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A high-efficiency gene silencing in plants using two-hit asymmetrical artificial MicroRNAs

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Summary

MicroRNAs (miRNAs) are small non-coding RNA molecules that play a crucial role in gene regulation. They are produced through an enzyme-guided process called dicing and have an asymmetrical structure with two nucleotide overhangs at the 3′ ends. Artificial microRNAs (amiRNAs or amiRs) are designed to mimic the structure of miRNAs and can be used to silence specific genes of interest. Traditionally, amiRNAs are designed based on an endogenous miRNA precursor with certain mismatches at specific positions to increase their efficiency. In this study, the authors modified the highly expressed miR168a in *Arabidopsis thaliana* by replacing the single miR168 stem-loop/duplex with tandem asymmetrical amiRNA duplexes that follow the statistical rules of miRNA secondary structures. These tandem amiRNA duplexes, called "two-hit" amiRNAs, were shown to have a higher efficiency in silencing GFP and endogenous PDS reporter genes compared to traditional "one-hit" amiRNAs. The authors also demonstrated the effectiveness of "two-hit" amiRNAs in silencing genes involved in miRNA, tasiRNA, and hormone signalling pathways, individually or in families. Importantly, "two-hit" amiRNAs were also able to over-express endogenous miRNAs for their functions. The authors compare "two-hit" amiRNA technology with CRISPR/Cas9 and provide a web-based amiRNA designer for easy design and wide application in plants and even animals.

Introduction

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate gene expression by cleaving target gene transcripts and/or inhibiting their translation (Schwarz *et al.*, 2003). During biogenesis, a typical miRNA precursor is processed into a miRNA/miRNA* duplex, with the miRNA strand being loaded into the AGO1-centered RNA-induced silencing complex (RISC) to interact with the target mRNA and the miRNA* strand being excluded from the RISC and degraded, a process known as asymmetrical assembly of RISC (Schwarz *et al.*, 2003). Artificial miRNA (amiRNA) is an artificially produced 21 nucleotide miRNA that targets gene transcripts for silencing in transgenic plants (Li *et al.*, 2013; Yu and Pilot, 2014). It is designed based on a pre-miRNA backbone by replacing the miRNA/miRNA* duplex with an amiRNA/amiRNA* duplex. Like the endogenous miRNA, the amiRNA/amiRNA* duplex is processed by DCL1 and the amiRNA is loaded into the RISC, while the passenger amiRNA* strand is expected to be degraded through the same mechanism of asymmetrical RISC assembly (Schwarz *et al.*, 2003). Traditional amiRNAs have been shown to be effective in silencing target genes in various cases (Li *et al.*, 2013; Yu and Pilot, 2014).

amiRNAs can effectively silence genes with fewer off-target effects, similar to endogenous miRNAs (Tang and Galili, 2004). However, the efficiency of gene silencing by amiRNAs may vary depending on the target positions and sequences on the target genes. Therefore, researchers have sought out various miRNA backbones and their specific miRNA duplex structures to improve silencing efficiency (Schwab *et al.*, 2006). In one study (Park *et al.*, 2009), two different amiRNAs targeting *AP1* and *CAL* genes were designed using the miR319a backbone and the WMD3 tool (Ossowski *et al.*, 2008). Another report used two different amiRNAs with two different backbones (miR319a and miR395a) to target different mRNAs (Liang *et al.*, 2012). Eamens *et al.* (2014) used amiRNAs expressed through the miR172a and miR159b backbones to silence several miRNAs. Fahim *et al.* (2012) replaced the five native miRNA sequences in the first five duplex arms of native miR395 in wheat with five amiRNAs designed to target WSMV. To ensure the silencing of specific genes, multiple candidate amiRNAs are often designed to target different regions

of the target gene through individual trials. Successful amiRNAs have been produced by mimicking specific duplex structures of endogenous miRNAs, such as miR172a, miR164a, miR165a, miR166g, miR167a, miR319a, miR395a, miR390a in Arabidopsis, and miR528 and miR390 in monocots (Alvarez *et al*., 2006; Carbonell *et al*., 2015; Ju *et al*., 2017; Lunardon *et al*., 2021; Warthmann *et al*., 2008). Synthetic trans-acting small interfering RNAs (syn-tasiRNAs) have been created by modifying the backbone of an endogenous tasiRNA precursor, such as TAS1, to silence genes of interest (Carbonell *et al*., 2014). However, there are no clear and consistent rules for the general design and application of amiRNAs in gene silencing (Singh and Das, 2021).

In this study, we developed an amiRNA production system using statistical structural rules based on numerous miRNA duplexes and a modified, unused pre-miRNA backbone of the highly expressed miR168a from Arabidopsis. In the modified miR168a precursor, we replaced the single miR168a/miR168a* duplex with tandem amiR/amiR* duplexes that have mismatches at two key positions, P1 and P12, counted from the 5′ end of the amiR strand. By analysing miRNA/miRNA* duplex secondary structures in plants and animals, we found that two asymmetrical key positions, P1 and P12, are pronouncedly mismatched in most natural cases. We applied this simple, conserved rule of asymmetrical mismatches at P1 and P12 to design amiRNAs and test their silencing efficacy.

We further duplicated the single amiRNA duplex structure into a "two-hit" tandem amiRNA structure for gene silencing and achieved nearly complete silencing of the target genes of different plant pathways in transgenic plants. In a comparative study of the gene-silencing effects between traditional single amiRNAs and two-hit amiRNAs, we quantitatively demonstrated that two-hit amiRNAs have higher gene-silencing efficiency than one-hit amiRNAs in targeting the constitutively expressed GFP reporter and an endogenous target, *PDS3*, in Arabidopsis. When these two-hit amiRNA structures were used to express endogenous miRNAs, robust production of the endogenous miRNAs was detected in transgenic plants with opposite phenotypes to miRNA-down-regulated plants using short tandem target mimic (STTM) (Yan *et al*., 2012). Finally, we developed and implemented a web-based amiRNA design tool for the application of such two-hit amiRNA technology in plants and even animals. Therefore, two-hit amiRNAs provide a powerful tool for gene silencing and for characterizing miRNA functions in a variety of plant species.

Results

Designing two-hit amiRNAs following the asymmetrical rules of endogenous miRNA structures

It is well known that miRNAs are structurally asymmetrical, with the miRNA strand preferentially being assembled into the RNA-induced silencing complex (RISC) while the miRNA* strand is excluded in most cases of miRNA biogenesis (Schwarz *et al*., 2003). To understand this asymmetry, we investigated the secondary structures of all miRNAs listed in miRBase (http://www.mirbase.org/) and obtained the general characteristics of the duplex structures of endogenous miRNAs (Figure 1a). We observed that miRNA duplexes contain a certain percentage of mismatches at positions 1 (P1) to 19 (P19) along the miRNA strand from the 5′ to the 3′ ends. In humans, 58% of miRNAs start with the preferred base "U" at P1 (Figure 1a), corresponding to 24% matches with the base "A", 12% mismatches with "G", 10% mismatches with "U", 6% mismatches with "C", and 6% bulges without any matches or mismatches. In contrast, 76% of Arabidopsis miRNAs start with the preferred base "U" at P1, corresponding to 60% matches with the base "A", 8% mismatches with "U", 4% mismatches with "G", 3% mismatches with "C", and 1% bulges without any matches or mismatches (Figure 1a). The remaining miRNAs, 42% in humans and 24% in Arabidopsis, start with bases other than "U" with their different percentages of pairs, mismatches, or bulges (Figure 1a). Next, we calculated the rate of mismatches at each location from P1 to P19 of all existing miRNA/miRNA* duplexes in key model organisms of animals and plants (Figure S1). We found that positional mismatches between a miRNA guider and its passenger strand have the highest probability at P1 (before the seed region) and at P12 (after the seed region). While this trend is quite clear in animals, it is largely followed in plant species as well (Figure S1). The two mismatch percentage peaks at P1 and P12 may represent the typical asymmetry of most endogenous miRNA duplexes, regardless of other variations (Figure S1). Therefore, we used these two typical mismatches as a universal approach for designing amiRNAs.

To select the mismatched bases on the amiR* strand corresponding to P1 and P12 on the amiR strand, we chose the bases with the highest probability of mismatching in amiR design. For example, if the first nucleotide of a miRNA is A at P1 in humans or Arabidopsis, the corresponding nucleotide on the miRNA* strand should also be A rather than T to create a mismatch on the duplex, since A has the highest probability of occurrence among all mismatched bases at that position. Similarly, if the twelfth nucleotide of a miRNA is G, the corresponding nucleotide on the miRNA* strand should be U rather than C (Figure 1a). Therefore, amiR and amiR* are designed such that mismatches are always present at P1 and P12, with bases chosen based on the highest probability of mismatching according to the statistical analysis (Figure 1a,b).

