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A new strategy for construction of artificial miRNA vectors in *Arabidopsis*

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Abstract MicroRNAs are a class of small RNAs that specifically suppress their target genes by transcript cleavage or/and translation repression. Natural miRNA precursors have been used for the backbones of artificial miRNA precursors, which can give rise to expected artificial miRNAs with which to repress specific target genes. Artificial miRNA technology is a powerful tool to silence genes of interest. However, it is costly and time-consuming to construct artificial miRNA precursors by the use of an overlapping PCR method. We describe a new strategy to construct artificial miRNAs. A miRNA gene consists of three components (upstream, stem-loop, and downstream regions). Upstream and downstream regions of a natural miRNA transcript were amplified in conjunction with the introduction of two suitable restriction sites in the amplicons, which were inserted into a plasmid to form a median vector. Production of an artificial miRNA vector was easily achieved by insertion of an artificial stem-loop into the median vector. The artificial miRNAs produced by this method efficiently repressed their target genes in *Arabidopsis*. In addition, two artificial miRNA constructs were expressed as one polycistron driven by the CaMV 35S promoter and their targets were suppressed simultaneously in *Arabidopsis*. Thus, artificial miRNAs are a powerful tool with which to analyze rapidly the functions of not only a single gene or multiple homologous genes, but also multiple non-homologous genes.

Keywords miRNA · Artificial miRNAs · *Arabidopsis*

Abbreviations

miRNA	MicroRNA
amiRNA	Artificial microRNA
pri-miRNA	Primary microRNA transcript
nt	Nucleotide
DCL1	Dicer-like 1
AGO1	Argonaute 1
FT	Flowering locus T
TRY	TRIPTYCHON
ETC	ENHANCER OF TRY AND CPC 2
CPC	CAPRICE
RNAi	RNA interference

Introduction

As genome sequencing technology rapidly develops, it becomes increasingly important to investigate the functions of predicted genes. Generally, it is easiest to analyze the functions of a certain gene by analysis of a loss-of-function mutant of this gene. However, searching for naturally occurring loss-of-function alleles or screening large collections of chemically induced or insertion mutants is time-consuming and expensive. It is also very difficult to saturate entire genomes by these approaches. Traditionally, in

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order to investigate the functions of genes without mutants, manipulation of gene expression has relied on either overexpression or RNA interference (RNAi). Transgene-mediated gene silencing by RNAi is a method used for functional studies through the inactivation of target genes.

Several related transgenic silencing approaches to repress target gene expression in plants have been developed, such as transcribed sense, anti-sense, or hairpin RNAs, which subsequently were all found to function through small RNAs that arise from double-stranded RNA precursors (Sen and Blau 2006). These small RNAs are named small interfering RNAs (siRNAs). Conventional siRNAs are processed from a perfect endogenous or exogenous double-stranded RNA by RNaseIII-like enzymes (Dicer or Dicer-like), which produce several double-stranded intermediates of ~21 nt in length (Elbashir et al. 2001). Interference induced by hairpin RNAs can efficiently decrease the abundance of genes of interest. However, hairpin RNA constructs often give rise to unexpected results. For example, a hairpin RNA often produces several siRNAs, which may lead to potential off-target effects. Therefore, hairpin RNA constructs are not the best choice for functional analysis of genes.

The characteristics of plant microRNAs (miRNAs) can make up for the disadvantages of siRNAs. miRNAs are a class of small RNAs that arise from an endogenous transcript (Bartel 2004). Generally, in plants each miRNA precursor is typically processed into a single, predominantly 21–24 nt RNA duplex miRNA/miRNA* by DCL1. Only miRNA (the guide strand) is successfully incorporated into the RNA-induced silencing complex, whereas miRNA* (the passenger strand) is eliminated (Wang 2010). miRNAs regulate their target transcripts by means of RNA cleavage or/and translation repression through imperfect complementary pairs. These characteristics enable plant miRNAs to specifically repress their targets. Recently, artificial miRNAs were confirmed to act in the same manner as natural miRNAs and this approach has been applied for the suppression of genes in *Arabidopsis*, *Oryza sativa*, and *Chlamydomonas reinhardtii* (Schwab et al. 2006; Alvarez et al. 2006; Warthmann et al. 2008; Molnar et al. 2009). Artificial miRNAs, as a new tool for the suppression of gene expression, can repress not only a single gene, but also several homologous genes. Given functional redundancy of homologous genes, it is usually difficult to analyze the function of a gene of interest, even though a single mutant is obtained. Thus, artificial miRNA technology will advance the process of reverse genetics research in future.

To construct an artificial miRNA, site-directed mutagenesis on precursors of endogenous miRNAs is performed using overlapping PCR (Schwab et al. 2006). Oligonucleotide primers are used to replace miRNA and miRNA* regions with artificial sequences. This approach involves four PCR reactions, which is often time-consuming because of failed PCR reactions and also raises the possibility of PCR-introduced errors. To shorten the construction time of an artificial miRNA and decrease the possibility of base mutation, in the present study we developed two median vectors containing two restriction sites. An artificial miRNA construct can be obtained by inserting an artificial stem-loop produced from one PCR reaction into the corresponding median vector. Experimental results revealed that artificial miRNA precursors constructed by this approach can be processed into mature artificial miRNAs, which then suppress expression of their targets in *Arabidopsis*. In addition, when two different artificial miRNA precursors were fused and expressed as one polycistron driven by the CaMV 35S promoter, both mature artificial miRNAs were produced and their respective target genes were suppressed simultaneously.

