Review

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Evolutionary history of plant microRNAs

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microRNAs (miRNAs) are short noncoding regulatory genes that perform important roles in plant development and physiology. With the increasing power of next generation sequencing technologies and the development of bioinformatic tools, there has been a dramatic increase in the number of studies surveying the miRNAomes of plant species, which has led to an explosion in the number of described miRNAs. Unfortunately, very many of these new discoveries have been incompletely annotated and thus fail to discriminate genuine miRNAs from small interfering RNAs (siRNAs), fragments of longer RNAs, and random sequence. We review the published repertoire of plant miRNAs, discriminating those that have been correctly annotated. We use these data to explore prevailing hypotheses on the tempo and mode of miRNA evolution within the plant kingdom.

Plant miRNAs are small genes with big potential but equally big problems

miRNAs are short (~21 nt) noncoding RNAs involved in post-transcriptional gene regulation through both degradative and nondegradative mechanisms [1,2]. In plants, miRNAs have been demonstrated to have an influential role in development [3] as well as tolerance and response to extrinsic stresses [4], including drought [5], temperature [6,7], salinity [8], oxidative stress [9], and exposure to UV radiation [10]. Hence, miRNAs are obvious targets for bioengineering to improve crop yield and food security, mitigating the impact of global climate change [11–13]. However, although there has been considerable research surveying the miRNA repertoire of individual plant species, and crop species in particular, a synthetic understanding of their systematic distribution has been compromised by specious annotation of putative miRNA loci resulting in the effective corruption of databases such as miRBase [14,15] (http://mirbase.org).

Our aim in this review is to evaluate published plant miRNAs to determine whether they meet the criteria required of annotation. We propose a phylogenetic framework for organising these data which predicts the miRNA repertoire of evolutionary lineages, including those that have yet to be studied. In this regard, we evaluate conventional perceptions of the tempo and mode of plant miRNA evolution and their role within plant organismal evolution, before considering this within the perspective of miRNA evolution more generally.

How to identify a plant miRNA

The criteria required to identify novel plant miRNAs are unambiguous. Initial surveys of the systematic distribution and diversity of miRNAs quickly led to a consensus on the best practice criteria required for correctly identifying novel miRNAs based on the distinctive nature of miRNA biogenesis [16–18]. In this regard, the role of the DCL1 enzyme, a member of the Dicer family that cleaves double-strand RNAs, is influential [19,20]. DCL1 is responsible for the cleavage of the hairpin structures, known as miRNA primary sequences (pri-miRNA), into short functional miRNA strands. For DCL1 to process a double-stranded RNA, there must be a high level of complementarity between the opposing arms of the hairpin [21]. DCL1-mediated cleavage is extremely precise, recognising the ~21 nt miRNA sequence with high fidelity [22]. Consequently, the pri-miRNA strands are cleaved with great precision 5′ of the mature miRNA sequence and 3′ of the miRNA* [23]. Cleavage on the 5′ and 3′ arms occurs with a positional offset of two nucleotides, giving rise to a characteristic overhang of two nucleotides on the 5′ arm [23]. Thus, identification of bona fide miRNAs requires the following five criteria (Figure 1). (i) The miRNA sequence must have a high degree of complementarity to the opposing arm. The necessary degree of complementarity of the functional part of the precursor is unclear but at a minimum there should be in excess of 15 nucleotides bonding with the opposing arm. (ii) The processed miRNA strands must show evidence of precise 5′ cleavage. The vast majority of the processed reads from each arm of the precursor should have their 5′ ends within a nucleotide of each other at the miRNA site. (iii) There is little or no heterogeneity in the sequences matching to the miRNA precursor. Evidence of such 'smearing', even if there is a greater accumulation of the purported miRNA strand, strongly suggests that a locus is an siRNA and not a miRNA. (iv) There must be evidence for the expression of the miRNA*. For validation of a miRNA family, this evidence can be obtained from any organism, and from any paralogue within the miRNA

