Biochemical and Biophysical Research Communications 406 (2011) 315–319

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Mini Review

Artificial microRNAs (amiRNAs) engineering – On how microRNA-based silencing methods have affected current plant silencing research

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ARTICLE INFO

Article history:
Received 26 January 2011
Available online 15 February 2011

Keywords:
amiRNAs
Gene knockouts
Model organism
Virus

ABSTRACT

In recent years, endogenous microRNAs have been described as important regulators of gene expression in eukaryotes. Artificial microRNAs (amiRNAs) represent a recently developed miRNA-based strategy to silence endogenous genes. amiRNAs can be created by exchanging the miRNA/miRNA* sequence within a miRNA precursor with a sequence designed to match the target gene, this is possible as long as the secondary RNA structure of the precursor is kept intact. In this review, we summarize the basic methodologies to design amiRNAs and detail their applications in plants genetic functional studies as well as their potential for crops genetic improvement.

1. Introduction

Eukaryotes use small non-coding RNAs in processes of post-transcriptional gene silencing (PTGS) to regulate gene expression, as well as to direct epigenetic modifications. These small RNAs interact with silencing complexes to recognize and modify complementary nucleic acids [1,2].

Small-interfering RNAs (siRNAs, 21–24 nucleotides long) and microRNAs (miRNAs, 21–22 nucleotides long) are the two types of small RNAs involved in PTGS with important differences in their biogenesis and target genes. siRNAs originate commonly from invading or aberrant nucleic acids and produce *cis*-acting silencing by targeting the same molecule from which they were derived. miRNAs instead originate from endogenous genes and are involved in *trans*-acting silencing of other endogenous genes [3,1]. miRNAs are usually transcribed by RNA polymerase II as long primary RNA transcripts (pri-miRNAs), which are normally capped at the 5′ end and polyadenylated at the 3′ end [4]. The pri-miRNA is processed in the nucleus by Dicer-like1 (*DCL1*) in plants, to liberate a 60–70 nt stem loop structure known as precursor miRNA (pre-miRNA) [3]. The pre-miRNA is then exported to the cytoplasm by an Exportin 5-dependent mechanism and further processed into a transient ~22 bp miRNA:miRNA* duplex (* indicates the passenger strand, which is not complementary to the RNA target) again by *DCL1* in plants [3]. The miRNA:miRNA* duplex is then loaded into the RNA-induced silencing complex (RISC). Only the mature miRNA strand is retained in the complex, while the miRNA* is degraded. The mature miRNA is used as a template to guide the silencing of complementary target mRNAs [5]. In plants, miRNAs display a high degree of complementary with their targets and produce silencing mostly through mRNA cleavage, however translational repression has also been documented [6].

2. Why amiRNAs were needed, design and advantages?

Prior to the development of amiRNAs, various strategies exploited the PTGS pathway to produce gene knockouts and study gene function. These strategies were mainly based on the production of siRNAs derived from dsRNAs introduced to the plant in various ways. One strategy known as virus-induced gene silencing which employs viral genomes as vectors to introduce dsRNAs to silence any desired gene in the plant to study the phenotype produced [7]. Although the approach has certain advantages, the main difficulty is to identify the appropriate virus to develop an adequate viral vector acting as a silencer without causing symptoms. Other alternatives based on the production of siRNAs include over-expression of the target gene (usually in antisense direction) by genetic transformation [8] and the construction of sense-antisense fragments of DNA, which can include an intron (hairpin) or not [9]. The large inserts employed in these approaches produces a diverse set of siRNAs from a complete dsRNA since the siRNA signal is amplifiable producing often multiple secondary siRNAs, increasing the risk of silencing undesired genes (off-targets) by fortuitous complementarity [10,11]. Additionally, transgene can be auto-silenced, thus losing activity over the target gene after some time or generations [12]. The extensive studies in miRNAs have of-

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doi:10.1016/j.bbrc.2011.02.045

fered a new alternative approach to effectively silence target genes circumventing these difficulties through the production of amiRNAs.

