Chapter 1
Artificial Small RNAs for Functional Genomics in Plants

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Abstract RNA interference (RNAi) is based on the sequence-specific degradation of target RNAs by highly complementary small RNAs (sRNAs), which can be engineered to selectively target genes of interest. In plants, artificial microRNAs (amiRNAs) and artificial/synthetic trans-acting small interfering RNAs (atasi/syntasiRNAs) are the two main classes of artificial small RNAs (art-sRNAs). Art-sRNAs are refined, highly specific, selective, and potent RNAi tool that has been extensively used in gene function studies and for crop improvement. Here we describe the biogenesis and function of art-sRNAs, and how they are designed and used to study the function of plant genes.

Keywords Artificial small RNA · Functional genomics · Plants · RNA silencing · Artificial microRNA · Artificial tasiRNA · Synthetic tasiRNA

1.1 Introduction

In the current genomic era, the use of high-throughput sequencing technologies has allowed the identification of the genes of a large number of organisms, including model and crop plants (Parinov and Sundaresan 2000; Morozova and Marra 2008). In this context, one of the main challenges of modern plant biology is the characterization of the function of the genes of relevant plant species. Typically, once a gene has been identified, its functional characterization is assessed by the generation of either gain- or loss-of-function mutant plants with enhanced or reduced/null gene activity, respectively (Kuromori et al. 2009). Historically, gain-of-function mutants have been generated mainly through the transgenic overexpression of the target gene using potent constitutive promoters such as *Cauliflower mosaic virus* (CaMV) 35S (Weigel et al. 2000), while loss-of-function mutants have been obtained through ethane methyl sulfonate (EMS) mutagenesis (Kim et al. 2006) or by T-DNA insertion

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(Azpiroz-Leehan and Feldmann 1997). All these approaches have been extensively used for decades, despite their randomness in the gene targeting process.

In recent years, efforts have seeked to develop technologies for more controlled and efficient gene targeting, mainly to generate loss-of-function mutant plants. Indeed, a plethora of tools for targeting either the DNA or the RNA of a given gene have been developed and applied successfully to plants in gene function studies. DNA targeting tools include technologies such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), meganucleases, and the clustered regularly interspaced short palindromic repeats [CRISPR/CRISPR-associated nuclease 9 (Cas9) system] (Teotia et al. 2016). RNA targeting tools have exploited endogenous sRNA-directed silencing pathways controlling gene expression, stress responses, and genome integrity. Classic RNA interference (RNAi) technologies such as virus-induced gene silencing (VIGS) or hairpin-based silencing rely on the expression of double-stranded RNA (dsRNA) or dsRNA-like precursors including sequences corresponding to the target transcript to trigger small interfering RNA (siRNA) production to silence complementary target sequences (Ossowski et al. 2008; Baykal and Zhang 2010). Despite their massive use, these strategies are not considered highly specific as the large populations of siRNAs generated from dsRNA precursors might accidentally target cellular transcripts with high sequence complementarity to that of certain siRNAs. More recently, a series of more refined "second-generation RNAi" strategies with high specificity have been developed and applied successfully in gene function studies and crop improvement (Carbonell 2017a). These strategies are based on the expression of plant artificial sRNAs (art-sRNAs). Here, we describe what art-sRNAs are, and how they are designed, produced, and used in gene function studies in plants.

1.2 Artificial sRNAs (Art-sRNAs)

Art-sRNAs are 21-nucleotide sRNAs designed to selectively target one or several RNAs with high specificity and efficacy, by exploiting endogenous sRNA pathways. The two main classes of plant art-sRNAs are described next.

1.2.1 Artificial microRNAs (amiRNAs)

In plants, microRNAs (miRNAs) arise from miRNA transcripts with imperfect self-complementary foldback structures transcribed from endogenous *MIRNA* genes (Fig. 1.1a). These miRNA foldbacks are processed by DICER-LIKE1 (DCL1) to generate miRNA duplexes. One of the strands of the duplex, the miRNA guide strand, is selectively loaded into a protein of the ARGONAUTE (AGO) family based on the identity of the 5' nucleotide of the sRNA and/or other sequence and structural

