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植物 miRNA 的特殊長度─生成與演化

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關鍵字：miRNA size、biogenesis、evolution

作者簡介

我是呂明軒，目前就讀於台北市建國高級中學。自從參加了中研院舉辦的高中生生命科學人才培育計畫，課堂中每樣新奇的知識莫不深深吸引我，更讓我期待親身體驗實驗室的生活，以近距離接觸這些看似遙不可及的高深學問。

這樣的生活，如今竟也一年了！回想初入實驗室時，熱情的教授和實驗室的學長姐們打破我對科學既有的想像，耐心帶領我學習實驗的技術，還有包羅萬象的科學討論。不在實驗室時，則是自行摸索程式的撰寫，分析「開檔就當機」的龐大資料庫，遇到不會用的函式，或是難以揪出的 bug，總在電腦面前奮鬥好幾小時，捨不得離開。實驗方面，也非總是順遂，像是 14K 大 plasmid 超難做的 cloning，還有整個進行超過半年的題目宣告失敗等等，這些經驗的累積都是高中生活中最難得的。

能夠參與這次的國際科學展覽會，最要感謝爸媽無條件的支持、教授與學姐全心投入的指導、還有老師與同學的鼓勵。不論結果如何，從資料分析、實驗操作，到撰寫報告、製作海報，還有實驗室生活的點滴，這都是我最豐盛的旅程。

摘要

基因靜默為各種生物普遍擁有的調控機制，是以 microRNA (miRNA)和 Dicer-Like (DCL)與 Argonaute (AGO)等數個蛋白質來完成。在植物，miRNA 大多是由 DCL1 截切前驅物所產生，因 DCL1 具有 21-nt 分子尺，所以絕大多數 miRNA 的長度為 21nt。然而，其他特殊長度卻也有一定比例的存在，且可能有特殊的功能。最新研究發現 22nt 的 miRNA 可誘發更多的小片段干擾核酸來造成大量而強烈的基因靜默；然而，一般 21nt 的 miRNA 卻無此功能。本研究以生物資訊的方法分析大量資料庫，並以分子生物學實驗佐證，探討特殊長度 miRNA 的生成、功能與演化。結果發現，20nt 的 miRNA 可藉由對稱性與非對稱性縮短產生，這是 RNA 結構研究的新發現。功能上，20nt 的 miRNA 與生長發育有關，22nt 的 miRNA 則多與逆境反應有關；在演化上，長度 20nt 的 miRNA 呈現高保守性，而大部分 22nt 的 miRNA 則呈現高度物種專一性，為演化快速的特殊長度。

Abstract

Gene silencing, a common regulatory mechanism for gene expression among organisms, is accomplished by micro-RNA (miRNA), Dicer-Like (DCL), and Argonaute (AGO) in plants. Because of the specificity of DCL1 on miRNA precursors and the 21-nt molecular ruler within DCL1, plant miRNAs are dominantly 21nt in size. However, miRNAs of other unique sizes also exist and may function differently. For instance, recent studies indicated that 22-nt miRNAs could amplify gene silencing by triggering secondary small interfering RNA biogenesis while most 21-nt miRNAs lack this ability. In this study, bioinformatics and molecular biology methods are used to study the biogenesis, functionality and evolutionary trends of unique-sized miRNAs in plants. Results show that 20-nt miRNAs could be produced if the miRNA precursors have symmetric or asymmetric sequence signatures. This discovery is new in the study of RNA structure. In terms of biological function, 20-nt miRNAs are related to plant development, whereas 22-nt miRNAs are mostly involved in stress responses. Evolutionarily, 20-nt miRNAs are highly conserved among diverse plant species, while 22-nt miRNAs are mostly frequent birth-and-death and species-specific families.

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I. Introduction

A. Literature Review

1. Gene silencing

Gene silencing, also known as RNA interference (RNAi), is a common regulatory mechanism for gene expression among diverse organisms, playing crucial roles in development (1, 2) and stress responses (3, 4). RNAi-based applications are extensively exploited, and some have been shown to be effective in treatment for tumors (5), Huntington's disease (6), and HIV (7, 8).

Small RNAs of 20~30nt, including microRNAs (miRNAs) and small interfering RNAs (siRNAs), are central to gene silencing pathways. They achieve gene silencing through complementary and sequence-specific base-pairing to their target nucleic acids (9). As in plants, DCL and AGO are two highly-conserved key protein molecules accomplishing gene silencing (10, 11).

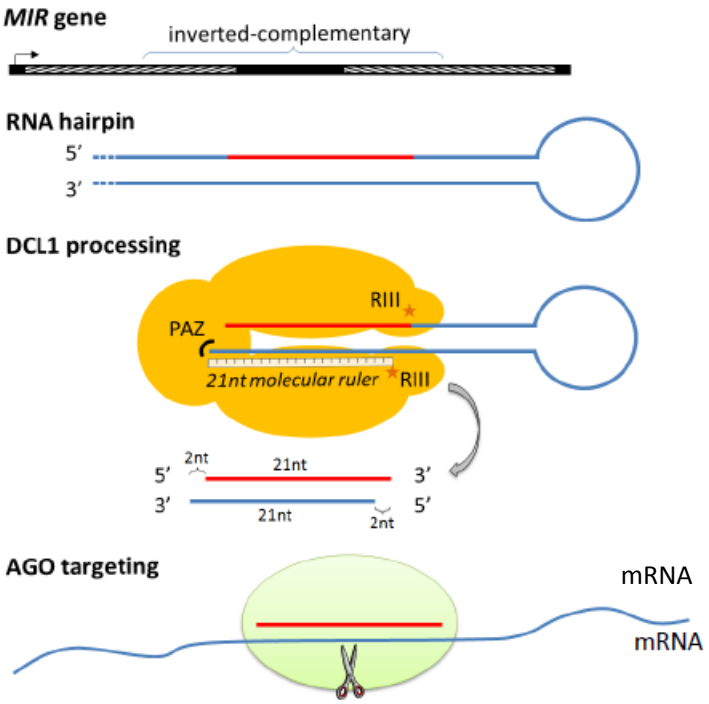


Fig. 1. The miRNA pathway in plants. The *MIR* gene with inverted-complementary sequence is transcribed and folded into a hairpin. Dicer-like 1 (DCL1) recognizes the RNA hairpin and processes it into a 21-nt miRNA duplex with 2-nt overhangs. The red line stands for the mature miRNA. The PAZ domain is responsible for the binding of the RNA 3' end and the stars next to RIII represent the RNaseIII catalytic center. The 21-nt molecular ruler is the physical distance between the PAZ domain and the RNaseIII catalytic center, which determines the miRNA size. The model was modified from (11).

DCL is an RNA endonuclease family that slices double-stranded RNA into fragments of small RNA duplexes, approximately 19~24nt in size. For miRNA biogenesis, inverted-complementary *MIR* transcripts are modified, and folded into hairpins assisted by a series of proteins (10). These miRNA precursors are predominantly substrates of DCL1, because of the high specificity of DCL1 on hairpin RNAs. In most cases, DCL1 generates 21-nt duplexes with 2-nt overhangs on the 3' end of both strands (11) (Fig.1).

AGO recruits one strand of the miRNA duplex to form the RNA-induced silencing complex (RISC) (12). Because of sequence specificity, the miRNA carried

by AGO acts as a probe and searches for complementary nucleic acid targets. In most cases, miRNA is incorporated into AGO1, which cleaves its target mRNAs to achieve downregulation of gene expression (Fig. 1). In addition, miRNAs, according to their 5' terminal nucleotides, as well as length, could be sorted into RISCs with different AGOs (13). A RISC of a different AGO, or even a RISC of different miRNAs with the same AGO, results in different regulations, such as RNA cleavage, translational inhibition or DNA methylation (9, 14-16).

2. Size of miRNA

Plant miRNAs are predominantly 21nt long. However, recent studies revealed that 22-nt miRNAs possess unusual capability in gene silencing (17-19). miRNAs of 22nt can direct target cleavage as well as trigger secondary siRNA production from the cleavage products. The secondary siRNAs can regulate the original target or other unrelated genes and thus amplify the effect of gene silencing. Interestingly, 21-nt miRNAs sharing the same sequence lack this ability, but they can still guide the cleavage on the same targets (Fig. 2).

The presence of a "bulge" in the miRNA duplex may account for the production of the special 22-nt miRNA (17, 18). miRNA precursors are folded into complementary hairpin RNAs, which often contain imperfect matches and even bulges. As described in Figure 1, the "molecular ruler" measures the length of "21nt", the physical distance between PAZ domain and RNaseIII catalytic center within DCL1 (20). A bulge on the miRNA strand does not expand the length of the miRNA duplex measured by DCL1; in other words, DCL1 ignores the bulge and thus produces 22-nt miRNA.

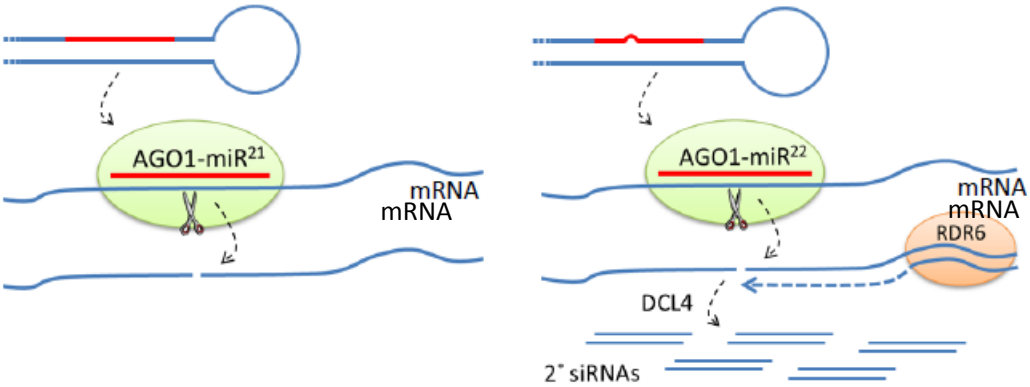


Fig. 2. The unique function of 22-nt miRNA in plants. DCL1-dependent 22-nt miRNA results from a bulge. The AGO1-miR^{21} complex cleaves an mRNA target of complementary sequence, whereas the AGO1-miR^{22} complex further recruits RNA-dependent RNA polymerase 6 (RDR6) to generate double-stranded RNAs, which are processed by DCL4, thus resulting in the production of secondary siRNAs. Thus 22-nt miRNA amplifies the effect of gene silencing.