amiRNAs are designed from an endogenous miRNA precursor which is used as a structural support in which the miRNA:miRNA* region is replaced with an specific miRNA complementary to the desired target sequence. The biogenesis of this new precursor will be the same as the endogenous one, as long as the secondary fold-back structure of the precursor is kept intact. Fig. 1 illustrates the process of amiRNA engineering for effective silencing of the target gene. Thus, for functional gene analysis, amiRNAs can be designed to target any gene of interest, even multiple genes, and an amiRNA sequence with little similarity to any undesired plant gene can be chosen to avoid off-target effects [13–15]. Since they are trans-acting, the silencing activity is stable through various generations [12,16]. Also it is possible to generate constructs expressing multiple and unrelated amiRNAs due to their small size as well as designing amiRNAs targeting specific alleles or splice forms for a given gene [17–19]. It has also been suggested that amiRNAs pose fewer biosafety or environmental problems when applied to agriculture than other strategies [20,21]. Other advantages include strand-specific silencing, tissue differential expression and the possibility of complementation with non-cleavable targets [13].

3. amiRNA studies in model organisms

3.1. Arabidopsis thaliana

Since early in their development in molecular biology model plant *A. thaliana*, amiRNAs were found to exert efficient silencing in plants and their advantages in terms of specificity, effectiveness have increased with research over the last few years [13,14,22]. As an indication of the potential of amiRNAs in *Arabidopsis* research, a library of amiRNAs was developed and is currently kept at Cold Spring Harbor Laboratories, where each of the estimated 22,000 *A. thaliana* genes are targeted by at least three amiRNAs [10].

In early functional studies in *A. thaliana*, amiRNAs were used to identify possible phosphatase components of the mechanism responsible for polar targeting of PIN (pin-formed) auxin transport proteins [23]. The sub-cellular localization and distribution of these proteins is essential to direct auxin silencing, thus affecting overall organ development [23]. Few components of the molecular mechanisms involved in PIN sub-cellular distribution were known. Three closely related regulatory A subunits of the protein phosphatase 2A (*PP2A*) complex (*PP2AA1*, *PP2AA2* and *PP2AA3*) were candidates to be involved in PIN signaling. It was however not possible to obtain triple mutants seedlings through conventional muta-

