Chapter 2
Recent Advancements in MIGS Toward Gene Silencing Studies in Plants

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Abstract In plants, RNA interference (RNAi) causes gene silencing in which small RNAs (sRNAs) inhibit gene expression by causing sequence-specific degradation of target transcripts. Several RNAi-based tools have been developed and optimized to study gene function and trait improvements in plants. One recent strategy based on miRNA-triggered secondary small interfering RNAs (siRNAs) through trans-acting siRNA (tasiRNA) pathway has been developed for efficient gene silencing. In plants, miRNA-mediated cleavage of noncoding *TAS* RNAs triggers production of tasiRNAs which cause downregulation of one or more target genes. MiRNA-induced gene silencing (MIGS) works on this module in which a single miRNA target site fused with a target gene fragment in a vector triggers production of tasiRNAs and subsequent target gene silencing in plant cells. This technology has been successfully employed to silence one or more target genes to study their role in plant development and stress response. It has gained much attention due to its ease of design and capacity to silence multiple paralogous genes simultaneously. Further, MIGS vector designing does not require whole genome information, making it suitable to be used in plant species which lacks this information. This chapter summarizes recent progress in MIGS and its application in gene function studies and trait improvements.

Keywords miRNA · MIGS · Gene silencing · tasiRNAs · phasiRNAs

2.1 Introduction

Since the discovery of plant small RNAs (sRNAs), RNA is at the center of plant functional genomics studies (Morris and Mattick 2014) and has paved the way for exploitation of sRNAs in deciphering gene function via gene silencing. In plants, sRNAs are produced from double-stranded RNA precursors by Dicer-like (DCL) enzymes (Axtell 2013). Plant sRNAs are mainly categorized into microRNAs (miRNAs) and small interfering RNAs (siRNAs). The former gets excised from

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partially double-stranded regions of hairpin transcripts, whereas siRNAs originate from perfectly complementary double-stranded RNAs (Yoshikawa 2013). After the formation of miRNA and siRNA duplex, one strand of each duplex is loaded onto an Argonaute (AGO) protein and a RNA-induced silencing complex (RISC) is formed in association with other protein factors. These RISCs are guided by the AGO-bound sRNAs to target complementary sequences and regulate target gene expression either by transcriptional silencing and/or translational inhibition or degradation of transcript (Wei et al. 2012; Voinnet 2009; Eamens et al. 2008).

In addition to this, miRNA and siRNA-directed cleavages produce sequence templates to generate double-stranded RNA (dsRNA) by the action of RNA-dependent RNA polymerases (RDRs), which are further processed by DCL enzymes to generate secondary siRNAs (Schwab and Voinnet 2010; Baulcombe 2007; Voinnet 2008). Some secondary siRNAs, generated from the end of dsRNA, originate from an AGO-catalyzed cleaved RNA at a miRNA target site are 21-nt phased siRNAs (phasiRNAs) (Axtell 2013; Rajeswaran et al. 2012). PhasiRNAs which are able to repress different target loci other than the loci of their origin are known as trans-acting siRNAs (tasiRNAs) (Axtell 2013).

In Arabidopsis, four gene families encoding trans-acting siRNA (tasiRNA) precursors (TAS) have been identified (Fei et al. 2013). TAS1, TAS2, and TAS4 precursors are targeted by 22-nt asymmetric miRNAs/AGO1 complex by one-hit mechanism to generate tasiRNAs in Arabidopsis and related species, whereas TAS3 precursors generate tasiRNAs triggered by 21-nt symmetric miR390/AGO7 complex by two-hit mechanism (Cuperus et al. 2011; Axtell et al. 2006).

Based on miRNA-triggered secondary siRNA biogenesis, three different classes of silencing tools have been developed—(i) artificial synthetic tasiRNA (atasiRNA/syn-tasiRNA), (ii) miRNA-induced gene silencing (MIGS), and (iii) artificial miRNA (amiRNA). These tools have been extensively used to induce gene silencing in plants (Carbonell 2019). MIGS has an advantage over other techniques due to its ease of design (de Felippes et al. 2012; de Felippes 2013) and capacity to silence multiple genes, simultaneously. Current chapter describes MIGS as an effective gene silencing technology and recent advancements in gene silencing studies in plants, employing this strategy.

