Targeting of genomic and negative-sense strands of viral RNA contributes to antiviral resistance mediated by artificial miRNAs and promotes the emergence of complex viral populations

Frida Mesel | Mingmin Zhao | Beatriz García | Carmen Simón-Mateo | Juan Antonio García

1Department of Plant Molecular Genetics, Centro Nacional de Biotecnología (CNB-CSIC), Campus Universidad Autónoma de Madrid, Madrid, Spain
2College of Horticulture and Plant Protection, Inner Mongolia Agricultural University, Hohhot, China

Correspondence
Carmen Simón-Mateo and Juan Antonio García, Department of Plant Molecular Genetics, Centro Nacional de Biotecnología (CNB-CSIC), Campus Universidad Autónoma de Madrid, 28049 Madrid, Spain. Emails: csimon@cnb.csic.es and jagarcia@cnb.csic.es

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Abstract
Technology based on artificial small RNAs, including artificial microRNAs (amiRNAs), exploits natural RNA silencing mechanisms to achieve silencing of endogenous genes or pathogens. This technology has been successfully employed to generate resistance against different eukaryotic viruses. However, information about viral RNA molecules effectively targeted by these small RNAs is rather conflicting, and factors contributing to the selection of virus mutants escaping the antiviral activity of virus-specific small RNAs have not been studied in detail. In this work, we transformed Nicotiana benthamiana plants with amiRNA constructs designed against the potyvirus plum pox virus (PPV), a positive-sense RNA virus, and obtained lines highly resistant to PPV infection and others showing partial resistance. These lines have allowed us to verify that amiRNA directed against genomic RNA is more efficient than amiRNA targeting its complementary strand. However, we also provide evidence that the negative-sense RNA strand is cleaved by the amiRNA-guided RNA silencing machinery. Our results show that the selection pressure posed by the amiRNA action on both viral RNA strands causes an evolutionary explosion that results in the emergence of a broad range of virus variants, which can further expand in the presence, and even in the absence, of antiviral challenges.

Keywords
amiRNA, Plum pox virus, potyviruses, PPV, RNA silencing, virus evolution, virus resistance

1 INTRODUCTION

RNA silencing, also known as RNA interference, consists of a set of mechanisms that regulate gene expression in all major eukaryotic lineages, which have in common the central role played by different populations of small RNAs (Baulcombe, 2005; Meister & Tuschl, 2004; Shabalina & Koonin, 2008). In general, these small RNAs are produced by cleavage of double-stranded or partially double-stranded RNAs by RNase III-like endonucleases, known as Dicer or Dicer-like (DCL). Then, they are loaded on Argonaute proteins and guide effector complexes, such as RNA-induced silencing complex (RISC), to their targets to repress gene expression.
RNA silencing is well known to be a key source of antiviral resistance in plants (Al-Kaff et al., 1998; Ratcliffe et al., 1997; Voinnet, 2001). Accumulation of virus-derived small interfering RNAs (siRNAs) associated with antiviral responses is a general feature of plant virus infections (Hamilton & Baulcombe, 1999; Szittya et al., 2002). Indeed, it has been demonstrated that antiviral resistance depends on cleavage of viral RNA by RISC guided by virus-specific siRNAs (Pantaleo et al., 2007). A similar antiviral mechanism has been demonstrated to operate in insects (Ding et al., 2004), and there is evidence that RNA silencing/RNA interference can also contribute to antiviral defence in mammals (Fang et al., 2021; Maillard et al., 2019; Poirier et al., 2021; Qiu et al., 2017). Engineering RNA silencing mechanisms has been a subject of numerous biotechnological studies aiming to provide antiviral protection to both plants and animals. In plants, the first and most exploited approach has been the transgene expression of long fragments of the viral genome, preferably as inverted repeats, able to induce the production of virus-specific siRNAs (Dong & Ronald, 2019; Dougherty & Parks, 1995; Simón-Mateo & García, 2011; Smith et al., 2000). This approach has yielded successful results, giving rise to useful commercial applications (Fuchs & Gonsalves, 2007; Lima Aragão, 2014; Scorzà et al., 2013; Yaling et al., 2020).

Despite the success of using the expression of long viral double-stranded RNA fragments to generate virus resistance in plants, this strategy poses some concerns about possible off-target effects and environmental risks derived from the possibility that viral sequences complement or recombine with nontarget viruses. Attempts to overcome these possible drawbacks have led to the development of systems targeting viral RNAs with only one or a few distinct small RNAs (García & Simón-Mateo, 2006). In a pioneer study, Niu et al. expressed in transgenic plants artificial microRNAs (amiRNAs) designed to target viral mRNAs by modifying the precursor of an endogenous microRNA (miRNA), miR159, and demonstrated their antiviral efficiency (Niu et al., 2006). Since then, expression of different amiRNAs, as well as other related approaches producing several virus-specific small RNAs, have demonstrated efficacy in different plants against a large variety of plant viruses (Carbonell et al., 2016; Cisneros & Carbonell, 2020).

While many amiRNAs have proven high antiviral efficiency, others did not, and only experimental testing was able to soundly distinguish one from the other (Carbonell, López et al., 2019; Kung et al., 2012). Moreover, viruses are usually able to escape the resistance posed by amiRNAs, even when this is highly effective (Lafforgue et al., 2011; Martínez et al., 2012). Viruses with single-stranded, positive-sense RNA genomes replicate through negative-sense RNA strand intermediates. Numerous studies have focused on targeting the genomic RNA of these viruses by amiRNAs. However, targeting the viral negative-sense RNA strand by RNA silencing machinery in general, and by amiRNAs in particular, has not attracted much attention. It has been suggested that viral RNA forming part of replication factories holds the negative-sense RNA strand protected from RNA silencing (Hong et al., 2021). In agreement with this hypothesis, it has been reported that the negative-sense strand of two mammalian positive-sense RNA viruses, hepatitis C virus and coxsackievirus B3, are not targeted by exogenous siRNAs (Lisowski et al., 2013; Schubert et al., 2007). By contrast, the results of Wilson and Richardson, and also those published by Simón-Mateo and García, were compatible with susceptibility to RNA silencing of hepatitis C virus (Wilson & Richardson, 2005) and plum pox virus (PPV) (Simón-Mateo & García, 2006) negative-sense RNA strands.

Plum pox virus is a plant virus species of the genus Potyvirus. In nature PPV infects trees of the genus Prunus, but it is also able to infect a wide range of experimental herbaceous hosts (Rodamilans et al., 2020). Potyviruses have a single-stranded, positive-sense RNA genome of approximately 10 kb encoding a genome-length polyprotein and a frameshift product derived from RNA polymerase slippage, which are processed by three viral proteases (Revers & García, 2015; Yang et al., 2021). In this work, we assessed the effect of the expression of different virus-specific amiRNAs on PPV infection. We searched for experimental evidence of the susceptibility of negative-sense RNA strands to RNA silencing, and compared the effectiveness of amiRNAs targeting genomic and negative-sense RNA strands. Moreover, we studied factors affecting the generation of particular virus mutants escaping amiRNA-derived resistance and the evolutionary potential of these mutants in the presence and in the absence of the selective pressure posed by the amiRNAs.