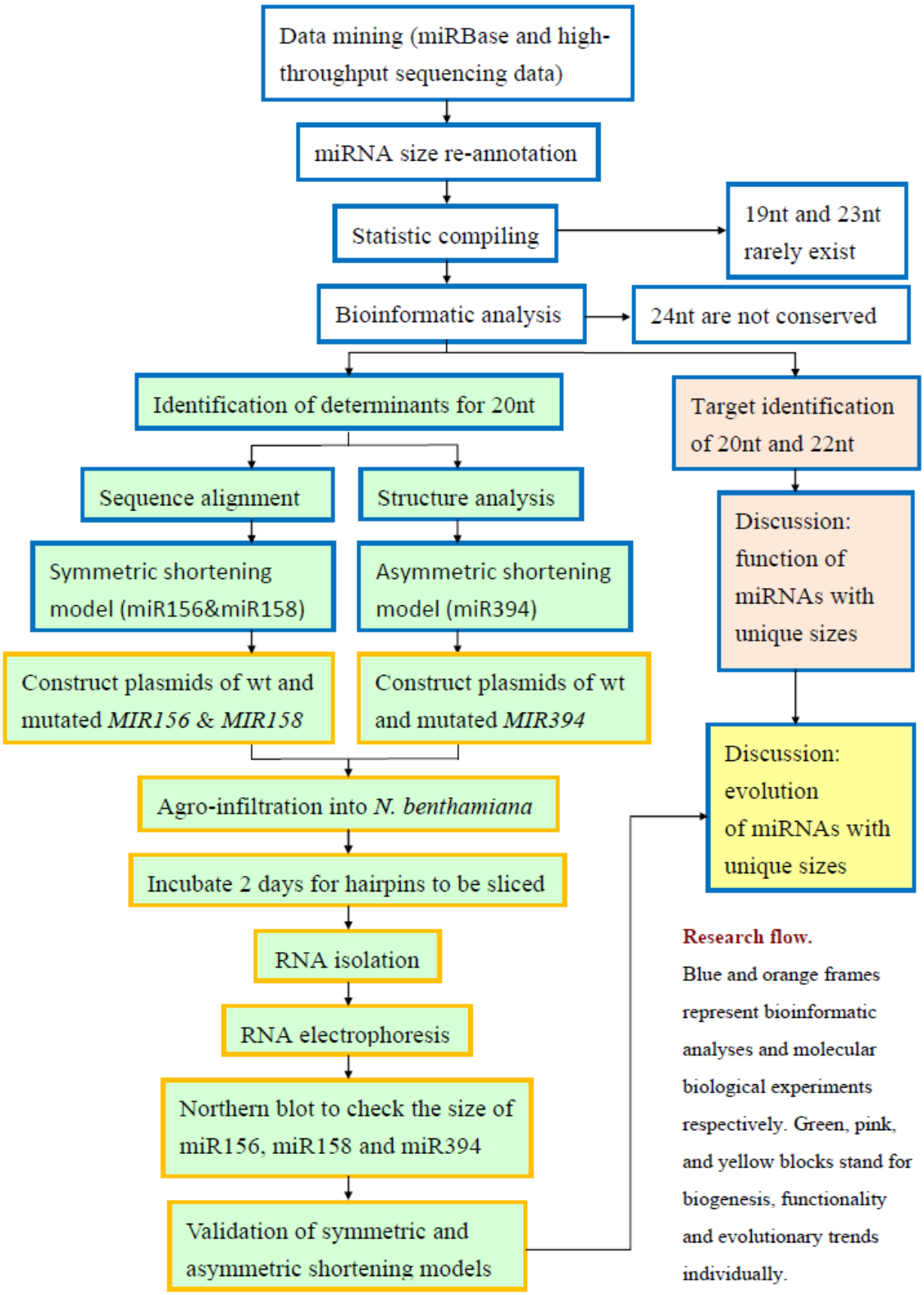
B. Motivation

"Size of miRNA" is a new focus in the study of gene silencing.

Typical DCL1 products are 21nt long. However, recent analyses of high-throughput small RNA transcriptome revealed the existence of miRNAs of other sizes ranging from 19 to 24nt, that surprisingly, are not rare in plants. However, how these unique sizes are produced and whether they influence the functions of the corresponding miRNAs is unclear. Here, bioinformatics and molecular biology methods are adopted to reveal the biogenesis, functionality, and evolutionary trends of plant miRNAs with unique sizes.

II. Materials and Methods

A. Research Flow



Research flow.
Blue and orange frames represent bioinformatic analyses and molecular biological experiments respectively. Green, pink, and yellow blocks stand for biogenesis, functionality and evolutionary trends individually.

B. Organisms

1. *Escherichia coli* (strain: DH5α)

2. *Agrobacterium tumefaciens* (strain: C58C1)

3. *Nicotiana benthamiana*

C. Methods

1. Bioinformatic analyses of plant miRNA size

   a. miRNA size re-annotation (Appendix A)

   ActivePerl was adopted for scripts to perform re-annotation of miRNA size. Mature miRNAs were mapped to miRNA precursors, both retrieved from miRBase 16.0(21), to obtain miRNA isoforms from 19 to 24nt that share the same sequences starting from the 5' end. sRNA reads combined from available GEO datasets (Appendix B) were then mapped to the isoforms with various sizes. The isoforms with the highest reads were then defined as the size of miRNA. The read numbers of re-annotated miRNAs should pass criteria of "more than 5 hits" and "5 hits more than all the other individual reads of isoforms". Having bulge or not was another auxiliary reference, to decide whether the miR/miR* is 22nt when needed.

   b. Distribution of miRNA sizes among species

   According to the re-annotated miRNA size, data were compiled to show the amount of unique miRNA loci of each size in a bar chart. For 20-nt and 22-nt miRNA families of Arabidopsis, sizes of the members and their orthologs in other nine plant species were shown.

c. Sequence alignment

Sequence alignment was performed by using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/) to reveal potential sequence features determining unique miRNA sizes.

2. Constructs of *MIR* backbones for miRNA shortening models (Fig.3)

MIR backbones lacking common sequence features discovered in the precursors of the 20-nt miRNA families, miR156, miR158 and miR394, were constructed by PCR-based site-directed mutagenesis (Appendix C), to test the shortening models. For *MIR156c* and *MIR158a*, the A-C mismatch right after the 3' end of miRNA was replaced by the G-C Watson-Crick pair. For *MIR394a*, the third nucleotide from the 3' end on the passenger strand was deleted to remove the bulge. Sequences are listed in Appendix D.

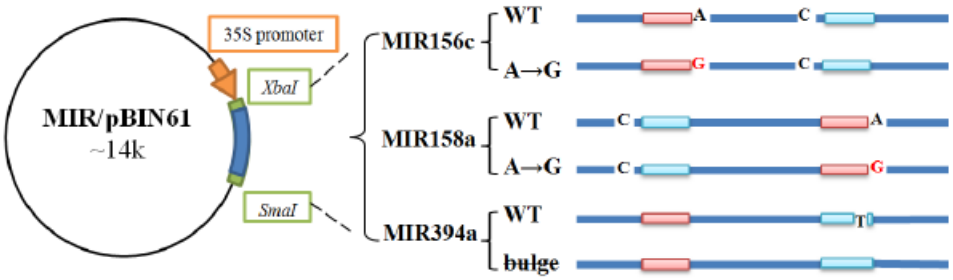


Fig. 3. Constructs of *MIR* backbones for testing miRNA shortening models. The blue strand represents the inserted DNA, and the red and light-blue blocks represent miRNA and miRNA* positions. The miR/miR* are paired and folded into hairpins when they are expressed. The red letters are the mutated sites. The mutation was accomplished by PCR-based site-directed mutagenesis (Appendix C).

3. *Agrobacterium*-mediated infiltration

Leaves of 3~4-week-old *N. benthamiana* (grown at 27℃, 14 hrs light daily) were infiltrated with *Agrobacterium tumefaciens* (strain C58C1, grown at 28℃ in LB containing 50 μg/ml kanamycin, 50 μg/ml rifampicin and 20 μM acetosyringone) carrying *MIR* backbones. Agrobacteria were precipitated by centrifugation at 5000 rpm for 10 min under 4℃, and then re-suspended with the buffer containing 10 mM $MgCl_2$ and 100μM acetosyringone. Agrobacteria were incubated in this medium for at least 2hrs at room temperature and then brought to $O.D._{600}$ = 1.0 for infiltration. The empty vector pBIN61 and the vector carrying GFP were used as negative and positive controls, respectively, for Agro-infiltration. Infiltrated tissues were collected after 2 days for total RNA extraction.

4. Small RNA northern blot analysis

Total RNA was extracted from infiltrated tissues with use of TRIZOL reagent (Invitrogen). Total RNA of 5~10 μg was separated by 15% TBE-urea denaturing polyacrylamide gels (Invitrogen) and transferred to N+ nylon membranes (GE Healthcare), with a transblot semidry transfer cell (Bio-Rad). Antisense DNA oligonucleotides of 20nt were used as probes (miR156c_as: 5'-GTGCTCACTCTCTTCTGTCA-3'; miR158a_as: 5'-TGCTTTGTCTACAT-TTGGGA-3'; miR394a_as: 5'-GGAGGTGGACAGAATGCCAA-3'). Probe labeling, blot hybridization, and film development were performed by Dr. Ho-Ming Chen, who possesses certification in processing experiments involving radioisotope.

III. Results

A. Re-annotation of miRNA size

miRBase (21) is a repertoire of published miRNA sequences and annotations from diverse organisms. Mis-annotation of miRNA size might occur when the annotations are solely based on sequence homology. Because the size of miRNAs was the focus of this study, we needed to obtain a reliable annotation of miRNA size. High-throughput sequencing data of plant small RNA downloaded from the Gene Expression Omnibus (22) were adopted for further curation of miRNA size obtained from miRBase 16.0. The 13 plant species used for data mining included *Physcomitrella patens*, *Selaginella moellendorffii*, *Triticum aestivum*, *Brachypodium distachyon*, *Zea mays*, *Oryza sativa*, *Solanum lycopersicum*, *Vitis vinifera*, *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Glycine max*, *Medicago truncatula*, and *Citrus sinensis*.