We generated the pOT2-Poly-Cis vector by inserting a 2x (dual)-35S promoter, a DNA fragment called Poly-Cis, and a 35S terminator into the pOT2 vector (Figures S2 and S3). The Poly-Cis region has a modified miR168a precursor backbone, which can be used to clone one or two amiR/amiR* duplexes, known as one-hit or two-hit amiRs, in place of the miR168a/miR168a* duplex. The biogenesis of the inserted amiRs of interest was verified in transient expression or in stable transgenic plants. We designed an amiR of 21 nucleotides with perfect or almost perfect sequence complementarity to the gene transcripts that we wanted to silence. The one-hit amiR was created by a single step of inverse polymerase chain reaction (PCR), while the two-hit amiRs in tandem were created as a single polycistron by two sequential back-to-back inverse PCRs, one cloned after the other into the amiR precursor on the pOT2 vector (Figure S3).

Two-hit amiRs demonstrate greater silencing efficiency than one-hit amiRs in gene silencing

In this study, we compared the gene silencing efficacy of one-hit and two-hit amiRs in *Arabidopsis thaliana* plants expressing green fluorescent protein (GFP). We used a vector system that expresses one or two amiRs in a polycistron, which has been previously shown to be effective in expressing one amiR at a time (Chi *et al*., 2014). When expressed under a 2x 35S promoter, both one-hit and two-hit amiRs silenced GFP, but there was no detectable significant difference in the GFP silencing efficiency due to the saturation effect of the over-expressed amiRs (Figures S4 and S5). To further compare the effectiveness of one-hit and two-hit amiRs, we expressed them under a dosage-

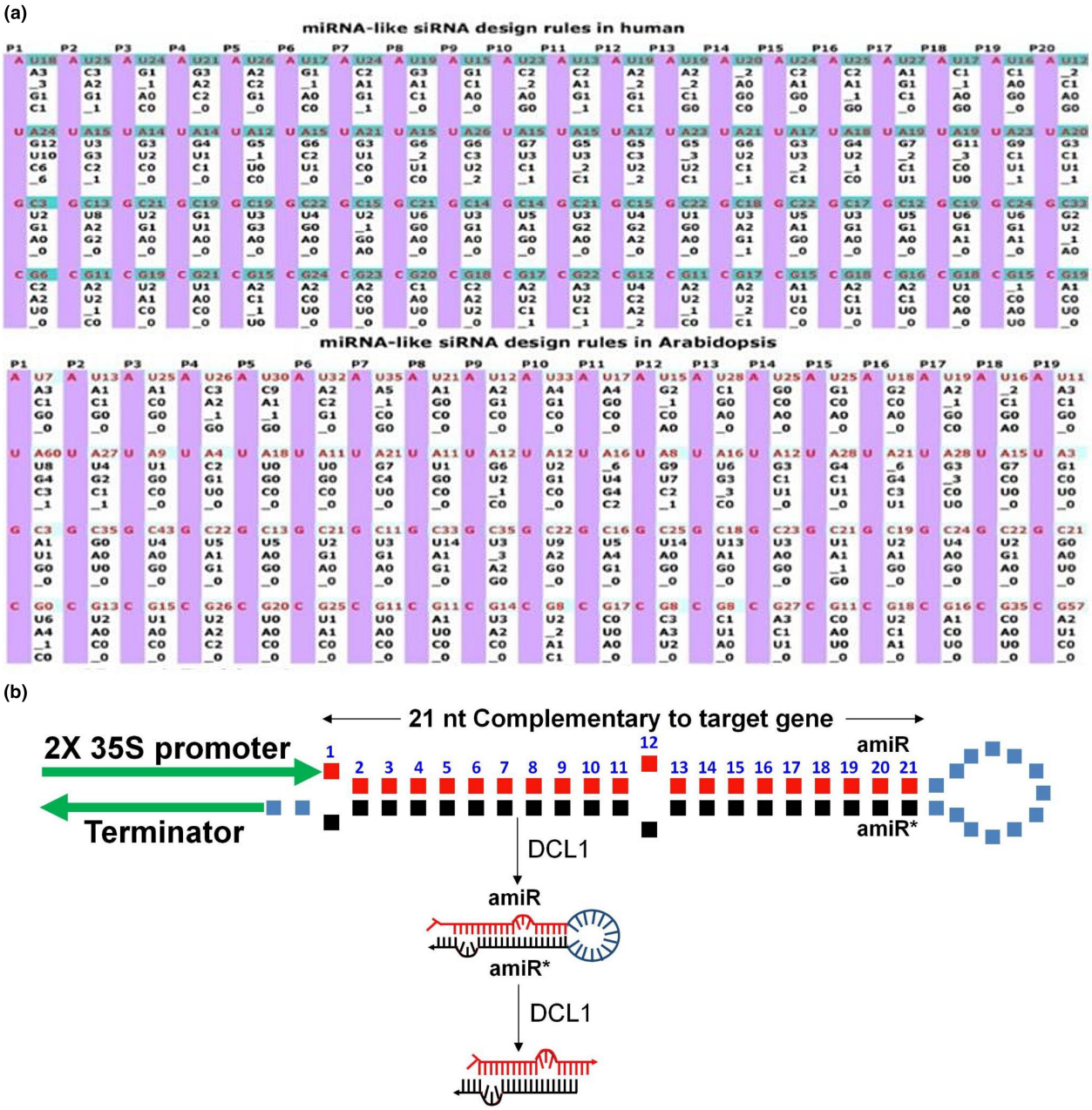

Figure 1 The amiR design strategy based on the statistical study of miRNA secondary structures in human and Arabidopsis. (a) A statistical display of the positional occurrence percentage of nucleotide bases in miRNAs from human (top) and Arabidopsis (bottom), two representative species of animals and plants, respectively. P1 to P20/19 represent the positions of nucleotides from 1 to 19/20 counted from the 5′ end of the mature miRNAs (guide strand). For each position, there are four possibilities on the miRNAs: A, U, G, and C. For each A/U/G/C (pink letters), there are five possibilities on the passenger (miRNA*) strand: A, U, G, C, and none (black letters). For example, in Arabidopsis, U at position 1 on the miRNA (guide strand) has five possibilities on the passenger strand: A, U, G, C, and __, with their respective percentage occurrence of 60, 8, 4, 3, and 1, designated as A60, U8, G4, C3, and __1. (b) A general structure of an amiR duplex showing a 21 nt amiR targeting a gene (in red squares), followed by a stem-loop region (in light blue squares) and amiR* (in black squares). The amiR* has a mismatch with nucleotides corresponding to position 1 and 12 in amiR. After the processing of the amiR duplex by DCL1, amiR (red) is loaded into RISC and amiR* (in black) is left out or degraded.

dependent β-oestradiol-inducible promoter. After induction with β-oestradiol, we found that two-hit amiRs were significantly more effective in silencing *GFP* than individual one-hit amiRs (Figure 2a–c). The expression of both one-hit and two-hit amiRs was induced by identical dosages of β-oestradiol in all transgenic lines (Figure 2c), indicating that two-hit amiRs induced better *GFP* silencing than one-hit amiRs under the same strength of transcriptional induction. These results provide quantitative evidence for the advantages of two-hit amiRs (Figure 3a) and support the proposed model that two-hit amiRs in a polycistron induce stronger gene silencing than one-hit amiR (Figure 3).