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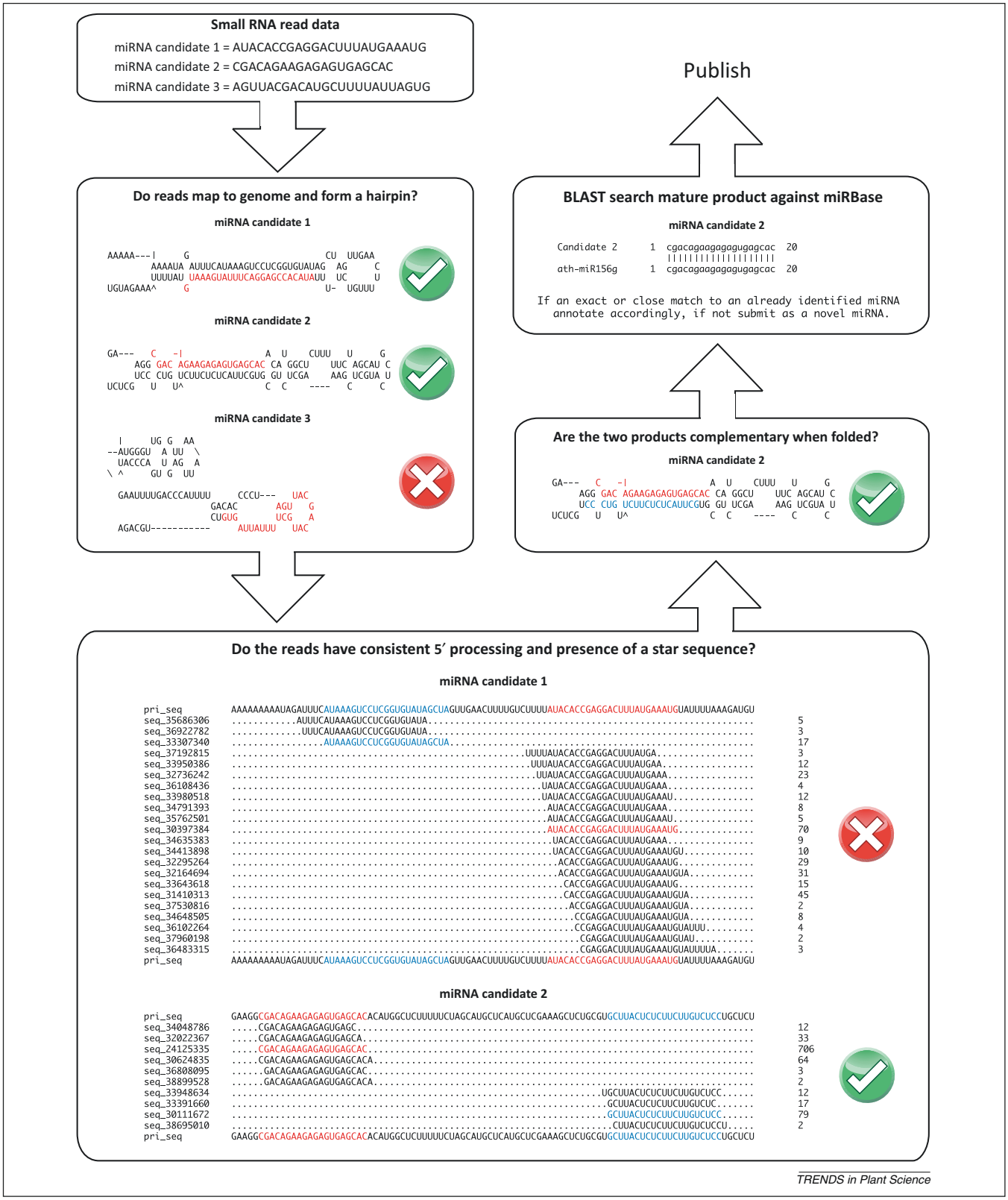

Figure 1. miRNA identification protocol. An outline of the correct protocol for identifying and annotating miRNAs from deep sequencing libraries, to ensure accuracy and fulfilment of the established annotation criteria [16–18]. Adapted from [41].

family. (v) There should be a two nucleotide overhang of the 5′ sequence from the 3′ sequence. Small variations in folding, or errors in folding prediction, may lead to a minor amount of heterogeneity.