Size annotations of 97 of 2,721 miRNAs were revised (Appendix A), on the basis of methods and criteria described in *Materials and Methods*. The following research was base on 11 re-annotated species, excluding *Triticum aestivum* and *Brachypodium distachyon*, since reported miRNAs of them are too few. The re-annotation improves the accuracy of miRNA size in miRBase to approximately 3.56%, which may contribute to future studies of miRNA sizes.

B. miRNA size distribution among species

Canonical miRNAs should be 21nt. A summary of miRNA size distribution according to unique *MIR* loci shown in Fig.4 proves this and provides additional perspectives. Three major points deduced from the results are:

First, 21nt is the most common size, occupying 70%~80% of all miRNAs in each

species. Sizes of 19 and 23nt are rare and only accumulate to < 3% of the total miRNA loci. These numbers agree with the premise that typical miRNAs are products of DCL1, a slicer possessing a 21-nt molecular ruler.

Second, the size of 24nt is abundant only in *O. sativa* and *G. max*, with percentages of about 20% and 40% respectively. However, bioinformatic survey of the sequence of 24-nt miRNAs in the 2 species revealed that they are neither overlapping nor similar. Therefore the 2 groups of 24-nt miRNAs in *O. sativa* and *G. max* may be heterologous. This implies that they are evolved independently and might be produced through a pathway different from the canonical miRNA pathway.

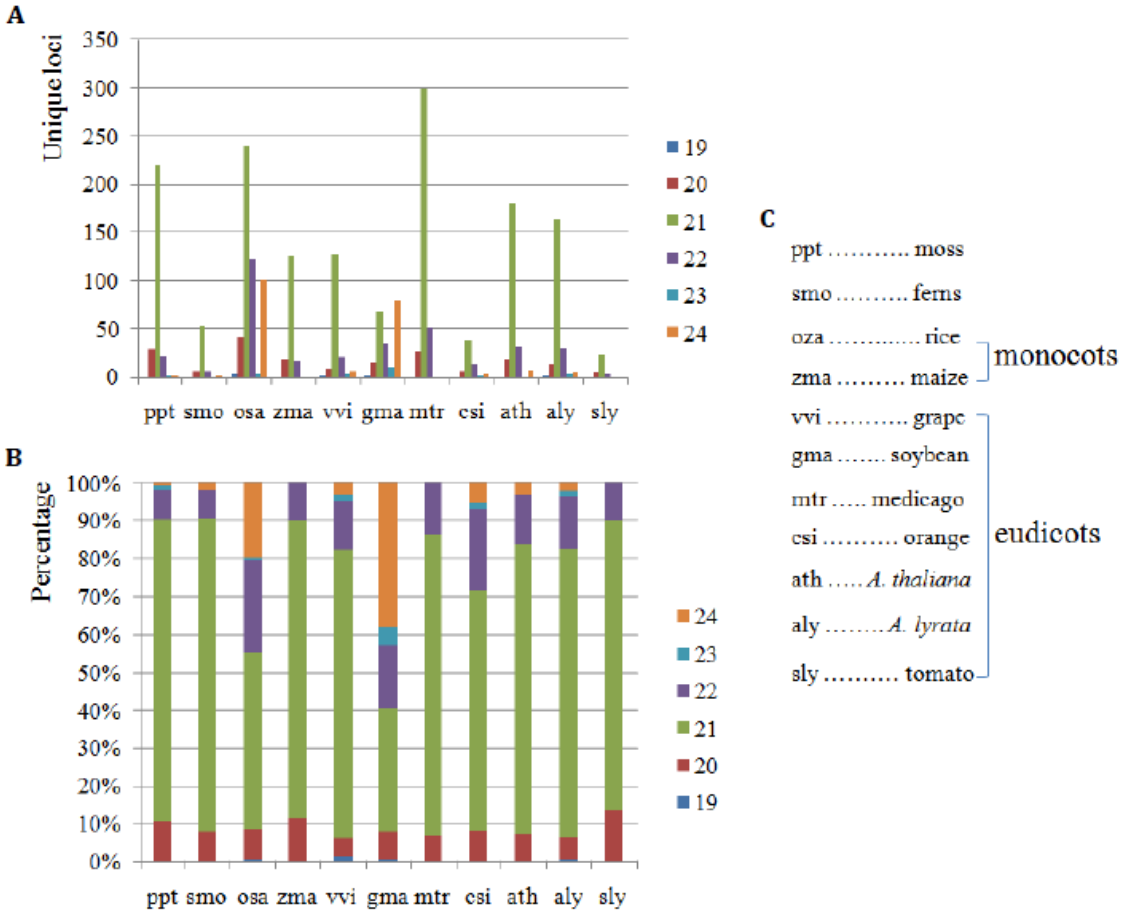


Fig. 4. miRNA size distribution among species. Results were expressed as numbers (A) and percentages (B) and are based on the amount of unique *MIR* loci instead of the expressed level in each species. This analysis includes all reported miRNAs deposited in miRBase. (C) Abbreviations of plant species.

Finally, among most plant species analyzed, 20- and 22-nt miRNAs each contribute approximately 10%~20% of the total miRNAs. The generally stable percentage implies that presence of 20-nt miRNAs, rather than being just a coincidence, might have a unique function in gene silencing. Moreover, how miRNAs smaller than 21nt are produced is still unclear.

C. Arabidopsis 20- and 22-nt miRNA families and their orthologs

With the re-annotated miRNA size data, we could identify members of 20- and 22-nt miRNA families. Families of 20- and 22-nt miRNAs identified in *A.thaliana* and *A. lyrata* with their orthologs in 9 other plant species are shown in Fig. 5.

miR156/157, miR158, miR319 and miR394 were identified to be 20-nt miRNA families in Arabidopsis (Fig.5A). Members of miR156, miR319 and miR394 also exist in other species and form large families. This finding indicates that 20-nt miRNAs mostly arose before the divergence of these species, have been kept under selection pressure till nowadays and have expanded into families with many variants. This observation, which concurs with the study of miRNA size distribution, suggests that the size of 20nt might be crucial for these miRNAs to execute their biological functions.

For 22-nt miRNA families, only miR393 and miR167 expanded into a family with several variants (Fig.5B). The number of 22-nt families is more than that of 20-nt families, yet most of the 22-nt families are small ones with only 1 or 2 variants. Moreover, their orthologs are mostly limited in 1 or 2 species. Therefore, Figure 5B shows that *A. thaliana* and *A. lyrata* own a group of 22-nt miRNAs by themselves in the "others" section with less than 3 variants. This finding implies that, unlike 20-nt miRNAs, most current 22-nt families arose at a later time after the divergence of these species.

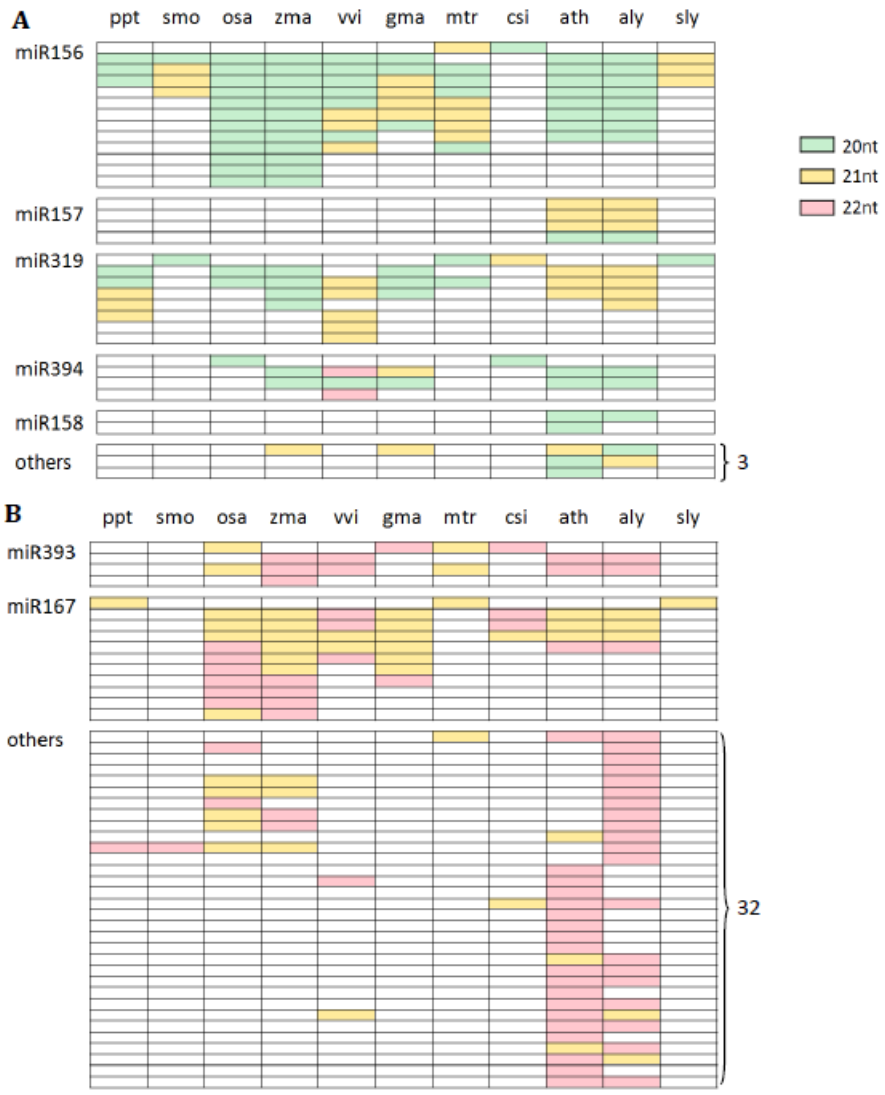


Fig. 5. Identification of 20- (A) and 22-nt (B) miRNA families in Arabidopsis and their orthologs in other plants. The sizes are presented in colors. Every section represents a miRNA family having not less than three variants, and families less than three variants are classified as “others”, except miR158.

