

Lin, S.S., Wu, H.W., Elena, S.F., Chen, K.C., Niu, Q.W., Yeh, S.D., Chen, C.C. and Chua, N.H. (2009) Molecular evolution of a viral non-coding sequence under the selective pressure of amiRNA-mediated silencing. *Plos Pathog.* **5**, e1000312.

Liu, Y.P., Haasnoot, J., ter Brake, O., Berkhout, B. and Konstantinova, P. (2008) Inhibition of HIV-1 by multiple siRNAs expressed from a single microRNA polycistron. *Nucleic Acids Res.* **36**, 2811–2824.

Lu, C., Meyers, B.C. and Green, P.J. (2007) Construction of small RNA cDNA libraries for deep sequencing. *Methods*, **43**, 110–117.

Maas, C., Simpson, C.G., Eckes, P., Schickler, H., Brown, J.W.S., Reiss, B., Salchert, K., Chet, I., Schell, J. and Reichel, C. (1997) Expression of intro modified nptII genes in monocotylenonous and dicotyledonous plants cells. *Mol. Breed.* **3**, 15–28.

Mallory, A.C., Reinhart, B.J., Jones-Rhoades, M.W., Tang, G.L., Zamore, P.D., Barton, M.K. and Bartel, D.P. (2004) MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5′region. *EMBO J.* **23**, 3356–3364.

Maroney, P.A., Chamnongpol, S., Souret, F. and Nilsen, T.W. (2007) A rapid, quantitative assay for direct detection of microRNAs and other small RNAs using splinted ligation. *RNA*, **13**, 930–936.

Matzke, M., Kanno, T., Daxinger, L., Huettel, B. and Matzke, A.J. (2009) RNA-mediated chromatin-based silencing in plants. *Curr. Opin. Cell Biol.* **21**, 367–376.

McCaskill, J.S. (1990) The equilibrium partition-function and base pair binding probabilities for RNA secondary structure. *Biopolymers*, **29**, 1105–1119.

Mi, S., Cai, T., Hu, Y., Chen, Y., Hodges, E., Ni, F., Wu, L., Li, S., Zhou, H., Long, C., Chen, S., Hannon, G.J. and Qi, Y. (2008) Sorting of small RNAs into Arabidopsis argonaute complexes is directed by the 5′ terminal nucleotide. *Cell*, **133**, 116–127.

Molnar, A., Bassett, A., Thuenemann, E., Schwach, F., Karkare, S., Ossowski, S., Weigel, D. and Baulcombe, D. (2009) Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*. *Plant J.* **58**, 165–174.

Niu, Q.W., Lin, S.S., Reyes, J.L., Chen, K.C., Wu, H.W., Yeh, S.D. and Chua, N.H. (2006) Expression of artificial microRNAs in transgenic Arabidopsis thaliana confers virus resistance. *Nat. Biotechnol.* **24**, 1420–1428.

Ossowski, S., Schwab, R. and Weigel, D. (2008) Gene silencing in plants using artificial microRNAs and other small RNAs. *Plant J.* **53**, 674–690.

Park, W., Zhai, J.X. and Lee, J.Y. (2009) Highly efficient gene silencing using perfect complementary artificial miRNA targeting AP1 or heteromeric artificial miRNA targeting AP1 and CAL genes. *Plant Cell Rep.* **28**, 469–480.

Pellegrineschi, A., Noguera, L.M., Skovmand, B., Brito, R.M., Velazquez, L., Salgado, M.M., Hernandez, R., Warburton, M. and Hoisington, D. (2002) Identification of highly transformable wheat genotypes for mass production of fertile transgenic plants. *Genome*, **45**, 421–430.

Qu, J., Ye, J. and Fang, R.X. (2007) Artificial microRNA-mediated virus resistance in plants. *J. Virol.* **81**, 6690–6699.

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W.S. and Khvorova, A. (2004) Rational siRNA design for RNA interference. *Nature Biotechnology*, **22**, 326–330.

Rotte, C. and Leustek, T. (2000) Differential subcellular localization and expression of ATP sulfurylase and 5′-adenylylsulfate reductase during ontogenesis of arabidopsis leaves indicates that cytosolic and plastid forms of ATP sulfurylase may have specialized functions. *Plant Physiol.* **124**, 715–724.

Schnippenkoetter, W.H., Martin, D.P., Willment, J.A. and Rybicki, E.P. (2001) Forced recombination between distinct strains of *Maize streak virus*. *J. Gen. Virol.* **82**, 3081–3090.

Schwab, R., Ossowski, S., Riester, M., Warthmann, N. and Weigel, D. (2006) Highly specific gene silencing by artificial microRNAs in Arabidopsis. *Plant Cell*, **18**, 1121–1133.

Seifers, D.L., Harvey, T.L., Martin, T.J. and Jensen, S.G. (1998) Partial host range of the high plains virus of corn and wheat. *Plant Dis.* **82**, 875–879.