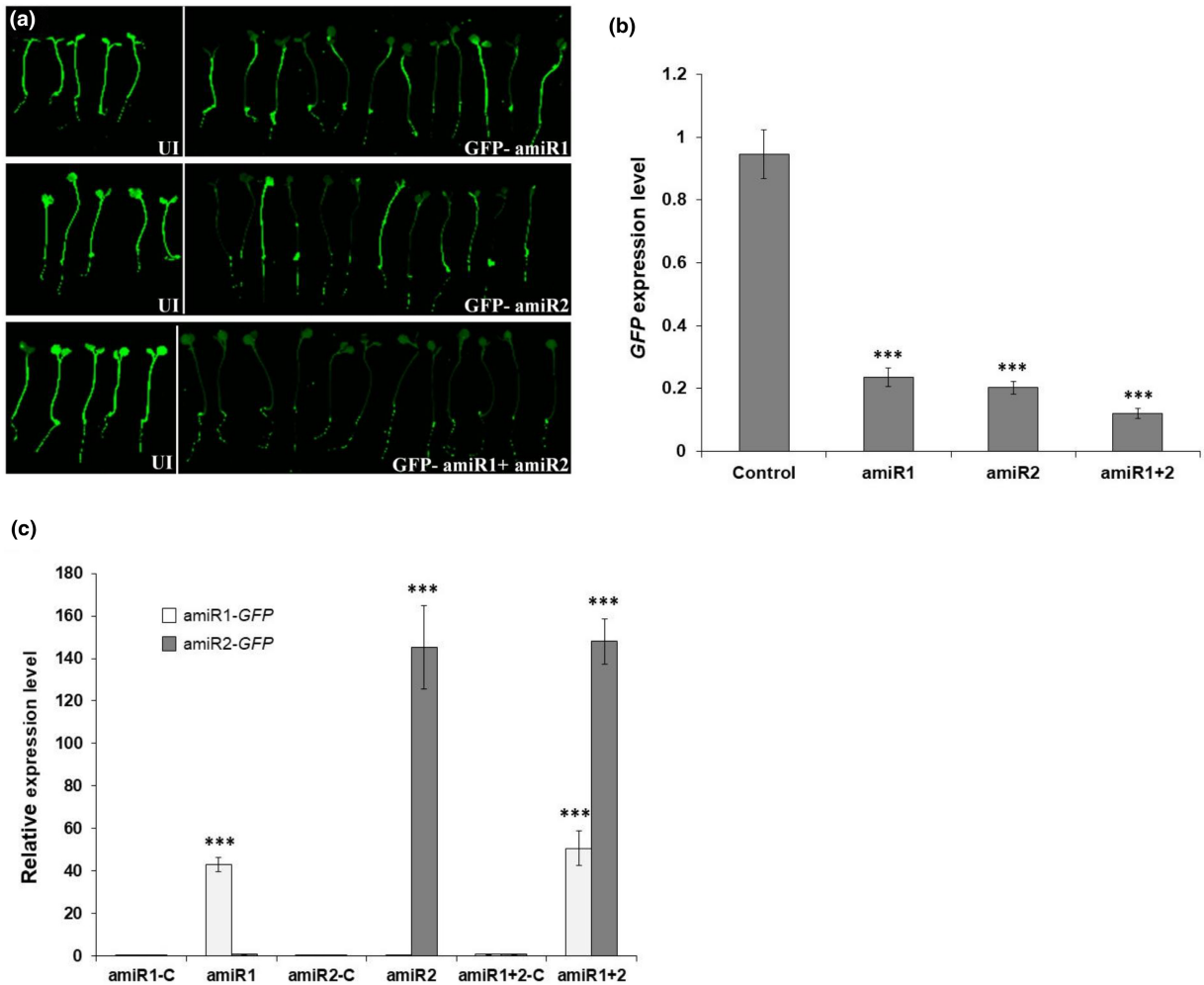

Figure 2 Comparison of the gene-silencing efficiency of one-hit and two-hit amiRs against *GFP* in Arabidopsis. (a) Representative images showing GFP fluorescence in control (uninduced-UI) plants and transgenic lines expressing one-hit (amiR1 or amiR2) or two-hit (amiR1+2) amiRs against *GFP*. (b) Real-time PCR analysis of *GFP* expression in control (uninduced) and beta-oestradiol-induced transgenic lines. (c) Quantitative stem-loop PCR analysis of amiR1 and amiR2 expression in control (uninduced: amiR1-C, amiR2-C, and amiR1+2-C) and beta-oestradiol-induced transgenic lines. Error bars represent ± SD. Asterisks denote statistically significant differences from the control by Student's *t* test: with *P* values as: ***$P < 0.001$.

In order to further demonstrate the improved efficiency of two-hit amiRs compared to one-hit amiRs in gene silencing, we examined their activity in *Arabidopsis thaliana* plants expressing one-hit and two-hit amiRs targeting the endogenous gene *PHYTOENE DESATURASE 3* (*PDS3*). We found that when expressed using a β-oestradiol-inducible promoter, two-hit amiRs were more effective at silencing the *PDS3* gene than one-hit amiRs, as evidenced by the bleaching of the leaves due to the lack of chlorophyll resulting from the absence of carotenoids, which are normally produced by *PDS3* (Qin *et al.*, 2007; Figure S6). This further supports the superiority of two-hit amiRs in gene silencing compared to one-hit amiRs in plant species.

Two-hit amiRs may switch between AGOs to effectively silence *AGO1*, *AGO7*, as well as *CTR1* genes in Arabidopsis

We aimed to investigate whether two-hit amiRs could direct gene silencing through AGO1 or other AGOs in Arabidopsis. To do so, we targeted the *AGO1* gene for silencing with two-hit amiRs. We found that the two-hit amiRs putatively silenced the *AGO1* gene, as evidenced by the range of severe phenotypes and abnormal development observed in the transgenic plants expressing the amiRs (Figure 4a). In addition, the expression of mature miRNAs, such as miR165/166 and miR168, which are typically loaded into the AGO1-guided RISC complex (Zhang and Zhang, 2012), was reduced in plants expressing the AGO1-amiR (Figure 4b). This suggests that *AGO1* was indeed knocked down by the two-hit amiRs in the transgenic plants.

We used two-hit amiRs to target the *AGO7* gene and successfully silenced its expression, as demonstrated by the production of *AGO7* amiRs and the reduction of *AGO7* and its mediated tasiRNAs, D7(+) and D8(+), as well as the increased expression of the tasiRNA target genes *ARF3* and *ARF4* (Figure 5). Previous research has shown that miR390 cleaves the TAS3 precursor RNA, which is then converted into double-stranded RNA and diced into tasiRNAs D7(+) and D8(+) by DCL4 and DRB4. These tasiRNAs subsequently inhibit the expression of *ARF2, ARF3*, and *ARF4* mRNAs (Marin *et al.*, 2010). The phenotype of

