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Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance

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Plant microRNAs (miRNAs) regulate the abundance of target mRNAs by guiding their cleavage at the sequence complementary region. We have modified an *Arabidopsis thaliana* miR159 precursor to express artificial miRNAs (amiRNAs) targeting viral mRNA sequences encoding two gene silencing suppressors, P69 of turnip yellow mosaic virus (TYMV) and HC-Pro of turnip mosaic virus (TuMV). Production of these amiRNAs requires *A. thaliana* DICER-like protein 1. Transgenic *A. thaliana* plants expressing amiR-P69^{159} and amiR-HC-Pro^{159} are specifically resistant to TYMV and TuMV, respectively. Expression of amiR-TuCP^{159} targeting TuMV coat protein sequences also confers specific TuMV resistance. However, transgenic plants that express both amiR-P69^{159} and amiR-HC-Pro^{159} from a dimeric pre-amiR-P69^{159}/amiR-HC-Pro^{159} transgene are resistant to both viruses. The virus resistance trait is displayed at the cell level and is hereditable. More important, the resistance trait is maintained at 15 °C, a temperature that compromises small interfering RNA–mediated gene silencing. The amiRNA-mediated approach should have broad applicability for engineering multiple virus resistance in crop plants.

Plants possess several innate mechanisms to resist viruses, one of which entails the production of dominant resistance gene products that can trigger hypersensitive response and systemic acquired resistance[1,2]. However, transgenic technology offers the possibility to genetically modify plants with genes encoding virus tolerance/resistance. Over the last two decades, several strategies have been developed mostly based on the concept of pathogen-derived resistance[3].

The coat protein gene of the tobacco mosaic virus (TMV) was used in the first demonstration of virus-derived, protein-mediated resistance in transgenic plants[4]. Transgenic tobacco plants expressing high TMV coat protein levels were resistant to TMV virions but less so to TMV RNA. This pioneering observation was quickly confirmed by similar results with other viral coat protein genes[5]. In addition, dominant-negative mutant forms of viral proteins, for example, replicase[6] and movement proteins[7], also confer virus resistance in transgenic plants.

RNA-mediated virus resistance can be brought about by expression of satellite RNA, defective interfering RNA or the noncoding region of viral genome RNAs, which interfere competitively with virus replication[7]. This type of resistance can also be accomplished by expression of viral sequences in the sense or antisense orientation[8,9] or in double-stranded forms[10]. In these cases, expression triggers degradation of both the transgene RNA and the corresponding viral RNA via post-transcriptional gene silencing.

The post-transcriptional gene silencing pathway targets double-stranded (ds)RNA for degradation by DICER-like (DCL) proteins in a sequence-specific manner through the production of small interfering (si)RNA. Whereas DCL2 cleaves dsRNAs from replicating virus[11], DCL3 cleaves dsRNAs derived from endogenous transcripts through the activity of RNA-dependent RNA polymerases 2 and 6 (refs. 12,13). The siRNAs produced are incorporated into RNA-induced silencing complexes (RISC), which guide cleavage of target RNAs. Because post-transcriptional gene silencing is a host antiviral defense mechanism, it is not surprising that viruses encode suppressor proteins that can block this function[2,14–16].

Recently, miRNAs have been identified as important regulators of gene expression in both plants and animals. miRNAs are single-stranded RNAs, 20–24 nucleotides (nt) in length, generated from processing of longer pre-miRNA precursors[17] by DCL1 in *A. thaliana*[11]. These miRNAs are recruited to the RISC complex. Using RNA:RNA base-pairing, miRNAs direct RISC in a sequence-specific manner to downregulate target mRNAs in one of two ways. Limited miRNA:mRNA base-pairing results in translational repression, which is the case with the majority of the animal miRNAs studied so far. By contrast, most plant miRNAs show extensive base-pairing to, and guide cleavage of, their target mRNAs[18]. In *A. thaliana*, miRNAs are known to be important regulators of plant developmental processes.

Previous reports have shown that alteration of several nucleotides within an miRNA 21-nt sequence does not affect its biogenesis[19]. Therefore, it may be possible to modify plant miRNA sequence to target specific transcripts, originally not under miRNA control. To explore this possibility we used the 273-bp backbone of pre-miRNA^{159a} to generate artificial pre-miRNAs^{159} (pre-amiRNAs^{159}) containing sequences complementary to two plant viruses TYMV and TuMV.

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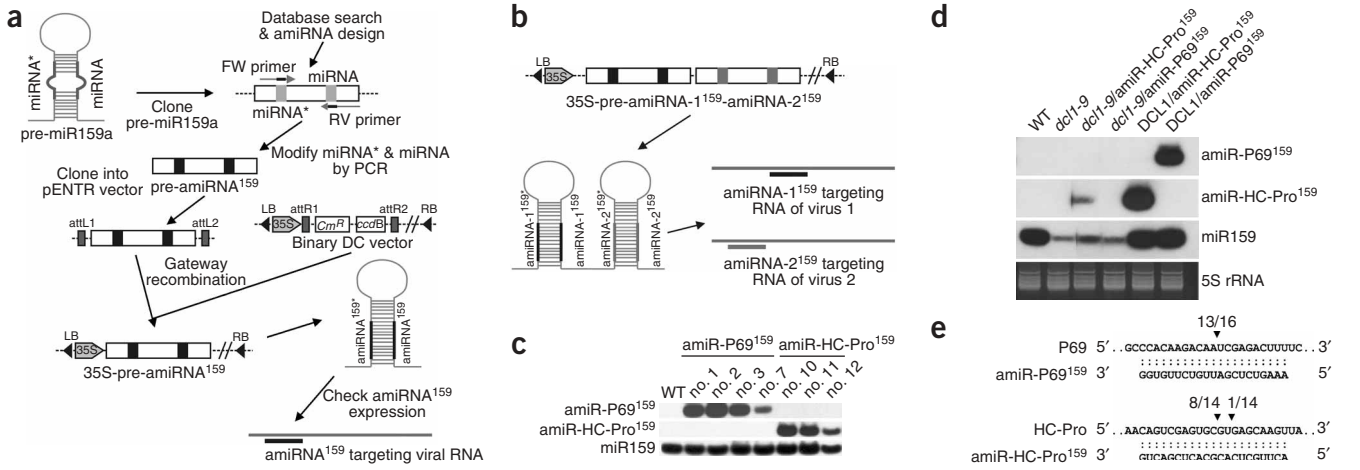

Figure 1 Design of amiRNA precursors and analysis of transgenic *A. thaliana* plants expressing amiRNAs. (**a**) Structure of pre-miR159a (273 nt) presented as a hairpin. Primers containing the amiRNA or amiRNA* sequences were used to replace miR159 and miR159* sequences in the pre-miRNA. Note that the amiRNA sequence is fully complementary to the amiRNA* sequence. The pre-amiRNA^{159} was subcloned into pENTR vector and then moved downstream of a 35S promoter in a binary destination vector (destination cassette, DC) by the Gateway recombination system. The pre-amiRNA^{159} precursor in the binary plasmid was tested for amiRNA^{159} expression in *N. benthamina* before being transferred into *A. thaliana*. (**b**) A binary vector carrying 35S-pre-amiR-P69^{159}/amiR-HC-Pro^{159}. In transgenic plants, this transgene can produce two different amiRNAs directed against two different viruses. (**c**) Transgenic plants carrying 35S-pre-amiR-P69^{159} or 35S-pre-amiR-HC-Pro^{159}. Four lines expressing amiR-P69^{159} (lines nos. 1, 2, 3 and 7) and three lines expressing amiR-HC-Pro^{159} (lines nos. 10, 11 and 12) were analyzed. The endogenous miR159 was used as a loading control. Each lane contained 10 μg total RNA. (**d**) Expression of amiRNAs requires DCL1. amiRNA transgenic plants were crossed with *dcl1-9* mutant and *dcl1-9* homozygous seedlings carrying 35S-pre-amiRNA transgenes were analyzed. Each lane contained 10 μg RNA. 5S rRNA and tRNAs were used as loading controls. (**e**) Mapping of cleavage site of P69 and HC-Pro mRNA by 5′RACE. The arrows indicate that 13/16 clones of P69 and 8/14 clones of HC-Pro were derived from products cleaved at the expected site. This cleavage site is located between nucleotides complementary to nucleotide 10 and 11 of the amiRNA. (**f**) Transgenic plants expressing amiR-TuCP^{159} or amiR-P69^{159}/amiR-HC-Pro^{159}. Three lines expressing amiR-TuCP^{159} lines (nos. 3, 11 and 15) and three lines expressing amiR-P69^{159}/amiR-HC-Pro^{159} lines (nos. 2, 5 and 9) were analyzed. The endogenous miR159 was used as a loading control. Each lane contained 10 μg total RNA.