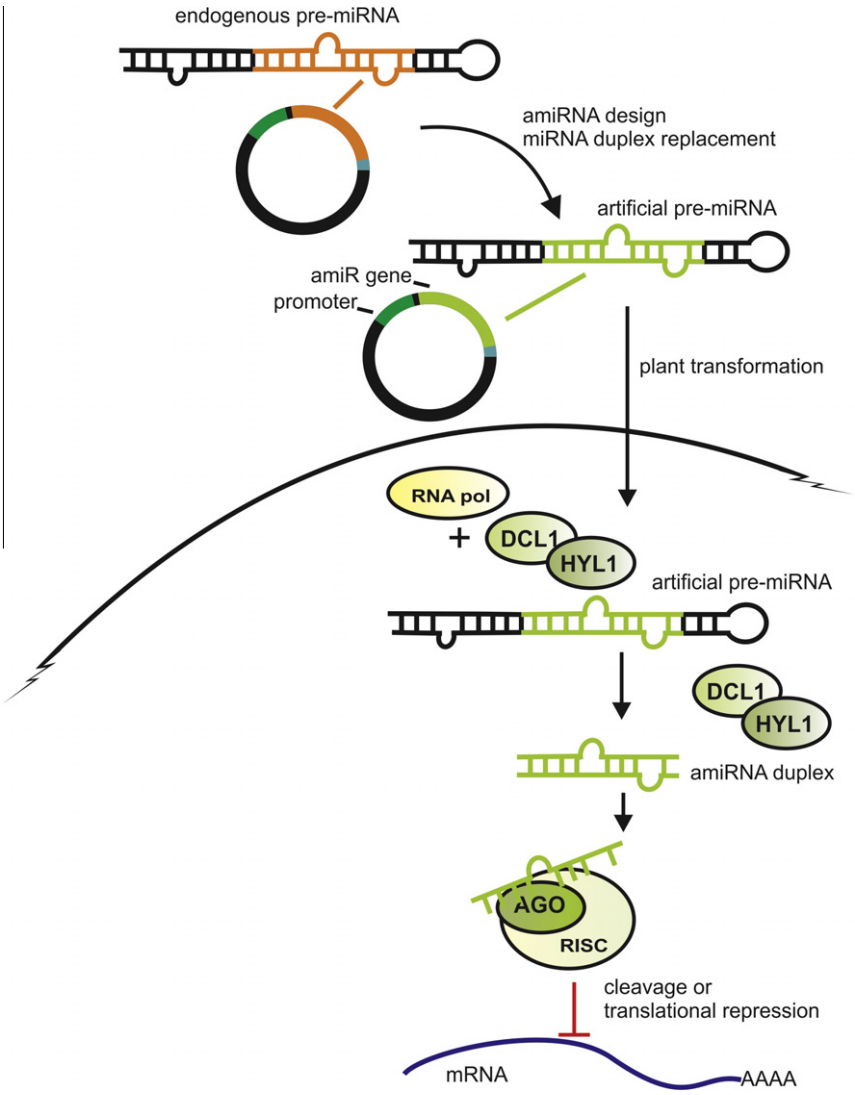

Fig. 1. Process of plant genetic transformation using amiRNAs.

tional and breeding strategies due to early developmental defects [23]. By developing transgenic lines with two estrogen inducible amiRNAs that simultaneously target all three *PP2A* genes it was possible to study the phenotype of the triple mutant, which showed more severe developmental defects than the single mutants [23]. This work shows how amiRNAs can be used to generate inducible silencing of closely related genes with great specificity and avoiding seedling lethality.

Very recently amiRNAs were used to elucidate the function of genes involved in circadian clock regulation in *A. thaliana*. A standardized method was elaborated for rapid assessment of gene function in circadian rhythms by combining transient expression of amiRNAs with a luciferase-based reporter of circadian clock activity in mesophyll protoplasts [24]. Using transient expression of amiRNAs coupled with luciferase-based reporter it was possible to recreate the loss-of-function phenotypes of genes *EARLY FLOWERING3* (*ELF3*), *ZEITLUPE* (*ZTL*) and *GIGANTEA* (*GI*) known to affect circadian cycling. In addition, it was possible to study the function of the *casein kinase II beta subunits* (*CKB*) gene family which are well-conserved regulators of eukaryotic circadian clocks [25]. A one-to-one and a cumulative target approach were used to target all four beta subunits using amiRNAs and highly specific results were obtained for all four *CKB* genes without off-targets, displaying a lengthened circadian period, in agreement with previous reports of a short period in *CKB* over-expresser's [24].

amiRNAs have been also successfully used to gain insights into pollen development. Arabinogalactan proteins (AGPs) are structurally complex proteoglycans present in plasma membranes and cell walls and perform diverse functions including developmental and structural patterning, and also affect plant sexual reproduction. Two very similar arabinogalactan genes, *AGP6*, and *AGP11* are pollen specific and play a major role in pollen development [26]. Levitin et al. [27] using RNAi lines showed that the reduction of the *AGP6* and of *AGP11* expression by RNAi caused inhibition of pollen tube growth and hampered pollen release. However in a recent work using amiRNA constructs to silence specifically *AGP6* and *AGP11* it has been hypothesized that the reduction in the fertilization is not due to pollen tube and stamen development but to the abortion of pollen grains during development [28]. Furthermore, using highly-specific amiRNA silencing it was shown that the *AGP6* and *AGP11* functions are redundant [28].

3.2. Oryza sativa

amiRNA studies started to spread to other plants, importantly, the mechanism was applied and standardized for functional studies in the model plant and crop *O. sativa* (rice). amiRNA engineering was successfully applied to Nipponbare (japonica) and IR64 (indica) rice varieties to target three different genes named *Phytoene desaturase* (*pds*, *Os03g08570*), *Spotted leaf 11* (*Spl11*, *Os12g38210*), and *Elongated uppermost internode1/CYP714D* (*Eui1*, *Os05g40384*). amiRNA-mediated silencing of these genes caused an albino phenotype (*pds*), spontaneous lesion formation in the absence of pathogens (*spl11*), and elongation of the uppermost internode at heading stage (*eui1*), thus displaying the same phenotypes as loss-of-function mutants, plus the effects were highly specific and stable in the progeny [29]. MicroRNAs were later used in rice to study *histone deacetylase* genes, important for overall gene expression regulation. Using down-regulation of target genes by amiRNAs, it was demonstrated that expression of rice *histone deacetylase* genes is tissue/organ/treatment specific [30].