Since these criteria were established [17,18], sequencing technology has improved dramatically, facilitating cheaper sequencing at orders of magnitude greater depth. Many of these original criteria were effectively idealistic

because it was not practically feasible to demonstrate phenomena such as cleavage precision and expression of the miRNA*. As a result, this led to the spurious identification of, for instance, siRNAs as miRNAs [24]. However, given the depth of read afforded by contemporary sequencing technology, these features can now be demonstrated trivially, hence they have become required elements for miRNA annotation [16,17,25].

An unfortunate consequence of this increase in the burden required for miRNA annotation is that public databases such as miRBase include putative miRNAs for which evidence is lacking for many of these annotation criteria [14,15]. Resequencing has demonstrated that many previously annotated miRNAs are siRNAs or fragments of long noncoding RNAs including tRNAs, mRNAs, and rRNAs [26]. Despite the increased economy and scale of deep sequencing technology, many contemporary surveys of plant miRNAs continue to regard many of the annotation criteria as optional, adding more incompletely justified and, therefore, potentially spurious miRNAs to public databases [15].

Separating the wheat from the chaff

We set out to assess the validity of annotated plant miRNAs. We evaluated the miRNA repertoires of all plant species available on miRBase v20 and, where possible, we also considered the sequencing data from the original publications when it had not been submitted to miRBase (see Table S4 in the supplementary material online). Additionally, we searched MirNest [27] (http://lemur.amu.edu.pl/share/php/mirnest/), an online repository for computationally predicted miRNAs. We considered these records in terms of the annotation criteria outlined above, paying particular attention to deep sequencing read data (where available) in attempting validation.

Our analysis considered putative miRNAs within their families, namely, the grouping of loci into families based on similarity in the sequence of the mature miRNA, based on the assumption that paralogues within a family are descended through duplication from a single orthologue. This assumption reduced the burden of evidence required for miRNA validation because miRNA* expression need only be demonstrated in one paralogue of that family across all species.

Putative miRNAs exhibiting complementarity pairing of 15 or fewer nucleotides between the opposing arms hairpin were deemed to have insufficiently robust structure to be validated as a miRNA, unless there was strong evidence from deep sequencing for precise cleavage. Evidence of heterogeneous 5′ processing, or an offset of greater than three nucleotides or less than one nucleotide between the 5′ and 3′ cleaved ends of the hairpin sequence, were also reasons for rejection of many putative miRNAs.

Finally, we conducted a BLAST search of the validated miRNAs against the genome sequence data that were available for each of the taxa. This allowed us to detect validated miRNAs in species where they had not been reported previously, establish the systematic distribution of miRNA families among extant plant species, and, ultimately, infer their evolutionary history within the plant kingdom.

Results

We examined 6172 miRNA genes annotated in miRBase v20 and found that 1993 (32.3%) lacked sufficient evidence to justify their annotation as genuine miRNAs (see Table S1 in the supplementary material online). Of these, the majority (61.1%) were rejected because they lacked evidence of miRNA* sequence expression for any paralogue within the respective family (112 families lacked evidence of miRNA* expression in their miRBase entry but it was available in their primary publication). A further 9.5% were rejected due to insufficient complementarity between opposing arms of the pri-miRNA and 29.4% of miRNA loci were rejected because of evidence of imprecise 5′ processing. At the family level, we rejected 1351 of the 1802 plant miRNA families on miRBase (Table 1; see Table S2 in the supplementary material online). The vast majority (95.8%) of the miRNA families that we reject have been described in just a single taxon. In some taxa, almost all such orphan miRNA families failed to fulfil the criteria required for annotation (e.g., 96.3% of orphan miRNAs in *Glycine max* and 89.2% in *Medicago truncatula*), invariably because of an absence of miRNA* expression data. MirNest contained 22 additional miRNAs that fulfilled the annotation criteria. BLAST searches of publicly available genome sequence data revealed 109 previously unidentified miRNA families in 20 taxa (see Table S3 in the supplementary material online). We provide a library of valid plant miRNAs in FASTA format at <http://palaeo.gly.bris.ac.uk/donoghue/>, which we hope will serve as a benchmark for future surveys of plant miRNAomes.