Materials and methods

Plant growth conditions

Arabidopsis thaliana (accession Columbia) seeds (obtained from the *Arabidopsis* Information Resource, http://www.arabidopsis.org) were surface sterilized with 20% bleach and washed three times with sterile water. Sterilized seeds were suspended in 0.1% agarose and plated on MS medium. Plates were vernalized in darkness for 2 days at 4°C and then transferred to a tissue culture room at 22°C under a 16 h-light/8 h-dark photoperiod. Potted plants were grown in growth chambers at 22°C and 75% humidity under a 16 h-light/8 h-dark photoperiod.

Vector construction and plant transformation

The upstream and downstream regions of MIR319a and MIR395a were amplified by PCR using the following primer pairs, with lower-case letters indicating the introduced restriction sites: for MIR319a upstream, AAAggatccCAAACACACGCTCGGACGCAT (*Bam*HI) and AAAgatatcATATATTCCTAAAACATCAATTC (*Eco*RV); for MIR319a downstream, AAAgaattcTTTGTATTCCAATTTTCTTGATTAA (*Eco*RI) and TTTaagcttCATGGCGATGCCTTAAATAAAG (*Hin*dIII); for MIR395a upstream, AAAggatccTTGGTCATCATCTTATGATCTC (*Bam*HI) and AAAgatatcTCCAACCAAGAATATTGTTTCTTGA (*Eco*RV); for MIR395a downstream, AAAgaattcTTCAACGGATTTCCGCAGGTC (*Eco*RI) and TTTaagcttCCGCTTATACGTATCTAACATTACG (*Hin*dIII). Next, the upstream and downstream products were inserted into the pBluescript KS plasmid to form

pAMIR319a and pAMIR395a, respectively. The stem-loop regions were amplified by the following primer pairs: for MIR319a, AAAgatatcAGAGAGCTTCCTTGAGTCCATT CAC (*Eco*RV) and TTTgaattcAGGGAGCTCCCTTCAG TCCAATC (*Eco*RI); for MIR395a, AAAgatatcCTAGAG TTCCTCTGAGCACTTCATTG (*Eco*RV) and TTTgaattc CGGGAGTTCCCCCAAACACTTCAGT(*Eco*RI); for amiR-ft in a MIR319a backbone, AAAgatatcGGACTCTTCCTT TTTAACCATTCACAGGTCGTGATATGATTCA (*Eco*RV) and AAAgaattcGGCCTCTTCCTTTATAACCAATCAAA GAGAATCAATGATCCA (*Eco*RI); for amiR-tri in a MIR395a backbone, AAAgatatcCTAGGCGAGCAGTGT CGAATGGGTTGGGGATACAATTTTTCTAAATG (*Eco*RV) and AAAgaattcCGGGGCGAGCAGTATCGAATGGGAT GGATAATCATTTAGAAAAATTG (*Eco*RI). These stem-loop sequences were inserted into pAMIR319a and pA-MIR395a to form the complete backbones, which then were cloned into pOCA30 binary vectors. The binary vectors were then transformed into *Agrobacterium tumefaciens* strain GV3101. *Arabidopsis* transformation was conducted by the floral dip method. Transgenic plants were selected on MS medium supplemented with 50 μg/mL kanamycin.

Determination of sulfate content

Leaves were harvested from 3 week-old plants for determination of sulfate ions. About 20 mg of fresh plant samples were immersed in 1 ml of 0.1 M HCl for 2 h at room temperature. After centrifugation at 12,000*g*, the supernatant was recovered and used for the determination of sulfate content by the turbidimetric method (Liang et al. 2010). Appropriate calibration curves were constructed, using potassium sulfate as a standard.

Northern blotting and real-time RT-PCR

Total RNA was isolated from plant tissues with the use of TRIzol reagent (Invitrogen). For high-molecular-weight RNA gel blot analysis, 20 μg total RNA was separated on a 1.5% agarose gel and transferred to a Hybond-N^+ membrane. Probes were labeled with [α-^{32}P]dATP using the Klenow fragment from *Escherichia coli* DNA polymerase I. Low-molecular-weight RNAs were separated by electrophoresis on denaturing 15% polyacrylamide gels, and miRNA gel blot hybridizations were performed as described previously (Liang et al. 2010). For small RNA gel blots, 20 μg total RNA was separated on a 15% polyacrylamide gel with 7 M urea. DNA oligonucleotides complementary to miRNAs or amiRNAs were end-labeled using T4 polynucleotide kinase and used for hybridizations.

