

Resistance to *Wheat streak mosaic virus* generated by expression of an artificial polycistronic microRNA in wheat

Muhammad Fahim^{1,2,†}, Anthony A. Millar², Craig C. Wood¹ and Philip J. Larkin^{1,*}

¹CSIRO Plant Industry, Canberra, ACT, Australia

²Division 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 T₁ 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 T₂ 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 antiviral 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	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA
		1 21	21 1	21 21	1 21	1 21
		Origin				
		Description				
(a)						
AF057533	Sydney 81	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA
1AF285169	Type Strain	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA
AF285170	El Batan	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA
AF454454	Czech	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA
AF454455	Turkish	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA
(b)						
AF511614	H955	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA
AF511615	H98	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA
AF511618	ID96	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA
AF511619	ID99	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA
AF511630	Mon96	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA
EU914917	Naghadeh	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA
EU914918	Sadat-Saher	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA
F511643	WA99	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA
FJ348358	WA94	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA
FJ348359	ARG2	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA
(c)						
Unpublished	ACT	AGCTCTCGCATAGAGATAAGC	TCGAGCAAGATCTTTCACACG	GAAGATCCATTATGTGCCGA	CCAGGAAGCAATTTCTGGTCA	CCGCGAAGCTCTTGCAAGTTA

WSMV, Wheat streak mosaic virus.

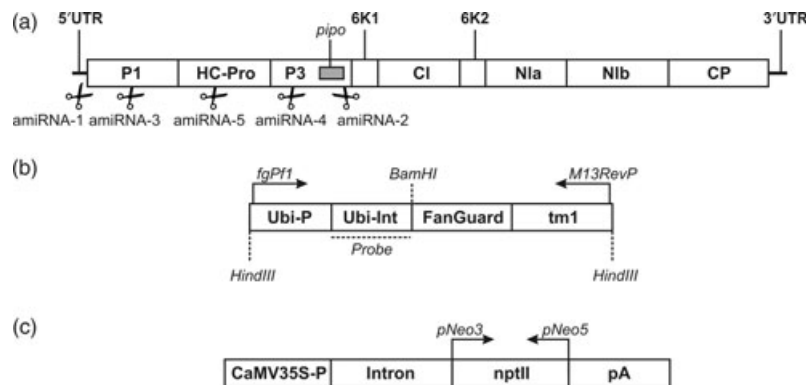


Figure 1 Structure of the *Wheat streak mosaic virus* (WSMV) genome (approximately 9400 nt), the target sites for amiRNAs and the *FGmiR395* transgene. (a) Genome map of WSMV showing the five conserved regions (indicated by scissors) targeted by amiRNAs, amiRNA-1 to amiRNA-5. (b) Design of *FGmiR395* 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.

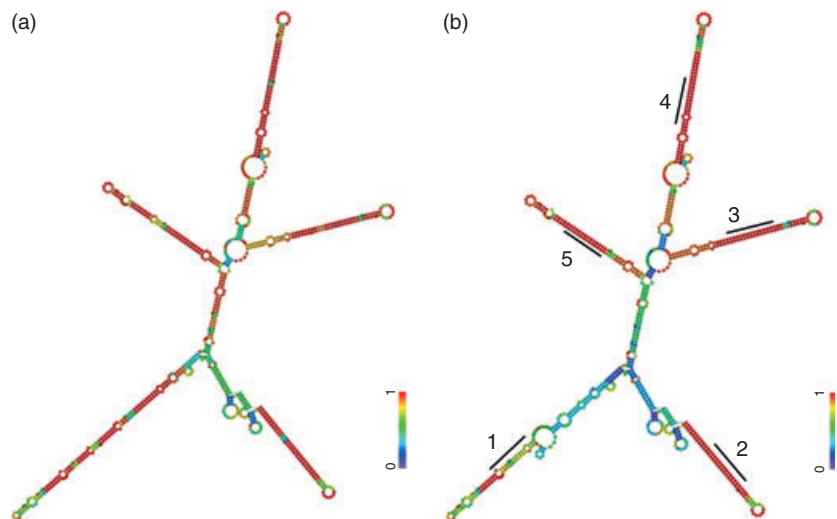


Figure 2 amiRNA Secondary Structure. A comparison of truncated *Osa-miR395* and *FGmiR395* secondary structures. (a) Predicted secondary structure of *miR395* truncated to include only the first five native miRNAs. (b) Predicted secondary structure of *FGmiR395* replacing the first five natural miRNA 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