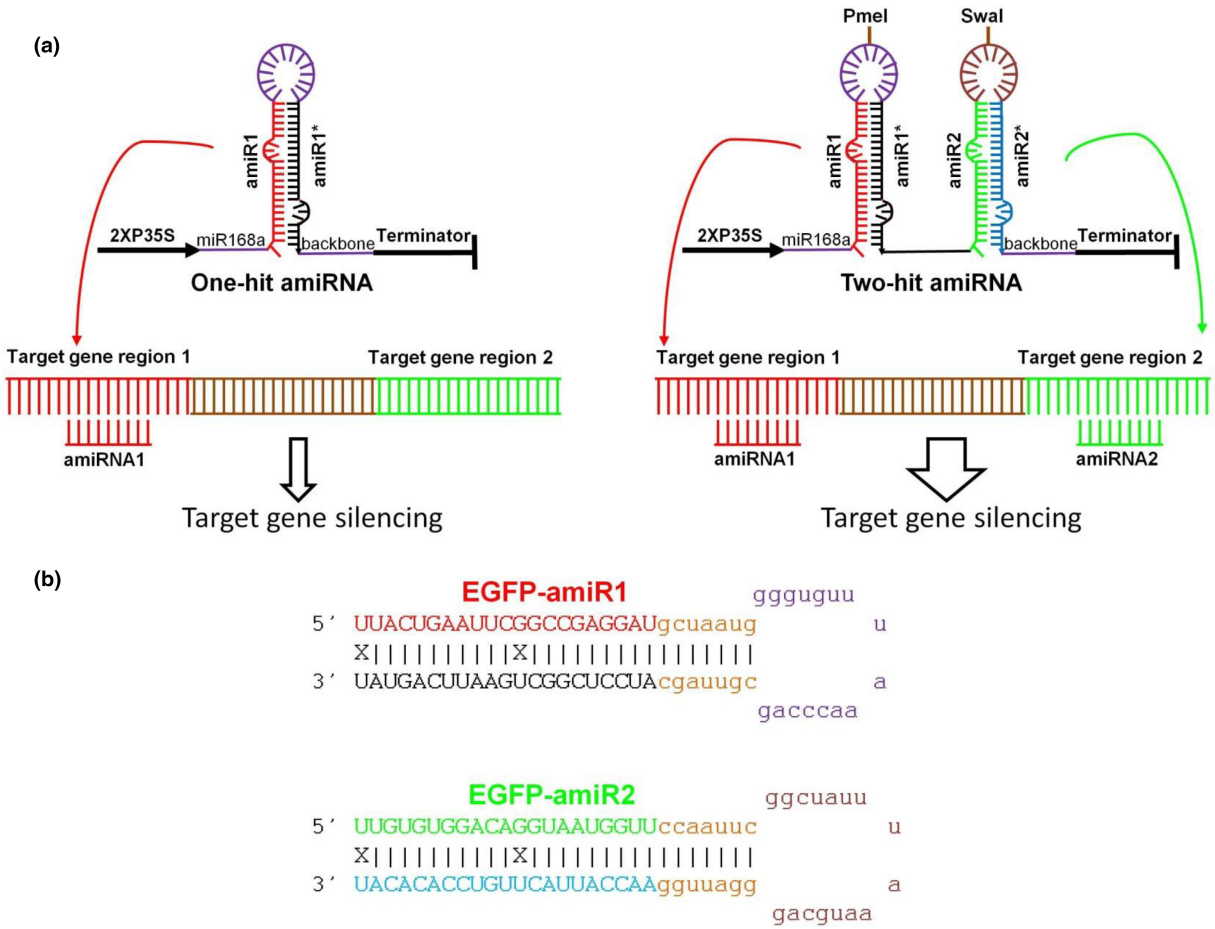

Figure 3 Efficacy of two-hit amiRs compared to one-hit amiRs in gene silencing. (a) One-hit or two-hit amiRs were cloned into the miR168a backbone through PCR and digestion with PmeI and/or SwaI. The amiRs were expressed using the strong 2x 35S promoter. Both beta-oestradiol-inducible and CaMV 35S promoters were used to express the amiRs for silencing *GFP*. Our data suggest that when expressed using the beta-oestradiol-inducible promoter, two-hit amiRs targeting two regions of an mRNA showed better gene silencing than one-hit amiRs targeting only a single region. (b) A depiction of two amiRs, amiR1 (in red) and amiR2 (in green) along with the stem-loop region (purple and brown) targeting *EGFP*. amiR1* (in black) and amiR2* (in blue) have mismatches corresponding to nucleotide 1 and 12 of amiR1 and amiR2, respectively.

plants expressing the *AGO7* two-hit amiRs resembled that of *AGO7* genetic mutants, supporting the silencing of *AGO7* mRNA by the two-hit amiRs.

To further evaluate the efficiency of two-hit amiRs in gene silencing, we targeted the *CONSTITUTIVE TRIPLE RESPONSE 1* (*CTR1*) gene, which is involved in the ethylene signalling pathway (Kieber *et al.*, 1993). The *ctr1* genetic mutant in Arabidopsis exhibits a dramatic phenotype, making it an ideal target for comparison. We found that silencing *CTR1* using two-hit amiRs resulted in a phenotype that was similar to or even stronger than that of the *ctr1* genetic mutant, including dwarfism, smaller rosette leaves, and late flowering (Figure S7). This suggests that two-hit amiRs are effective in silencing the *CTR1* gene in Arabidopsis.

Two-hit amiRs can be used to silence multiple genes within a family simultaneously

We evaluated the efficiency of two-hit amiRs in silencing multiple genes of a family by selecting conserved target regions and designing specific amiRs for each gene. In Arabidopsis, we successfully applied these two-hit amiRs to silence the SnRK2 gene family, including *SnRK2.2*, *SnRK2.3*, and *SnRK2.6* (Figure S8b; Cai *et al.*, 2014). We also used two-hit amiRs to simultaneously knock down all five members of the SINAT gene family (Figure S8a; Yang *et al.*, 2017). In addition, we employed two-hit amiRs to silence the *ARF7* and *ARF19* genes, resulting in plants with narrow leaves and a lack of lateral roots (Figure 6a,b). This phenotype matched the previously reported *arf7; arf19* double mutant (Wilmoth *et al.*, 2005). Furthermore, we successfully silenced multiple *ARF* genes (*ARF14, 15, 20, 21, 22* and *23*) by concatenating two-hit amiR transcriptional units, resulting in a series of severe phenotypes that had not been previously reported (Figure S9).

Using the two-hit amiR vector system to overexpress endogenous miRNAs

The two-hit amiR vector system has been demonstrated to be an effective method for increasing the biogenesis of miRNAs in plants through the overexpression of endogenous miRNAs. In this study, we utilized the two-hit amiR vector system to significantly increase the expression of endogenous miR169 in tomato and miR166 in rice. As shown in Figure 7, the expression of miR169 in

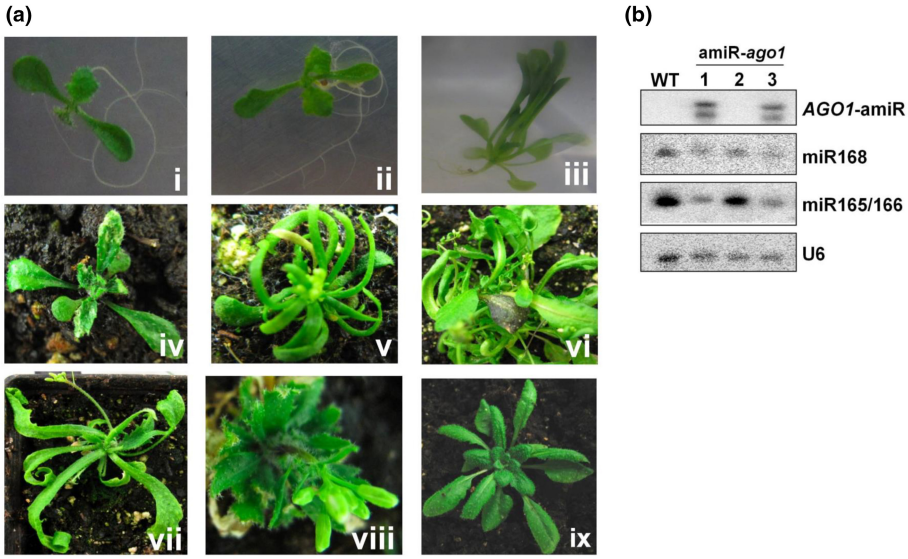

Figure 4 The knockdown of *AGO1* by two-hit amiRs. (a) Transgenic plants expressing amiRs against *AGO1* display a range of severe phenotypes (i–viii) compared to the wild type (WT) plant (ix). (b) Northern blot analysis of total RNA from transgenic plants expressing amiRs against *AGO1* shows expression of amiRs, reduced expression of mature miR168 (middle panel), and reduced expression of miR165/166 (lower panel). Line-2 behaves like WT, perhaps due to transgene silencing. When *AGO1* expression is reduced in transgenic plants, the accumulation of mature miRNAs, which depend on the stability of the AGO1-RISC complex, is also reduced. The band size of *AGO1*-amiR, miR168, and miR165/166 corresponds to 21 nt. The two bands in the case of *AGO1*-amiR may be the result of processing by different Dicer-like proteins. U6 serves as an internal control.

tomato was dramatically increased and its target gene, *NF-YA1*, was down-regulated. In rice, the overexpression of miR166 was successful, as indicated in Figure S10. This approach has also been previously used to overexpress miR165/166 in Arabidopsis (Ji *et al*., 2011), although the methods for doing so were not described in that study. In all of these cases, we employed a modified version of the two-hit amiR vector system in which the precursor of the endogenous miRNA was replaced with the cloned miRNA and mismatches were introduced at positions corresponding to P1 and P12 of the miRNA strand. This approach resulted in a significant increase in miRNA expression and the down-regulation of target genes, such as *NF-YA1* in the case of miR169 in tomato.

A comparison of the silencing efficacy of the Two-Hit amiR system with other amiR systems

In this study, we aimed to evaluate the effectiveness of the miR168-based amiR system for silencing target genes. To do this, we expressed the same amiR used by Weigel's group using miR172 or miR319 backbones. The amiRs were designed according to our rules and targeted the *TRY* and *YAB* genes using the same target region as described previously (Schwab *et al*., 2006). We found that the *lfy-2* amiR, which had previously been shown to be ineffective in silencing *LFY* (Schwab *et al*., 2006), was also ineffective in silencing *LFY* using our design. However, the two-hit amiR construct comprising *lfy-1* and *lfy-2* amiRs designed according to our rules demonstrated better silencing efficacy than the amiR designs used by Schwab *et al*. (2006) in the tested miR168 backbone (Figure 8). The same was true for the tested two-hit amiRs targeting *TRY* and *YAB* genes. We also found that Weigel's *lfy-1* amiR exhibited significantly better silencing efficacy in the miR172 backbone (Schwab *et al*., 2006). To investigate whether adding an extra U at the 5′ end of the amiR would increase its silencing efficacy, we added an extra 'T' to the amiRs targeting *YAB* and *TRY* genes. The *try-1*/*try-2* amiR with or without the extra U did not show any differences in silencing efficacy, but the amiR with the extra U targeting *YAB* showed significantly better silencing than the one without it (Figure 8, S11 and data not shown). The amiRs targeting *YAB1* and *YAB2*, as designed by Schwab *et al*. (2006), showed 40% and 20% penetrance, respectively. A two-hit amiR construct expressing *yab-1* and *yab-2* amiRs showed 45% penetrance, while a two-hit construct with a *YAB1* amiR with an extra U showed 75% phenotypic penetrance (Figure 8 and data not shown).