Simon-Mateo, C. and Garcia, J.A. (2006) MicroRNA-Guided processing impairs Plum pox virus replication, but the virus readily evolves to escape this silencing mechanism. *J. Virol.* **80**, 2429–2436.

Sivamani, E., Brey, C.W., Dyer, W.E., Talbert, L.E. and Qu, R.D. (2000) Resistance to *Wheat streak mosaic virus* in transgenic wheat expressing the viral replicase (NIb) gene. *Mol. Breed.* **6**, 469–477.

Sivamani, E., Brey, C.W., Talbert, L.E., Young, M.A., Dyer, W.E., Kaniewski, W.K. and Qu, R.D. (2002) Resistance to *Wheat streak mosaic virus* in transgenic wheat engineered with the viral coat protein gene. *Transgenic Res.* **11**, 31–41.

Slykhuis, J.T. (1955) *Aceria tulipae* Keifer (Acarina, Eriophyidae) in relation to the spread of wheat streak mosaic. *Phytopathology*, **45**, 116–128.

Smith, N.A., Singh, S.P., Wang, M.B., Stoutjesdijk, P.A., Green, A.G. and Waterhouse, P.M. (2000) Gene expression – total silencing by intron-spliced hairpin RNAs. *Nature*, **407**, 319–320.

Szittya, G., Silhavy, D., Molnar, A., Havelda, Z., Lovas, A., Lakatos, L., Banfalvi, Z. and Burgyan, J. (2003) Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. *EMBO J.* **22**, 633–640.

Takeda, A., Iwasaki, S., Watanabe, T., Utsumi, M. and Watanabe, Y. (2008) The mechanism selecting the guide strand from small RNA duplexes is different among argonaute proteins. *Plant Cell Physiol.* **49**, 493–500.

Vaucheret, H., Vazquez, F., Crete, P. and Bartel, D.P. (2004) The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev.* **18**, 1187–1197.

Wagaba, H., Basavaprabhu, P.L., Jitender, Y.S., Nigel, T.J., Alicai, T., Baguma, Y., Settumba Mukasa, B. and Fauquet, C.M. (2010). Testing the efficacy of artificial microRNAs to control cassava brown streak disease. Second RUFORUM Biennial Meeting 20–24 September 2010, Entebbe, Uganda, 287–291.

Wang, M.B. and Waterhouse, P.M. (2000) High-efficiency silencing of a beta-glucuronidase gene in rice is correlated with repetitive transgene structure but is independent of DNA methylation. *Plant Mol. Biol.* **43**, 67–82.

Warthmann, N., Chen, H., Ossowski, S., Weigel, D. and Herve, P. (2008) Highly specific gene silencing by artificial miRNAs in rice. *PLoS ONE*, **3**, e1829.

Waterhouse, P.M., Graham, H.W. and Wang, M.B. (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc. Natl Acad. Sci. USA*, **95**, 13959–13964.

Wen, R.H. and Hajimorad, M.R. (2010) Mutational analysis of the putative pipo of soybean mosaic virus suggests disruption of PIPO protein impedes movement. *Virology*, **400**, 1–7.

Westerhout, E.M., ter Brake, O., Ooms, M., Vink, M., Das, A.T. and Berkhout, B. (2005) HIV-1 can evade RNAi-mediated inhibition by altering the secondary structure of its RNA genome. *J. Biotechnol.* **118**, S69–S69.

Wilson, J.A. and Richardson, C.D. (2005) Hepatitis C Virus replicons escape RNA interference induced by a short interfering RNA directed against the NS5b coding region. *J. Virol.* **79**, 7050–7058.

Xu, P., Zhang, Y., Kang, L., Roossinck, M.J. and Mysore, K.S. (2006) Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants. *Plant Physiol.* **142**, 429–440.

Zeng, Y., Wagner, E.J. and Cullen, B.R. (2002) Both natural and designed micro RNAs technique can inhibit the expression of cognate mRNAs when expressed in human cells. *Mol. Cell*, **9**, 1327–1333.

Zhang, X., Li, H., Zhang, J., Zhang, C., Gong, P., Ziaf, K., Xiao, F. and Ye, Z. (2011). Expression of artificial microRNAs in tomato confers efficient and stable virus resistance in a cell-autonomous manner. *Transgenic Res.* **20**, 569–581.

Zhao, T., Wang, W., Bai, X. and Qi, Y. (2009) Gene silencing by artificial microRNAs in Chlamydomonas. *Plant J.* **58**, 157–164.

Zuker, M. and Stiegler, P. (1981) Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. *Nucleic Acids Res.* **9**, 133–148.

Supporting information

Additional Supporting information may be found in the online version of this article:

Table S1 Oligo sequences used in miRTect analysis of amiRNA accumulation in immune transgenics.