3.3. Physcomitrella patens

Silencing through amiRNAs has also been used in genetic research of lower land plants. In the case of the moss *P. patens*, a first attempt to evaluate amiRNA processing consisted in two amiRNAs designed to target the genes *PpFtsZ2-1*, essential for chloroplast division, and *PpGNT1*, encoding an N-acetylglucosaminyltransferase. These amiRNAs successfully silenced the target mRNAs producing the expected loss-of-function phenotypes. Notably in these essays, transient production of siRNAs from cleaved target products was detected (indicating miRNA signal amplification) however this seemed to have no effect on target specificity [31]. Both *Physcomitrella* amiRNA's were expressed from the *Arabidopsis* miR319a pre-miRNA, showing high conservation of pre-miRNA processing across the plant kingdom [31]. It was later shown that the processing mechanism for pre-miR319 in *Physcomitrella*, both native and artificial, consists of a loop-first processing mechanism, opposed to the general loop-last processing of many pre-miRNAs, this new mechanism may contribute to the high efficiency of miR319-based amiRNA strategies [32].

miRNAs processing in *Physcomitrella* have gained much interest since new mechanisms of miRNA-mediated regulation were recently uncovered, with the aid of amiRNAs. The authors introduced the amiRNA targeting *PpGNT1* into a *Physcomitrella* mutant lacking *DCL1* expression, and surprisingly, reduced expression of *PpGNT1* was observed without mRNA cleavage or translational repression, instead the amiRNA was able to bind to the mRNA in a non-cleavable manner and subsequently direct DNA methylation of the corresponding gene [33]. It is yet to be determined how prevalent this mechanism of action is among plants.

3.4. Chlamydomonas reinhardtii

The work in the unicellular *Chlamydomonas* has shed further lights onto amiRNA specificity and efficiency, and the ongoing work in this model is expected to unveil the role of miRNAs in basal plant processes and to give insights into plant miRNAs evolution. In two jointly published works, *Chlamydomonas* genes were successfully silenced by amiRNAs, using the endogenous pre-miRNA1162 as backbone Zhao et al. silenced *Chamydomonas MAA7* and *RBCS1/2* genes coding for the tryptophan synthase β-subunit and the rubisco small subunit, respectively [16]. Molnar et al. [12] successfully silenced the *cytochrome c oxidase* subunit (*COX90*), *Phytoene synthase* (*PSY*) and *Dicer-like 1* (*DCL1*) genes using the endogenous pre-miRNA1157. By both approaches it was possible to obtain the desired loss-of-function phenotypes and, importantly, the short generation time of *C. reinhardtii* allowed an extensive assessment of amiRNA stability; in these studies amiRNA silencing was maintained for as long as six months or 500 generations [12,16].

Further insights into amiRNA specificity have been achieved in *C. reinhardtii* while studying the functions of three different *hydrogenase-like* genes *HydA1*, *HydA2*, and *HYD3*, which share some sequence similarity. By designing amiRNAs specifically targeting non-conserved regions in these genes was possible to differentiate each protein function without off-target silencing. It was thus revealed that *HydA1* represents the major hydrogenase activity under anaerobic conditions, the role of *HydA2* is not yet completely clear and *HYD3* is needed for normal growth since it is required for the activity of cytosolic Fe-S enzymes [34].

4. amiRNAs and potential applications in plant immunity

amiRNAs also hold a potential great impact in plant pathology, mainly, the use of amiRNAs to engineer plants resistant to viruses has been extensively explored [10,18]. In an early work, amiRNAs were designed (based on an *A. thaliana* miR159 precursor) to target viral mRNA sequences coding two silencing suppressors: P69 of the turnip yellow mosaic virus (TYMV) and HC-Pro of the turnip mo-

saic virus (TuMV). Transgenic *A. thaliana* plants expressing amiR-P69 and amiR-HC-Pro were specifically resistant to TYMV and TuMV, respectively, indicating that the expression of amiRNAs can successfully confer specific resistance to plant viruses [18].

Also, in *Nicotiana tabacum* amiRNAs were effectively used to target mRNAs encoding the silencing suppressor *HC-Pro* of potato virus Y (PVY) and the TGBp1/p25 (p25) of potato virus X (PVX) [35].

