6 Artificial Small RNA-based Strategies for Effective and Specific Gene Silencing in Plants

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6.1 Introduction

In plants, small RNAs (sRNAs) function in diverse RNA silencing pathways to regulate development, control genome integrity and protect against viruses (Borges and Martienssen, 2015). MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) are the two main classes of plant sRNAs acting in post-transcriptional gene silencing pathways. Both associate with an ARGONAUTE (AGO) protein to target and silence highly sequence-complementary transcripts through direct AGO-mediated endonucleolytic cleavage or through their translational repression (Axtell, 2013). MiRNAs and siRNAs differ in their biogenesis pathways as well as in the spectrum of their target transcripts. MiRNAs arise from endogenous miRNA transcripts with imperfect self-complementary foldback structures processed by DICER-LIKE1 (DCL1), and target other cellular transcripts. SiRNAs originate from transposons, centromeres or exogenous nucleic acids such as transgene inverted repeats or viral RNAs, are processed by DCL2, DCL3 or DCL4 and usually silence the transcript from which they derive. *Trans*-acting siRNAs (tasiRNAs) are a particular subclass of plant siRNAs that are produced in a sophisticated way. In *Arabidopsis thaliana*, the cleavage of a transcript from the *TAS* family by a miRNA/AGO complex triggers the RNA-DEPENDENT RNA POLYMERASE6 (RDR6)-dependent synthesis of double-stranded RNA (dsRNA) from one of the cleavage products. DCL4 sequentially processes the dsRNA into 21 nucleotide (nt) tasiRNA duplexes in register with the miRNA-guided cleavage site. In all cases, one strand of the miRNA or tasiRNA duplex (named the 'guide strand') is selectively incorporated into an AGO protein to direct specific silencing of cognate transcripts, while the other strand (named the 'star strand', or *) is generally degraded (Bologna and Voinnet, 2014).

Endogenous sRNA-directed silencing pathways have been exploited in plants to selectively silence genes of interest in gene function studies, and to generate antiviral resistance or other type of crop improvement. Classic RNA interference (RNAi) approaches such as virus induced gene silencing (VIGS) or hairpin-based silencing are based on the expression of dsRNA or dsRNA-like precursors. These sequences

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  Plant Gene Silencing: Mechanisms and Applications (ed. T. Dalmay)

correspond to the target transcript to trigger the DCL-dependent production of siRNAs which will silence complementary target sequences (Ossowski *et al*., 2008; Baykal and Zhang, 2010). In VIGS, a sequence corresponding to the target transcript is inserted into a viral genome. Replication of the virus in a susceptible host triggers the synthesis by endogenous RDRs of dsRNAs of viral sequence, including those derived from the target RNA sequence that was inserted in the VIGS construct. In hairpin-based silencing, a transgene containing an inverted repeat sequence of the target transcript is introduced into plants and expresses a hairpin RNA (hpRNA) with a characteristic stem-loop secondary structure. Unfortunately, and despite their massive use in recent years, both approaches have a relatively high risk of inducing undesired off-target effects, as the populations of siRNA species produced from dsRNAs or hpRNAs may lead to the accidental targeting of other cellular transcripts sharing high sequence complementarity with that of certain siRNAs.

6.2 Plant Artificial Small RNAS

The limited specificity of classic plant RNAi approaches was overcome by the recent development of 'second-generation RNAi' strategies based on artificial sRNAs. These strategies include artificial microRNAs (amiRNAs) and synthetic tasiRNAs (syn-tasiRNAs). In both cases the artificial sRNA is designed to specifically silence the desired target(s) with no off-target effects, and is produced *in planta* by expressing a functional miRNA or tasiRNA precursor with modified miRNA/miRNA* or tasiRNA sequences, respectively. Details of the biogenesis, action and application of both classes of plant artificial sRNAs are provided below.

6.2.1 Artificial microRNAs

AmiRNAs have been the most extensively used plant artificial sRNAs. AmiRNAs are generated *in planta* by expressing amiRNA transgenes that include the sequence of a plant miRNA precursor in which the endogenous miRNA sequence is substituted by the sequence of the designed amiRNA (Fig. 6.1). Other sequences of the miRNA precursor (including the miRNA*) are also modified to preserve the original secondary structure of the miRNA precursor and allow its accurate processing by DCL1 to produce the 21 nt amiRNA duplex. The amiRNA guide strand is usually designed with a 5´U to associate with AGO1 and direct the endonucleolytic cleavage or the translational repression of target genes (Li *et al*., 2013; Tiwari *et al*., 2014; Yu and Pilot, 2014).