Evaluating the Off-Target effects of asymmetric Two-Hit amiRs

In this study, we evaluated the off-target effects of two-hit asymmetric amiRs by analysing the expression of the top ten predicted off-targets for each amiR that targets *LFY* and *TRY* genes, as determined by psRNA (https://www.zhaolab.org/psRNATarget/) (Dai and Zhao, 2011). The results showed a slight down-regulation of *LFY* expression, accompanied by a clear phenotype, likely due to both target cleavage and translational repression. The two-hit *try-1* and *try-2* amiRs effectively down-regulated *TRY* and *CPC* genes (see Figure S12).

The expression of the ten predicted off-target genes for each amiR was also analysed. The results indicated that the *lfy-1* amiR had no off-target effects, although there was a slight reduction in the expression of *LFY*. In contrast, the *lfy-2* amiR slightly down-regulated *LFY* and exhibited one off-target. Overall, the *try-1* and *try-2* two-hit amiRs significantly down-regulated *TRY* and *CPC* genes, with each amiR effectively down-regulating its predicted target, as demonstrated by the observed phenotypic changes (Figure S12). These findings suggest that the two-hit asymmetric amiR system is a powerful tool for gene silencing, with a high degree of specificity and effectiveness in down-regulating target

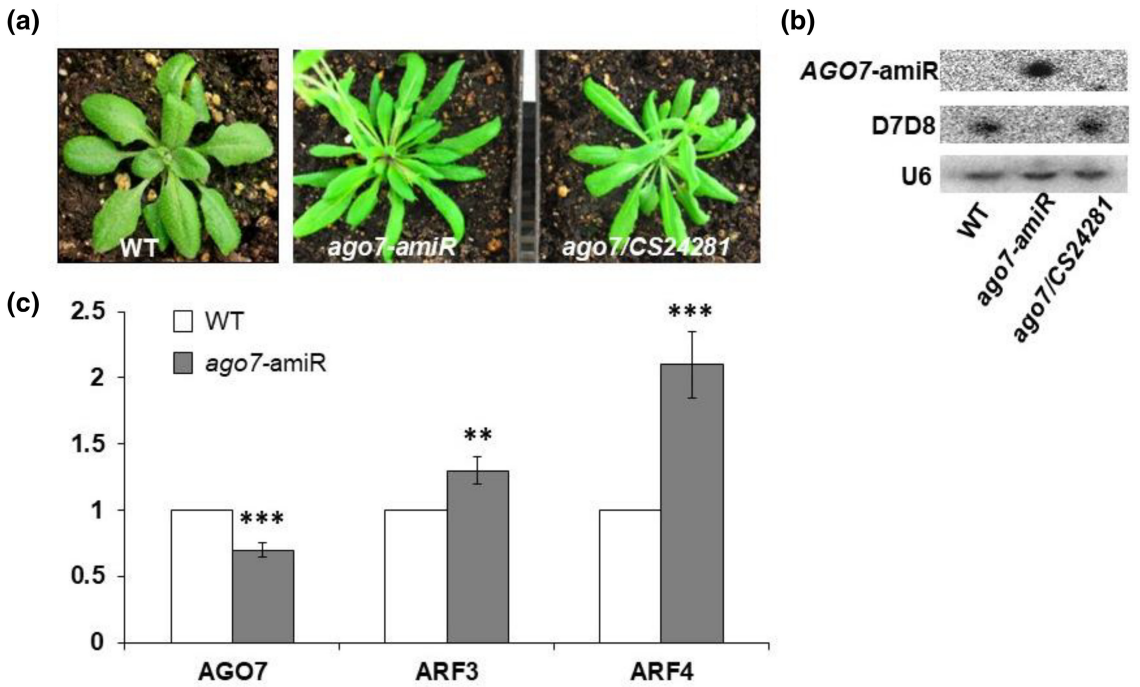

Figure 5 Knockdown of *AGO7* by two-hit amiRs. (a) amiR-*ago7* transgenic plant showing a phenotype similar to the *ago7* genetic mutant (CS24281). (b) Northern blot analysis of total RNA from transgenic plants expressing amiRs against *AGO7*. The amiR-*ago7* transgenic plant shows reduced accumulation of ta-siRNAs D7+ and D8+ which are dependent on miR390 for their biogenesis. miR390 is specifically loaded onto the AGO7-guided RISC complex. When *AGO7* expression is reduced, the expression of mature miR390 is also reduced, which further reduces the expression of D7/D8 ta-siRNAs. The band size of *AGO7*-amiR, D7 and D8 corresponds to 21 nt. (c) Real-time PCR analysis of *AGO7, ARF3*, and *ARF4* expression in the *ago7*-amiR transgenic line compared to the wild type. AGO7 expression is reduced in the *ago7*-amiR plant, with a concomitant increase in *ARF3* and *ARF4* expression. As explained above, the reduced expression of D7/D8 ta-siRNAs increases the expression of *ARF3* and *ARF4*. The TAS3 ta-siRNAs D7 and D8 target *ARF3* and *ARF4* and reduce their expression. The *ago7*-amiR plants show reduced expression of *TAS3* ta-siRNAs and therefore accumulate more *ARF3* and *ARF4* transcripts. Error bars represent ±SD. WT, wild type. Asterisks denote statistically significant differences from the WT by Student's *t* test: with *P* values as: $^{**}P < 0.01$; and $^{***}P < 0.001$.

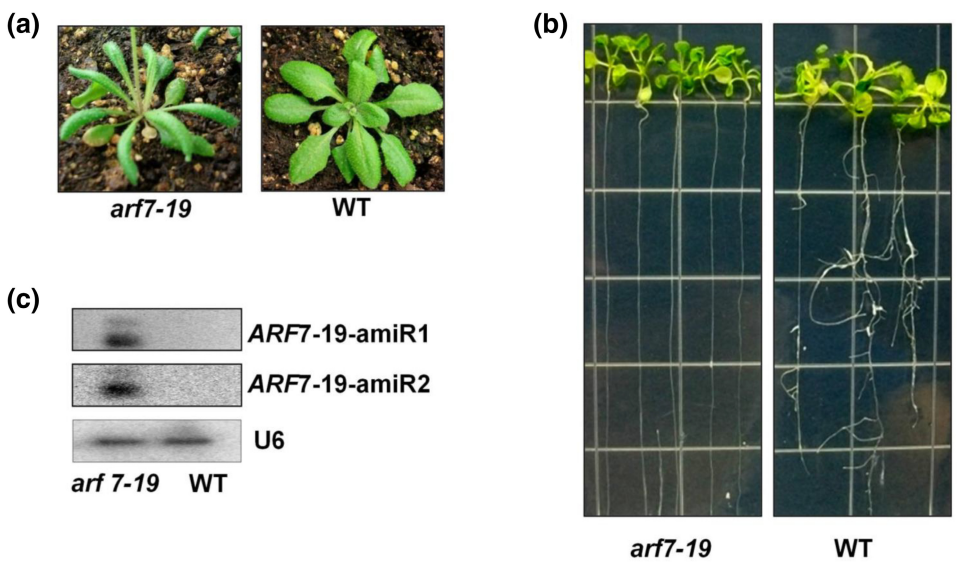

Figure 6 Gene silencing of *ARF7-19* by two-hit amiRs. Transgenic plants expressing amiRs against *ARF7-19* exhibit narrow leaves (a) and lack of lateral roots (b) compared to wild-type (WT) plants. (c) Northern blot analysis of total RNA from transgenic plants expressing amiRs against *ARF7-19*. The band size for *ARF7-19* amiR1 and amiR2 is 21 nt.

