Artificial microRNAs and their applications in plant molecular biology

MicroARNs artificiales y sus aplicaciones en biología molecular de plantas

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ABSTRACT

Artificial microRNAs (amiRNAs) are modified endogenous microRNA precursors in which the miRNA:miRNA* duplex is replaced with sequences designed to silence any desired gene. amiRNAs are used as part of new genetic transformation techniques in eukaryotes and have proven to be effective and to excel over other RNA-mediated gene silencing methods in both specificity and stability. amiRNAs can be designed to silence single or multiple genes, it is also possible to construct dimeric amiRNA precursors to silence two non-related genes simultaneously. amiRNA expression is quantitative and allows using constitutive, inducible, or tissue-specific promoters. One main application of amiRNAs is gene functional validation and to this end they have been mostly used in model plants; however, their use can be extended to any species or variety. amiRNA-mediated antiviral defense is another important application with great potential for plant molecular biology and crop improvement, but it still needs to be optimized to prevent the escape of viruses from the silencing mechanism. Furthermore, amiRNAs have propelled research in related areas allowing the development of similar tools like artificial trans-acting small interference RNAs (ta-siARNs) and artificial target mimicry. In this review, some applications and advantages of amiRNAs in plant molecular biology are analyzed.

Key words: miRNAs, amiRNAs, viruses, gene silencing, RNA interference.

RESUMEN

Los microARNs artificiales (amiARNs) consisten en precursores de microARNs (miARNs) nativos en los que se reemplazan las secuencias del dúplex miARN:miARN* con secuencias diseñadas para silenciar cualquier gen deseado. Los amiARNs son utilizados en transformación genética de eucariotas y han mostrado ser efectivos, superando otros métodos de silenciamiento génico mediado por ARN en cuanto a especificidad y estabilidad. Los amiARNs pueden ser diseñados para silenciar uno o múltiples genes, su expresión es cuantitativa y permite la utilización de promotores constitutivos, inducibles o tejido-específicos. Una de sus principales aplicaciones es la validación funcional de genes, y para este fin han sido utilizados hasta ahora principalmente en plantas modelo, pero su uso puede extenderse a cualquier especie o variedad. Otra aplicación con gran potencial es la defensa contra virus mediada por amiARNs, sin embargo, aun debe ser optimizada para prevenir el escape de los virus al mecanismo de silenciamiento. Los amiARNs han impulsado además la investigación en áreas relacionadas y el desarrollo de herramientas como los ta-siARNs (*trans actins small interference RNAs*) artificiales y la imitación de blancos o *target mimicry*. En esta revisión se analizan algunas de las aplicaciones y ventajas de los amiARNs en biología molecular de plantas.

Palabras clave: miARNs, amiARNs, virus, silenciamiento génico, ARN de interferencia.

Introduction

Eukaryotes use post transcriptional gene silencing (PTGS) as a mechanism of gene regulation and natural defense against invasive nucleic acids, transposons, and other highly repetitive genomic sequences (Bartel, 2004). This mechanism is mediated by different kinds of small RNAs, among which the most widely studied are microRNAs (miRNAs) and small interference RNAs (siRNAs).

miRNAs are molecules of approximately 22 nt originating from nuclear genes. A gene codifying for a miRNA (*MIRNA*) is first transcribed by the RNA polymerase II in a primary miRNA (pri-miRNA) whose size can range from 100 nt to several kilobases (kb). It is then processed to an intermediate RNA called miRNA precursor (pre-miRNA) by Dicer-like 1 (DCL1) in plants. Pre-miRNAs have a characteristic secondary "hairpin-like" structure, with high and negative fold-free energy, these can have widely varying sizes, commonly between 70 and 400 nt. Then, the pre-miRNA is processed to amiRNA:miRNA* duplex again by DCL1 in plants. The duplex is recruited by the RNA-Induced Silencing Complex (RISC) where the strand with the least stability in the 5' extreme (the mature miRNA) will be retained by an argonaut protein (AGO), while the passenger strant (miRNA*) will be degraded, then this

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complex will guide the cleavage ortranslational repression of messenger RNAs complementaryto the mature miRNA (*targets*) (Bartel, 2004; Zhang *et al*., 2006; Zhu, 2008).