Table S2 Families used in segregation analysis in T_2.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Supplementary Table: 1. Oligo sequences used in miRTect analysis of amiRNA accumulation in immune transgenics. The bridging oligos were synthesised and reaction was carried out using manufacturer's protocol. The oligos without * were used to detect the guide strands. The oligos with * were used to detect passenger strands. The tag sequence GAATGTCATAAGCG is common in all bridging oligos and is known as the *detection oligo*.

amiRNA	amiRNA-specific oligo	Bridging oligo
amiRNA-1	gcttatctctatgcgagagct	GAATGTCATAAGCGgcttatctctatgcgagagct
amiRNA-1*	agctctcgcatagagataagc	GAATGTCATAAGCGagctctcgcatagagataagc
amiRNA-2	cgtgtgaaagatcttgctcga	GAATGTCATAAGCGcgtgtgaaagatcttgctcga
amiRNA-2*	tcgagcaagatctttcacacg	GAATGTCATAAGCGtcgagcaagatctttcacacg
amiRNA-3	ccaggaagcattttctggtca	GAATGTCATAAGCGccaggaagcattttctggtca
amiRNA-3*	tgaccagaaaatgcttcctgg	GAATGTCATAAGCGtgaccagaaaatgcttcctgg
amiRNA-4	ccgcgaacgtcttgcaagtta	GAATGTCATAAGCGccgcgaacgtcttgcaagtta
amiRNA-4*	taacttgcaagacgttcgcgg	GAATGTCATAAGCGtaacttgcaagacgttcgcgg
amiRNA-5	gaagattccattatgtgccga	GAATGTCATAAGCGgaagattccattatgtgccga
amiRNA-5*	tcggcacataatggaatcttc	GAATGTCATAAGCGtcggcacataatggaatcttc

Supplementary Table 2. Families used in segregation analysis in T_2

+R = *FGmiR395* carrying, resistant; +MR = transgene, moderately resistant;

+S = transgene, susceptible; -S = transgene null, susceptible. Symptoms are scored on a 0-4 scale.

Event	Phenotype	n=	Plant Height (cm)	Symptoms			
				7dpi	14dpi	21dpi	28dpi
FGP4a	FGmiR395+R	15	42.4±1.2	0	0	0	0
	FGmiR395+MR	2	26±0.7	0	1.7±0.0	2.5±0.1	2.3±0.9
	FGmiR395+S	7	14.5±0.8	0.2 ± 0.25	1±0.1	2.1±0.2	3.2±0.2
	FGmiR395-S	8	13.2±1.2	0.8±0.2	2.1±0.1	3.3±0.09	3.5±0.1
FGP6	FGmiR395+R	13	46.4±1.03	0	0	0	0
	FGmiR395-S	13	14.6±1.4	0.5±0.2	1.5±0.2	2.2±0.2	3.2±0.1
FGP8c	FGmiR395+R	30	45.5±1.0	0	0	0	0
	FGmiR395+MR	7	28.5±1.8	0	1.3±0.09	2.1±.1	2.6±0.2
	FGmiR395+S	14	16.9±1.0	0	1.7±0.1	2.6±0.1	3.1±0.09
	FGmiR395-S	19	14.2±0.7	0.5±0.1	2.0±0.1	2.9±0.1	3.7±0.09
FGP15a	FGmiR395+R	32	47.0±0.8	0	0	0	0
	FGmiR395+MR	5	21.2±0.7	0	1±0.25	1.9±0.09	2.5±0.1
	FGmiR395-S	14	15.0±1.5	0.2±0.1	1.71±0.2	3.03±0.1	3.5±0.1
FGP18	FGmiR395+MR	17	26.1±1.3	0.1±0.1	1.4±0.2	2.1±0.1	3.44±0.1
	FGmiR395-S	21	14.2±0.9	0.8±0.1	1.9±0.1	3.04±0.1	3.7±0.08

Chapter 7
Conclusions and Recommendations

Chapter 7. Conclusions and Recommendations

WSMV is a newly discovered virus in Australia and is an emerging risk to wheat production. The current losses due to WSMV in Australia have been estimated to be around $62 million per annum, however, under favourable climatic conditions, the virus can potentially play havoc with wheat production in Australia and around the world, especially in the Great Plains of the North America.