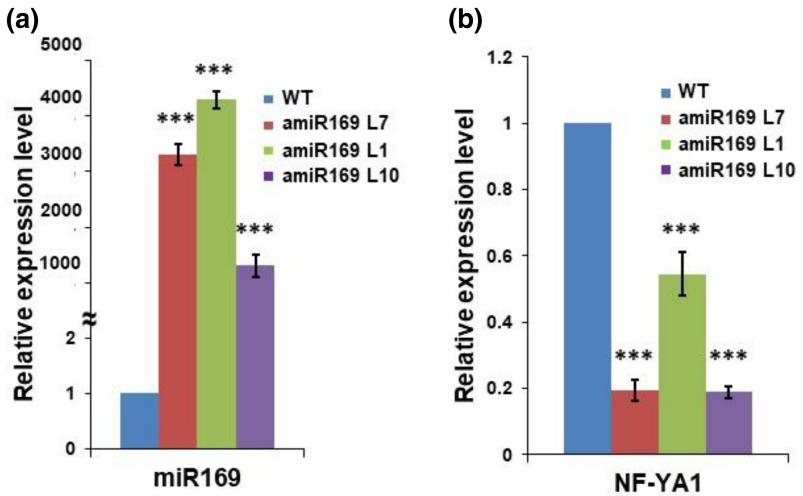

Figure 7 The effectiveness of using two-hit amiRs technology to overexpress miR169 and functionally silence its target gene, NF-YA1, in tomato plants. The abundance of miR169 was significantly increased in the transgenic lines expressing amiR169 compared to the wild-type plants, as demonstrated by stem-loop qRT-PCR (a) and Real-time PCR analysis of the miR169 target gene *NF-YA1* showed a significant suppression of its expression in the plants overexpressing amiR169 (b). Error bars represent ±SD. Asterisks denote statistically significant differences from the WT by Student's *t* test: with *P* values as: ***$P < 0.001$.

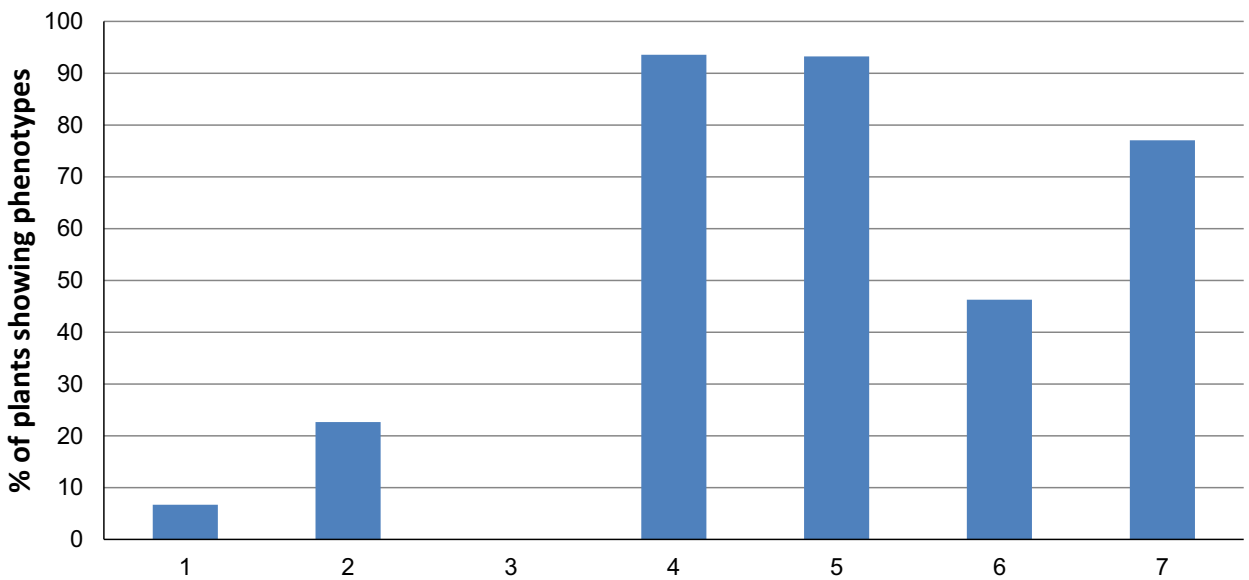

Figure 8 Percentage of plants showing phenotypes transformed with the following constructs: 1. Two-hit with *lfy-1/lfy-2* of Weigel's design; 2. Two-hit with *lfy-1/lfy-2* of Tang lab design; 3. Two-hit with *lfy-2/lfy-2* of Tang lab design (a negative control); 4. Two-hit with *try-1/try-2* of Tang's lab design; 5. Two-hit with *try-1/try-2* (with U) of Tang's lab design; 6. Two-hit with *yab-2/yab-1* of Tang's lab design; 7. Two-hit with *yab-2/yab-1* (with U) of Tang's lab design. Note: An extra 'T' was added to the amiRs targeting *YAB* and *TRY* genes which formed two-hit amiRs with 'U'.

genes. It is important to carefully evaluate the potential off-target effects of these amiRs in order to ensure accurate and reliable results.

The development of a web-based tool for designing highly functional asymmetrical amiRs

A web-based tool has been developed for designing highly functional asymmetrical amiRs, small RNA molecules that specifically inhibit the expression of target genes (http://www.mirnainfo.com/miRNADesignOne.aspx). The tool allows users to design amiRs using either the Arabidopsis miR168a backbone (http://www.smallrna.mtu.edu/Tang_Website/submit.htm) or any other endogenous miRNA backbone (http://www.mirnainfo.com/miRNADesignOne.aspx). To design amiRs using an endogenous miRNA backbone, users first select a biological species from the list provided and enter the name and sequence of the targeted gene in the text editor, including only the characters A, a, U, u, G, g, C, and c and a sequence of 21 RNA nucleotides (Figure S13). They can then choose whether mismatches in the amiR duplex should occur at maximum or minimum frequency and select

specific positions on the duplex to have mismatches or bulges. Finally, users can choose whether the guider and passenger strands should be joined by an endogenous miRNA loop, with a dropdown box listing all available loops from endogenous miRNAs if the 'with a loop' option is selected. The tool will provide the secondary structures of the amiR precursor based on the user's selections, allowing for the design of highly effective asymmetrical amiRs (Figure S14).

The process of designing amiRs using the Arabidopsis miR168a backbone begins by inputting the target gene sequence into the tool, which generates an amiR candidate that is perfectly complementary to the target sequence and then designs a passenger strand (amiR*) based on the candidate amiR. The resulting amiR/amiR* pair will have mismatched bases at positions 1 and 12 of the candidate amiR from the 5′ to 3′ direction. To be acceptable, the amiR candidate must be perfectly or almost perfectly base-paired with the target gene's 21-nt sequence and have a uracil residue at position 1 from the 5′ end. A whole genome BLAST search is then conducted to ensure that the candidate amiR does not have a high likelihood of binding to off-target genes. If the candidate amiR passes these criteria, PCR primers for inverse PCR amplification of the plasmid pOT2-poly-Cis, which contains a 2× 35S promoter, a modified pri-miR168 backbone, and a CaMV terminator (plasmid available at https://www.addgene.org/Guiliang_Tang/), are designed by the tool. The sequences of the designed PCR primers and probes for the amiRs used in this study can be found in Table S1.

Discussion

Designing asymmetrical amiRs for target gene silencing

The first generation of amiRs were hairpin structures fully complementary to the target gene, similar to siRNAs in animals (Paddison *et al.*, 2002). The second generation of amiRs were simply mimics of endogenous miRNAs (Schwab *et al.*, 2006). While both types of amiRs have been used for gene silencing in plants and animals, mimics of endogenous miRNA structures have several advantages and have been widely used for the past decade. In most cases, the design of these second-generation amiRs involves either mimicking the secondary structures of endogenous miRNAs or using algorithms based on mutational studies. However, the efficiency of these amiRs can vary, so several evaluation systems have been established to assess their effectiveness. These include protoplast-based evaluations (Li *et al.*, 2013) and Agrobacterium-mediated transient expression systems using tobacco leaves (Bhagwat *et al.*, 2013). Therefore, designing effective amiRs is crucial for successful gene silencing, minimizing off-target effects, and reducing the workload of generating gene-silenced transgenic plants in studies (Teotia *et al.*, 2016).

In this study, we sought to design the next generation of amiRs by studying the secondary structures of numerous endogenous miRNAs in order to understand why these molecules are stable and effective at regulating the expression of their target genes across generations during evolution. We hypothesized that there must be certain structural patterns that contribute to their ability to silence genes. Previous research in Drosophila and humans has shown that endogenous miRNA duplexes are largely asymmetrical, allowing for a highly asymmetrical and preferred assembly of the RNA-induced silencing complex (RISC) using the miRNA strand rather than the miRNA* strand (Schwarz *et al.*, 2003). We found that plant endogenous miRNAs also have similar asymmetry in their miRNA/miRNA* duplexes, resulting in asymmetrical RISC assembly with the miRNA strand. We further discovered that this asymmetry can be simplified to base mismatches at positions P1 and P12. AmiRs designed according to these rules demonstrated sustained gene-silencing effects in our studies, overcoming the limitations of traditional hairpin structures or simple mimicry of endogenous amiRs on a case-by-case basis.

