Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1779 (2008) 725–734
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbagrm

Review

Evolution of microRNAs and their targets: Are all microRNAs biologically relevant?

Michael J. Axtell *

Department of Biology and Huck Institutes of the Life Sciences, Pennsylvania State University, University Park, PA 16802, USA

Received 4 January 2008; received in revised form 8 February 2008; accepted 8 February 2008
Available online 10 March 2008

Abstract

MicroRNAs (miRNAs) are defined by their precise processing from a longer stem-loop precursor and by their subsequent ability to direct the regulation of target RNAs distinct from the miRNA precursor. Several lines of evidence suggest that miRNAs arose at least twice during eukaryotic evolution from an ancestral, pan-eukaryotic small RNA producing molecular machinery, though alternative scenarios cannot be ruled out. A handful of plant miRNAs are strongly expressed, widely conserved among plants, and have identical targets in long-diverged species; most of these very well conserved miRNA–target relationships involve DNA-binding transcription factors with suspected roles in developmental control. In contrast, a much greater number of plant miRNAs are weakly expressed, poorly conserved, and have few if any readily identifiable targets. These miRNAs appear to be evolutionarily "transient", and many of them may be of little to no selective value. However, this ever-changing cast of transient miRNAs could provide a reservoir of potentially useful miRNAs from which new regulatory interactions sometimes are selected.
© 2008 Elsevier B.V. All rights reserved.

Keywords: microRNA; miRNA; Evolution; Plant; Target

1. Introduction

MicroRNAs are a gene family united not by a common sequence, nor by a common phenotypic output, but by a unique mode of biogenesis and molecular mechanism. Putting aside species- and kingdom-specific variations, all microRNAs (miRNAs) are processed from single-stranded precursor RNAs which form a stem-loop secondary structure [1]. This stem-loop is recognized and accurately processed by specialized endonucleases of the Dicer protein family to yield a discrete ~21 nt duplex termed the miRNA/miRNA* duplex [2–6]. One of these two strands, the miRNA, is then coupled to a second endonuclease in the Argonaute family of proteins [7–11]. Ultimately, the mature miRNA serves as a guide to direct Argonaute proteins to target RNAs based upon Watson–Crick base-pairing between the miRNA and target. In most circumstances, associations between Argonaute proteins and miRNA-selected targets lead to "repression" of the target, either via Argonaute-catalyzed hydrolysis of the target RNA's phosphodiester backbone [12,13] or through other mechanisms [14]. Thus, in contrast to the molecular entities typically thought of as "genes", miRNAs in isolation do not make a phenotypic contribution: It is only through their regulatory interactions with targets that they exert a phenotypic influence. In this review I shall focus on the evolution of miRNAs and their targets, emphasizing knowledge gained from plant systems. Particular attention is paid to a) the possible origins of miRNA-mediated gene regulation in plants, b) the identities and origins of plant miRNAs and their relationships to their targets, c) and the conservation of miRNA–target relationships in plants. Finally, I review the evidence for the recently proposed hypothesis that many miRNAs are "orphans" lacking biologically relevant targets, and subject to evolutionarily rapid decay via neutral drift.

2. siRNAs: An overview

miRNAs are not the only type of small RNA produced and utilized by the Dicer/Argonaute system. Short interfering RNAs (siRNAs) are produced from long perfectly double-stranded RNA (dsRNA) precursors by Dicer enzymes, bound to

* Tel.: +1 814 867 0241; fax: +1 814 863 1357.
E-mail address: mja18@psu.edu.

1874-9399/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagrm.2008.02.007

Argonaute proteins, and used to guide the repression of target RNAs. The first observations of siRNAs were those produced from "silenced" transgenes in plants as well as siRNAs produced against an invading plant virus [15]. The phenomenon of RNA interference (RNAi), where introduction of dsRNA results in the strong suppression of any identical mRNAs [16] is directly attributable to siRNA production, as shown initially by biochemical experiments using *Drosophila melanogaster* extracts [17–19]. The ability to convert dsRNA to repressive siRNAs appears to be nearly universal in eukaryotes: Besides multicellular eukaryotes, a diverse group of unicellular eukaryotes also silence genes when presented with a homologous dsRNA stimulus, including the green alga *Chlamydomonas reinhardtii* [20], the trypanosome *Trypanosoma bruceii* [21], the unicellular fungi *Cryptococcus neoformans* [22] and *Schizosaccharomyces pombe* [23], and the slime mold *Dictyostelium discoideum* [24], to name a few. Indeed, very few eukaryotes clearly lack the capacity to repress genes using siRNAs; these include the brewer's yeast *Saccharomyces cerevisiae* and certain species of trypanosome pathogens whose genomes encode neither Dicer nor Argonaute [25,26]. The generally pan-eukaryotic distribution of the Dicer/Argonaute-based siRNA pathway suggests that it arose early in eukaryotic evolution and has persisted in most extant lineages.

Natural selection did not produce the siRNA silencing pathway to enable modern genetic research. Instead, it likely arose as a primitive, nucleic acid based immune system. This hypothesis is particularly well supported in plants, where siRNAs produced against RNA viruses have been shown to accumulate during infections [15]. Strikingly, almost every plant virus inspected in detail encodes a protein which functions to prevent siRNA function, strongly suggesting that viruses without strong counter-measures against the siRNA pathway are not viable (reviewed in [27]). Genetic ablation of certain components of the siRNA pathway in plants can also increase susceptibility to viral infection [28–32]. Insects can also use the siRNA pathway for viral defense [33,34] but innovations in adaptive immunity have largely rendered siRNAs obsolete for viral defense in vertebrates. siRNAs are also deployed by many organisms to repress endogenous genomic parasites, such as transposons: Mutations in the *Caenorhabditis elegans* siRNA machinery lead to mobilization of transposons [35,36], and many endogenously produced plant siRNAs arise from interspersed repetitive elements [37–39].

3. The origins of miRNAs

In contrast to the pan-eukaryotic siRNAs, the phylogenetic distribution of miRNAs is more restricted. Until 2007, miRNAs were only known in plants, animals, and certain DNA viruses which infect animals. There are systematic differences between plant and animal miRNAs:

3.1. Production of miRNAs

Production of animal miRNAs is a two-step process where the primary miRNA transcript (the pri-miRNA) is first cleaved in the nucleus by the RNAse III Drosha, liberating a short stem-loop RNA (the pre-miRNA; [6]). This cleavage defines the position of one end of the miRNA/miRNA* duplex. After nuclear export of the pre-miRNA-mediated by Exportin-5 [40–42], Dicer catalyzes excision of the miRNA/miRNA* duplex in the cytosol. In contrast, plant miRNA/miRNA* duplexes are completely liberated in the nucleus by a single Dicer protein, known as DCL1 in *Arabidopsis thaliana* [43–45].

3.2. Pri-miRNA secondary structures

The stem-loop secondary structures which give rise to miRNA/mRNA* duplexes, and eventually mature miRNAs, are quite uniform in size throughout animals, with a typical separation of ~11nts between the base of the stem and the position of Drosha cleavage, and a uniform loop length [46,47]. In contrast, the sizes of plant stem-loops vary dramatically between different miRNAs [48–50]. These differences seem likely to reflect different modes of pri-miRNA recognition by the miRNA processing machineries of plants and animals.

3.3. Modes of target repression

Many animal miRNAs interact with target mRNAs via short complementary regions located predominately within 3′ untranslated regions (3′-UTRs). Complementarity to a short 7 or 8 nt region of the miRNA (the "seed") is often enough to confer measurable repression [51–53]. Such seed-based miRNA–target interactions repress translation of the target without using Argonaute-catalyzed hydrolysis; repression is likely conferred by interfering with the initiation of translation and promoting subsequent de-adenylation and de-stabilization of the target — though the precise mechanism remains controversial (a concise review of this controversy may be found in [14]). In contrast, most known plant miRNAs have much more extensive complementarity to their targets, and are repressed by Argonaute-catalyzed cleavage [12,54–56]. The cleaved target sites in plants can be found throughout the mature transcript, with most occurring within open reading frames.