However these strategies faced an obstacle when thought to be applied in agriculture because viral genomes evolve a lot faster than plant miRNAs and can evade amiRNA targeting by mutating the targeted region [17,36]. Possible solutions include expressing multiple amiRNAs directed against multiple regions of the virus, especially highly conserved regions.

In this way, amiRNAs have also been successfully engineered as potential viral suppressors against cucumber mosaic virus (CMV), a tomato pathogen. In early works, it was shown that an amiRNA targeting sequences coding for the silencing suppressor 2b of the CMV could efficiently inhibit *2b* gene expression and confer effective resistance to CMV infection in transgenic tobacco plants [20,37]. Recently, transgenic tomato lines were developed expressing two amiRNAs targeting the highly conserved 3′ untranslated region (UTR) and the coding sequence shared by the RNA-dependent RNA polymerase (RdRP) 2a protein and the suppressor 2b protein genes of CMV [38]. Simultaneous targeting of these conserved regions resulted in effective and cell-autonomous resistance in tomato against CMV [38]. There are currently ongoing efforts to use amiRNAs to produce durable resistance against virus in various important crops, for example, cassava [39].

amiRNAs have also been used to gain insights into the molecular mechanisms underlying different aspects of plant immunity. They were important in *A. thaliana* to understand autoimmune responses as observed by hybrid necrosis [40]. An NBS–LRR protein encoding gene was thought to be responsible for epistatic allele-specific interactions involved in hybrid necrosis. Hybrids containing the *DM1* allele of this gene show an autoimmune necrosis response and when silenced by amiRNAs a normal phenotype was restored, thus proving for the first time a role for resistance-like genes in autoimmune responses [40]. Recently in *Glycine max* (soybean) an amiRNA was used to silence the *Glyma18g02680.1* gene which encodes for an LRR-kinase protein (a disease resistance gene homolog) located in the *Rhg1* locus. The *Rhg1* locus is associated with a quantitative trait locus (QTL) with significant contribution to soybean cyst nematode resistance (SCN, caused by *Heterodera glycines*) resistance. However when the *Glyma18g02680.1* gene locus was silenced by an amiRNA, the reduced expression of the LRR-kinase protein did not alter significantly the resistance to SCN. Complementation studies also suggest a non-essential role for this gene in SCN-soybean interaction [41].

5. amiRNAs advances in crops research

There is currently a lot of ongoing work using amiRNAs as the tool of choice for functional genetic studies, and they are spreading rapidly to important crops and to other plants. In wheat, for example, amiRNAs were used to silence the *5-methylcytosine glycosylase* gene responsible for the endosperm-specific demethylation and transcriptional activation, named *DEMETER.* amiRNAs were specifically expressed under the control of an endosperm-specific promoter to eliminate low molecular weight glutenins (gLMWs) from the developing endosperm in wheat transformed knock outs [42]. Recently amiRNAs have been effectively used in elucidating the role of the *flotillin-like* gene family (*FLOTs*) in symbiotic interactions in *Medicago truncatula*, the model leguminose to study nitrogen fixation. amiRNAs were used to target *FLOT2* and *FLOT4*, which are regulated during nodulation. Silenced *FLOT2* and *FLOT4* lines are defective in nodule formation and function and are unable to fix nitrogen, therefore illustrating the potential roles of these in nodule formation and nitrogen fixation [43].

As was mentioned, amiRNAs may represent fewer biosafety risks when compared with other silencing strategies, this is due to the fact that the strategy does not use viral vectors and that the insert size is very small reducing the probability of recombination or horizontal transfer. Recently, biosafety advantages of amiRNAs has been broaden since they have been proposed as a tool to solve a crucial problem for genetically modified (GM) crop cultivation: transgene containment. One way to prevent GM crop populations from crossing with wild populations is by rendering the transgenic male plant sterile, or unable to produce functional pollen. Control systems as cytoplasmic male-sterility (CMS) and genic male-sterility (GMS), are usually used for this purpose as well as for commercial production of high-quality hybrid seeds, these systems are however non-reversible. Recently, male sterility has been achieved using amiRNAs targeting TBP-associated factors (TAFs) encoding genes, these transcription factors are crucial for many developmental aspects including pollen production. Toppino et al. [44] silenced two TAF genes highly expressed in anthers in eggplant (*Solanum melongena*), with amiRNAs under control of the anther tissue specific promoters pTA29 and pNTM1. Additionally the system was made reversible by the ethanol inducible expression of an amiRNA-insensitive form of the TAF target genes [44]. Notably it was developed in a non-model plant and can easily be applied in other plants.