In previous web-based amiR design tools such as WMD3 (Ossowski *et al.*, 2008), it was suggested that amiRs with mismatches outside the seed region with the target sequence could be effective, but the reasoning behind this was not clear. In contrast, we designed highly asymmetrical amiRs with mismatches at just two positions, P1 and P12 of the amiR, by altering the corresponding bases on the amiR*. This led to a higher incorporation of the amiR than the amiR* into the RNA-induced silencing complex (RISC) for amiR biogenesis and the formation of the active silencing complex (Figure S1). The inclusion of a mismatch at position 1 was also supported by previous studies showing that an amiR containing a 5′ mismatch with its amiR* and having perfect complementarity to the target region can have specific and efficient gene silencing (Chi *et al.*, 2014; Park *et al.*, 2009). The inclusion of a conserved mismatch at position 12 in the design seemed to further enhance the asymmetry and silencing efficiency of the amiR-guided RISC assembly against the target genes.

Endogenous miRNAs often complement and cleave their target gene transcripts extensively. In contrast, synthetic small interfering RNAs (siRNAs) are designed to fully complement and cleave the target gene transcript for highly efficient silencing (Tang *et al.*, 2003), which supports our approach to amiR design. This perfect base-pairing between amiR and target does not trigger the production of secondary small RNAs (Guo *et al.*, 2014; Park *et al.*, 2009). In cases where a gene family with multiple members is being silenced, not all the conserved regions are identical, and therefore an amiR targeting this conserved region may not always be fully complementary to all members. G-U wobbles between the amiR and the target can be introduced in the design process in these cases (Schwartz and Blobel, 2003). In this study, we preferred to design amiRs with a uracil (U) residue at position 1 at the 5′ end, because most endogenous miRNAs start with a "U". Although adenine (A) has been suggested as the tenth base of an amiR by other designers (Eamens *et al.*, 2014; Ossowski *et al.*, 2008), this is not essential in our design.

Enhancing gene silencing with two-hit amiRs: *GFP* amiRs Provide a quantitative evaluation

In this study, we aimed to improve the efficiency of amiR-mediated gene silencing by designing highly asymmetrical amiRs using mismatches at positions P1 and P12 for robust production of amiRs and by using two amiRs in tandem (two-hit) to target an mRNA at different locations for improved accessibility (Figure 2). Our results demonstrate that two-hit amiRs have a better quantitative silencing effect than one-hit amiRs, as shown in our experiments using two-hit *GFP* amiRs to target the transgenic reporter gene GFP. When the GFP mRNA was targeted by weakly expressed amiRs under the dosage-dependent β-oestradiol-inducible promoter, two-hit amiRs showed a clear advantage in terms of silencing efficiency compared to one-hit amiRs. This finding provides the first quantitative evidence for the hypothesis that two-hit amiRs are more effective than one-hit amiRs in our newly designed amiR technology. This improved efficiency may

be due to the increased accessibility of the target mRNA, as well as the increased production of amiRs, which may lead to a higher likelihood of successful silencing. It is worth noting that the target site accessibility and amiR production are both crucial factors in amiR-mediated gene silencing, and optimizing both aspects may further enhance the efficiency of this gene silencing method.

In this study, we demonstrated the efficacy of two-hit amiRs in gene silencing by comparing their performance to one-hit amiRs in silencing transgenic reporter genes as well as endogenous genes in Arabidopsis. Two-hit amiRs were able to effectively silence the *PDS* gene and other endogenous genes, such as *AGO1, AGO7, CTR1*, *ARFs*, SnRK2s, and SINAT family genes. In addition, we used two-hit amiRs to over-express endogenous miRNAs, providing a complementary approach to the STTM technology in functional genomics of small RNAs/miRNAs (Yan *et al*., 2012). These results highlight the potential of two-hit amiRs as a powerful tool for gene silencing and functional genomics studies.

Two-Hit asymmetrical amiRs target *AGO1* gene to study sorting of AGO proteins

The silencing effects of two-hit amiRs were observed in the targeting of *AGO1*, a key gene involved in endogenous miRNA and amiR biogenesis. When *AGO1* was targeted by two-hit amiRs, transgenic Arabidopsis plants displayed a range of phenotypes, including butterfly-like shapes in plant leaf and stem architecture and dwarf plants with comb-teeth-shaped rosette leaves (Figure 4a). These phenotypes suggest that *AGO1* was silenced by the two-hit amiRs. It is possible that the amiRs were sorted to AGO proteins other than AGO1 in this case, or that there was residual activity of AGO1 in sorting amiRs due to incomplete silencing. Interestingly, the expression of endogenous miRNAs such as miR168 and miR165/166 was dramatically reduced in *amiR-ago1* transgenic plants. This suggests that, unlike amiRs, endogenous miRNAs such as miR168 and miR165/166 are unable to be sorted to AGO proteins other than AGO1 after losing protection from degradation. These observations indicate that two-hit asymmetrical amiRs have distinct structural properties from endogenous miRNAs in their sorting to various AGO proteins. While amiRs with P1 and P12 mismatches can be sorted to AGO proteins other than AGO1, endogenous miRNAs such as miR168 and miR165/166 have more structural restrictions for AGO1 due to their highly regulated functions in plant development. This specificity may be important in the context of evolution and plant functions. Thus, varying amiR secondary structures, such as those with mismatches at positions other than P1 and P12, may help to uncover the role of miRNA secondary structures in sorting to specific AGO proteins, such as miR165/166 sorting to AGO1 and/or AGO10 (Dalmadi et al., 2021), and miR390 sorting to AGO7, among others.

Utilizing amiRs as a complementary approach to CRISPR/Cas9-Mediated gene knockouts for functional studies

While CRISPR/Cas9 technology has greatly advanced the ability to create gene knockouts, amiRs remain a valuable tool for gene functional studies due to their ability to create knockdowns rather than complete gene deletions. This can be particularly useful for studying essential genes, as null alleles created by CRISPR may be lethal. Additionally, amiRs can be used to simultaneously knockdown multiple genes within a family, a task that may be more challenging to accomplish with CRISPR. While CRISPR-mediated knockouts are permanent, amiRs can transiently and reversibly knockdown target genes, in addition to creating stable transgenics. Therefore, amiRs can serve as a complementary approach to CRISPR/Cas9-mediated loss of gene function studies.

Designing Two-Hit amiRs using simple rules based on the secondary structures of endogenous plant and animal miRNAs

The design of two-hit amiRs follows distinct, statistics-based rules that differ from traditional approaches and are based on the secondary structures of duplex regions of endogenous plant and animal miRNAs available from miRBase. These rules are simple and robust, largely excluding the need to mimic the specific duplex structures of endogenous miRNAs, allowing for the creation of highly effective amiRs with fewer sequence requirements. In this study, we introduced tandem amiR duplexes, referred to as "two-hit" amiRs, which are structurally similar but base-wise distinct and can target single or multiple genes of a family for effective silencing. We found that two-hit amiRs have a higher gene-silencing efficiency than traditional single amiR structures in targeting the constitutively expressed *GFP* and endogenous *PDS* reporter in Arabidopsis.

The Two-Hit amiR vector system: A robust approach for gene silencing and overexpression of endogenous miRNAs in a wide range of plant species

The two-hit amiR vector system is an effective method for not only silencing genes with improved efficiency, but also for overexpressing endogenous miRNAs to enhance the depletion of miRNA target genes. This complements loss-of-function studies using short tandem target mimics (STTMs). Using two-hit amiRs, we have generated stable transgenic lines in Arabidopsis that silence the SnRK2 gene involved in ABA signalling and SINAT E3 ligases in Brassinosteroid (BR) signalling pathways (Cai *et al*., 2014; Yang *et al*., 2017). We have also used this approach to overexpress miR165/166 in Arabidopsis with genetic stability to study their role in sorting between AGO1 and AGO10 in terminal flowering (Ji *et al*., 2011). Traditionally, endogenous miRNAs have been overexpressed by cloning and expressing the entire miRNA precursor using a 35S promoter (Gao *et al*., 2016; Li *et al*., 2014; Zhang and Li, 2013). However, the new generation of two-hit amiRs allows for the simple overexpression of endogenous miRNAs in Arabidopsis, rice, and tomato using the same AtmiR168a precursor backbone, demonstrating the applicability of two-hit amiRs in a wide range of species. Many of the two-hit amiR transgenic plants generated in Arabidopsis, rice, tobacco, and tomato show stable inheritance of their characterized features from generation to generation, making two-hit amiRs a useful tool for plant functional genomics and breeding. To facilitate the use of two-hit amiRs, we have developed two new-generation amiR designers for effective gene silencing and functional genomics of miRNAs in plants. In summary, the two-hit amiR vector system provides a powerful technology for plant functional genomics.

