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Improving the Effectiveness of Artificial MicroRNA (amiR)-Mediated Resistance against *Turnip Mosaic Virus* by Combining Two amiRs or by Targeting Highly Conserved Viral Genomic Regions

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A drawback of recent antiviral therapies based on the transgenic expression of artificial microRNAs (amiRs) is the ease with which viruses generate escape mutations. Here, we show two alternative strategies for improving the effectiveness of resistance in plants. First, we expressed two amiRs complementary to independent targets in the viral genome, and second, we designed amiRs complementary to highly conserved RNA motifs in the viral genome.

The transgenic expression of 21-nucleotide (nt)-long artificial microRNAs (amiRs) complementary to viral genomes has been proposed as a new antiviral strategy. Niu et al. (1) used the pre-miRNA159a precursor to engineer amiR159-HCPro, complementary to the HC-Pro cistron of *Turnip mosaic virus* (TuMV; genus *Potyvirus*, family *Potyviridae*). Transgenic expression of this amiR in *Arabidopsis thaliana* conferred high levels of specific resistance. Similarly, a gene-silencing mechanism (RNA interference [RNAi]) has been used in *in vitro* assays as an antiviral therapeutic to inhibit the replication of several human viruses (2–4). However, a major issue of these amiR-based antiviral therapies has been the emergence of escape mutant viruses (2, 5–8). These escape variants differ from the wild-type virus by at least one point mutation in the 21-nt target, leading to imperfect matching with the amiR. To evaluate the effectiveness of amiR159-HCPro-mediated resistance in plants, Lafforgue et al. (6) performed an evolution experiment in which multiple independent lineages of TuMV were founded with an ancestral viral clone and allowed to evolve and diversify by serial passages in two different hosts. The first host was a wild-type *A. thaliana* strain, and the second one was a partially resistant transgenic *A. thaliana* strain that expressed the amiR at subinhibitory concentrations (line 10-4). Periodically, the evolving populations were used to challenge the resistance of another transgenic *A. thaliana* line (line 12-4), which was remarkably resistant to the ancestral TuMV. It was found that all lineages, regardless of the plant genotype where they evolved, accumulated mutations in the amiR target and acquired the capacity to successfully infect 12-4 plants. However, viruses evolving in line 10-4 broke resistance approximately seven times faster than viruses evolving in wild-type *A. thaliana* (6). A subsequent deep-sequencing study of some of the evolved TuMV lineages confirmed that substantial nucleotide diversity was generated at each position of the amiR target along the evolution phase and that the alleles finally breaking the resistance were randomly chosen among the many coexisting ones. The frequency of mutant alleles potentially breaking resistance was significantly higher in virus lineages replicating in the 10-4 line than in wild-type *A. thaliana* (9). In all cases, resistance-breaking mutations were more common in central positions of the target than in others (e.g., positions 11 and 12) (6). In retrospect, the relatively ease of overcoming the resistance was most likely related to the existence of sequence variations in the 21-nt target of the amiR in TuMV natural populations (G. Lafforgue and S. F. Elena, unpublished results), suggesting that this genomic region is not under strong purifying selection.

The expression of multiple amiRs targeting conserved motifs in viral genomes has also been shown to be a promising solution for both negative (10)- and positive (11)-sense plant RNA viruses, as well as to human retroviruses such as HIV-1 (12), although in the latter case, cross-resistance to the expression of two amiRs was readily shown (13). Here, we have explored two alternative methods to improve the effectiveness of amiR-mediated resistance. In both methods, we tried to minimize the possibility of emergence of escape mutants. Although the generation of an escape mutant is certainly central to the effectiveness of the resistance, factors such as target accessibility (14, 15) and amiR levels (6) should also play an important role. We assume that these additional factors exert a similar effect in all our experiments. The first strategy consisted of combining two amiRs in a single transgenic plant, each one targeting a different sequence in the TuMV genome. The first amiR was the above-mentioned amiR159-HCPro, and the second one was amiR159-TuCP (1), which is complementary to the sequence 5′-GCCAAGAUACGAGCAGAGAGU-3′ within the TuMV CP cistron (positions 8987 to 9007; GenBank accession number AF530055.2). We argued that this approach would expand resistance effectiveness in inverse proportion to the product of the probabilities of generating an escape mutant per amiR target. The mutation rate for the amiR159-HCPro 21-nt target was estimated to be 5.545×10^{-5} per replication event (16); thus, assuming a mutation rate for the amiR159-TuCP target in the same range, the probability of generating the double mutant in a single replication event would be in the range 10^{-9} to 10^{-10}. The second strategy, which still relied on the expression of a single amiR, was designed to target an RNA sequence under strict purifying selection. In