D. Biogenesis of unique-sized miRNA

Unique-sized miRNA may be lengthened or shortened 21-nt DCL1 products. It was reported previously that a bulge may cause asymmetric lengthening (17, 18). However, this feature explains only some of the lengthened cases. Thus, this study hopes to discover the factors contributing to shortening of miRNAs. Symmetric and asymmetric shortening mechanisms were revealed by sequence analysis and further experimentally

validated.

1. Symmetric shortening

From the identification of 20-nt miRNA families, among the 10 plant species analyzed here except tomato, miR156 exhibited high conservation of size. Sequence alignment of *MIR156* foldbacks were performed to reveal their common features (Appendix E).

The A-C mismatch right after the 3’ end of the 20-nt miR156 is highly conserved, as was the C-G Watson-Crick pair or U-G pair after the A-C mismatch (Fig.6A). The specific site of the A-C mismatch is tantamount to the last base pair of a canonical 21-nt miRNA which is processed in a stem-to-loop fashion. This conserved sequence signature is special because, although mature miRNAs depend on their sequences to function, the flanking sequences of mature miRNAs on the hairpins are usually less conserved (23). In contrast, most members defined as 21nt via size re-annotation in miR156 family lack this signature. In addition to the *MIR156* family, the signature is also found in *ppt-MIR477c*.

For miR158, which is also 20nt long, a similar signature of an A-C mismatch was found at an equivalent position (Fig.6B). However, unlike miR156, miR158 locates at the 3’ arm of the *MIR* foldback. Recent study showed several cases of non-canonical loop-to-stem *MIR* processing of DCL1 (24). Although the processing direction of *MIR158* has not been studied, *MIR158* may be processed in a loop-to-stem fashion. Thus, similar to that in *MIR156*, the A-C mismatch could serve as a potential signature responsible for shortening of miR158.

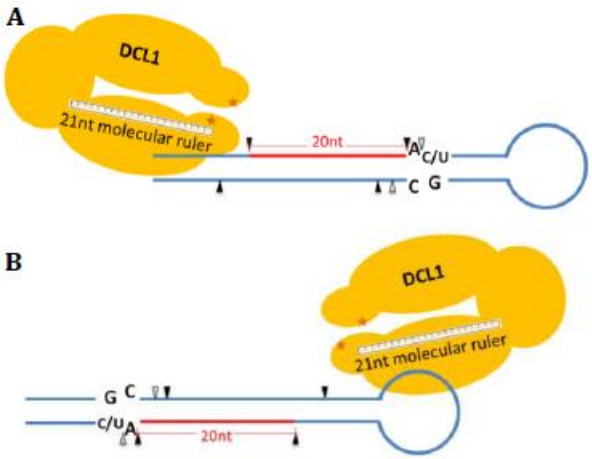


Fig. 6. Symmetric shortening model. The black triangle represents the actual cleavage sites, and the gray the predicted sites according to the 21-nt molecular ruler. Stem-to-loop processing is seen in *MIR156* families (A) and loop-to-stem processing is discovered in *MIR158* families (B).

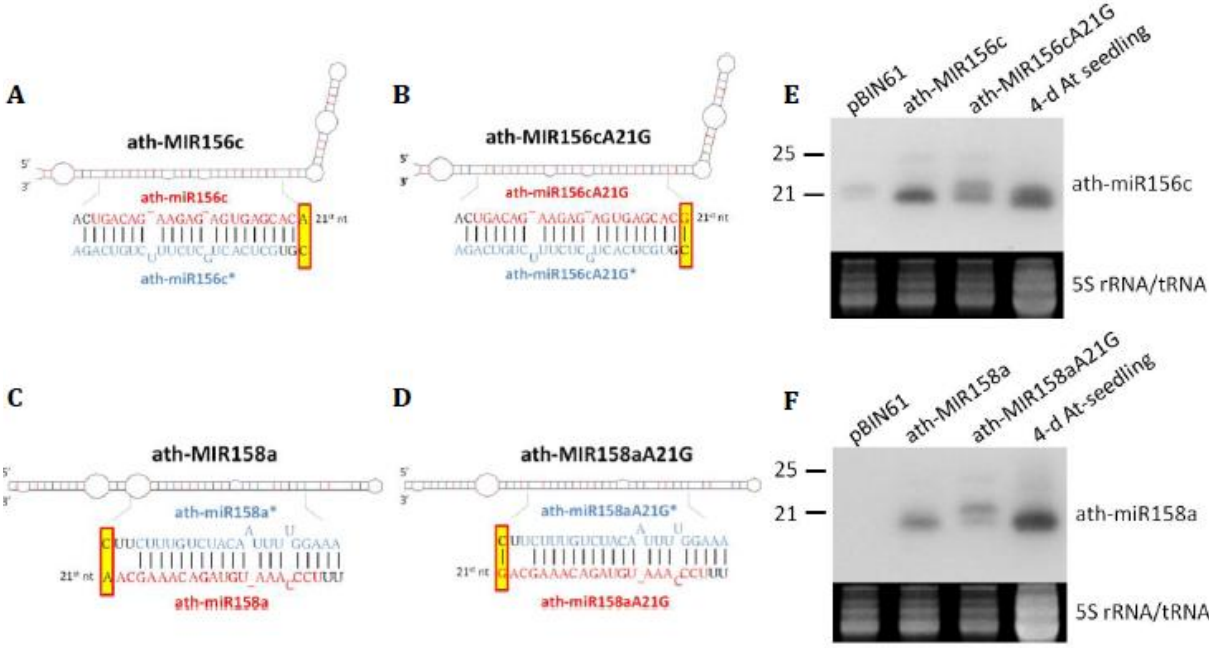


Fig. 7. Validation of the symmetric model. Predicted foldback structures for *MIR156c* (A) and artificial *MIR156cA21G* (B). Predicted foldback structures for *MIR158a* (C) and artificial *MIR158aA21G* (D). Northern blot analysis of artificial *MIR156cA21G* (E) and *MIR158aA21G* (F).

From the sequence alignment of *MIR156* and similar structure features shared between *MIR156* and *MIR158*, a hypothesis of symmetric shortening is proposed: When DCL1 encounters an A-C mismatch at the 21st nucleotide of a miRNA on a hairpin, it slices the hairpin 1nt before the predicted site on both miR and miR* strands based on the 21-nt molecular ruler.

To test this hypothesis, mutated constructs of *MIR156c* and *MIR158a* lacking the A-C mismatch were designed (Fig.7A-D). The A-C mismatch was changed into a G-C Watson-Crick perfect match, and thus the mutants were named *MIR156cA21G* and *MIR158aA21G*. Mature miRNA products of 21nt were expected to be generated from them.

Nicotiana benthamiana transient assays were performed for validation. Constructs of *MIR156c, MIR158a, MIR156cA21G* and *MIR158aA21G* were each inserted into vector pBIN61 after the constitutive 35S promoter. Infiltration of *Agrobacterium tumefaciens* carrying the recombinant plasmids expressed the wild type and mutated *MIR* gene fragments in *N. benthamiana*, whose expression of the miR156 and miR158 family during the experiments was low enough to distinguish endogenous miRNAs from exogenous miRNAs. After 2 days of *N. benthamiana* endogenous DCL1 processing, the hairpins of *MIR156c, MIR158a, MIR156cA21G* and *MIR158aA21G* were sliced to produce mature miRNAs.

Small RNA northern blot analyses revealed that *MIR156cA21G* and *MIR158aA21G* produced longer 21-nt miRNAs additional to the original 20-nt miRNAs (Fig.7E and F). A possible explanation is that the alteration of A-C mismatch at the 21st nucleotide of miR156/158 may extend the miRNA produced to a certain degree, which is nearly while less than 1nt. Because the size of miRNA should be integers, both 20- and 21-nt miR156cA21G and miR158aA21G may be yielded.

2. Asymmetric shortening

To understand whether structural characters of 20-nt miRNA precursors affect the shortening, hairpin structures of these families was observed. Surprisingly, a

bulge, which was a signature previously revealed to be responsible for lengthening in the case of 22-nt miRNA (17-19), was found to be conserved in the 20-nt miR394 family. Interestingly, the conserved bulge was specifically found on the third nucleotide from the 3’ overhang of the miR394* and this bulge doesn’t alter the size of miR394* but rather cause the shortening of miR394.

Considering the processing of MIR foldback by DCL1, a hypothesis of specific asymmetric structure causing shortening was formed: When DCL1 processes an MIR foldback, once the site before the miRNA is sliced, the bulge on the 3’ overhang of the complementary strand is no longer constrained by the force of the hydrogen bond from flanking base pairs and therefore extends (Fig.8A-C). Thus, the 2-nt overhang on the 3’ end becomes 3nt. Because DCL1 measures 21nt from the free 3’ end, the 3-nt overhang thus results in an asymmetric miRNA duplex of 20nt:21nt (Fig.8D). The same asymmetric signature was also discovered in other foldbacks that produce 20-nt ath-miR775*, osa-miR159cdef and vvi-miR3633a.

Mutated construct of *MIR394a* without the bulge was therefore designed and named *MIR394aΔbulge* (Fig.9A and B), to test the hypothesis. Products of 21-nt miR394 were expected to be produced once the bulge was removed. This model was also validated by the *N. benthamiana* transient assays described previously.

Small RNA northern blot analysis revealed that the size of miR394aΔbulge increased to 21nt as for normal DCL1 products (Fig.9C). This finding supports the hypothesis that a bulge could shorten an miRNA if it is present on the 3’ overhang of the opposite strand of the shortened one.