Both TYMV and TuMV can infect Brassica and non-Brassica hosts inflicting significant economic damages worldwide[20–23]. TYMV is a tymovirus with an RNA genome that encodes three proteins, one of which is a 69-kDa movement protein with silencing suppressor function[24]. TuMV, a potyvirus, encodes a large polyprotein, which is post-translationally processed into several smaller proteins[25]. HC-Pro, a viral proteinase with multiple functions, acts as a silencing suppressor. Because viral suppressors play an important role in counteracting host defense, we chose to design amiRNAs to target these sequences. In addition, we have also designed amiRNAs to target the coat protein sequences of TuMV.

In this study, we demonstrate that transgenic plants can generate two amiRNAs from pre-amiRNAs—amiR-P69^{159} and amiR-HC-Pro^{159} with sequences complementary to P69 and HC-Pro coding sequence, respectively. Furthermore, when inoculated with the specific virus, these plants displayed specific resistance even at low temperatures that inhibit post-transcriptional gene silencing. In addition, expression of a dimeric amiRNA precursor, which can generate both amiR-P69^{159} and amiR-HC-Pro^{159}, conferred resistance to both viruses. During the preparation of this manuscript, Schwab *et al.*[26] and Alvarez *et al.*[27] reported the successful downregulation of plant genes by amiRNAs targeting individual genes or groups of endogenous genes. These amiRNAs were constructed using precursors of miR164b, miR172a and miR319a as backbones. Furthermore, plum pox virus chimeras bearing endogenous miRNA target sequences displayed reduced infectivity in plants[28]. The use of synthetic miRNAs and multiple miRNA genes to downregulate gene expression has also been reported in animal and human cells[29–33].

RESULTS

Expression of amiRNAs in transgenic *A. thaliana*

Preliminary analysis showed that miRNA159 was expressed at high levels in all *A. thaliana* organs (data not shown). We therefore chose the 273-nt pre-miR159a hairpin structure (**Fig. 1a**) as a backbone for the construction and expression of amiRNAs termed amiRNA^{159}. The designs of pre-amiRNAs and expression of amiRNAs in transgenic plants are shown (**Fig. 1**).

Using oligonucleotide-directed mutagenesis, we replaced the 21-nt sequence of miR159 with the synthetic sequence 5′-AAAGUCUCG AUUGUCUUGUGG-3′ (amiR-P69^{159}) or 5′-ACUUGCUCACGCAC UCGACUG-3′ (amiR-HC-Pro^{159}) directed against a region of the mRNA encoding the suppressor P69 or HC-Pro, respectively. We obtained expression of amiR-HC-Pro^{159} and amiR-P69^{159} in wild-type transgenic lines (**Fig. 1a,c**) but their expression, like that of the endogenous miR159, was much reduced in *dcl1-9*, implicating DCL1 in their biogenesis (**Fig. 1d**). Four amiR-P69^{159} (nos. 1, 2, 3 and 7) and three amiR-HC-Pro^{159} (nos. 10, 11 and 12) independent transgenic lines were chosen for further experimentation.

Cleavage of P69 and HC-Pro mRNA

To see if the amiRNAs can mediate cleavage of target RNAs, we infiltrated *Nicotiana benthamiana* leaves with Agrobacterial cells carrying plasmids containing the constructs pBA-amiR-P69^{159}/pBA-P69-HA, pBA-amiR-HC-Pro^{159}/pBA-HC-Pro-HA, pBA002/pBA-P69-HA or pBA002/pBA-HC-Pro-HA. The empty vector pBA002 was used as a negative control. In 13 out of 16 clones analyzed, P69 mRNA cleavage

occurred at the expected position (nucleotide 224) located between the two nucleotides complementary to nucleotide 10 and 11 of the amiR-P69^{159} (**Fig. 1e**). Three other cleavage products with 5′ ends further downstream (at nucleotide 254, 285 and 403) were recovered, suggesting *A. thaliana* XRN4 exonuclease activity[34,35] (data not shown). No rapid amplification of 5′ complementary DNA ends (5′RACE) product was obtained from the negative control sample. Similar results were obtained in an HC-Pro mRNA cleavage experiment with 8 out of 14 clones analyzed having the correct cleavage at nucleotide 840 of the HC-Pro mRNA (complementary to nucleotides 10 to 11 of the triggering amiR-HC-Pro^{159}) whereas one clone showed cleavage at nucleotide 842 (**Fig. 1e**). The 5′ ends of the other 5 clones were detected at nucleotide 876, 878, 879, 913 and 1030 of the HC-Pro mRNA (data not shown). These data showed that both amiRNAs can mediate precise cleavage of their target viral RNAs.

Specificity of amiRNA

Using BLAST search we failed to find any *A. thaliana* genes having complementary sequences of more than 15-nt over the 21-nt sequence of amiR-P69^{159} or amiR-HC-Pro^{159}. The PATMATCH (ver 1.1) program on the TAIR website (http://www.arabidopsis.org/) also did not uncover any endogenous *A. thaliana* transcripts as possible targets when the parameter was set as 0 mismatch. However, 6 *A. thaliana* transcripts can potentially form imperfect pairing (mismatch and deletion) with amiR-P69^{159} and amiR-HC-Pro^{159} when three mismatches were allowed (see **Supplementary Fig. 1a,b** online).

