

1 **Dec 12, 2008**

2 **Molecular evolution of a viral non-coding sequence under the selective**
3 **pressure of amiRNA-mediated silencing**

4 Shih-Shun Lin^{1,2,6}, Hui-Wen Wu^{1,4,6}, Santiago F. Elena³, Kun-Chun Chen⁴, Qi-Wen Niu¹,
5 Shyi-Dong Yeh⁴, Chin-Chih Chen^{4,5}, Nam-Hai Chua^{1,7}

6

7 1. Laboratory of Plant Molecular Biology, Rockefeller University, 1230 York Ave, New
8 York, New York, 10065, USA.

9 2. Present address: Institute of Biotechnology, National Taiwan University, 81
10 Chang-Xing ST., Taipei, 106, Taiwan.

11 3. Instituto de Biología Molecular y Celular de Plantas (CSIC-UPV), Campus UPV CPI
12 8E, Ingeniero Fausto Elio s/n, 46022 València, Spain.

13 4. Department of Plant Pathology, National Chung Hsing University, 250 Kuo-Kuang
14 Road, Taichung, Taiwan 402.

15 5. Division of plant Pathology, Agricultural Research Institute, Wu-Feng, Taichung,
16 Taiwan 41301

17 6. These authors contributed equally to this work.

18 7. Corresponding author: N.H. Chua; Telephone: +1-212-327-8126; Fax:
19 +1-212-327-8327; E-mail: chua@mail.rockefeller.edu

20

21

22 Manuscript information: Abstract, 264 words; Author summary, 162; Main text, 3969
23 words (Introduction, 751 words; Results, 1962 words; Discussion, 1256 words);
24 Materials and methods, 994 words; 6 figures; 2 tables; 31 References.

25

1 Abstract

2 Plant microRNAs (miRNA) guide cleavage of target mRNAs by DICER-like proteins
3 thereby reducing mRNA abundance. Native precursor miRNAs can be redesigned to
4 target RNAs of interest and one application of such artificial microRNA (amiRNA)
5 technology is to generate plants resistant to pathogenic viruses. Transgenic Arabidopsis
6 plants expressing amiRNAs designed to target the genome of two unrelated viruses were
7 resistant, in a highly specific manner, to the appropriate virus. Here, we pursued two
8 different goals. First, we confirmed that the 21-nt target site of viral RNAs is both
9 necessary and sufficient for resistance. Second, we studied the evolutionary stability of
10 amiRNA-mediated resistance against a genetically plastic RNA virus, TuMV. To
11 dissociate selective pressures acting upon protein function from those acting at the RNA
12 level we constructed a chimeric TuMV harboring a 21-nt, amiRNA target site in a
13 non-essential region. In the first set of experiments designed to assess the likelihood of
14 resistance breakdown, we explored the effect of single nucleotide mutation within the
15 target 21-nt on the ability of mutant viruses to successfully infect amiRNA-expressing
16 plants. We found non-equivalency of the target nucleotides, which can be divided into
17 three categories depending on their impact in virus pathogenicity. In the second set of
18 experiments, we investigated the evolution of the virus mutants in amiRNA-expressing

1 plants. The most common outcome was the deletion of the target. However, when the
2 21-nt target was retained, viruses accumulated additional substitutions on it, further
3 reducing the binding/cleavage ability of the amiRNA. The pattern of substitutions within
4 the viral target was largely dominated by G to A and C to U transitions.

5

6 **Author summary**

7 RNA viruses are well known for their tremendous capacity to evolve, a characteristic that
8 threatens the development of effective antiviral strategies. A new antiviral strategy was
9 recently proposed to control plant RNA viruses that relied on the expression in plants of
10 artificial microRNAs (amiRNAs) targeting short sequences of 21-nt in the viral genome.
11 Here, we have evaluated the likelihood that changes in the 21-nt target sequence would
12 result in resistance breakdown. We found that changes at different sites in the target had
13 different consequences on the ability of the virus to evade amiRNA surveillance. Then,
14 we evolved viruses with a single substitution within the target under the selective
15 pressure imposed by amiRNAs but without any selective pressure at the protein level. We
16 found extra mutations accumulated in the target that further reduced base pairing with the
17 amiRNA. These results showed that when allowed to replicate, RNA viruses would
18 readily generate genetic variability that would facilitate evasion from the plant innate

1 immune response.

2

1 Introduction

2 Plant miRNAs regulate the abundance of target mRNAs by guiding their cleavage at the
 3 sequence complementary region. Previous reports have shown that changes of several
 4 nucleotides within a miRNA 21-nt sequence do not affect its biogenesis and maturation [1,
 5 2]. This finding raises the possibility to redesign the miRNA sequence to target specific
 6 transcripts, originally not under miRNA control. Such artificial miRNAs have been
 7 produced in dicotyledonous [3-5] and monocotyledonous plants [6] using different
 8 pre-miRNAs as backbones. We have successfully demonstrated that redesigned artificial
 9 miRNAs (amiRNAs) are biologically active and can be used to confer specific virus
 10 resistance in transgenic plants [4]. The pre-miR159a precursor was used to generate two
 11 amiRNA¹⁵⁹s (amiR¹⁵⁹-P69 and amiR¹⁵⁹-HC-Pro) with sequence complementary to the
 12 RNA genome of two plant viruses, *Turnip yellow mosaic virus* (TYMV) and *Turnip*
 13 *mosaic virus* (TuMV), respectively. The amiR-P69 was designed to target sequences
 14 encoding the P69 suppressor of TYMV whilst amiR¹⁵⁹-HC-Pro would target sequences
 15 for the HC-Pro silencing suppressor of TuMV. Transgenic lines carrying both
 16 35S-pre-amiR¹⁵⁹-P69 and 35S-pre-amiR¹⁵⁹-HC-Pro transgenes can express the
 17 appropriate amiRNA at high levels and showed specific resistance to either TYMV and
 18 TuMV, depending on the expression of the cognate amiRNA [4]. Specific resistance to

1 TuMV was also seen with plants expressing amiR¹⁵⁹-TuCP directed against the TuMV
2 coat protein (CP) gene [4].

3 In animal systems, RNA interference (RNAi), a gene-silencing mechanism similar
4 to that of miRNA, has been used in clinical trials as antiviral therapeutics to inhibit
5 replication of several human pathogenic viruses (reviewed in [7]). As demonstrated for
6 HIV-1, a major problem of RNAi-mediated antiviral therapies is the emergence of
7 resistant virus variants, which differ from the wild type virus by having fixed point
8 mutations in the target sequence leading to imperfect matching; these mutant viruses are
9 not properly processed by the enzymatic silencing machinery [8-12]. Some mismatches
10 within the target sequence are tolerated by the RNAi machinery whereas other
11 mismatches, such as those in the central region (position 9 to 11) of the target sequence,
12 compromise RNAi-guided antiviral therapies [13, 14]. However, all these studies suffer
13 from the drawback of having a superimposition of two different selective forces: on the
14 one hand, purifying selection acts at the protein level (i.e., the necessity of maintaining a
15 functional protein) and, on the other hand, diversifying selection acts at the RNA
16 sequence level favoring mutant genomes capable of evading RNA silencing.

17 Mallory et al (2004) have used an *in vitro* wheat germ system to assay for critical
18 positions within a miRNA target site needed for efficient plant mRNA cleavage [15].

1 Analysis of scanning mutants revealed that mismatches at the center and the 3' end of the
2 miRNA are more tolerated compared to mismatches at the 5' region [15]. Recently, the
3 molecular mechanism of RISC-mediated RNA cleavage has been investigated by *in vitro*
4 reconstitution assays using human RISC [16-18]. It was found that the accessibility of
5 RNA target site correlates directly with the RNA cleavage efficiency, indicating that
6 RISC is unable to unfold structured RNA. In the course of target recognition, RISC
7 transiently contacts single-stranded RNA nonspecifically and promotes siRNA-target
8 RNA annealing. Furthermore, the 5' portion of the siRNA within RISC creates a
9 thermodynamic threshold that determines the stable association of RISC and the target
10 RNA. Furthermore, in addition to this clear position-effect, overall destabilization of
11 the double strand structure has little effect on RNAi activity until an energy threshold is
12 reached, beyond of which a negative correlation exist between stability and
13 RNAi-mediated inhibition [14].