6. amiRNA as effective endogenous miRNA regulator

Interestingly, amiRNAs where recently used to silence endogenous miRNAs in *Arabidopsis* [45]. amiRNAs where specifically designed to target miRNA families miR159 and miR164, which target the *GAMYB*-like family of transcription factors and the CUC and NAC transcription factors, respectively. These families are expressed by multiple loci and the mature protein shows high sequence conservation. The amiRNA-mediated silencing was exerted over all members of each family [45]. The transformed plants showed, as expected, increased target mRNA expression and phenotypes resembling previously reported miRNA knockout mutants [45]. In these essays amiRNAs cleaved not the processed mature miRNAs but the stem-loop pre-miRNAs, suggesting that miRNA-mediated silencing can occur in the nucleus or that pre-miRNAs are exported to the cytoplasm.

Furthermore, amiRNAs were designed to silence miRNAs in a loci-specific manner by aiming to target specific non-shared regions of miRNA precursors [45]. These results show how amiRNAs can be used as a reliable strategy to elucidate the function of new miRNAs, and additionally differentiate the function of individual members of a miRNA family.

Acknowledgments

Gaurav Sablok thanks Key Lab of Horticultural Plant Biology (MOE), Huazhong Agricultural University. Tatiana Tatarinova would like to thank the University of Glamorgan's Research Investment Scheme for supporting this project. Financial support for Camilo López and Alvaro Perez comes from Dirección de Investigaciones sede Bogota (Universidad Nacional), Colciencias, and Ministerio de Agricultura de Colombia.

References

[1] W. Filipowicz, L. Jaskiewicz, F.A. Kolb, R.S. Pillai, Post-transcriptional gene silencing by siRNAs and miRNAs, Curr. Opin. Struc. Biol. 15 (2005) 331–341.