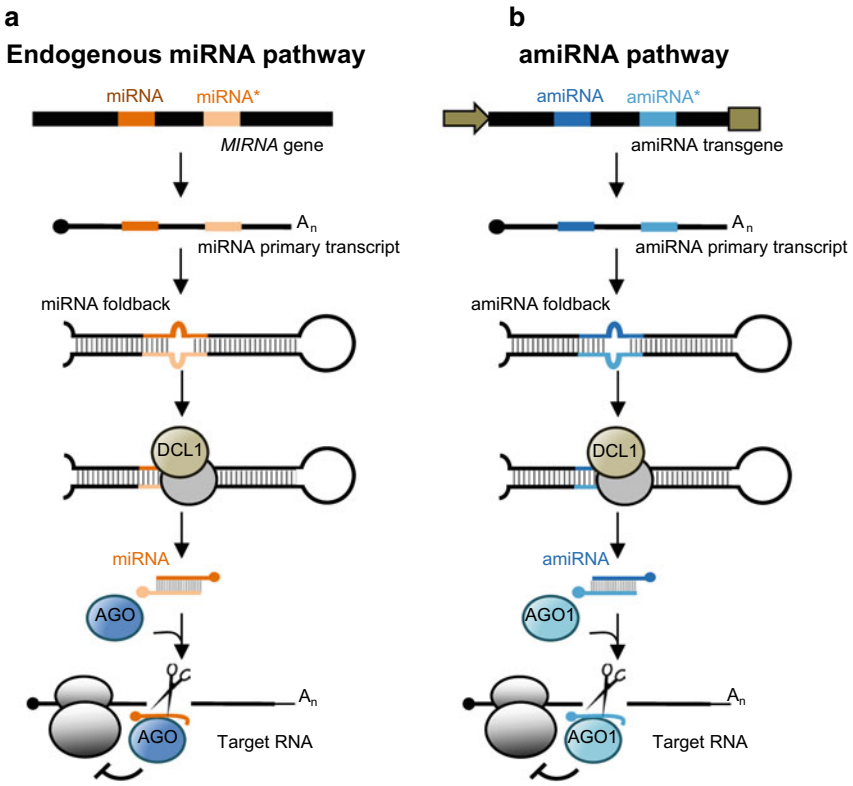

Fig. 1.1 Endogenous and artificial miRNA pathways in plants. Left, endogenous miRNA pathway. A *MIRNA* gene is represented in black with native miRNA/miRNA* sequences in dark and light orange, respectively. Right, the amiRNA pathway. An amiRNA transgene is introduced into plants, and includes exogenous promoter and terminator sequences (gold arrow and box, respectively), and the sequence of a plant miRNA precursor (in black) in which the original miRNA/miRNA* sequences have been substituted by the amiRNA/amiRNA* sequences (in dark and light blue, respectively). The transgene expresses an amiRNA primary transcript which is processed into an amiRNA foldback. A rationale amiRNA design requires that the amiRNA foldback preserves the original secondary structure of the endogenous precursor, and that the amiRNA guide strand contains a 5'U nucleotide to favor its association with AGO1 to silence highly complementary transcripts

features of the sRNA duplex and the AGO (Takeda et al. 2008; Mi et al. 2008; Montgomery et al. 2008a; Zhu et al. 2011; Zhang et al. 2014b), while the other strand (the star *) is usually degraded. The miRNA guides the AGO to bind and silence highly sequence complementary RNAs either by direct slicing or by repressing their translation (Fig. 1.1a) (Bologna and Voinnet 2014; Carbonell 2017b).

Artificial miRNAs (amiRNAs) are typically expressed *in planta* from transgenes including a miRNA precursor in which the original miRNA/miRNA* sequences have been substituted by the amiRNA/amiRNA* sequences (Fig. 1.1b). The amiRNA transgene is transcribed into a primary transcript that follows the canonical miRNA

biogenesis pathway. Importantly, amiRNAs are designed to contain a 5' U that favors AGO1 loading and subsequent silencing of cognate transcripts (Fig. 1.1b) (Carbonell 2017a). Typically, amiRNAs have been used to target a single target transcript, although other methodologies for co-expressing multiple amiRNAs from a single construct have also been reported. These include the expression of multiple amiRNAs from different precursors in tandem (Kung et al. 2012; Liang et al. 2012; Zhang et al. 2018a) or polycistronic precursors (Fahim et al. 2012; Kis et al. 2016).

1.2.2 Artificial/Synthetic Trans-Acting Small Interfering RNAs (atasi/syn-tasiRNAs)

Trans-acting siRNAs (tasiRNAs) are a particular subclass of plant sRNAs that arise from transcripts of *TAS* genes in *Arabidopsis thaliana.* The biogenesis pathway of endogenous tasiRNA is initiated by the cleavage of a *TAS* transcript by a miRNA/AGO complex, which triggers the recruitment of RNA-DEPENDENT RNA POLYMERASE6 (RDR6) to synthesize dsRNA from one of the cleavage products (Fig. 1.2a) (Allen et al. 2005; Rajagopalan et al. 2006). The dsRNA is sequentially processed by DCL4 into 21 nucleotide (nt) tasiRNA duplexes in register with the miRNA-guided cleavage site (Yoshikawa et al. 2005; Montgomery et al. 2008b). As for miRNAs, the guide strand is selectively loaded into an AGO protein to direct the silencing of highly sequence complementary RNAs (Fig. 1.2a) (Yoshikawa et al. 2005; Deng et al. 2018).

Artificial/synthetic tasiRNAs (atasiRNAs/syn-tasiRNAs) are produced in plants expressing a transgene containing a *TAS* precursor in which a subset of the native tasiRNA sequences has been substituted by several syn-tasiRNA sequences in tandem (Fig. 1.2b) (Zhang 2014; Carbonell 2017a). The atasiRNA/syn-tasiRNA transgene is transcribed into a primary transcript that follows the canonical tasiRNA biogenesis pathway. AtasiRNAs/syn-tasiRNAs, as described for amiRNAs, are designed to contain a 5' U to favor association with AGO1 and lead to the silencing of one or multiple highly sequence complementary transcripts (Fig. 1.2b) (Carbonell 2017a). Typically, syn-tasiRNA constructs are used to co-express multiple syn-tasiRNAs targeting different sites in the same transcript (de la Luz Gutierrez-Nava et al. 2008) or transcripts from different genes (Carbonell et al. 2014, 2019a, b; Chen et al. 2016; Carbonell and Daros 2017).

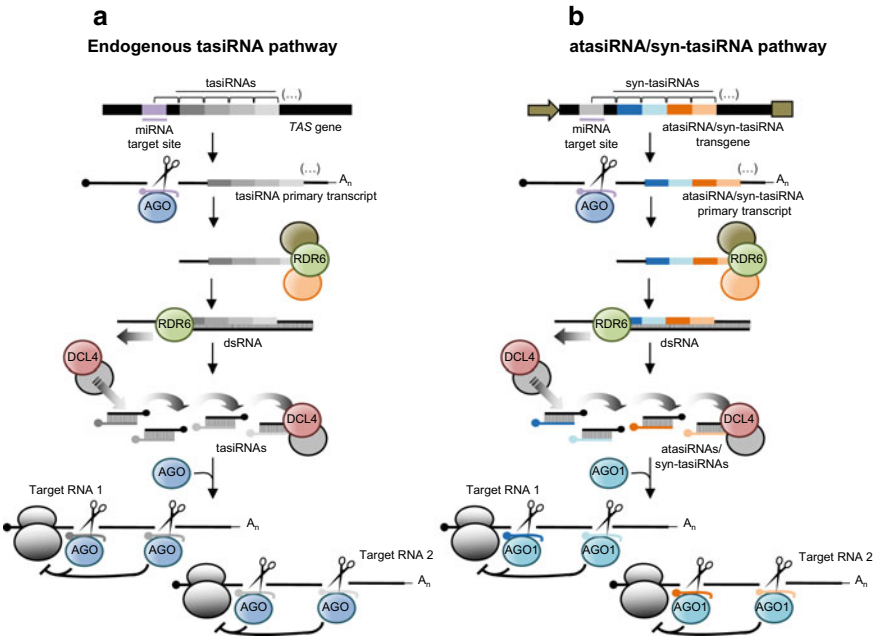

Fig. 1.2 Endogenous and artificial/synthetic tasiRNA pathways in plants. **a** The tasiRNA pathway. **b** The artificial/synthetic tasiRNA pathway. An atasiRNA/syn-tasiRNA transgene, containing a plant *TAS* precursor in which a subset of the original tasiRNA sequences has been substituted by several 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