14 Here, we first investigated whether the 21-nt of an amiRNA target site is both
15 necessary and sufficient for amiRNA-mediated specific resistance. Second, we were
16 interested in identifying critical positions within the target site for this resistance. Third,
17 we have explored the patterns of sequence polymorphism of viral sequences that evolve
18 under the only selective pressure of amiRNA-mediated silencing. To address these issues,

1 we established a heterologous-virus resistance system using a TuMV-GFP viral vector to
2 carry a non-essential 21-nt sequence of the *P69* gene targeted by amiR¹⁵⁹-P69. This
3 heterologous-virus system allows us to modify any nucleotide within the 21-nt target site
4 without altering virus coding sequences and thus without affecting replication and activity.
5 In other words, this heterologous system allows separating the selective pressure imposed
6 by protein functionality from the selective pressure imposed at the sequence level by
7 RNA silencing. The 21 scanning mutant viruses were inoculated on amiR¹⁵⁹-P69 plants
8 and the proportion of transgenic plants that became infected was used to determine the
9 importance of the mutated nucleotide position within the amiRNA target site.

10

11 **Results**

12 **Construction of the 21-nt target site in TuMV**

13 We have previously demonstrated that a 21-nt amiRNA, with sequence complementary to
14 a viral sequence, can mediate cleavage of target viral RNA and confer resistance on
15 transgenic plants [4]. However, it was not known whether the 21-nt viral target site,
16 complementary to the amiRNA sequence, was sufficient for specific resistance. To this
17 end, we constructed a *green fluorescence protein (GFP)* gene carrying a 21-nt sequence
18 (5'-CCACAAGACAAUCGAGACUUU-3') of the TYMV *P69* gene at its 3'-end and

1 inserted the *GFP-P69_{21nt}* fusion gene in between the *NIb* and *CP* genes to generate a
 2 TuMV-P69 chimeric virus (Fig. 1). As a control, we mutated 4 nts (position 9 to 12 from
 3 the 3'-end; underline) of the target 21-nt sequence
 4 (5'-CCACAAGACCUGAGAGACUUU-3') to give *GFP-P69_{21ntm}*, which was inserted
 5 in the same position of the viral genome to generate the TuMV-P69m chimeric virus (Fig.
 6 1D).

7 The presence of two TuMV NIa protease cleavage sites (CVYHQA) at both the N-
 8 and C-termini of the GFP-P69_{21nt} fusion protein allows the release of GFP plus a 7 amino
 9 acid C-terminal extension (PQDNRDF) from the TuMV-GFP viral polyprotein (Fig. 1C
 10 & D). Virus infection can be easily confirmed and followed by monitoring GFP signals
 11 from infected tissues.

12

13 **amiRNA-mediated specific resistance to heterologous virus**

14 We have previously shown that transgenic *Arabidopsis thaliana* plants expressing
 15 amiR-P69 can specifically target the *P69* gene of TYMV and displayed specific
 16 resistance to TYMV [4] although these plants remained susceptible to heterologous virus
 17 (TuMV-GFP) infection (Fig. 2A, top second panel). Figure 2 shows that insertion of the
 18 21-nt sequence of the TYMV *P69* gene into TuMV-GFP, which was targeted by

1 amiR¹⁵⁹-P69, rendered these amiR¹⁵⁹-P69 plants resistant to TuMV-GFP-P69 (Fig. 2A,
 2 top third panel). Control experiments showed that the amiR¹⁵⁹-P69 plants remained
 3 sensitive to TuMV-GFP-P69m (Fig. 2A, top fourth panel), which carried 4 mutations in
 4 the central region of the 21-nt site of the P69 gene. Systemic leaves of amiR¹⁵⁹-P69 plants
 5 displayed GFP fluorescence when inoculated with TuMV-GFP or TuMV-GFP-P69m (Fig.
 6 2B, top second and forth panels), but no GFP signal was detected upon TuMV-GFP-P69
 7 inoculations (Fig. 2B, top third panel).

8 Plants expressing amiR¹⁵⁹-HC-Pro were resistant to chimeric TuMV-GFP,
 9 TuMV-GFP-P69 and TuMV-GFP-P69m and no GFP was detected on systemic leaves of
 10 inoculated plants (Fig.2 B, bottom panels). These results were expected since all these 3
 11 chimeric viruses contained the HC-Pro gene targeted by amiR¹⁵⁹-HC-Pro [4].

12

13 **Expression of amiR¹⁵⁹-P69 in *Nicotiana benthamiana* confers resistance to**

14 **TuMV-P69**

15 Next, we established the heterologous virus resistance system in *N. benthamiana* and
 16 tested amiRNA-mediated resistance efficiency. Fig 3A shows amiR¹⁵⁹-P69 expression
 17 levels in 4 independent transgenic *N. benthamiana* lines (#1, 2, 3, and 4). Progeny plants
 18 of these lines were challenged with TuMV-GFP, TuMV-GFP-P69 or TuMV-GFP-P69m.

The GFP signal produced by infection with TuMV-GFP or TuMV-GFP-P69m can be detected at 4 dpi (early stage of symptom development) (Fig. 3B). As expected, at 7 dpi, transgenic *N. benthamiana* plants expressing amiR¹⁵⁹-P69 were resistant to TuMV-GFP-P69 but susceptible to TuMV-GFP and TuMV-GFP-P69m (Fig 3C). Plants that were sensitive to virus infection showed severe wilting symptoms (Fig. 3C). These results, which were very similar to those obtained with *A. thaliana* transgenic plants, provided further confirmation that the targeted 21-nt site is necessary and sufficient for specific amiRNA-mediated specific resistance. In addition, the results also suggested that pre-amiR¹⁵⁹-P69, which is a modified form of the *Arabidopsis* miR¹⁵⁹ precursor, can be processed by *N. benthamiana* plants to produce functional amiR¹⁵⁹-P69 to confer virus resistance.

Scanning mutagenesis of the 21-nt sequence targeted by amiR¹⁵⁹-P69

As the 4 nt mutation on the central positions of the target sequence (position 8 to 12) compromised specific resistance, we decided to further investigate nucleotide positions within this 21-nt sequence that are critical for amiRNA-mediated resistance. Note that the sequence can be systematically altered without affecting essential viral gene functions because the amiR¹⁵⁹-P69 target sequence is non-essential to the TuMV-GFP-P69 chimeric

1 virus.

2 Accordingly, we generated a series of mutants by making all possible synonymous
3 scanning substitutions within the 21-nt sequence of *P69* in the background of
4 TuMV-GFP-*P69* (Fig. 4A). Each A of the *P69* viral sequence that pairs to a U of
5 amiR¹⁵⁹-*P69* was changed to a C to create a C:U mismatch; each C and G of the viral
6 sequence was changed to an A to create A:G and A:C mismatches; and each U of the viral
7 sequence was changed to a C to create C:A mismatches.

8 A total of 21 mutant viruses with single nt substitution from the 1st to the 21st
9 position of the target site were used to challenge non-transgenic WT and amiR¹⁵⁹-*P69* *N.*
10 *benthamiana* plants. The proportion of inoculated amiR¹⁵⁹-*P69* plants that showed visible
11 symptoms after inoculation, i.e. pathogenicity, was used as a measure of the importance
12 of the mutated nucleotide within the 21-nt target site in amiR¹⁵⁹-*P69*-mediated specific
13 resistance.

14 We used TuMV-GFP, TuMV-GFP-*P69*, and TuMV-GFP-*P69m* as controls. Whereas
15 WT plants were susceptible to TuMV-GFP-*P69* no symptoms developed in amiR¹⁵⁹-*P69*
16 plants even at 10 dpi (Table 1, and Fig. 4B bottom third panel). By contrast, TuMV-GFP
17 and TuMV-GFP-*P69m* elicited 100% infection on WT as well as amiR¹⁵⁹-*P69* transgenic
18 tobacco plants, and these infected plants displayed symptoms at 5 dpi (Table 1, and Fig.

1 4B bottom second and forth panel).