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TABLE 1 Summary of the infectivity experiments

Genotype of target plant	No.[a] of plants inoculated or infected with inocula from genotype				Frequency of infection[b] (95% CI[c])
	Wild type		10-4		
	Inoculated	Infected	Inoculated	Infected	
Wild-type	301	166	311	155	5.244×10^{-1} ($4.849–5.640 \times 10^{-1}$)
amiR159-HCPro (12-4)	522	9	537	26	3.393×10^{-2} ($2.290–4.496 \times 10^{-2}$)
amiR159-HCPro/amiR159-TuCP	539	0	475	0	$\leq 9.843 \times 10^{-4}$ ($0–3.655 \times 10^{-3}$)
amiR159-CPb	535	0	533	0	$\leq 9.346 \times 10^{-4}$ ($0–3.471 \times 10^{-3}$)

[a] Numbers resulted from adding up the six experimental blocks.
[b] Estimates derived pooling data for both sources of inocula, as no overall difference between them was observed.
[c] Computed using Wald's formula (22).

other words, even if produced at the above rate, any mutation in this sequence will render the virus nonviable. To this end, we identified a 29-nt sequence stretch that is strictly conserved among 95 TuMV natural worldwide isolates (full genomes available at GenBank) (Lafforgue and Elena, unpublished). This conserved sequence is in the 3′ end of the CP cistron, which in another potyvirus (*Tobacco etch virus*) has been shown to be involved in the formation of RNA secondary structures essential for genome replication (17). This new amiR, named amiR159-CPb, is complementary to the sequence 5′-UCGCAUGUAACUUUUCGCAU U-3′ (positions 9323 to 9343; GenBank accession number AF530055.2). New transgenic plants were generated by the floral dip method as described by Niu et al. (1), and all seeds used were homozygous from T4 generations. High and consistent expression of the corresponding amiRs was confirmed by Northern blot analyses.

A large stock of infectious extract was obtained from *Nicotiana benthamiana* plants inoculated with a plasmid containing TuMV cDNA (6, 18). This amplification step was necessary to overcome the low efficiency of *A. thaliana* infection by the TuMV cDNA. The extract was obtained by grinding infected tissues in a mortar with liquid N_2 and 20 volumes of extraction buffer (50 mM potassium phosphate [pH 7.0], 3% polyethylene glycol 6000). Large numbers of wild-type and 10-4 *A. thaliana* plants were inoculated with 5 μl buffer containing 10% Carborundum, which was applied to three different leaves by gentle rubbing with a cotton swab soaked in the extract. After inoculation, plants were maintained in a growth chamber (16 h of light at 25°C and 8 h of dark at 24°C). Infected plants from each genotype were pooled and used to prepare two new infectious inocula: wild-type and 10-4. Our previous results showed that amplification in *N. benthamiana* followed by a single passage in *A. thaliana* was enough to generate substantial genetic variability in the amiR targets (6, 9). Each infectivity experiment consisted of the inoculation, as described above, of a number of wild-type, amiR159-HCPro (line 12-4), amiR159-HCPro/amiR159-TuCP, and amiR159-CPb *A. thaliana* plants. Six independent infectivity experiments were performed.