The rejection of almost one-third of annotated plant miRNA loci in miRBase, and three-quarters of the miRNA families, confirms previous assertions [14,15] that the database has been corrupted by spurious data. As pessimistic as this appears, our results may underestimate the number of spuriously identified miRNAs in miRBase because some entries are supported by extremely limited evidence. However, many of the miRNAs that we reject for lack of evidence of miRNA* sequence expression may yet be validated by resequencing. However, they cannot be considered valid miRNAs based on the evidence currently available.

A role for 'Tree-Thinking' in the study of plant miRNAs

miRNA families are defined on evolutionary principles and it has been generally accepted that there is some degree of conservation in the evolution of plant miRNAs [28–30]. Nevertheless, there have been few attempts to organise the distribution of miRNAs among plant species within an evolutionary framework [29,31,32]. Rather, the presence or absence of miRNA families among plant species has been conveyed through increasingly unwieldy tables that fail to exploit the predictive power of tree-based approaches to organising phylogenetic data [33]. Because miRNAs are inherited through vertical descent, it is possible to infer the miRNAome of common ancestors using phylogenetic approaches and, therefore, predict the minimal miRNA repertoire of hitherto unsampled lineages (Figure 2).

Although it has been argued that few miRNAs are deeply conserved and that the majority of miRNA families

Table 1. An outline of the miRNA families listed on miRBase v20 that were rejected due to lacking sufficient evidence for confident annotation[a]

Reason for rejection	Number of families rejected (% rejected)
No reported star	779 (43.2%)
Available read data do not support mature/ star	65 (3.6%)
Heterogeneous processing	423 (23.5%)
Improper mature/ star offset	60 (3.3%)
Improper precursor structure	89 (4.9%)
Reported products do not map to genome	2 (0.1%)
No experimental support for annotation	97(5.4%)
Total invalid	**1351 (75.0%)**
Total valid	**451 (25.0%)**
Taxon	**microRNA family present**
Embryophyta	156, 159, 160, 166, 167, 171, 390, 408, 477, 530, 535
Tracheophyta	396
Euphyllophyta	168, 169, 172
Spermatophyta	162, 164, 393, 394, 395, 397, 398, 399, 482, 2950
Angiospermae	827
Monocotyledones	437, 444, 528, 2275
Poales	1432, 1878, 5566
Eudicotyledones	403, 828, 2111, 3627, 3630, 4376, 4414

[a]In addition, the complement of miRNA families present in each of the major plant groups is listed, as inferred from the distribution in sampled taxa.

have evolved recently [29,34,35], a phylogenetic perspective demonstrates precisely the reverse. Many miRNA families have been inherited from the ancestral embryophyte (Figure 2, node a), and another large suite of miRNA families encountered among angiosperms evolved in the ancestral spermatophyte (Figure 2, node d). Few miRNA families appear to have evolved within the lineage leading to angiosperms after it separated from gymnosperms (Figure 2, node f). Instead many new miRNA families evolved among the angiosperms, within component lineages after the divergence of the extant flowering plants from their last common ancestor.

Based on the current knowledge of miRNA family distribution and lineage divergence dates [36], it is also possible to establish evolutionary rates for the innovation of miRNA families (Figure 3). The rate of accumulation of families is high in the embryophyte stem lineage (Figure 2, node a; 'bryophytes' and vascular plants), after it separated from its nearest green algal relatives, and in the spermatophyte stem lineage (Figure 2, node d; gymnosperms plus angiosperms) after it separated from the pteridophyte lineage (ferns). Rates of miRNA family innovation appear approximately equivalent elsewhere within plant phylogeny with the exception of the angiosperms where there is a marked increase in the rate of innovation after the divergence of the monocot and eudicot evolutionary lineages (Figure 2, nodes g and h, respectively). However, due to the disparity in depth of coverage between the different flowering plants species, it is impossible to undertake a proper comparison of the number of families present. For example, the most highly studied genus is *Arabidopsis*, and it is unlikely to be a coincidence that the highest number of miRNA families has been recorded from this genus [37].