2 | RESULTS

2.1 | amiRNAs directed toward various targets in the PPV genome protect against viral infection with variable effectiveness

In a previous work of our laboratory, we used a transient agroinfiltration assay to investigate the antiviral activity of different amiRNAs constructed in the backbone of the Arabidopsis thaliana mir159 precursor (manuscript in preparation). As a result of this study, we selected three amiRNAs with efficient antiviral activity, amiR-C, amiR-D, targeting the nuclear inclusion b protein (Nlb) and coat protein (CP) coding sequences, respectively, and amiR-H, targeting the 3′ noncoding region (NCR) (Figure 1) to be expressed in transgenic Nicotiana benthamiana plants.

Plants were transformed with the single constructs pre-amiR-C, pre-amiR-D, and pre-amiR-H and with a double construct pre-amiR-C+D, cloned in the pMD132 vector. As a control, plants were also transformed with the empty vector. Five plants derived from self-fertilization of each initially transformed plant were challenged by agroinoculation with pBin-PPV-NK-GFP, an infectious PPV cDNA clone expressing green fluorescent protein (GFP) as a visual marker. Each transgenic line was initially classified as resistant when none of its plants were systemically infected, susceptible when all plants were infected, and partially resistant when some plants were infected and others were not. According to these criteria, most amiR-C, amiR-D, and amiR-C+D lines were resistant, although there were also lines partially resistant and susceptible (Table 1). By contrast, all amiR-H and V lines were susceptible (Table 1).
The genomic stability of the viral progeny of some infected plants was assessed by reverse transcription (RT)-PCR amplification and sequencing. Mutations in the amiRNA target sequence were detected in plants of the amiR-C and amiR-D partially resistant lines analysed (Figure 2). Mutations were also detected in plants of amiR-H susceptible lines, although in this case the wild-type virus

### Table 1: Response to PPV of F1 generation plants from transgenic lines of Nicotiana benthamiana transformed with different artificial microRNA constructs

| Construct  | Totala | Resistantb | Partially resistantb | Susceptibleb |
|------------|--------|------------|----------------------|--------------|
| amiR-C     | 7      | 4, 11, 12, 14 | 2 (2/5)c | 3, 8         |
| amiR-D     | 11     | 1, 4, 5, 6, 7, 8, 9, 10, 13 | 2 (1/5); 11(4/5) | 7, 14        |
| amiR-C+D   | 11     | 3, 4, 6, 9, 10, 12, 13, 16, 17 |            |              |
| amiR-H     | 7      | 1, 2, 4, 5, 6, 7, 8 |            |              |
| Vector     | 6      | 1, 2, 3, 4, 5, 6 |            |              |

*aTotal number of analysed lines.

*bLines with the indicated phenotype: Resistant, none of the inoculated plants was infected; Partially resistant, some plants were infected, but others were not; Susceptible, all inoculated plants were infected. Lines selected for analysis of the F2 generation are highlighted in bold.

*cNumber of systemically infected plants/number of inoculated plants.
continued being the predominant species (Figure 2). These results indicate that antiviral activity of amiRNAs even poses a significant selection pressure in plants unable to resist virus infection.

To assess the phenotype stability of the amiRNA-expressing lines, F₂ plants were inoculated as in the initial challenge experiment. The results, summarized in Table 2, revealed a very high stability of the different resistance phenotypes in all cases. As in F₁ infected plants, the viral progeny of most infected F₂ amiRNA-expressing plants also had mutations in the amiRNA targets, confirming the selective pressure caused by amiRNA expression.

2.2 | A wide range of mutations at the amiRNA target facilitate the escape of PPV from amiRNA antiviral action

The initial genetic analysis of the PPV populations that accumulated in plants expressing different amiRNAs was conducted by immunocapture (IC)-RT-PCR and sequencing of a fragment of the viral genome including the amiRNA target (Figure 2).

Direct sequencing of the cDNA fragments amplified by IC-RT-PCR from the leaf tissue of amiRNA-expressing plants showed cases of chromatograms with complex two-peak patterns. This could either indicate plants coinfected with wild-type virus and a viral species with several mutations or plants coinfected with multiple viral species with different mutations. To discriminate between these possibilities, the products of RT-PCR amplification were cloned in a bacterial plasmid and several clones were sequenced. Clones with wild-type sequence were not found. In two plants, clones corresponding to only one viral species were found, suggesting that this species was highly predominant (although not exclusive) in the viral population of these plants. However, in other plants the coexistence of several virus variants with up to five mutations was demonstrated. We also detected in plant D 11.2 a viral species that, in addition to the two point mutations already observed in the direct sequencing of the RT-PCR product, showed an insertion of three adenines.
insertion of three adenines (clone i, Figure 2). Sequence analysis of the individual clones showed that most of the viral species escaping the antiviral activity of amiRNAs and capable of providing strong resistance had two or three mutations, suggesting that a single mismatch is not enough to efficiently prevent the action of these amiRNAs.

Considering as a whole the viral progenies of the different transgenic plants that express any PPV-specific amiRNA, the first thing that stands out is the wide variety of mutations detected. While no mutations were observed outside the predicted amiRNA targets, new variants were found with nucleotide changes in 15 of their 21 amiRNA positions (Figure 2 and Table 3). However, these mutations were not homogenously distributed. They were more abundant in the central region, where they would be expected to affect cleavage by RISC, and in the 3’ terminal region, the most relevant for recognition of the predicted mature amiRNA strand (Axtell, 2013). But there were also mutations located between positions 1 and 8, expected to be mainly relevant for the recognition of the negative-sense strand of viral RNA by the amiRNA star strand.

The reasoning behind the design of the C+D construct was the presumption that the virus would have more difficulty to escape from its action because it would need to introduce mutations simultaneously in both amiRNA targets, C and D. Despite this, in some plants PPV did in fact escape from the weak resistance of the C+D 7 line by mutations in one or both of the two targets (Figure 2).

### 2.3 | Transgenic N. benthamiana amiR-Di plants, designed to produce a mature amiRNA strand directed against the viral negative-sense RNA strand, show less resistance to PPV than amiR-D plants

To gain insight into the antiviral effectiveness of amiRNA strands directed against the negative-sense RNA strand, N. benthamiana plants were transformed with the construct pre-amiR-Di. This construct encodes a pre-miRNA assembled in the backbone of pre-miR159 that includes the same PPV sequence as amiR-D, but inversely inserted, so that the predicted mature and star amiRNA strands are complementary to the negative-sense and genomic viral RNAs, respectively (Figure 3).

Four plants derived from self-fertilization of each initially transformed plant were challenged by agroinoculation with pBin-PPV-NK-GFP. As a control of resistance, plants belonging to three amiR-D lines that had shown different degrees of protection against PPV in the previous experiment were also inoculated. Plants transformed with the empty vector were used as a positive control of infection (Table 4). The amiR-D lines confirmed their high level of anti-PPV resistance. By contrast, all amiR-Di plants developed systemic infection, although generally with some delay compared to the control plants. These results provide clear evidence that expression of amiR-D from the standard pre-miRNA is much more effective at suppressing PPV infection than expression of amiR-Di from the inverted construct.

### 2.4 | Predicted mature amiRNAs accumulate in greater abundance in amiR-D and amiR-Di transgenic plants

To verify the predominant accumulation of predicted amiR-D and amiR-Di strands and to compare the efficacy of amiR-Di and amiR-D transgene expression, the levels of accumulation of both amiRNA strands in two transgenic lines of each construct were assessed by northern blot analysis (Figure 3c).