2 WT plants were 100% susceptible to all 21 scanning mutant viruses and symptoms
 3 appeared at 5 dpi (data not shown), indicating that the single nt substitutions on the target
 4 21-nt sequence did not affect mutant virus replication nor movement. On the other hand,
 5 these mutant viruses showed variable pathogenicity after inoculation on amiR¹⁵⁹-P69
 6 plants (Table 1, and Fig 4B top panels & bottom first panel). Fifteen mutants showed
 7 pathogenicity values that were significantly greater than zero (Table 1). For these
 8 pathogenic mutants, the percentage of infected plants ranged from 8.33% (m7) to 92.86%
 9 (m9). Mutants were classified according to their pathogenicity using a 2-step cluster
 10 analysis. The minimum number of clusters into which the mutants can be significantly
 11 partitioned was three (Bayesian weight 98.44%; Kruskal-Wallis test: $H = 17.739$, 2 d.f., P
 12 < 0.001). Fig. 4C assigns mutants to the different clusters. The first cluster (green bars in
 13 Fig. 4C), is characterized by positions causing low pathogenicity, with an average value
 14 of 6.91 ± 0.59 %, suggesting that these sites are non critical for resistance. These low
 15 pathogenicity mutants are scatter along the entire 21-nt region. The second cluster
 16 contains mutants of intermediate pathogenicity (yellow bars in Fig. 4C), with an average
 17 value of 36.36 ± 4.71 %, suggesting that these positions are moderately important for
 18 amiRNA-mediated resistance. Most of these medium effect mutants are located between

1 nucleotides 10 and 18, with the exception of m2, which is located at the 3' end of the
 2 target sequence. Finally, the third cluster contains those mutants with a greater likelihood
 3 of resistance breakdown (red bars in Fig. 4C). On average, these large effect mutants have
 4 81.85 ± 4.14 % pathogenicity, highlighting their importance in amiRNA-mediated
 5 resistance. These important sites mostly congregate on the 3' third of the target sequence,
 6 plus m9 and m12 which are located in the center of the sequence.

7 In good agreement with the pathogenicity data, symptoms elicited by these mutants
 8 were generally delayed in comparison with TuMV-GFP (Table 1). For the 9 small-effect
 9 mutants, the median delay in symptom development was two days, for the medium effect
 10 mutants, two days, and for the large-effect mutants, only one day.

11

12 **Evolution of viral genomes that causes breakdown of amiR¹⁵⁹-P69-mediated** 13 **resistance**

14 We sought to gain deeper insights into the question of why different substitutions within
 15 the 21-nt target sequence overcame the amiRNA-mediated resistance to different degrees.

16 To address this issue, we recovered viral RNAs from symptomatic leaves of amiR¹⁵⁹-P69
 17 plants and analyzed the 21-nt target sequence on the viruses by RT-PCR and sequencing.

18 Several possibilities could account for the resistance breakdown. (1) The scanning

mutation could affect amiR¹⁵⁹-P69-mediated cleavage to different degrees in amiR¹⁵⁹-P69
 plants. (2) The scanning mutant virus could undergo rapid evolution accumulating
 additional mutation(s) within the target site to further increase the number of mismatches
 and consequently the ability to replicate in presence of the amiR¹⁵⁹-P69. (3) Since the
 21-nt sequence and the *GFP* gene are non-essential for virus survival, the surviving
 mutant virus could undergo in-frame deletions in this region that would render the virus
 unrecognizable by the amiR¹⁵⁹-P69. To discriminate amongst these possibilities, we
 designed two primers (PTuNIb-8671 and MTuCP-8982) to amplify an 1136 bp DNA
 fragment including a partial *Nib* gene, *GFP* gene, the target 21-nt sequence, and a partial
CP gene (Fig. 1B). This primer set can be used to check for any possible deletion within
 the *GFP*-21nt sequence. The recovery of lower molecular mass PCR fragment(s) would
 indicate deletion of this region. In addition, we designed another primer set (PXFP-532
 and MTuCP-8982) to amplify a 482-bp fragment that included a partial *GFP* gene and a
 partial *CP* gene (Fig. 1B). This 482-bp DNA fragment can be used to analyze sequences
 surrounding and within the 21-nt target site.

Figure 5A top panel shows that several virus sequences recovered from
 symptomatic amiR¹⁵⁹-P69 plants contained deletion of the *Nib*-*CP* gene, such as
 TuMV-P69m2 (lanes 3, 4 and 7) and TuMV-P69m3 (lanes 10 and 13). In addition, several

1 viruses, such as TuMV-P69m5-13, -15, and -19 contained partially-deleted 21-nt
 2 sequence (Fig. 5B). Moreover, TuMV-P69m5-15 also accumulated two additional
 3 mutations on positions 6 and 8 (Fig. 5B).

4 We selected viruses with no deletion on the *Nib-CP* gene region and sequenced the
 5 *GFP-CP* gene regions (Fig. 5A, bottom panel). Our results showed that virus sequences
 6 recovered from symptomatic amiRNA plants contained additional mutations within the
 7 21-nt target site (Table 2). These scanning mutant viruses have 1 - 3 additional mutation(s)
 8 on the 21-nt target site and most of additional mutations introduced additional
 9 mismatches (Table 2). Only 11 out of the 21 positions showed additional mutations.
 10 These 11 positions are 3, 4, 6, 8, and 10 - 16 (Fig. 5C). Positions 8, 14 and 16 were of
 11 little importance for amiRNA mediated resistance (see above), whereas all other 8
 12 positions had either a moderate or a large effect on the likelihood of escaping the
 13 amiRNA-mediated resistance. All together, our results indicate that up to 2 mutations on
 14 critical positions within the 21-nt sequence can overcome specific resistance.

15 Interestingly, we found that 40 out of 55 observed additional mutations were
 16 transitions. Over 50% of the additional mutations in positions 3, 4, 6, 8, 11, and 15 were
 17 transition mutations. For example, there were 100% U → C or C → U transitions in
 18 position 3 and 4. In position 15, the G → A transition represented 88.89% of all observed

mutations at this particular site. Moreover, there were 50 to 66.67% of G → A transitions
 at positions 6, 8, and 11. This result is not surprising, since it is well known that virus
 coding regions show an excess of transitions over transversions [19, 20]. Three reasons
 can account for this bias: (i) the underlying mechanisms of mutation render transitions
 easier than transversions, (ii) the redundancy of the genetic code is expected to make the
 average effect of a transition smaller than the average effect of a transversion, and (iii)
 RNA editing by deaminase-like enzymes have been shown to induce transition mutations
 in single-stranded regions of certain viral genomes [21, 22]. Our results show that
 transitions rather than transversions also mainly accumulate in viral sequences, such as
 that of the target of amiR¹⁵⁹-P69, which are not under the selective constrain imposed by
 being a coding sequence. Furthermore, not all transitions are equally represented in Table
 2, since G → A (17/40) and C → U (14/40) are significantly over represented ($\chi^2 =$
 12.600, 3 d.f., $P = 0.006$). This bias amongst transitions is expected if the viral RNA was
 edited by cytidine deaminase enzymes.

Discussion

A 21-nt sequence is necessary and sufficient for amiRNA specific resistance

Here, we have developed a heterologous-virus resistance system to study and identify

1 critical positions of amiRNA target site for amiRNA-mediated resistance. The
2 amiR¹⁵⁹-P69 transgenic plant were resistant to TYMV, but not to TuMV (a
3 heterologous-virus), because there was no sequence homology with amiR¹⁵⁹-P69 on the
4 TuMV viral genome [4]. However, the chimeric heterologous-virus TuMV-P69 carrying
5 the 21-nt sequence of *P69* gene cannot infect amiR¹⁵⁹-P69 plants because of
6 amiR¹⁵⁹-P69-mediated cleavage. By contrast, the TuMV-P69m virus with mutations on
7 the central region within this sequence is sufficient to prevent amiRNA-mediated
8 cleavage on the viral RNA and compromise specific virus resistance. These results
9 indicated that the 21-nt target site is portable and is necessary and sufficient to confer
10 virus resistance.