As a consequence of the work assembled in this thesis, both conventional and transgenic options for the control of this serious viral pathogen are available to the wheat industry in Australia and in the rest of the world. We found that resistance sources available in *Wsm1* (an alien translocation), *Wsm2* (CO960293-2) and c2652 are effective in protecting against WSMV under the agro-climatic condition of Australia. However, wheat genotypes carrying any of these genes, are still susceptible to other viruses such as *Barley yellow dwarf virus* (BYDV), *Cereal yellow dwarf virus* (CYDV), *High plains virus* (HPV) and a recently discovered mite-vectored virus, *Triticum mosaic virus* (TriMV). However, the stability and strength of the WSMV resistance in the wheat/*Th. intermedium* partial amphiploids leads to the conclusions that multiple genes on multiple chromosomes are involved in these stable and strong resistances. This illustrates the potential advantage of gene stacking for more effective resistance. It would be of great value to generate new resistance translocations to wheat from these amphiploids and broaden the opportunities for gene stacking which should ultimately increase the strength and longevity of protection. In the medium term opportunities for gene stacking for WSMV resistance are already available: *Wsm1*, is available on chromosomes 4D and 4A; *Wsm2* is on 3B; c2652 is on the A or B genome. Although more tightly linked markers for *Wsm2* and c2652 would be highly desirable, the markers developed for the various *Wsm1* translocations along with the heat stability of

Wsm2-CA745 and c2652, may already create an opportunity for gene stacking. Once intercrossed, segregants with both genes (c2652 and *Wsm1*, or CA745 and *Wsm1*) might be distinguished by the presence of a *Wsm1* marker and the presence of heat stable resistance. Despite this theoretical possibility, breeders concerned with this disease will welcome the mapping of c2652 resistance and development of tightly linked molecular markers for both c2652 and *Wsm2*.

Apart from exploring natural resistance sources we developed transgenic strategies against WSVM. The recent reports of successful use of amiRNAs for resistance in plants, has opened a new chapter in transgenic resistance against plants. Although long hairpin RNAi strategies are very attractive from a biotechnology application perspective, they pose a greater risk of unintended silencing of off-target genes in the host, compared to amiRNA. Furthermore, long hpRNA approaches are seen by some as posing a risk in the field of heterologous recombination with other virus genomes and resulting in new virus biotypes. Low temperatures can also compromise the efficacy RNAi silencing strategies. AmiRNAs are likely to address these issues. However, further work is necessary to understand the implications of replacing native mature miRNA sequences with amiRNAs. We anticipate ongoing improvements in the understanding of miRNA biogenesis and design of amiRNA to further enhance the utility for virus resistance and engineering other agronomically important traits.

The work reported demonstrates that immunity in wheat has been achieved to WSMV from both the long hairpin ds-RNA and the amiRNA strategies. A greater proportion of transgenics with hpRNA were immune compared to amiRNA, and by that measure hpRNA was the more robust approach. However, I conclude that amiRNA based viral resistance, especially polycistronic amiRNA as advocated here, deserves and needs further in depth studies to improve the amiRNA efficiency. An even better comparison would be achieved if the long hpRNA was designed to cover the same regions as the

amiRNA used in this study. The use of miR395 and similar miRNA clusters as a carrier of multiple amiRNAs can be used to target multiple regions of the one virus (as here), multiple viruses, or multiple endogenous mRNA species. Mixed viral infections are common in the field especially in fruits and vegetables, thus there is a need to target highly conserved regions of multiple viruses using polycistronic amiRNA or hpRNA. Therefore, combined immunity from the one transgene to WSMV, BYDV, CYDV, HPV and TriMV is an exciting prospect once GM wheat finds its way into the market place.

Appendices

Appendix I

A new source of resistance against wheat streak mosaic virus (WSMV) derived from doubled haploid (DH) spring wheat line C2652

Steve Haber[1], **Muhammad Fahim[2]**, Ligia Ayala-Navarrete[2], P.J. Larkin[2] and Dallas L. Seifers[3]
[1]Cereal Research Centre, Agriculture & Agri-Food Canada, Winnipeg, MB R3T 2M9 Canada
[2]CSIRO Plant Industry, Canberra ACT 2601, Australia;
[3]Kansas State University, Agricultural Research Center–Hays, Hays, KS 67601-9228;.

E-mail: Steve.Haber@agr.gc.ca

ABSTRACT

Host genetic resistance can completely prevent losses from infection with wheat streak mosaic virus (WSMV). The hitherto available sources, however, suffer from one or more of the following deficiencies: a) loss of effectiveness at higher growing temperatures; b) linkage to inferior quality or yield; and c) unsuitability for simple introgression into durum wheat germplasm. We describe here a new source of resistance, designated c2652, which appears to avoid these defects. Its effectiveness at higher temperatures, in particular, meets Australian needs better than other sources. Individual plants with near-immunity to seedling infection with WSMV were fortuitously discovered in C2652, an otherwise susceptible doubled haploid (DH) experimental line of hard red spring bread wheat developed at the Cereal Research Centre in Winnipeg. Back-assay titration showed that true-breeding lines derived from these individuals expressed immunity to seedling infection at 18 C, but mild symptoms and moderate virus titres at 24 C, similar to the expression of resistance originating from CO960293-4. With repeated selection in temperature regimes above 22 C, lines were derived that performed better than CO960293-4 in temperature regimes above 18 C and one of these (CA 745) remained resistant to an Australian WSMV isolate for 30 days in a regime of 26 C day, 18 C night, longer than lines with resistance conferred by *Wsm1* or CO960293-2. The resistance derived from the selected, WSMV-resistant sub-lines of c2652 was introgressed into both hexaploid and tetraploid elite germplasms using simple backcrossing regimes and was inherited in a manner consistent with that of a single dominant gene.