In contrast, siRNAs originate from transgenes, viruses, and transposons and form perfectly complementary double-strand RNA precursors (dsRNAs) (Aravin *et al*., 2003; Filipowicz *et al*., 2005). siRNAs silence the same gene from which they come and, from a siRNA precursor multiple siRNAs can be generated and these can be transmitted among cells (Bartel, 2004).

miRNAs have been identified as important gene expression regulators in plants and animals (Jones-Rhoades *et al*., 2006). Among the different functions established for miRNAs in plants, there are physiological aspects as important as morphogenesis and organ polarity, identity of floral organs and flowering time, hormone signaling, transition from juvenile to adult states, reproduction, and response to different kinds of biotic and abiotic stress (Dugas and Bartel, 2004; Jones-Rhoades *et al*., 2006; Mallory and Baucheret, 2006).

miRNAs have been studied extensively during recent years, among other reasons because by it being a very elegant and precise mechanism of gene regulation, its manipulation through different methods would offer new possibilities for genetic improvement of eukaryotes. One way of using the miRNAs path to alter the expression of certain genes is by employing artificial miRNAs (amiRNAs), which are precursors of endogenous miRNAs modified for silencing any desired target. This work will analyze some applications of artificial amiRNAs in plants, including functional validation of genes and defense against viruses.

Design of artificial microRNAs

Artificial miRNAs are designed from a precursor of an endogenous miRNA, used as structural support and in which the region of the mature miRNA is replaced with a specific amiRNA sequence complementary to the desired target sequence (Ossowski *et al*., 2008; Park *et al*., 2009). The procedure was first developed in animals (Zeng *et al*., 2002) and then in plants (Parizotto *et al*., 2004). A miRNA precursor can be modified without affecting its natural processing, as long as the same secondary structure is maintained (Parizotto *et al*., 2004; Vaucheret *et al*., 2004). For this, we must replace the mature miRNA sequence along with that of the complementary strand or miRNA* without changing structural aspects like non-complementary regions (mismatches) orbulges (Ossowski *et al*., 2008).

The first step in designing amiRNAs consists of selecting an amiRNA sequence complementary to the desired gene, which must be optimal regarding specificity and effectiveness (Schwab *et al*., 2010). If what is sought is to silence one or multiple genes, it is necessary to verify that there is no random mating with other genome regions. To avoid these types of situations, it is ideal to have an annotation of the genome or a significant collection of Expressed Sequence Tags (ESTs) of the studied species. Optimizing by effectiveness is mainly based on the calculation of hybridization energy between the amiRNA and the desired target, ideally the amiRNAs with the lowest hybridization energy, less than -30 kcal/mol should be chosen (Schwab *et al*., 2010). There is a quick and easy way of designing amiRNAs by using the MicroRNA Designer (WMD) web application (Schwab *et al*., 2006), which has been widely used in recent years (Khraiwesh *et al.,* 2008; Ossowski *et al*., 2008; Warthmann *et al*., 2008; Molnar *et al*., 2009).

To ensure amiRNA effectiveness, there are other considerations based on *in vivo* mating of endogenous miRNAs with their targets. Total complementarity is preferred of the ~21nt of the amiRNA with the target; if there are mismatches, these should never be in positions 10 or 11 (5'-3' sense), given that this is the cleavage site of the miRNA: target pair, also, mismatches should preferably not be in the 5' region (Mallory *et al*., 2004; Schwab *et al*., 2006). Additionally, some authors consider it important to introduce *mismatches* in the 3' region (Moissiard *et al*., 2007; Khraiwesh *et al*., 2008). It is preferable for the miRNA to have an A or a U in position 10 and a U in the first position given that these nucleotides are overrepresented in natural plant miRNAs (Mallory *et al*., 2004; Reynolds *et al.,* 2004; Schwab *et al*., 2006). Also, a higher AU content in the 5' extreme and greater GC content in the 3' extreme yields instability to the 5' extreme, which is considered necessary to incorporate the mature strand to the RISC complex (Schwab *et al*., 2006).

There might also be important effects caused by the mRNA structure next to the targeted region; thereby, we must bear in mind the energy of the miRNA union to the target, as well as the energy needed to "open" the union site (Kertesz *et al*., 2007; Warthmann *et al*., 2008).