Since their initial applications (Alvarez *et al*., 2006; Schwab *et al*., 2006) amiRNAs produced from different miRNA precursors have been used in multiple plant species – including eudicots, monocots, mosses or algae – (Table 6.1) to silence endogenous genes and non-coding RNAs, reporter transgenes and viruses (reviewed in Tiwari *et al*., 2014). AmiRNAs are typically designed to target a single transcript, but in some cases it is possible to design amiRNAs to target multiple transcripts simultaneously in cases where these share enough sequence similarity (e.g. members of the same gene family). For example, a single amiRNA was used

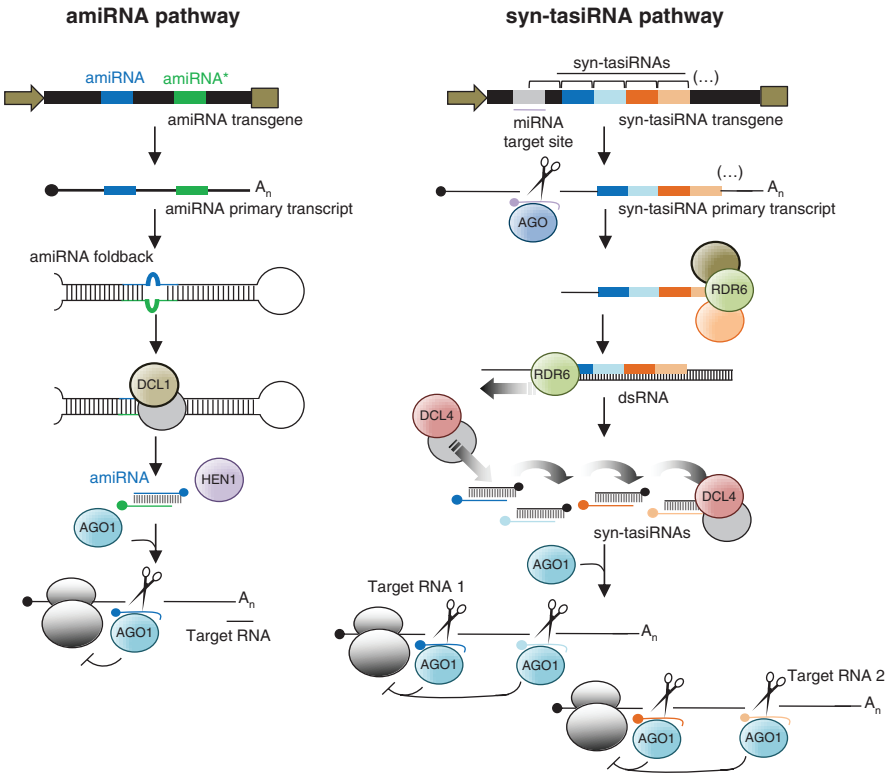

Fig. 6.1. Artificial sRNA pathways in plants. Left, the amiRNA pathway. An amiRNA transgene, containing a plant miRNA precursor in which the original miRNA/miRNA* sequences have been substituted by the amiRNA/amiRNA* sequences, is introduced into plants to express an amiRNA primary transcript processed into an amiRNA foldback. A rational amiRNA design requires that the amiRNA foldback preserves the original secondary structure of the endogenous precursor. DCL1 further processes the amiRNA foldback to produce the amiRNA duplex methylated by HEN1. The 5´U amiRNA strand is usually incorporated into AGO1 to silence highly complementary transcripts by direct slicing or by repressing their translation. Right, syn-tasiRNA pathway. A syn-tasiRNA transgene, containing a plant *TAS* precursor in which a subset of the original tasiRNA sequences has been substituted by several syn-tasiRNA sequences in tandem, is introduced into plants to express a syn-tasiRNA primary transcript. An endogenous miRNA cleaves this primary transcript, a process that triggers the recruitment of RDR6 complexes to synthesize a dsRNA from one of the cleavage products. DCL4 processes the dsRNA into phased tasiRNA duplexes in 21 nt register with the miRNA cleavage site. Syn-tasiRNA guide strands with a 5´U are incorporated into AGO1 to direct specific silencing of sequence-unrelated target transcripts at one or multiple sites.

to downregulate three *MYB* transcripts (*TRIPTYCHON* (*TRY*), *CAPRICE* (*CPC*) and *ENHANCER OF TRIPTYCHON AND CAPRICE2* (*ETC2*)) and induce high numbers of clustered trichomes in *A. thaliana* rosette leaves (Schwab *et al.*, 2006; Liang *et al.*, 2012; Carbonell *et al.*, 2014). Multiple amiRNAs can be expressed from a

Table 6.1. Endogenous precursors used to produce artificial sRNAs in plants.