Submitted to Plant Disease

Appendix II

Multi-gene silencing using a single polycistronic artificial miRNA

Srinivas Belide[1,3], James R Petrie[1], Pushkar Shrestha[1], **Muhammad Fahim[2,4]**, Qing Liu[1], Craig C Wood[2], Surinder P Singh[1]

[1] Food Futures National Research Flagship, CSIRO Plant Industry, PO Box 1600, Canberra, ACT 2601, Australia.
[2] CSIRO Plant Industry, PO Box 1600, Canberra, ACT 2601, Australia.
[3] Department of Biotechnology, Sreenidhi Institute of Science and Technology, Yamnampet, Ghatkesar, Hyderabad-501301, AP, India.
[4] Department of Microbiology, Hazara University Mansehra,KPK, Pakistan.

Abstract

Background: Various post transcriptional gene silencing (PTGS) strategies have been developed and exploited to study gene function or develop disease resistance. The recently-developed artificial microRNA (amiRNA) strategy is an alternative method of effectively silencing target genes although it tends to be effective against single gene targets only.

Results: *Arabidopsis thaliana* (ecotype Columbia) was transformed with a modified polycistronic *Oryza sativa* miR395 gene that targets three *A. thaliana* genes, FAD2, FAE1 and FATB, generating amiRFAD2FAE1FATB. Single amiR159b-based constructs targeting these genes were also transformed into plants. Fatty acid profiles from transgenic homozygous T_3 seeds reveal that polycistronic amiRFAD2FAE1FATB silenced these multiple genes simultaneously although not as effectively as single amiR159b-based constructs.

Conclusions: It is possible to use engineered polycistronic miRNAs for simultaneously down-regulation of multiple endogenes in seeds, in this case FAD2, FAE1 and FATB.

Submitted to Plant Methods

Appendix III

Pathways to the development of 'perennial wheat' for Australian environments.

Hayes, R., Newell, **M., Fahim**, M., Norton, M., Newberry, M., DeHaan, L.R., Cox, T.S., Jones, S.S., Murphy, K.M., Wade, L.J., Larkin, P.J.

CSIRO Plant Industry, Canberra ACT 2601, Australia;
Department of Microbiology, Hazara University Mansehra, KPK, Pakistan.

Cereal plants with a perennial habit would offer advantages in production stability and environmental sustainability. Breeding programs in the northern hemisphere have produced derivatives with perennial potential following crosses involving bread wheat and perennial wheatgrasses (*Thinopyrum or Lophopyrum spp.*). This study evaluated the performance of 67 of these hybrid derivatives in Australia. Field experiments were conducted at Cowra and Woodstock in the mixed cropping zone of NSW. All hybrid derivatives were significantly later in their maturity than locally adapted wheats. All partial amphiploids (2n=56) derived from *Th. intermedium* or *Th. ponticum* were highly resistant to wheat streak mosaic virus and most were also very resistant to stripe and leaf rust. Good resistance to current Australian races of stem rust were rarer. Nine amphiploid (2n=56) entries regrew and produced grain in the second season. Importantly it was shown that there is a strong correlation between ability to regrow and the retention of at least seven pair of extra chromosomes, presumably from the perennial donor. Aneuploids with less wheatgrass chromosomes were not capable of regrowth. The regrowth of plots of the best lines at Woodstock was similar to the regrowth of perennial rye (*Secale montanum*). Further research is required to strengthen perenniality, improve adaptation and survival through the harsh Australian summers, improve grain yields, confirm the beneficial depth of the root systems, and measure the quantity, quality and impact of forage cuts. We see a potential role for such stable amphiploids as dual purpose graze and grain crops. Realistic schemes to generate adapted breeding material are suggested.