3.4. Conservation of miRNAs

Some animal miRNAs are conserved in widely divergent animal taxa: The seminal example is *let-7*, which is identical in vertebrates, arthropods, and roundworms [57]. Many other animal miRNAs are conserved across similar phylogenetic distances only in the seed region [58]. Similarly, many plant miRNAs are identical across large evolutionary distances [59,60]. However, with two questionable exceptions (see below), there are no miRNAs which are *shared* between animals and plants.

The extensive differences between animal and plant miRNAs in their biogenesis, modes of target repression, and patterns of conservation have led to the suggestion that plant and animal miRNAs arose independently by co-opting a pre-existing, universal system for siRNA production based upon Dicer and Argonaute proteins [1]. This hypothesis has the advantage of simultaneously explaining why the core

components of the miRNA pathway (Dicers and Argonautes) are identical between the two kingdoms but why the details of the miRNAs themselves are consistently different. However, this simple two origin hypothesis for the origins of miRNAs has been complicated by several independent studies. Arteaga-Vazquez et al. [61] described two miRNAs, miR854 and miR855, which are present in both plants and animals and are derived from LTR-retrotransposons. The implication of this finding is that miR854 and miR855 are ancient miRNAs which were present in the last common ancestor of modern plants and animals, and that miRNAs could have only arisen only once in eukaryotic evolution. Curiously, two independent experiments which collectively sequenced over a million *A. thaliana* small RNAs [37,49] failed to recover miR854 or miR855; however, the regions surrounding the miR854 and miR855 loci have numerous, *RDR2*-dependent 24nt small RNAs matching broad regions of both polarities of the genome. Additionally, the predicted secondary structures of *A. thaliana* miR854 and miR855 are strikingly different than those of other more conventional miRNAs. Altogether, these data suggest that miR854 and miR855 are transposon-associated siRNA-producing loci mis-annotated as miRNAs. Thus, the conservation of these loci likely reflects the ubiquity of both LTR-retrotransposons in eukaryotic genomes and of siRNAs deployed as transposon counter-measures. More convincing evidence for miRNA expression has been reported in the unicellular green alga *C. reinhardtii* [62,63]. The *Chlamydomonas* miRNAs are reminiscent of plant miRNAs in their high complementarity to targets, their propensity to direct target cleavage rather than translational repression, and their variable stem-loop sizes. However, none of the *Chlamydomonas* miRNAs identified to date are conserved in either land plants or animals. The social amoeba *D. discoideum* also expresses miRNAs distinct in sequence from known plant and animal miRNAs from stem-loops of variable length; in this case the mode of targeting has not been determined [64]. Thus, while the two origin hypothesis for miRNAs remains an attractive vehicle for explaining the systematic differences between plant and animal miRNAs, the recent observations of miRNAs in single-celled eukaryotes necessitate more complex patterns of miRNA evolution. Further small RNA discovery and analysis from diverse eukaryotes will undoubtedly shed light on this question.

4. Conservation of plant miRNA–target relationships

From the outset, it was recognized that many miRNAs found in the dicot *Arabidopsis* had potential homologs in rice and other monocotyledonous plants [50,65], and computational analyses predicted that these miRNAs might regulate homologous targets in both species [66,67]. In a seminal report, Floyd and Bowman [60] showed that the extent of miRNA–target conservation in plants was much deeper. The *A. thaliana* miR166 miRNA regulates a small clade of *homeodomain leucine-zipper III* (*HD-ZIPIII*) transcription factor mRNAs — this regulation is critical for adaxial/abaxial patterning of lateral organs and for the maintenance of the shoot apical meristem [68–70]. Analysis of *HD-ZIPIII* cDNAs from a diverse assembly of land plants demonstrated that the miR166 complementary site was conserved at all three codon positions, indicating selection for nucleic acid sequence rather than for amino acid sequences [60]. Thus, the regulation of *HD-ZIPIII* transcripts by the miR166 miRNA was both present in the last common ancestor of all or most land plants, and has persisted unchanged in multiple, long-diverged lineages. Subsequent studies have found that these results are typical for some plant miRNA families. Using microarrays, Axtell and Bartel [59] showed that several miRNA families first identified in *A. thaliana* had ancient origins in land plant phylogeny — several families were expressed in flowering plants, gymnosperms, and ferns, and a few were detected in the even more divergent lycopods and mosses. Importantly, in every case where targets of these ancient miRNAs could be experimentally verified, they were homologous to the known *A. thaliana* target [59]. Further computational prediction and experimental verification of conserved miRNA targets in mosses [71,72], gymnosperms [73], and diverse angiosperms [74–77] confirmed the generality of this observation: In plants, conservation of a miRNA generally implies conservation of a regulatory relationship with a specific set of targets.

Conserved miRNA families typically have many paralogs within a single genome which produce identical mature miRNAs. In *A. thaliana*, conservation of flanking protein-coding regions implies that some of these paralogs arose from tandem or segmental duplication events [78]. Because they are conserved in mosses and lycopods, many of the conserved miRNAs are more than ten times older than the ~40 million year resolution limit of the technique used to discern these duplication events, implying that local and large-scale genome duplications have contributed to copy number expansion of miRNAs for long periods of time. As for protein-coding genes, copy number expansion could have allowed opportunities for the diversification of expression patterns; this hypothesis is supported by MPSS-inferred pri-miRNA expression patterns [78] and by the differential expression and partial non-redundancy of the three *A. thaliana* miR164 paralogs [79]. In contrast, the observation that most non-conserved plant miRNAs are generally single copy loci [48,49,72] could be explained by the lack of time a recently born miRNA would have had to undergo copy number expansion by duplication events.

What processes might account for the extreme conservation of miRNA–target relationships in plants? The question can be divided into two parts: Why do the sequences of the miRNAs and of their target sites remain essentially fixed, and why have the regulatory relationships themselves been so indispensable? The first is easy to rationalize. Because miRNA–target recognition requires a high degree of base-pairing between miRNAs and their targets, sequence changes in the miRNA must be accompanied by compensatory changes in the target to retain base-pairing. Most miRNAs have more than one target, which further compounds the requirement for simultaneous, compensatory mutations to preserve the integrity of the regulatory interaction. The requirement for a high degree of complementarity between plant miRNAs and their targets thus acts as a stabilizer preventing sequence drift even over long periods of evolutionary time. The second question, why some of these miRNA–target relationships are so indispensable in the first

place, is much more difficult to answer without further studies in multiple, divergent species.

Despite the difficulties mentioned above, there are a few cases where coordinated sequence divergence appears to have obscured the evolutionary relationships between ancient miRNAs and their targets. Angiosperm miR390 targets a small family of *TAS3* non protein-coding mRNAs; certain siRNAs produced from the miR390-cleaved *TAS3* transcripts can regulate the expression of *Auxin Response Factor* (*ARF*) mRNAs [80]. In the moss *Physcomitrella patens*, miR390 also targets a small family of non protein-coding transcripts which go on to produce secondary siRNAs, but neither the target transcripts nor the resultant siRNAs have any sequence similarity to the angiosperm *TAS3* loci [81,82]. Nonetheless, some of the moss siRNAs function to target *ARF*s similar to those targeted by angiosperm *TAS3*-derived siRNAs [72], implying that the miR390-*TAS3*-*ARF* regulatory cascade is ancient, but has diverged in sequence in different lineages. Similar divergent evolution may also be possible for miRNA sequences themselves. *A. thaliana* miR168 regulates the expression of the *Argonaute1* (*AGO1*) transcript, and functions as a negative feedback regulator of the miRNA pathway [83,84]. Microarray experiments indicate that miR168 is conserved and expressed in angiosperms, gymnosperms, and ferns, but not detectable in mosses [59]. The moss-specific miR904 also targets three *AGO1* homologs in *P. patens*, but does not share any sequence identity with miR168 [72]. One possible interpretation of this observation is that the miR168-*AGO1* and miR904-*AGO1* regulatory interactions diverged from an ancient common ancestor. However for both of these examples, the hypothesis that the observed regulatory interactions arose from independent events (via convergent evolution) cannot be ruled out. Regardless of origins, these examples demonstrate that even miRNA–target interactions which initially appear to be lineage-specific can be sequence-divergent examples of common regulatory functions.