Artificial sRNA	Precursor	Species tested	Original reference
amiRNA	*Ath-miR159a*	*Arabidopsis thaliana*	Niu *et al*., 2006
		Nicotiana benthamiana	Mitter *et al*., 2016
		Nicotiana tabacum	Mitter *et al*., 2016
		Solanum lycopersicum	Zhang *et al*., 2011
	Ath-miR159b	*Arabidopsis thaliana*	Eamens *et al*., 2011
	Ath-miR164a	*Arabidopsis thaliana*	Alvarez *et al*., 2006
		Solanum lycopersicum	Alvarez *et al*., 2006
	Ath-miR164b	*Nicotiana tabacum*	Alvarez *et al*., 2006
	Ath-miR168a	*Solanum tuberosum*	Bhagwat *et al*., 2013
	Ath-miR169d	*Arabidopsis thaliana*	Liu *et al*., 2010
	Ath-miR171a	*Arabidopsis thaliana*	Qu *et al*., 2007
	Ath-miR172a	*Arabidopsis thaliana*	Schwab *et al*., 2006
	Ath-miR319a	*Arabidopsis thaliana*	Schwab *et al*., 2006
		Catharanthus roseus	Li *et al*., 2013
		Corchorus olitorius	Shafrin *et al*., 2015
		Glycine max	Melito *et al*., 2010
		Helianthus annuus	Li *et al*., 2013
		Medicago sativa	Verdonk and Sulllivan, 2013
		Nicotiana benthamiana	Li *et al*., 2013
		Nicotiana tabacum	Vu *et al*., 2013
		Petunia hybrida	Guo *et al*., 2014
		Phaeodactylum tricornutum	Kaur and Spillane, 2015
		Physcomitrella patens	Khraiwesh *et al*., 2008
		Solanum lycopersicum	Fernandez *et al*., 2009
		Solanum melongena	Toppino *et al*., 2011
		Solanum tuberosum	Wyrzykowska *et al*., 2016
		Vitis vinifera	Jelly *et al*., 2012
		Zea mays	Li *et al*., 2013
	Ath-miR390a	*Arabidopsis thaliana*,	Montgomery *et al*., 2008a
		Nicotiana benthamiana	Montgomery *et al*., 2008a
	Ath-miR395a	*Arabidopsis thaliana*	Liang *et al*., 2012
	Cre-miR1157	*Chlamydomonas reinhardtii*	Molnar *et al*., 2009
	Cre-miR1162	*Chlamydomonas reinhardtii*	Zhao *et al*., 2009
	Ghb-miR169a	*Nicotiana benthamiana*	Ali *et al*., 2013
	Gma-miR159a	*Glycine max*	Yamada *et al*., 2014
	Hvu-miR171	*Hordeum vulgare*	Kis *et al*., 2015
		Nicotiana benthamiana	Kis *et al*., 2015
	Lgi-miR166a	*Lemna minor*	Canto-Pastor *et al*., 2015
	Mpo-miR160	*Marchantia polymorpha*	Flores-Sandoval *et al*., 2015
	Mtr-miR159b	*Medicago truncatula*	Devers *et al*., 2013
	Osa-miR390	*Brachypodium distachyon*	Carbonell *et al*., 2015
	Osa-miR395	*Triticum aestivum*	Fahim *et al*., 2012
	Osa-miR528	*Oryza sativa*	Warthmann *et al*., 2008

Continued

Table 6.1. Continued.

Artificial sRNA	Precursor	Species tested	Original reference
	Ptc-miR408	*Populus trichocarpa*	Shi *et al*., 2010
	Skr-miR166	*Marchantia polymorpha*	Flores-Sandoval *et al*., 2015
	Sly-miR159	*Nicotiana tabacum*	Vu *et al*., 2013
		Solanum lycopersicon	Vu *et al*., 2013
	Sly-miR168a	*Nicotiana tabacum*	Vu *et al*., 2013
		Solanum lycopersicon	Vu *et al*., 2013
	Vvi-miR166f	*Nicotiana benthamiana*	Roumi *et al*., 2012
syn-tasiRNA	*Ath-TAS1a*	*Arabidopsis thaliana*	Felippes and Weigel, 2009
	Ath-TAS1c	*Arabidopsis thaliana*	de la Luz Gutierrez-Nava *et al*., 2008
		Nicotiana benthamiana	Montgomery *et al*., 2008b
	Ath-TAS3a	*Arabidopsis thaliana*	Montgomery *et al*., 2008a
		Nicotiana benthamiana	Montgomery *et al*., 2008a

single construct when they are produced from an endogenous polycistronic precursor. For instance, rice *Osa-miR395* precursor containing five stem-loop structures, each of which produces a functional miRNA, was used to produce five different amiRNAs and induce resistance to *Wheat streak virus* in wheat (Fahim *et al*., 2012). Alternatively, multiple amiRNAs can be produced from a single transcript containing the same precursor sequence in tandem (Liang *et al*., 2012). In all cases, the expression of multiple amiRNAs in a single plant favours multi-targeting and can be used to enhance the silencing of a particular target (if multiple amiRNAs target different sites in the same target transcript) and/or to silence different sequence-unrelated target transcripts (if each amiRNA targets a different sequence-unrelated target transcript).

6.2.2 Synthetic trans-acting siRNAs

Syn-tasiRNA transgenes include the sequence of a *TAS* precursor in which a region corresponding to various endogenous tasiRNAs is substituted by a fragment containing multiple syn-tasiRNA sequences (Fig. 6.1). When transcribed, the syn-tasiRNA primary transcript is cleaved by a miRNA/AGO complex, and one of the cleaved products is used by RDR6 as a template for dsRNA synthesis. DsRNA is processed by DCL4 in several syn-tasiRNA duplexes in register with the miRNA cleavage site. Twenty-one nt syn-tasiRNA guide strands designed to have an AGO1-preferred 5´U direct silencing of cognate transcript(s) (Fig. 6.1). Initially, syn-tasiRNAs were used in *A. thaliana* when expressed from *TAS1a* (Felippes and Weigel, 2009), *TAS1c* (de la Luz Gutierrez-Nava et al., 2008; Montgomery et al., 2008b) and *TAS3a* precursors (Montgomery *et al*., 2008a) (Table 6.1) to silence single genes (for a recent review see Zhang, 2014). However, the main advantage of the syn-tasiRNA approach is the possibility of multiplexing multiple syn-tasiRNAs in the same construct, which allows multi-targeting, as shown recently. For example, the simultaneous targeting of *TRY/CPC/ETC2* and

sequence-unrelated *FLOWERING LOCUS T (FT)* endogenous transcripts in transgenic *A. thaliana* expressing two different *TAS1c*-based syn-tasiRNAs produced the expected combined phenotype of increased clustering of trichomes in rosette leaves and delay in flowering (Carbonell *et al*., 2014). In another recent study, five different syn-tasiRNAs expressed transgenically from *TAS3a* precursors in *A. thaliana* were used against *Turnip mosaic virus* and *Cucumber mosaic virus*, two sequence-unrelated RNA viruses, to confer multiple antiviral resistance (Chen *et al*., 2016). Importantly, *TAS1/TAS2*- and *TAS3a*-based syn-tasiRNA biogenesis depends on the presence of miR173 and miR390a, respectively. Thus, because miR173 is unique to *A. thaliana* (and close relatives), it must be co-expressed with *TAS1/TAS2*-based syn-tasiRNA transgenes to trigger syn-tasiRNA biogenesis in species different from *A. thaliana*.