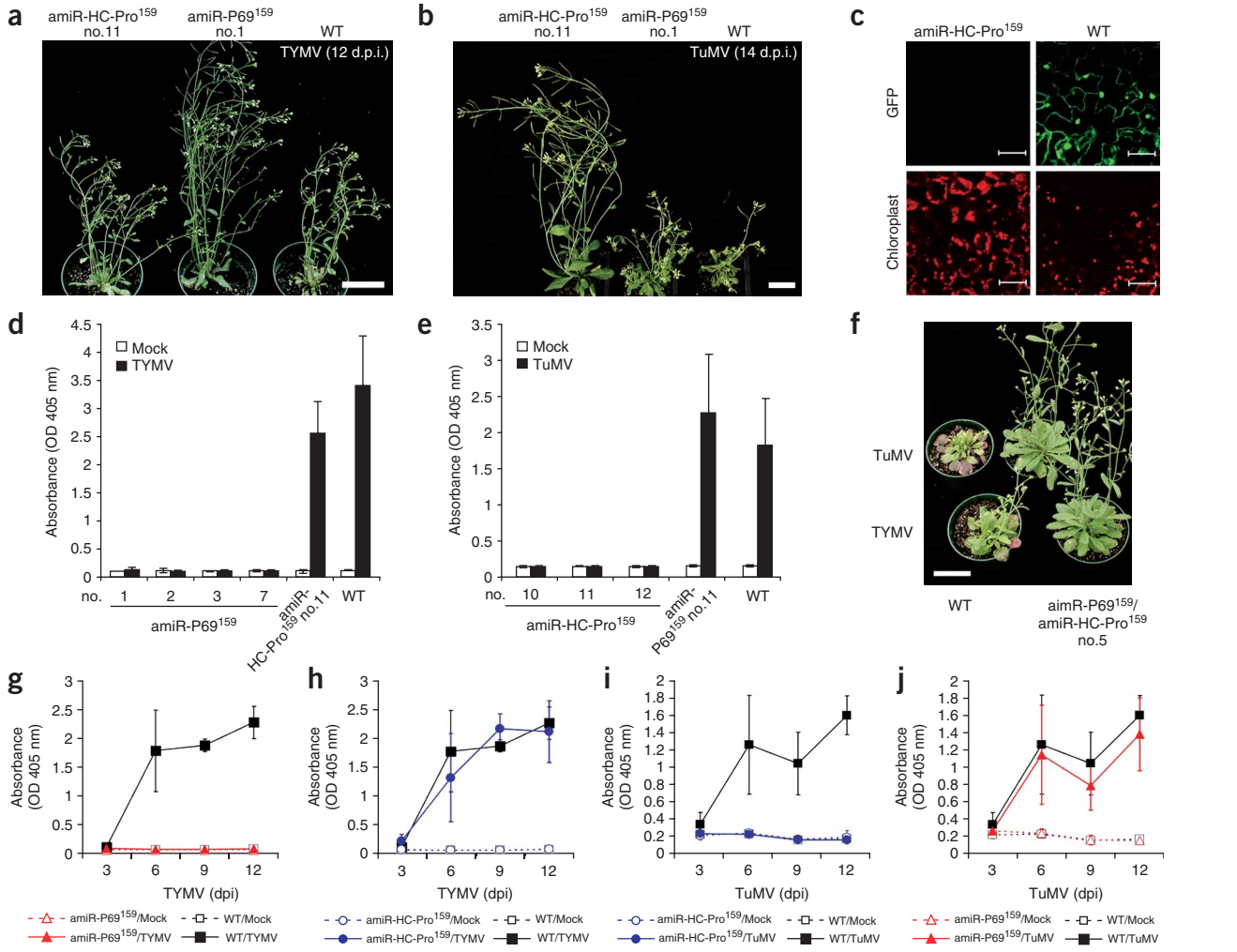

Figure 2 Transgenic plants expressing amiR-P69^{159} and amiR-HC-Pro^{159} are resistant to TYMV and TuMV infection, respectively. (**a**) Plants were inoculated with TYMV and photographs taken 12 d.p.i. Bar, 4 cm. (**b**) Plants were inoculated with TuMV and photographs taken 14 d.p.i. Bar, 3 cm. (**c**) TuMV-GFP infection and cell-to-cell movement of the chimeric virus on inoculated leaves. Entire inoculated leaves of wild-type and transgenic plants were examined by confocal microscopy. Inoculated wild-type leaves always displayed fluorescence due to replication of the GFP-virus, whereas transgenic leaves of amiR-HC-Pro^{159} plants never showed any fluorescence even when the entire leaf was examined. Bar, 50 μm. (**d**) ELISA detection of TYMV coat protein in different transgenic and wild-type plants. Readings were taken after 3 h of substrate hydrolysis. Bars represent standard deviations and $N = 22$. (**e**) ELISA detection of TuMV coat protein in different transgenic and wild-type plants at 14 d.p.i. Readings were taken after 30 min of substrate hydrolysis. Bars show standard deviations. $N = 12$. (**f**) Transgenic plants expressing amiR-P69^{159}/amiR-HC-Pro^{159} are resistant to TYMV and TuMV infection. Plants were inoculated with TYMV or TuMV and photographs taken 14 d.p.i. Bar, 4 cm. (**g**,**h**) Time course of TYMV titer in various transgenic lines (amiR-P69^{159} no. 1 and amiR-HC-Pro^{159} no. 11) and wild-type plants. Six d.p.i. transgenic amiR-HC-Pro^{159} no. 11 and wild-type plants were systemically infected by TYMV. Bars represent standard deviations and $N = 92$. (**i**,**j**) Time course of TuMV titers in various transgenic lines (amiR-P69^{159} no. 1 and amiR-HC-Pro^{159} no. 11) and wild-type plants. Bars represent standard deviation and $N = 41$.

Table 1 Infectivity assay of amiR-HC-Pro^{159} and amiR-P69^{159} transgenic plants challenged with TYMV inocula

	Nº infected/inoculated plants					
	Experiment 1			Experiment 2		
Transgenic plant	Line	u[a]	R (%)[b]	Line	u	R (%)
amiR-P69^{159}	1[c]	0/3	100	1-1[d]	0/32	100
	2	0/6	100	2-5	0/22	100
	3	0/6	100	3-1	0/32	100
	7	0/6	100	7-1	0/32	100
amiR-HC-Pro^{159}	10	5/6	16.7	10-4	30/31	3.2
	11	6/6	0	11-3	32/32	0
	12	5/5	0	12-4	29/30	3.3
WT		16/17	5.9		61/61	0

[a]Upper noninoculated leaves were analyzed by ELISA. The nominator indicates the number of ELISA-positive plants and the denominator the total number of plants in the experiment. [b]R, number of resistant plants as a percentage of the total plant population. [c]The amiR-P69^{159} lines (nos. 1, 2, 3 and 7) and amiR-HC-Pro^{159} lines (nos. 10, 11 and 12) are T_2 progeny containing a mixture of homozygotes and hemizygotes with respect to the transgene. [d]The amiR-P69^{159} lines (nos. 1-1, 2-5, 3-1 and 7-1) and amiR-HC-Pro^{159} lines (nos. 10-4,11-3 and 12-4) are homozygous T_3 progeny. WT, wild type.

To check if the two amiRNAs can also downregulate *A. thaliana* transcripts with such imperfect matches, we monitored their expression levels in wild-type and transgenic plants by RT-PCR. We also examined possible effects on genome-wide expression by hybridizations to Affymetrix ATH1 arrays. Our analysis showed that none of the *A. thaliana* genes expressed in wild type were downregulated by either of the amiRNAs (see **Supplementary Table 1** online). Expression of five of the putative target genes was not affected by the amiRNAs (see **Supplementary Fig. 1c**,**d** online). The sixth putative target gene (AT1G69320) was not expressed either in wild-type plants or in transgenic plants by genome array analysis or by RT-PCR. This is likely due to its low transcript abundance (see **Supplementary Table 1** online). In addition to this molecular analysis, we found that transgenic plants expressing the amiRNAs displayed normal morphology and growth rate. The flowering time was unaltered, the flowers were fertile and the siliques contained seeds of the normal amount. Although these results suggest that amiR-P69^{159} and amiR-HC-Pro^{159} can specifically target viral RNA but not endogenous transcripts, we can not exclude the possibility that transcripts expressed only under specific conditions, for example, abiotic stress, might be affected.

TYMV resistance of amiR- P69^{159} plants

The cleavage of P69 mRNA mediated by amiR-P69^{159} suggested the possibility of using this amiRNA to mediate cleavage of viral RNA to confer resistance. We selected four amiR-P69^{159} transgenic lines (nos. 1, 2, 3 and 7) and challenged progeny plants with TYMV or buffer. Wild-type plants and three amiR-HC-Pro^{159} lines (nos. 10, 11 and 12) were used as controls.