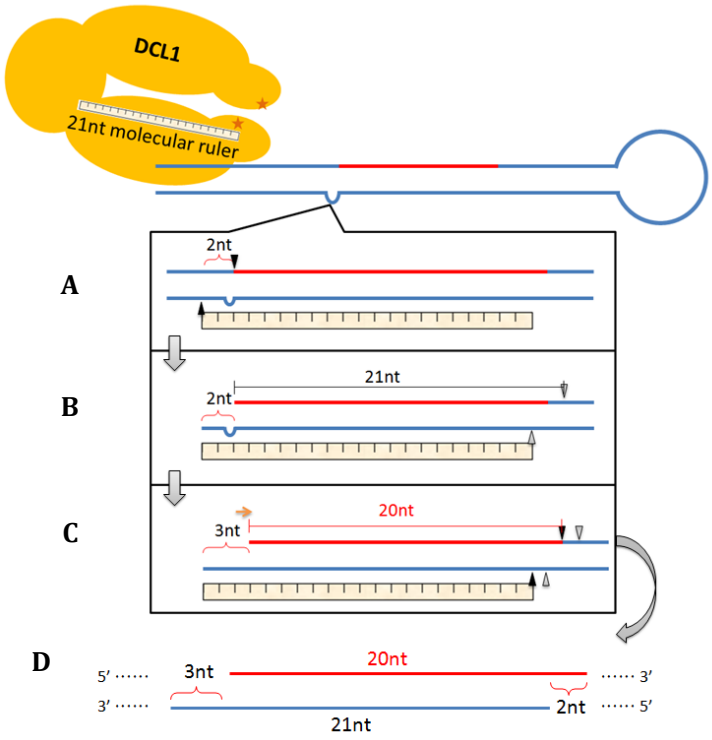


Fig. 8. Asymmetric shortening model. The symbols are the same as used in Fig. 6. (A) A bulge is on the specific site. (B) DCL1 slices the hairpin, and the bulge exposes. (C) The bulge extends the 3' overhang to 3nt. (D) The asymmetric product is a 20nt:21nt miRNA duplex.

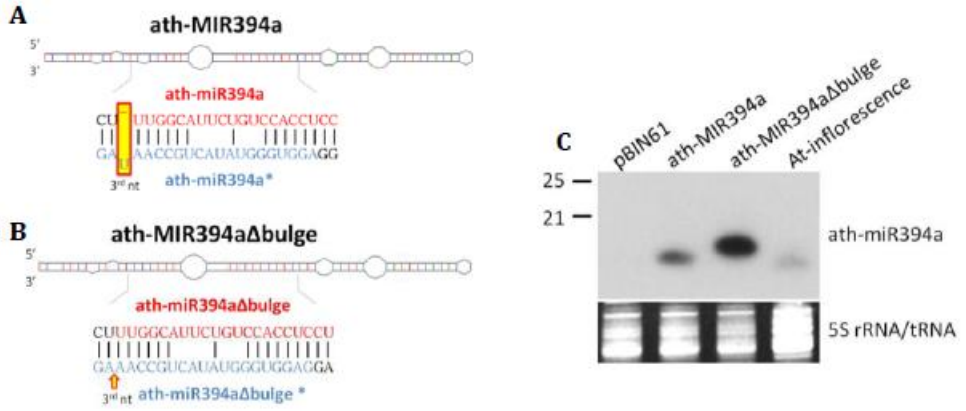


Fig. 9. Validation of the asymmetric model. Predicted foldback structures for MIR394a (A) and artificial MIR394Δbulge (B). (C) Northern blot analyses of artificial MIR394Δbulge.

With the lengthening mechanism known already and shortening mechanisms newly discovered in this study, biogenesis of more unique-sized miRNAs based on sequence

characteristics in their foldback precursors can be better understood. For instance, structure and sequence analyses reveal that ath-miR156c* contains 2 bulges and an A-C mismatch on the required site, thus causing 2-nt lengthening and 1-nt shortening, which should result in 1-nt net lengthening, so the size should increase to 22nt. Indeed, 22-nt miR156c was revealed by deep sequencing analyses in *A. thaliana*.

E. Biological function of unique-sized miRNA

The functions of miRNAs have been studied previously, yet the connection of the function of target genes and miRNA size has not been linked. In this and previous studies, miRNAs of sizes of 20 or 22nt were identified. Targets of these unique-sized miRNAs were then sought to determine whether they possess biological functions distinct from the more commonly observed 21-nt miRNAs. For this purpose, miRNA target genes were arranged into 20- or 22-nt miRNA groups. miRNA families of 20-nt are mostly involved in the regulation of development, whereas 22-nt miRNA families preferentially contribute to stress responses in plants (Appendix F).

For 22-nt miRNAs, the unique functionality of amplifying gene silencing by triggering secondary siRNA production may benefit the plant with rapid and effecient responses to stresses, such as temperature, nutrition, salinity, UV, bacteria and virus infection, and mechanical injuries. For 20-nt miRNAs, why they preferentially regulate genes controlling developmental processes remains to be studied.

IV. Discussion

1. 19-, 23- and 24-nt miRNA

miRNAs of 19nt and 23nt are extremely rare and may be a transit status in the fluid of evolution. Additionally, the 24-nt size is not conserved among plants.

2. 20- and 22-nt miRNA

Among plant species, both 20- and 22-nt miRNAs occupy a certain percentage of all miRNAs, approximately 10% each. A model of the evolutionary trends of 20- and 22-nt miRNA was proposed (Fig.10).

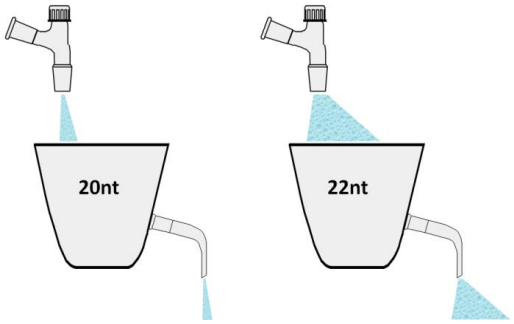


Fig. 10. Model of evolutionary trends of 20- and 22-nt miRNA families.

a) Inflow

For the control of inflow, the birth of these unique-sized miRNA may be crucial. For 20-nt miRNAs, the opportunity for a mutation creating a new variant is relatively slimmer than that for 22-nt miRNAs, because 20-nt miRNAs need an A-C mismatch or a bulge on specific sites, whereas 22-nt miRNAs could result from a bulge on nearly any site. The inflow of 22-nt miRNA therefore may be more affluent than that of 20-nt miRNA.

b) Outflow

On the other hand, the outflow represents those eliminated by selection of evolution. miRNAs of 22nt can amplify the effect of gene silencing, which is an efficient pathway if it acts appropriately. However, birth of 22-nt miRNA may also

be a lethal mutation, owing to their amplification effects. Thus, the outflow of 22-nt miRNA could be greater than that of 20-nt miRNA.

In summary, 22-nt miRNAs should belong to frequent birth-and-death families, whereas 20-nt miRNAs are mostly highly conserved families. This model can also be supported by the phenomenon of 20-nt miRNA families being larger and more conserved among plants, whereas 22-nt miRNA families are smaller and mostly species-specific.

V. Conclusions

1. Size of 97 miRNAs in miRBase were re-annotated.

2. 21-nt miRNAs are the main class, whereas 20- and 22-nt miRNA remain at a certain percentage approximately 10% each, among plant species.

3. Symmetric and asymmetric shortening models explain the biogenesis of 20-nt miRNAs.

4. 20-nt miRNA families mostly function in developmental regulation, whereas 22-nt miRNA families are mostly related to stress responses.

5. 20-nt miRNA families are mostly highly conserved among plants, whereas 22-nt miRNA families are mostly species-specific and frequent birth-and-death families.

VI. References

A. Websites

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VII. Appendices

A. miRNA size re-annotation

The re-annotation was performed following two criteria: highest reads >5, highest reads – other reads >5. Having a bulge or not was another auxiliary reference to determine whether the miR or miR* was 22nt when needed. 97 revised entries in miRBase are listed below:

miR/miR* name	re-annotation	miRBase
>aly-miR156d* MIMAT0017404 Arabidopsis lyrata miR156d*	24	21
>aly-miR157a* MIMAT0017414 Arabidopsis lyrata miR157a*	21	22
>aly-miR161.1* MIMAT0017443 Arabidopsis lyrata miR161.1*	21	19
>aly-miR165a MIMAT0017456 Arabidopsis lyrata miR165a	21	20
>aly-miR165a* MIMAT0017455 Arabidopsis lyrata miR165a*	21	20
>aly-miR165b MIMAT0017458 Arabidopsis lyrata miR165b	21	20
>aly-miR169a* MIMAT0017486 Arabidopsis lyrata miR169a*	20	21
>aly-miR172a* MIMAT0017521 Arabidopsis lyrata miR172a*	20	21
>aly-miR172e* MIMAT0017529 Arabidopsis lyrata miR172e*	21	20
>aly-miR3437* MIMAT0017706 Arabidopsis lyrata miR3437*	21	20
>aly-miR3446* MIMAT0017728 Arabidopsis lyrata miR3446*	21	24
>aly-miR390b* MIMAT0017536 Arabidopsis lyrata miR390b*	20	21
>aly-miR862-3p MIMAT0017654 Arabidopsis lyrata miR862-3p	24	21
>ath-miR773 MIMAT0003932 Arabidopsis thaliana miR773	22	21
>ath-miR776 MIMAT0003935 Arabidopsis thaliana miR776	22	21
>bdi-miR169b MIMAT0012189 Brachypodium distachyon miR169b	20	21
>csi-miR167a MIMAT0018498 Citrus sinensis miR167a	22	21
>csi-miR167b MIMAT0018454 Citrus sinensis miR167b	22	21
>csi-miR172a MIMAT0014076 Citrus sinensis miR172a	21	20
>gma-miR1507b MIMAT0010080 Glycine max miR1507b	22	21
>gma-miR1509b MIMAT0011201 Glycine max miR1509b	22	21
>gma-miR1510a-3p MIMAT0007368 Glycine max miR1510a-3p	21	23
>gma-miR1510a-5p MIMAT0017338 Glycine max miR1510a-5p	21	24
>gma-miR1511 MIMAT0007369 Glycine max miR1511	21	20
>gma-miR1512 MIMAT0007370 Glycine max miR1512	21	22
>gma-miR1514a MIMAT0007372 Glycine max miR1514a	22	21
>gma-miR1535 MIMAT0007398 Glycine max miR1535	21	19
>gma-miR156b MIMAT0001692 Glycine max miR156b	20	21