After total RNA was digested with DNase I (Fermentas), reverse transcription was carried out in accordance with the manufacturer's protocol (Fermentas). First-strand cDNA derived from 0.5 μg total RNA was used for amplification of target genes by the following gene-specific primers: for *FT*, CTGCGGAGGAAGAAGACTTTAGA and ACTATA GGCATCATCACCGTTCG; for *TRY*, TAATGGATAAC ACTGACCGTCGT and CTGCTATCAAATCCCACCTA TCA; for *CPC*, TGTTTCGTGTCTTCAGATTAGTTCG and CGAGTTTATACATCCGAGAAATGAG; for *ECT2*, CGGTCCCAGTCTTAGGCAAACTA and GCCTTTCTT CCTACGACTCTTCC; for *ACT2*, TGTGCCAATCTAC GAGGGTTT and TTTCCCGCTCTGCTGTTGT. PCR reactions were performed with SYBR® Premix Ex Taq™ (Tli RNaseH Plus) on a Roche LightCycler® 480 real-time PCR system in accordance with the manufacturer's instructions. *ACT2* (At3g18780) was used as an internal control in real-time RT-PCR. The resulting data are presented as the mean ± SE of three technical replicates. Student's *t* test was used to compare the means of two populations.

A simple protocol for construction of an artificial miRNA

A suitable artificial miRNA for a gene of interest can be designed by WMD3 (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi; Schwab et al. 2006). In theory, an artificial miRNA can be processed from any natural miRNA precursor as a backbone. Based on MIR319a and MIR395a transcripts, we constructed two median vectors, pA-MIR319a and pAMIR395a. When MIR319a was chosen for the backbone of an amiRNA of interest, two specific primers were designed based on the MIR319a stem-loop (Fig. 1b; Online Resource 1). The resulting PCR products were digested with the restriction enzymes *Eco*RV and *Eco*RI and then inserted into the pAMIR319a vector to form an artificial miRNA construct. When MIR395a was used for the backbone, an artificial stem-loop was obtained by primer extension (Fig. 1b; Online Resource 1). Similarly, the PCR products were digested and subcloned into the pAMIR395a vector with *Eco*RV and *Eco*RI. For the expression of an artificial miRNA, the construct was driven by the CaMV 35S or a gene-specific promoter. A detailed protocol is available as Online Resource 1.

Results

Generation of two median vectors

Generally, a miRNA primary transcript consists of upstream, stem-loop, and downstream regions (Fig. 1a). Upstream and downstream regions are crucial for processing of a miRNA precursor in *Arabidopsis* (Song et al. 2010). Conventionally, the construction of an artificial

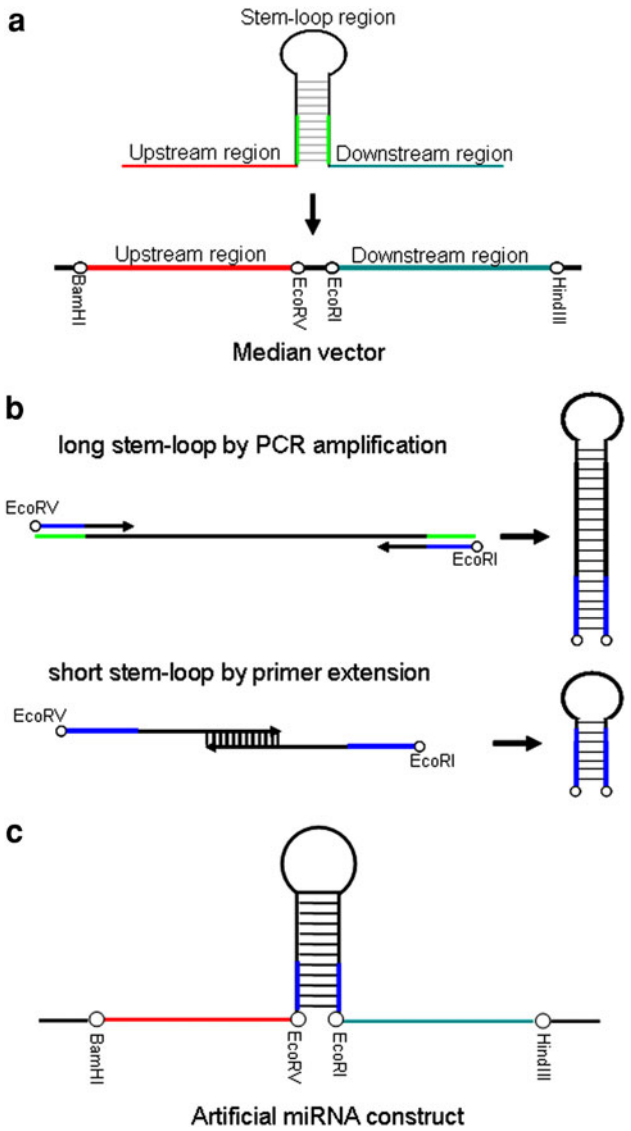

Fig. 1 Strategy of artificial miRNAs construction. **a** Construction of a median vector. The upstream and downstream regions of an endogenous miRNA transcript are amplified with primers that contain different restriction sites and inserted into the multiple cloning site (MCS) of the pBluescript KS plasmid to form a median vector. **b** Generation of artificial miRNA stem-loops. A long stem-loop is amplified by a pair of specific primers designed on the basis of the natural miRNA stem-loop. A short stem-loop is amplified by a primer extension-based method (a pair of primers with 15–20 nt complementary to each other are used for annealing and extension to produce the stem-loop sequences). **c** Production of artificial miRNA constructs. An artificial miRNA construct is produced by insertion of an artificial stem-loop in the corresponding median vector. **a**, **b** and **c** The *blank circles* indicate restriction sites, *green lines* indicate natural miRNA or miRNA*, and *blue lines* indicate artificial miRNA or miRNA*