A strategy similar to syn-tasiRNAs was described and named MIGS (for MiRNA Induced Gene Silencing) (Felippes *et al*., 2012). In this case, a transgene including a fragment of the target gene fused to an upstream miR173 target site was expressed in *A. thaliana* and induced the accumulation of tasiRNAs derived from the target gene sequences. These tasiRNAs have been shown to direct effective silencing of the desired target (Felippes *et al*., 2012). It is important to clarify that the MIGS technology does not produce authentic syn-tasiRNAs, but rather generates a series of non-designed tasiRNAs which can induce undesired off-target effects, as observed in a recent study (Han *et al*., 2015).

6.3 Design of Plant Artificial Small RNAS

The main difference between plant artificial sRNAs and other sRNAs produced in certain RNAi approaches is that the former are designed to be specific for the intended target(s). The rationale for design of artificial sRNAs must optimize both the effectiveness and the specificity of the designed sRNA. First, an effective artificial sRNA is required to have high sequence complementarity with the target RNA. Although the exact base-pairing requirements for productive sRNA/target RNA interactions are not fully known, it is generally accepted that mismatches within the sRNA seed region (nts 2–14) drastically reduce the sRNA activity, while mismatches in positions 1 or 14–21 have a much more moderate effect. Second, the artificial sRNA must be highly specific and silence the intended target(s) exclusively. The specificity of an artificial sRNA is assessed through the analysis of all possible base-pairing interactions between the candidate artificial sRNA and the complete set of cellular transcripts. These off-target analyses are only possible for species with annotated transcriptomes or expressed sequence tag (EST) collections, and thus must be computational. The two main tools used for plant artificial sRNA design are WMD3 (from Web MicroRNA Designer 3) (Ossowski *et al*., 2008; Schwab *et al*., 2010) and P-SAMS (from Plant Small RNA Maker Suite) (Fahlgren *et al*., 2016), and are described below.

WMD3 (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) is an amiRNA designer tool initially implemented for *A. thaliana* (Schwab *et al*., 2006) but which has now been extended to more than 200 species. It has been extensively used as a general amiRNA designer tool, and also to obtain the sequences of the six oligonucleotides

necessary for amiRNA cloning in *Ath-miR319a*, *Osa-miR528* or *cre-miR1157* precursors (Schwab *et al*., 2006; Warthmann *et al*., 2008; Molnar *et al*., 2009). The user inputs the ID or sequence of the target transcript, selects the plant species where the amiRNA is expressed, submits the job and several hours later receives an e-mail with a link to a web page where a list of candidate amiRNAs is displayed. Candidate amiRNAs are ranked based on a cumulative score which depends on different empirical rules regarding the hybridization energy of the amiRNA/target RNA base-pairing, the base-pair composition of the amiRNA and the specificity criteria. WMD3 recommends selecting an amiRNA with: (i) no mismatches at positions 2–12 when paired to the desired target; (ii) one or two mismatches at positions 18–21 to avoid transitivity (due to priming and extension by RDRs) and preserve specificity; (iii) hybridization energy of the binding between the amiRNA and the target between –35 and –40 kcal/mole; and (iv) amiRNA preferably targeting the coding region, as these are usually better annotated than the untranslated regions (UTRs). Based on these criteria, the user selects the preferred amiRNA sequence which is manually introduced in the Oligo tool to obtain the sequence of the oligonucleotides necessary for cloning.

P-SAMS (http://p-sams.carringtonlab.org) was developed more recently, and contains two applications, the P-SAMS amiRNA Designer and P-SAMS Syn-tasiRNA Designer, for the simple and automated design of amiRNAs and syn-tasiRNAs, respectively (Fahlgren *et al*., 2016). Key features of P-SAMS are its simplicity and speed. It has a user-friendly, modern interface and wizard-assisted navigation which guides the user through the whole design process. Simple questions are answered by the user to advance to the next step of the design process, with help boxes appearing when requested. Median job time for single-targeting amiRNA design is around 3 min, which represents a considerable improvement in speed compared to other tools. The on-screen results page includes the sequence of the designed sRNA, the sequence of the two oligonucleotides required for cloning into compatible *AtMIR390a*- or *AtTAS1c*-based B/c vectors (see next section) and a summary of the off-targeting analysis. P-SAMS outputs designed sRNAs as 'Optimal Results' or as 'Suboptimal Results' if they have or do not have off-targets, respectively. Up to three Optimal and/or Suboptimal results are displayed with no specific ranking criteria. Regarding the computational design of artificial sRNAs, P-SAMS first catalogues all target sites that do not contain a 15-nt sequence from positions 6–20 perfectly matching a transcript not contained in the input set. An sRNA is then designed to target each target site from the input transcript with the additional criteria that the sRNA has: (i) an AGO1-preferred 5´U nucleotide; (ii) a C in position 19 to produce a star strand with an AGO1 non-preferred 5´G, thus limiting the competition for AGO1 association with the guide strand; and (iii) an intentional mismatch with the target transcript at position 21 to reduce transitivity.