We found that plants of the highly resistant amiR-D 7 line accumulated a small, but clearly detectable, amount of its mature strand, complementary to the viral genomic RNA, whereas the star strand was undetectable by this technique, as were also both amiRNA strands in plants of the low-resistance amiR-D 11 line. Analysis of the two amiR-Di lines (1 and 6) showed that the predicted mature amiRNA, complementary to the negative-sense strand of the viral RNA, was indeed the strand most accumulated, whereas the predicted star strand was not detected.
Although limitations of the assay did not allow for an exact quantification, the densitometry of the amiRNA signals and of known amounts of oligodeoxynucleotides loaded as control indicated that amiR-Di plants accumulated a greater amount of the mature strand (same polarity as viral genomic RNA) than the mature strand in the amiR-D plants (complementary to viral genomic RNA) with a higher expression level. These data indicate not only that the proportion of amiRNA strands directed to the genomic and antigenomic viral RNAs had been successfully flipped with the new transgene, but also that levels of amiRNA accumulation in amiR-Di plants were even higher than those conferring strong resistance against PPV in amiR-D plants. Thus, the lack of effective resistance of amiR-Di plants could suggest that the negative-sense strand of viral RNA is not accessible to amiRNA-directed RNA silencing or that the cleavage of this strand does not have a significant antiviral effect.

### Table 3: Frequency of mutations at different positions of PPV-specific amiRNA target

| amiRNA | N* | amiRNA targets (genomic RNA sequence) |
|--------|----|---------------------------------------|
| C      |    | A A A A A G A U G G A A A A G C C C C G U A |
| D      |    | A U G A A A A A G C A U A C A U G C C A A |
| H      |    | C C U G G G U G A G A G U C U A A U C A U |

| Position in the amiRNA target | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|-------------------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|====|
| C                             | 6 | 1 | 1 | 1 | 3 | 1 | 1 | 1 | 3 | 3 |
| C+D**                        | 6 | 2 | 3 | 1 | 4 | 3 |
| D                             | 10 | 2 | 3 | 1 | 4 | 3 |
| D+D**                       | 6 | 2 | 2 |
| H                             | 3 | 1 | 6 |

*a Number of different viral species detected in transgenic plants expressing this amiRNA shown in Figure 2.

*b Number of plants expressing this amiRNA in which mutations at this position were detected, as shown in Figure 2; the grey intensity reflects the frequency of mutations at this position.

2.5 | **amiR-Di expression has an antiviral effect that promotes the emergence of PPV variants with mutations in the amiRNA target**

Despite amiRNA expression not providing effective resistance against PPV in amiR-Di plants, the delayed progression of infection in these transgenic plants could reflect a weak antiviral activity, able to provoke the emergence of escape mutants. This possibility was tested by IC-RT-PCR amplification and sequencing of a genome fragment including the amiR-D/amiR-Di target of the viral progeny of some of these plants.

The sequencing chromatograms showed multiple double, and even triple, peaks in the amiRNA target sequence, suggesting the accumulation of several viral species (data not shown). To determine the sequence of individual viral species coexisting in each plant, in this case, instead of cloning the RT-PCR products in bacterial plasmids as we did in the analysis of viral progenies of amiR-D plants, we used the MiSeq technique for high-throughput amplicon sequencing (Figures 4 and 5).

Around 100,000 specific reads were obtained for each sample. In plants transformed with the empty vector used as a control, approximately 90% of the reads corresponded to the wild-type virus sequence (Figure 4b); the rest of reads represented multiple low-represented (often only one time) sequence variants, each with point mutations distributed along the complete amplicon, without preferences for the amiRNA target. Without a more detailed study, we could not discriminate whether these minority sequences corresponded to background noise or they reflected the quasispecies structure of the viral population.

Reads of the remaining samples revealed a complex composition of the viral populations in all amiR-Di plants analysed. The wild-type virus was always a minority and two to five viral species were represented in more than 4%. Moreover, quite a few more species were detected with a minor representation, but clearly above the background noise, and concentrated on the amiRNA target (Figure 4). Unlike the abundance of species with more than one mutation found in the viral populations of plants expressing amiR-C and amiR-D (Figure 2), the majority species accumulating in any of the amiR-Di plants had a single mutation. This result agrees with the lower level of resistance raised by the amiR-Di construct, allowing viruses with single mutations to escape. We also included two F₂ plants descendant of plant D11.4 in this experiment (Figure 4b). Apparently, the resistance of these plants was weaker than in other amiR-D plants, because in one of them the wild-type virus was largely predominant, and in the other one, although minority species with double mutations were detected, the two majority species showed single mutations.

Because amiRNA activity is mainly conditioned by the interaction of its 5′ region with the 3′ region of the target, mutations facilitating the escape from the antiviral action in amiR-D and amiR-Di plants would be expected to be very different. However, patterns of
changes observed in the two types of plants were very similar, with most affecting the central region of the amiRNA targets (Figure 5). A notable difference is that, whereas mutations in the 3′ region of the amiRNA target in the genomic RNA (that is predicted as relevant for recognition by the abundant amiR-D mature strand) were selected in some amiR-D plants, this type of mutation was never observed in viral progenies of amiR-Di plants. On the other hand, the number of mutations affecting the 3′ region of the amiRNA target in the negative-sense strand of viral RNA (the 5′ one in the genomic RNA) was similar in amiR-D and amiR-Di plants, despite an expected greater relevance of this region for resistance in amiR-Di plants (in which the amiRNA that recognizes the viral negative-sense strand is the highly abundant mature amiR-Di) than in amiR-D plants (in which the viral negative-sense strand would be targeted by the minority amiR-D star strand).

Alignment of the detected mutations showed that the most frequently mutated residues in the amiR-D and amiR-Di plants, G9300 and A9299, respectively, are displaced by one nucleotide (Figure 5). Bearing in mind that the sites expected for the cleavages directed by amiR-D and amiR-Di are also displaced by one nucleotide (Figure 3b), position 9 of the target in genomic RNA (position 15 in the negative-sense strand) would appear to be especially relevant for the activity

**FIGURE 3** Reversal of the amiR-D duplex to build the amiR-Di construct. (a) Predicted effects of the two chains of amiR-D and amiR-Di on the two strands of PPV RNA. (b) Schematic representation of the precursors of amiR-D and amiR-Di built using pre-miR159 of Arabidopsis thaliana as backbone. The expected amiRNA targets in the genomic RNA and in the negative-sense RNA strand numbered according to Lain et al. (1989) are shown in the lower part of the panel. Black and empty arrowheads signal the predicted cleavage sites of RISC loaded with amiR-D or amiR-Di, respectively. amiRNA and viral RNA sequences with the polarity of the genomic RNA are highlighted in dark blue, and the sequences of the opposite polarity are shown in light blue. amiRNA nucleotides that do not match with the viral sequence are shown in red. (c) Accumulation of amiR-D and amiR-Di in different Nicotiana benthamiana transgenic lines. Samples of small RNAs from young leaves of F₁ generation plants (lines Di) or of F₂ generation plants descendant of the indicated F₁ plants (lines D) were subjected to northern blot analysis. Two biological replicas of amiR-D and amiR-Di plants, each composed of two plants, were separately analysed in two gels. As negative control, a sample of plants transformed with the empty vector (V) was also included in the analysis. Known amounts of 21-nucleotide (nt) oligodeoxynucleotides with the sequence of mature amiR-D and amiR-Di (#3222 and #3218, respectively) and its star strands (#3224 and #3220, respectively) mixed with a sample of small RNAs of wild-type N. benthamiana were loaded as calibration standards. The membranes were sequentially incubated with [32P]-labelled 19-nt oligodeoxynucleotides #3250 (upper panels) and #1935 (middle panels), with an intermediate stripping step, to detect the indicated amiRNA strands. In the lower panel, the gel stained with ethidium bromide shows tRNAs and 5S rRNA as loading control.
of amiR-D and amiR-Di, regardless of which nucleotide that occupies this position.