Figure 2 shows responses of wild-type and transgenic plants to virus challenge. Twelve days post-inoculation (d.p.i.), wild-type plants and amiR-HC-Pro^{159} plants (line no. 11) showed diffused chlorotic local lesions on inoculated leaves and yellow mosaics on systemic leaves (**Fig. 2a** and **Supplementary Fig. 2a** online) characteristic of TYMV-induced symptoms[14]. Moreover, these infected plants developed shorter inflorescence internodes (see **Supplementary Fig. 2b** online), the inflorescences displayed early senescence and flowers had pollination defects. By contrast, amiR-P69^{159} plants (line no. 1) appeared normal, like mock-inoculated plants of all genotypes (wild type and transgenic) (**Fig. 2a** and **Supplementary Fig. 2a**,**b** online). Similar results were also obtained for amiR-P69^{159} transgenic line nos. 2, 3 and 7 after TYMV inoculation confirming that TYMV resistance can be seen in all four independent lines.

Extracts of systemic leaves from individual plants were assayed by enzyme-linked immunosorbent assays (ELISA) using polyclonal antibodies to the TYMV coat protein. The TYMV coat protein was undetectable in inoculated amiR-P69^{159} plants indicating virus resistance. By contrast, TYMV coat protein was present at high levels in the susceptible wild-type and amiR-HC-Pro^{159} plants (**Fig. 2d**) consistent with the appearance of virus symptoms (**Fig. 2a** and **Supplementary Fig. 2a**,**b** online). TYMV-inoculated wild-type and amiR-HC-Pro^{159} plants contained high TYMV coat protein levels, which were not detected in mock-inoculated plants of all genotypes and in TYMV-inoculated amiR-P69^{159} plants (see **Supplementary Fig. 2c** online).

In wild-type plants, coat protein was not detectable at 3 d.p.i. but accumulated rapidly to high levels at 6 d.p.i. and then the level reached a plateau thereafter (**Fig. 2g**,**h**). TYMV-inoculated amiR-HC-Pro^{159} plants continued to accumulate TYMV coat protein until 12 d.p.i. before leveling off. No accumulation of TYMV coat protein was found in amiR-P69^{159} plants at all time points, indicating virus resistance.

We also monitored symptoms development and TYMV coat protein accumulation on upper noninoculated leaves in two independent experiments (**Table 1**). In the first experiment, T_2 progeny (homozygotes and hemizygotes) of amiR-P69^{159} plants (nos. 1, 2, 3 and 7) and of amiR-HC-Pro^{159} plants (nos. 10, 11 and 12) were analyzed along with wild-type plants. Most of the wild-type plants (16 out of 17), all of the amiR-HC-Pro^{159} plants derived from two independent lines (nos. 11 and 12) and 5 out of 6 plants of amiR-HC-Pro^{159} line no. 10 showed systemic yellow mosaic symptom and accumulated TYMV coat protein. The percent resistance is around 0 to 16.7%. By contrast, all amiR-P69^{159} lines showed 100% resistance to TYMV. In the second experiment, T_3 progeny (homozygotes) of amiR-P69^{159} plants (line nos. 1-1, 2-5, 3-1 and 7-1) and of amiR-HC-Pro^{159} plants (line nos. 10-4, 11-3 and 12-4) were challenged with TYMV. The percent resistances of wild-type and amiR-HC-Pro^{159} plants were around 0–3.3%, but T_3 progeny of

Table 2 Infectivity assay of amiR-HC-Pro^{159} and amiR-P69^{159} transgenic lines challenged with TuMV inocula

		Nº infected/inoculated plants					
		Experiment 1		Experiment 2		Experiment 3	
Transgenic plant	Line	u[a]	R (%)[b]	u	R (%)	u	R (%)
amiR-P69^{159}	1[c]	11/12	8.3	8/8	0	18/19	5.3
	2	ne[d]		8/8	0	16/17	5.9
	3	ne		5/6	16.7	ne	
	7	ne		5/5	0	ne	
amiR-HC-Pro^{159}	10	ne		0/12	100	0/24	100
	11	0/12	100	0/12	100	0/17	100
	12	ne		0/9	100	ne	
WT		11/12	8.3	25/33	24.2	41/41	0

[a]Upper noninoculated leaves were analyzed by ELISA. The nominator indicates the number of ELISA-positive plants and the denominator the total number of plants in the experiment. [b]R, number of resistant plants as a percentage of the total plant population. [c]The amiR-P69^{159} lines (nos. 1, 2, 3 and 7) and amiR-HC-Pro^{159} lines (nos. 10, 11 and 12) are T_2 progeny containing a mixture of homozygotes and hemizygotes with respect to the transgene. [d]ne, not examined. WT, wild type.

Table 3 Infectivity assay of amiR-P69^{159}/amiR-HC-Pro^{159} transgenic plants challenged with inocula of TYMV, TuMV or both viruses

		Nº infected/inoculated plants							
		Single infection[a]				Co-infection[b]			
	Line	u_{TuMV}[c]	R[d] (%)	u_{TYMV}	R (%)	u_{TuMV}	R (%)	u_{TYMV}	R (%)
amiR-P69^{159}/amiR-HC-Pro^{159}	2[e]	0/17	100	0/17	100	0/35	100	0/35	100
	5	0/16	100	0/17	100	0/34	100	0/34	100
	9	0/23	100	0/25	100	0/43	100	0/43	100
amiR-P69^{159}	7-1[f]	23/23	0	0/25	100	35/36	2.8	0/36	100
amiR-HC-Pro^{159}	10-4	0/23	100	23/23	0	0/32	100	32/32	0
WT		16/16	0	19/20	5	33/37	10.8	37/37	0

[a]Single infection with TuMV or TYMV inocula. [b]Co-infection with mixed TuMV and TYMV inocula. [c]Upper noninoculated leaves were analyzed by ELISA. The nominator indicates the number of ELISA-positive plants and the denominator the total number of plants in the experiment. [d]R, number of resistant plants as a percentage of the total plant population. [e]The amiR-P69^{159}/amiR-HC-Pro^{159} lines (nos. 2, 5 and 9) are T_2 progeny containing a mixture of homozygotes and hemizygotes with respect to the transgene. [f]amiR-P69^{159} line no. 7-1 and amiR-HC-Pro^{159} line no. 10-4 are homozygous T_3 progeny. WT, wild type.

amiR-P69^{159} plants displayed 100% resistance to TYMV. Together, the results show that the virus resistance trait can be transmitted to the next generation.

TuMV resistance of amiR-HC-Pro^{159} plants

Next, we inoculated the same set of transgenic plants with TuMV using wild-type plants as controls. Fourteen days after inoculation, vegetative growth of amiR-P69^{159} (line no.1) and wild-type plants showed severe developmental abnormalities, including stunting and reduced internodal distances (**Fig. 2b** and **Supplementary Fig. 3a** online). TuMV-infected amiR-P69^{159} and wild-type plants showed narrow sepals that failed to encase developing flowers. Moreover, the flowers were often sterile because of short anthers that failed to pollinate (see **Supplementary Fig. 3b** online), and siliques were small and appeared abnormal (see **Supplementary Fig. 3c** online). These symptoms were characteristics of plants infected with TuMV[15]. By contrast, transgenic amiR-HC-Pro^{159} plants (line no. 11) were resistant to TuMV infection and displayed normal inflorescence, flower and silique development, like mock-inoculated plants of all genotypes.

amiR-HC-Pro^{159} and wild-type plants were also challenged with a TuMV virus carrying a GFP gene. GFP signal was seen on inoculated and systemic leaves of wild-type but not those of amiR-HC-Pro^{159} plants (**Fig. 2c**). These results indicate the amiRNA-mediated resistance can block viral RNA replication in infected cells, and viruses can not move from cell to cell nor translocate systemically to upper leaves.