6.4 Engineering Artificial Small RNA Constructs

The selection of the endogenous precursor to be used to express the artificial sRNAs is a critical step when engineering plant vectors for artificial sRNA cloning.

The ideal precursor should accumulate to high levels and be processed accurately in multiple plant species for broad, efficient and specific gene silencing. In addition, the sequence and/or structural features of a given precursor may benefit a particular cloning strategy, while the particular processing pathway of a given precursor could determine the accuracy of its processing. For example, *Ath-miR390a* was selected as the preferred precursor for amiRNA cloning and expression in eudicots because: (i) the *MIR390* family is deeply conserved in different plant species, hence *Ath-miR390a* is likely to be processed accurately in multiple species; (ii) miR390 precursors are processed base to loop, which should favour a more accurate processing, resulting in reduced off-target effects compared to precursors that follow a multi-step loop to base processing such as *Ath-miR319a*; and (iii) it contains a short stem-loop region (Fig. 6.2A) compared to other conserved miRNA precursors, facilitating the synthesis of the amiRNA insert (see below).

6.4.1 AmiRNA cloning

The methodology most used for amiRNA cloning was initially described for cloning amiRNAs in *Ath-miR319a* precursors (Schwab *et al*., 2006), and later adapted for generating *Osa-miR528*- (Warthmann *et al*., 2008) and *Cre-miR1157*-based (Molnar *et al*., 2009) amiRNA constructs (Table 6.1). In this strategy, PCR-based mutagenesis is used to amplify the sequence of the miRNA precursor while substituting the original miRNA/miRNA* sequence with the corresponding amiRNA/amiRNA* sequence. Six amiRNA-specific oligonucleotides are needed in three different PCRs to amplify the complete amiRNA precursor sequence in three pieces when using a plasmid containing the wild-type miRNA precursor sequence. After gel purifying the three PCR fragments, an additional recombinant PCR with two generic oligonucleotides binding at the ends of the precursor and including the three PCR purified fragments produces the final amplicon containing the whole amiRNA precursor sequence. This DNA fragment is digested with specific restriction enzymes and cloned into an intermediate vector linearized with the same restriction enzymes. The DNA fragment containing the amiRNA precursor is finally transferred by restriction enzyme digestion and DNA ligase ligation into the expression vector of interest.

More recent methodologies for amiRNA cloning have aimed to reduce the number of reactions and treatments for the generation of amiRNA constructs, and are listed in Table 6.2. In all cases, a sequence of the precursor lacking the miRNA/loop/miRNA* region was already introduced in the cloning plasmid. Of particular interest is the simple, fast and cost-effective cloning strategy recently described for high-throughput generation of amiRNA constructs for efficient gene silencing in eudicots (Carbonell *et al*., 2014) and monocots (Carbonell *et al*., 2015). This strategy is based on the Golden Gate cloning method (Engler *et al*., 2008), uses positive insert selection for zero-background cloning and eliminates PCR steps, gel-based DNA purifications, restriction digestion and sub-cloning of amiRNA inserts (Table 6.2). The amiRNA insert is synthesized by annealing two overlapping and partially complementary oligonucleotides obtained with the P-SAMS tool