>gma-miR156f MIMAT0018318 Glycine max miR156f	21	22
>gma-miR162 MIMAT0007353 Glycine max miR162	21	20
>gma-miR169d MIMAT0018330 Glycine max miR169d	22	23
>gma-miR172d MIMAT0011199 Glycine max miR172d	21	24
>gma-miR172e MIMAT0011200 Glycine max miR172e	21	24
>gma-miR172f MIMAT0018333 Glycine max miR172f	21	20
>gma-miR2109 MIMAT0010086 Glycine max miR2109	21	20
>gma-miR390a-3p MIMAT0007360 Glycine max miR390a-3p	21	20
>gma-miR393 MIMAT0007362 Glycine max miR393	22	21
>gma-miR394a MIMAT0018341 Glycine max miR394a	21	20
>gma-miR396e MIMAT0018345 Glycine max miR396e	21	22
>gma-miR4413 MIMAT0018338 Glycine max miR4413	21	20
>gma-miR482b MIMAT0018286 Glycine max miR482b	21	22
>mtr-miR1509 MIMAT0010034 Medicago truncatula miR1509	22	21
>mtr-miR169i MIMAT0011110 Medicago truncatula miR169i	22	21
>mtr-miR169n MIMAT0011122 Medicago truncatula miR169n	22	21
>mtr-miR169o MIMAT0011123 Medicago truncatula miR169o	22	21
>mtr-miR2597 MIMAT0013287 Medicago truncatula miR2597	22	21
>osa-miR1318 MIMAT0009135 Oryza sativa miR1318	21	20
>osa-miR1423 MIMAT0005957 Oryza sativa miR1423	24	21
>osa-miR1429-3p MIMAT0005963 Oryza sativa miR1429-3p	22	21
>osa-miR1429-5p MIMAT0009208 Oryza sativa miR1429-5p	24	21
>osa-miR1432 MIMAT0005966 Oryza sativa miR1432	22	21
>osa-miR1437 MIMAT0005988 Oryza sativa miR1437	21	22
>osa-miR156k MIMAT0001020 Oryza sativa miR156k	20	21
>osa-miR156l MIMAT0001021 Oryza sativa miR156l	20	21
>osa-miR159a.2 MIMAT0009200 Oryza sativa miR159a.2	20	21
>osa-miR159f MIMAT0001027 Oryza sativa miR159f	20	21
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>osa-miR167i MIMAT0001044 Oryza sativa miR167i	22	21
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>osa-miR2125 MIMAT0011194 Oryza sativa miR2125	20	24
>osa-miR319a MIMAT0001028 Oryza sativa miR319a	21	20

>osa-miR319b MIMAT0001029 Oryza sativa miR319b	21	20
>osa-miR396f MIMAT0010070 Oryza sativa miR396f	23	22
>osa-miR437 MIMAT0001586 Oryza sativa miR437	24	21
>osa-miR530-5p MIMAT0006787 Oryza sativa miR530-5p	21	20
>ppt-miR1038-5p MIMAT0005146 Physcomitrella patens miR1038-5p	21	20
>ppt-miR319d.1* MIMAT0004325 Physcomitrella patens miR319d.1*	21	20
>ppt-miR390c* MIMAT0004327 Physcomitrella patens miR390c*	20	21
>ppt-miR477h MIMAT0005053 Physcomitrella patens miR477h	20	19
>ppt-miR894 MIMAT0004365 Physcomitrella patens miR894	21	20
>tae-miR156 MIMAT0018208 Triticum aestivum miR156	20	21
>vvi-miR167a MIMAT0005670 Vitis vinifera miR167a	22	21
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>vvi-miR393a MIMAT0006557 Vitis vinifera miR393a	22	21
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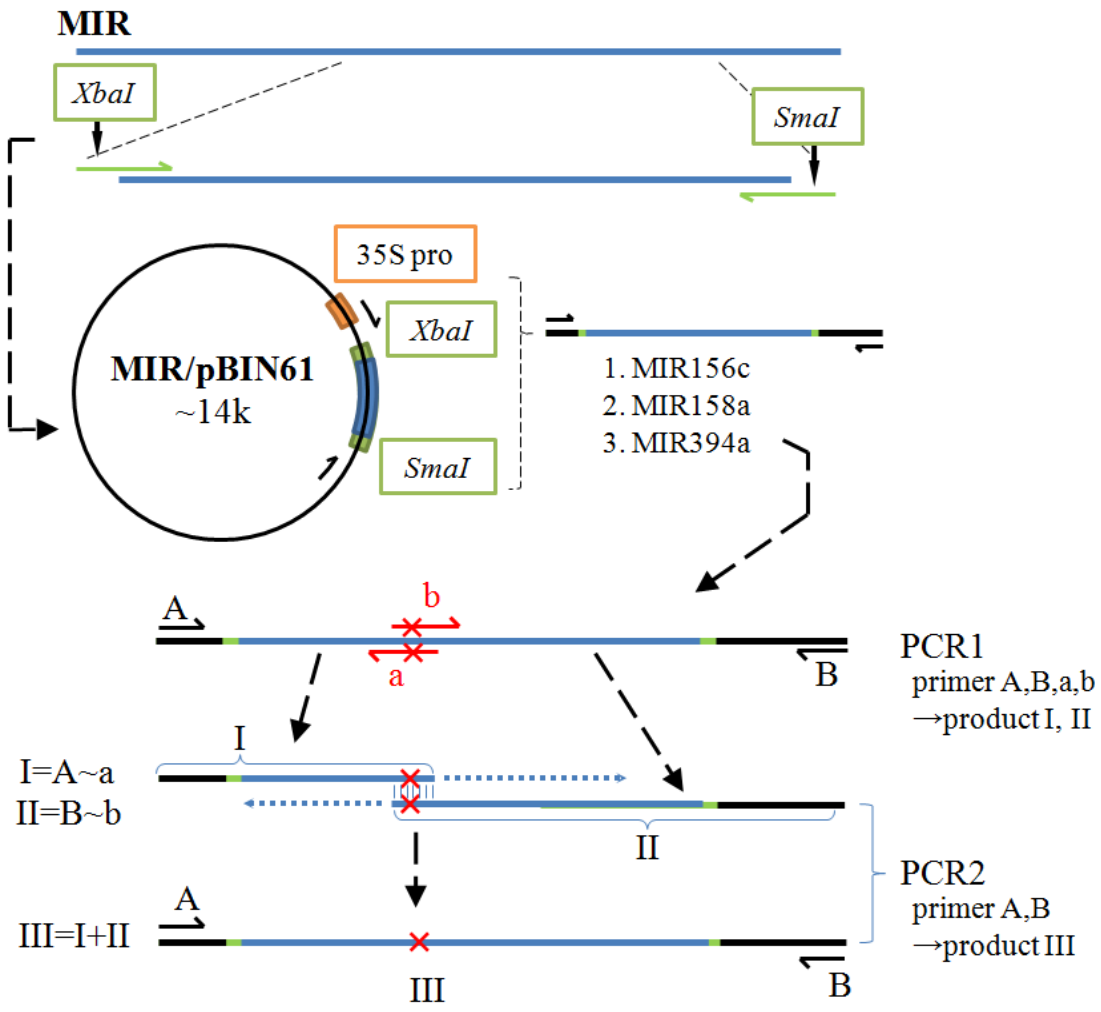
>zma-miR172c MIMAT0001390 Zea mays miR172c	21	20
>zma-miR172d MIMAT0001388 Zea mays miR172d	21	20

B. GEO database used in this study

GEO Accession	Organism	Tissue	Age	Instrument model	Date
GSM451894	*Arabidopsis lyrata*	rosette leaves	8 weeks	Illumina Genome Analyzer	May, 2010
GSM518389	*Arabidopsis lyrata*	flowers	stage 1-12	454 GS FLX	May, 2010
GSM518390	*Arabidopsis lyrata*	flowers	stage 1-12	454 GS FLX	May, 2010
GSM518391	*Arabidopsis lyrata*	seedlings	14 days	454 GS FLX	May, 2010
GSM518392	*Arabidopsis lyrata*	seedlings	14 days	454 GS FLX	May, 2010
GSM518429	*Arabidopsis lyrata*	rosette leaves		Illumina Genome Analyzer	May, 2010
GSM518430	*Arabidopsis lyrata*	flowers	stage 1-12	Illumina Genome Analyzer	May, 2010
GSM518431	*Arabidopsis lyrata*	flowers	stage 1-12	Illumina Genome Analyzer	May, 2010
GSM118372	*Arabidopsis thaliana*	flowers	4 weeks	454 GS FLX	Nov, 2006
GSM118373	*Arabidopsis thaliana*	rosette leaves	6 weeks	454 GS FLX	Nov, 2006
GSM118374	*Arabidopsis thaliana*	seedlings	6 days	454 GS FLX	Nov, 2006
GSM118375	*Arabidopsis thaliana*	siliques	2 months	454 GS FLX	Nov, 2006
GSM406302	*Brachypodium distachyon*	young spike (reproductive)	mature	Illumina Genome Analyzer II	Jun, 2009
GSM406303	*Brachypodium distachyon*	root, shoot, leaf (vegetative)	mature	Illumina Genome Analyzer II	Jun, 2009
GSM455228	*Citrus sinensis*	fruits	170 days after flowering	Illumina Genome Analyzer	Sep, 2010
GSM543393	*Glycine max*	immature seed coats	dissected from seeds of 50-75 mg fresh weight	Illumina Genome Analyzer	May, 2010
GSM543394	*Glycine max*	immature seed coats	dissected from seeds of 50-75 mg fresh weight	Illumina Genome Analyzer	May, 2010
GSM543395	*Glycine max*	immature cotyledons	dissected from seeds of 50-75 mg fresh weight	Illumina Genome Analyzer	May, 2010
GSM543396	*Glycine max*	immature seed coats	dissected from seeds of 50-75 mg fresh weight	Illumina Genome Analyzer	May, 2010
GSM346592	*Medicago truncatula*	aerial part (including stems and leaves)	9 weeks	Illumina Genome Analyzer	Dec, 2008
GSM346593	*Medicago truncatula*	aerial part (including stems and leaves)	9 weeks	Illumina	Dec, 2008
GSM387472	*Medicago truncatula*	mature nodules	1-week old seedling inoculated for 1 month	454 GS FLX	Jul, 2009
GSM387473	*Medicago truncatula*	root tips (1-2 cm)	10-12 days post-germination	454 GS FLX	Jul, 2009
GSM278571	*Oryza sativa*	seedlings and de-husked rice grains	1-5 days after fertilization grains	Illumina	Aug, 2008
GSM278572	*Oryza sativa*	seedlings and de-husked rice grains	6-10 days after fertilization grains	Illumina	Aug, 2009