1.3 Design, Production, and Validation of Art-sRNA Constructs

Despite the extensive use of art-sRNAs during the last decade, the design, production, and validation of art-sRNA constructs for plants has been a tedious process until very recently. The development of a series of high-throughput methodologies to generate art-sRNA constructs in a time- and cost-effective manner allows the efficient use of these tools in gene functional studies.

1.3.1 Design of Plant Art-sRNAs

Plant art-sRNAs are designed to be highly effective and highly specific with the help of automated web tools such as WMD3 (from Web MicroRNA Designer 3, http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) (Ossowski et al. 2008), amiRNA Designer (http://www.cs.put.poznan.pl/arybarczyk/AmiRNA) (Mickiewicz et al. 2016), micro RNA Designer (http://www.smallrna.mtu.edu/Tang_Website/submit.htm), and P-SAMS (from Plant Small RNA Maker Suite, http://p-sams.carringtonlab.org/) (Fahlgren et al. 2016). To account for high efficacy, these tools generally design art-sRNAs with extensive sequence complementarity with the target RNA. Despite the rules governing productive sRNA/target RNA interactions are not fully understood, it is well known that (i) the degree of silencing induced by an art-sRNA positively correlates with the degree of base-pairing between the sRNA and the target RNA (Liu et al. 2014), and (ii) mismatches included in the sRNA "seed region" (nucleotides 2–14) drastically decrease the activity of the sRNA (Schwab et al. 2006; Fahlgren and Carrington 2010). In any case, the efficacy of a given art-sRNA is difficult to predict, as the in vivo accessibility of target sites can be limited if they form highly structured conformations or if they are occupied by RNA-binding proteins. To account for high specificity, design tools assess the specificity of each art-sRNA in a given plant species by analyzing all possible base-pairing interactions between the candidate art-sRNA and the complete set of cellular transcripts of this species. Thus, the transcriptome of this particular species must be available, and, ideally, well-annotated.

To date, P-SAMS is the only web tool allowing for the design of the two classes of plant art-sRNAs: amiRNAs and atasiRNAs/syn-tasiRNAs, through its P-SAMS amiRNA Designer and P-SAMS Syn-tasiRNA Designer applications, respectively (Fahlgren et al. 2016). Briefly, P-SAMS has a user-friendly interface combined with a wizard-assisted navigation through simple questions that the user answers during the design process. An FAQ page addresses usual questions and contains video tutorials describing the different types of designs. Job times for designs are relatively short compared to other tools. For example, typical median job time for single-targeting amiRNA design is approximately 3 min. Results are displayed on-screen and include the sequence of up to three "optimal" art-sRNA and/or up to three "suboptimal" art-sRNAs if off-targets are predicted or not, respectively, as well as the sequence of the two oligonucleotides required for cloning into compatible "b/c" vectors (see section below). If the off-target filtering is activated, P-SAMS starts by cataloguing all target sites not containing a 15 nucleotide sequence from positions 6–20 perfectly matching a transcript not included in the input set. Then, an art-sRNA with the following sequence features is designed to target each target site from the input transcript: (i) the art-sRNA contains a 5'U nucleotide that favors AGO1 association, (ii) position 19 of the art-sRNA is a C to generate a star strand with an AGO-non preferred 5'G, and (iii) position 21 of the art-sRNA does not base-pair with the target transcript to reduce chances of triggering transitivity.

1.3.2 Generation of Art-sRNA Constructs

To generate an art-sRNA construct, a DNA fragment corresponding to the amiRNA insert has to be introduced in a plasmid including the plant precursor sequence flanked by regulatory promoter and terminator sequences. The selection of an appropriate art-sRNA precursor to clone and express the art-sRNA is a critical step, as it will actually influence both the cloning procedure and the in vivo activity of the art-sRNA. Moreover, it is recommended to use an evolutionary conserved precursor that most likely will be accurately processed in a large number of plant species (Carbonell 2017a).

Regarding the cloning, the generation of art-sRNA constructs can be a tedious process of several days. For example, classic methodologies for amiRNA cloning involved a large number of steps such as various PCRs, gel purifications, restriction and ligation reactions, subcloning, etc. (Schwab et al. 2006; Warthmann et al. 2008; Molnar et al. 2009). One of the reasons is that some of the amiRNA precursors used were excessively long, and thus not well adapted for an easy cloning. More recent technologies have been developed for high-throughput cloning of art-sRNAs mainly by reducing the number of steps during cloning (Chen et al. 2009; Yan et al. 2011, 2012; Carbonell et al. 2014; Hu et al. 2014; Li et al. 2014b; Luo et al. 2018; Carbonell 2019a). For example, the *Ath-MIR390a* and *Osa-MIR390* precursors from the well-conserved *MIR390* family were selected to clone and express amiRNAs in eudicot and monocot species, respectively, due to their short size compared to other miRNA precursors, that facilitated the synthesis and cloning of the amiRNA insert in zero-background cloning/expression "B/c" vectors containing a modified version of the *MIR390* precursor interrupted by a *ccd*B cassette flanked by two inverted *Bsa*I sites (Carbonell et al. 2014, 2015). AmiRNA inserts are obtained by annealing two partially complementary and overlapping oligonucleotides containing the amiRNA/stem-loop/amiRNA* region, and present 4 nucleotide 5' overhangs compatible with those resulting from the *Bsa*I digestion of the "B/c" vector. AmiRNA inserts are directly cloned into "B/c" vectors in a 5 min digestion–ligation reaction in the presence of *Bsa*I and T4 DNA ligase (for a detailed description see [Carbonell 2019a]). A similar strategy was developed for generating atasi/syntasiRNA constructs (Carbonell et al. 2014; Carbonell 2019a). The development of these types of high-throughput methodologies to generate art-sRNA constructs should definitely facilitate the use of the art-sRNA technology in functional genomics studies.