The designed amiRNA and its amiRNA* sequence must replace the miRNA:miRNA* duplex from an endogenous pre-miRNA, some recommended precursors are MIR319a from *Arabidopsis*, MIR528 from rice, and MIR1157 from *Chlamydomonas reinhardtii* (Tab. 1). Once we have the cloned precursors in plasmids, the sequences may be in-

corporated to the precursors, through guided mutagenesis or overlapping PCR (Schwab *et al*., 2010). An alternative for replacing the miRNA:miRNA* sequences is the method developed by Molnar *et al*. (2009), which involves the use of double-strand DNA oligonucleotides to replace DNA from the precursor in the plasmid by using restriction enzymes. This process, however, may be restricted to small miRNA precursors and has only been implemented until now in *C. reinhardtii* (Molnar *et al*., 2009).

A terminator sequence and a promoter sequence must also be added to the plasmid with the precursor. Silencing mediated by amiRNAs occurs in quantitative manner. Constructions containing strong promoters are highly expressed; promoters like 35S from *Cauliflower mosaic viruses* (CMV) have been broadly used (Ossowski *et al*., 2008; Zhao *et al*., 2008; Schwab *et al*., 2010). However, it is possible to also use inducible or tissue-specific promoters (Schwab *et al*., 2006). amiRNA can then be inserted in the plant via traditional genetic transformation methods like *Agrobacterium*-mediated transformation (Schwab *et al*., 2010).

It is recommended that studies on transformed plants confirm the reduction of transcripts of the desired gene through RT-PCR (Schwab *et al*., 2010); RACE-PCR could also be used to verify that the mRNA scission occurs in the desired site (Kasschau *et al*., 2003; Álvarez *et al*., 2006; Schwab *et al.,* 2010).

Silencing of endogenous amiRNA-mediated genes

One of the most common ways to characterize gene function is by analyzing individuals with loss-of-function alleles or mutant genes, in this way the gene's biological activity is inferred from the resulting phenotype (Lukowitz *et al.,* 2000). Several tools have been successful for this type of approach in plants like transpose on tagging, insertional mutagenesis, and tilling, which have been applied successfully, producing extensive and detailed collections of these types of mutants in various species (Alonso and Ecker, 2006; Molnar *et al*., 2009; Schwab *et al*., 2010). Nevertheless, saturating complete genomes through this approach requires very large populations of the desired specie and the strategy is normally restricted to certain genetic "contexts" or varieties. Besides, the method can be limited by selectivity in the mutation process and commonly does not permit partial or regulated function loss, which is necessary for studying genes when their null alleles are lethal (Warthmann *et al*., 2008, Molnar *et al*., 2009, Schwab *et al*., 2010).

Alternative methods to obtain loss of gene function are based on RNA-mediated gene silencing (interference RNA or PTGS). RNA transgenes are dominant and can be applied to any variety and to species in which extensive collections of mutants are not yet available. Also, these approaches allow simultaneous silencing of related genes, and gene silencing in an inducible or tissue specific manner (Ossowski *et al*., 2008; Warthmann *et al*., 2008).

Common methods of gene silencing are based on the production of dsRNAs that will produce siRNAs to silence the desired gene. The most usual ones consist of 1) the overexpression of a transgene, usually antisense, from the gene sought to be silenced (co-suppression or antisense RNA) (Jorgensen *et al*., 2006), 2) the sense and antisense expression for the gene to be silenced thus forming a hairpin RNA or hpRNA (Watson *et al*., 2005), or 3) the insertion of the gene to be silenced in genomes of viruses inoculated on the plant which generate dsRNAs during their replication, this strategy is better known as virus-induced gene silencing (VIGS) (Lu *et al*., 2003). Although these tools have been broadly used for gene validation and discovery, they still have some difficult ties hindering their broader use in species of agronomic interest (Warthmann *et al*., 2008). Among its main disadvantages, there are specificity and stability. These strategies normally use inserts of relatively long size (>200 base pairs), which can generate a great number of siRNAs complementary to distinct regions of the desired gen. Long inserts will most probably generate siRNAs with effective silencing, but will also possibly generate siRNAs complementary or partially complementary to unwanted genome regions (Schwab *et al*., 2006; Khraiwesh *et al*., 2008; Warthmann *et al*., 2008; Molnar *et al*., 2009). The vast number of possible siRNAs generated by a transgene makes it difficult to predict the unwanted targets (Schwab *et al*., 2006). It is estimated that between 50 and 70% of the genes in an organism can produce siRNAs complementary to unwanted targets if they are used in interference RNA methods (Xu *et al.,* 2006). Furthermore, the transgenes can be self-silenced, resulting in loss of silencing after several generations (Zhao *et al.,* 2008; Molnar *et al*., 2009).