Figure 2 clarifies the effect that disparities in taxonomic sampling have on our understanding of the evolution of miRNAs. Entire divisions have either no coverage (Hepatophyta, Anthocerophyta, Psilophyta, Sphenophyta, Cycadophyta, Gingkophyta, and Gnetophyta) or only a single taxon has been surveyed (Bryophyta, Lycophyta,

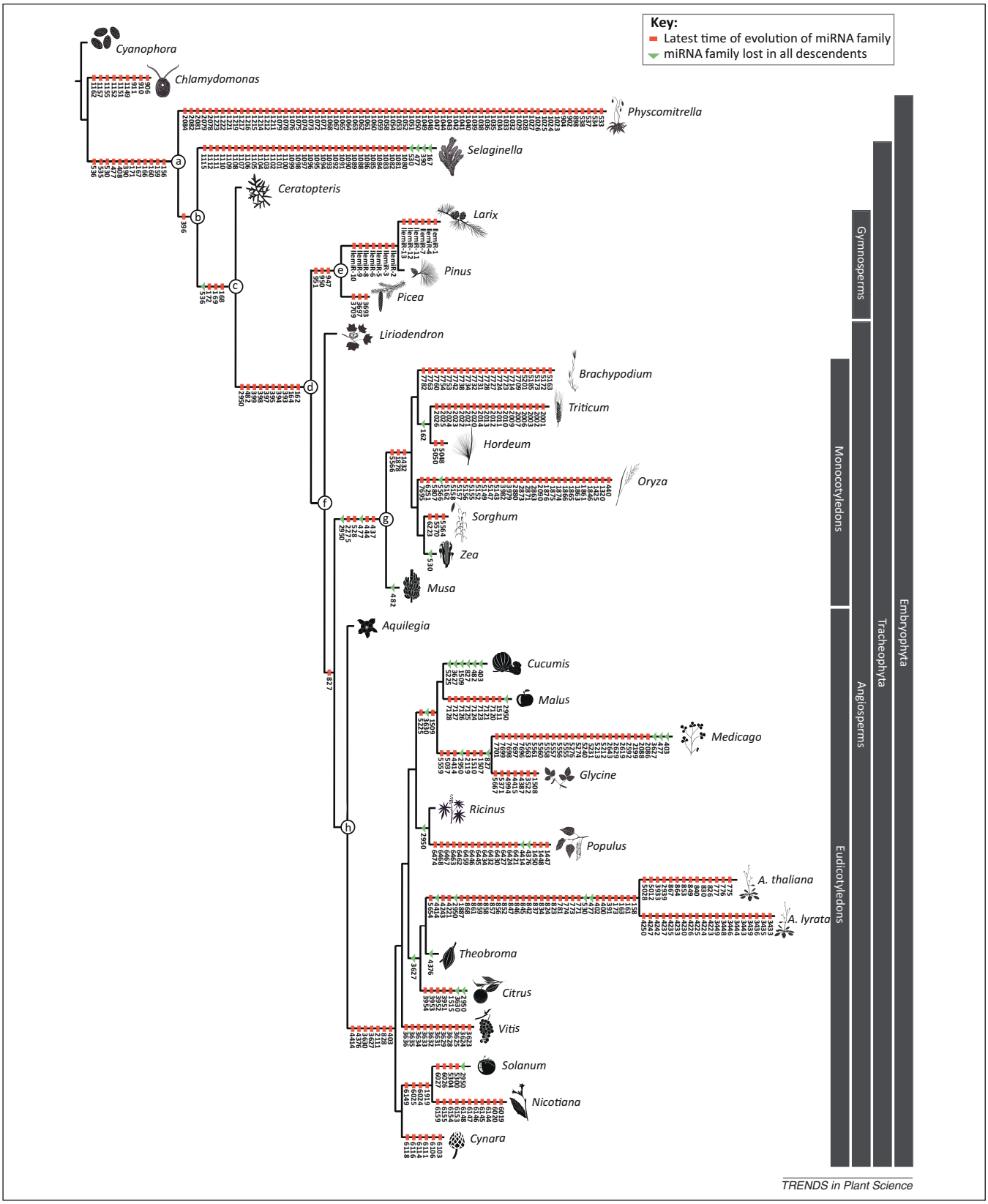

Figure 2. The acquisition of validated miRNA families in taxa across 31 taxa representing a broad coverage of the plant kingdom. The observed distribution of families in these taxa is utilised to infer which node each family is likely to have evolved at. Node labels: (a) Embryophyta, (b) Tracheophyta, (c) Euphyllophyta, (d) Spermatophyta, (e) Gymnospermae, (f) Angiospermae, (g) Monocotyledons, and (h) Eudicotyledons. The established plant phylogeny is used (Angiosperm Phylogeny Website. Version 12, July 2012; http://www.mobot.org/MOBOT/research/APweb/) and the majority of data are drawn from miRBase v20, with additional miRNA families that fulfil the annotation criteria included for *Musa* [46], *Triticum* [10], *Larix* [47], *Liriodendron* [31], and *Ceratopteris* [31].