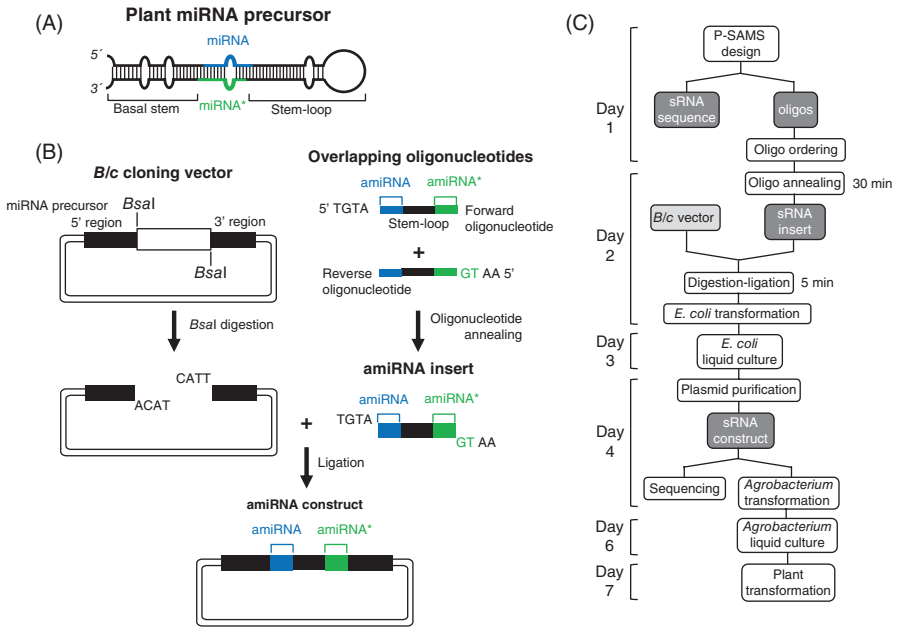

Fig. 6.2. High-throughput cloning of plant artificial sRNAs in B/c vectors (*Bsa*I/*ccd*B or 'B/c' vectors). A, Diagram of a canonical plant miRNA precursor. B, Diagram of the main steps for amiRNA cloning in B/c vectors containing a modified version of *Ath-miR390a* precursor that includes a *ccd*B cassette flanked by two inverted *Bsa*I sites. The amiRNA insert obtained after annealing the two partially complementary and overlapping oligonucleotides has 5´ overhangs compatible with those resulting from the *Bsa*I digestion of the B/c cloning vector where it is inserted directionally. The DNA fragments corresponding to the miRNA precursor, the amiRNA and the amiRNA* are in black, dark grey and light grey, respectively. C, Flowchart of the main steps from sRNA design to plant transformation using P-SAMS and B/c cloning vectors. (Adapted from Carbonell *et al*., 2014.)

and containing the amiRNA/stem-loop/amiRNA* sequence, and has 4-nt specific 5´ overhangs for direct cloning in the corresponding amiRNA vector (Fig. 6.2B). The amiRNA cloning vector or 'B/c' (from *Bsa*I/*ccd*B) vector contains a truncated miRNA precursor sequence whose miRNA/stem-loop/amiRNA* region was replaced by a DNA cassette containing the *ccd*B gene (Bernard and Couturier, 1992) flanked by two *Bsa*I sites (one inverted with respect to the other) to have both *Bsa*I recognition sites outside of the miRNA precursor sequence (Fig. 6.2B). AmiRNA inserts are ligated directionally into B/c cloning vectors in a short 5 min digestion-ligation reaction including the amiRNA cloning vector, the amiRNA insert, *Bsa*I restriction enzyme and T4 DNA ligase. An aliquot of the digestion-ligation reaction is used to transform a *ccd*B-sensitive *Escherichia coli* strain (e.g. DH5α). Typically, all colonies growing on plates containing the appropriate bacterial resistance antibiotic for selection are positive. One or two colonies are usually sent for sequencing to confirm that the sequence of the insert does not contain any mutation that could have accumulated during the oligonucleotide synthesis.

Table 6.2. Published methods to generate plant artificial sRNA constructs.

Artificial sRNA	Plant use	Precursor	Cloning in expression vector	Positive bacterial selection/ direc-tional cloning[a]	Number of reactions/ treatments[b]	Types of reactions/ treatments[c]	Reference	Software for oligonucleotide design[d]
amiRNA	*Chlamydomonas*	*Cre-miR1157*	+	–/–	7	OA, PR, P, DP, L	Molnar *et al*., 2009	WMD3
			+	–/+	5	OA, PR, D, L	Hu *et al*., 2014	–
	Eudicots	*Ath-miR159a*	–	–/+	6	PCR, PR, D, L, R	Niu *et al*., 2006	–
			–	GFP/+	6	PCR, PR, R, M	Yan *et al*., 2011a	–
		Ath-miR159b	+	LacZ/–	5	PCR, PR, D, L	Eamens *et al*., 2011	amiRNA primer designer
		Ath-miR164a	–	GFP/–	6	PCR, PR, R, M	Yan *et al*., 2011a	–
		Ath-miR319a	–	? /–	11	PCR, PR, D, L	Schwab *et al*., 2006	WMD3
			–	LacO/–	5	PCR, PR, D, L, M	Yan *et al*., 2011b	–
			–	GFP/–	6	PCR, PR, R, M	Yan *et al*., 2011a	–
			–	–/–	6	PCR, PR, D, L	Liang *et al*., 2012	–
			+	–/–	3	PCR, PR, D, L	Wang *et al*., 2012	–
			+	*ccd*B/–	6	D, K, PCR, PR, L	Zhou *et al*., 2013	–
		Ath-miR390a	+	*ccd*B/+	2	OA, D-L	Carbonell *et al*., 2014	P-SAMS
		Ath-miR395	–	–/–	6	PCR, PR, D, L	Liang *et al*., 2012	–
	Monocots	*Osa-miR390a*	+	*ccd*B/+	2	OA, D-L	Carbonell *et al*., 2015	P-SAMS

Continued

Table 6.2. Continued.

Artificial sRNA	Plant use	Precursor	Cloning in expression vector	Positive bacterial selection/ direc-tional cloning[a]	Number of reactions/ treatments[b]	Types of reactions/ treatments[c]	Reference	Software for oligonucleotide design[d]
		Osa-miR528	–	?/–	11	PCR, PR, D, L	Warthmann *et al*., 2008	WMD3
			+	*ccd*B/–	3	PCR, D, L	Chen *et al*., 2009	–
			+	–/–	4	OA, PCR, D, L	Wang *et al*., 2010	–
			+	–/–	6	PCR, PR, D, L	Yan *et al*., 2012	–
			+	*ccd*B/–	6	D, K, PCR, PR, L	Zhou *et al*., 2013	–
			+	*ccd*B/+	2	OA, D-L	Li *et al*., 2014	–
syn-tasiRNA	*A. thaliana*	*Ath-TAS1c*	+	*ccd*B/+	2	OA, D-L	Carbonell *et al*., 2014	P-SAMS