amiRNAs become a gene silencing alternative with all the advantages of previous methods of PTGS-mediated silencing and with additional advantages regarding specificity and durability. Successful experiments of amiRNAs-mediated gene silencing have been conducted in dicotyledonous (*Arabidopsis*, tomato, tobacco) monocotyledonous (rice), mosses (*Physcomitrella patens*), and algae (*C. reinhardtii*) (Tab. 1). Different precursors have been used and it has been shown that precursors from different plants can

be expressed in others sharing the same miRNA family, producing effective silencing that permits standardizing methods for broad phyologenetic groups (Álvarez *et al*., 2006; Khraiwesh *et al*., 2008).

amiRNAs can be designed and optimized to silence one or several genes with similar sequences, including tandem genes, (Álvarez *et al*., 2006; Schwab *et al.,* 2006; Choi *et al*., 2007) and have a specificity as high as that of natural plant miRNAs, without formation of secondary siRNAs and without non-autonomous effects (Parizotto *et al.,* 2004; Schwab *et al*., 2005; Warthmann *et al*., 2008). Given that an amiRNA produces a unique miRNA with the capacity to silence a specific target, potential unwanted targets can be predicted and avoided from the design (Schwab *et al*., 2010). In addition, amiRNA transgenes are dominantly inherited and remain stable and active in the progeny (Warthmann *et al*., 2008; Liu and Chen, 2010). In *Chlamydomonas* amiRNA activity has remained for up to six months (Zhao *et al.,* 2008) and 500 generations (Molnar *et al*., 2009). In all, these advantages allow amiRNAs to be considered the most suitable strategy to generate transgenic plants and improve crops (Liu and Chen, 2010).

As with siRNA transgenes, amiRNAs can be expressed in constitutive or inducible manner or via specific tissue according to the promoter used; it has even been possible to generate amiRNAs under the control of the promoter from the same gene sought to be silenced (Schwab *et al.,* 2006). Also, the small size of the precursors used for amiRNAs (<300 base pairs) has allowed for two functional amiRNAs to be simultaneously produced from the same transgene, permitting for two unrelated genes to be simultaneously silenced (Niu *et al*., 2006; Lin *et al*., 2009). Given their specificity, amiRNAs can be designed to silence specific alleles or specific forms (E.g., produced via alternative splicing) of certain genes (Ossowski *et al*., 2008; Schwab *et al*., 2010).

There is yet no systematic study comparing and showing the advantages of amiRNAs over other interference RNA methods, although Qu *et al*. (2007) reported a case in which amiRNAs were more effective than hpRNAs in producing silencing. The rate of success of amiRNA-mediated silencing, inferred from published studies, may vary from 90% (Álvarez *et al*., 2006; Niu *et al*., 2006; Choi *et al*., 2007; Mathieu *et al.,* 2007; Qu *et al.,* 2007; Schwab *et al*., 2006) to 75% (Ossowski *et al.,* 2008). The reasons why some genes cannot be silenced by amiRNAs are not yet clear (Park *et al*., 2009; Schwab *et al*., 2010). A possible explanation is the difficulty in accessing the target mRNA by the RISC complex (Ameres *et al*., 2007; Kertesz *et al.,* 2007; Warthmann *et al*., 2008; Molnar *et al*., 2009). This difficulty may, nevertheless, be compensated by thermodynamically modeling the RNA-RNA interactions during the design, as previously described. Another yet unproven possibility may be through negative regulation, such that the silencing produced is compensated with higher rates of transcription that overcome the amiRNA activity (Ossowski *et al*., 2008).

An additional consideration when working with amiRNAs must be the fact that, although cleavage of the messenger RNA is the predominant mode of action for miRNAs in plants, mRNA translational inhibition has also been reported without knowing yet any adequate way of determining in which instances it occurs (Brodersen *et al.,* 2008). When amiRNA-mediated translational inhibition occurs, the silencing and the phenotypic effects will not be different from those given by mRNA cleavage (Jones-Rhoades *et al.,* 2006; Ossowski *et al*., 2008). However, this activity may not be monitored and quantified through usual RT-PCR methods (Kasschau *et al.,* 2003; Schwab *et al.,* 2010).