miRNA backbone by the overlapping-PCR-based method requires multiple PCR reactions to generate the expected site-mutated fragment (Schwab et al. 2006; Online Resource 2), which is time-consuming and does not always readily succeed. In addition, the resulting PCR products often contain nucleotide mutations or deletions. Thus, a minimal number of PCR reactions is required for rapid construction of a correct artificial miRNA backbone. Given that construction of an artificial miRNA backbone only requires the substitution of a natural stem-loop for an artificial one, the upstream and downstream regions of a natural miRNA gene can be subcloned into the pBluescript KS plasmid with restriction sites to form a median vector. An artificial backbone can be generated by inserting an artificial miRNA stem-loop (Fig. 1b) with restriction sites into the vector (Fig. 1c).

MIR319a has been used previously as a backbone of artificial miRNAs (Schwab et al. 2006). In our protocol, we amplified a 404 bp genomic DNA sequence containing a miR319a precursor and inserted it into pBluescript KS to create the template vector (this vector was used as the template for amplification of upstream, downstream, and stem-loop regions, as well as an artificial stem-loop). This fragment is sufficient for the production of a mature miR319 in *Arabidopsis* (Schwab et al. 2006). Two restriction sites (*Eco*RI and *Eco*RV) with four complementary bases for each other were chosen and used to maintain the original secondary structure of MIR319a (Fig. 2a). Upstream and downstream regions of MIR319a were amplified with separate pairs of primers containing the two restriction sites and the regions were subcloned into the pBluescript KS plasmid to form pAMIR319a. To investigate whether the introduction of restriction enzymes sites affects the production of mature miRNAs, the MIR319a stem-loop was amplified and inserted into pA-MIR319a with *Eco*RI and *Eco*RV to generate an artificial MIR319a. The natural MIR319a and artificial MIR319a (AMIR319a) were introduced into the binary vector downstream of the *Cauliflower mosaic virus* (CaMV) 35S promoter and then introduced into wild-type *Arabidopsis* by *Agrobacterium*-mediated transformation. By kanamycin screening, 36 and 40 plants transgenic for 35S::MIR319a and 35S::AMIR319a, respectively, were obtained. All transgenic plants displayed a similar phenotype with undulate leaves (Fig. 2c). Northern blotting analysis indicated that transcripts from both natural and artificial MIR319a constructs were processed into mature miR319 (Fig. 2b). These results revealed that the introduction of two restriction sites into the natural MIR319a backbone did not disrupt the processing of mature miR319.

MIR319a contains a long (172 bp) stem-loop structure (Fig. 2a). To confirm whether this strategy is also effective for modification of other endogenous miRNA transcripts with a short stem-loop, we constructed another median vector, pAMIR395a, by the same method. The stem-loop of MIR395a was 73 bp long (Fig. 3a), which could be directly synthesized or easily produced by primer extension. As for MIR319a, both natural MIR395a and artificial