Perhaps the most striking observation with regard to the conservation of plant miRNA–target regulatory interactions is the identity of the targets themselves. Of the seven miRNA families which are identical in *A. thaliana* and *P. patens*, six either directly or indirectly target DNA-binding transcription factors in both species (Table 1; [72]). Each of these transcription factor targets can be expected to directly regulate the transcription of scores of downstream genes. Thus, the small number of very ancient miRNA–target interactions sit on the top of potentially extensive networks of gene expression. Most of these ancient miRNA targets have been experimentally demonstrated to play a role in angiosperm development, including the *SPB*-box targets of miR156 [56,85–87], the *ARF* targets of miR160 [88–90], the *HD-ZIPIII* targets of miR166 [68–70,91,92], the *TCP* targets of miR319 [93,94], and the *ARF* targets regulated indirectly via miR390 by siRNA intermediates [95–99]. Thus, the best available evidence suggests that the most conserved of the plant miRNAs function to control cascades of gene expression via transcription factor targets with the ultimate purpose of regulating plant development and morphology. How these conserved regulatory interactions have been recruited to assist in the huge diversity of land plant developmental strategies is an important and exciting topic of research.

5. The birth of plant miRNAs and their regulatory assignments may be linked

How do new miRNAs arise, and how do they acquire their target assignments? In plants, an attractive and well supported hypothesis put forward by Allen et al. [100] suggests that the birth of new miRNAs and their target specificities are inextricably linked. The precursor sequences for two miRNAs, miR161 and miR163, were observed to have long regions of similarity to their respective target mRNAs. The miRNA loci and the target genes were also closely linked within the *A. thaliana* genome. The observations suggested a duplication and divergence model for miRNA biogenesis where the critical initiating event was the duplication of a portion of a gene to create an inverted repeat locus. If transcribed, the inverted repeat would produce stem-loop RNA which could be recognized by Dicer-like proteins and used to produce small RNAs; by definition, these small RNAs would have perfect identity to the originating locus and thus be capable of regulation. Over time, mutational drift was predicted to erode the extended similarities between the originating locus and the inverted repeat, until only a small section (the miRNA) remained. Examples such as miR161 and miR163, which are physically linked to their target loci and retain extended complementarity, were postulated to be examples of "young" miRNAs in the middle of this process [100], a contention supported by their lack of conservation in other plant species.

Further support for this hypothesis has come from analyses of low-abundance, non-conserved miRNAs in *A. thaliana*. Out

Table 1
Ancient plant miRNA gene families and their targets

miRNA family	*A. thaliana* loci	*P. patens* loci	*A. thaliana* targets	*P. patens* targets
miR156	12	3	*SBP*-box transcription factors	*SBP*-box transcription factors
miR159/319	6	5	*MYB*/*TCP* transcription factors	*MYB* transcription factors
miR160	3	9	*ARF* transcription factors	*ARF* transcription factors
miR166	9	13	*HD-ZIPIII* transcription factors	*HD-ZIPIII* transcription factors
miR171	4	2	*GRAS* transcription factors	*GRAS* transcription factors
miR390	2	3	*ARF* transcription factors (via ta-siRNA intermediates)	*ARF* and *AP2* transcription factors (via ta-siRNA intermediates)
miR408	1	2	Plastocyanin-like genes	Plastocyanin-like genes

of a large number of poorly conserved *A. thaliana* miRNAs Rajagopalan et al. [49] found six whose precursors had extended similarity to their predicted target genes. Similarly, Fahlgren et al. [48] found that 16 out of 48 non-conserved *A. thaliana* miRNAs analyzed had significant identity to one or more mRNAs even when setting aside the central miRNA and miRNA∗ sequences. In many of these cases, the predicted targets of the mature miRNAs were also the genes displaying extended complementarity to the miRNA precursors, supporting the inverted repeat hypothesis for the origins of plant miRNAs and their targets. However, in several other cases, the predicted targets of non-conserved miRNAs were different from the presumed originating loci, suggesting that miRNAs born of a duplication of one locus can evolve to target another. Two non-conserved miRNAs, miR822 and miR839 also fulfilled another specific prediction of the inverted repeat hypothesis originally set forward by [100]; both of them produced "clouds" of small RNAs from scattered positions along their stem-loops instead of the discrete miRNA/miRNA* duplexes typical of canonical miRNAs [49]. Also unlike most miRNAs, miR822 and miR839 are dependent upon on an siRNA-associated Dicer (*DCL4*), rather than the miRNA-associated *DCL1* for their accumulation. This suggests that these miRNAs may not have yet acquired the structural features needed to facilitate recognition by the presumably more specific DCL1. These observations are consistent with Allen and colleagues' postulated early stages of miRNA evolution prior to the eventual selection of a single, discrete regulatory miRNA sequence [100]; however, only miR822 has been shown to have extended complementarity to its targets — the targets of miR839, if any, remain obscure. In summary, the observation of several *A. thaliana* miRNAs whose precursors have extended complementarity to their targets and which appear to be species-specific supports the notion that miRNA birth can proceed by inverted duplication of future target genes. The fact that canonical plant miRNAs, whose precursors bear no resemblance to their targets outside of the mature miRNA, typically have multiple targets from the same clade of a gene family also supports this hypothesis.

6. Diminishing returns in miRNA discovery: The case for neutral miRNA expression

The small RNA populations of eukaryotes are complex both in terms of total diversity (the number of distinct RNAs expressed) and abundance — at least five orders of magnitude separate the most abundant small RNAs from the least in multiple species. Although subtractive hybridization strategies which could potentially be used for normalization of small RNA libraries have been reported [101], they have yet to be widely implemented. For plants, as in animals, the first efforts to sequence endogenous small RNAs analyzed relatively modest numbers of reads [50,65,102], and thus the initially identified miRNAs were those which were most abundant in readily isolated tissues. These most abundant miRNAs had several characteristics which were initially taken to be diagnostic of all miRNAs, including conservation in multiple species, precise excision of a single miRNA/miRNA* duplex from the primiRNA transcript, the existence of targets possessing a single site with near-perfect complementarity anywhere within the mature target message, and the miRNA-directed cleavage of those targets [12,50,55]. The application of next generation DNA sequencing technology to small RNA discovery, pioneered by Lu et al. [38] has radically changed this view. In *A. thaliana*, the bulk of miRNA diversity, but not abundance, is accounted for by miRNA families which tend to be weakly expressed, poorly conserved, and to have few, if any, verifiable or predicted targets [48,49]. A similar situation is found in the moss *P. patens* and the lycophyte *Selaginella moellendorffii* (though the targets of the *S. moellendorffii* miRNAs have yet to be predicted or verified; [72]). In rice, large-scale sequencing of small RNAs has also been reported [103,104]; the preponderance of "sparse clusters" of small RNA expression in the rice genome is suggestive of a similar pattern of highly diverse, non-conserved miRNAs. Analysis of the "discovery trajectories" of miRNA discovery in multiple species (Fig. 1) reveals two key features: One, as pointed out by Rajagopalan et al. [49] for *A. thaliana*, there is no sign of a plateau in miRNA discovery — that is, further increases in the depth of sampling of small RNA expression seem likely to enable discovery of yet more weakly expressed miRNA families. The second, and perhaps more intriguing observation, is that in every species thus far examined, the most deeply conserved miRNAs are also among the most highly expressed (Fig. 1).