GSM455965	*Oryza sativa*	seedlings		Illumina Genome Analyzer	Nov, 2009
GSM520640	*Oryza sativa*	seedlings		Illumina Genome Analyzer	Mar, 2010
GSM571077	*Oryza sativa*	seedlings	12 days	Illumina Genome Analyzer	Aug, 2010
GSM115095	*Physcomitrella patens*	protonemata	7 days	performed as described by Axtell et al. (2006) Cell 127: 565-577	Aug, 2006
GSM115096	*Physcomitrella patens*	protonemata	14 days	performed as described by Axtell et al. (2006) Cell 127: 565-577	Aug, 2006
GSM115097	*Physcomitrella patens*	mature gametophores and sporophytes	~60 days	performed as described by Axtell et al. (2006) Cell 127: 565-577	Aug, 2006
GSM313212	*Physcomitrella patens*	protonemata	10 days	Illumina Genome Analyzer	Dec, 2008
GSM313213	*Physcomitrella patens*	protonemata	10 days	Illumina Genome Analyzer	Dec, 2008
GSM304985	*Solanum lycopersicum*	fruits	developmental stages F1-3, F3-5, F5-7, F7-11, F11-14	454 high throughput pyrosequencing	Aug, 2008
GSM304986	*Solanum lycopersicum*	leaves	mature	454 high throughput pyrosequencing	Aug, 2008
GSM176654	*Selaginella moellendorffii*	above-ground tissues (stems and leaves)		454 high throughput pyrosequencing	Jun, 2007
GSM406301	*Triticum aestivum*	root, shoot, leaf, young spike	mature	Illumina Genome Analyzer II	Jun, 2009
GSM458927	*Vitis vinifera*	stems and leaves	during vegetative start	Illumina Genome Analyzer	Apr, 2010
GSM458928	*Vitis vinifera*	tendrils	during vegetative start	Illumina Genome Analyzer	Apr, 2010
GSM458929	*Vitis vinifera*	inflorescences	during vegetative start	Illumina Genome Analyzer	Apr, 2010
GSM458930	*Vitis vinifera*	berries	during vegetative start	Illumina Genome Analyzer	Apr, 2010
GSM306487	*Zea mays*	immature ears with the floral tissue including young tassels 2-3 cm long	68 days	Illumina Genome Analyzer	Oct, 2008
GSM433620	*Zea mays*	leaves	6-week-old seedlings	Illumina Genome Analyzer II	Dec, 2009
GSM433621	*Zea mays*	female inflorescence (ears)	10 and 11 weeks	Illumina Genome Analyzer II	Dec, 2009
GSM433622	*Zea mays*	Male inflorescence (tassels)	8 weeks	Illumina Genome Analyzer II	Dec, 2009

C. PCR-based site-directed mutagenesis



The blue strands represent fragments of *MIR* gene. Restriction sites used for cloning are shown in green, and sequences on vector are in black. The red represents mutated sites. I ~ III represent the PCR products for each of the consecutive experimental steps.

D. Sequence of *MIR156c*, *MIR158a* and *MIR394a*

Sequences in italics represent restriction enzyme sites. Bold type highlighted in yellow represents the miRNA, and sequences highlighted in gray represents the miRNA*. The sharp arrows represent the primers used to retrieve the *MIR* genes with enzyme sites, and the solid arrows represent the primers used for site-directed mutagenesis. The nucleotides in red are the mutated sites: A mutated to G, and T deleted.

1. *MIR156c*

5’-GAC*TCTAGA*AAAAGCCTCAGATCTAACTCCAACACCTTCAAAGTCTGC
CTCCTTTCCAATCTTCTTTCTTCTGTTCGATCTCTAATCTCAGAATTTGTGT
CGGTAAGGTAAAGGTGATAATGAGTGATGACTGATGAGGGAGTTTTGGGA
CAAATTTTAAGAGAAACGCATAGAAAC**TGACAGAAGAGAGTGAGCAC**A
CAAAGGCACTTTGCATGTTCGATGCATTTGCTTCTCTTGCG**TGCTCACTG**
CTCTATCTGTCAGATTCCGGCTCCGATTCGGTCCCGGTCACGTTTTCTTC
TTCTAATTGTGTTCCCATCTCTCACTTTCTCTCTCATGTGTTCTTCATCTCTC
AAAGGTAAATTAAATACGATCTGATAATATCTGTATGAAGATTGTTCTTATT
TGCTGCCATTGATTTGGTTCCCAATTGCATGCGAGTTTGCTTTTTGTATTTT
CCTTTGTTGAACTTTATATTTTATGAGATCATTAGTATCGAAAGCCTAATCT
ATGAGTTTAAGGCGTGGTTAAGTGAGATTAACATGGGACCATTAAC*CCCG*
*GG*CAC-3’

2. *MIR158a*

5’-CTTCTAGGGTT*TCTAGA*TTGTATACCCTAGATAAGCATCCTATAAAGTAA
ACACAAGTACTTGCAGAGACTTTAGATTAGAGGGCTAGCGACTGCAGAA
GAAGAGTAACACGTCATCTCTGTGCTT**CTTTGTCTACAATTTTGGAAA**A
AGTGATGACGCCATTGCTCTT**TCCCAAATGTAGACAAAGCA**ATACCGTG
ATGATGTCGTGGAGATTTTGTTGAGATGCTACGAGCGTGTAAATGTTTCAT
GTTTGTTGTTTGATTTTACTGACTGCTGATTTCTTTTTTTCTTGGATCTCGC
TCAAAACTAGATCCTCACTACAGAGCATAACTGTTGATTTTTCTTATTCCA
GATCTTGCTCTGAACAAGGGCATCTAAAGTCACAGATGCGTGTGATTATT
G*CCCGGG*CAC-3’

3. *MIR394a*

5’-GAC*TCTAGA*TAAGCCAAGCTTATATAGCCCGTCATAAAGAGAACTCATC
TGCCTCTCTCTCAATACCAATAAATATCACCACCGTCCTTCTCTCCTATCAC
TATTCAATCTATCGCAAACTCCTTTATGTCTCTCCAATTTTATGAGAGGGTT
TCCTTCAAGAACACAGTAAAATAGATTGGATCTTTAAACTTTTGTTCCTTT
TCATGAGGGTTTGACAAAGATTTTCTTACAGTCATCT**TTGGCATTCTGTC**
CACCTCCTTCTATACATATATGCATGTGTATATATATATGCGTTTCGTGTGAA
AGAAGG**AGGTGGGTATACTGCCAA**T**AG**AGATCTGTTAGGGCTTCTTCGT
AAAACCCTCTTGATCTATATACAAGTTTATCAATGTCTTGAATTTGACGATC
TTGATTTGTTTCTTGGCGATGAAGAGCCTCGATCTAGGGTAATTTTACGAT
TAACCATTTTCTTGATTCTCACATCTTTGATTCTCTCGATTCTTTTTGTTTGA
TTTCATATATAAAGTTCGTTTCTAATATTGATCCTTCACTG*CCCGGG*CAC-3’

E. *MIR156* sequence alignment

Sequence alignment was performed to research potential sequence features determining size alterations in miRNAs. The aligned sequences are miR156 members re-annotated as 20nt. Yet mistakes may still occur in situations when read numbers of the variants cannot be discriminated, because they share the same sequence in size 19nt and 20nt and therefore share the same read numbers.