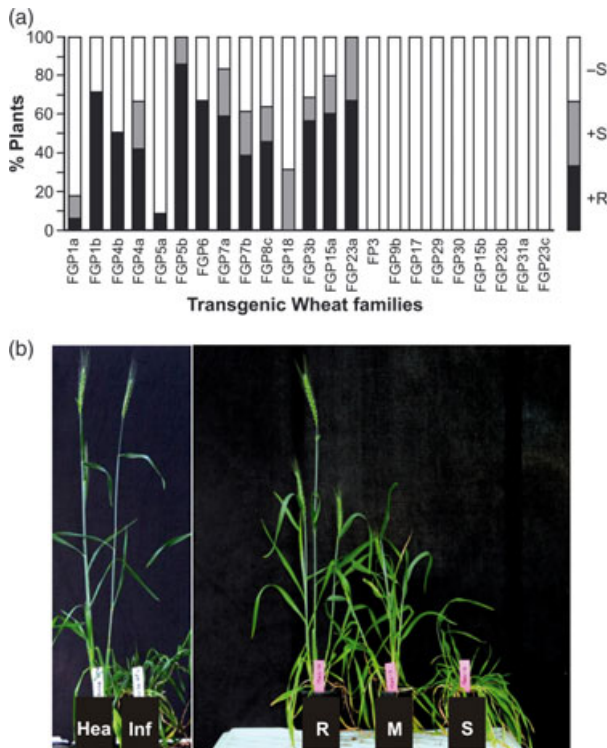


Figure 3 Preliminary assessment of resistance in T₁ families of FGmiR395 expressing transgenic wheat. (a) Wheat streak mosaic virus ELISA-based bioassay analysis of resistance in segregating populations. Virus levels were detected by double-antibody sandwich enzyme-linked immunosorbent assay at 14 d 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 T₁ 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

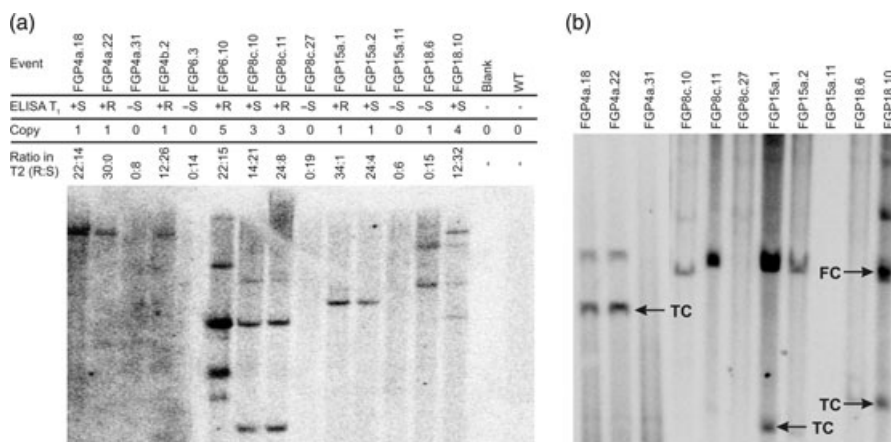


Figure 4 Southern blot analysis of families in T₂ generation. (a) *Bam*HI digested wheat DNA for transgene copy number. (b) *Hind*III-digested wheat DNA for transgene copy size. FC is full copy insert size 3 KB; TC is truncated copy inserts. Plant FGP18.6 is labelled -S by PCR and bioassay; it has FG 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