In preparation

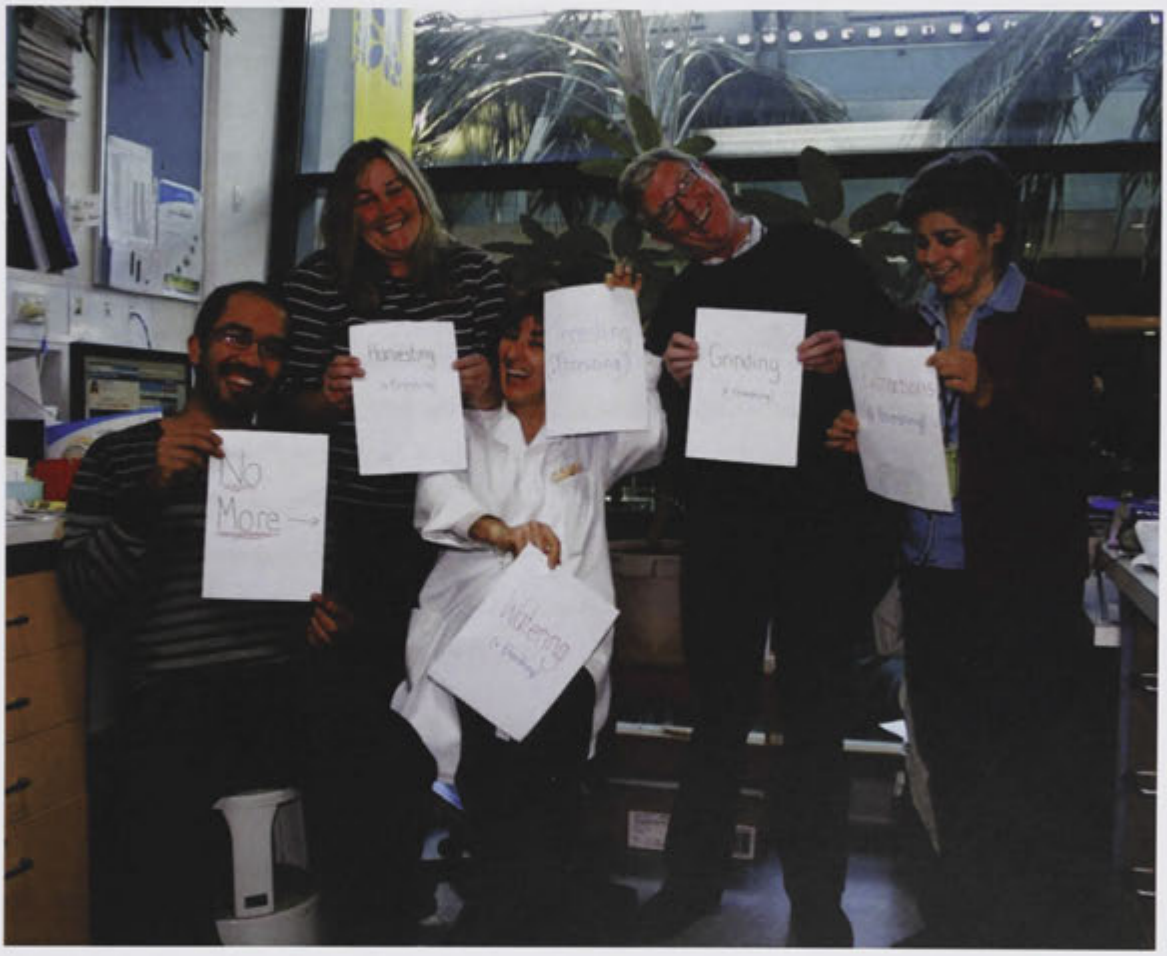
Appendix IV

Calculating Delta G for hpRNA -The miR Mate Algorithm-

```
print "enter file number ";
$num=<STDIN>;
chomp $num;

$dg = "deltaG" . $num . '.txt';


open(OUTFILE, "> $dg") or die("could not open file out \n");

$seqName = "";
$silseq = "1";
$numSeqs = 0;
$targseq = "GCGCCGAGGTGAAGTTCGA";
$revt = "CGCGGCTCCACTTCAAGCT";


while ($silseq){

print "      TARGET SEQUENCE = $targseq \n";
print "REV C TARGET SEQUENCE = $revt \n";
print "      Enter  sequence : ";

$silseq=<STDIN>;
chomp $silseq;

if ($silseq eq "exit"){exit}

$silseq =~ tr/acgtuU/ACGTTT/;
$revtarg = reverse($revt);



print "      SilenceSEQUENCE = $silseq \n";


            open(TMPFILE, "> minFE.tmp") or die("could not open file
minFE.tmp\n");

            $silseqcomp = reverse($silseq);
            #$silseqcomp =~ tr/ACGT/TGCA/;

            $hp = $targseq . "AAAA" . $revtarg;
print "  perfect match hp  = $hp\n";
            print TMPFILE "$hp\n";
            close TMPFILE;

open(TMPFILE2, "> minFE2.tmp") or die("could not open file minFE2.tmp\n");



            $hp2 = $targseq . "AAAA" . $silseqcomp;
print "imperfect match hp2 = $hp2\n";

            print TMPFILE2 "$hp2\n";
            close TMPFILE2;
```

```
$results = `C:/Perl/RNAfold.exe < minFE.tmp`;
$results =~ s/\n/ /;



if ($results=~ /(\(\-)/) {
my $match = $&;

$mfe = $';
chomp $mfe;
chop $mfe;



$results2 = `C:/Perl/RNAfold.exe < minFE2.tmp`;
$results2 =~ s/\n/ /;
#print $results2;


#print "\n $results2 \n";
$res1 = substr($results2, 0,42);
$res2 = substr($results2, 43,43);
print "         tested hp2 = $res1 \n";
print "         tested hp2 = $res2 \n";


if ($results2=~ /(\(\-)/) {


print " Perfect Free energy : $mfe";



$mfe2 = $';
chomp $mfe2;
chop $mfe2;
print "Free energy of new miR: $mfe2\n";


$ratio = ($mfe2/$mfe);

print OUTFILE " $hp \n $hp2 \n $res1 \n $res2 \n $mfe \n $mfe2 \n $ratio
\n\n\n";


}
}
}


print "why are you stopping here? \n";
close OUTFILE;
```