TuMV coat protein was not detected in mock-inoculated plants of all genotypes and in TuMV-inoculated amiR-HC-Pro^{159} plants indicating TuMV resistance. On the other hand, TuMV coat protein was present at high levels in TuMV-infected wild-type and amiR-P69^{159} plants (**Fig. 2e** and **Supplementary Fig. 3d** online) consistent with the appearance of virus symptoms (**Fig. 2b** and **Supplementary Fig. 3a-c** online).

In a time course experiment, amiR-P69^{159} and wild-type plants showed no detectable TuMV coat protein at 3 d.p.i. but it accumulated rapidly to high levels at 6 d.p.i. The TuMV titer decreased slightly at 9 d.p.i. but higher TuMV titer was detected at 12 d.p.i. (**Fig. 2i,j**). This result indicates that amiR-P69^{159} and wild-type plants were susceptible to TuMV infection. No accumulation of TuMV coat protein was found in amiR-HC-Pro^{159} plants at all time points, indicating TuMV resistance.

We monitored symptoms of upper noninoculated leaves and performed ELISA assays of leaf extracts in three independent experiments (**Table 2**). Plants of T_2 progeny (homozygotes and hemizygotes) of amiR-P69^{159} (line nos. 1, 2, 3 and 7) and amiR-HC-Pro^{159} (line nos. 10, 11 and 12) and wild-type control plants were analyzed. The percent resistance of wild-type and amiR-P69^{159} plants was ~0–24.2%. By contrast, all of the amiR-HC-Pro^{159} lines showed 100% resistance to TuMV in all three experiments. These results confirm the resistance of amiR-HC-Pro^{159} plants to TuMV.

In addition, we found that amiR-TuCP^{159} transgenic plants (line nos. 3, 11 and 15), which produced amiR-TuCP^{159} (5′-ACUCUCUGC UCGUAUCUUGGC-3′) targeting the TuMV coat protein sequences, showed complete resistance to TuMV (**Fig. 1f** and **Supplementary Table 2** online).

Transgenic plants resistant to both viruses

Transgenic plants expressing either amiR-P69^{159} or amiR-HC-Pro^{159} were resistant only to the specific virus targeted by the amiRNA. However, we observed breakdown of this specific resistance in a few transgenic plants co-inoculated with both viruses (see **Supplementary Table 3** online). This occasional breakdown is not surprising because when produced at sufficiently high levels the gene silencing suppressor of the nontargeted virus (P69 of TYMV or HC-Pro of TuMV) would interfere with the host miRNA machinery thereby blocking the targeting amiRNA activity. To generate transgenic plants with double resistance against both viruses, we constructed a dimeric amiRNA precursor (pre-amiR-P69^{159}/amiR-HC-Pro^{159}) by ligating pre-amiR-P69^{159} and pre-amiR-HC-Pro^{159} and expressing the dimer in a single Pol II transcription unit (**Fig. 1b**). Expression of both amiRNAs was detected in three independent transgenic lines (nos. 2, 5 and 9) carrying the dimeric pre-amiRNA (**Fig. 1f**). No viral symptoms were detected in these lines upon infection with either TuMV or TYMV or when co-inoculated with both viruses (**Fig. 2f**). These double resistance results were confirmed by ELISA analysis (**Table 3**).

Maintenance of virus resistance of amiRNA plants at 15 °C

Sense viral RNA-mediated resistance to cymbidium ringspot virus breaks down at 15 °C because of the sensitivity of post-transcriptional gene silencing to low temperatures[36]. To see whether the amiRNA plants would maintain their virus resistance at 15 °C, we grew wild-type and transgenic plants at 15 °C for 3 weeks. We found that

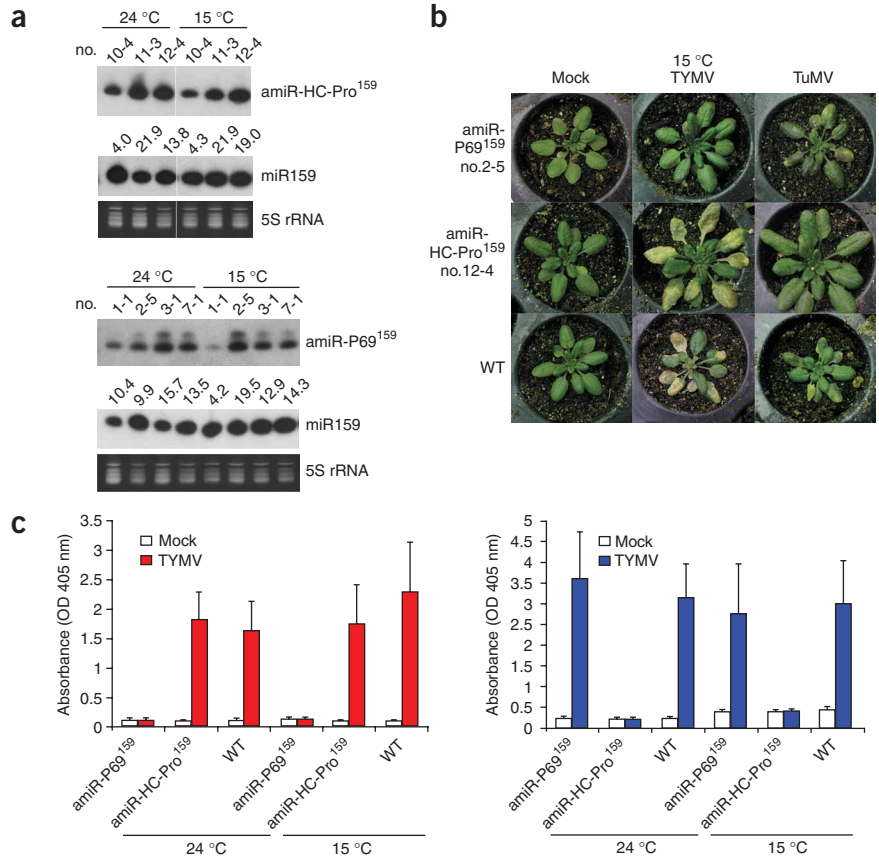

Figure 3 Effect of temperature on amiRNA production and virus resistance. (**a**) Expression of amiRNAs at 24 °C and 15 °C. Homozygous T_3 amiR-P69^{159} and amiR-HC-Pro^{159} transgenic plants were grown at 24 °C and 15 °C. Each lane contained 10 μg total RNA. 5S rRNA and tRNAs were used as loading controls. Transgenic line numbers are given on the top panels and numbers between the two panels represent signal strengths of amiRNA relative to miR159. (**b**) Symptom development and virus resistance phenotype of amiR-P69^{159} and amiR-HC-Pro^{159} transgenic plants at low temperatures. (**c**) amiR-P69^{159} and amiR-HC-Pro^{159} transgenic plants were resistant to TYMV (left panel) and TuMV (right panel), respectively, at low temperatures. Two transgenic amiR-HC-Pro^{159} lines (nos. 10-4 and 11-3), two transgenic amiR-P69^{159} lines (nos. 1-1 and 2-5) and wild-type plants were used. For each line, 12 individual plants were inoculated with TuMV or TYMV whereas 4 individual plants were inoculated with buffer as controls (Mock). After 2 weeks systemic leaves were collected for ELISA analyses using an antibody against TuMV coat protein or TYMV coat protein. Bars represent standard deviation and $N = 24$.

amiRNA levels at 15 °C were comparable to those at 24 °C (**Fig. 3a**). In addition, expression of endogenous miRNAs (miR165, miR164, miR159 and miR167) and trans-acting (ta-) siRNA (si255) was also not significantly affected at 15 °C (see **Supplementary Fig. 4** online). TYMV symptoms were more severe at 15 °C than at 24 °C. Severe yellowing and necrosis were visible on inoculated leaves of amiR-HC-Pro^{159} and wild-type plants at 10 d.p.i. (**Fig. 3b**). On the other hand, only mild chlorosis appeared on TuMV-inoculated leaves of amiR-P69^{159} and wild-type plants at the same temperature. For both types of transgenic plants, amiRNA-mediated specific virus resistance was maintained at 15 °C (**Fig. 3b**,**c**).