[a]Refers to the first vector used to clone the insert containing the amiRNA/amiRNA* sequence. ?, depends on the intermediate cloning vector of choice; *ccd*B, toxin from the *ccd* system of *Escherichia coli*; GFP, green fluorescent protein; lacO, operator site involved in the transcriptional regulation of the lac operon of *E. coli*; lacZ, structural gene of the lac operon coding β-galactosidase

[b]In each case the number was estimated by counting the basic reactions or treatments preceding bacterial transformation and described in the standard protocol detailed in the given reference

[c]D, digestion; DP, dephosphorylation; K, Klenow fill-in; L, ligation; M, mating; OA, oligo annealing; P, phosphorylation; PCR, polymerase chain reaction; PR, purification; R, recombination

[d]amiRNA primer designer is available at http://sydney.edu.au/science/molecular_bioscience/waterhouse/amiR_new/amiRcat2.html, P-SAMS at http://p-sams.carringtonlab.org, and WMD3 at http://wmd3.weigelworld.org

Importantly, non-linearized B/c cloning vectors with no amiRNA insert will express the *ccd*B toxin and will not propagate in *ccd*B-sensitive *E. coli* strains.

B/c amiRNA vectors contain the *Ath-miR390a* (Carbonell *et al*., 2014) or the *Osa-miR390* (Carbonell *et al*., 2015) precursor for use in eudicots or monocots, respectively (Table 6.3). Most amiRNA B/c vectors are expression vectors, with a unique combination of bacterial and plant antibiotic resistance genes. Others are intermediate Gateway-compatible entry vectors used for cloning the amiRNA insert, and subsequently recombine the complete amiRNA precursor sequence to the preferred Gateway expression vector containing a promoter, terminator or other features of choice (Table 6.3). In summary, this methodology based on a new generation of B/c amiRNA vectors is simple, fast and cost-effective compared to other described strategies for cloning amiRNAs. Indeed, the whole process from amiRNA design to plant transformation can be completed in just 1 week (Fig. 6.2C).

6.4.2 Syn-tasiRNA cloning

Initial syn-tasiRNA cloning methods (de la Luz Gutierrez-Nava *et al*., 2008; Montgomery *et al*., 2008a; Felippes and Weigel, 2009) were not adapted for efficient cloning. Similar to amiRNA B/c vectors, a new generation of syn-tasiRNA vectors also named 'B/'c was recently developed for high-throughput cloning of syn-tasiRNAs (Carbonell *et al*., 2014) (Table 6.3). B/c syn-tasiRNA vectors include a modified *TAS1c* gene from *A. thaliana* whose region including endogenous tasiRNAs D3[+] and D4[+] was substituted with a *ccd*B cassette flanked with two *Bsa*I sites, in the same configuration described for B/c amiRNA vectors. B/c syn-tasiRNA vectors allow the multiplexing of several syn-tasiRNA sequences in a single construct, which represents an attractive option when multiple and unrelated sequences need to be targeted. The cloning methodology is very similar to that described for generating B/c-based amiRNA constructs. Briefly, two overlapping and partially complementary oligonucleotides containing the syn-tasiRNA sequences are designed with the P-SAMS tool. The syn-tasiRNA insert results from the annealing of both oligonucleotides, contains two 4-nt specific 5´ overhangs and is cloned directionally in a B/c syn-tasiRNA vector in a 5 min digestion-ligation reaction (Table 6.2). Several *Ath-TAS1c*-based B/c syn-tasiRNA expression vectors were developed, as well as a Gateway-compatible entry vector (Table 6.3).

6.5 Validation of Artificial Small RNA Constructs

The accuracy of the processing of artificial sRNA precursors has typically been evaluated by Northern blot analysis only, but should also be analysed by other complementary and more sensitive technologies such as sRNA deep sequencing. An accurate processing of the precursor results in the accumulation of the artificial sRNA as a single RNA species revealed by Northern blot hybridization, and in the sequencing of a high proportion of reads corresponding to the desired artificial sRNA sequence and not to other sequences derived from the precursor. For example, the accuracy of processing *Ath-miR390a/Osa-miR390* amiRNA and

Table 6.3. New generation of plant 'B/c' vectors for high-throughput cloning of amiRNAs and syn-tasiRNAs.

Artificial sRNA	Plant use	Vector	Bacterial antibiotic resistance	Plant antibiotic resistance	Gateway use	Promoter	Terminator
amiRNA	Eudicots	*pENTR-AtMIR390a-B/c*	Kanamycin	–	Donor	–	–
		pFK210B-AtMIR390a-B/c	Spectinomycin	BASTA	–	*CaMV 35S*	*rbcS*
		pMDC123SB-AtMIR390a-B/c	Kanamycin	BASTA	–	*CaMV* 2x35S	*nos*
		pMDC32B-AtMIR390a-B/c	Kanamycin	Hygromycin	–	*CaMV* 2x35S	*nos*
	Monocots	*pENTR-OsMIR390-B/c*	Kanamycin	–	Donor	–	–
		pH7WG2-OsMIR390-B/c	Spectinomycin	Hygromycin	–	*Os Ubiquitin*	*CaMV*
		pMDC123SB-OsMIR390-B/c	Kanamycin	BASTA	–	*CaMV* 2x35S	*nos*
		pMDC32B-OsMIR390-B/c	Kanamycin	Hygromycin	–	*CaMV* 2x35S	*nos*
syn-tasiRNA	*Arabidopsis*[a]	*pENTR-AtTAS1c-B/c*	Kanamycin	–	Donor	–	–
		pMDC123SB-AtTAS1c-B/c	Kanamycin	BASTA	–	*CaMV* 2x35S	*nos*
		pMDC32B-AtTAS1c-B/c	Kanamycin	Hygromycin	–	*CaMV* 2x35S	*nos*