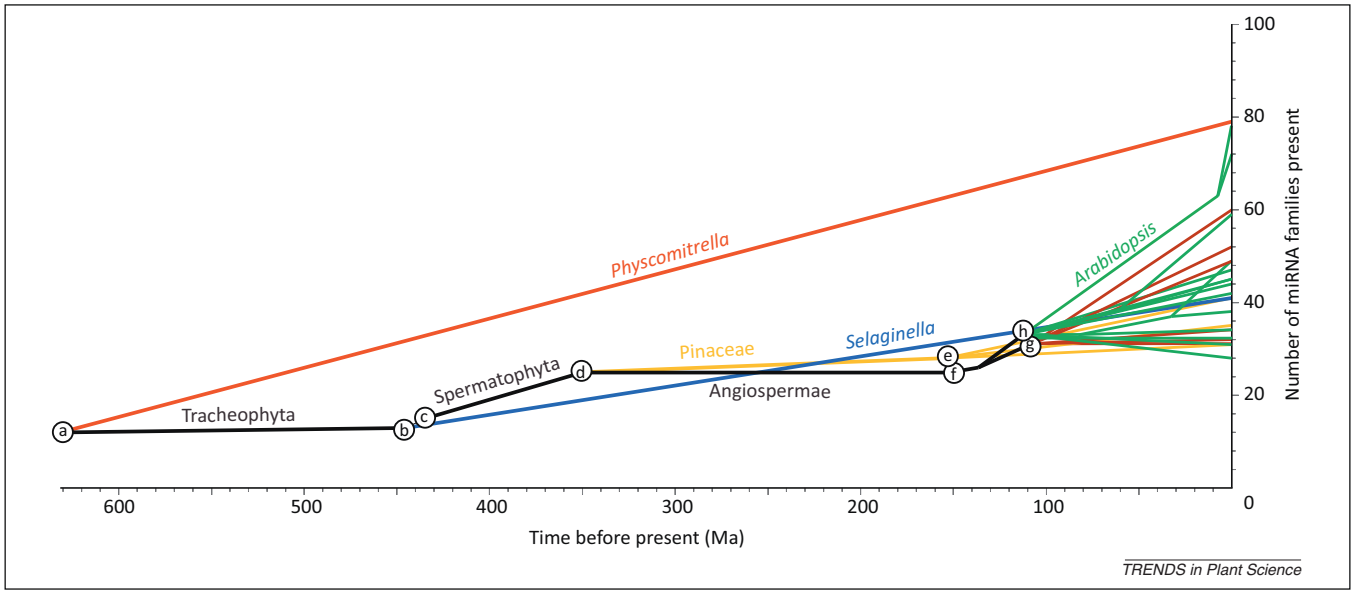

Figure 3. The rate of acquisition of miRNA families over geological time. Divergence dates are consistent with both molecular data and the fossil record [36,48]. Node labels: (a) Embryophyta, (b) Tracheophyta, (c) Euphyllophyta, (d) Spermatophyta, (e) Gymnospermae, (f) Angiospermae, (g) Monocotyledons, and (h) Eudicotyledons. Each line produced at the point of the monocot (brown) and eudicot (green) divergence represents an angiosperm species, and illustrates both the disparity in coverage between the angiosperm and rest of the plant kingdom, and the differences in coverage within the angiosperms.

and Pteridophyta). In the latter case, this creates the illusion that few miRNA families are conserved in these lineages, whereas the reality is that these miRNAs appear species-specific simply because they are the only species studied so far within their fundamental evolutionary lineage. Inevitably, denser taxonomic sampling will reveal that the majority of these miRNAs have evolved much earlier than the origin of the species.