11 Because of the genome organization and proteolytic processing strategy of
12 potyvirus, TuMV can express GFP when a cDNA for this protein is inserted in-frame
13 between the *NIb* and *CP* genes. The encoded GFP protein contains two NIa proteinase
14 cleavage sites (CVYHQ/A) at the N- and the C-terminus such that GFP can be released
15 from the viral polyprotein by proteolytic processing. In addition, the additional 21-nt
16 target site that encodes seven amino acids is also nonessential for TuMV. Therefore, any
17 modification on the GFP gene and the 21-nt target site would not affect the chimeric
18 TuMV as evidenced by its ability to infect plants and stably replicate.

1

2 **Critical position for amiRNA-mediated resistance**

3 Using an *in vivo* assay we identified critical positions on the 21-nt target sequence for
4 RISC-amiRNA-mediated cleavage. Scanning mutations on the 21-nt target site of the
5 challenging chimeric virus showed different degree of resistance breakdown on
6 amiR¹⁵⁹-P69 transgenic plants. Based on the proportion of amiR¹⁵⁹-P69 plants that
7 become susceptible, we defined critical, moderately critical and non-critical positions on
8 the 21-nt sequence. Positions 3 - 6, 9, and 12, are found to be critical for resistance
9 because chimeric virus with mutations at these sites were pathogenic, on average, on
10 ~82% of amiR¹⁵⁹-P69 plants. Positions 2, 10, 11, 13, 15, and 18 are classified to be
11 moderately critical mutations giving average pathogenicity of ~36% in transgenic plants.
12 The remaining positions are classified as non-critical for resistance since mutants at these
13 sites were only pathogenic in less than 7% of inoculated plants. In summary, most critical
14 positions are localized on sequences complementary to the 5' portion of the amiRNA
15 whereas the moderate critical positions are mainly localized on the central region of the
16 target site. The exception being position 18, which is complementary to the 18th
17 nucleotide on 3' portion of the amiRNA, and was also moderately important for
18 amiRNA-mediated resistance. These results are consistent with those obtained with *in*

1 *vitro* miRNA-mediated cleavage using a wheat germ system [15]. All together, results
2 suggest that the 5' portion of the miRNA is more important in governing the specificity of
3 miR165/166 regulation [15]. Furthermore, the “two-state model” for RISC-mediated
4 target recognition also proposes that the specific interaction between RISC and the
5 substrate is initiated via the 5' portion of siRNA, as the 3' portion is less favorably
6 structured to undergo base pairing before the initial recognition of a mRNA target [16].

7

8 **Possible mode of breakdown induced by critical-position mutation**

9 Representative results showed that several virus sequences recovered from symptomatic
10 amiRNA plants contained deletions and additional mutations within the 21-nt target site.
11 This observation is consistent with the hypothesis that mutations in certain critical
12 positions within the target site reduced amiRNA-mediated cleavage efficiency (Fig. 6A).
13 The reduced RISC activity allowed certain mutant viruses to escape amiRNA-mediated
14 cleavage, allowing them to replicate and complete an infectious cycle (Fig. 6B). During
15 subsequent virus replication, additional mutations or deletions of the target sequence
16 would be positively selected because they would escape from the amiRNA-mediated
17 specific resistance more efficiently (Fig. 6B). Indeed, the effect of miRNA-mediated
18 cleavage was drastically alleviated in transgenic plants expressing the silencing

1 suppressor P1/HC-Pro. Chimeric *Plum pox virus* (PPV) carrying an endogenous miRNA
2 target site can also overcome the resistance by deletion and mutation on the 21-nt target
3 sequence [23]. Finally, deletions, in addition to point mutations, are also a very common
4 pathway taken by HIV-1 to escape from RNAi-based therapy in cell culture experiments
5 [9, 10]. In general, these deletions have a major impact on the local RNA secondary
6 structure, creating new hairpin structures not accessible to the siRNAs [10].

7 In some deletion mutant viruses recovered from breakdown plants, the entire *GFP*
8 gene, along with small portions of the *Nib* gene C-terminus or the *CP* gene N-terminus,
9 has been deleted from the viral genome (data not shown). These results suggest that
10 TuMV can tolerate small deletions in the *Nib* or *CP* gene. In addition, deletions in
11 between the *GFP* gene and the target site (Fig. 5B) may be triggered by
12 polymerase-jumps on repeat sequence (ACAA).

13 Widespread plant miRNA-directed translational repression as an important
14 miRNA-mediated regulatory mechanism in plants has recently been reported [24].
15 Imperfect pairing with central mismatches in small RNA-target hybrids promotes
16 translational repression because it excludes slicing [24]. This observation suggests the
17 possibility that imperfect pairing between the amiRNA and mutant target sequences might
18 lead to translational repression rather than viral RNA cleavage. In contrast to the catalytic

1 effects of amiRNA-mediated viral RNA cleavage, translational repression requires
2 stoichiometric amounts of amiRNAs and therefore is not as efficient. Inefficient
3 translation inhibition might allow residual virus replication and progeny virus can still
4 escape the repression by fixing changes in the target sequence.

5 In this study, we have provided evidence that the 21-nt target site is necessary and
6 sufficient for amiRNA specific resistance and we have also identified several positions on
7 the target site that are critical for this resistance. These results are clearly important for
8 future design of amiRNA-mediated virus resistance. Highly conserved regions on viral
9 genomes should be selected as target sites to minimize the likelihood of fixation of
10 mutations responsible for resistance breakdown, because these mutations might affect
11 viral protein function and hence have a negative impact on virus fitness and survival.
12 Furthermore, several amiRNAs targeting different conserved regions on a viral genome
13 could be co-expressed in transgenic plants to minimize the chances of resistance
14 breakdown. Finally, the heterologous-viral system described here also can be used for
15 viral evolution studies in the future.

16

17 **Patterns of molecular evolution in the amiR¹⁵⁹-P69 target**

18 As we have highlighted several times here, the amiR¹⁵⁹-P69 target sequence inserted in

the TuMV-GFP genome is functionally neutral. This has allowed us to separate selective pressures acting on the protein level from those acting on the RNA level. Consequently, the patterns of molecular evolution should be different. Not surprisingly, and in agreement with previous data obtained with other viruses, we have observed that most of the mutations fixed within the target were transition mutations [20]. We consider it striking that 77.50% of these fixed transitions were of the type $G \rightarrow A$ and $C \rightarrow U$. These transitions are from the particular type induced by cellular cytidine deaminases (CDAs) involved in innate immune responses to viral infection [25], a phenomenon particularly well described for HIV-1 and other retroviruses [22, 26] but never before on an RNA virus. This observation prompted us to hypothesize that as an antiviral strategy plants may have an RNA-editing system that induces hypermutagenesis in viral genomes. *A thaliana* contains a family of nine paralogous genes that are annotated as CDAs owing to their homology to *CDA1* [27]. These nine genes are good candidates to explore whether their gene products possess cytidine deaminase activity and whether they are indeed involved in plant antiviral defense.

Materials and Methods

Plant material and growth conditions

Two amiRNA transgenic *A. thaliana* lines, amiR¹⁵⁹-P69 and amiR¹⁵⁹-HC-Pro, were used in this study [4]. Plants of *N. benthamiana* were transformed with *Agrobacterium tumefaciens* containing the pBA-amiR¹⁵⁹-P69 plasmid by standard methods. T2 transgenic *N. benthamiana* (a mixture of homozygotes and hemizygotes) were analyzed for transgene and miRNA levels and 4 independent lines (#1, 2, 3, and 4) with high amiR¹⁵⁹-P69 expression levels were selected for virus challenge experiments. Seeds were surface-sterilized and chilled at 4 °C for 2 d before being placed on Murashige and Skoog (MS) medium with/without antibiotics or sowed directly 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).

11

12 Northern blot hybridizations

Ten µg of total RNA was resolved in a 15% polyacrylamide/1× TBE (8.9 mM Tris, 8.9 mM boric acid, 20 mM EDTA)/8M urea gel and blotted to a Hybond-N+ membrane (Amersham). DNA oligonucleotides with the exact reverse-complementary sequence to miRNAs were end-labeled with ³²P-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

1 signals were detected by autoradiography. In each case, the probe contained the exact
2 antisense sequence of the expected miRNA to be detected.