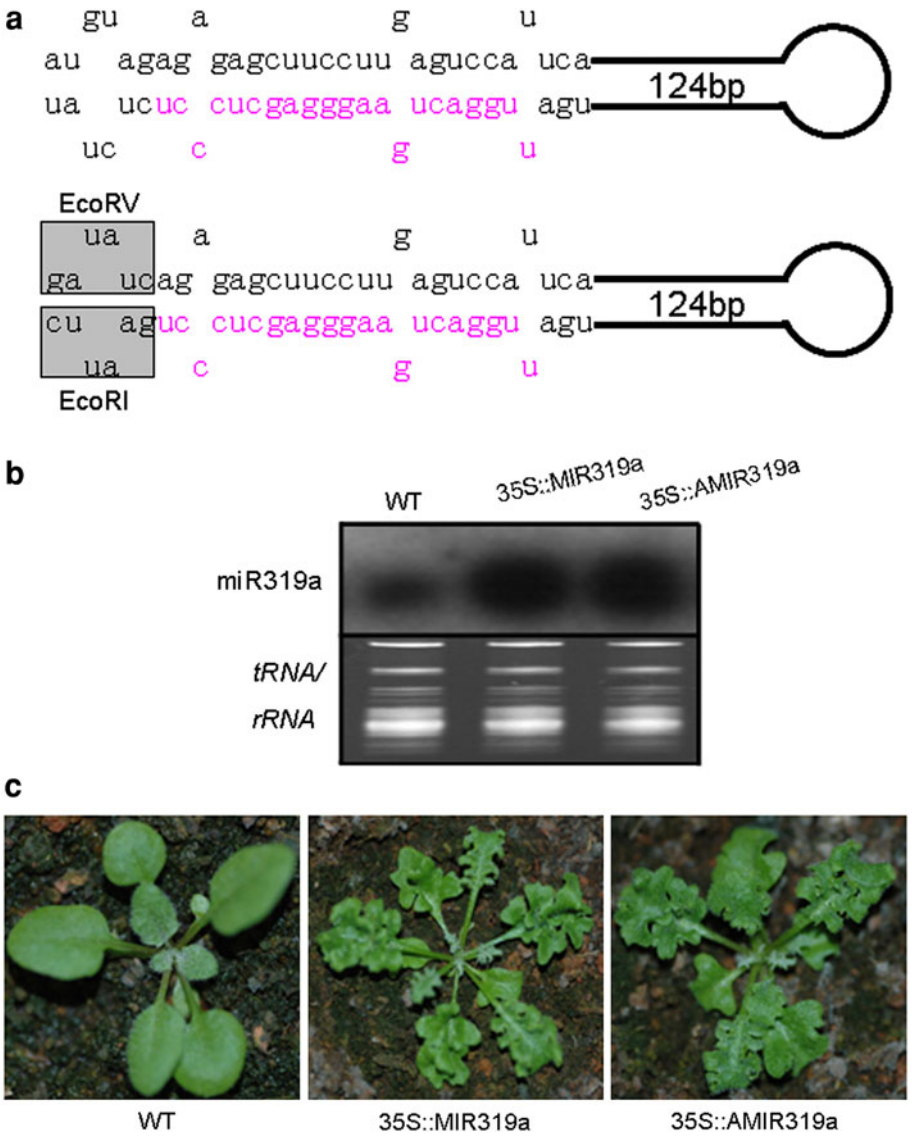

Fig. 2 Establishment of the median vector pAMIR319a. **a** Two restriction sites (*Eco*RV and *Eco*RI) were introduced into the MIR319a stem-loop ends. **b** Detection of mature miR319a by Northern blotting. Total RNA (20 μg) extracted from leaves was used for hybridization. **c** MIR319a and AMIR319a transgenic plants showed similar phenotypes

MIR395a transgenic plants highly expressed miR395 (Fig. 3b). A recent study revealed that overexpression of miR395 resulted in sulfate overaccumulation in *Arabidopsis* leaves (Liang et al. 2010). Therefore, we measured the sulfate content in transgenic and wild-type plants, which demonstrated that both MIR395a and AMIR395a transgenic plants accumulated more sulfate in leaves than the wild-type plants did (Fig. 3c). Taken together, our results suggested that the introduction of two suitable restriction enzyme sites into an endogenous miRNA backbone did not disrupt the production of mature miRNAs. This stem-loop insertion strategy will improve the efficiency of artificial miRNAs construction.

amiR-ft produced from pAMIR319a efficiently suppressed its single target *FT*

Artificial miRNAs have been used for specific silencing of target genes in *Arabidopsis*, rice and *Chlamydomonas reinhardtii* (Alvarez et al. 2006; Schwab et al. 2006; Warthmann et al. 2008; Molnar et al. 2009). The *FT* gene encodes a protein that controls flowering time in *Arabidopsis*, and loss-of-function of the gene strongly delays the flowering time (Koornneef et al. 1991). An artificial miRNA, amiR-ft, has been used previously for the suppression of *FT* (Schwab et al. 2006). To investigate whether our median vectors could be used for expression of amiR-ft, the stem-loop of amiR-ft (Fig. 4a) was amplified by a pair of specific primers (the primers containing the restriction sites, *Eco*RI and *Eco*RV, were designed on the basis of the artificial miRNA sequence and MIR319a stem-loop structure; Online Resource 1) with the template vector as a template. The amplified fragment was inserted into the pAMIR319a vector to form an amiR-ft construct that was then fused with the CaMV 35S promoter to yield 35S::AMIR-ft. In the 28 independent transgenic lines we examined, all 35S::AMIR-ft transgenic plants had not

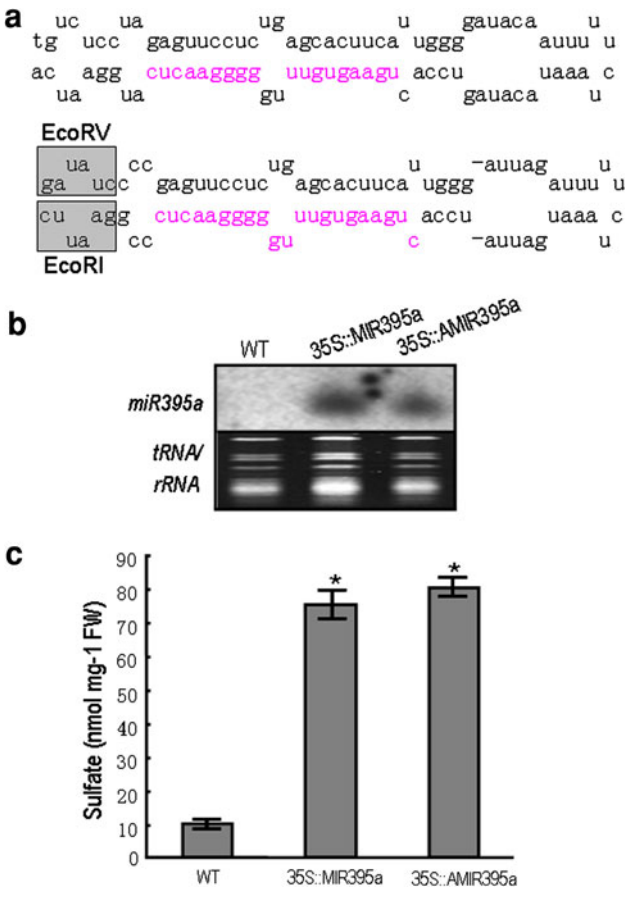

Fig. 3 Establishment of the median vector pAMIR395a. **a** Two restriction sites (*Eco*RV and *Eco*RI) were introduced into the MIR395a stem-loop ends. **b** Detection of mature miR395a by Northern blotting. Total RNA (20 μg) extracted from leaves was used for hybridization. **c** Both 35S::MIR395a and 35S::AMIR395a transgenic plants accumulated a high sulfate content in leaves compared with the wild type. *Values* marked by an *asterisk* are significantly different from the corresponding wild-type value with Student's *t* test ($P < 0.01$; $n = 10$)