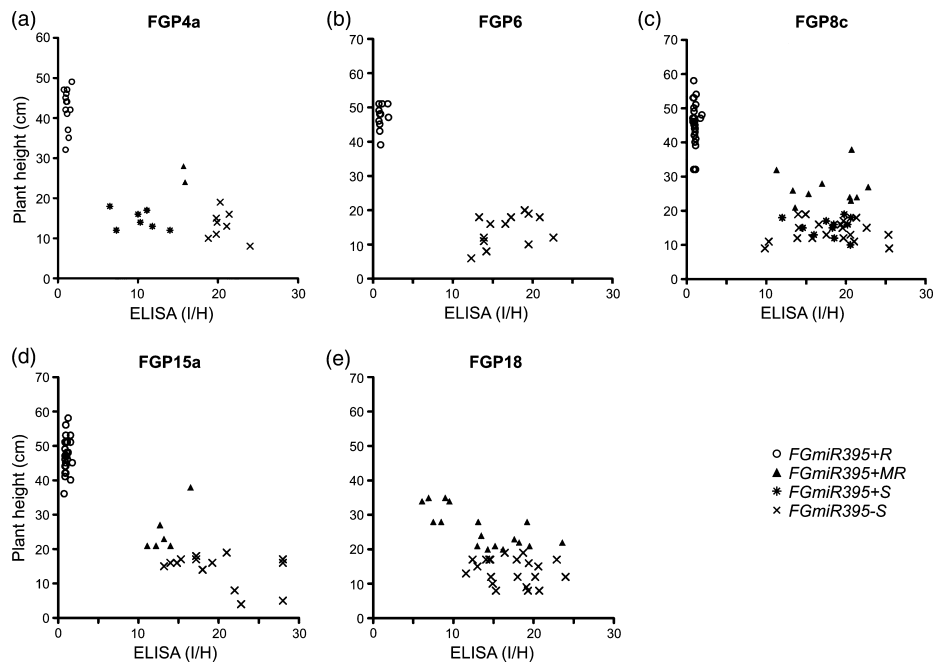


Figure 5 Segregation of resistance in FGP families expressing *FGmiR395* transgene. Virus levels were detected by double-antibody sandwich enzyme-linked immunosorbent assay at 28 d p.i. postinoculation and expressed as ratio of inoculated and healthy control. The plant height (in cm) was measured at 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 T₁ and T₂. Analysis of six selected FGmiR395 families in T₁ and T₂. Recorded for T₁ are the results of Southern hybridisation, segregation for genomic PCR, and resistance. Segregation for resistance in T₂ is also shown for a series of selected derivative families. Selected T₂ families were derived as shown from T₁ 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		
	FGmiR395 Copy no. Southern blot (examples Figure 4a)	FGmiR395 no. of loci (segregation)	PCR <i>ptII</i> + : –	PCR <i>GmiR395</i> + : –	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 4a.22 4a.31	+S +R –S	20 : 15 29 : 0 0 : 8
FGP6	4	–	17 : 9	13 : 173	13 : 13	6.3 6.10	–S +R	0 : 14 22 : 15
FGP8c	3	1	49 : 21	51 : 19	30 : 21	8c.10 8c.11 8c.27	+S +R –S	14 : 21 24 : 8 0 : 19
FGP15a	1	1	39 : 13	39 : 13	32 : 7	15a.1 15a.2 15a.11	+R +S –S	34 : 1 24 : 4 0 : 6
FGP18	3	–	26 : 12	17 : 21	0 : 38	18.6 18.10	–S +S	0 : 15 12 : 32

WSMV, *Wheat streak mosaic virus*.

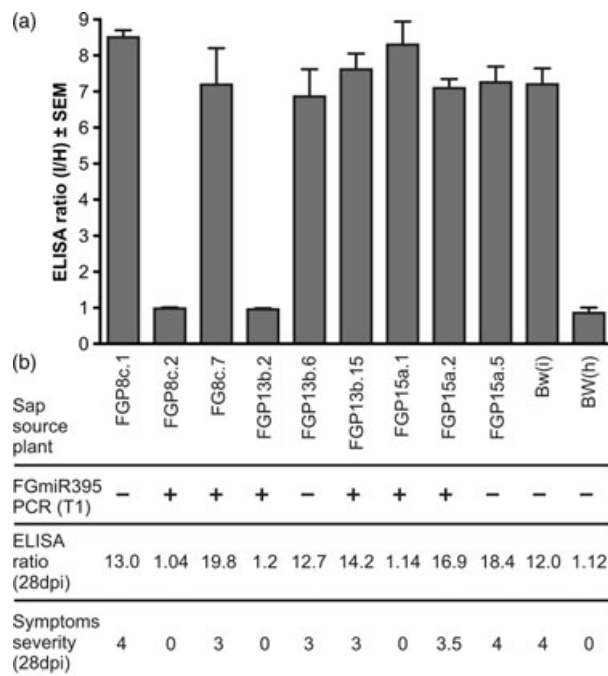


Figure 6 Virus transmission from the inoculated transgenic plants onto Bobwhite26. Sap was extracted from inoculated transgenic plants at 1/10 (w/v). Each sap extract was inoculated onto three wild-type BW26 plants. At 28 d p.i., samples were collected and processed for *Wheat streak mosaic virus* ELISA. (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 T₁ 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 T₂ 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 T₁ 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.

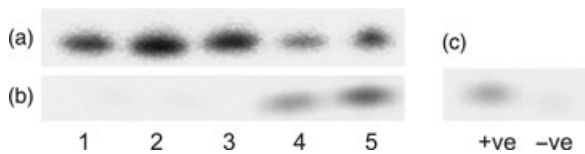


Figure 7 Expression analysis of *Wheat streak mosaic virus*-specific amiRNAs: Levels of the amiRNAs in immune transgenic segregant FGP4a.22 were examined using the MiRtect IT splinted ligation assay. Assays with bridge oligonucleotides specific for (a) amiRNA, (b) amiRNA*, and (c) internal controls. The numbers in the bottom panel correspond to amiRNA-1 to -5, respectively.