However, it is not only the position in the target that defines the preferred mutations to facilitate escape from amiR-D and amiR-Di actions. In both the viral progenies of amiR-D and amiR-Di plants, transitions emerged more frequently than transversions (Figure 5). However, the example that best illustrates limitations to viral mutational capacity for protection against amiRNA antiviral activity is the conservation of A9297 in the viral populations of all the amiR-D- and amiR-Di-expressing plants analysed (Figure 2). The simplest explanation for this observation is that the triplet of which A9297 is part (AAA) (Figure 2) encodes a lysine, and its replacement by other nucleotides results in a stop codon or codons encoding glutamic acid or glutamine, whose protein product we guess would not be functional.

### Table 4 Susceptibility to PPV infection of transgenic lines expressing amiR-D or amiR-Di

| Systemic infection | |
|-------------------|---|
| Di 1<sup>a</sup> | 4/4<sup>b</sup> (9 dpi) |
| Di 2 | 1/4 (8 dpi), 1/4 (9 dpi), 2/4 (11 dpi) |
| Di 3 | 4/4 (9 dpi) |
| Di 5 | 3/4 (9 dpi), 1/4 (11 dpi) |
| Di 6 | 3/4 (8 dpi), 1/4 (9 dpi) |
| D 2.6<sup>c</sup> | 0/4 |
| D 7.3 | 0/4 |
| D 11.4 | 1/5 (8 dpi), 2/5 (9 dpi) |
| V 1.6<sup>d</sup> | 4/4 (8 dpi) |

<sup>a</sup> amiR-Di transgenic lines whose F<sub>1</sub> generation plants were analysed.  
<sup>b</sup> Number of plants that started showing green fluorescent protein (GFP) expression at the indicated time (days postinoculation, dpi)/number of inoculated plants.  
<sup>c</sup> amiR-D plants whose F<sub>2</sub> generation descendants were analysed.  
<sup>d</sup> Plant transformed with the empty vector whose F<sub>2</sub> generation descendants were analysed.

The above results show that PPV infection of amiRNA-expressing plants with weak antiviral resistance provokes the emergence of multiple viral species, which in many cases coexist in the same plant. However, the example that best illustrates limitations to viral mutational capacity for protection against amiRNA antiviral activity is the conservation of A9297 in the viral populations of all the amiR-D- and amiR-Di-expressing plants analysed (Figure 2). The simplest explanation for this observation is that the triplet of which A9297 is part (AAA) (Figure 2) encodes a lysine, and its replacement by other nucleotides results in a stop codon or codons encoding glutamic acid or glutamine, whose protein product we guess would not be functional.

### 2.6 The negative-sense strand of PPV RNA is cleaved in transgenic plants expressing amiR-Di on the expected site for RISC processing directed by this amiRNA

The conjunction that (a) both amiR-D and amiR-Di have antiviral activity, although that of amiR-D is more powerful; (b) in viral progenies of plants that express amiR-D and amiR-Di, mutations arise in similar positions at the central and 5′ (genomic orientation) regions of the target; and (c) mutations in the 3′ region (genomic orientation) were only observed in viral progenies of amiR-D plants suggests that, although targeting the genomic strand is more effective, both the genomic and the negative-sense strands are accessible to the action of the amiRNAs. To obtain more direct evidence for amiRNA activity on the negative-sense strand of PPV RNA, we conducted a rapid amplification of 5′ cDNA ends (5′-RACE) experiment programmed to identify cleavage sites in this viral RNA.

For this analysis we chose the Di 6.3-infected plant, which, according to the MiSeq data, accumulated a significant amount of wild-type virus, providing enough perfectly complementary target RNA, coexisting with mutated viral species, revealing effective selective pressure of the amiRNA (Figure 4a). To maximize the amount of viral RNA without mutations in the amiRNA target, for the 5′-RACE analysis we used agroinfiltrated leaves collected at 9 days postinoculation. Although this was a relatively early time point, the infection was already well established, as revealed by the fluorescence of the GFP marker. As a negative control we used the tissue of an infected plant transformed with the empty vector, collected in equivalent conditions.

The first step of the 5′-RACE technique involves ligation of an adapter to the 5′ end of the RNA fragments accumulated in the analysed plant. Next, cDNA is synthesized with a primer complementary to the negative-sense strand of the viral RNA, and a DNA fragment is amplified by PCR using primers that hybridize with the adapter and an RNA sequence close to the target of the amiR-Di mature strand (Figure S2). Agarose gel electrophoresis of the plant amiR-Di 6.3 PCR product showed a major band with the mobility expected for a fragment derived from the cleavage of amiR-Di-loaded RISC in the negative-sense strand of viral RNA (Figure 6a). A similar band was not observed in the sample of the control plant.

To precisely determine the 5′ ends of the amplified viral RNA fragments, PCR products of expected size were purified from the agarose gel and cloned in a bacterial plasmid, and the sequence of several clones was determined by Sanger sequencing. In nine of the 11 clones from sample Di 6.3 analysed, the adapter was ligated to the sequence corresponding to the cleavage site in the negative-sense strand of the viral RNA predicted for RISC loaded with mature amiR-Di (Figure 6b). In four of these nine clones we detected a nucleotide change in the amiRNA target corresponding to a G9300A mutation in the viral genome, showing that a perfect match in the central region of the complementarity region is not essential for cleavage. As expected, the sequence of clones of the control sample identified different 5′ ends located outside the amiRNA target. These results imply that the antigenomic RNA strand of PPV can be the target of an amiRNA. In addition, the precise location of the major cutting site in the predicted position confirms the correct processing of the pre-amiR-Di to give rise to the expected amiRNA duplex.

### 2.7 Mutations selected under the selective pressure of amiRNA can be maintained in wild-type plants

The above results show that PPV infection of amiRNA-expressing plants with weak antiviral resistance provokes the emergence of multiple viral species, which in many cases coexist in the same
plant. But it is important to know the genetic stability and competitiveness of these viral species when propagated in the same environment in which they were generated and also in different environments. With this aim, the viral progeny that emerged under the weak selective pressure of amiR-Di was propagated in different plants and the genetic composition of the new resulting viral populations was characterized by MiSeq high-throughput sequencing.

The study was performed with the highly complex viral population generated in plant Di 6.3 (Figure 4a). As a first approach, four N. benthamiana plants transformed with the empty vector (V plants) were inoculated with a leaf extract of plant Di 6.3, and the viral progenies of the newly infected plants were analysed by MiSeq sequencing. In none of the V plants the number of reads corresponding to the wild-type virus was higher than in the parental
amiR-Di-expressing plant (Figure 7). This implies that at least one of the mutations imposed under the amiRNA selective pressure does not cause a significant decrease in fitness in the absence of this pressure. The composition of the viral populations of the four V plants inoculated with the Di 6.3 extract differed markedly from that of this plant and among them (Figure 7). A decrease in population
complexity was observed in all V plants compared to the plant used as inoculum. However, considering the viral population of all the plants together, the explosion in variability generated by the antiviral action of amiR-Di was not significantly reduced after propagation in the absence of selective pressure.

Focusing on more specific aspects, the most abundant species in the parental Di 6.3 population (mutation G9294A) only significantly accumulated in one of the four plants expressing amiRNA, and in that plant it was a minority species (Figure 7). This suggests that the G9294A mutation, which causes a substitution of glutamic acid with lysine (Figure 2), very effectively enables the virus to escape the amiR-Di antiviral action but at some cost to fitness.