1.3.3 In Vivo Validation of Art-sRNA Constructs

Despite a thorough web tool-assisted design and subsequent cloning into a well-established expression vector, the correct activity of a given art-sRNA construct cannot be taken for granted. A first step to validate in vivo an art-sRNA construct

is to check that the art-sRNA accumulates *in planta* as a single sRNA species of the correct size. This can be evaluated by combining Northern blot hybridization with deep sequencing analysis (Carbonell et al. 2014, 2015). The accuracy of the processing of the art-sRNA precursor typically results in the accumulation of the art-sRNA as a single species in Northern blot analysis, and in the overrepresentation in sRNA libraries of reads corresponding to the art-sRNA compared to reads mapping to other precursor positions. In the case of syn-tasiRNA constructs including multiplexed syn-tasiRNAs, sRNA libraries are used to confirm the correct phasing of syn-tasiRNAs (Carbonell et al. 2014). Indeed, a rapid assessment of in vivo art-sRNA accumulation can be done by transiently expressing the art-sRNA construct in *Nicotiana benthamiana* leaves (Yu and Pilot 2014).

The second validation step is to assess the art-sRNA efficacy in silencing its corresponding target(s). Ideally, the efficacy of the art-sRNA can be inferred visually if target silencing leads to an obvious phenotype, which may be quantitative. If not, target gene silencing can be analyzed by measuring target RNA levels by quantitative RT-PCR, and art-sRNA cleavage sites are mapped by 5' RLM-RACE (Schwab et al. 2006). Alternatively, genome-wide transcriptome profiling through RNA sequencing can be used both to quantify target RNA accumulation and art-sRNA specificity (Carbonell et al. 2015). Very recently, degradome analysis has also served to check sRNA specificity (Singh et al. 2019), although through an MiRNA-Induced Gene Silencing (MIGS) strategy (Felippes et al. 2012), where the specificity of generated siRNAs is not controlled (Carbonell 2019b). In any case, it is important to consider that art-sRNA constructs can be easily screened and validated in *N. benthamiana* transient assays to select the most effective for stable expression in transgenic plants (Yu and Pilot 2014; Carbonell et al. 2019a, b). Alternatively, amiRNA efficacy can be assessed in epitope-tagged protein-based amiRNA (ETPamiR) screens, where target transcript encoding epitope-tagged proteins are co-expressed with amiRNA candidates in protoplasts (Li et al. 2013, 2014a).

1.4 Examples of Art-SRNAs Used in Gene Function Studies in Plants

Art-sRNAs, mainly amiRNAs, have been extensively used to silence genes in a wide range of plant species, from model plants to ornamentals and crops. A list of the precursors successfully used to express art-sRNAs in different plant species is presented in Table 1.1.

Despite art-sRNAs have been widely used for crop improvement, including the generation of antiviral resistance, a major use of this technology has focused on silencing plant genes in order to study their function. Here, we will describe just a few representative examples on how art-sRNAs can accelerate gene function discovery.

Table 1.1 Examples of uses of artificial sRNA precursors in plants

Artificial sRNA	Plant Species	Common name	Precursor used	References
amiRNA	*Arabidopsis thaliana*	Thale cress	*Ath-MIR159a* *Ath-MIR159b* *Ath-MIR164a* *Ath-MIR169d* *Ath-MIR171a* *Ath-MIR172a* *Ath-MIR319a* *Ath-MIR390a* *Ath-MIR395a*	Niu et al. (2006) Eamens et al. (2011) Alvarez et al. (2006) Liu et al. (2010) Qu et al. (2007) Schwab et al. (2006) Schwab et al. (2006) Montgomery et al. (2008a) Liang et al. (2012)
	Brachypodium distachyon	Purple false brome	*Osa-MIR390-AtL* *Osa-MIR528*	Carbonell et al. (2015) Smertenko et al. (2020)
	Catharanthus roseus	Madagascar periwinkle	*Ath-MIR319a*	Li et al. (2013)
	Chlamydomonas reinhardtii	Green algae	*Cre-MIR1157* *Cre-MIR1162*	Molnar et al. (2009) Zhao et al. (2009)
	Corchorus olitorius	Jute mallow	*Ath-MIR319a*	Shafrin et al. (2015)
	Fragaria Vesca	Strawberry	*Fve-MIR166*	Li et al. (2019)
	Glycine max	Soybean	*Ath-MIR319a*	Melito et al. (2010)
	Helianthus annuus	Sunflower	*Ath-MIR319a*	Li et al. (2013)
	Hordeum vulgare	Barley	*Hvu-MIR171*	Kis et al. (2016)
	Lemna minor	Duckweed	*Lgi-MIR166a*	Canto-Pastor et al. (2015)
	Malus domestica	Apple	*Mdo-MIR156h*	Charrier et al. (2019)
	Medicago sativa	Alfalfa	*Ath-MIR319a*	Verdonk and Sulllivan (2013)

(continued)

Table 1.1 (continued)