flowered by the time wild-type plants had already produced many siliques (Fig. 4c), which indicated that the flowering time of transgenic plants was dramatically delayed. We further detected the expression levels of amiR-ft by Northern blotting with anti-sense amiR-ft sequences as the probe. As shown in Fig. 4b, 35S::AMIR-ft transgenic plants overexpressed amiR-ft, whereas no signal was detected in wild-type plants. Since amiR-ft was designed to target the *FT* gene, we determined the expression of *FT* transcripts. As expected, *FT* transcription was strongly decreased in 35S::AMIR-ft plants compared with that in wild-type plants (Fig. 4d). These results suggested that the amiR-ft construct generated from pAMIR319a was efficient for the production of amiR-ft, which then suppressed *FT* expression and led to a delayed flowering time in 35S::AMIR-ft plants.

amiR-tri expressed from pAMIR395a silenced its three homologous target genes

Target genes of most *Arabidopsis* miRNAs usually belong to the same gene families (http://asrp.cgrb.oregonstate.edu/db/microRNAfamily.html). Similarly, artificial miRNAs can also be used to target multiple homologous genes. amiR-tri was designed to target three *MYB* family genes, *TRY*, *ETC2*, and *CPC* (Fig. 5a), which are involved in the formation of *Arabidopsis* trichomes (Schellmann et al. 2002; Kirik et al. 2004). To generate an amiR-tri construct in the MIR395a backbone, the amiR-tri stem-loop was amplified with a pair of specific primers (the primers containing the restriction sites, *Eco*RI and *Eco*RV, were designed on the basis of the artificial miRNA sequence and MIR395a stem-loop structure, Online Resource 1) by a primer extension-based method (Gou et al. 2007). The amiR-tri stem-loop was inserted into the pAMIR395a vector, then the amiR-tri construct was fused with the CaMV 35S promoter to generate 35S::AMIR-tri. Out of 18 independent 35S::AMIR-tri transgenic lines, 17 transgenic plants produced clustered trichomes on the leaf surface (Fig. 5b). Northern blotting was used to assess amiR-tri accumulation in transgenic plants. The results indicated that amiR-tri was overaccumulated in 35S::AMIR-tri plants, but not in wild-type plants (Fig. 5c). In accordance with this result, the predicted target genes, *ETC2* and *CPC*, were dramatically down-regulated in 35S::AMIR-tri plants, whereas the expression of *TRY* decreased weakly (Fig. 5d). The phenotype of 35S::AMIR-tri plants phenocopied that of *try–cpc* double mutants (Schellmann et al. 2002), which indicated that amiR-tri was expressed by the pAMIR395a vector and it successfully silenced its three homologous target genes.

Two different artificial miRNAs can be expressed as one polycistron

In animals, about 40–50% of predicted miRNA genes are located within clusters (Altuvia et al. 2005; Kim et al. 2009). Certain animal miRNA clusters, which show co-regulated expression, can simultaneously target genes that encode different functionally related proteins (Lee et al. 2002; Tanzer and Stadler 2004). In *Arabidopsis*, four miRNA clusters were transcribed as polycistron precursors and ath-miR859-774 was confirmed to be coexpressed and to form both mature miRNA species (Merchan et al. 2009). To explore whether two artificial miRNAs produced from pAMIR319a and pAMIR395a could be transcribed simultaneously, the amiR-ft construct was fused with the amiR-tri construct to form the 35S::AMIR-tri-ft construct (Fig. 6a). Twelve independent transgenic plants were obtained, of which all displayed similar phenotypes of