2.2 Biogenesis of MicroRNA-Triggered Secondary siRNAs

The tasiRNA pathway involves two different mechanisms, named as the “one-hit” and “two-hit” models (Fei et al. 2013). In the one-hit model, a 22-nt asymmetric miRNA directs cleavage of a tasiRNA precursor transcript by AGO1 (Fig. 2.1). Then, RDR6 along with a suppressor of gene silencing 3 (SGS3) catalyzes to synthesize a complementary RNA strand to form dsRNA molecule from the 3’ end of the cleaved product. Finally, endonuclease DCL4 cleaves the dsRNA every 21 nt from the first cleavage point to generate a population of secondary siRNAs (Yoshikawa et al. 2005). It has been reported that very few 22-nt asymmetric miRNAs trigger

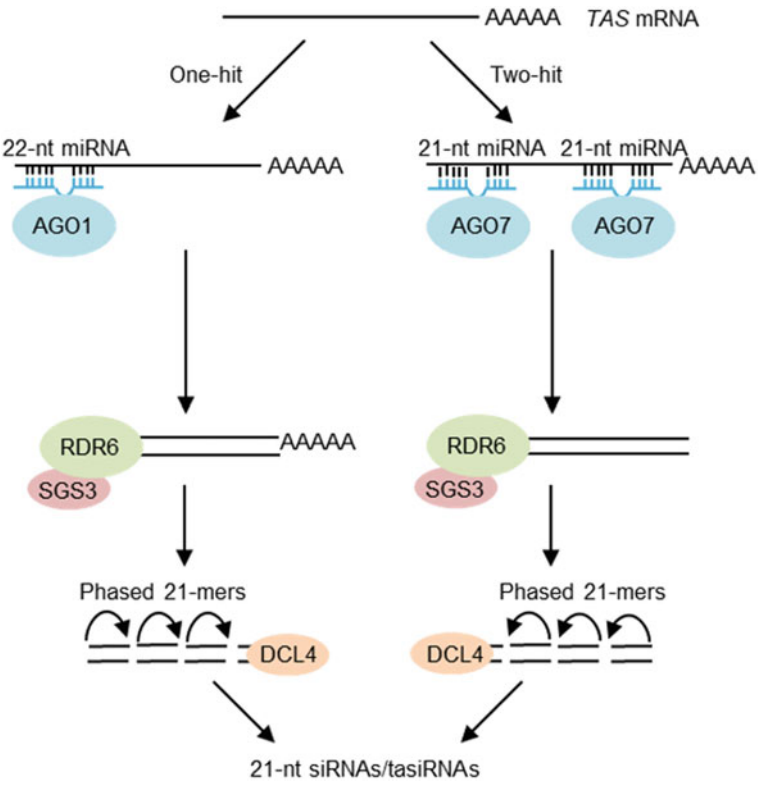

Fig. 2.1 Biogenesis of miRNA-triggered secondary siRNAs. In "one-hit" model, TAS transcript is targeted and cleaved by 22-nt miRNA/AGO1 complex whereas in "two-hit" model TAS transcript is targeted by two numbers of 21-nt miRNA/AGO7 complex and cleaved by miRNA/AGO7 complex closer to 3' end. Both the models recruit RDR6 and SGS3 for dsRNA synthesis and DCL4 for the production of 21-nt tasiRNAs

tasiRNAs production (Chen et al. 2010; Cuperus et al. 2010). In the two-hit model, the tasiRNA precursor transcript is targeted by two 21-nt miRNA390/AGO7 complexes (Fig. 2.1). The miRNA-AGO cleaves the target transcript closer to the 3' end whereas the other target site remains intact. Then RDR6 and SSG3 act to form dsRNA from the AGO-miRNA cleavage site to the site bound by other complex and finally DCL4 processes the dsRNA for 21-nt tasiRNAs that are phased with respect to the cleaved end (Axtell et al. 2006). The produced tasiRNAs target specific complementary RNAs in *trans* to degrade those targeted RNAs. Both one-hit and two-hit tasiRNA pathways have been exploited to design different constructs to knockdown target genes in plants.