What mechanisms could account for the systematic correlation between weak expression, poor conservation, and difficult to identify targets among plant miRNAs? Weak expression coupled with poor conservation could be explained by lineage-specific miRNAs which have recently evolved to fulfill a

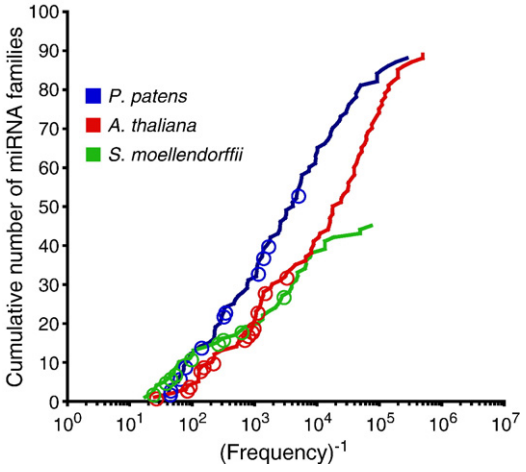

Fig. 1. miRNA discovery trajectories in three plant species. The cumulative number of distinct miRNA families discovered as a function of their abundance in wild-type tissues is plotted for the flowering plant *Arabidopsis thaliana* (red), the lycopod *Selaginella moellendorffii* (green), and the moss *Physcomitrella patens* (blue). Circles indicate miRNA families conserved between at least two of the three species — note that the conserved miRNAs in all three species are strongly biased toward higher abundances. *A. thaliana* data was a combination of the wild-type small RNAs of Kasschau et al. [37] and those of Rajagopalan et al. [49], while the *P. patens* and *S. moellendorffii* data were from Axtell et al. [72].

specialized role in a narrowly defined tissue or temporal condition. Indeed, the evolutionarily recent *A. thaliana* miR824 is expressed specifically within developing guard cell precursors where it functions to regulate the *MADS*-box transcription factor *AGL16* — this regulation is important for regulating the density of stomatal complexes on *A. thaliana* leaves [105]. miR824 is an example of a "young" miRNA under the inverted repeat hypothesis since its precursor possesses extended complementarity to the target *AGL16* mRNA [48]. Conversely, miRNAs for which targets are difficult or impossible to predict may obey rules for target interaction different than those which are currently understood. The atypical interactions with non-protein-coding RNAs demonstrated for both miR390 [81] and miR399 [106] suggest that alternative targeting rules do exist. But even if there does to prove to be a much larger set of plant miRNAs which use alternative rules for selection of targets, there is no obvious reason why these alternative rules would be strongly correlated with both poor conservation and weak expression. Indeed, both miR399 and miR390 are well-conserved. A simple null hypothesis which currently cannot be rejected for many of the poorly expressed, seemingly target-less, and non-conserved miRNAs is that they are selectively neutral — that is, their expression is of no biological consequence.

The hypothesis that at least some plant miRNAs could be of no biological significance is supported by several pieces of circumstantial evidence. The first, as pointed out by several publications [48,49,72], is that a huge diversity of miRNAs in multiple plant species are of very recent evolutionary vintage, implying that birth of new miRNAs is a frequent occurrence. Correspondingly, the "death" rate of such miRNAs must also be very high, because by definition these lineage-specific miRNAs are not found outside of the species in which they were first isolated. The observation that, in general, lineage-specific miRNAs have fewer, if any, confidently predicted targets [48,49,72] also argues in favor of their neutrality with regard to natural selection. The weak expression levels also might reflect a sort of "transcriptional ambivalence" with regard to these loci as would be expected if they were of little or no biological utility.

Several well described processes can easily be envisioned to conspire to create a vast pool of short-lived, largely neutral miRNAs. First, the hypothesis of inverted repeat origins for miRNA loci of Allen et al. [100] need not be restricted to protein-coding loci; inverted repeats likely occur everywhere in eukaryotic genomes with some frequency. Indeed, naive searches of plant genomes for simple inverted repeats find many millions [66]. Second, plant genomes, like those of animals, support expansive low-level transcription not only from canonical polI, polII, and polIII genes, but also from huge areas of presumably "intergenic" DNA [107,108]. Thus, pervasive low-level transcription of the numerous inverted repeats could easily give rise to a large diversity of stem-loop RNAs, many of which could be recognized as substrates for the miRNA biogenesis machinery. Most of the resulting miRNAs would not have enough sequence identity to regulate any targets, and in the absence of the selective pressure that a useful target regulatory interaction would confer, could be expected to be subject to rates of mutational drift typical of neutral DNA. This mutational drift would rapidly erode the ability of the locus to produce a Dicer-recognizable stem-loop, soon leading to the death of the miRNA. A permanent pool of individually transient miRNAs could form a sequence-diverse reservoir from which new miRNA-regulatory interactions could occasionally be recruited and retained [48,49,72].

7. Evolution of miRNA targets in animals

Do the lessons learned from plant miRNA–target relationships apply in animals? Like in plants, there is a core set of animal miRNAs which are conserved across highly divergent phyla, including *let-7* [57] and many others [109]. Analyses of orthologous 3′-UTRs clearly indicate that some miRNA–target interactions have been preferentially retained between related animal species. In fact, examination of conserved miRNA complementary sites in the 3′-UTRs of relatively closely related animals has greatly facilitated miRNA target predictions in vertebrates [110–114], insects [115–120], and roundworms [58,121–123]. However, there is little evidence for the rigid and extremely ancient miRNA–target relationships which typify the deeply conserved plant miRNAs. For instance, Grun et al. [116] compared the predicted targets of several miRNAs conserved between insects and vertebrates, and found only a small proportion of them were orthologous — many more targets were unique either to insects or to vertebrates. The implication is that though many animal miRNAs predate the emergence of disparate phyla, their regulatory targets have differentially evolved over time. Some of this may be explained by the different modes of target regulation between plants and animals: As few as seven nucleotides of complementarity are often sufficient for miRNA-mediated regulation in animals, and many such complementary sites occur in the relatively unconstrained 3′-UTRs. Thus, rather than being locked into fixed miRNA–target relationships, the 3′-UTRs of animal mRNAs may be capable of "sampling" regulation by different combinations of miRNAs over evolutionary time via mutational drift. In support of this hypothesis are observations indicating that tissue-specific miRNAs have played a major role in the evolution of 3′-UTR sequences of tissue-specific mRNAs [124,125]. Perhaps because the relationships between established animal miRNAs and their targets are so labile, no evidence of extended complementarity between animal miRNA precursors and an animal target has been reported. As in plants [39,49], a few *C. elegans* loci which produce small RNAs from near-perfect inverted repeats have been reported [58]; while these may be interpreted as "young" miRNAs, it remains unclear whether they were "born" with a pre-existing relationship to targets. Similar to plants, however, are data suggesting that there might be a vast array of poorly expressed, lineage-specific miRNAs in humans [126,127]. Analyses of *Drosophila* miRNAs are also reminiscent of the plant situation: miRNAs which are less conserved among 12 sequenced *Drosophila* genomes tend to have weak expression and have fewer predictable targets [117]. These observations are consistent with the hypothesis that animal

genomes also contain reservoirs of evolutionary transient miRNAs. Because the base-pairing requirements for biologically relevant target interactions are so reduced in animals compared to plants, it may be easier for recently evolved miRNAs of arbitrary sequence to become incorporated into a useful regulatory interaction in animals as compared to plants.

8. Conclusions and prospects

The understanding of miRNA evolution and targeting has blossomed in the last few years. A handful of miRNA–target relationships likely to be involved in developmental control are invariant in land plants separated by long periods of evolutionary time. Determining how these invariant molecular control elements have been utilized for vastly different phenotypic outputs is a major goal of future research. The observation of a large diversity of lineage-specific miRNAs which exhibit rapid birth and death rates raises the possibility that many miRNAs are "orphans" lacking biologically relevant targets, or lacking targets altogether. As this hypothesis can only be supported by negative data (i.e. the lack of a demonstrated function), it is quite difficult to conclusively demonstrate. Thus, it should instead be treated as a null hypothesis which can be rejected upon experimental confirmation of a biological role for any individual non-conserved miRNA. Methods which have served well for the more conserved miRNAs should be equally applicable to non-conserved miRNAs: In particular, miRNA over-expression followed by expression profiling [56] and inhibition of miRNA function using mimics [106] would seem to be especially promising avenues for attempting to reject the null hypothesis for non-conserved miRNAs. Piecing together how this shadow world of transient miRNAs interacts with the more obviously functional miRNA–target relationships will continue to be an exciting endeavor.

Acknowledgements

I apologize to colleagues whose work was not discussed due to size constraints. Many of the broad ideas on miRNA and target evolution explored in this review were strongly influenced by the work of E. Allen, N. Fahlgren, and J. Carrington, as well as the work of R. Rajagopalan and D. Bartel. I also thank T. Eshoo for critical comments on this manuscript, and the anonymous referees for constructive suggestions. Research in the author's laboratory is supported by the National Science Foundation.