Artificial sRNA	Plant Species	Common name	Precursor used	References
	Nicotiana benthamiana	–	*Ath-MIR159a* *Ath-MIR319a* *Ath-MIR390a* *Ghb-MIR169a* *Hvu-MIR171* *Vvi-MIR166f* *Vvi-MIR319e*	Mitter et al. (2016) Li et al. (2013) Montgomery et al. (2008a) Ali et al. (2013) Kis et al. (2016) Roumi et al. (2012) Castro et al. (2016)
	Nicotiana tabacum	Tobacco	*Ath-MIR159a* *Ath-MIR164b* *Ath-MIR319a* *Sly-MIR159* *Sly-MIR168a*	Mitter et al. (2016) Alvarez et al. (2006) Vu et al. (2013) Vu et al. (2013) Vu et al. (2013)
	Marchantia polymorpha	Liverwort	*Mpo-MIR160*	Flores-Sandoval et al. (2016)
	Medicago truncatula	Barrelclover	*Mtr-MIR159b*	Devers et al. (2013)
	Oryza sativa	Rice	*Osa-MIR528*	Warthmann et al. (2008)
	Petunia hybrida	Garden petunia	*Ath-MIR319a*	Guo et al. (2014)
	Phaeodactylum tricornutum	Marine diatom	*Ath-MIR319a*	Kaur and Spillane (2015)
	Physcomitrella patens	Spreading earthmoss	*Ath-MIR319a*	Khraiwesh et al. (2008)
	Populus trichocarpa	Poplar	*Ptc-MIR408*	Shi et al. (2010)
	Solanum lycopersicum	Tomato	*Ath-MIR159a* *Ath-MIR164a* *Ath-MIR319a* *Ath-MIR390a* *Sly-MIR159* *Sly-MIR168a*	Zhang et al. (2011) Alvarez et al. (2006) Fernandez et al. (2009) Carbonell et al. (2019a) Vu et al. (2013) Vu et al. (2013)
	Solanum melongena	Eggplant	*Ath-MIR319a*	Toppino et al. (2011)

(continued)

Table 1.1 (continued)

Artificial sRNA	Plant Species	Common name	Precursor used	References
	Solanum tuberosum	Potato	*Ath-MIR168a* *Ath-MIR319a*	Bhagwat et al. (2013) Wyrzykowska et al. (2016)
	Vitis vinifera	Grape	*Ath-MIR319a*	Jelly et al. (2012)
	Triticum aestivum	Wheat	*Osa-MIR395*	Fahim et al. (2012)
	Whitania somnifera	Ashwagandha	*Ath-MIR159a*	Singh et al. (2016)
	Zea mays	Maize	*Ath-MIR319a*	Li et al. (2013)
atasiRNA/ syn-tasiRNA	*Arabidopsis thaliana*	Thale cress	*Ath-TAS1a* *Ath-TAS1c*	Felippes and Weigel (2009) de la Luz Gutierrez-Nava et al. (2008)
	Nicotiana benthamiana	–	*Ath-TAS1c* *Ath-TAS3a*	Montgomery et al. (2008b) Montgomery et al. (2008a)
	Solanum lycopersicon	Tomato	*Ath-TAS1c*	Carbonell et al. (2019a)

1.4.1 Artificial MiRNAs

Besides their extensive biotechnological use in crop improvement (Kamthan et al. 2015), amiRNAs have been broadly used to silence plant genes in functional studies in both model and crop plants (Sablok et al. 2011; Tiwari et al. 2014) (see Table 1.2).

1.4.1.1 Silencing of Coding Genes

A major problem to assign gene functions in plants is the presence of large gene families, which cause functional genetic redundancies and partial or complete functional overlap among closely related genes, as observed in the Arabidopsis genome (2000). Indeed, this may be the reason for the absence of visible phenotypes in single mutants. In this scenario, and because amiRNAs can target both single and multiple gene family members, amiRNA-based tools for screening the functionally redundant gene space were developed. First, a computationally derived library of 22,000 genome-wide family-specific amiRNAs was synthesized in multiple sub-libraries, each targeting defined functional protein classes (Hauser et al. 2013). For example, this amiRNA collection was used to encover novel morphological seed germination mutants for amiRNAs targeting zinc-finger homeodomain transcription factors

Table 1.2 Examples of uses of amiRNAs to study gene function in plants

Plant species	Target(s)[a]	Gene function studied	References
Arabidopsis thaliana	576 transcription factor genes	Redundancy in transcription factors	Jover-Gil et al. (2014)
	All *A. thaliana* protein-coding genes	Functional redundancy of Arabidopsis genes	Hauser et al. (2013)
		Identification of genes involved in CO_2 and abscisic acid responses	Hauser et al. (2019)
	Homologous genes with subclades of transporter families	Transport of signaling molecules	Zhang et al. (2018b)
	Ath-ADK	Adenosine kinase role in cytokinin interconversion	Schoor et al. (2011)
	Ath-AGP6/11	Role of arabinogalactan proteins in pollen development	Coimbra et al. (2009)
	Ath-CaM1	Senescence and abscisic acid response	Dai et al. (2018)
	Ath-CH42	Movement of the silencing signal	de Felippes et al. (2011)
	Ath-CHS	Asymmetric 22-nt miRNA role trigger widespread RNA silencing	McHale et al. (2013)
	AthCIPK16	Identification of a protein kinase involved in Na + exclusion	Roy et al. (2013)
	Ath-CKB, *Ath-ELF3*, *Ath-GI*, *Ath-ZTL*	Circadian clock regulation	Kim and Somers (2010)
	Ath-ERF102, *Ath-ERF104*	Cold stress	Illgen et al. (2020)
	Ath-cpHSC70-1/2	Involvement of heat shock proteins in chloroplast development	Latijnhouwers et al. (2010)

(continued)

Table 1.2 (continued)

Plant species	Target(s)[a]	Gene function studied	References
	Ath-FAD2, *Ath-FAE1*, *Ath-FATB*	Seed oil composition content	Belide et al. (2012)
	Ath-FT	Molecular mechanisms of flowering	Schwartz et al. (2009) Yeoh et al. (2011)
	Ath-H2AZ	Role of the SWR1 complex in flowering and development	Choi et al. (2007)
	Ath-IPMI-SSU1	Role of *Ath-IPMI-SSU1* in growth and development	Imhof et al. (2014)
	Ath-LNP1, Ath-LNP1/2	ER cisternae formation	Kriechbaumer et al. (2018)
	Ath-MAS2	Involvement of *MAS2* in 45S ribosomal DNA silencing	Sánchez-García et al. (2015)
	Ath-MIR159, Ath-MIR164	Specific functions of different amiRNA family members	Eamens et al. (2011)
	Ath-MIR408	Functional characterization of *MIR408*	Zhang and Li (2013)
	Ath-MYB14	Identification of genes involved in freeze tolerance	Chen et al. (2013)
	Ath-NB-LRR	Role of NB-LRR in autoinmune responses like hybrid necrosis	Bomblies et al. (2007)
	Ath-PHB, *Ath-REV*	microRNA sorting into AGOS	Zhang et al. (2014b)
	Ath-PP2AA1/2/3	Identification of phosphatase components in polar targeting of PIN auxin transport proteins	Michniewicz et al. (2007)
	Ath-PPPC4	New function in salt tolerance	Wang et al. (2012)