2.3 Gene Silencing Technologies Based on tasiRNA Pathway

Based on tasiRNA pathway, atasiRNA/syn-tasiRNA approach was first developed to study gene silencing. In this approach, the endogenous tasiRNAs in a TAS precursor is substituted by a fragment containing one or more atasiRNAs/syn-tasiRNA sequences (Carbonell 2019). When these constructs are transferred to plants, engineered transcripts are cleaved by miRNA/AGO complex and one of the cleaved fragments is converted to dsRNA, which is processed further into 21-nt phased tasiRNAs. Then atasiRNA/syn-tasiRNA-guided strands are incorporated into AGO1 to induce silencing of one or more transcripts (Carbonell 2019). Several attempts have been made to efficiently knockdown one or multiple genes in gene function studies in Arabidopsis (de la Luz Gutiérrez-Nava et al. 2008; Montgomery et al. 2008a, b; Carbonell et al. 2014) by using this technology. The major advantage of this approach is a possibility of producing several atasiRNAs from one TAS precursor, which can target different target sequences at different locations (Carbonell et al. 2014). Another advantage of this technique is less chance of off-targeting. But, to design atasiRNA molecules to specifically downregulate certain genes, the entire genome information is necessary to minimize the chances of unwanted silencing (Ossowski et al. 2008).

The artificial miRNA (amiRNA) constructs are prepared by incorporating amiRNA and amiRNA* sequences in place of endogenous miRNA and miRNA* in a miRNA precursor. Upon transformation into plants, amiRNAs of the desired sequence get accumulated as a result of which the endogenous target transcripts would get silenced (Schwab et al. 2006). This approach has demonstrated high specificity and ability to silence multiple genes, however the constructs designing needs multiple steps of PCR to replace mature miRNA in a precursor backbone with amiRNA (Schwab et al. 2006; Ossowski et al. 2008). McHale et al. (2013) have successfully used amiRNA approach to knockdown *CHALCONE SYNTHASE* (CHS) in Arabidopsis.

Another approach based on tasiRNA pathway is termed as miRNA-induced gene silencing (MIGS), which was firstly used in Arabidopsis by expressing a target gene fused to an upstream miR173 target sequence (de Felippes et al. 2012). When MIGS constructs are introduced into plants, the miR173-mediated cleavage triggers the synthesis of secondary siRNAs which promote silencing of related target genes. MIGS can be used to knockdown multiple genes simultaneously by using a single vector by linking of different gene fragments, each with one miRNA target site (de Felippes et al. 2012). The major advantage of MIGS technology is that genome information is not required to design the constructs to be used in different plant species. But the risk of off-targeting is a major concern in employing this technology.

2.4 MicroRNA-Induced Gene Silencing (MIGS) and Its Advantages

Initially MIGS constructs were designed by taking miR173 target site followed by the target gene fused downstream of that (Fig. 2.2) (de Felippes et al. 2012). miR173, an asymmetric 22-nt miRNA, is able to trigger production of tasiRNAs from the fused transcript (Chen et al. 2010; Cuperus et al. 2010). The miR173/AGO1 complex guides the cleavage of the transcript which triggers RDR6-dependent synthesis of dsRNA and subsequent processing by DCL4 to release phased tasiRNAs. These tasiRNAs target the endogenous genes for efficient silencing. Since miR173 is absent in non-Arabidopsis species, so miR173 co-expression is required along with MIGS transgenes to induce tasiRNA production (de Felippes et al. 2012).