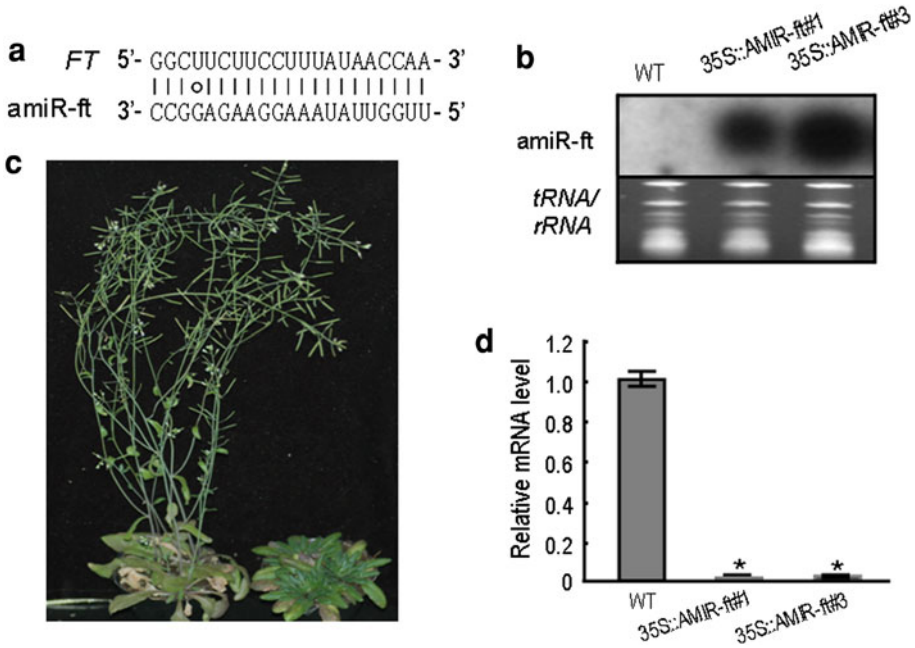

Fig. 4 Expression of amiR-ft with the pAMIR319a vector. **a** The predicted complex of amiR-ft and its single target, *FT*. **b** Examination of amiR-ft by Northern blotting. Total RNA (20 μg) extracted from leaves was used for hybridization. **c** The 35S::AMIR-ft plants (*right*) showed a delayed flowering phenotype under long-day conditions. **d** Determination of *FT* transcripts by real-time quantitative PCR. *Values* marked by an *asterisk* are significantly different from the corresponding wild-type value with Student's *t* test ($P < 0.01$; $n = 3$)

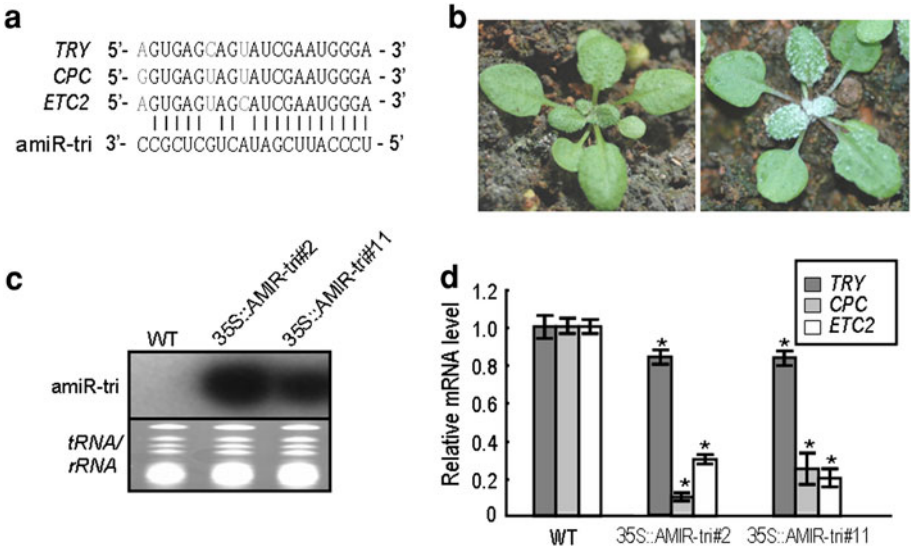

Fig. 5 Expression of amiR-tri with the pAMIR395a vector. **a** The predicted complex of amiR-ft and its three homologous target genes. **b** The 35S::AMIR-tri plants (*right*) produced clustered trichomes on the leaf surface. **c** Examination of amiR-tri by Northern blotting. Total RNA (20 μg) extracted from leaves was used for hybridization. **d** Determination of *TRY*, *CPC*, and *ETC2* transcripts by real-time quantitative PCR. *Values* marked by an *asterisk* are significantly different from the corresponding wild-type value with Student's *t* test ($P < 0.01$; $n = 3$)

increased trichome density on the leaf surfaces and significantly delayed flowering time (Fig. 6c). Clearly, these phenotypes were a combination of the phenotypes of 35S::AMIR-ft and 35S::AMIR-tri transgenic plants. As expected, both amiR-ft and amiR-tri were overexpressed in 35S::AMIR-tri-ft plants (Fig. 6b). Consistent with these results, the target transcripts of both amiR-ft and amiR-tri were down-regulated in 35S::AMIR-tri-ft plants (Fig. 6d). These results showed that two different artificial miRNAs could be transcribed as one polycistron that was then processed into two mature artificial miRNAs.