PhD – *down under*



Plant Virus Research Group- Plant Industry CSIRO 2007-2011

Right to left:

Dr. Ligia Ayala-Navarette: The driving force behind my understanding of Plant virology

Dr. Philip Larkin: I owe him this successful project in every possible way.

Anna Mechanicos: My strongest support as a friend and mentor

Jenny Gibson: An excellent wheat breeder and cheerful friend

Myself: whose love for plant virology made this project more of a hobby than a PhD.

What exactly made me choose *"back of bourke"* or "The *down-under"* over other countries for my doctoral degree remains a mystery?. The same goes for the expressing mixture of feelings for years spent in Canberra in few paragraphs. The journey had been full of rich experiences in personal and professional ways that enabled me to discover more of myself and the world around me.

The first most wonderful experience of my stay remained to be Phil Larkin - in the capacity of a friend. Phil had been an inspirational human with whom I discussed matters in life around us and around the world at countless informal sessions of coffee, tea and lunch. His friendly approach with refined touch of humour and exemplary supervision remains the driving force behind success of this project. I extend my vote of thanks for his amazing family with whom I shared lunches and dinners on several occasions. The second best part of my stay was having the pleasure of multicultural friends with whom I enjoyed numerous social and academic events from several caving trips to an adrenaline-driven Skydiving; from a Bushwalking to Kayaking and a countless photoshoots. Nonetheless, Wednesday night "Hogwart's Dinners" at University House remains another best experience of my stay at The Australian National University. I thank my mates for sharing greatest moments of laughter making the sometimes monotonous life in Canberra into a *"no worries mite"*.

I would specifically remain in debt to the wonderful company of my dearest friends Acram Latip, Ankit Jalan, S.M. Akramus Salehin, Cong Phuoc Hyunh , David Fettling, Ezzat Alhalabi & Maha Zohbi, Faham Abdus, Fatima Naim, Jane Flanagan(with thanks to her parents and a loving, caring aunt Julie Flanagan), Jie-Lian Beh, Ju lynn Ong, Junaid Ghani (Dingo), Nicolas Lema and Penelope Coulter; for their greatest understanding, thought provoking discussions (evolution, creation, religion and politics) over countless coffees, lunches and dinners, and their wonderful friendship during the course of my stay in Australia. It would not be exaggeration to claim that Hafiz Aziz-ur-Rahman, Khalid Chauhan, Raja Ajmal Jahangeer, Shah Faisal and Muhammad Atif and their families, were my Pakistan in Australia. Thanks is a small word for their wonderful company and mateship, Pakistani dinners and Oz BBQs and regular monday coffees at Pajenka's, God's and Degree Cafe.

It was a pleasure to have interacted with Dr. John Richards (ANU), Drs. John Watson, Tony Pryor, Ming-Bo Wang, Mick Ayliff, Peter Ryan, Craig Wood and Qing Luo at (CSIRO). I gratefully acknowledge their advices and fruitful

discussions throughout my stay in Australia. I am very thankful to Anna Mechanicos, CSIRO not only for the wonderful technical and professional assistance but also her kind encouragement at a personal and social level through various stages of this research. Staying in Plant Industry CSIRO was a great experience and the space in the (Plant Industry CSIRO) tea room I shared with people (too numerous to thank individually) would remain one of the best experience of my stay.

Working part-time at the National Museum of Australia in Canberra enhanced my experience of *"Down-Under"* and I got to see the real Aussies up close at a different level. I thank my work colleagues for making my stay unforgettable, I thank them all with special note to Kristine, Simon, Graham, Michelle, Sameh, Troy, Lasiate, and Peter.

Though, never dared not to touch *"Vegemite"*, I did try *"throwing shrimps on the barbie"*, nonetheless I carry with me a treasure of *"Gud-eye Mite"*, *"waddayareckon"*, *"owyeragoin"*, and much more.