DISCUSSION

We show here that *A. thaliana* transgenic plants carrying a 35S-pre-amiRNA can express mature, 21-nt amiRNAs at high levels by a process that requires DCL1. Moreover, the amiRNAs can mediate target viral RNA cleavage to confer virus resistance. The resistance is highly specific to the amiRNA/virus pair and therefore can not be explained by the induction of some general host resistance mechanism by the amiRNAs. The resistance is detected at the cell level and is hereditable.

The amiRNA-mediated approach has several advantages. First, no viral cDNA fragment is used and any conserved or specific 21-nt antisense sequence from the viral RNA genome can be chosen[26]. In addition, by computer prediction, it should be possible to select a sequence with no extensive homology to any *A. thaliana* genes. Second, the 21-nt sequences of miR159 and miR159* (the complementary strand to miR159 in pre-miR159) can be easily replaced with any artificial sequences targeting any viral RNAs by PCR. Third, a binary plasmid carrying the 35S-pre-amiRNA can be first assayed by transient expression in *N. benthaminiana* leaves for amiRNA expression and cleavage efficiency of target mRNA. As the amiRNA gene is dominant, the resistance phenotype can be screened at the T_1 or even T_0 generation.

Comparison of the four different TYMV strains (BL, J04373, X07441 and X16378) showed that the 21-nt target sequence of amiR-P69^{159} is fully conserved, suggesting that the amiR-P69^{159} plants should be resistant to all the strains (see **Supplementary Fig. 5** online). Similarly, the amiR-HC-Pro^{159} plants should be resistant to at least 36 out of the 78 TuMV strains, whose HC-Pro genes show full sequence complementarity to the 21-nt sequence of amiR-HC-Pro^{159} (see **Supplementary Fig. 5a**,**b** online).

It is generally recognized that not all siRNA species against a given mRNA target are equally effective and some siRNAs show limited efficacy because of extensive positional effects, for example, secondary structure, along the mRNA[37]. A similar situation may also apply to amiRNA-mediated cleavage of target mRNAs or viral RNAs. Although we have not extensively investigated this possibility we found that amiRNA directed against a 21-nt sequence of the viral RNA encoding the TuMV coat protein was as effective as that against the HC-Pro sequence supporting the general utility of this method.

Low temperatures are known to inhibit accumulation of siRNAs in insect, plant and mammalian cells[36,38,39] and both virus- and transgene-triggered RNA-silencing become attenuated[36]. It is, therefore, not surprising that siRNA-mediated virus resistance breaks down at low temperatures[36]. By contrast, amiRNA expression levels are similar between 15 °C and 24 °C and the amiRNA plants continue to resist virus at 15 °C, suggesting that target RNA cleavage still occurs at this temperature. In *Brassicaceae*, cold-season bi-annuals such as turnips require 15–18 °C for optimum growth. As both TYMV and TuMV infect Brassica crops even at these low temperatures, the amiRNA-mediated strategy should prove useful for engineered virus resistance of low-temperature crop plants.

A number of environmental concerns have been raised regarding the large scale use of virus-resistant transgenic plants[5] irrespective of

whether the resistance is mediated by RNA or protein. Potential risks include[5,40–43]: (i) possible recombination between the virus-derived transgene and nontarget viruses; (ii) possible transmission by unrelated viruses, through trans-encapsidation or enhanced seed or pollen transmission; (iii) possible synergy with unrelated viruses; (iv) possible gene flow from transgenic pollen to weedy relatives; and (v) possible production of new allergens or toxic proteins in transgenic plants. With the exception of iv, these potential risks can be minimized by the amiRNA-mediated approach, which uses only a short, 21-nt sequence.

The importance of suppressors in viral pathogenesis has prompted us to exemplify the efficacies of the amiRNA-mediated virus-specific resistance strategy by first targeting suppressor-encoding sequences of two different RNA viruses. Subsequently, we found that transgenic plants expressing amiR-TuCP^{159}, which targets the TuMV coat protein sequence, are also resistant to TuMV infection. These observations, along with the generality of miRNA action, suggest that the amiRNA strategy should be broadly applicable to other viruses as well. Under field conditions, virus may evade the amiRNA surveillance mechanism and overcome the specific resistance by mutations. However, in the case of suppressor-specific amiRNAs, mutations within conserved sequences may attenuate suppressor function and weaken virus virulence[44]. Moreover, this potential problem of possible resistance breakdown can be mitigated by coexpressing several amiRNAs targeting different sequences of the same virus.

As mixed infection is common in the field, we have challenged amiRNA transgenic plants with both TYMV and TuMV. We observed a breakdown of specific resistance for some transgenic plants in co-infection experiments. Several factors, including the relative rate of virus replication and the efficiency of viral RNA cleavage, are expected to influence the outcome of such experiments. A more rapid replication of the nontargeted virus and subsequent accumulation of its silencing suppressor would inhibit the host miRNA machinery and block cleavage of the targeted viral RNA by its cognate amiRNA. Therefore, it is not surprising to see a breakdown of specific virus resistance in some transgenic plants. However, any possible breakdown of specific virus resistance because of a prior infection by an unrelated virus could be obviated by expressing in the same plant amiRNAs targeting two different viruses. Indeed, we show here that transgenic plants expressing both amiR-P69^{159} and amiR-HC-Pro^{159} were resistant to co-infection by TYMV and TuMV. Taken together, our observations indicate the possibility to engineer broad spectrum resistance to several viruses by co-expression of appropriately designed multiple amiRNAs.

METHODS

Construction of artificial pre-amiRNAs. A 273-bp fragment containing the entire sequence of the *A. thaliana* miR159a (see below) was cloned by PCR amplification using primers miR159-F1 (5′-CACCACAGTTTGCTTATGTCG GATCC-3′) and miR159-*Xma*I-R1 (5′-TGA*CCCGGG*ATGTAGAGCTCCCTT CAATCC-3′). The miR159-*Xma*I-R1 contains 18 of 21 nucleotides of the mature miR159 (underlined) and an introduced *Xma*I site (italic). The PCR fragment was cloned into pENTR vector (Invitrogen) according to the manufacturer's instructions to obtain pENTR-pre-miR159a. Mutagenesis of pre-miR159a was performed by PCR with amiR-HC-Pro^{159}-F1 and amiR-HC-Pro^{159}-R1 primers. The amiR-HC-Pro^{159}-F1 primer (5′-AAGAT*AGATCT*TG ATCTGACGATGGAAGCAGTCGAGTGCGTGAGCAAGTCATGAGTTGAGCA GGGTA-3′) contains a *Bgl*II site (italic) and the amiR-HCPro^{159*} sequence (underlined). The amiR-HC-Pro^{159}-R1 (5′-AAGA*CCCGGGA*TGCAGTCGA GTGCGTGAGCAAGTGAAGAGTAAAAGCCATTA-3′) contains an *Xma*I site (italic) and the mature amiR-HC-Pro^{159} reverse complementary sequence (underlined).