(continued)

Table 1.2 (continued)

Plant species	Target(s)[a]	Gene function studied	References
	Ath-PPR4	Trans-splicing of *rps12* chloroplast transcripts	Lee et al. (2019)
	Ath-SAUR19-24	Cell expansion	Spartz et al. (2012)
	Ath-SEP3	Role of DNA polymerase δ in the deposition of epigenetic marks, development, and flowering	Iglesias et al. (2015)
	Ath-snRK2	Involvement of SnRK2s in BIN2-modulated abscisic acid responses	Cai et al. (2014)
	Ath-TAS1c	tasiRNA biogenesis	Cuperus et al. (2010)
	Ath-TAS1c-A388U	AGO2-mediated target slicing	Carbonell et al. (2012)
	Ath-TAS2	tasiRNA biogenesis	Yoshikawa et al. (2013)
	Ath-U11/U12-31 K	Role *of Ath-U11/U12-31 K* in U12 intron splicing and plant development	Kim et al. (2010)
	Ath-U11/u12-65 K	Role of *Ath-U11/u12-65 K* in U12 intron splicing and plant development	Jung and Kang (2014)
	Ath-XCT	*RESISTANCE TO POWDERY MILDEW8.1*-based immunity	Xu et al. (2017)
	GFP	Pollen development	Grant-Downton et al. (2013)
		sRNA movement	Slotkin et al. (2009)
Brachypodium distachyon	*Bdi-MAP20*	Metaxylem pit development and drought recovery	Smertenko et al. (2020)

(continued)

Table 1.2 (continued)

Plant species	Target(s)[a]	Gene function studied	References
	Bdi-GT43B2	Xylan biosynthesis and seedling survival	Petrik et al. (2020)
Chlamydomonas reinhardtii	*Chr-CDPK3*	Regulation of flagellar biogenesis by a calcium-dependent protein kinase	Liang and Pan (2013)
	Chr-HSF1	Identification of genes involved in thermotolerance	Schmollinger et al. (2010)
	Chr-HydA1, Chr-HydA2, Chr-HYD3	Hydrogenase activity	Godman et al. (2010)
	Chr-MDAR1	Tolerance to photooxidative stress	Yeh et al. (2019)
	Chr-PEPC1/2	Role in fatty acid accumulation	Wang et al. (2017)
Chysanthemum morifolium	*Cmo-BBX8*	Flowering time	Wang et al. (2020b)
Glycine max	*Gma-Rhg1*	Identification of genes involved in resistance to cyst nematode	Melito et al. (2010)
	Gma-tRF001, *Gma-tRF003*	Nodulation regulation by rhizobial tRFs	Ren et al. (2019)
Medicago truncatula	*Mtr-FLOT2, Mtr-FLOT3, Mtr-FLOT4*	Flotillin requirement for bacterial infection	Haney and Long (2010)
Nicotiana benthamiana	–	microRNA sorting into AGOs	Zhang et al. (2014b)
	Ath-DRB1	microRNA sorting	Eamens et al. (2009)
	Nbe-SACPD-A/B, Nbe-SACPD-A/B/C, Nbe-SACPD-C	Ovule development	Zhang et al. (2014a)
	Nbe-siPPase	Involvement of viroid-derived sRNAs in symptom development	Eamens et al. (2014)
	Ppy-LUC	Functionality of intron-derived miRNAs	Shapulatov et al. (2018)
		Secondary siRNA production	Manavella et al. (2012)

(continued)

Table 1.2 (continued)

Plant species	Target(s)[a]	Gene function studied	References
Nicotiana tabacum	*Nta-CHS*	Hairy root metabolism	Hidalgo et al. (2017)
	Nta-FLS	Resistance to insects	Misra et al. (2010)
	Nta-siPPase	Involvement of viroid-derived sRNAs in symptom development	Eamens et al. (2014)
Oriza sativa	*Osa-A2*	Grain yield, shoot growth, and nitrogen level	Loss Sperandio et al. (2020)
	Osa-Eui1	Elongation of the uppermost internode at heading stage	Warthmann et al. (2008)
	Osa-GLP2-1	Seed dormancy	Wang et al. (2020a)
	Osa-HDAC1, *Osa-HDAC2* *Osa-HDAC3*	Histone deacetylation	Hu et al. (2009)
	Osa-PLL3, *Osa-PLL4*	Pollen development in rice panicles	Zheng et al. (2018)
	Osa-Spl11	Lesion formation in the absence of pathogen	Warthmann et al. (2008)
Physcomitrella patens	*Ppa-FtsZ2-1, Ppa-GNT1*	Chloroplast division, miRNA processing	Khraiwesh et al. (2008)
Populus tomentosa	*Ptr-SS3*	Secondary growth	Li et al. (2020)
Populus trichocarpa	*Ptr-PAL2/4/5, Ptr-PAL1/3*	Identification of phenylalanine ammonia lyase (PAL) genes	Shi et al. (2010)
Solanum lycopersicum	*Sly-CLC-b, Sly-RPS3a*	Cleavage of endogenous transcripts by viroid-derived sRNAs	Adkar-Purushothama et al. (2017)
Solanum melongene	*Sme-TAF10* *Sme-TAF13*	Male sterility	Toppino et al. (2011)
Solanum tuberosum	*Stu-CBP89*	Molecular mechanisms of drought tolerance	Pieczynski et al. (2013)
	Stu-PPO1, Stu-PPO2, Stu-PPO3, Stu-PPO2/3, Stu-PPO2/3/4, Stu-PPO1/2/3/4	Individual contribution of different PPO genes in total PPO protein activity	Chi et al. (2014)
Whitania somnifera	*Wso-SGTL1/2/3*	Role of sterol glycosyltransferases, antibacterial resistance	Singh et al. (2016)