[2] C.A. Kidner, R.A. Martienssen, The developmental role of microRNA in plants, Curr. Opin. Plant Biol. 8 (2005) 38–44.
[3] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[4] Y. Lee, M. Kim, J. Han, K.-H. Yeom, S. Lee, S.H. Baek, V.N. Kim, MicroRNA genes are transcribed by RNA polymerase II, EMBO J. 23 (2004) 4051–4060.
[5] M.W. Jones-Rhoades, D.P. Bartel, B. Bartel, MicroRNAs and their regulatory roles in plants, Annu. Rev. Plant Biol. 57 (2006) 19–53.
[6] B. Yu, H. Wang, Translational inhibition by micrornas in plants, in: R.E. Rhoads (Ed.), MiRNA Regulation of the Translational Machinery, Springer, Berlin Heidelberg, 2010, pp. 41–57.
[7] R. Lu, A.M. Martin-Hernandez, J.R. Peart, I. Malcuit, D.C. Baulcombe, Virus-induced gene silencing in plants, Methods 30 (2003) 296–303.
[8] R.A. Jorgensen, N. Doetsch, A. MÇoLler, Q. Que, K. Gendler, C.A. Napoli, A Paragenetic Perspective on Integration of RNA Silencing into the epigenome, its role in the biology of higher plants, Cold Spring Harb. Symp. Quant. Biol. 71 (2006) 481–485.
[9] C.A. Helliwell, P.M. Waterhouse, Constructs and methods for hairpin RNA-mediated gene silencing in plants, in: R.E. David (Ed.), Methods in Enzymol. RNA Interference, Academic Press, 2005, pp. 24–35.
[10] R. Schwab, S. Ossowski, M. Riester, N. Warthmann, D. Weigel, Highly Specific Gene Silencing by Artificial MicroRNAs in *Arabidopsis*, Plant Cell 18 (2006) 1121–1133.
[11] P. Xu, Y. Zhang, L. Kang, M.J. Roossinck, K.S. Mysore, Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants, Plant Physiol. 142 (2006) 429–440.
[12] A. Molnar, A. Bassett, E. Thuenemann, F. Schwach, S. Karkare, S. Ossowski, D. Weigel, D. Baulcombe, Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*, Plant J. 58 (2009) 165–174.
[13] S. Ossowski, R. Schwab, D. Weigel, Gene silencing in plants using artificial microRNAs and other small RNAs, Plant J. 53 (2008) 674–690.
[14] E.A. Parizotto, P. Dunoyer, N. Rahm, C. Himber, O. Voinnet, In vivo investigation of the transcription, processing, endonucleolytic activity, and functional relevance of the spatial distribution of a plant miRNA, Genes Dev. 18 (2004) 2237–2242.
[15] W. Park, J. Zhai, J.Y. Lee, Highly efficient gene silencing using perfect complementary artificial miRNA targeting AP1 or heteromeric artificial miRNA targeting AP1 and CAL genes, Plant Cell Rep. 28 (2009) 469–480.
[16] T. Zhao, W. Wang, X. Bai, Y. Qi, Gene silencing by artificial microRNAs in *Chlamydomonas*, Plant J. 58 (2008) 157–164.
[17] S.S. Lin, H.W. Wu, S.F. Elena, K.C. Chen, Q.W. Niu, S.D. Yeh, C.C. Chen, N.H. Chua, Molecular evolution of a viral non-coding sequence under the selective pressure of amiRNA-mediated silencing, PLoS Pathog. 5 (2009) e1000312.
[18] Q.W. Niu, S.S. Lin, J.L. Reyes, K.C. Chen, H.W. Wu, S.D. Yeh, N.H. Chua, Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance, Nat. Biotechnol. 24 (2006) 1420–1428.
[19] R. Schwab, S. Ossowski, N. Warthmann, D. Weigel, Directed gene silencing with artificial microRNAs, Methods Mol. Biol. 592 (2010) 71.
[20] C.G. Duan, C.H. Wang, R.X. Fang, H.S. Guo, Artificial microRNAs highly accessible to targets confer efficient virus resistance in plants, J. Virol. 82 (2008) 11084–11095.
[21] Q. Liu, Y.Q. Chen, A new mechanism in plant engineering: the potential roles of microRNAs in molecular breeding for crop improvement, Biotechnol. Adv. 28 (2010) 301–307.
[22] Y. Kurihara, Y. Watanabe, Arabidopsis micro-RNA biogenesis through Dicer-like 1 protein functions, Proc. Natl. Acad. Sci. USA 101 (2004) 12753–12758.
[23] M. Michniewicz, M.K. Zago, L. Abas, D. Weijers, A. Schweighofer, I. Meskiene, M.G. Heisler, C. Ohno, J. Zhang, F. Huang, R. Schwab, D. Weigel, E.M. Meyerowitz, C. Luschnig, R. Offringa, J. Friml, Antagonistic regulation of PIN phosphorylation by *PP2A* and PINOID directs auxin flux, Cell 130 (2007) 1044–1056.
[24] J. Kim, D.E. Somers, Rapid assessment of gene function in the circadian clock using artificial microRNA in *Arabidopsis* mesophyll protoplasts, Plant Physiol. 154 (2010) 611–621.