PCR amplification of the miR159a precursor using the above primers and pENTR-pre-miR159a DNA as a template generated a DNA fragment that was subsequently digested with *Bgl*II and *Xma*I. The *Bgl*II-*Xma*I fragment was inserted into pENTR-pre-miR159a to generate pENTR-pre-amiR-HC-Pro^{159}. The point mutations introduced rendered the miRNA:miRNA* pair fully complementary. Gateway system (Invitrogen) procedures were used to transfer the amiR-HC-Pro^{159} precursor to the plant binary Gateway destination vector pBA-DC-myc[45] generating pBA-pre-amiR-HC-Pro^{159}, in which the pre-amiR-HC-Pro^{159} sequence is placed downstream of a 35S promoter.

In the case of amiR-P69^{159} two forward oligonucleotides amiR-P69^{159}-F1 and F2 and two reverse oligonucleotides amiR-P69^{159}-R1 and R2, were designed to change the miR159a sequence to a synthetic sequence targeting the P69 mRNA coding sequence from TYMV. The primer amiR-P69^{159}-F1 (5′-GGAAGCCACAAGACAATCGAGACTTTCATGAGTTGAGCAGGGTA-3′) contains the synthetic amiR-P69^{159*} sequence (underlined). The primer amiR-p69^{159}-F2 (5′-TCGAT*AGATCT*TGATCTGACGAT**GGAAGCCACAAGA CAATCGAGA**-3′) contains a partial amiR-P69^{159}-F1 sequence (bold) and a *Bgl*II site (italic).

The primer amiR-P69^{159}-R1 (5′-CCACAAGACAATCGAGACTTTGAAG AGTAAAAGCCATTAA-3′) contains the synthetic amiR-P69^{159} sequence (underlined). The primer amiR-P69^{159}-R2 (5′-CCCTTTGA*CCCGGG*ATG**C CACAAGACAATCGAGACTTT**-3′) contains a partial amiRP69^{159}-R1 sequence (bold) and an *Xma*I site (italic).

Two rounds of PCR were performed to amplify a 227-bp DNA fragment that contains the amiR-P69^{159} and amiR-P69^{159*} sequences. Plasmid pENTR-pre-miR159a (as a template) and primers amiR-P69^{159}-F1 and amiR-P69^{159}-R1 were used in the first round PCR. In the second round, the PCR product of the first round was used as a template along with primers amiR-P69^{159}-F2 and amiR-P69^{159}-R2 to produce the 227-bp DNA fragment. After digestion with *Bgl*II and *Xma*I, the DNA fragment was cloned into *Bgl*II-*Xma*I–digested pENTR-pre-miR159a vector to generate pENTR-pre-amiR-P69^{159}. Gateway system procedures were used again to transfer the amiR-P69^{159} precursor to pBA-DC-myc generating pBA-pre-amiR-P69^{159}. In this vector the pre-amiR-P69^{159} sequence was placed downstream of a 35S promoter.

To construct amiRNA against the TuMV coat protein sequence, we designed amiRTuCP-F1; (5′-AAGAT*AGATCT*TGATCTGACGATGGAAGGCCAAGAT ACGAGCAGAGAGTCATGAGTTGAGCAGGGTA-3′) contains synthetic amiR-TuCP^{159*} sequence (underlined) and a *Bgl*II site (italic) and amiR-TuCP^{159}-R1 (5′-AAGA*CCCGGG*ATGGCCAAGATACGAGCAGAGAGTGAAGAGTAAAAG CCACCA-3′) contains synthetic amiR-TuCP^{159} sequence (underlined) and an *Xma*I site (italic) to change the miR159a sequence to a synthetic sequence targeting the TuMV coat protein sequence (8988 nt–9008 nt). The PCR fragment was first digested with *Bgl*II and *Xma*I before being cloned into *Bgl*II-*Xma*I–digested pENTR vector to generate pENTR-pre-amiR-TuCP^{159}. The pre-amiR-TuCP^{159} fragment was transferred to a binary vector containing a 35S promoter by Gateway recombination to generate pBA-pre-amiR-TuCP^{159}.

To construct the dimeric pre-amiR-P69^{159}-amiR-HC-Pro^{159}, we ligated the two PCR fragments of amiR-P69^{159} and amiR-HC-Pro^{159} with compatible cohesive ends obtained by previous digestions with *Spe*I and *Avr*II. The ligation product was used as a template for a second round of PCR with amiR-P69^{159}-F2 and amiR-HC-Pro^{159}-R1 primers. The fragment obtained from the second PCR was digested with *Bgl*II and *Xma*I and cloned into pENTR vector to generate pENTR-pre-amiR-P69^{159}-amiR-HC-Pro^{159}. The dimeric amiRNA precursor was placed downstream of a 35S promoter by transfer into a binary vector using Gateway recombination to generate pBA-pre-amiR-P69^{159}–amiR-HC-Pro^{159}.

Construction of cDNA encoding viral suppressors. Full length cDNAs encoding P69 and HC-Pro were cloned from infectious clones of TYMV[46] and TuMV (Yeh, S.D., unpublished data), respectively, by PCR. For the P69 cDNA, the primers were P69-F1 (5′-CACCATGAGTAACGGCCTTCC-3′) and P69-R1 (5′-ATCGGTGTCGGGGGCGCTGCCGTAGTC-3′). For the HC-Pro gene, the primers were HC-F1 (5′-CACCATGAGTGCAGCAGGAGCCAAC-3′), and HC-R1 (5′-TCCGACACGGTAGTGTTTTAAGCTTGA-3′). The P69 cDNA and the HC-Pro cDNA were cloned into the pENTR/D vector (Invitrogen) before being transferred to the pBA-DC-HA vector[45] by recombination using

the LR Clonase enzyme (Invitrogen) and generating pBA-P69-HA and pBA-HC-Pro-HA, respectively. Both viral suppressor genes were fused at the 3′ end with DNA sequences encoding hemagglutinin (HA) and the fusion gene placed downstream of a 35S promoter.

Plant material and growth conditions. Plants of *A. thaliana* ecotype Columbia (Col-0) were transformed with *Agrobacterium tumefaciens* containing the pBA-amiR-P69^{159} or pBA-amiR-HC-Pro^{159} plasmid by the floral dip method[47]. T_2 transgenic plants (a mixture of homozygotes and hemizygotes) were analyzed for transgene and miRNA levels and four independent lines of each construct were used for virus challenge experiments. In addition, homozygous T_3 progeny plants were used for some experiments. Homozygous amiR-P69^{159} plants (no. 2-5) and amiR-HC-Pro^{159} (no. 12-4) were crossed with *dcl1-9*. *dcl1-9* mutant carrying 35S-pre-amiRNA transgene were selected from T_2 progeny and analyzed. Seeds were surface sterilized and chilled at 4 °C for 2 d before either growth on Murashige and Skoog (MS) medium with/without antibiotics or planting on Florobella potting compost/sand mix (3:1). Plants were maintained in a growth room (16 h light/8 h darkness, 20 to 25 °C). For low temperature treatments, seedlings were grown on MS medium or soil at 15 °C in a 16 h light/8 h darkness cycle for 3 weeks.

Transient expression by agro-infiltration of *N. benthamiana*. Four constructs, pBA-pre-amiR-HC-Pro^{159}, pBA-pre-miR-P69^{159}, pBA-P69-HA and pBA-HC-Pro-HA, were used to infiltrate *N. benthamiana* leaves by agro-infiltration[16,48]. Two days after infiltration, total RNA was extracted from the infiltrated leaves using the RNeasy Plant Mini Kit (QIAGEN) and analyzed by 5′RACE.

Analysis of *in vivo* miRNA-directed cleavage. To identify products of miRNA-directed cleavage, we used the First Choice RLM-RACE Kit (Ambion) in 5′RACE experiments. We used 2 μg total RNA for direct ligation to the RNA adaptor without further processing of the RNA sample. Subsequent steps were done according to the manufacturer's directions. PCR fragments obtained from 5′RACE experiments were cloned into the pPCR-Script AMP SK(+) vector (Stratagene) and individual clones were analyzed by DNA sequencing.