3

4 **Construction of infectious clones of TuMV-GFP-P69 and TuMV-GFP-P69m**

5 **chimeric viruses**

6 The TuMV infectious clone (p35STuMV-GFP) comprises of a 35S promoter and the
7 full-length cDNA of TuMV-GFP. The *GFP* gene was inserted between *NIb* and *CP* genes.
8 This chimeric TuMV-GFP virus was used as a surrogate wild type virus and as a
9 backbone for construction of various chimeric recombinant viruses described here.

10 We fused the 21-nt sequence (5'-CCACAAGACAAUCGAGACUUU-3') of the
11 TYMV P69 gene targeted by amiR¹⁵⁹-P69 to the 3' end of the *GFP* gene. The GFP-P69
12 fusion sequence was then inserted in between the *NIb* and *CP* genes to generate the
13 p35STuMV-GFP-P69 infectious clone. As a control, the central 4 nts (underlined) of the
14 21-nt target sequence (5'-CCACAAGACCUGAGAGACUUU-3') was mutated to give
15 GFP-P69m which was also inserted in the same position of the virus to generate
16 p35STuMV-GFP-P69m.

17

18 **Scanning single nucleotide mutagenesis on the amiR¹⁵⁹-P69 target site**

1 As the 21-nt target sequence is in a non-essential region of the TuMV-GFP-P69 it can be
2 altered without affecting essential viral gene function. We performed serial single nt
3 mutagenesis from the 1st-nt to the 21st-nt of the target site on the p35STuMV-GFP-P69
4 infectious clone by PCR mutagenesis and the resulting series of scanning mutants were
5 confirmed by sequencing. A total of 21 single-nt substitution recombinant viruses were
6 generated. Based on the mutation position, the recombinant viruses were named
7 TuMV-P69mX, in which X refers to the mutation position. For example, the mutant with
8 substitution on the 1st-nt of the target site was named TuMV-P69m1.

9

10 **Protocol of challenge inoculation with recombinant viruses and pathogenicity** 11 **estimation**

12 To evaluate the efficiency of amiRNA-mediated specific resistance toward wild type and
13 mutant viruses, we have established a standard protocol for virus challenge inoculation
14 and quantitative evaluation of pathogenicity. Our overall aim was to reduce the time for
15 virus maintenance and propagation in host plants so as to minimize possible virus
16 evolution. All recombinant viruses were propagated from DNA infectious clones.
17 Aliquots of 20 µL, containing 1 µg of DNA in sterilized water, were mechanically
18 applied onto carborundum-dusted leaves of *Chenopodium quinoa* Willd with a sterilized

1 glass spatula. Seven days post-inoculation (dpi), local lesions were obtained on
 2 inoculated leaves. Viruses were then isolated from single lesions and transferred to *N.*
 3 *benthamiana* for amplification. Four dpi leaves of *N. benthamiana* with viral infection
 4 symptoms were used as the source of inoculum to challenge WT and amiR¹⁵⁹-P69 *N.*
 5 *benthamiana* plants for evaluation of virus pathogenicity (i.e., frequency of break-down).
 6 Twenty amiR¹⁵⁹-P69 plants were used for each experiment, and the experiments were
 7 repeated 3 times. Resistance efficiency of amiR¹⁵⁹-P69 plants challenged with
 8 recombinant viruses were compared with those obtained with control viruses, including
 9 TuMV-GFP, TuMV-GFP-P69 and TuMV-GFP-P69m.

10 Pathogenicity was evaluated between two and four times for each one of the 21
 11 TuMV-P69mX recombinant viruses. Count data from experiments that were statistically
 12 homogeneous were pooled into a single set, whereas experiments that behaved as outliers
 13 were removed from the dataset.

14

15 **Sequence analysis of the 21-nt target region**

16 In plants displaying symptoms, it was important for us to verify the sequence of the 21-nt
 17 target site to ensure that no other mutations had occurred to confound our results. To this
 18 end, total RNA was extracted from infected leaf tissues using the Trizol reagent

1 (Invitrogen). One μ g total RNA was used for reverse-transcriptional polymerase-chain
2 reaction (RT-PCR) with PTuNIb-8671 (5' - GAACCAGCTCAAGAGGATCT-3') and
3 MTuCP-8982 (5' - GCCACTCTCTGCTCGTATCTTGGCACGCGC-3') for amplification
4 of the viral region between the partial *NIb* and the *CP* genes (Fig. 1B). The PCR
5 fragments then were analyzed by sequencing.

6

7 **Statistical analyses**

8 The pathogenicity of different recombinant viruses was estimated as the frequency of
9 infected plants out of the total number of inoculated plants. The LaPlace's point estimator
10 for the Binomial frequency parameter was used instead of the commonly used maximum
11 likelihood estimator [28]. The LaPlace method provides a more robust estimate of the
12 Binomial parameter for small sample sizes [29]. Binomial 95% confidence intervals (CI)
13 were also computed.

14 TuMV-P69mX recombinant viruses were classified into groups of similar
15 pathogenicity using the two-step cluster analysis [30]. In brief, this method classifies data
16 in groups that minimize the within-group variance whilst maximizing the among-groups
17 variance. The method starts with the simplest model (i.e., all viruses are equally
18 pathogenic) and computes its likelihood; then, it classifies the mutants into two clusters

1 and computes the likelihood of this model; finally, it does the same for three clusters, four
2 clusters and up to 21 clusters (i.e., each site behaves in a different way and no
3 classification is possible). For each model, Schwarz's Bayesian information criterion
4 (*BIC*) was used as a measure of the goodness-of-fit and the model with the lowest *BIC*
5 was considered to be the best one [31].

6

7 **Acknowledgments**

8 S.-S. Lin was supported by a fellowship from Ministry of Education, Taiwan. H.-W. Wu
9 and K.-C. Chen were supported by fellowships from National Science Council, Taiwan
10 (NSC-96-2752-B-005-002-PAE and NSC-94-2752-B-005-001-PAE). We thank Dr. Enno
11 Krebbers, Bobby Williams, Carl Falco and Barbara Mazur for helpful suggestions and
12 stimulating discussions. This work was supported in part by a grant from DUPONT to
13 NHC and a Human Frontier Science Program grant RGP0012/2008 to SFE and NHC.

14

15 **Author Contributions**

16 N.-H.C., and S.-S.L conceived and designed the experiments. S.-S.L., H.-W.W., and
17 K.-C.C. constructed all mutant viruses. Q.-W.N. generated the transgenic plants. S.-S.L.,
18 and H.-W.W performed the virus challenge and related experiments and collected the

1 results. S.F.E. performed the statistic analysis. S.-D.Y., and C.-C.C. provided specific
2 strain of TuMV-GFP infectious clone and advice on experimental design. All authors
3 discussed the results and commented on the manuscript, which was written by N.-H.C.,
4 S.-S.L., and S.F.E.

5

References

1. Guo, H.S., Xie, Q., Fei, J.F., and Chua, N.H. (2005). MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for *Arabidopsis* lateral root development. *Plant Cell* 17, 1376-1386.
2. 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.
3. Alvarez, J.P., Pekker, 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.
4. 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 Biotech* 24, 1420-1428.
5. 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.
6. Warthmann, N., Chen, H., Ossowski, S., Weigel, D., and Hervé, P. (2008). Highly specific gene silencing by artificial miRNAs in rice. *PLoS ONE* 3, e1829.
7. Haasnoot, J., Westerhout, E.M., and Berkhout, B. (2007). RNA interference against viruses: strike and counterstrike. *Nat. Biotech.* 25, 1435-1443.
8. 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.
9. 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.
10. Westerhout, E.M., Ooms, M., Vink, M., Das, A.T., and Berkhout, B. (2005). HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. *Nucl. Acids Res.* 33, 796-804.
11. Sabariego, R., Giménez-Barcons, M., Tapia, N., Clotet, B., and Martínez, M.A. (2006). Sequence homology required by human immunodeficiency virus type 1 to escape from short interfering RNAs. *J. Virol.* 80, 571-577.
12. von Eije, K.J., ter Brake, O., and Berkhout, B. (2008). Human immunodeficiency virus type 1 escape is restricted when conserved genome sequences are targeted by RNA interference. *J. Virol.* 82, 2895-2903.

13. Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. (2001). Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J.* 20, 6877-6888.
14. Westerhout, E.M. and Berkhout, B. (2007). A systematic analysis of the effect of target RNA structure on RNA interference. *Nucl. Acids Res.* 35, 4322-4330.
15. Mallory, A.C., Reinhart, B.J., Jones-Rhoades, M.W., Tang, G., 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.
16. Ameres, S.L., Martinez, J., and Schroeder, R. (2007). Molecular basis for target RNA recognition and cleavage by human RISC. *Cell* 130, 101-112.
17. Martinez, J., and Tuschl, T. (2004). RISC is a 5' phosphomonoester-producing RNA endonuclease. *Gene Dev.* 18, 975-980.
18. Zhang, X., Yuan, Y.R., Pei, Y., Lin, S.S., Tuschl, T., Patel, D.J., and Chua, N.H. (2006). *Cucumber mosaic virus*-encoded 2b suppressor inhibits *Arabidopsis* Argonaute1 cleavage activity to counter plant defense. *Gene Dev.* 20, 3255-3268.
19. Burch, C.L., Guyader, S., Samarov, D., and Shen, H. (2007). Experimental estimate of the abundance and effects of nearly neutral mutations in the RNA virus ϕ 6. *Genetics* 176, 467-476.
20. Haydon, D., Knowles, N., and McCauley, J. (1998). Methods for the detection of non-random base substitution in virus genes: models of synonymous nucleotide substitution in picornavirus genes. *Virus Genes* 16, 253-266.
21. Bishop, K.N., Holmes, R.K., Sheehy, A.M., and Malim, M.H. (2004). APOBEC-mediated editing of viral RNA. *Science* 305, 645.
22. Cullen, B.R. (2006). Role and mechanism of action of the APOBEC3 family of antiretroviral resistance factors. *J. Virol.* 80, 1067-1076.
23. Simón-Mateo, C., and García, 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.
24. Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y.Y., Sieburth, L., and Voinnet, O. (2008). Widespread translational inhibition by plant miRNAs and siRNAs. *Science* 320, 1185-1190.
25. Conticello, S.G., Thomas, C.J., Petersen-Mahrt, S.K., and Neuberger, M.S. (2005). Evolution of the AID/APOBEC family of polynucleotide (deoxy)cytidine deaminases. *Mol. Biol. Evol.* 22, 367-377.
26. Harris, R.S., and Liddament, M.T. (2004). Retroviral restriction by APOBEC

- 1 proteins. Nat. Rev. Immunol. 4, 868-877.
- 2 27. Vincenzetti, S., Cambi, A., Neuhard, J., Schnorr, K., Grelloni, M., and Vita, A.
- 3 (1999). Cloning, expression, and purification of cytidine deaminase from
- 4 *Arabidopsis thaliana*. Protein Expr. Purif. 15, 8-15.
- 5 28. Chew, V. (1971). Point estimation of the parameter of the binomial distribution. The
- 6 American Statistician 25, 47-50.
- 7 29. Lewis, J.R., and Sauro, J. (2006). When 100% really isn't 100%: improving the
- 8 accuracy of small-sample estimates of completion rates. J. Usability Studies 3,
- 9 136-150.
- 10 30. Abonyi, J., and Feil, B. (2007). Cluster analysis for data mining and system
- 11 identification. Birkhäuser *Basel, Switzerland*.
- 12 31. Posada, D., and Buckley, T.R. (2004). Model selection and model averaging in
- 13 phylogenetics: advantages of Akaike information criterion and Bayesian approaches
- 14 over likelihood ratio tests. Sys. Biol. 53, 793-808.

15

Table 1. Pathogenicity of -nt substitution of chimeric TuMV-GFP viruses on amiR¹⁵⁹-P69 plants

	N° infected/ inoculated plants	Days of delay in symptoms relative to TuMV-GFP	Pathogenicity (± 95 CI) (%)
TuMV-GFP	58/58	0	98.33±3.12
TuMV-P69	0/58	Never	1.67±3.12
TuMV-P69m	48/48	0	98.00±3.72
TuMV-P69-			
m1	2/40	4	7.14±8.45
m2	8/30	2	28.13±15.34*
m3	37/40	1	90.48±9.34*
m4	33/50	1	65.38±12.75*
m5	40/46	1	85.42±10.15*
m6	33/40	1	80.95±11.92*
m7	5/70	3	8.33±6.65*
m8	1/40	2	4.76±7.38
m9	38/40	2	92.86±8.45*
m10	23/48	1	48.00±13.60*
m11	18/48	1	38.00±13.24*
m12	37/48	1	76.00±11.78*
m13	11/30	2	37.50±16.37*
m14	6/30	3	21.88±14.27*
m15	7/30	2	25.00±14.84*
m16	3/58	2	6.67±6.74
m17	3/49	3	7.84±7.84
m18	27/48	2	56.00±13.52*
m19	2/46	2	6.25±7.47
m20	3/40	1	9.52±9.34*
m21	1/40	2	4.76±7.38

* Pathogenicity values significantly greater than zero.

1 **Table 2. Additional mutations on the 21-nt target fixed during virus evolution**

amiR ¹⁵⁹ -P69 seq.		21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
		G	G	U	G	U	U	C	U	G	U	U	A	G	C	U	C	U	G	A	A	A
		:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Targeted Seq		C	C	A	C	A	A	G	A	C	A	A	U	C	G	A	G	A	C	U	U	U
TuMV-P69-	#																					
m2	1											C					A				C	
	4						A										C		U		C	
	6																A				C	
	7											G	C								C	
m3	4								G												C	
	11						A														C	
	10									U											C	
	13														A						C	
	14										C										C	
m4	4																		A		C	
	6						A												A		C	
	11														A				A			
	14									U									A		C	
	15						A												A			
	16														U				A			
m6	1						A										A					
	7																A		U			
	5														A							
	10										C											
	13						U															
m7	1															C			U			
	2															C		A				
	13								A							C						
m10	2										U		C									
	3												C						U			
	4						C						C									
	5						A						C						U			
m11	1											C							U			
	8											C			A							

[illegible]

1 ^aM2, M3, M4, ..., M18 = mutant virus that single mutated on targeted 21-nt seq.

2 ^bA.M.F. = frequency of additional mutation

3

1 **Figure legends**

2 **Figure 1.** Schematic representations of infectious clones of chimeric *Turnip mosaic virus*
3 (TuMV). (A) Schematic representation of TuMV-GFP infectious clone carrying a GFP
4 gene inserted between the *NIb* and *CP* genes. (B) Arrows represent the positions and
5 orientations of primers used for RT-PCR. The primer sets PTuNIb-8671/MTuCP-8982
6 and PXFP-532/MTuCP-8982 were used to amplify the NIb-CP and GFP-CP regions,
7 respectively. (C & D) Schematic diagrams showing the 21-nt sequence of P69 and P69m
8 in the chimeric viruses TuMV-P69 and TuMV-P69m, respectively. Predicted base pairing
9 of the 21-nt target RNA sequence (top strand) and amiR¹⁵⁹-P69 (bottom strand) are
10 shown below the amino acid sequence of the TuMV-GFP poly-protein.



1 **Figure 2.** A 21-nt sequence targeted by amiRNA is necessary and sufficient to confer
2 virus resistance. (A) amiR¹⁵⁹-P69 and amiR¹⁵⁹-HC-Pro transgenic *Arabidopsis* plants
3 were mock-inoculated or inoculated with TuMV-GFP, TuMV-P69 or TuMV-P69m. As
4 controls, the same transgenic lines were inoculated with TuMV-GFP. Photographs were
5 taken at 12 dpi. Bar = 0.5 cm. (B) GFP fluorescence of systemic leaves of plants infected
6 with chimeric viruses. Leaves were examined by fluorescence microscopy. Leaves of
7 sensitive amiR¹⁵⁹-P69 plants displayed green fluorescence due to replication of
8 GFP-virus, whereas no green fluorescence was detected in leaves of resistant
9 amiR¹⁵⁹-HC-Pro or amiR¹⁵⁹-P69 plants. Bar = 2 cm.