Not so young miRNAs

It has been argued that, because the majority of plant miRNAs appear to be species specific, they must have evolved recently [29,34,35,38,39], that most of these miRNAs are likely to lack any function, and that they will soon be lost through neutral selection [35]. Our analysis reveals two fundamental errors in these arguments. First, although it may be true that the species in which a novel miRNA family is identified may itself be young, if that species is the sole representative of an otherwise unsampled evolutionary lineage, then we have no constraint on the antiquity of the miRNA family except that it evolved after that species lineage separated from the nearest living species or lineage whose miRNAome has been assayed.

This is both most obvious and extreme in *Physcomitrella patens*, which is the only representative of the moss evolutionary lineage to have its miRNA repertoire studied. *P. patens* has been described to encode 67 miRNA families that are not encountered in any other plant species studied so far. However, it does not follow that these miRNA families are exclusive to the species *P. patens*. Rather, they are the net sum of the miRNA families that evolved in the lineage leading to *P. patens* after it separated from the last common ancestor that it shared with tracheophytes (vascular plants, including the lycophytes, pteridophytes, gymnosperms, and angiosperms; Figure 3, node a). Through this episode lasting half a billion years, some of the miRNA families will have evolved very early and some much more recently, interpretations that can be reconciled by assaying the miRNAome of members of the moss lineage that are related by degree to *P. patens*.

Similarly, even within the comparatively intensively sampled angiosperms, those species that have been investigated for their miRNAs are all relatively distantly related. They are representative of their evolutionary lineages, not merely of their species. Indeed, we only have approximate knowledge of species-specific miRNAs in *Arabidopsis* where both *Arabidopsis thaliana* and *Arabidopsis lyrata* have been investigated [37]. These two species appear to have 14 and 20 new miRNAs, respectively (Figure 2). However, our survey revealed that spuriously identified, incompletely annotated, and/or lowly expressed novel miRNAs are most common in individual species. It is likely that resequencing efforts will significantly diminish the inventory of truly species-specific miRNAs.

Nothing unique about model systems

It has often been noted that miRNA families are distributed very unevenly among evolutionary lineages, with species of *Arabidopsis*, *Oryza*, and *Glycine* having evolved a disproportionately large number of miRNA families. However, given the phylogenetic perspective that we provide, the distribution of miRNA families appears to be approximately proportional to the antiquity of the evolutionary lineages assayed. For instance, the miRNAomes of the moss *P. patens* and the eudicot *A. thaliana* have both been well characterised. In the half billion years of independent evolutionary history since they last shared a common ancestor, these distantly related evolutionary lineages have evolved comparable numbers of novel miRNA families (67 and 66, respectively; Figure 2).

Less well-characterised taxa, such as *Nicotiana* and *Selaginella*, appear to have fewer miRNAs but this is almost certainly an artefact of incomplete sampling. Where comparisons have been made within angiosperms to highlight the considerable variability of miRNA evolution at the individual gene level, for example, between rice (*Oryza sativa*) and papaya (*Carica papaya*), it has been noted that rice has gained 126 genes while losing only one, whereas papaya has gained only eight genes and lost 25 [35]. However, rice and papaya differ dramatically in the extent to which their miRNAs have been studied; a Web of Science search (search string Topic=([rice | papaya]) AND Topic=(microRNA OR miRNA) yields 602 versus ten published studies, respectively. Bioinformatic approaches could overcome this if the level of gene sequencing was comparable for both taxa. However, whereas rice has enjoyed effectively complete genome sequencing and annotation, the papaya genome has been the subject of only very low (3×) sequence coverage [40]. Previous studies in animals have shown that the ability to identify miRNAs bioinformatically is correlated to the level of genome sequencing, with low coverage genomes missing up to 32% of the expected miRNA repertoire, whereas completely sequenced genomes were missing <3% [41]. Consequently, for comparisons to be made between the miRNA repertoires of any two species, care should be taken to ensure that sampling bias does not affect interpretations of the available data.