In spite of the potential of the amiRNA method, it has only been used until now in model species (Tab. 1), particularly in *Arabidopsis thaliana*, for which there are even efforts to have a library of amiRNAs covering all the annotated genes (Ossowski *et al*., 2008), as there is for humans and mice (Chang *et al*., 2006). Nevertheless, it is only a matter of time for this strategy to expand to other species. Among the *Arabidopsis* genus amiRNAs have already been used in wild varieties or in non-model lines (Bomblies *et al.,* 2007). Likewise, given the novelty of microRNAs, most studies conducted have concentrated on showing their suitability using genes with already known functions and with those for which we already have null mutants (Tab. 1).

Some successful and novel functional analysis experiments have been conducted on *A. thaliana*. For example, by using the tissue-specific expression of amiRNAs, Mathieu *et al.* (2007) showed that the FT protein and not its mRNAis the mobile signal to induce flowering. Choi *et al.* (2007) used amiRNAs to demonstrate that *H2AZ* genes are necessary to activate gene transcription in similar manner as in yeast. Finally, Bomblies *et al.* (2007) used amiRNAs to show that NBS-LRR type genes *AT5G41740/AT5G41750* are involved in the necrosis produced in *Arabidopsis* hybrid crosses as an autoimmune response.

amiRNA-mediated defense against viruses

One of the applications of amiRNAs with the greatest agricultural could be the defense against viruses. Interference RNA methods had already been used in plants to increase

TABLE 1. Endogenous plant genes silenced using amiRNAs.

Specie	Silenced gene	Function	Pre-miRNA	References
A. thaliana	ARF	Auxin-response factor	ath-miR164b	Álvarez *et al.* (2006)
A. thaliana	NGA	Trancription factor	ath-miR164a	Álvarez *et al.* (2006)
A. thaliana	AT5G41740/AT5G41750	NBS-LRR, autoinmune response	ath-miR-319a	Bomblies *et al.* (2007)
A. thaliana	H2AZ	Trancription factor	ath-miR-319a	Choi *et al.* (2007)
A. thaliana	PP2AA	Phosphatase, auxin distribution	ath-miR-319a	Michniewicz *et al.* (2007)
A. thaliana	GFP (transgen)	Green fluorescent protein	ath-miR171	Parizotto *et al.* (2004)
A. thaliana	AP1	Floral identity	ath-miR-319a	Park *et al.* (2009)
A. thaliana	CAL	Floral identity	ath-miR-319a	Park *et al.* (2009)
A. thaliana	CRC	Adaxial polarity regulation	ath-miR319a, ath miR172a	Schwab *et al.* (2006)
A. thaliana	GUN4	Chlorophyll biosynthesis	ath-miR319a, ath miR172a	Schwab *et al.* (2006)
A. thaliana	INO, YAB3	Floral organs distribution	ath-miR319a, ath miR172a	Schwab *et al.* (2006)
A. thaliana	LFY	Floral identity	ath-miR319a, ath miR172a	Schwab *et al.* (2006)
A. thaliana	SEP1-4, SHP1-2, AP1, CAL	Floral organs distribution	ath-miR319a, ath miR172a	Schwab *et al.* (2006)
A. thaliana	SOC1, MAF1-3, ANR1	Nutrient uptake and flowering time	ath-miR319a, ath miR172a	Schwab *et al.* (2006)
A. thaliana	TRY, CPC, ETC2	Trichome regulation	ath-miR319a, ath miR172a	Schwab *et al.* (2006)
A. thaliana	FT	Flowering time	ath-miR319a, ath miR172a	Schwab *et al.* (2006), Mathieu *et al.* (2007), Schwartz *et al.* (2009)
C. reinhardtii	COX90	Cytochrome oxidase c subunit	cre-miR1157	Molnar *et al.* (2009)
C. reinhardtii	DCL1	Small RNA processing	cre-miR1157	Molnar *et al.* (2009)
C. reinhardtii	PSY	Phytoenesinthase	cre-miR1157	Molnar *et al.* (2009)
C. reinhardtii	MAA7	Tryptophan synthesis	cre-miR1162	Zhao *et al.* (2008)
C. reinhardtii	RBCS1/2	Rubisco small subunit	cre-miR1162	Zhao *et al.* (2008)
N. tabacum	ARF	Auxin-response factor	ath-miR164b	Álvarez *et al.* (2006)
O. sativa	Eui1	Internode elongation	osa-miR528	Warthmann *et al.* (2008)
O. sativa	Pds	Phytoenedesaturase	osa-miR528	Warthmann *et al.* (2008)
O. sativa	Spl11	Lesion formation	osa-miR528	Warthmann *et al.* (2008)
P. patens	FtsZ2-1	Chloroplast division	ath-miR319a	Khraiwesh *et al.* (2008)
P. patens	GNT1	N-acetilglucosaminiltransferase	ath-miR319a	Khraiwesh *et al.* (2009)
S. lycopersicum	ARF	Auxin-response factor	ath-miR164b	Álvarez *et al.* (2006)
S. lycopersicum	NGA	Transcription factor	ath-miR164a	Álvarez *et al.* (2006)