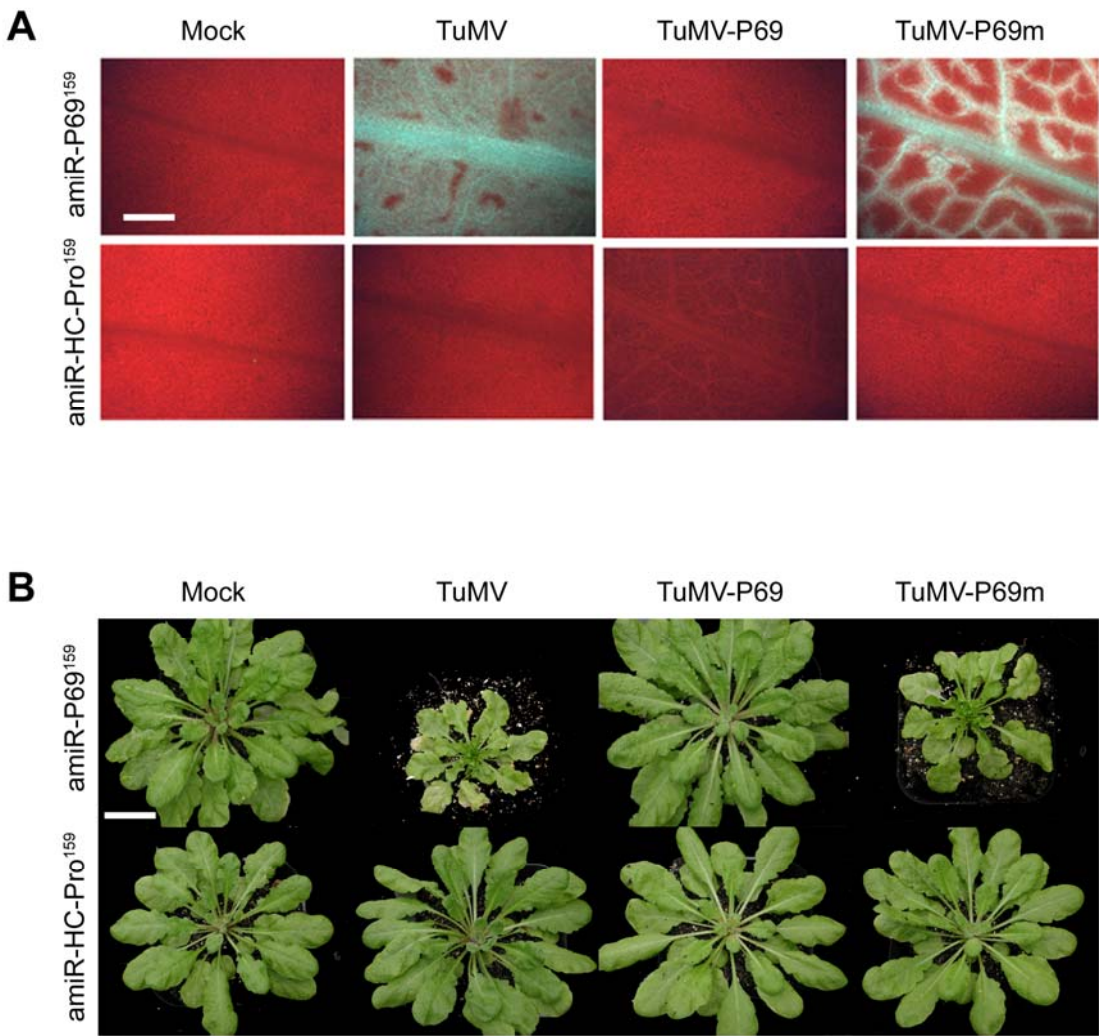


Fig. 2. Lin et al.

1

2

Figure 3. Transgenic *N. benthamiana* plants expressing amiR¹⁵⁹-P69 are resistant to infection by chimeric TuMV virus. (A) amiR¹⁵⁹-P69 expression levels of transgenic *N. benthamiana* plants carrying 35S-pre-amiR¹⁵⁹-P69. Four independent lines (# 1, 2, 3, and 4) were analyzed. 5S rRNAs were used as a loading control. (B) Early infection of chimeric TuMV viruses monitored by UV excitation. (C) amiR¹⁵⁹-P69 *N. benthamiana* plants were resistant to TuMV-P69 but susceptible to TuMV-P69m or TuMV-GFP.

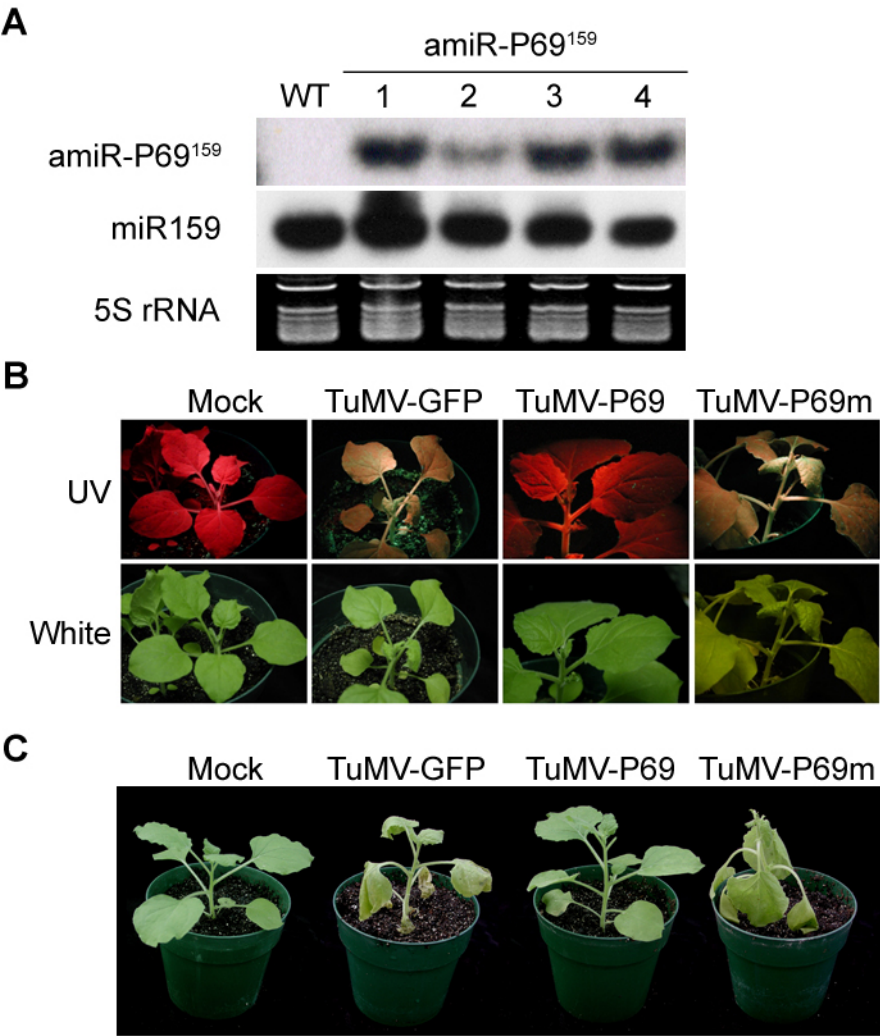


Fig. 3. Lin et al.

Figure 4. Scanning mutagenesis of the amiR¹⁵⁹-P69 target site on TuMV-P69 chimeric virus. (A) A schematic representation of the 21 scanning mutants with substitution of single nucleotide within the 21-nt sequence targeted by amiR¹⁵⁹-P69. (B) Representative amiR¹⁵⁹-P69 *N. benthamiana* plants displaying different degree of breakdown when inoculated with the scanning mutants. The ratio in each panel indicates the number of susceptible amiR¹⁵⁹-P69 plants amongst 20 plants challenged. (C) A summary of critical positions within the amiR¹⁵⁹-P69 target site. The 21-nt RNA sequence is shown on the X-axis. Numbers below the sequence indicate the positions of amiR¹⁵⁹-P69 starting from the 5' end. The degree of resistance breakdown was represented as the percent of inoculated plants with viral disease symptoms. Red bars represent critical positions for resistance; yellow bars represent positions of moderate importance; green bars represent positions of minimal influence in resistance-breakdown.