The use of Two-Hit amiRs as a complementary approach to other amiR designs

The use of two-hit amiRs as a complementary approach to other amiR designs has been demonstrated through their improved silencing efficiency compared to other amiR designs tested in the miR168 backbone. The *lfy-1* amiR, designed by Weigel's group, displayed improved phenotypic penetrance when expressed

under the miR172a backbone, but showed the same efficiency as the miR168 backbone when expressed through the miR319a backbone. This difference in efficiency may be due to the miR172a backbone inducing additional translational repression in addition to cleavage of the *LFY* target mRNA.

It has recently been shown that the presence of a specific GC-rich sequence signature within the miRNA/miRNA* region is necessary for both precise miRNA biogenesis and efficient amiR mRNA targeting (Narjala *et al*., 2020). This finding highlights the importance of considering the secondary structure of the miRNA/miRNA* region in the design of effective amiRs. The off-target effect observed in the *try-1* and *try-2* amiRs may be the result of the indirect effect of down-regulating *TRY/CPC*-regulated regulatory networks, as many of the tested targets showed higher expression than in the wild-type plants.

Overall, the use of two-hit amiRs as a complementary approach to other amiR designs has the potential to improve the efficiency and effectiveness of gene silencing in a variety of applications. Further research into the mechanisms underlying the improved performance of two-hit amiRs and the development of new design strategies may lead to even more powerful tools for functional genomics research.

Experimental procedures

Vector construction

The construction of the shuttle vector pOT2-Poly-Cis, which contains an *A. thaliana* miR-168a gene and a dual 35S promoter and 35S terminator, has been previously reported (Bhagwat *et al*., 2013; Tang *et al*., 2012). The pri-miR168a DNA was cloned into the pOT2 plasmid and fused with a dual 35S promoter at its 5′ end and a 35S terminator at its 3′ end. To create two-hit amiRs using pOT2-Poly-Cis as a template, inverse PCR was performed with a pair of long primers (each 80 nucleotides) that are in opposite directions to each other (Figure S2). These long primers replaced the natural miR168a/miR168a* regions with specifically designed amiR/amiR* sequences for silencing the target gene(s). Two sets of amiR/amiR* were cloned into the backbone of the precursor miR168a. PmeI and SwaI restriction sites were introduced in the primer pairs for amiR1 and amiR2, respectively. For a two-hit construct, two inverse PCRs were carried out sequentially, with one plasmid containing amiR1 serving as a template for the other containing amiR2, to clone both amiRs in the same construct. For a one-hit amiR, only one inverse PCR was necessary, either for amiR1 or amiR2. All plasmids were verified by the introduced unique PmeI and SwaI sites and subsequently sequenced to confirm the intended construction with the desired sequences.

To transform plants using Agrobacterium, the amiRs, the CaMV d35S (2x enhanced) promoter, the 35S terminator, and CamR selection marker were subcloned into a modified binary vector, pFGC5941-PacI or pCambia 1300-PacI, using inverse PCR with primers containing PacI restriction sites and using the pOT2-d35S-two-hit-amiRs-35 T as a template to delete the plasmid origin. The PCR product and the vector pFGC5941, both containing a unique PacI site, were ligated together after digestion with PacI. To clone GFP amiRs fused with the β-oestradiol promoter, another set of primers were designed with AscI and PacI restriction sites in the forward and reverse primers, respectively. The PCR product amplified the poly-cis region containing the amiRs and was digested with AscI and PacI and then ligated into the AscI/PacI-digested pER8 vector (Zuo *et al*., 2000). All recombinant plasmids were transformed into the *Agrobacterium tumefaciens* strains GV3101 (pMP90) or EHA105 for plant transformation.

Plant materials and growth conditions

All *A. thaliana* lines used in this study were derived from the Columbia-0 (Col-0) background. The GFP142 lines, which express GFP in the ecotype C24, were used for GFP-related experiments (Dalmay *et al*., 2000). *A. thaliana* seeds were surface sterilized with 20% bleach and washed five times with sterile water. The sterilized seeds were suspended in 0.1% agarose and plated on MS medium with 1% sucrose. Plates containing Col-0 and C24 seeds were vernalized in darkness for 3 and 5 days, respectively, at 4 °C. After stratification, the plates were kept under lights at 22 °C with 50% relative humidity in controlled growth chambers, under a long-day (16 h, 100 μmol/m^2/s) white light photoperiod. Potted plants were grown in growth chambers at 22°C and 75% humidity under a 16 h-light/8 h-dark photoperiod. Rice (*Oryza sativa* ssp. japonica cv. Nipponbare) was grown in tissue culture media supplemented with various nutrients and plant hormones at 25–28 °C, under light. Tomato (*Solanum lycopersicum* L. cv. Micro-Tom) was grown at 22–25 °C.

Transformation of Arabidopsis, rice and tomato

Agrobacterium tumefaciens GV3101 harbouring the pFGC5941 or pER8/pCambia1300 vectors were used to transform Arabidopsis plants using a simplified floral dip method (Narusaka *et al*., 2010) or tissue culture methods for rice (Teotia *et al*., 2017) and tomato (Smith *et al*., 1990). Plants displaying severe phenotypes were selected for further analysis. Plants transformed with amiRs against GFP in the inducible vector pER8 were selected on MS medium containing hygromycin (30 μg/mL). After germination, 6-day-old seedlings were transferred to MS medium containing hygromycin and 2 μM β-oestradiol for an additional 3 days, after which the seedlings were used for GFP expression analysis or RNA extraction.

Total RNA isolation, northern blot, real-time PCR and phenotypic analysis

Endogenous miRNA or two-hit amiR over-expression was confirmed by stem-loop qRT-PCR or northern blot, while gene knockdown was confirmed by qRT-PCR. At least three independent lines were analysed for each construct. Total RNA was extracted from plants using Trizol reagent according to the manufacturer's instructions. For gel-blot analysis, total RNA was resolved on a 15% PAGE gel under denaturing conditions (8 M urea). Blots were hybridized using 32P end-labelled oligonucleotide probes complementary to miRNAs and amiRs as described (Yan *et al*., 2012).

For real-time PCR analysis of mRNAs, total RNA was isolated from three biological replicates of each genotype. cDNA was prepared from total RNA using High-Capacity cDNA Kit (Applied Biosystems). qRT-PCR was performed with an Applied Biosystems step one instrument using the 2x SYBR Green PCR Master mix (Applied Biosystems), according to the manufacturer's instructions. *ACTIN2* gene was used as an internal control. cT values were obtained by normalizing to the internal control and then comparing the normalized values to those of control plants. The relative levels of gene expression were calculated using $2^{-\Delta\Delta CT}$ Method (Livak and Schmittgen, 2001).

To measure the levels of miRNAs and amiRs, stem-loop PCR was performed on total RNA as previously described

(Varkonyi-Gasic *et al*., 2007). Real-time PCR was then carried out and analysed in the same way as for mRNAs. The sequences of the probes and primers used can be found in Table S1.

1–3 representative plants each from at least three independent lines from T0 or T1 generations and the controls were taken for each construct for all analyses related to northern and real-time PCR. For expression analyses of GFP, around 10–12 representative seedlings of each construct were pooled together for RNA isolation. For determining the percentage of plants showing phenotypes, for each construct, we analysed about 75–100 plants in Figure 8; more than 300 seedlings in Figures 2a and S6 and at least 50 plants for all other analyses.

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Conflict of interest

The authors declare no conflicts of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 The percentage of positional mismatches between a miRNA guider and a passenger strand at positions 1 to 20.
Figure S2 A map of the pOT2-poly-Cis vector.
Figure S3 The sequence of the pOT2-Poly-Cis region.
Figure S4 The two-hit amiRs targeting the *GFP* transcript.
Figure S5 Arabidopsis GFP142 plants transformed with amiRs against *GFP*.
Figure S6 Two-hit amiRs are more effective in silencing *PDS3* than one-hit amiRs when expressed by an inducible promoter.
Figure S7 Arabidopsis plants transformed with two-hit amiRs against *CTR1*.
Figure S8 Silencing of multiple genes of a family by two-hit amiRs.
Figure S9 Arabidopsis plants transformed with two-hit amiRs against *ARF12, 14, 15, 20, 21, 22*, and *23*.
Figure S10 Overexpression of amiR165/166 in rice using two-hit amiRs technology.
Figure S11 Representative phenotypes of plants with the following constructs.
Figure S12 Expression analysis of predicted targets of amiRs.
Figure S13 A walk-through to design amiRs through the web-tool.
Figure S14 A preview of a miRNA designer.
Table S1 Primers and probes used in the study.