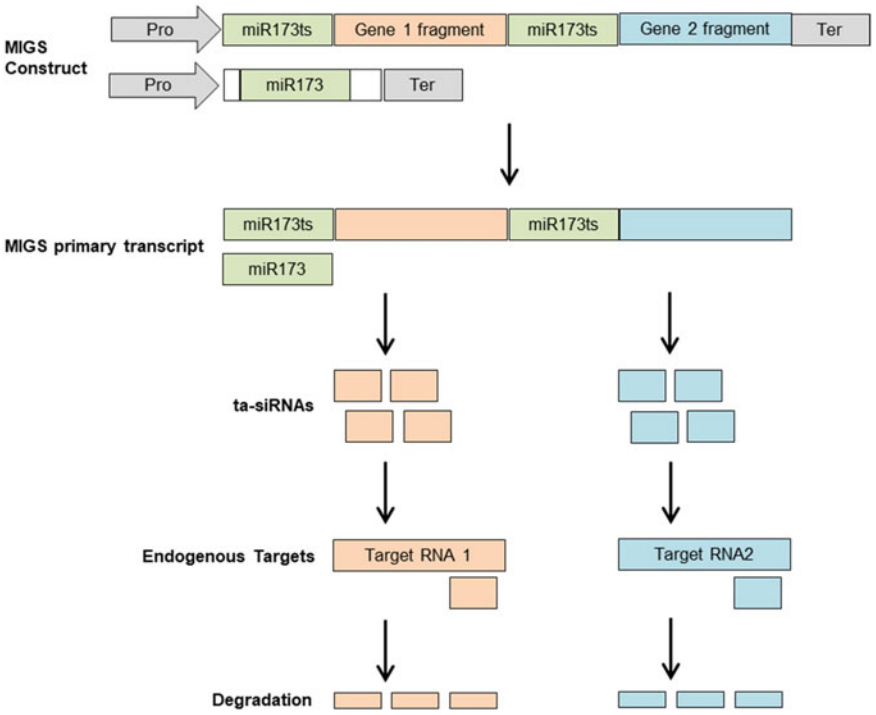

Fig. 2.2 A model MIGS construct and silencing mechanism. A MIGS construct can be prepared by placing a miRNA target sequence (e.g., miR173ts) in front of a target gene fragment. Multiple combinations can be used in a single cassette to downregulate multiple genes. Upon transformation into plant cells, it undergoes to form a long transcript having miR173ts and complementary transcript to each gene fragment. Binding of miR173 to the miR173ts triggers production of tasiRNAs and subsequent silencing of endogenous complementary genes by tasiRNAs. Pro (promoter), Ter (terminator)

Other 22-nt miRNAs like miR1514a.2 have also been reported to trigger tasiRNA production in soybean and the MIGS constructs have efficiently demonstrated to knockdown target genes in soybean (Jacobs et al. 2016). MiR390 which triggers production of tasiRNAs by two-hit model from TAS3 transcripts has also been used to prepare MIGS construct (Felippes and Weigel 2009). Since miR390 associates only with AGO7, miR390-based MIGS would be limited to AGO7 expression site, that is only in the vascular system (Montgomery et al. 2008b).

MIGS has major advantages over other technologies due to its easiness of design (de Felippes et al. 2012; de Felippes 2013). With one-step PCR, one target gene can be fused downstream of a miRNA target site which can trigger tasiRNA production. MIGS is very much effective in co-silencing multiple genes through a single vector construct by linking different target fragments, each with one miRNA target site, thus saving time. Sequence similarity between different target genes is not necessary since specific MIGS module is generated for each target separately (de Felippes et al. 2012). Since this technology does not require genome-wide data, it can be used in gene silencing studies of plant species which lacks this information (de Felippes 2019). The predictable pattern of produced siRNAs and specific expression profiles of miRNA triggers add extra level of control in MIGS compared to other gene silencing technologies (Jacobs et al. 2016).