Table 1 shows a summary of the results of the infectivity experiments. The following binomial logistic regression equation was fitted to the infectivity data using generalized linear models: $\log[I_{ijkl}/(1 - I_{ijkl})] = \beta_0 + \beta_i SP_i + \beta_j TP_j + \beta_{ij}(SP \times TP)_{ij} + \beta_{ijk}B_{ijk} + \varepsilon_{ijkl}$, where *SP*, *TP*, and *B* are the genotypes of the plants used as sources of inocula, the genotypes of the plants used as targets (Table 1), and the experimental block (nested within the interaction $SP \times TP$), respectively. I_{ijkl} corresponds to the *l*th observation of infectivity made in the *k*th experimental block for inoculum *j* and in the target plant *i*. The linear predictor had coefficients β, and each observation is subjected to experimental error ε_{ijkl}. The model significantly fitted the observed data (omnibus goodness-of-fit test: $\chi^2 = 1247.002$, 47 df, $P < 0.001$). First, we found no significant differences among experimental blocks ($\chi^2 = 20.908$, 17 df, $P = 0.230$), and thus we pooled data from different blocks for making Table 1. Second, the genotype of the target plants had a highly significant overall effect on infectivity ($\chi^2 = 7556.643$, 3 df, $P < 0.001$), meaning that infectivity varies among the different genotypes. Third, we found no overall differences among inocula generated in the wild type (WT) and in 10-4 plants ($\chi^2 = 0.000$, 1 df, $P = 1.000$). However, the significant interaction term ($\chi^2 = 400.617$, 3 df, $P = 0.028$) highlights a subtler effect of inoculum that depends on the genotype of the target plant. Indeed, this effect was due to the higher infectivity of inocula prepared from 10-4 plants for 12-4 plants than inocula prepared from WT plants (see below).

The last column in Table 1 shows the frequency of infection computed using Laplace's rule-of-succession method (19). This is a Bayesian probabilistic method that takes into consideration *a priori* knowledge about the possible outcome of any individual trial: success and failure are both likely events, though the probabilities of each outcome are largely unbalanced. The method offers the advantage that for finite sample sizes it does not assign a zero probability to the most unlikely outcome; in this sense, the method provides an upper limit value. TuMV populations showed the largest infectivity in wild-type plants, followed by 12-4 plants. In contrast, the infectivity in the two new transgenic plants was at best 34 to 36 times lower than in 12-4 plants expressing a single amiR targeting a non-strictly conserved RNA sequence. Therefore, we conclude that by combining more than one amiR into the same transgenic plant, or by designing amiRs taking into consideration natural variation of putative target sequences, the likelihood of breaking amiR-mediated resistance by a polymorphic viral population is dramatically reduced. We have previously shown that TuMV populations generated in 10-4 plants contain ca. 100-fold more mutants able to escape from the amiR159-HCPro than those generated in WT plants and thus break resistance more often (9). Indeed, Table 1 confirms this observation, showing that the frequency of infected plants is significantly higher for the 10-4 inocula (Fisher's exact test, $P = 0.005$). Given this observation, one may think that for this TuMV population the situation would be similar to that in infected plants expressing only the amiR159-TuCP and therefore showing a higher infectivity in the amiR159-HCPro/amiR159-TuCP plants than the TuMV

population generated in WT plants. Table 1 shows that this is not the case and that both viral populations behave equally. There are two possible explanations. First, amiR159-TuCP confers a very robust resistance, and second, the amiR159-HCPro escape mutants contained in the population still represents a very minor fraction (less than 10% of that found in the study in reference 9) and thus are not necessarily transmitted during mechanical inoculation (20). An additional consideration is that mutant genotypes escaping amiR159-HCPro may display too low a fitness to face the second amiR159-TuCP amiR.

In our experiments, neither of the two new strategies proved to be more effective than the other against escape mutants; thus, the choice would entirely depend on the availability of strictly conserved RNA sequences in a virus' genome.

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