(continued)

Table 1.2 (continued)

Plant species	Target(s)[a]	Gene function studied	References
	Wso-CYP85A69	Role in triterpenoids biosynthesis	Sharma et al. (2019)
Zea mays	*Zma-ZCN*	Molecular mechanisms of flowering	Meng et al. (2011)

[a]*ADK, ADENOSINE KINASE*; *AGP, ARABINOGALACTAN PROTEIN*; *BBX8*, *B-BOX PROTEIN8*; *CaM1, CALMODULIN1*; *CBP89, CAP-BINDING PROTEIN89*; *CDPK3, CALCIUM-DEPENDENT PROTEIN KINASE3*; *CH42, CHLORINA42*; *CHS*, *CHALCONE SYNTHASE*; *CIPK16, CBL-INTERACTING PROTEIN KINASE16*; *CKB, CASEIN KINASE II BETA*; *CLC-b, CHLORIDE CHANNEL PROTEIN b*; *cpHSC70, CHLOROPLASTIC HEAT SHOCK PROTEIN70*; *DRB1, DOUBLE-STRANDED RNA-BINDING PROTEIN1*; *ELF3, ELONGATION FACTOR3*; *ERF102/104*, *ETHYLENE RESPONSE FACTOR102/104*; *Eui1, ELONGATED UPPERMOST INTERNODE1*; *FAD2, FATTY ACID DESATURASE2*; *FAE1, FATTY ACID ELONGASE1*; *FATB, FATTY ACYL-ACP THIOESTERASE B*; *FLOT, FLOTILLIN-LIKE*; *FLS, FLAVONOL SYNTHASE*; *FT, FLOWERING LOCUS T*; *FTsZ2,* gene encoding a plastidial division protein; *GFP, GREEN FLUORESCENT PROTEIN*; *GI, GIGANTEA*; *GLP2-1*, *GERMIN-LIKE PROTEIN2-1*; *GNT1*, gene encoding an N-acetylglucosaminyltransferase*; GT43B2*, ortholog of wheat GT43-4 xylan synthase scaffolding protein; *H2AZ,* nucleosomal histone H2A variant Z*; HDAC, HISTONE DEACETYLASE*; *HSF1, HEAT SHOCK FACTOR1*; *HYD3, HYDROGENASE-LIKE PROTEIN3*; *HydA1/A2, FeFe-HYDROGENASEA1/A2*; *IPMI-SSU1, ISOPROPYLMALATE ISOMERASE SMALL SUBUNIT1*; *LNP1/2, LUNAPARK1/2*; *LUC, LUCIFERASE*; *MAP20*, *MICROTUBULE-BINDING PROTEIN20*; *MAS2*, *MORPHOLOGY OF AGO1-52 SUPPRESSED2*; *MDAR1, MONODEHYDROASCORBATE REDUCTASE1*; *MYB14, MYELOBLASTOSIS14*; *NB-LRR, NUCLEOTIDE-BINDING-SITE-LEUCINE-RICH-REPEAT*; *PAL, PHENYLALANINE AMMONIA-LYASE*; *PEPC1/2, PHOSPHOENOLPYRUVATE CARBOXYLASE1/2*; *PHB, PHAVOLUTA*; *PLL, PECTATE LYASE-LIKE; PP2A, PROTEIN PHOSPHATASE2A*; *PPO, POLYOHENOL OXIDASE*; *PPPC4, BACTERIAL-TYPE PHOSPHOENOLPYRUVATE CARBOXYLASE*; *PPR4, PENTATRICOPEPTIDE REPEAT*; *Rhg1,* gene conferring resistance to *Heterodera glycines*; *REV, REVOLUTA*; *RPS3a*, *RIBOSOMAL PROTEIN S3a*; *SACPD, STEAROL-ACYL CARRIER PROTEIN DESATURASE*; *SAUR*, *SMALL AUXIN UP RNA*; *SEP3*, *SEPALLATA3*; *SGTL, STEROL CLYCOSYLTRANSFERASE*; *siPPase*, *SOLUBLE INORGANIC PIROPHOSPHATASE*; *snRK2, snf1-RELATED KINASE2S*; *SPL11, SPOTTED LEAF11*; *SS3*, *SUCROSE SYNTHASE3*; *TAF, TBP-ASSOCIATED FACTORS*; *TAS, TRANS-ACTING SIRNA*; *tRF, RHIZOBIAL tRNA-DERIVED FRAGMENT*; *XCT, XAP5 CIRCADIAN TIMEKEEPER; ZCN, ZEA MAYS CENTRORADIALIS*; *ZTL, ZEITLUPE*

(Hauser et al. 2013), and more recently, to generate a seed resource for screening functional redundant genes and isolation of new mutants impaired in carbon dioxide and abscisic acid (Hauser et al. 2019). Another effort to simplify the analysis of gene function between gene families was the generation of a collection of amiRNAs targeting groups of paralogs encoding transcription factors by Jover-Gil and collaborators (Jover-Gil et al. 2014). In this case, 338 amiRNA-expressing Arabidopsis lines were generated, each of which expressed an amiRNA designed to simultaneously inactivate a set of two to six paralogous transcription factors. This collection was used to identify 21 amiRNAs causing vegetative leaf morphological phenotypes (Jover-Gil et al. 2014).