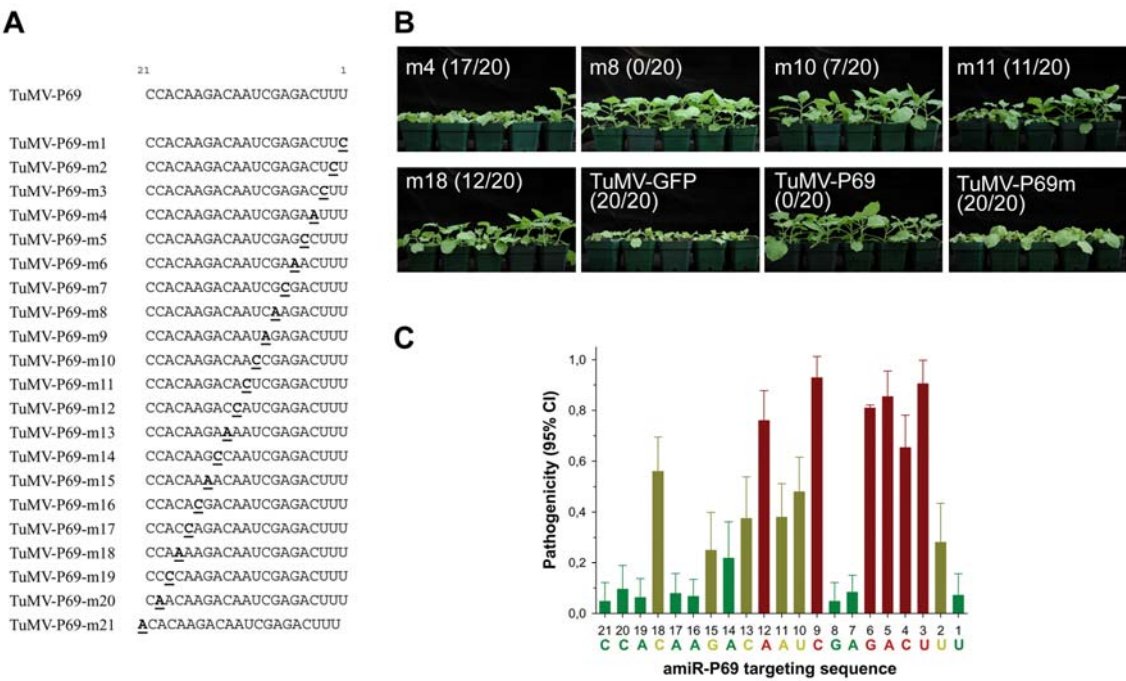


Fig. 4. Lin et al.

1

2

Figure 5. Sequence analysis of chimeric TuMV viruses recovered from susceptible amiR¹⁵⁹-P69 transgenic plants. (A) Representative RT-PCR results of chimeric TuMV viruses derived from susceptible transgenic plants infected with m1, m2, and m3. The Nib-CP (top panel) and GFP-CP regions (bottom panel) of scanning mutants virus TuMV-P69m1 (m1; lane 1), TuMV-P69m2 (m2; lanes 2-7), and TuMV-P69m3 (m3; lanes 8-14) were checked for deletion of the 21-nt target sequence by RT-PCR. (B) Representative results of chimeric TuMV viral sequences with deletion in the 21-nt target site. The sequence of TuMV-P69 was used as the standard sequence (gray box) and the 21-nt target site was underlined. Representative sequences of three scanning mutant viruses TuMV-P69m5-13, 15, and 19 from susceptible plants were aligned. Nucleotide mutation in position 5 was in bold and indicated with an arrow. Additional mutations were marked by asterisks. (C) Frequency of additional mutation on the 21-nt target site. The X-axis shows the 21-nt sequence on TuMV-P69. Numbers below indicate the positions of amiR¹⁵⁹-P69 starting from the 5' end. Bars show frequency of additional mutations in scanning mutant viruses recovered from susceptible plants.

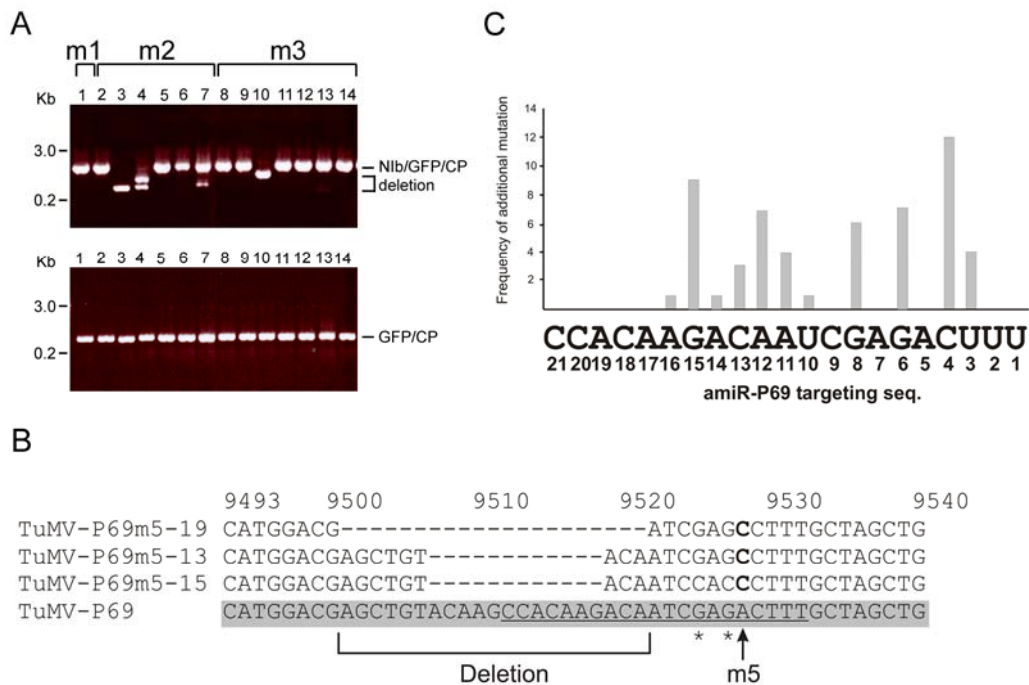


Fig. 5 Lin. et al.

1

2

Figure 6. A working model to explain breakdown of amiRNA-mediated resistance by virus mutation. (A) Complete sequence complementarity between the 21-nt target site and the amiRNA. (B) TuMV-P69m9 is a mutant virus with a single mutation (underlined) on position 9 of the target site. As this position is critical the mutation causes a decrease in the cleavage efficiency of TuMV-P69m9 viral RNAs allowing some viral RNAs to escape the amiRNA-mediated surveillance. The surviving TuMV-P69m9 virus rapidly undergoes evolution collecting additional mutations on the target site. The next generation of mutated viruses with additional mutations can overcome the amiRNA-mediated resistance.

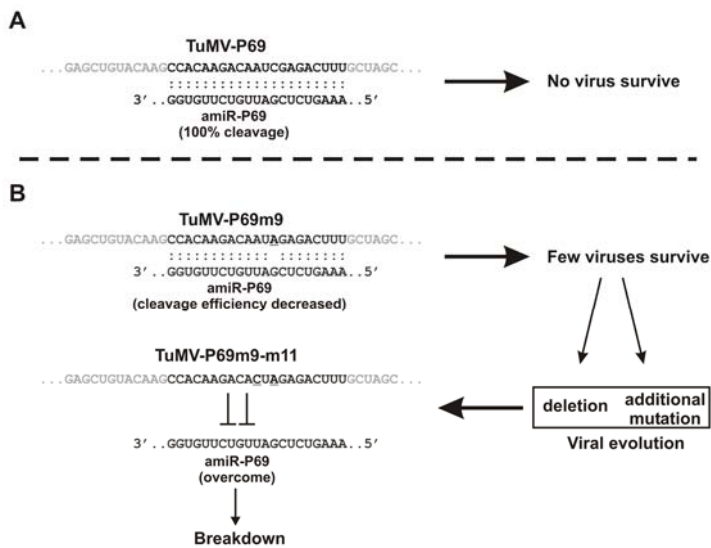


Fig. 6 Lin et al.