[a]As miR173 is a non-conserved miRNA, a construct expressing miR173 has to be co-expressed with the syn-tasiRNA construct to trigger syn-tasiRNA biogenesis in species other than *Arabidopsis thaliana* (or close relatives)
CaMV, *Cauliflower mosaic virus*; nos, nopaline synthase; *Os*, *Oryza sativa*; *rbcS*, Rubisco small subunit

Ath-TAS1c/Ath-TAS3a syn-tasiRNA precursors was confirmed by both methodologies (Montgomery *et al*., 2008a; Montgomery *et al*., 2008b; Carbonell *et al*., 2014; Carbonell *et al*., 2015). In particular, sRNA library analysis was also used to confirm the correct phasing of *Ath-TAS1c*- and *Ath-TAS3a*-derived syn-tasiRNAs (Montgomery *et al*., 2008a; Montgomery *et al*., 2008b; Carbonell *et al*., 2014). In contrast, sRNA deep sequencing analyses in amiRNA-expressing *Petunia* revealed that multiple sRNA species of different size are generated from different regions of *Ath-miR319a* precursors (Guo *et al*., 2014), and that many of these sRNAs meet the required criteria for amiRNA design (Schwab *et al*., 2006) and therefore are very likely to induce off-target effects. It is suggested that the reason for the accumulation of these additional sRNAs is the multi-step loop to base processing of Ath-miR319a precursors by DCL1 (Guo *et al*., 2014).

The efficacy of individual sRNA candidates can easily be screened in *N. benthamiana* transient assays by co-agroinfiltrating each artificial sRNA with the target RNA and quantifying target silencing (Yu and Pilot, 2014). The most effective amiRNAs are then selected for stable expression in transgenic plants. Indeed, a positive correlation was observed between the activity of amiRNAs tested transiently in *N. benthamiana* and their activity in amiRNA-expressing *A. thaliana* transgenic lines, which supports the use of this type of preliminary assay (Yu and Pilot, 2014). Another alternative to test artificial sRNA efficacy is the epitope-tagged protein-based amiRNA (ETPamiR) screens. In these assays, target mRNAs encoding epitope-tagged proteins are constitutively or inducibly co-expressed in protoplasts with amiRNA candidates targeting single or multiple genes (Li *et al*., 2013; Li *et al*., 2014). Finally, when using syn-tasiRNAs, testing multiple amiRNAs in a rapid assay first (e.g. agroinfiltration in *N. benthamiana*) is suggested, then selection of the most effective and expression of them all in the same construct as syn-tasiRNAs (Carbonell *et al*., 2016).

The quantification of target gene silencing is usually done by quantitative RT-PCR analysis of target RNAs preferentially using oligonucleotides spanning the amiRNA-small RNA-guided cleavage site, as it is still considered that amiRNA effects are mostly evident at the transcript level. Indeed, amiRNA-guided cleavage sites can be mapped by 5´RLM-RACE analysis. The specificity of plant artificial sRNAs has been evaluated in just a few cases. In particular, amiRNA specificity was confirmed for *Ath-miR319a*-based amiRNAs in *A. thaliana* by genome-wide expression profiles (Schwab *et al*., 2006) and, more recently, for *Osa-miR390*-based amiRNAs in *B. distachyon* by genome-wide transcriptome profiling combined with 5´RLM-RACE analysis (Carbonell *et al*., 2015).

6.6 Conclusions and Future Challenges

Despite the recent emergence of the potent CRISPR (from 'Clustered Regularly Interspaced Short Palindromic Repeats') knock-out technology for genome editing, plant artificial sRNAs should still be used extensively in the next few years because of their unique features:

1. Plant artificial sRNAs are highly specific.
2. sRNA-based silencing is a knock-down approach that allows the study of genes whose complete knock-out causes lethality.

3. A fine-tuned regulation of artificial sRNA activity either by adjusting the artificial sRNA spatio-temporal activity (e.g. using tissue-specific and/or inducible promoters), efficacy (e.g. by varying the degree of base-pairing of the artificial sRNA with the target RNA) or accumulation (e.g. varying promoter strength) allows for the generation of an allelic series for a knock-down gene. For instance, the generation of an allelic series for flowering time could be very important economically for certain crops.
4. A single artificial sRNA construct should allow for the targeting of duplicated genes (and gene families), antisense transcripts or individual isoforms.
5. Plant artificial sRNAs can be used to engineer disease resistance against pathogens with RNA genomes such as viruses.

In conclusion, the recent development of high-throughput cloning strategies and automated design tools for the generation of plant artificial sRNA constructs should accelerate gene function studies and crop improvement (for a recent review see Kamthan *et al*., 2015). However, a better knowledge of the rules governing the biogenesis, efficacy and mode of action (cleavage or translation al repression) of endogenous sRNAs will undoubtedly help to further improve and refine plant artificial sRNA-based approaches.

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