their defenses against viruses (Waterhouse *et al.,* 1998; Helliwell and Waterhouse, 2005), in spite of presenting the difficulties mentioned in the case of endogenous genes: poor specificity due to the high probability of unwanted targets and low stability due to self-silencing.

Plants naturally use PTGS to defend themselves against viruses, mainly by siRNA production (Ding *et al*., 2004; Mahmood-ur-Rahman *et al*., 2008), and until now, a natural role of plant miRNAs for defense against viruses has not been found, as it has been observed in animals (Lecellier *et al*., 2005). Several authors suggest that plant miRNAs have a potential for defense against viruses (Llave, 2004; Dunoyer and Voinnet, 2005; Lecellier *et al*., 2005; Simón-Mateo and García, 2006; Lu *et al*., 2008) and bioinformatics analyses supports this hypothesis (Pérez-Quintero *et al.,* 2010).

By using amiRNAs, it has been possible to show that the miRNA path can work efficiently in defense against viruses. Transgenic *A. thaliana* and *N. benthamiana* plants were created with resistance to *Turnip mosaic virus* (TMV), *Turnip yellow mosaic virus* (TuMV), and *Cucumber mosaic virus* (CMV) using amiRNAs aimed against sequences of these viruses (Tab. 2). Inthese cases, it was shown that resistance occurs at cellular level and is inheritable and that amiRNAs can successfully block virus replication and avoid its mobility and translocation (Niu *et al*., 2006). It has also been shown that it is possible

to create transgenic plants expressing dimeric amiRNAs aimed against two types of viruses resulting in plants resistant to a broad viral spectrum (Niu *et al.,* 2006; Lin *et al.,* 2009).

As with amiRNAs aimed against endogenous genes, accessibility to the target mRNA of the virus can affect amiRNA effectiveness (Niu *et al*., 2006; Simón-Mateo and Garcia, 2006; Duan *et al.,* 2008). The secondary structure of the long viral mRNA *in vivo* is difficult to predict and model. Duan *et al.* (2008) suggested an experimental method to design effective amiRNAs ensuring target mRNA accessibility. For this, a test is carried out by inoculating wild and mutant *Arabidopsis* plants lacking DCLs (proteins responsible for processing small RNAs) with the unwanted viral RNA, viral RNA cleavage sites are compared and, thus, sensitive sites to siRNA-mediated cleavage are identified; these sites could then be used to design amiRNAs against the virus analyzed.

There are other aspects to consider when applying amiRNAs as a strategy to increase plant resistance against viruses. First, many viruses codify silencing suppressors that directly interfere with the miRNA machinery (Llave, 2004; Zhang *et al*., 2006; Duan *et al.,* 2008). The relatively simple solution of designing amiRNAs aimed against silencing suppressors has proven to be effective (Niu *et al*., 2006; Qu *et al.,* 2007). The second objection and perhaps the most important is that viral genomes evolve much faster than plant miRNAs, and it has been noted that eventually viruses can evade amiRNA attacks through mutations or deletions in the target region (Simón-Mateo and García, 2006; Lin *et al.,* 2009). This has also been an obstacle in developing gene therapy against viruses like HIV in humans (Das *et al*., 2004; Westerhout *et al*., 2005). It is felt that the key to overcome this obstacle lies in the "polymeric strategy", *i.e*., create transgenic plants with amiRNAs aimed against several regions of the same virus (and even of several viruses), preferably using highly conserved regions (Niu *et al.,* 2006; Duan *et al.,* 2008; Lin *et al.,* 2009). The effectiveness of this strategy has not yet been shown in plants.

amiRNA sin defense against viruses offer as an advantage the preventive feature of this type of defense, meaning that the plant can be resistant to viruses without ever having found them (Lu *et al*., 2008). Besides, it has been suggested that amiRNA-mediated silencing would pose less problems regarding bio-safety and environmental security with respect to other strategies (Liu and Chen, 2010), bearing in mind that the size of the inserts is relatively small, reducing the probabilities for horizontal transfer of genes and that viral genomes are not used in the transformation. Production of new allergens or toxic proteins in transgenic plants is equally less likely than with other strategies (Niu *et al*., 2006). However, studies have not been conducted to extensively assess the safety of this strategy.