Northern blot hybridizations. Total RNA was extracted from leaves using the Trizol reagent (Invitrogen). Ten μg total RNA was resolved in a 15% polyacrylamide/1× TBE (8.9 mM Tris, 8.9 mM boric acid, 20 mM EDTA)/8 M urea gel and blotted to a Hybond-N$^+$ membrane (Amersham). DNA oligonucleotides with the exact reverse-complementary sequence to miRNAs were end-labeled with ^{32}P-γ-ATP and T4 polynucleotide kinase (New England Biolabs) to generate high specific activity probes. Hybridization was carried out using the ULTRAHyb-Oligo solution according to the manufacturer's directions (Ambion), and signals were detected by autoradiography. In each case, the probe contained the exact antisense sequence of the expected miRNA to be detected.

Microarray analysis. Microarray analyses using the Affymetrix ATH1 platform were performed with three biological replicates using wild-type plants and amiR-P69^{159} and amiR-HC-Pro^{159} transgenic plants. Seedlings were grown on 1× MS medium with 1% sucrose for 14 d. One μg of total RNA was used for reverse transcription using MessageAmp II aRNA kits (Ambion) and 15 μg of labeled cRNA for hybridization. GeneChip hybridization and scanning were performed at the Genomic Resource Center, Rockefeller University, New York (http://www.rockefeller.edu/genomics).

Statistical analysis of microarray data. Statistical analysis of microarray data was performed by Genespring GX 7.3.1 software (Agilent). Normalized expression estimates were obtained using GC robust multiarray average (gcRMA) and present calls for genes in control (wild type) were obtained. All present genes were filtered by 1.5-fold change of expression level in the amiRNA transgenic lines. The Welch-*t*-test (variances not assumed equal) was used for parametric test. The $P \leq 1\%$ of Benjamini and Hochberg False Discovery Rate was adjusted for multiple testing correction.

Viruses and challenge inoculations of plants. *N. benthamiana* leaves were inoculated with TuMV YC5 strain (GenBank AF530055)[49] and chimeric TuMV-GFP contains a GFP gene inserted in between the NIb and the coat protein gene (Yeh, S.D., unpublished data). Two weeks later infected leaf tissues (1 g) containing 900 ng/mg tissue of TuMV viral RNA were extracted in 20 ml of 50 mM potassium phosphate buffer (pH 7.0), and the extract used as a TuMV inoculum. Leaves of 4-week-old plants of wild-type *A. thaliana* (col-0) were inoculated with TYMV Blue Lake strain (TYMV BL) (GenBank AF035403)[46] and 1 week later leaf tissues (1 g) containing 690 ng/mg tissue of TYMV viral RNA were extracted with 10 ml of 50 mM potassium phosphate buffer (pH 7.0). The extract was used as a TYMV inoculum. T_2 (amiR-P69^{159} nos. 1, 2, 3 and 7; amiR-HC-Pro^{159} nos. 10, 11 and 12) or T_3 (amiR-P69^{159} nos. 1-1, 2-5, 3-1 and 7-1; amiR-HC-Pro^{159} nos. 10-1, 11-3 and 12-4) plants of different transgenic lines expressing either amiR-P69^{159} or amiR-HC-Pro^{159} were grown in a greenhouse for 4 weeks (5- to 6-leaf stage). Plants were dusted with 600-mesh carborundum on the first to fourth leaf and then gently rubbed with 200 μl TuMV or TYMV inoculum. wild-type (col-0) plants were used as controls. Equal volumes of TuMV and TYMV inocula were mixed and used for co-infection experiments. All inoculated plants were kept in a greenhouse (23–28 °C) and development of symptoms was monitored daily for 2 weeks for TuMV experiments and 1 week for TYMV experiments. TuMV-GFP inoculated leaves were examined 5–7 d.p.i. with an LSM 510 confocal microscope (Zeiss).

ELISA and time course of virus accumulation. Leaf tissues (10 mg) from different systemic leaves of each plant infected with TuMV or TYMV were collected at 0, 3, 6, 9 and 12 d.p.i., and assayed by indirect ELISA using a polyclonal antiserum to the TuMV coat protein[49] or the TYMV coat protein (Loewe Biochemica). Goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Amersham Biosciences) was used as a secondary antibody and *p*-nitrophenyl phosphate (Sigma) was used as a substrate for color development. Results were recorded by measuring absorbance at 405 nm using a VERSAmax Tunable Microplate Reader (Molecular Devices).

Western blot analyses. Systemic leaves from inoculated plants were homogenized in 20 volumes (wt/vol) of denaturing buffer (50 mM Tris-HCl, pH 6.8, 4% SDS, 2% 2-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue). After incubation at 100 °C for 5 min, extracts were clarified by centrifugation at 8,000*g* for 3 min. Total proteins were separated by SDS gel electrophoresis and western blots were analyzed using polyclonal antiserum to TuMV coat protein or TYMV coat protein. Gels were stained with Coomassie brilliant blue R250 and levels of the large subunit of RUBISCO (molecular mass, 55 kDa) were used as loading controls.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

N.-H.C. and J.L.R. first conceived the idea of using amiRNAs to regulate gene expression. Q.-W.N., S.-S.L. and J.L.R. designed the amiRNAs. Q.-W.N. generated the transgenic plants. S.-S.L, Q.-W.N., K.-C.C. and H.-W.N. performed the virus challenge and related experiments. S.-D.Y. provided specific strains of TuMV and TuMV-GFP and advice on experimental design. All authors discussed the results and commented on the manuscript, which was written by N.-H.C. and S.-S.L.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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Erratum: Rainbow biotech—South Africa's emerging sector

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Nat. Biotechnol. **24, 1313–1316 (2006); published online 2 November 2006.**

In the version of the article initially published, on page 1316, paragraph 4, line 17, the interest rate of the eGoliBio incubator is erroneous. Instead of 3%, the rate is actually prime rate less 3%, which is ~10%. The text should read "They also have access to Rand 100,000 ($12,600) loans at prime less 3% interest-rate…."

Corrigendum: Reversal of mouse hepatic failure using an implanted liver-assist device containing ES cell–derived hepatocytes

Alejandro Soto-Gutiérrez, Naoya Kobayashi, Jorge David Rivas-Carrillo, Nalu Navarro-Álvarez, Debaio Zhao, Teru Okitsu, Hirofumi Noguchi, Hesham Basma, Yashuhiko Tabata, Yong Chen, Kimiaki Tanaka, Michiki Narushima, Atsushi Miki, Tadayoshi Ueda, Hee-Sook Jun, Ji-Won Yoon, Jane Lebkowski, Noriaki Tanaka & Ira J Fox
Nat. Biotechnol. **24, 1412–1419 (2006); published online 5 November 2006; corrected after print 7 February 2007.**

In the version of the article initially published, the fifth author's name is misspelled. The correct spelling is Debiao Zhao. The error has been corrected in the HTML and PDF versions of the article.

Corrigendum: Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance

Qi-Wen Niu, Shih-Shun Lin, Jose Luis Reyes, Kuan-Chun Chen, Hui-Wen Wu, Shyi-Dong Yeh & Nam-Hai Chua
Nat. Biotechnol. **24, 1420–1428 (2006); published online 22 October 2006; corrected after print 7 February 2007.**

In the version of the article initially published, in the Author Contributions, the initials of the last author responsible for the virus challenge and related experiments are H.-W.W. and not H.-W.N., as originally indicated. In Figure 2e, the amiR-P69^{159} line is line number 1 and not line number 11, as originally indicated. The error has been corrected in the PDF version of the article.