Similarly, the second most abundant species in parental plant Di 6.3 (mutation A9299G), which was by far the majority in the amiR-Di plants infected with wild-type PPV taken together (Figure 4a), was not the predominant species in any of the four V plants inoculated with extract from the Di 6.3 plant (Figure 7). This could therefore constitute another example of a species well adapted to propagate under the selective pressure of amiR-Di, but whose competence is reduced in the absence of this pressure. Surprisingly, in this case the A9299G mutation is silent.

The predominant species in three of the four Di 6.3-inoculated V plants, which has mutation G9300U, was only the fourth most abundant species in the Di 6.3 parental plant (Figure 7). This viral species was not represented by more than 12.5% of the MiSeq reads in any of the amiR-Di plants inoculated with wild-type PPV in the former experiment (Figure 4a). Therefore, it could be concluded that the G9300U mutation may not be as effective as other mutations at protecting the virus from the antiviral activity of amiR-Di. However, the alanine-to-serine substitution that it provokes would not have a negative effect on multiplication of the virus when the amiRNA is no longer present, or this could even be positive.

### 2.8 The complexity of viral populations generated in transgenic plants expressing an amiRNA may increase when propagating in the same transgenic line

To assess how the viral population selected to escape from the antiviral activity of an amiRNA evolves when propagated under the same selective pressure, six plants of the F1 generation of the amiR-Di 6 line were inoculated with a leaf extract from the Di 6.3 plant (Figure 7). This could therefore constitute another example of a species well adapted to propagate under the selective pressure of amiR-Di, but whose competence is reduced in the absence of this pressure. Surprisingly, in this case the A9299G mutation is silent.

The predominant species in three of the four Di 6.3-inoculated V plants, which has mutation G9300U, was only the fourth most abundant species in the Di 6.3 parental plant (Figure 7). This viral species was not represented by more than 12.5% of the MiSeq reads in any of the amiR-Di plants inoculated with wild-type PPV in the former experiment (Figure 4a). Therefore, it could be concluded that the G9300U mutation may not be as effective as other mutations at protecting the virus from the antiviral activity of amiR-Di. However, the alanine-to-serine substitution that it provokes would not have a negative effect on multiplication of the virus when the amiRNA is no longer present, or this could even be positive.
populations of all newly infected plants than in the population from the plant used as inoculum. This is especially striking in the plants Di 6.7 and Di 6.10, in which the vast majority of reads corresponded to a single mutated viral species. Even so, some plants retained a wide variety of viral species.

In contrast to what was observed in the passage to plants that do not express amiRNAs, in which A9299G and, especially, G9294A mutations lost some relevance (see above), species with one of these mutations were the most abundant in four of the six amiR-Di 6 plants infected with the Di 6.3 viral population (Figure 7). This result is in agreement with the conclusion mentioned above that these mutations are highly effective at facilitating the escape from amiR-Di activity, although probably with some fitness cost. The almost exclusive presence in plant Di 6.10 of the viral species with the G9300U mutation, the most prevalent in plants that do not express amiRNAs, suggests that the better fitness of this viral species could also be relevant in the competition that takes place under the selective pressure of amiR-Di. On the other hand, the most represented species in the Di 6.5 plant was the one with the mutation A9302G, a species that was only the fifth most abundant in the Di 6.3 inoculum. The strong enrichment of this viral species in the Di 6.5 plant, while undetectable in the other three plants infected with the same inoculum, suggests a significant contribution of genetic drift in the evolution of the viral population generated under the selective pressure imposed by amiRNA expression.

As in the viral populations of plants from the initially infected amiR-Di lines, in those generated by the passage between amiR-Di 6 plants the viral species with a single mutation continued being a majority (Figures 4 and 7). However, in this case some species with two mutations accumulated at very high levels (Figure 7). This observation implies that although a single mutation is sufficient to escape from the weak antiviral action of amiR-Di, a second mutation in the amiRNA target facilitates, as in the case of amiR-D, the infection.

Altogether, the results suggest that selective pressures to escape from amiRNA activity and to preserve the function of the affected protein together with genetic drift favour an increase in the diversity generated in plants expressing amiRNAs at a global level over successive passages, although the complexity of the viral population of each individual plant can be reduced.

3 | DISCUSSION

The first evidence that opened up the possibility of using RNA silencing machinery to block the expression of plant genes with high specificity by means of amiRNAs dates back to 2006 (Alvarez et al., 2006; Schwab et al., 2006). In the same year, Niu et al. (2006) demonstrated that the expression of amiRNAs could be employed to interfere with viral infections. Subsequently, multiple works on different combinations of viruses and host plants have demonstrated the wide applicability of this antiviral strategy (for a review, Cisneros & Carbonell, 2020). However, not all amiRNAs directed against viral targets are able to efficiently block viral multiplication, and the properties that confer efficacy to antiviral amiRNAs are largely unknown. Moreover, the evolutionary outcome of the selective pressure imposed by antiviral amiRNAs has been barely explored. In this work, an analysis of the response to PPV of several N. benthamiana transgenic lines expressing PPV-specific amiRNAs has unveiled the relative effectiveness of targeting amiRNAs to viral genomic and negative-sense RNA strands and allowed us to characterize factors that condition the emergence of a broad range of mutants escaping amiRNA-mediated antiviral resistance. Our results also illustrate how the evolutionary explosion initiated in amiRNA-expressing plants can progress even in the absence of amiRNA antiviral challenges.

In concordance with previous reports, we observed notable differences in the susceptibility to PPV among the different amiRNA-expressing transgenic lines, even among lines transformed with the same amiRNA construct. The quantification of amiRNA accumulation in two amiR-D lines showed a positive correlation between amiRNA expression levels and the degree of resistance (Figure 3c), which agrees with data described in other works (Carbonell, Lison, et al., 2019; Kung et al., 2012; Niu et al., 2006; Qu et al., 2007). However, differences in amiRNA accumulation cannot explain the low antiviral efficiency of amiR-H, because this amiRNA accumulates in transgenic plants at levels comparable to those of amiR-C and amiR-D (Figure S3). We could think that amiR-H is less efficient than amiR-C and amiR-D because the former targets a noncoding region, while the others target RNA sequences encoding essential proteins. The fact that the genomic RNA of potyviruses is a poly-cistronic mRNA, meaning that the effect of its processing by RISC should be similar regardless of the cleavage point, argues against this possibility. An attractive hypothesis is that the reduced activity of amiR-H could be a consequence of the low accessibility of the target to RISC, as suggested previously for artificial small RNAs targeting human immunodeficiency virus (Westerhout et al., 2005) and cucumber mosaic virus (Duan et al., 2008). In this regard, we would like to emphasize that a structured region shown to be involved in RNA replication includes part of the 3′ NCR of the RNA of another potyvirus, tobacco etch virus (Haldeman-Cahill et al., 1998). This structure, also probably present in PPV RNA, but not yet delimited, could obstruct the access of RISC to amiRNA targets located in the 3′ NCR such as that of amiR-H.