References

[1] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[2] A. Grishok, A.E. Pasquinelli, D. Conte, N. Li, S. Parrish, I. Ha, D.L. Baillie, A. Fire, G. Ruvkun, C.C. Mello, Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing, Cell 106 (2001) 23–34.
[3] G. Hutvagner, J. McLachlan, A.E. Pasquinelli, E. Balint, T. Tuschl, P.D. Zamore, A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA, Science 293 (2001) 834–838.
[4] R.F. Ketting, S.E. Fischer, E. Bernstein, T. Sijen, G.J. Hannon, R.H. Plasterk, Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*, Genes Dev. 15 (2001) 2654–2659.
[5] N.C. Lau, L.P. Lim, E.G. Weinstein, D.P. Bartel, An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*, Science 294 (2001) 858–862.
[6] Y. Lee, C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim, V.N. Kim, The nuclear RNase III Drosha initiates microRNA processing, Nature 425 (2003) 415–419.
[7] N. Baumberger, D.C. Baulcombe, *Arabidopsis* ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 11928–11933.
[8] J. Liu, M.A. Carmell, F.V. Rivas, C.G. Marsden, J.M. Thomson, J.J. Song, S.M. Hammond, L. Joshua-Tor, G.J. Hannon, Argonaute2 is the catalytic engine of mammalian RNAi, Science 305 (2004) 1437–1441.
[9] G. Meister, M. Landthaler, A. Patkaniowska, Y. Dorsett, G. Teng, T. Tuschl, Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs, Mol. Cell 15 (2004) 185–197.
[10] Y. Qi, A.M. Denli, G.J. Hannon, Biochemical specialization within *Arabidopsis* RNA silencing pathways, Mol. Cell 19 (2005) 421–428.
[11] J.J. Song, S.K. Smith, G.J. Hannon, L. Joshua-Tor, Crystal structure of Argonaute and its implications for RISC slicer activity, Science 305 (2004) 1434–1437.
[12] C. Llave, Z. Xie, K.D. Kasschau, J.C. Carrington, Cleavage of *Scarecrow-like* mRNA targets directed by a class of *Arabidopsis* miRNA, Science 297 (2002) 2053–2056.
[13] S. Yekta, I.H. Shih, D.P. Bartel, MicroRNA-directed cleavage of HOXB8 mRNA, Science 304 (2004) 594–596.
[14] N. Standart, R.J. Jackson, MicroRNAs repress translation of m7Gpppp-capped target mRNAs in vitro by inhibiting initiation and promoting deadenylation, Genes Dev. 21 (2007) 1975–1982.
[15] A.J. Hamilton, D.C. Baulcombe, A species of small antisense RNA in posttranscriptional gene silencing in plants, Science 286 (1999) 950–952.
[16] A. Fire, S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, C.C. Mello, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, Nature 391 (1998) 806–811.
[17] S.M. Elbashir, W. Lendeckel, T. Tuschl, RNA interference is mediated by 21- and 22-nucleotide RNAs, Genes Dev. 15 (2001) 188–200.
[18] S.M. Hammond, E. Bernstein, D. Beach, G.J. Hannon, An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells, Nature 404 (2000) 293–296.
[19] P.D. Zamore, T. Tuschl, P.A. Sharp, D.P. Bartel, RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals, Cell 101 (2000) 25–33.
[20] M. Fuhrmann, A. Stahlberg, E. Govorunova, S. Rank, P. Hegemann, The abundant retinal protein of the *Chlamydomonas* eye is not the photoreceptor for phototaxis and photophobic responses, J. Cell Sci. 114 (2001) 3857–3863.
[21] H. Ngo, C. Tschudi, K. Gull, E. Ullu, Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 14687–14692.
[22] H. Liu, T.R. Cottrell, L.M. Pierini, W.E. Goldman, T.L. Doering, RNA interference in the pathogenic fungus *Cryptococcus neoformans*, Genetics 160 (2002) 463–470.
[23] M. Raponi, G.M. Arndt, Double-stranded RNA-mediated gene silencing in fission yeast, Nucleic Acids Res. 31 (2003) 4481–4489.
[24] H. Martens, J. Novotny, J. Oberstrass, T.L. Steck, P. Postlethwait, W. Nellen, RNAi in *Dictyostelium*: the role of RNA-directed RNA polymerases and double-stranded RNase, Mol. Biol. Cell 13 (2002) 445–453.
[25] L. Aravind, H. Watanabe, D.J. Lipman, E.V. Koonin, Lineage-specific loss and divergence of functionally linked genes in eukaryotes, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 11319–11324.
[26] D.F. Smith, C.S. Peacock, A.K. Cruz, Comparative genomics: from genotype to disease phenotype in the leishmaniases, Int. J. Parasitol. 37 (2007) 1173–1186.
[27] M.B. Wang, M. Metzlaff, RNA silencing and antiviral defense in plants, Curr. Opin. Plant. Biol. 8 (2005) 216–222.