2.5 Gene Silencing Studies in Plants Using MIGS

For the first time de Fellipes successfully employed MIGS to induce gene silencing of four Arabidopsis genes (*AGAMOUS*, *EARLY FLOWERING 3*, *FLOWERING LOCUS T*, *LEAFY*) in *A. thaliana* (de Felippes et al. 2012). Using MIGS technology Benstein et al. (2013) efficiently silenced *Arabidopsis* phosphoglycerate dehydrogenase1 (PGDH1) to study its role in Phosphoserine pathway, which has an important function in plant development. Using MIGS2.1 vector, silencing was successfully induced in C-terminally encoding protein1 (*CEP1*) gene in *Medicago trancatula,* which plays a major role in root development (Imin et al. 2013). To characterize the role of *Phosphoserine Aminotransferase1* (PSAT1) in serine biosynthesis pathway and its role in plant growth, Wulfert and Krueger (2018) used MIGS technology to downregulate *AtPSAT1* gene and obtained strong growth inhibition in both shoots and roots of *PSAT1*-silenced lines. Starch composition of rice was altered by silencing granular-bound starch synthase (GBSS) gene by using MIGS which effectively reduced amylose content in rice endosperm (Zheng et al. 2018). The authors also compared silencing efficiency of different MIGS constructs and found that the silencing efficiency was related to the selection of MIGS interfering target sites and specificity of the target genes. The target sites with high sequence homology found to be more efficient in interfering certain genes (Zheng et al. 2018). To study the role of microProteins in flowering behavior, Graeff et al. (2016) successfully reduced the expression levels of microProteins miP1a and miP1b using MIGS in *Arabidopsis thaliana.* In petunia, MIGS efficiently induced gene silencing of chalone synthase

(CHS) and phytone desaturase (PDS) and resulted in albino plants (Han et al. 2015). The authors carried out deep sequencing and concluded that processing of miRNA precursor in petunia is different from Arabidopsis. Zhao et al. (2015) demonstrated antiviral resistance in *Nicotiana benthamiana* targeting 3' noncoding region or capsid protein-coding region of *Plum pox virus* (PPV) RNA by producing siRNAs through MIGS. MIGS was successfully employed to achieve viral resistance in tobacco and tomato by downregulating two RNAi suppressor proteins, AC2 and AC4, of geminivirus Tomato leaf curl New Delhi virus (ToLCNDV) (Singh et al. 2015). The authors used miR390-based MIGS vector to achieve silencing of target genes.

Jacobs et al. (2016) identified nine tasiRNA loci in soybean and experimentally validated corresponding targets by silencing a transgenic GFP gene and two endogenous genes by developing transgenic hairy roots and plants. The authors demonstrated the use of another 22nt-miRNA, miR1514 in constructing MIGS vectors. MIGS triggered by miR1514a.2 was successfully tested by silencing nodulation factor receptor kinase 1α (NFR) and putative cytochrome P450 CYP51G1 in soybean hairy roots and whole plants (Jacobs et al. 2016) (Table 2.1).

Table 2.1 Application of MIGS in gene silencing studies of model and crop plants

miRNA trigger	Plant species	Targets[a]	References
miR173	*Arabidopsis thaliana*	CH42	Felippes and Weigel (2009)
		AG, ELF3, FT, LFY	de Felippes et al. (2012)
		PGDH1	Benstein et al. (2013)
		miP1a, miP1b	Graeff et al. (2016)
		PSAT1	Wulfert and Krueger (2018)
	Medicago truncatula	CEP1	Imin et al. (2013)
	Nicotiana benthamiana	PPV	Zhao et al. (2015)
	Petunia hybrida	CHS, PDS	Han et al. (2015)
	Oryza sativa	GBSS, LAZY1, PDS, ROC5	Zheng et al. (2018)
miR390	*Arabidopsis thaliana*	CH42	Felippes and Weigel (2009)
	Nicotiana tabacum *Solanum lycopersicum*	ToLCNDV, ToLCGV	Singh et al. (2015)
miR1514a.2	*Glycine max*	NFR1α, P450 CYP51G1	Jacobs et al. (2016)