The evolution of plant miRNAs

A key feature of miRNA evolution is that once evolved, families are rarely lost and, as such, this high level of conservation between taxa was exploited as an ancillary criterion for miRNA annotation [17,18]. However, not all miRNAs are equally conserved and it has been argued that more ancient miRNA families have a higher level of conservation than younger families [32]. Our phylogenetic perspective on miRNA evolution reveals that of the 36 observed secondary losses of miRNA families, 21 of these were in families that evolved prior to the origin of the spermatophytes (Figure 2), suggesting that older families may not be more highly conserved.

It is clear that new miRNA families are not integrated into gene regulatory networks at a continuous rate over plant evolutionary history but, rather, in distinct episodes of miRNAome expansion [e.g., in the ancestral embryophyte (Figure 2, node a) and ancestral spermatophyte (Figure 2, node d)], with long intervening periods of stasis. It is tempting to suggest that these bursts of miRNA innovation are associated causally with major episodes of plant phenotypic evolution, such as in the adaptation of plants to life on land. Indeed, expansion in the number of miRNA families within the ancestral angiosperm lineage (Figure 2, node f) has been implicated in the origin of the flowering plants [35], although our analysis shows that this apparent coincidence is an artefact of the paucity of miRNA studies in pteridophytes and gymnosperms. Evidently, not all of the major events in plant evolution can be explained by the origin of new miRNA families, although because miRNAs are nevertheless known to play an essential role in the development of the flower, [42,43] co-option of existing miRNA families to perform novel functions may also play a crucial role in the evolution of novel phenotypes.

The relation between miRNA and phenotypic evolution in plants contrasts with this same relation in animal evolutionary history where the innovation of miRNA families is a constant process [44]. This has been evidenced by the observation that novel miRNA families characterise almost every major animal clade surveyed to date [41]. Animal evolution also exhibits a positive relation between the sum of miRNA families and proxies for phenotypic complexity [45]. This relation does not appear to hold in plants, not least since, as we have shown, the moss *P. patens* and eudicot *A. lyrata* have acquired a comparable repertoire of miRNAs.

Concluding remarks and future perspectives

Our review of the plant miRNA database has shown that very many fail to meet the criteria required for miRNA annotation. However, many of the miRNA families that we failed to validate were rejected due to absence of evidence, rather than evidence that they failed to meet validation criteria. In particular, the identification of miRNA* expression was the major reason for the rejection of families. Conversely, many families were not rejected but have read counts so low that it is difficult to assess the precision of 5′ processing with any confidence. This paucity of evidence is particularly acute in important crop species including maize (*Zea mays*) and soybean (*Glycine max*) (Figure 2). It is important, therefore, that the validity of these candidate miRNAs is more fully evaluated through a systematic programme of miRNAome resequencing.

The phylogenetic framework within which we organise the database of valid plant miRNAs provides not only a powerful perspective within which to trace the evolution of miRNA families but also to predict the miRNAome of unstudied plant species. Although angiosperms represent the majority of plant biodiversity, to gain a more complete understanding of plant miRNA evolution it is essential that there is a rebalancing of sampling to more fully represent the systematic breadth of the plant kingdom. Targeted sequencing of hitherto neglected lineages that represent the majority of plant evolutionary history is therefore urgently needed. This will afford a better perspective on the role that miRNAs have played in developmental evolution within the plant kingdom.

Finally, given their high levels of conservation and interspersed locations across the genome, miRNAs may serve as a simple proxy for tracing major events in the evolution of plant genomes. For instance, paralogy within miRNA families affords a ready insight into the phylogenetic timing and sequence of whole genome duplication events in plant evolutionary history, as well as revealing the roles that miRNA evolution play in responding to genome obesity following whole genome duplication events. This will require the establishment of a coherent systematic classification scheme for paralogy groups within miRNA families, to replace current schemes that serve more to obscure than to reveal homology.

Research into the evolution of the plant miRNAome holds great potential for bioengineering as well as uncovering the role of these regulatory factors in organismal

evolution. However, these prospects can only be achieved if greater effort is expended by researchers and referees in ensuring that the published record is not further corrupted by the description of spurious miRNAs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tplants.2013.11.008.

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