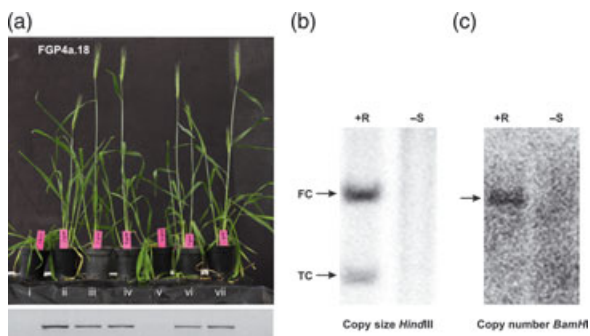


Figure 8 Segregation of resistance in T_2 progeny of T_1 individual FGP4a.18: (a) Inoculated T_2 plants and FGmiR395 PCR results. (i) and (v) transgene-negative susceptibles; (ii) transgene carrying moderately resistant; (iii) (iv) (vi) and (vii) transgene-carrying immunes. (b) Southern blot for copy 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 led 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.

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

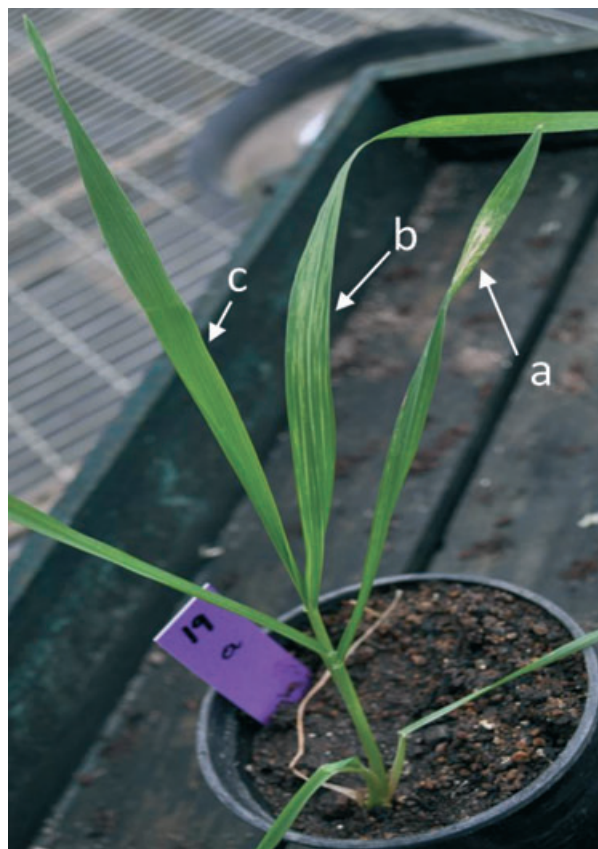


Figure 9 Recovery phenotype. (a) Inoculated leaf (b) leaf showing symptoms (c) newly emerged healthy leaf. In this transgenic FGP13b.2 individual, we inoculated the leaf with spray gun (necrotic spots shows mechanical injury resulting from spray inoculation); at 14 d p.i., the plants showed symptoms 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).

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 T₁ 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 fourth 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 re-inoculation 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 *FGmiR395*, 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 T₁ plants was used to inoculate T₂ progeny. Not only did the resistant transgenic continue to be resistant to these inoculations, but progeny from some +S T₁ 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 T₂ 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 T₁ and T₂ 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; Khraiweh *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 (Szittyta *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; Szittyta *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 algorithm 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 T₀ 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'-TGCAGCATC-TATTCATATGC-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'-GGCTATTGGCTATGACTG-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 T₁ and T₂ 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 T₁ plants, the inoculum was prepared from virus-infected non-transgenic BW26; for the analysis of T₂ 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 $A_{405\text{nm}}$ 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 *nptII* 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*HI in *FGmiR395* (Figures 1b and 4). DNA was digested with *Hind*III 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 μ L of solution using Nanodrop (Thermo Scientific, Wilmington). The splinted ligation was performed on the purified fraction with miRtect-ITTM 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 P³²-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 GAllx 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 Nia 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.
- Irasena, N., Supavongwong, 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.
- Khraiweh, 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-Engle, 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 monocotyledonous 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 Khvorov, 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'-adenylsulfate 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 (N1b) 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.
- Szittyá, 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., Nigal, 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 T₂.

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.