In the previous examples, amiRNA-expressing lines were obtained by introducing an amiRNA transgene into the plant genome. However, besides the standard expression of amiRNAs in plants through transgenes, *aMIRNA* precursors have also been successfully expressed from several plant DNA viruses through the so-called MIR-VIGS approach. Because viruses move throughout the plant, amiRNAs were also expressed systemically and silencing effects were visible in all those tissues the virus could invade. In all cases, plant DNA viruses used in MIR-VIGS belong to the genus Begomovirus, family Geminiviridae, and include *Cabbage leaf curl virus* (CaLCuV) (Tang et al. 2010), *Cotton leaf crumple virus* (CLCrV) (Gu et al. 2014), and the viral satellite DNA vector of *Tomato yellow leaf curl China virus* (TYLCCNV) (Ju et al. 2017). Although these vectors have been used mainly to silence endogenous reporter genes, they may constitute a useful tool for functional genomics in plants.

Finally, a recent report by Zhang and colleagues has offered new improvements in the amiRNA technology aimed to increase the levels of amiRNA-induced silencing (Zhang et al. 2018a). First, the authors developed a system in which the amiRNA was embedded into a portable intron within a fluorescent reporter. The basis of this system is that both the fluorescent reporter and amiRNA are produced from the same transcript, and thus the fluorescent reporter serves as a visible surrogate for checking amiRNA efficacy in vivo. And second, efficient multiplexing of several amiRNAs in the same construct was achieved by adding various amiRNA precursors in tandem, each of which was flanked by tRNA-processing sites.

1.4.1.2 Silencing of Non-coding Genes

AmiRNAs have also been used to silence endogenous *MIRNA* genes, to study the function of new miRNAs or to differentiate the function of individual members of a *MIRNA* family. Eamens and co-workers first reported the use of amiRNAs in Arabidopsis to target one or multiple miRNA family members, by targeting the mature miRNA or precursor stem–loop sequence, respectively (Eamens et al. 2011). Interestingly, these results suggest that sRNA-guided cleavage function could occur not only in the cytoplasm but also in the nucleus, thus providing new insights in the mechanisms of sRNA-mediated silencing. In another study, also in Arabidopsis, silencing of endogenous *MIR408* by amiRNAs caused impaired plant growth and

highlighted the importance of miR408 accumulation level for proper plant vegetative development (Zhang and Li 2013).

1.4.2 Artificial/Synthetic tasiRNAs

AtasiRNAs/syn-tasiRNAs have been used to study gene function and improve crops (Zhang 2014). They were first employed a decade ago to study the biogenesis of tasiRNAs from *TAS* transcripts in Arabidopsis (de la Luz Gutierrez-Nava et al. 2008). In most cases, expressed atasi/syn-tasiRNAs targeted genes with visible loss-of-function phenotypes such as *PHYTOENE DESATURASE* (*PDS*), *GREEN FLUORESCENT PROTEIN* (*GFP*), or *CHLORINA42* (*CH42*). Seminal findings included the observation that (i) AGO1-miR173 complexes initiate phased siRNA formation in plants (Montgomery et al. 2008b), (ii) miR390 associates exclusively with AGO7, (iii) miR390-AGO7 complexes function in distinct cleavage or non-cleavage modes at two target sites in *TAS3a* transcripts (Montgomery et al. 2008a), and (iv) tasiRNAs have a greater range in cell nonautonomous movement compared to miRNAs (de Felippes et al. 2011). A summary of representative examples of use of atasi/syn-tasiRNA in gene function is shown in Table 1.3.

More recently, atasi/syn-tasiRNA tools have been used to confer enhanced antiviral resistance (Chen et al. 2016; Carbonell and Daros 2017; Carbonell et al. 2019b), because the multitargeting of viral RNAs with multiple atasi/syn-tasiRNAs from a single construct limits virus ability to mutate target sites and escape the resistance (Carbonell et al. 2016, 2019a).

1.5 Concluding Remarks and Future Perspectives

Still in the genome editing era, art-sRNA-based RNAi tools offer a variety of advantages for functional genomic studies in order to dissect the function of any desired gene or gene network. Art-sRNAs (i) are highly specific, (ii) allow the functional study of genes whose complete knock-out is lethal, (iii) allow the study of genes in a spatio-temporal manner, as target silencing can be induced at specific times and/or at specific places by using inducible and/or tissue-specific promoters, respectively, (iv) should allow the fine-tuned regulation of target transcript levels to generate an allelic series for a knock-down gene, (v) can target duplicated genes (and gene families), antisense transcripts or individual isoforms, and (vi) can be multiplexed in single constructs for multisilencing. Moreover, the development of high-throughput methodologies to generate art-sRNA constructs should definitely facilitate the use of art-sRNA-based tools not only in gene function studies but also in obtaining next generation crops.

Table 1.3 Examples of uses of artificial/synthetic tasiRNAs to study gene function in plants

Plant species	Target(s)[a]	Gene function studied	References
Arabidopsis thaliana	*Ath-CH42*	Movement of silencing signal	de Felippes et al. (2011)
		TAS1a-derived tasiRNA biogenesis	Felippes and Weigel (2009)
		TAS3-derived tasiRNA biogenesis	de Felippes et al. (2017)
	Ath-FAD2	*TAS1c*-derived tasiRNA biogenesis	de la Luz Gutierrez-Nava et al. (2008)
	Ath-FT	Modulation of flowering time	López-Dolz et al. (2020)
	Ath-PDS	*TAS1c*-derived tasiRNA biogenesis	Montgomery et al. (2008b)
		TAS2-derived tasiRNA biogenesis	Yoshikawa et al. (2013)
		TAS3-derived tasiRNA biogenesis	Montgomery et al. (2008a)
Nicotiana benthamiana	*Ath-PDS*	*TAS1c*-derived tasiRNA biogenesis	Montgomery et al. (2008b)
		AGO7 miRNA loading; *TAS3a*-derived tasiRNA biogenesis	Montgomery et al. (2008a)
	Cme-ARF3	Identification of melon *TAS3* locus	Cervera-Seco et al. (2019)
	GFP	*TAS1c*-derived tasiRNA biogenesis	Montgomery et al. (2008b)
	Nbe-SU	Chlorophyll synthesis	López-Dolz et al. (2020)

[a]*ARF3, AUXIN RESPONSE FACTOR3; CH42, CHLORINA42; FAD2, FATTY ACID DESATURASE2; PDS, PHYTOENE DESATURASE; SU, SULPHUR*

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