Tue Mar 19 09:47:20 EST 2013

b25698941 Last Updated: 15-11-2012 Created: 09-03-2012 Revisions: 2

LANG engEnglish CAT DATE 09-03-2012 BCODE3 gGNS FUND
SKIP 0 BIB LVL mMONOGRAPH COUNTRY acaAustralian Cap. Territory
LOC'N mz MENZIES MAT TYPE tANU THESES

MARC Leader #####ntm a22#####Ia 45e0

008 120309s2011 aca m 000 0 eng d
100 1 Fahim, Muhammad
245 1 0 Natural and engineered resistance to wheat streak mosaic virus (Tritimovirus: potyviridae) /|c Muhammad Fahim.
260 |c2011.
300 x, 153 leaves.
520 3 Wheat streak mosaic virus (WSMV) is a new virus of wheat crop in Australia. Discovered in the Australian Capital Territory (ACT) in 2003, the virus has put Australian commercial bread wheat at a risk of major losses. Although, the virus is naturally transmitted by Wheat curl mites (WCM), some of the Australian farmin community expressed concerns that grazing of early sown, dual-purpose wheat for winter forage may have a role in the spread of WSMV. We probed this issue in a series of experiments with housed sheep grazing on WSMV infected wheat plants. However, we find no evidence for the suggestion that grazing sheep spread the WSM between plants in a grazed wheat crop as a consequence of the grazing process itself.
520 3 |bWe tested for natural resistance against WSMV in diverse germplasm including three different known resistance sources in cultivated wheat. Previously reported resistances were effective against the Australian isolate of WSMV. Some accession of these resistances were ineffective at higher temperatures (all Wsm1 and most Wsm2 accessions); some were reported to have linked negative agronomic traits (most accessions of Wsm1). Two exceptions were c2652 and Wsm2 accession CA745 which were very effective at controlled higher temperatures (28°C), in the glasshouse, and also protected plants from symptoms and yield loss following WSMV mechanical inoculation in the field, making these two sources particularly useful in the relatively warm Australian agro-climate. New molecular markers were developed for the various derivatives of Wsm1 resistance that should help speed u the breeding of resistance into wheat cultivars. These Wsm1 markers are now being used by CSIRO for breeding Wsm1-resistance into elite wheat cultivars.
520 3 |bFurthermore, we developed and tested two independent transgenic strategies base on intron-hairpin RNA (ihpRNAi) and artificial microRNAs (amiRNA). Both strategie were effective in conferring immunity in transgenic wheat to mechanically inoculated WSMV. We classified this resistance as immunity by four criteria: no disease symptoms were produced; Enzyme linked immunosorbent assay (ELISA) reading were as in un-inoculated plants; viral sequences could not be detected by RT-PCR from leaf extracts; and leaf extracts failed to give infections in susceptible plants when used in test-inoculation experiments. We developed ihpRNA or RNAi based immune transgenic wheat by designing an RNAi construct to target the Nuclea inclusion protein 'a' (NIa) gene of WSMV. The Northern and Southern blot hybridization analysis indicated the ihpRNA transgene integrated into the wheat genome and was processed into typical 21-24 nucleotide long siRNAs and correlated with immunity in transgenic plants. In order to achieve amiRNA immunity, we designed five artificial microRNAs (amiRNA) against different portions of the WSM genome, utilising published miRNA sequence and folding rules; these amiRNAs were incorporated into five duplex arms of the polycistronic rice primary microRNA (pri-miR395) and transformed into wheat. Southern blot hybridisation showed that the transgene was stably integrated into the wheat genome and processed into smal RNAs, both correlating with transgenic resistance against WSMV.
520 3 |bAs a consequence of the work described in this thesis, the wheat industry in Australia and abroad has both conventional and transgenic options for the control of this serious viral pathogen.
502 Thesis (Ph.D.) -- Australian National University, 2011.

i28215382 Last Updated: 15-01-2013 Created: 09-03-2012 Revisions: 9

COPY #	1	LCHKIN	- -	LOANRULE	0
CAT STAT	0	# RENEWALS	0	STATUS	4MENZIES PROC
ICODE2	gGEN NON SUBJ	# OVERDUE	0	INTL USE	0
I TYPE	80Theses	ODUE DATE	- -	COPY USE	0
PRICE	$0.00	IUSE3	0	IMESSAGE	
OUT DATE	- -	RECAL DATE	- -	OPACMSG	
DUE	- -	TOT CHKOUT	0	YTDCIRC	0
PATRON#	0	TOT RENEW	0	2YRCIRC	0
LPATRON	0	LOC'N	8mz MENZIES thesis		

098 +2431095

b +2431095
x AUTHORISATION DATED:
x Ph.D. 201
x Issue to non-ANU
x Copying by individuals
x Copying for institutions
x Supervisor :
x Department :
x Colour of binding :
x Sent for shelving
x INFORMATION BELOW IS CONFIDENTIAL
x Author's address :
x CD-ROM made :
x CD-ROM disc provided by candidate:
x Copies sold :