A method suggested to improve the efficiency and durability of the resistance mediated by amiRNAs consists of the coexpression of several molecules with different targets in viral RNA (Carbonell, Lison, et al., 2019; Fahim et al., 2012; Kis et al., 2016; Kung et al., 2012; Lafforgue et al., 2013). In our study, we coexpressed amiR-C and amiR-D from the amiR-C+D transgene. The high level of resistance of many lines expressing amiR-C or amiR-D alone, and the small number of plants of each line analysed, prevented us from drawing definitive conclusions. Even so, our results suggest that escaping from amiR-C+D activity could be somewhat more difficult than escaping from amiR-C or amiR-D alone, given that, although in the analysis of the F1 generation the percentages of amiR-C, amiR-D, and amiR-C+D lines showing strong resistance were similar (Table 1), in the analysis of the F2 generation of some of the lines defined as
immune in the first assay (one amiR-C, three amiR-D, and four amiR-C+D lines), no escapes were observed in any of the four amiR-C+D lines, while two plants from the amiR-C line and one plant from one of the three amiR-D lines were infected by viral species with mutations in the amiRNA target (Table 2).

In general, it is assumed that although miRNAs are produced as a duplex, only one of them, the one named the mature strand, is functional. However, some studies show that the star strand (also called the passenger strand) of some miRNAs can also have relevant activity (Devers et al., 2011; Guo & Lu, 2010; Manavela et al., 2013; Takeda et al., 2008; Zhang et al., 2011). For this reason, the design of amiRNAs aimed at silencing cellular mRNAs is concerned with avoiding potential off-target effects of the star strands (Cisneros & Carbonell, 2020; Fahlgren et al., 2016). In the case of amiRNAs designed to provide resistance against positive-sense RNA viruses, the main objective has been to target the genomic RNA by the amiRNA mature strand, ignoring the possibility that the amiRNA star strand could attack the viral negative-sense RNA strand (Cisneros & Carbonell, 2020). Whereas in two studies on positive-sense animal RNA viruses (Lisowski et al., 2013; Schubert et al., 2007), treatment with siRNAs targeting antigenomic RNA did not exert a significant antiviral effect, a previous study of Wilson and Richardson (2005) showed signs of cutting on the negative-sense RNA strand of hepatitis C virus as a result of the administration of appropriate small RNAs. Moreover, results from our laboratory have shown that a natural miRNA caused selective pressure on a recombinant PPV whose negative-sense RNA included the target of this miRNA (Simón-Mateo & García, 2006). To compare the effects of amiRNAs targeting genomic RNA and its complementary strand, we analysed transgenic plants expressing amiR-D and amiR-Di, which differed in the orientation in which the same viral sequence was inserted in the backbone of pre-miR159 (Figure 3a,b). For both artificial constructs, the predicted mature strand was accumulated with ample preference (Figure 3c). Plants expressing amiR-D, whose mature strand is complementary to viral genomic RNA, were much more resistant to PPV than amiR-Di plants, which produce a mature amiRNA of opposite polarity (Tables 1 and 4). This clearly indicates that guiding RISC to genomic RNA is more effective at promoting antiviral resistance than guiding it to the negative-sense RNA strand. However, the existence of a selective pressure associated with amiR-Di expression suggests that targeting the antigenomic RNA could also have antiviral activity.

For plant miRNAs, the amiRNA region whose pairing with the target RNA is most relevant for its activity encompasses nucleotides 2 to 13 (9 to 20 of the target sequence) (Axtell, 2013). However, a report of Mallory et al. showed that disturbing pairing in the central region of the target had a much lower effect than disturbing pairing in the region closer to the miRNA 5’ end (Mallory et al., 2004). Therefore, it is striking that the positions preferred by PPV to escape the action of both amiR-D and amiR-Di, as well as those of amiR-C and amiR-H, are central (Figures 2, 4, and 5 and Table 3). The selection of mutations in positions of the 3’ region of amiR-C and amiR-D, even outside the extended seed region 2–13 defined by Axtell (2013) (Figures 2 and 5 and Table 3), is also surprising. These latter mutations would not be expected to affect the activity of the mature amiRNAs on the genomic RNA, but they could interfere with that of the star strand on the negative-sense strand of the viral RNA. Thus, the selection of mutations in the 3’ region of the target could imply a contribution of the activity of the star strand to the resistance conferred by the amiRNA.

Of note, while escape mutants of turnip mosaic virus (TuMV) selected in plants expressing a virus-specific amiRNA also accumulated mutations preferentially in the central region of the target (Lafforgue et al., 2011), mutations selected in recombinant variants of PPV that included targets of endogenous miRNAs in the genomic RNA or in its complementary strand were mainly located in positions faced with the 5’ region of the miRNA (Simón-Mateo & García, 2006), that is, the most relevant region for silencing according to Mallory et al. (2004).

A plausible explanation for all these results is that in the case of amiRNAs, although the accumulation of the star strand is very low (not detected by northern blot for amiR-D and amiR-Di, Figure 3), it can still contribute to antiviral resistance. In this scenario, mutations in the central region of the target, although possibly not optimal to disturb the action of mature amiRNA, would have the advantage of also interfering with the star strand activity. By contrast, most endogenous miRNAs would have been selected to have extremely unstable star strands aiming to minimize off-target effects; for this reason, in the case of PPV recombinants, the selected mutation would be the most appropriate to simply prevent the activity of endogenous mature miRNA.

This model assumes that antigenomic viral RNA is accessible to the machinery of miRNA-mediated silencing. The symmetric location of the mutations selected in PPV recombinants with miRNA targets inserted in the positive- or in the negative-sense strand of viral RNA (Simón-Mateo & García, 2006) strongly supports this assumption. The definitive demonstration that the negative-sense strand of viral RNA is accessible to miRNA-guided RISC was provided by the detection by RACE of specific cleavages at the expected site in the target of amiR-Di in plants expressing this amiRNA (Figure 6). However, the much weaker resistance of plants expressing amiR-Di compared to those expressing amiR-D (discussed above) suggests that the processing of the negative-sense RNA strand is much less efficient than that of the genomic RNA. This would justify the apparent inefficiency of siRNAs on antigenomic RNA that has been described for animal RNA viruses (Lisowski et al., 2013; Schubert et al., 2007).

The selection of mutations that facilitate the escape of resistance caused by amiRNAs is affected by other factors apart from the contribution they make to weakening the interaction with the amiRNA. The ease with which a mutation is generated and potential negative trade-off effects on viral replication also influence its potential success. Therefore, it is not surprising that in the spectrum of mutations found in PPV species that escaped the action of the different amiRNAs, transitions strongly predominated (Figures 2 and 4), because this type of mutation occurs more easily than transversions (Denver et al., 2004). Similar preference of transitions over transversions was detected, and discussed in depth, by Lafforgue et al. in TuMV mutants escaping amiRNA-provided resistance (Lafforgue
It is important to note that the more disturbing effect of transversions on the miRNA-target RNA interaction should increase the contribution of these mutations to the ability of the virus to escape; however, the aforementioned greater prevalence of transitions among escape mutants suggests that this factor is not too relevant.

It is also clear that inclusion of two mutations is more difficult than incorporating a single one. Moreover, two mutations are more likely to disturb viral replication than one. Accumulation of several mutations would therefore only be expected to take place when a single mismatch is not enough to prevent the amiRNA action. This does not always seem to be the case, as Laforgue et al. mainly found viral species with a single mutation in the TuMV progeny of plants expressing amiR159-HCP0 (Lafforgue et al., 2011). We also found the same in the PPV progeny of plants that express amiR-Di (Figure 4). However, one mutation does not appear to be enough for an optimal escape, as deduced by the frequent accumulation of PPV species with two mutations in the initial infection of plants expressing amiR-D (Figure 2), and after one passage in plants that express amiR-Di (Figure 7).