[28] N. Bouche, D. Lauressergues, V. Gasciolli, H. Vaucheret, An antagonistic function for *Arabidopsis* DCL2 in development and a new function for DCL4 in generating viral siRNAs, EMBO J. 25 (2006) 3347–3356.
[29] A. Deleris, J. Gallego-Bartolome, J. Bao, K.D. Kasschau, J.C. Carrington, O. Voinnet, Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense, Science 313 (2006) 68–71.
[30] J.A. Diaz-Pendon, F. Li, W.X. Li, S.W. Ding, Suppression of antiviral silencing by cucumber mosaic virus 2b protein in *Arabidopsis* is associated with drastically reduced accumulation of three classes of viral small interfering RNAs, Plant Cell 19 (2007) 2053–2063.
[31] P. Mourrain, C. Beclin, T. Elmayan, F. Feuerbach, C. Godon, J.B. Morel, D. Jouette, A.M. Lacombe, S. Nikic, N. Picault, K. Remoue, M. Sanial, T.A. Vo, H. Vaucheret, *Arabidopsis* SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance, Cell 101 (2000) 533–542.
[32] Z. Xie, L.K. Johansen, A.M. Gustafson, K.D. Kasschau, A.D. Lellis, D. Zilberman, S.E. Jacobsen, J.C. Carrington, Genetic and functional diversification of small RNA pathways in plants, PLoS Biol. 2 (2004) E104.
[33] K.M. Keene, B.D. Foy, I. Sanchez-Vargas, B.J. Beaty, C.D. Blair, K.E. Olson, RNA interference acts as a natural antiviral response to O'nyong-nyong virus (Alphavirus; *Togaviridae*) infection of *Anopheles gambiae*, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 17240–17245.
[34] R.P. van Rij, M.C. Saleh, B. Berry, C. Foo, A. Houk, C. Antoniewski, R. Andino, The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in *Drosophila* melanogaster, Genes Dev. 20 (2006) 2985–2995.
[35] T. Sijen, R.H. Plasterk, Transposon silencing in the *Caenorhabditis elegans* germ line by natural RNAi, Nature 426 (2003) 310–314.
[36] H. Tabara, M. Sarkissian, W.G. Kelly, J. Fleenor, A. Grishok, L. Timmons, A. Fire, C.C. Mello, The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*, Cell 99 (1999) 123–132.
[37] K.D. Kasschau, N. Fahlgren, E.J. Chapman, C.M. Sullivan, J.S. Cumbie, S.A. Givan, J.C. Carrington, Genome-wide profiling and analysis of *Arabidopsis* siRNAs, PLoS Biol. 5 (2007) e57.
[38] C. Lu, S.S. Tej, S. Luo, C.D. Haudenschild, B.C. Meyers, P.J. Green, Elucidation of the small RNA component of the transcriptome, Science 309 (2005) 1567–1569.
[39] X. Zhang, I.R. Henderson, C. Lu, P.J. Green, S.E. Jacobsen, Role of RNA polymerase IV in plant small RNA metabolism, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 4536–4541.
[40] M.T. Bohnsack, K. Czaplinski, D. Gorlich, Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs, RNA 10 (2004) 185–191.
[41] R. Yi, Y. Qin, I.G. Macara, B.R. Cullen, Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs, Genes Dev. 17 (2003) 3011–3016.
[42] E. Lund, S. Guttinger, A. Calado, J.E. Dahlberg, U. Kutay, Nuclear export of microRNA precursors, Science 303 (2004) 95–98.
[43] Y. Kurihara, Y. Watanabe, *Arabidopsis* micro-RNA biogenesis through Dicer-like 1 protein functions, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 12753–12758.
[44] I. Papp, M.F. Mette, W. Aufsatz, L. Daxinger, S.E. Schauer, A. Ray, J. van der Winden, M. Matzke, A.J. Matzke, Evidence for nuclear processing of plant micro RNA and short interfering RNA precursors, Plant Physiol. 132 (2003) 1382–1390.
[45] M.Y. Park, G. Wu, A. Gonzalez-Sulser, H. Vaucheret, R.S. Poethig, Nuclear processing and export of microRNAs in *Arabidopsis*, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 3691–3696.
[46] J. Han, Y. Lee, K.H. Yeom, J.W. Nam, I. Heo, J.K. Rhee, S.Y. Sohn, Y. Cho, B.T. Zhang, V.N. Kim, Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex, Cell 125 (2006) 887–901.
[47] L.P. Lim, N.C. Lau, E.G. Weinstein, A. Abdelhakim, S. Yekta, M.W. Rhoades, C.B. Burge, D.P. Bartel, The microRNAs of *Caenorhabditis elegans*, Genes Dev. 17 (2003) 991–1008.
[48] N. Fahlgren, M.D. Howell, K.D. Kasschau, E.J. Chapman, C.M. Sullivan, J.S. Cumbie, S.A. Givan, T.F. Law, S.R. Grant, J.L. Dangl, J.C. Carrington, High-throughput sequencing of *Arabidopsis* microRNAs: evidence for frequent birth and death of MIRNA genes, PLoS ONE 2 (2007) e219.
[49] R. Rajagopalan, H. Vaucheret, J. Trejo, D.P. Bartel, A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*, Genes Dev. 20 (2006) 3407–3425.
[50] B.J. Reinhart, E.G. Weinstein, M.W. Rhoades, B. Bartel, D.P. Bartel, MicroRNAs in plants, Genes Dev. 16 (2002) 1616–1626.
[51] J. Brennecke, A. Stark, R.B. Russell, S.M. Cohen, Principles of microRNA-target recognition, PLoS Biol. 3 (2005) e85.
[52] J.G. Doench, P.A. Sharp, Specificity of microRNA target selection in translational repression, Genes Dev. 18 (2004) 504–511.
[53] E.C. Lai, Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation, Nat. Genet. 30 (2002) 363–364.
[54] K.D. Kasschau, Z. Xie, E. Allen, C. Llave, E.J. Chapman, K.A. Krizan, J.C. Carrington, P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function, Dev. Cell 4 (2003) 205–217.
[55] M.W. Rhoades, B.J. Reinhart, L.P. Lim, C.B. Burge, B. Bartel, D.P. Bartel, Prediction of plant microRNA targets, Cell 110 (2002) 513–520.
[56] R. Schwab, J.F. Palatnik, M. Riester, C. Schommer, M. Schmid, D. Weigel, Specific effects of microRNAs on the plant transcriptome, Dev. Cell 8 (2005) 517–527.
[57] A.E. Pasquinelli, B.J. Reinhart, F. Slack, M.Q. Martindale, M.I. Kuroda, B. Maller, D.C. Hayward, E.E. Ball, B. Degnan, P. Muller, J. Spring, A. Srinivasan, M. Fishman, J. Finnerty, J. Corbo, M. Levine, P. Leahy, E. Davidson, G. Ruvkun, Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA, Nature 408 (2000) 86–89.
[58] J.G. Ruby, C. Jan, C. Player, M.J. Axtell, W. Lee, C. Nusbaum, H. Ge, D.P. Bartel, Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in *C. elegans*, Cell 127 (2006) 1193–1207.
[59] M.J. Axtell, D.P. Bartel, Antiquity of microRNAs and their targets in land plants, Plant Cell 17 (2005) 1658–1673.
[60] S.K. Floyd, J.L. Bowman, Gene regulation: ancient microRNA target sequences in plants, Nature 428 (2004) 485–486.
[61] M. Arteaga-Vazquez, J. Caballero-Perez, J.P. Vielle-Calzada, A family of microRNAs present in plants and animals, Plant Cell 18 (2006) 3355–3369.
[62] A. Molnar, F. Schwach, D.J. Studholme, E.C. Thuenemann, D.C. Baulcombe, miRNAs control gene expression in the single-cell alga *Chlamydomonas reinhardtii*, Nature 447 (2007) 1126–1129.
[63] T. Zhao, G. Li, S. Mi, S. Li, G.J. Hannon, X.J. Wang, Y. Qi, A complex system of small RNAs in the unicellular green alga *Chlamydomonas reinhardtii*, Genes Dev. 21 (2007) 1190–1203.
[64] A. Hinas, J. Reimegard, E.G. Wagner, W. Nellen, V.R. Ambros, F. Soderbom, The small RNA repertoire of *Dictyostelium discoideum* and its regulation by components of the RNAi pathway, Nucleic Acids Res. 35 (2007) 6714–6726.
[65] W. Park, J. Li, R. Song, J. Messing, X. Chen, CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*, Curr. Biol. 12 (2002) 1484–1495.
[66] M.W. Jones-Rhoades, D.P. Bartel, Computational identification of plant microRNAs and their targets, including a stress-induced miRNA, Mol. Cell 14 (2004) 787–799.
[67] R. Sunkar, T. Girke, P.K. Jain, J.K. Zhu, Cloning and characterization of microRNAs from rice, Plant Cell 17 (2005) 1397–1411.
[68] J.F. Emery, S.K. Floyd, J. Alvarez, Y. Eshed, N.P. Hawker, A. Izhaki, S.F. Baum, J.L. Bowman, Radial patterning of *Arabidopsis* shoots by class III HD-ZIP and KANADI genes, Curr. Biol. 13 (2003) 1768–1774.
[69] A.C. Mallory, B.J. Reinhart, M.W. Jones-Rhoades, G. Tang, P.D. Zamore, M.K. Barton, D.P. Bartel, MicroRNA control of *PHABULOSA* in leaf development: importance of pairing to the microRNA 5′ region, EMBO J. 23 (2004) 3356–3364.
[70] J.R. McConnell, J. Emery, Y. Eshed, N. Bao, J. Bowman, M.K. Barton, Role of *PHABULOSA* and *PHAVOLUTA* in determining radial patterning in shoots, Nature 411 (2001) 709–713.