[a]Abbreviations: CH42, CHLORINA 42; AG, AGAMOUS; ELF3, EARLY FLOWERING 3; FT, FLOWERING LOCUS T; LFY, LEAFY; PGDH1, PHOSPHOGLYCERATE DEHYDROGENASE 1; miP1a, microProtein 1a; miP1b, microProtein 1b; PSAT1, PHOSPHOSERINE AMINOTRANSFERASE 1; CEP1, C-TERMINALLY ENCODED PEPTIDE 1; PPV, Plum pox virus; CHS, CHALCONE SYNTHASE; PDS, PHYTOENE DESATURASE; GBSS, GRANULE BOUND STARCH SYNTHASE 1; LAZY1, shoot gravitropism gene; ROC5, RICE OUTERMOST CELL-SPECIFIC 5; ToLCNDV, Tomato leaf curl New Delhi virus; ToLCGV, Tomato leaf curl Gujarat virus; NFR1α, NODULATION FACTOR KINASE 1α; P450 CYP51G1, putative cytochrome P450 CYP51G1

2.6 Limitations of MIGS and Steps to Overcome the Limitations

There is some risk of off-targeting associated with MIGS. All the population of siRNAs produced from MIGS construct is capable of silencing targeted sequences. There is a possibility that the non-intended targets which share sequence similarities may get silenced (de Felippes 2019). And also, few tasiRNAs derived from MIGS load into AGO1 and others either load to different AGOs or get degraded (Carbonell 2019).

In case of MIGS, the tasiRNAs form in a phased manner due to miRNA-triggered cleavage and are highly predictable (Allen et al. 2005; Montgomery et al. 2008b; de Felippes 2013; Felippes and Weigel 2009). The formation of phased tasiRNAs can be predicted by bioinformatics softwares like pssRNAMINER, tasiRNAdb, and SoMART, which could be used for minimizing off-target gene silencing (Pandey et al. 2015). The use of endogenous miRNAs in MIGS vector construction is also useful to minimize the off-target effects of exogenous miRNA expression (Jacobs et al. 2016).

Like other silencing tools, a variety of siRNAs are produced from the template dsRNA. To choose gene specificity in MIGS, the gene fragments selected, should share little sequence similarity and in that case selecting untranslated regions (UTRs), among related homologs, is a better choice (Wesley et al. 2001). Also, small gene fragments will be ideal for MIGS, ensuring predictability of phased tasiRNAs (Montgomery et al. 2008b; Felippes and Weigel 2009).

2.7 Conclusions

Nowadays genome editing like CRISPR/Cas9 has been widely used to mutagenize or edit gene sequence in plants (Belhaj et al. 2015; Rinaldo and Ayliffe 2015) to generate gene knock-out lines. But gene silencing technologies, used for gene function studies, are much simpler to use. Complete knock-out of a gene, as in the case of CRISPR/Cas9, may be lethal to plants, and cannot be recovered, but gene silencing technologies allow incomplete gene knockdowns to study the function of a gene. This technology allows tissue-specific gene silencing and also silencing of multiple genes. Furthermore, the technologies can be improved by overcoming the limitations and possible applications in plant functional genomics studies. With the advancement of plant genomics, comparative studies can be performed and with the availability of different computer programs, potential effective siRNAs and dsRNAs can be designed and analyzed to minimize off-target effects (Naito and Ui-Tei 2012; Naito et al. 2005).

MIGS is a miRNA-mediated RNA interference technology which has emerged recently. It has gained importance due to its simple construction steps, high specificity and efficiency in gene silencing. The ability to effectively silence multiple unrelated

genes using a single vector is an added advantage. Still it needs to be refined in terms to reduce off-targeting of the genes. The role and molecular mechanisms of recently identified phasiRNAs in different plant species needs to be explored. A deeper understanding of miRNA-triggered tasiRNA biogenesis, mode of action, and targeting efficacy is needed for better use of this technology in gene silencing studies and crop improvement.

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