The comparison of viral species selected in amiR-C- and amiR-D-expressing plants illustrates how restrictions for escaping due to side effects of the escape mutations depend on the amiRNA target. Mutations that do not change the amino acid sequence predominated among the PPV species that escaped from amiRNA activity (Figure 2). By contrast, we did not observe a preference for silent mutations in the PPV species accumulating in amiR-D-expressing plants (Figure 2). These results suggest that the sequence of the Nib protein encoded by the amiR-C target has more functional restrictions than that of the CP encoded by the amiR-D target. This does not mean that all the residues that amiR-D targets can be mutated without side effects, as clearly shown by the apparently extreme wrestling of mutations of A9297, which would introduce a stop codon or a change from lysine to glutamine or glutamic acid in the CP (Figure 5). In any case, all our data together support the view that, as a consequence of the wide range of possible mutations in the amiRNA target capable of facilitating viral evasion, restrictions in changes caused by functional requirements of the virus at the RNA or protein level have little effect on the durability of amiRNA-mediated resistance.

We paid special attention to how viral species selected in plants expressing antiviral amiRNAs can evolve in wild-type plants and in plants that maintain the same selective pressure that promoted their emergence. Assuming a decrease in fitness associated with the mutations introduced to escape the action of amiR-Di, it is expected that a small percentage of original PPVs present in the viral progeny of plant Di 6.3 (Figures 4 and 7) would exhibit increased proliferation in wild-type plants. On the contrary, wild-type PPV continued being a minority in all wild-type N. benthamiana plants inoculated with leaf extract of plant Di 6.3 (Figure 7), which demonstrates a high fitness of at least some of the escape mutants. This does not mean that all mutations were neutral. The majority species in the viral progeny of plant Di 6.3 (mutation G9294A) almost disappeared from the four wild-type plants inoculated with that viral population (Figure 7). Conversely, the virus species with the mutation G9300U that was not predominant in the initial inoculum was the most abundant in three of these plants (Figure 7). This suggests that natural selection plays an important role in the evolution of the Di 6.3 viral population in wild-type plants, favouring accumulation of the best-fitted viral species. On the other hand, the fact that the virus with the mutation G9300A, which is a minority or absent in three of the wild-type plants, was the most abundant species in another (Figure 7) suggests that genetic drift contributes to the evolutionary dispersion of viral species emerging as a response to the antiviral activity of amiRNAs.

The result of the passage of a viral population selected in plants that express an amiRNA to other plants of the same transgenic line suggests that the virus continues evolving in this second infection to more efficiently avoid the selective pressure of the amiRNA (Figure 7). This adaptation can involve the introduction of a second mutation, as is the case with some highly abundant viral species detected after the viral population that arose in plant Di 6.3 evolved in the other Di 6 plants. In other cases, the initial predominant species was replaced after the passage by another that was only a minority in the first infected plant. The majority accumulation of the same viral species (mutation A9299G) in three of the six Di 6 plants inoculated with leaf extract of the infected plant Di 6.3 is solid evidence that natural selection forces are contributing to shape the viral population that emerged under the amiRNA action. As mentioned above, these forces combine selective pressures derived from the antiviral activity of the amiRNA and from the possible deleterious effects caused by the mutations that allow the virus to escape. However, the fact that the mutation preferentially selected in Di 6 plants (A9299G) is different from that preferred in wild-type plants (G9300U) firmly suggests that escaping amiRNA activity is the main selective force in amiRNA-expressing plants. On the other hand, the virus with the A9299G mutation was not the species that most accumulated in three Di 6 plants inoculated with the Di 6.3 extract. In each of these plants one viral species with a different mutation (G9294A, G9300U, and A9302G) accumulated with a very high pre-eminence (Figure 7). This finding provides further evidence for a contribution of genetic drift to the evolution of the viral species that overcome amiRNA-derived resistance. In the viral passage, as inoculum we used a raw extract of systematically infected leaves, with a very high absolute viral population size, which should favour competition over genetic drift as the evolutionary force. However, the probable contribution of genetic drift that we found could be justified by the results of several evolutionary studies that suggest that effective population sizes are much lower than the absolute sizes as a result of the existence of bottlenecks (García-Arenal et al., 2003; Zwart et al., 2011).

On the whole, all these results show that, both in wild-type plants and in those expressing the amiRNA, natural selection produces a decrease in the intraplant complexity of the population of escape mutants initially generated. However, genetic drift stochastically favours different mutants in different plants. The final result is that, after escaping resistance, the wild-type viral species (or quasispecies) is not replaced by a single virulent mutant, but by a large set of different virulent species (or quasispecies). It is reasonable to postulate that this multiplication of genetic resources could help the virus to adapt better to different hosts and environments.
4 | EXPERIMENTAL PROCEDURES

4.1 | Plants

Seeds of wild-type *N. benthamiana* were germinated on sterile vermiculite. Seeds of transgenic *N. benthamiana* were germinated in vitro in Murashige and Skoog (MS) medium containing 1% sucrose and 40 mg/L hygromycin. Seedlings of approximately 1 cm were transferred to soil substrate mixed with vermiculite (3:1) and cultured in a greenhouse maintained at 16 h light with supplementary illumination and at 19–23°C.

4.2 | Bacteria and plasmids

*Escherichia coli* DH5α was used for cloning and plasmid purification. For leaf infiltration and plant transformation, we made use of *Agrobacterium tumefaciens* C58C1 (pTiB653).

The amiRNA precursors (pre-amiRNAs) used in this work had as backbone the pre-miRNA159 of *A. thaliana* (Atpre-miRNA159), of 273 nucleotides (nt), which include a partially double-stranded stem of 21 nt formed by pairing of the mature miRNA and its star strand, with two unpaired nucleotides (Figure S1). They were obtained by replacing the duplex with PPV-derived 21-nt complementary sequences (Figure S1). The sequence of the duplex located in the 3′-terminal region of the pre-miRNA (which is expected to constitute the mature amiRNA) was exactly complementary to the target RNA, while the two 3′-terminal nucleotides of the 5′-proximal sequence could not be paired with the viral RNA because the two 3′-terminal residues of miR159* were maintained to facilitate the correct processing of the pre-amiRNA (Figure S1).

pGEM-miR159, which contains the cDNA sequence of the endogenous Atpre-miR159 flanked by the attB1 and attB2 recombinant sites of the Gateway system cloned in the pGEM-T vector (Promega), was used as template for all amiRNA constructs. pGEM-amiR-C, pGEM-amiR-D, and pGEM-amiR-H were obtained by replacing the sequence of the miR159 duplex by those of amiR-C, amiR-D, and amiR-H in pGEM-miR159. Then, the amiRNA sequences of these plasmids were transferred to the destination plasmid pMDC32 (Curtis & Grossniklaus, 2003) using the Gateway technology, yielding pMDC-amiR-C, pMDC-amiR-D, and pMDC-amiR-H. For construction of pGEM-amiR-Di, DNA fragments were amplified by PCR using pGEM-amiR-D as template and oligodeoxynucleotides #2858 and #2859 as primers, which included the complementary sequence of the mature amiR-Di strand (Table S1). PCR products were digested with BglII and Xmal and inserted in the large fragment of pGEM-miR159 digested with the same enzymes.