[71] T. Arazi, M. Talmor-Neiman, R. Stav, M. Riese, P. Huijser, D.C. Baulcombe, Cloning and characterization of micro-RNAs from moss, Plant J. 43 (2005) 837–848.
[72] M.J. Axtell, J.A. Snyder, D.P. Bartel, Common functions for diverse small RNAs of land plants, Plant Cell 19 (2007) 1750–1769.
[73] S. Lu, Y.H. Sun, H. Amerson, V.L. Chiang, MicroRNAs in loblolly pine (*Pinus taeda* L.) and their association with fusiform rust gall development, Plant J. (2007).
[74] A. Barakat, K. Wall, J. Leebens-Mack, Y.J. Wang, J.E. Carlson, C.W. Depamphilis, Large-scale identification of microRNAs from a basal eudicot (*Eschscholzia californica*) and conservation in flowering plants, Plant J. 51 (2007) 991–1003.
[75] J.H. Ko, C. Prassinos, K.H. Han, Developmental and seasonal expression of *PtaHB1*, a *Populus* gene encoding a class III HD-Zip protein, is closely associated with secondary growth and inversely correlated with the level of microRNA (miR166), New Phytol. 169 (2006) 469–478.
[76] S. Lu, Y.H. Sun, R. Shi, C. Clark, L. Li, V.L. Chiang, Novel and mechanical stress-responsive MicroRNAs in *Populus trichocarpa* that are absent from *Arabidopsis*, Plant Cell 17 (2005) 2186–2203.
[77] R. Velasco, A. Zharkikh, M. Troggio, D.A. Cartwright, A. Cestaro, D. Pruss, M. Pindo, L.M. Fitzgerald, S. Vezzulli, J. Reid, G. Malacarne, D. Iliev, G. Coppola, B. Wardell, D. Micheletti, T. Macalma, M. Facci, J.T. Mitchell, M. Perazzolli, G. Eldredge, P. Gatto, R. Oyzerski, M. Moretto, N. Gutin, M. Stefanini, Y. Chen, C. Segala, C. Davenport, L. Dematte, A. Mraz, J. Battilana, K. Stormo, F. Costa, Q. Tao, A. Si-Ammour, T. Harkins, A. Lackey, C. Perbost, B. Taillon, A. Stella, V. Solovyev, J.A. Fawcett, L. Sterck, K. Vandepoele, S.M. Grando, S. Toppo, C. Moser, J. Lanchbury, R. Bogden, M. Skolnick, V. Sgaramella, S.K. Bhatnagar, P. Fontana, A. Gutin, Y. Van de Peer, F. Salamini, R. Viola, A high quality draft consensus sequence of the genome of a heterozygous grapevine variety, PLoS ONE 2 (2007) e1326.
[78] C. Maher, L. Stein, D. Ware, Evolution of *Arabidopsis* microRNA families through duplication events, Genome Res. 16 (2006) 510–519.
[79] P. Sieber, F. Wellmer, J. Gheyselinck, J.L. Riechmann, E.M. Meyerowitz, Redundancy and specialization among plant microRNAs: role of the *MIR164* family in developmental robustness, Development 134 (2007) 1051–1060.
[80] E. Allen, Z. Xie, A.M. Gustafson, J.C. Carrington, microRNA-directed phasing during *trans*-acting siRNA biogenesis in plants, Cell 121 (2005) 207–221.
[81] M.J. Axtell, C. Jan, R. Rajagopalan, D.P. Bartel, A two-hit trigger for siRNA biogenesis in plants, Cell 127 (2006) 565–577.
[82] M. Talmor-Neiman, R. Stav, L. Klipcan, K. Buxdorf, D.C. Baulcombe, T. Arazi, Identification of *trans*-acting siRNAs in moss and an RNA-dependent RNA polymerase required for their biogenesis, Plant J. 48 (2006) 511–521.
[83] H. Vaucheret, A.C. Mallory, D.P. Bartel, AGO1 homeostasis entails coexpression of MIR168 and AGO1 and preferential stabilization of miR168 by AGO1, Mol. Cell 22 (2006) 129–136.
[84] H. Vaucheret, F. Vazquez, P. Crete, D.P. Bartel, The action of *ARGONAUTE1* in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development, Genes Dev. 18 (2004) 1187–1197.
[85] G. Chuck, A.M. Cigan, K. Saeteurn, S. Hake, The heterochronic maize mutant *Corngrass1* results from overexpression of a tandem microRNA, Nat. Genet. 39 (2007) 544–549.
[86] M. Gandikota, R.P. Birkenbihl, S. Hohmann, G.H. Cardon, H. Saedler, P. Huijser, The miRNA156/157 recognition element in the 3′ UTR of the *Arabidopsis* SBP box gene *SPL3* prevents early flowering by translational inhibition in seedlings, Plant J. 49 (2007) 683–693.
[87] G. Wu, R.S. Poethig, Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target SPL3, Development 133 (2006) 3539–3547.
[88] P.P. Liu, T.A. Montgomery, N. Fahlgren, K.D. Kasschau, H. Nonogaki, J.C. Carrington, Repression of *AUXIN RESPONSE FACTOR10* by microRNA160 is critical for seed germination and post-germination stages, Plant J. 51 (2007) 133–146.
[89] A.C. Mallory, D.P. Bartel, B. Bartel, MicroRNA-directed regulation of *Arabidopsis AUXIN RESPONSE FACTOR17* is essential for proper development and modulates expression of early auxin response genes, Plant Cell 17 (2005) 1360–1375.
[90] J.W. Wang, L.J. Wang, Y.B. Mao, W.J. Cai, H.W. Xue, X.Y. Chen, Control of root cap formation by MicroRNA-targeted auxin response factors in *Arabidopsis*, Plant Cell 17 (2005) 2204–2216.
[91] J. Kim, J.H. Jung, J.L. Reyes, Y.S. Kim, S.Y. Kim, K.S. Chung, J.A. Kim, M. Lee, Y. Lee, V. Narry Kim, N.H. Chua, C.M. Park, microRNA-directed cleavage of ATHB15 mRNA regulates vascular development in *Arabidopsis* inflorescence stems, Plant J. 42 (2005) 84–94.
[92] L. Williams, S.P. Grigg, M. Xie, S. Christensen, J.C. Fletcher, Regulation of *Arabidopsis* shoot apical meristem and lateral organ formation by microRNA miR166g and its AtHD-ZIP target genes, Development 132 (2005) 3657–3668.
[93] N. Ori, A.R. Cohen, A. Etzioni, A. Brand, O. Yanai, S. Shleizer, N. Menda, Z. Amsellem, I. Efroni, I. Pekker, J.P. Alvarez, E. Blum, D. Zamir, Y. Eshed, Regulation of *LANCEOLATE* by miR319 is required for compound-leaf development in tomato, Nat. Genet. 39 (2007) 787–791.
[94] J.F. Palatnik, E. Allen, X. Wu, C. Schommer, R. Schwab, J.C. Carrington, D. Weigel, Control of leaf morphogenesis by microRNAs, Nature 425 (2003) 257–263.
[95] N. Fahlgren, T.A. Montgomery, M.D. Howell, E. Allen, S.K. Dvorak, A.L. Alexander, J.C. Carrington, Regulation of *AUXIN RESPONSE FACTOR3* by TAS3 ta-siRNA affects developmental timing and patterning in *Arabidopsis*, Curr. Biol. 16 (2006) 939–944.
[96] D. Garcia, S.A. Collier, M.E. Byrne, R.A. Martienssen, Specification of leaf polarity in *Arabidopsis* via the *trans*-acting siRNA pathway, Curr. Biol. 16 (2006) 933–938.
[97] C. Hunter, M.R. Willmann, G. Wu, M. Yoshikawa, M. de la Luz Gutierrez-Nava, S.R. Poethig, *Trans*-acting siRNA-mediated repression of *ETTIN* and *ARF4* regulates heteroblasty in *Arabidopsis*, Development 133 (2006) 2973–2981.
[98] I. Pekker, J.P. Alvarez, Y. Eshed, Auxin response factors mediate *Arabidopsis* organ asymmetry via modulation of *KANADI* activity, Plant Cell 17 (2005) 2899–2910.
[99] A. Sessions, J.L. Nemhauser, A. McColl, J.L. Roe, K.A. Feldmann, P.C. Zambryski, *ETTIN* patterns the *Arabidopsis* floral meristem and reproductive organs, Development 124 (1997) 4481–4491.
[100] E. Allen, Z. Xie, A.M. Gustafson, G.H. Sung, J.W. Spatafora, J.C. Carrington, Evolution of microRNA genes by inverted duplication of target gene sequences in *Arabidopsis thaliana*, Nat. Genet. 36 (2004) 1282–1290.
[101] M. Boccara, A. Sarazin, B. Billoud, V. Jolly, R. Martienssen, D. Baulcombe, V. Colot, New approaches for the analysis of *Arabidopsis thaliana* small RNAs, Biochimie 89 (2007) 1252–1256.
[102] C. Llave, K.D. Kasschau, M.A. Rector, J.C. Carrington, Endogenous and silencing-associated small RNAs in plants, Plant Cell 14 (2002) 1605–1619.
[103] C. Johnson, L. Bowman, A.T. Adai, V. Vance, V. Sundaresan, CSRDB: a small RNA integrated database and browser resource for cereals, Nucleic Acids Res. 35 (2007) D829–D833.
[104] K. Nobuta, R.C. Venu, C. Lu, A. Belo, K. Vemaraju, K. Kulkarni, W. Wang, M. Pillay, P.J. Green, G.L. Wang, B.C. Meyers, An expression atlas of rice mRNAs and small RNAs, Nat. Biotechnol. 25 (2007) 473–477.
[105] C. Kutter, H. Schob, M. Stadler, F. Meins Jr., A. Si-Ammour, MicroRNA-mediated regulation of stomatal development in *Arabidopsis*, Plant Cell 19 (2007) 2417–2429.
[106] J.M. Franco-Zorrilla, A. Valli, M. Todesco, I. Mateos, M.I. Puga, I. Rubio-Somoza, A. Leyva, D. Weigel, J.A. Garcia, J. Paz-Ares, Target mimicry provides a new mechanism for regulation of microRNA activity, Nat. Genet. 39 (2007) 1033–1037.
[107] P. Kapranov, J. Cheng, S. Dike, D.A. Nix, R. Duttagupta, A.T. Willingham, P.F. Stadler, J. Hertel, J. Hackermuller, I.L. Hofacker, I. Bell, E. Cheung, J. Drenkow, E. Dumais, S. Patel, G. Helt, M. Ganesh, S. Ghosh, A. Piccolboni, V. Sementchenko, H. Tammana, T.R. Gingeras,

RNA maps reveal new RNA classes and a possible function for pervasive transcription, Science 316 (2007) 1484–1488.