Perspectives on research with amiRNAs in plants

The field of plant transformation with amiRNAs is quite novel and we are perhaps far from obtaining plants with desirable agronomical traits through this strategy. Applications in functional gene validation are more immediate and the method is already being accepted as an usual and effective tool in research. There are still many aspects to be clarified on the function of amiRNAs in plants, among others, the way of evaluating and differentiating mRNA cleavage and translational inhibition. For the case of resistance to viruses, we must evaluate the durability of such in transgenic plants obtained with the polymer strategy.

Research with amiRNAs has also influenced development of related techniques like artificial trans-acting small interference RNAs (ta-siRANs) orata-siRNAs (Gutiérrez-Nava *et al*., 2008). ta-siRNAs are a third type of small RNA involved in PTGS whose processing and activity involves siRNA and miRNAs machinery. ta-siRNAs are coded by nuclear genes, whose transcript mRNAis a target for miRNAs,

TABLE 2. Studies using silencing mediated amiARNs in virus defense in plants.

Species	Virus	Viral region	Reference
A. thaliana	*Turnip yellow mosaic virus* (TuMV)	P69	Niu *et al.* (2006), Lin *et al*. (2009)
A. thaliana	*Turnip mosaic virus* (TMV)	HC-PRO	Niu *et al*. (2006)
A. thaliana	*Cucumber mosaic virus* (CMV)	3 _ UTR	Duan *et al.* (2008)
A. thaliana	*Turnip yellow mosaic virus P69* (TuMV-P69) *Chimeric*	P69	Lin *et al.* (2009)
N. benthamiana	*Cucumber mosaic virus* (CMV)	2b (Silencing suppressor)	Qu *et al*. (2007)
N. benthamiana	*Turnip mosaic virus* (TMV)	P69	Lin *et al.* (2009)
N. benthamiana	*Turnip yellow mosaic virus P69* (TuMV-P69) *Chimeric*	P69	Lin *et al.* (2009)

one of the mRNA segments resulting from miRNA scission will produce a dsRNA that will then be processed to produce several siRNAs, which will have as target nuclear genes different to that from which they originate; hence, it is said they act in *trans*. Gutiérrez-Nava *et al.* (2008) managed to replace the siRNA region in the ta-siRNA coding gene TASc1 (which requires activity from miRNA miR173 to produce siRNAs), successfully silencing the gene from the Fatty Acid Desaturase 2 (FAD2). Gutiérrez-Nava *et al.* (2008) stated that with this strategy multiple genes could be silenced with a sole construct in a simpler manner than with amiRNAs and that for this strategy the secondary structure considerations are not critical.

Likewise, another promising strategy is target mimicry, which consists of inserting a non-cleavable RNA that will act in non-productive manner with a miRNA inhibiting its activity (Franco-Zorrilla *et al.*, 2007). This strategy will permit modulating the level of endogenous microRNAs and allow greater expression of genes that are normally under microRNA regulation and, thus, perhaps improve some agronomic features. This application, however, has not yet been extensively evaluated (Franco-Zorrilla *et al.*, 2007; Liu and Chen, 2010).

The discovery of gene silencing mechanisms mediated by small RNAs, and their implication in defense processes against viruses and regulation of endogenous genes in plants has permitted designing new approaches like amiRNAs to modify gene expression in plant sand, as a result, allow functional gene validation, without needing large populations or completely sequenced genomes for their application. This is of special importance in countries like Colombia where efforts are currently underway for genetic improvement of plants with practically unknown genomes. amiRNAs will surely contribute largely to the development of plant genetics being that it is a simple and effective mechanism, with advantages on specificity, stability, and environmental safety; besides, it is expected that it will allow the development of improved varieties in crops of agricultural interest in a world where increased population and climate change phenomena make up heavy pressure towards safe, innovative, and productive means of agriculture.

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