Discussion

Like natural miRNAs, artificial miRNAs can specifically repress their target genes (Schwab et al. 2006), thus artificial miRNAs are a powerful tool for the suppression of gene expression in plants. Plant miRNA stem-loops are longer and more heterogeneous than those of their animal counterparts (Voinnet 2009). A plant pri-miRNA contains three regions, namely upstream, stem-loop, and downstream regions. Bioinformatic analysis of pri-miRNA structures and physical mapping of initial cleavage sites revealed that the first loop-distal cleavage occurs at a

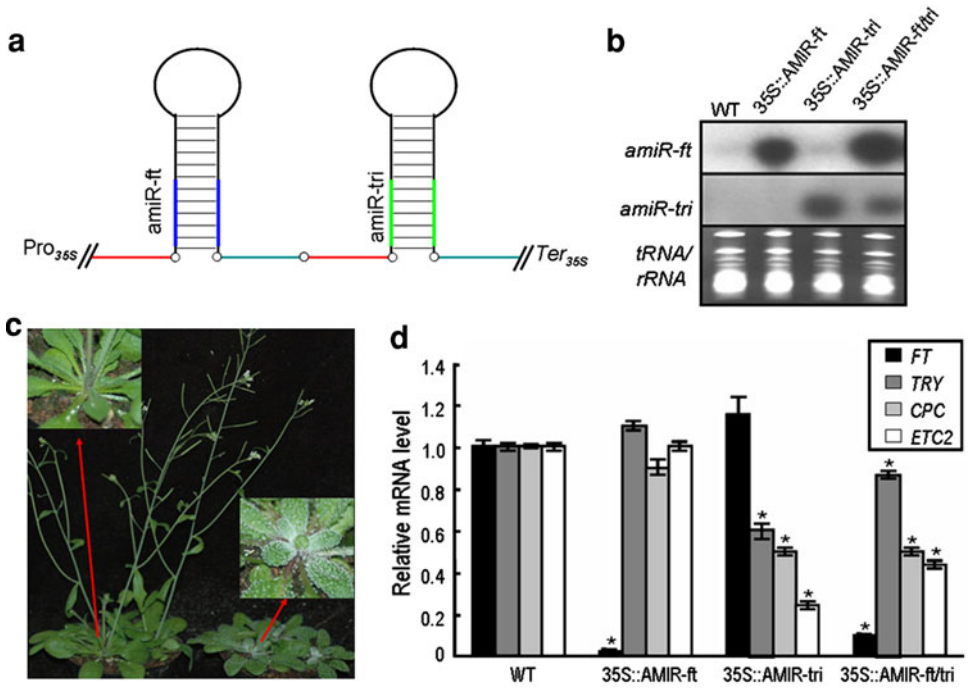

Fig. 6 Two different artificial miRNAs expressed as one polycistron. **a** A schematic diagram of construction of an expression vector containing two artificial miRNA precursors. The *blank circles* indicate restriction sites. **b** Examination of amiR-ft and amiR-tri by Northern blotting. Total RNA (20 μg) extracted from leaves was used for hybridization. **c** Phenotypes of 35S::AMIR-ft/tri transgenic plants. 35S::AMIR-ft/tri plants (*right*) showed the phenotypes of delayed flowering and clustered trichomes on the leaf surface. **d** Real-time quantitative RT-PCR analysis of *FT*, *TRY*, *CPC*, and *ETC2*. *Values* marked by an *asterisk* are significantly different from the corresponding wild-type value with Student's *t* test ($P < 0.01$; $n = 3$)

distance of ~15 nt from an unpaired region in *Arabidopsis* (Song et al. 2010). Hence, not only the stem-loop region but also upstream and downstream regions are crucial for the process of miRNA maturation. To ensure efficient processing of an artificial miRNA, a long backbone containing upstream and downstream sequences is required. The overlapping-PCR-based method has been used to produce an artificial miRNA construct (Schwab et al. 2006). However, this method involves four PCR reactions, which increases the risk of sequence mutation and prolongs the miRNA construction process.

In this study, we describe a new strategy to produce an artificial miRNA construct. Two median vectors (pAMIR319a and pAMIR395a) were constructed that can be used as the backbones of artificial miRNAs in *Arabidopsis*. Both vectors contain two restriction sites (*Eco*RI and *Eco*RV), which allows the insertion of a stem-loop that includes an artificial miRNA sequence. Because upstream and downstream regions of a natural miRNA transcript have been cloned into the median vectors, only one stem-loop sequence from one PCR reaction is required for the production of an artificial miRNA construct. This method improves the efficiency of construction of artificial miRNA vectors.

The overexpression of artificial miRNAs designed to target genes of interest can result in robust and strong phenotypes that resemble those of plants with mutations in the respective target genes. Our experiments confirmed that artificial miRNAs constructed by the use of pAMIR319a or pAMIR395a can be expressed successfully and the transgenic plants phenocopied the mutants of target genes, which implied that these two vectors are effective for the processing of mature artificial miRNAs in *Arabidopsis*. Artificial miRNAs as a tool of gene suppression are convenient and rapid for the reverse genetics research. Given that two different plant miRNAs driven by the same promoter can be transcribed simultaneously (Merchan et al. 2009), we also attempted to construct two different artificial miRNAs as a polycistron driven by the CaMV 35S promoter. The results revealed that these two artificial miRNAs were expressed simultaneously in *Arabidopsis* and the transgenic plants' phenotypes resembled those of their respective target gene mutants. In plants, many homologous genes with high sequence similarity often are closely linked on chromosomes, which blocks the construction of double mutants or multiple mutants, and thus increases the difficulty of analyzing the function of these genes. To design a multiple-target artificial miRNA for these homologous genes is a suitable approach. In addition, when two non-homologous genes are closely linked, two artificial miRNAs can be coexpressed to repress the expression of these two genes, which mimics the double mutants. Therefore, the application of artificial miRNAs will contribute to progress in gene function analysis by reverse genetics.

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