[108] K. Yamada, J. Lim, J.M. Dale, H. Chen, P. Shinn, C.J. Palm, A.M. Southwick, H.C. Wu, C. Kim, M. Nguyen, P. Pham, R. Cheuk, G. Karlin-Newmann, S.X. Liu, B. Lam, H. Sakano, T. Wu, G. Yu, M. Miranda, H.L. Quach, M. Tripp, C.H. Chang, J.M. Lee, M. Toriumi, M.M. Chan, C.C. Tang, C.S. Onodera, J.M. Deng, K. Akiyama, Y. Ansari, T. Arakawa, J. Banh, F. Banno, L. Bowser, S. Brooks, P. Carninci, Q. Chao, N. Choy, A. Enju, A.D. Goldsmith, M. Gurjal, N.F. Hansen, Y. Hayashizaki, C. Johnson-Hopson, V.W. Hsuan, K. Iida, M. Karnes, S. Khan, E. Koesema, J. Ishida, P.X. Jiang, T. Jones, J. Kawai, A. Kamiya, C. Meyers, M. Nakajima, M. Narusaka, M. Seki, T. Sakurai, M. Satou, R. Tamse, M. Vaysberg, E.K. Wallender, C. Wong, Y. Yamamura, S. Yuan, K. Shinozaki, R.W. Davis, A. Theologis, J.R. Ecker, Empirical analysis of transcriptional activity in the *Arabidopsis* genome, Science 302 (2003) 842–846.

[109] L.F. Sempere, C.N. Cole, M.A. McPeek, K.J. Peterson, The phylogenetic distribution of metazoan microRNAs: insights into evolutionary complexity and constraint, J. Exp. Zoolog. B. Mol. Dev. Evol. 306 (2006) 575–588.

[110] B. John, A.J. Enright, A. Aravin, T. Tuschl, C. Sander, D.S. Marks, Human microRNA targets, PLoS Biol. 2 (2004) e363.

[111] A. Krek, D. Grun, M.N. Poy, R. Wolf, L. Rosenberg, E.J. Epstein, P. MacMenamin, I. da Piedade, K.C. Gunsalus, M. Stoffel, N. Rajewsky, Combinatorial microRNA target predictions, Nat. Genet. 37 (2005) 495–500.

[112] B.P. Lewis, C.B. Burge, D.P. Bartel, Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets, Cell 120 (2005) 15–20.

[113] B.P. Lewis, I.H. Shih, M.W. Jones-Rhoades, D.P. Bartel, C.B. Burge, Prediction of mammalian microRNA targets, Cell 115 (2003) 787–798.

[114] X. Xie, J. Lu, E.J. Kulbokas, T.R. Golub, V. Mootha, K. Lindblad-Toh, E.S. Lander, M. Kellis, Systematic discovery of regulatory motifs in human promoters and 3′ UTRs by comparison of several mammals, Nature 434 (2005) 338–345.

[115] A.J. Enright, B. John, U. Gaul, T. Tuschl, C. Sander, D.S. Marks, MicroRNA targets in *Drosophila*, Genome Biol. 5 (2003) R1.

[116] D. Grun, Y.L. Wang, D. Langenberger, K.C. Gunsalus, N. Rajewsky, microRNA target predictions across seven *Drosophila* species and comparison to mammalian targets, PLoS Comput. Biol. 1 (2005) e13.

[117] J.G. Ruby, A. Stark, W.K. Johnston, M. Kellis, D.P. Bartel, E.C. Lai, Evolution, biogenesis, expression, and target predictions of a substantially expanded set of *Drosophila* microRNAs, Genome Res. 17 (2007) 1850–1864.

[118] A. Stark, J. Brennecke, R.B. Russell, S.M. Cohen, Identification of *Drosophila* m,icroRNA targets, PLoS Biol. 1 (2003) E60.

[119] A. Stark, P. Kheradpour, L. Parts, J. Brennecke, E. Hodges, G.J. Hannon, M. Kellis, Systematic discovery and characterization of fly microRNAs using 12 *Drosophila* genomes, Genome Res. 17 (2007) 1865–1879.

[120] A. Stark, M.F. Lin, P. Kheradpour, J.S. Pedersen, L. Parts, J.W. Carlson, M.A. Crosby, M.D. Rasmussen, S. Roy, A.N. Deoras, J.G. Ruby, J. Brennecke, H.F. Curators, B.D. Project, E. Hodges, A.S. Hinrichs, A. Caspi, B. Paten, S.W. Park, M.V. Han, M.L. Maeder, B.J. Polansky, B.E. Robson, S. Aerts, J. van Helden, B. Hassan, D.G. Gilbert, D.A. Eastman, M. Rice, M. Weir, M.W. Hahn, Y. Park, C.N. Dewey, L. Pachter, W.J. Kent, D. Haussler, E.C. Lai, D.P. Bartel, G.J. Hannon, T.C. Kaufman, M.B. Eisen, A.G. Clark, D. Smith, S.E. Celniker, W.M. Gelbart, M. Kellis, M.A. Crosby, B.B. Matthews, A.J. Schroeder, L. Sian Gramates, S.E. St Pierre, M. Roark, K.L. Wiley Jr, R.J. Kulathinal, P. Zhang, K.V. Myrick, J.V. Antone, W.M. Gelbart, J.W. Carlson, C. Yu, S. Park, K.H. Wan, S.E. Celniker, Discovery of functional elements in 12 *Drosophila* genomes using evolutionary signatures, Nature 450 (2007) 219–232.

[121] C.S. Chan, O. Elemento, S. Tavazoie, Revealing posttranscriptional regulatory elements through network-level conservation, PLoS Comput. Biol. 1 (2005) e69.

[122] S. Lall, D. Grun, A. Krek, K. Chen, Y.L. Wang, C.N. Dewey, P. Sood, T. Colombo, N. Bray, P. Macmenamin, H.L. Kao, K.C. Gunsalus, L. Pachter, F. Piano, N. Rajewsky, A genome-wide map of conserved microRNA targets in *C. elegans*, Curr. Biol. 16 (2006) 460–471.

[123] Y. Watanabe, N. Yachie, K. Numata, R. Saito, A. Kanai, M. Tomita, Computational analysis of microRNA targets in *Caenorhabditis elegans*, Gene 365 (2006) 2–10.

[124] K.K. Farh, A. Grimson, C. Jan, B.P. Lewis, W.K. Johnston, L.P. Lim, C.B. Burge, D.P. Bartel, The widespread impact of mammalian microRNAs on mRNA repression and evolution, Science 310 (2005) 1817–1821.

[125] A. Stark, J. Brennecke, N. Bushati, R.B. Russell, S.M. Cohen, Animal microRNAs confer robustness to gene expression and have a significant impact on 3′UTR evolution, Cell 123 (2005) 1133–1146.

[126] I. Bentwich, A. Avniel, Y. Karov, R. Aharonov, S. Gilad, O. Barad, A. Barzilai, P. Einat, U. Einav, E. Meiri, E. Sharon, Y. Spector, Z. Bentwich, Identification of hundreds of conserved and nonconserved human microRNAs, Nat. Genet. 37 (2005) 766–770.

[127] E. Berezikov, F. Thuemmler, L.W. van Laake, I. Kondova, R. Bontrop, E. Cuppen, R.H. Plasterk, Diversity of microRNAs in human and chimpanzee brain, Nat. Genet. 38 (2006) 1375–1377.