[25] B. Maier, S. Wendt, J.T. Vanselow, T. Wallach, S. Reischl, S. Oehmke, A. Schlosser, A. Kramer, A large-scale functional RNAi screen reveals a role for CK2 in the mammalian circadian clock, Genes Dev. 23 (2009) 708–718.
[26] L.G. Pereira, S. Coimbra, H. Oliveira, L. Monteiro, M. Sottomayor, Expression of arabinogalactan protein genes in pollen tubes of *Arabidopsis thaliana*, Planta 223 (2006) 374–380.
[27] B. Levitin, D. Richter, I. Markovich, Z. Moriyah, Arabinogalactan proteins 6 and 11 are required for stamen and pollen function in *Arabidopsis*, Plant J. 56 (2008) 351–363.
[28] S.Ç. Coimbra, M.Ç. Costa, B. Jones, M.A. Mendes, L.G. Pereira, Pollen grain development is compromised in *Arabidopsis agp6 agp11* null mutants, J. Exp. Bot. 60 (2009) 3133–3142.
[29] N. Warthmann, H. Chen, S. Ossowski, D. Weigel, P. Hervé, Highly specific gene silencing by artificial miRNAs in rice, PLoS ONE 3 (2008) e1829.
[30] Y. Hu, F. Qin, L. Huang, Q. Sun, C. Li, Y. Zhao, D. Zhou, Rice histone deacetylase genes display specific expression patterns and developmental functions, Biochem. Biophys. Res. Commun. 388 (2009) 2266–2271.
[31] B. Khraiwesh, S. Ossowski, D. Weigel, R. Reski, W. Frank, Specific gene silencing by artificial microRNAs in *Physcomitrella patens*: an alternative to targeted gene knockouts, Plant Physiol. 148 (2008) 684–693.
[32] C. Addo-Quaye, J.A. Snyder, Y.B. Park, Y. Li, R. Sunkar, M.J. Axtell, Sliced microRNA targets and precise loop-first processing of MIR319 hairpins revealed by analysis of the *Physcomitrella patens* degradome, RNA 15 (2009) 2112–2121.
[33] B. Khraiwesh, M.A. Arif, G.I. Seumel, S. Ossowski, D. Weigel, R. Reski, W. Frank, Transcriptional control of gene expression by microRNAs, Cell 140 (2010) 111–122.
[34] J.E. Godman, A. Molnár, D.C. Baulcombe, J. Balk, RNA silencing of hydrogenase(-like) genes and investigation of their physiological roles in the green alga *Chlamydomonas reinhardtii*, Biochem. J. 431 (2010) 345–351.
[35] T. Ai, L. Zhang, Z. Gao, C.X. Zhu, X. Guo, Highly efficient virus resistance mediated by artificial microRNAs that target the suppressor of PVX and PVY in plants, Plant Biol., 2010. doi:10.1111/j.1438-8677.2010.00374.x.
[36] C. Simón-Mateo, J.A. García, MicroRNA-guided processing impairs Plum pox virus replication, but the virus readily evolves to escape this silencing mechanism, J. Virol. 80 (2006) 2429–2436.
[37] J. Qu, J. Ye, R. Fang, Artificial microRNA-mediated virus resistance in plants, J. Virol. 81 (2007) 6690–6699.
[38] X. Zhang, H. Li, J. Zhang, C. Zhang, P. Gong, K. Ziaf, F. Xiao, Z. Ye, Expression of artificial microRNAs in tomato confers efficient and stable virus resistance in a cell-autonomous manner, Transgenic Res. (2010) 1–13.
[39] J.R. Thompson, M. Tepfer, Testing the efficacy of artificial microRNAs to control cassava brown streak disease, J. Gen. Virol. 90 (2009) 2293–2298.
[40] K. Bomblies, J. Lempe, P. Epple, N. Warthmann, C. Lanz, J.L. Dangl, D. Weigel, Autoimmune response as a mechanism for a Dobzhansky-Muller-type incompatibility syndrome in plants, PLoS Biol. 5 (2007) e236.
[41] S. Melito, A. Heuberger, D. Cook, B. Diers, A. MacGuidwin, A. Bent, A nematode demographics assay in transgenic roots reveals no significant impacts of the Rhg1 locus LRR-Kinase on soybean cyst nematode resistance, BMC Plant Biol. 10 (2010) 104.
[42] R. Brew-Appiah, S. Rustgi, M. Claar, G. Langen, K. Kogel, D. Weigel, D.V. Wettstein, Artificial microRNAs for silencing wheat proteins causing celiac disease, in: R.A.T. Brew-Appiah (Ed.), Plant Biology 2010, American Society of Plant Biologists, Montreal, Canada, 2010.
[43] C.H. Haney, S.R. Long, Plant flotillins are required for infection by nitrogen-fixing bacteria, Proc. Natl. Acad. Sci. USA 107 (2010) 478–483.
[44] L. Toppino, M. Kooiker, M. Lindner, L. Dreni, G.L. Rotino, M.M. Kater, Reversible male sterility in eggplant (*Solanum melongena* L.) by artificial microRNA-mediated silencing of general transcription factor genes, Plant Biotechnol. J., 2010. doi:10.1111/j.1467-7652.2010.00567.x.
[45] A.L. Eamens, C. Agius, N.A. Smith, P.M. Waterhouse, M.B. Wang, Efficient Silencing of Endogenous MicroRNAs Using Artificial MicroRNAs in *Arabidopsis thaliana*, Mol. Plant, 2010. doi:10.1093/mp/ssq061.