To build pGEM-amiR-CiD, coding sequences of pre-amiR-C and pre-amiR-D were individually amplified by PCR using the plasmids pGEM-amiR-C and pGEM-amiR-D as templates and the oligodeoxynucleotide pairs #1191/#1748 (amiR-C) and #1749/#1194 (amiR-D) (Table S1) as primers. The amiR-C and amiR-D PCR products were digested with the restriction enzyme pairs BglII/Spel and Spel/Xmal, respectively, and inserted by a triple ligation in the large fragment of BglII/Xmal-digested pGEM-miR159. For pMDC-amiR-CiDi assembly, pGEM-amiR-CiD was digested with EcoRV, and its amiRNA sequence was transferred first to the entry vector pDONR207 and then to the destination vector pMDC32 using the BP Clonase II and LR Clonase kits (Thermo Fisher Scientific).

4.3 | Plant transformation

*N. benthamiana* plants were transformed with the different pre-amiRNA constructs cloned in pMDC32 using the leaf disc method of Horsch et al. (1985). Small pieces of leaves of young *N. benthamiana* plants were sterilized with bleach and precultured for 2 days on plates with Murashige and Skoog (MS) medium containing 1 μg/ml 6-benzylaminopurine, 0.1 μg/ml 1-naphthaleneacetic acid, and 1% sucrose solidified with 0.6% agar (regeneration medium).

The explants were incubated for 15 min in a culture of *A. tumefaciens* carrying the appropriate plasmids induced with 150 μM acetosyringone and, after slight drying, incubated in solid regeneration medium for 2 days under low-intensity light. Next, explants were washed with MS medium containing 500 μg/ml carbenicillin, slightly dried, and transferred to plates with solid regeneration medium containing 40 μg/ml hygromycin and 250 μg/ml cefotaxime, followed by incubation at 25°C under 14 h light/10 h dark cycles with weekly transfers to fresh medium.

Shoots emerged after several weeks of incubation, and after reaching a size of approximately 0.5 cm they were transferred to MS medium with 1% sucrose and 40 μg/ml hygromycin solidified with 0.6% agar. After rooting, seedlings were transferred to soil and protected with plastic for acclimatization in the greenhouse. After 4–5 days, the plastic was removed and culture of the plants was continued as described above.

4.4 | Quantification of the accumulation of amiRNAs

For northern blot analyses, small RNAs were purified from leaf tissue following a modified version of the method of Lagrimini et al. (1987) in which the small RNAs that remained soluble after precipitation with 8 M LiCl were recovered by precipitation with ethanol and subsequent centrifugation. Small RNAs were fractionated in 12% polyacrylamide gels containing 8 M urea and transferred to Hybond-N+ membranes (GE Healthcare). RNAs were fixed to the membrane by UV irradiation, washed with ULTRAhyb Oligo Buffer (Ambion), and hybridized for 16 h at 43°C in the same solution with 100 ng of a specific [32P]-labelled oligodeoxynucleotide. The labelling was done with [32P]-ATP (5 μCi, 3,000 Ci/mmol) (Perkin Elmer) and T4 polynucleotide kinase (Thermo Fisher Scientific). Labelled probes were purified in Illustra MicroSpin G-25 columns (GE Healthcare). After hybridization, membranes were washed with a 2× SSC solution (0.3 M NaCl, 30 mM sodium citrate) containing 0.2% SDS at
OD 600 of 1. After 3 h of incubation at room temperature, the cell 2- morpholinoethanesulfonic acid, and 150 systemically infected leaves was ground to fine powder in liquid ni-
tissue) and tissue debris was removed by centrifugation. Two leaves ture with 5 mM sodium phosphate buffer pH 7.5 (2 ml per gram of tissue), and 150μM acetylsyringone at an OD\textsubscript{600} of 1. After 3 h of incubation at room temperature, the cell suspension was infiltrated in the abaxial lamina of two young leaves making pressure with a 1-ml syringe. For virus inoculation with extracts of infected plants, tissue from systemically infected leaves was ground to fine powder in liquid nitrogen using a mortar and pestle. The powder was mixed to homo-
tissue fragments containing amiR- D/Di, amiR- C, and amiR- H, re-
sequence from both ends of the DNA fragment, generating two li-
the amplified fragments were purified by agarose gel electrophoresis and extraction with the QIAEX II Gel Extraction kit (Qiagen) and cloned in the vector pUC19 (Yanisch-Perron et al., 1985) linearized with Smal, and individual clones were se-
quencing universal primers.

For high-throughput sequencing, the viral genome fragments amplified by IC-RT-PCR from PPV-infected plants were analysed by the MiSeq paired-end 2 × 300 nt sequencing system (Illumina), fol-
sequences per sample. The reads coinciding from both ends of the DNA fragment, generating two li-
reads as the value of nucleotide changes introduced by chance or as background derived from the quasispecies nature of the viral population. Species that appeared in only one of the two libraries of the same sample, although they were detected with more than 100 reads, were also eliminated as possible artefacts.

4.7 | Detection of ends of viral RNA fragments using 5′-RACE

To identify cleavage sites of amiRNA-loaded RISC in the viral RNA, we used a slightly modified version of the classic 5′-RACE method (Anonymous, 2005) (Figure S2).

Total RNA was purified with TRIzol reagent from leaves agroin-
Oligodeoxynucleotides used as probes or as calibration

4.5 | Virus inoculation

PPV-NK-GFP, a recombinant PPV expressing GFP (Fernández-
transformed with the plasmid pBIN-PPV-NK-GFP (Lucini, 2004) was resuspended in a solution containing 10mM MgSO\textsubscript{4}, 10 mM 2-morpholinoethanesulfonic acid, and 150μM acetylsyringone at an OD\textsubscript{600} of 1. After 3 h of incubation at room temperature, the cell suspension was infiltrated in the abaxial lamina of two young leaves making pressure with a 1-ml syringe. For virus inoculation with extracts of infected plants, tissue from systemically infected leaves was ground to fine powder in liquid nitrogen using a mortar and pestle. The powder was mixed to homo-
tissue fragments containing amiR- D/Di, amiR- C, and amiR- H, re-
sequence from both ends of the DNA fragment, generating two li-
the amplified fragments were purified by agarose gel electrophoresis and extraction with the QIAEX II Gel Extraction kit (Qiagen) and cloned in the vector TOPO- TA (Thermo Fisher Scientific) following the instructions of the supplier. Individual clones were sub-
using the primers employed for their amplification. In some cases, cDNA fragments were cloned in the vector pUC19 (Yanisch-Perron et al., 1985) linearized with Smal, and individual clones were se-
quencing universal primers.

4.6 | Analysis of viral progenies

Viral cDNA fragments were amplified from infected plants by IC-
RT-PCR. For immunocapture, leaf tissue was homogenized with sodium phosphate buffer pH 7.5 (2 ml per gram of tissue), and 50μl of extract was incubated for 2 h at 37°C or overnight at 4°C in tubes previously coated with anti-PPV CP IgGs prepared from rabbit immune serum as described by Steinbuch and Audran (1969). Next, after three washes with phosphate-buffered saline containing 0.05% Tween 20, RT-PCRs were carried out with the One Step Titan PCR System kit (Roche) using the primer pairs #1669/#1668, #1328/#1329, and #1253/#1254 (Table S3) for amplifying viral genome fragments containing amiR- D/Di, amiR- C, and amiR- H, re-
respectively. The amplified fragments were purified by agarose gel electrophoresis and extraction with the QIAEX II Gel Extraction kit (Qiagen) and subjected to Sanger sequencing by Macrogen Europe.
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CONFLICT OF INTEREST

No conflicting relationship exists for any author.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

ORCID

Mingmin Zhao https://orcid.org/0000-0002-4816-0949
Carmen Simón-Mateo https://orcid.org/0000-0002-4251-0821
Juan Antonio García https://orcid.org/0000-0002-4861-9609

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