1. Flanking sequence of miR156

Sequences highlighted in gray are mature miRNA, which is 20nt. The position of the A (adenine, red letters highlighted in yellow) right after the 3' end of the 20-nt miRNA is tantamount to the last nucleotide of a normal 21-nt miRNA. This signature is common in *MIR156* families among plants, which is quite special because the sequence outside the mature miRNA duplex usually varies. The C (cytosine, red letters highlighted in green) after the A also appears frequently. These conserved features are potential factors contributing to the shortening of 21- to 20-nt miRNAs.

```
aly-MIR156a     5'--- AAA-CUGACAGAAGAGAGUGAGCACACAAAGG ---3'
aly-MIR156b     5'--- AAAACUGACAGAAGAGAGUGAGCACAUGCAGG ---3'
aly-MIR156c     5'--- AAA-CUGACAGAAGAGAGUGAGCACACAAAGG ---3'
aly-MIR156d     5'--- GAAGUUGACAGAAGAGAGUGAGCACACAAAGG ---3'
aly-MIR156e     5'--- GGAGGUGACAGAAGAGAGUGAGCACACAUGGU ---3'
aly-MIR156f     5'--- GAUGGUGACAGAAGAGAGUGAGCACACAUGGU ---3'
aly-MIR156g     5'--- GAAGGCGACAGAAGAGAGUGAGCACACAUGGC ---3'
aly-MIR156h     5'--- AAUGUUGACAGAAGAAAGAGAGCACA-ACCUG ---3'
ath-MIR156a     5'--- AAA-CUGACAGAAGAGAGUGAGCACACAAAGG ---3'
ath-MIR156b     5'--- AAAACUGACAGAAGAGAGUGAGCACAUGCAGG ---3'
ath-MIR156c     5'--- AAA-CUGACAGAAGAGAGUGAGCACACAAAGG ---3'
ath-MIR156d     5'--- GAAGUUGACAGAAGAGAGUGAGCACACAAAGG ---3'
ath-MIR156e     5'--- GGAGGUGACAGAAGAGAGUGAGCACACAUGGU ---3'
ath-MIR156f     5'--- GAUGGUGACAGAAGAGAGUGAGCACACAUGGU ---3'
ath-MIR156g     5'--- GAAGGCGACAGAAGAGAGUGAGCACACAUGGC ---3'
ath-MIR156h     5'--- AAUGUUGACAGAAGAAAGAGAGCACA-ACCUG ---3'
csi-MIR156      5'--- GCUACUGACAGAAGAGAGUGAGCACA-CGCAG ---3'
gma-MIR156a     5'--- GAGGCUGACAGAAGAGAGUGAGCACAUGCUAG ---3'
gma-MIR156b     5'--- CAUGUUGACAGAAGAGAGAGAGCACA-ACCCG ---3'
gma-MIR156g     5'--- UUUGUUGACAGAAGAUAGAGAGCACA-GG-UG ---3'
mtr-MIR156b     5'--- GGAGGUGACAGAAGAGAGUGAGCACACAUGGU ---3'
mtr-MIR156c     5'--- GGUGGUGACAGAAGAGAGUGAGCACACAUGGU ---3'
mtr-MIR156d     5'--- GAAAUUGACAGAAGAGAGUGAGCACAUAGAC- ---3'
mtr-MIR156i     5'--- GAUAUUGACAGAAGAGAGUGAGCACA-UGCUG ---3'
osa-MIR156a     5'--- GAGGGUGACAGAAGAGAGUGAGCACACGUGGU ---3'
osa-MIR156b     5'--- AGGUCUGACAGAAGAGAGUGAGCACACACGGU ---3'
osa-MIR156c     5'--- GAGGCUGACAGAAGAGAGUGAGCACACAUGGU ---3'
osa-MIR156d     5'--- GAGAUUGACAGAAGAGAGUGAGCACACGGCGU ---3'
osa-MIR156e     5'--- CGAGGUGACAGAAGAGAGUGAGCACACGGCCG ---3'
osa-MIR156f     5'--- --AGUUGACAGAAGAGAGUGAGCACACAGCGG ---3'
osa-MIR156g     5'--- --GGCUGACAGAAGAGAGUGAGCACACAGCGG ---3'
osa-MIR156h     5'--- AUUGUUGACAGAAGAGAGUGAGCACACGGCGC ---3'
osa-MIR156i     5'--- ---GGUGACAGAAGAGAGUGAGCACACGGCCG ---3'
osa-MIR156j     5'--- AUUGUUGACAGAAGAGAGUGAGCACACGGCGC ---3'
osa-MIR156k     5'--- AGUGAUGACAGAAGAGAGAGAGCACA-ACCCG ---3'
osa-MIR156l     5'--- GGAGCCGACAGAAGAGAGUGAGCAUAUAUAGU ---3'
ppt-MIR156a     5'--- GGGAGUGACAGAAGAGAGUGAGCACAGCUGAG ---3'
```

```
ppt-MIR156b     5'--- GGGAGUGACAGAAGAGAGUGAGCACGGUUGCG ---3'
ppt-MIR156c     5'--- GGGAGUGACAGAAGAGAGUGAGCACAGUUGAG ---3'
smo-MIR156a     5'--- UGGC--GACAGAAGAGAGUGAGCACACGGGGC ---3'
vvi-MIR156a     5'--- -AUGUUGACAGAAGAGAGGGAGCACA-ACCCA ---3'
vvi-MIR156b     5'--- AAAACUGACAGAAGAGAGUGAGCACACAGAGG ---3'
vvi-MIR156c     5'--- AUAGGUGACAGAAGAGAGUGAGCACACAUGGU ---3'
vvi-MIR156d     5'--- GAGACUGACAGAAGAGAGUGAGCACAUGCAGG ---3'
vvi-MIR156e     5'--- GUAGGUGACAGAGGAGAGUGAGCACUCAUGGU ---3'
zma-MIR156a     5'--- CUAACUGACAGAAGAGAGUGAGCACACAGCGG ---3'
zma-MIR156b     5'--- AGGUUUGACAGAAGAGAGUGAGCACACACGGU ---3'
zma-MIR156c     5'--- AAAGCUGACAGAAGAGAGUGAGCACACAUGGU ---3'
zma-MIR156d     5'--- GAGAUUGACAGAAGAGAGUGAGCACACGGCGC ---3'
zma-MIR156e     5'--- CGCGGUGACAGAAGAGAGUGAGCACACGGCCG ---3'
zma-MIR156f     5'--- GUGGCUGACAGAAGAGAGUGAGCACGCAUCGG ---3'
zma-MIR156g     5'--- --GGCUGACAGAAGAGAGUGAGCACGCAUCGG ---3'
zma-MIR156h     5'--- CGCGGUGACAGAAGAGAGUGAGCACACGGCCG ---3'
zma-MIR156i     5'--- GGCGGUGACAGAAGAGAGUGAGCACACGGCCG ---3'
zma-MIR156j     5'--- AGCGAUGACAGAAGAGAGAGAGCACA-ACCCA ---3'
zma-MIR156k     5'--- GAGAUUGACAGAAGAGAGCGAGCACCUGGCGC ---3'
zma-MIR156l     5'--- ----GUGACAGAAGAGAGUGAGCACACGGCCG ---3'
```

2. Flanking sequence of miR156*

Sequences highlighted in gray are mature miRNA*, which is also 1-nt shortened than expected. The position of the C (cytosine, red letters highlighted in yellow) is complementary to the A on the other strand, which forms an A-C mismatch. The G (guanine, red letters highlighted in green) pairing with the C on the other strand also appears frequently. In this study, the A-C mismatch is mutated into the G-C Watson-Crick perfect match, to test the possibility of this signature contributing to symmetric shortening of miRNA.

```
aly-MIR156a     5'--- CUUGCGUGCUCACUGCUCUUUCUGUCA-GAUUCCG ---3'
aly-MIR156b     5'--- -GUGCGUGCUCACCUCUCUUUCUGUCA-GUUGCUU ---3'
aly-MIR156c     5'--- CUUGCGUGCUCACUGCUCUAUCUGUCA-GAUUUCG ---3'
aly-MIR156d     5'--- UUUGCGUGCUCACUC-UCUUUCUGUCAUAACUUCU ---3'
aly-MIR156e     5'--- UAUGUGUGCUUACU-CUCUCUCUGUCACCCC-UUC ---3'
aly-MIR156f     5'--- UAUGUGUGCUCACU-CUCUAUCUGUCACCCCCUUC ---3'
aly-MIR156g     5'--- UCUGCGUGCUUACU-CUCUUCUUGUCUCCUCCUGC ---3'
aly-MIR156h     5'--- GGAGUGUGCUCUCUUUCCUU-CUGCCACCAUCAUC ---3'
ath-MIR156a     5'--- CUUGCGUGCUCACUGCUCUUUCUGUCA-GAUUCCG ---3'
ath-MIR156b     5'--- -GUGCGUGCUCACCUCUCUUUCUGUCA-GUUGCCU ---3'
ath-MIR156c     5'--- CUUGCGUGCUCACUGCUCUAUCUGUCA-GAUUCCG ---3'
ath-MIR156d     5'--- UUUGCGUGCUCACUC-UCUUUUUGUCAUAACUUCU ---3'
ath-MIR156e     5'--- UAUGUGUGCUUACU-CUCUCUCUGUCACCCC-U-- ---3'
ath-MIR156f     5'--- UAUGUGUGCUCACU-CUCUAUCCGUCACCCCCUUC ---3'
ath-MIR156g     5'--- UCUGCGUGCUUACU-CUCUUCUUGUCUCCUGCUCU ---3'
ath-MIR156h     5'--- GGAGUGUGCUCUCUUUCCUU-CUGCCACCAUCAUU ---3'
csi-MIR156      5'--- GGUGCGUGCUCGCUCCUCUU-CUGUCAGCGUCAUU ---3'
gma-MIR156a     5'--- --UGCGUGCUCACUUCUCUAUCUGUCA-GCUUCCC ---3'
gma-MIR156b     5'--- GGAGUGUGCCCUCUCUUCCU-CUGUCAUCAUCACA ---3'
gma-MIR156g     5'--- GUUUUGUGCUCUCUAU-CUU-CUGUCAAUGUACUU ---3'
mtr-MIR156b     5'--- UAUGUGUGCUCACU-CUCUAUCUGUCACCCC-AU- ---3'
mtr-MIR156c     5'--- UAUAUGUGCUUACU-CUCUAUCUGUCACCCC-AC- ---3'
mtr-MIR156d     5'--- UUUGCGUGCUCACUCAUCUUUCUGUCA-AAUUCC- ---3'
mtr-MIR156i     5'--- GGUGCGUGCUCACUUCUCUUUCUGUCAUCUUAU-- ---3'
osa-MIR156a     5'--- UACGCGUGCUCACUUCUCUCUCUGUCACCUCC--- ---3'
osa-MIR156b     5'--- UGUGCGUGCUCACU-CUCUAUCUGUCAGCCGUUCA ---3'
osa-MIR156c     5'--- UAUGUGUGCUCACUUCUCUCUCUGUCAGCCA-UUU ---3'
osa-MIR156d     5'--- GCCGCGUGCUCACUCCUCUUUCUGUCACCCUCUUU ---3'
osa-MIR156e     5'--- GCCGCGUGCUCACUGCUCUUUCUGUCAUCCGGUGC ---3'
```

```
osa-MIR156f     5'--- GCUGCGUGCUCACUUCUCUUUCUGUCAGCU----- ---3'
osa-MIR156g     5'--- GUUGCGUGCUCACUUCUCUCUCUGUCAGCU----- ---3'
osa-MIR156h     5'--- GCCGCGUGCUCGCUCCUCUUUCUGUCAGCAUCUC- ---3'
osa-MIR156i     5'--- GCCGCGUGCUCACUGCUCUGUCUGUCAUC------ ---3'
osa-MIR156j     5'--- GCCGCGUGCUCGCUCCUCUUUCUGUCAGCAUCUCU ---3'
osa-MIR156k     5'--- GCCGUGUGCUCUCUGAUCUAUCUGUCAUUGCCGUC ---3'
osa-MIR156l     5'--- UAUAUGUGCUCACUUCUCUUUCUGUCAGCAAAUUA ---3'
ppt-MIR156a     5'--- CAUGUGUGCUCACU-CUCUUCCUGUCGCACCUCUC ---3'
ppt-MIR156b     5'--- CAAGUGUGCUCACU-CUCUUCAUGUCGCGCCUCUC ---3'
ppt-MIR156c     5'--- GAAGUGUGCUCACU-CUCUUCAUGUCACGCCUCUC ---3'
smo-MIR156a     5'--- UUCGCGUGCUCACU-CUCUGUCUGUCAGCUGUGCC ---3'
vvi-MIR156a     5'--- GGAGUGUGCUCUCUCUUCUU-CUGUCAUCAUCACA ---3'
vvi-MIR156b     5'--- -UUGCGUGCUCAUUUCUCUUUCUGUCA-GCUUCCA ---3'
vvi-MIR156c     5'--- UCUGCGUGCUCACU-----CUCUAUCUGUCA-UCU ---3'
vvi-MIR156d     5'--- --UGCGUGCUCACCUCUCUUUCUGUCA-GCUUCAG ---3'
vvi-MIR156e     5'--- UCUGUGUGCUUACU-CCCUAUCUGUCACCCC-UCA ---3'
zma-MIR156a     5'--- GCUGCGUGCUCACUUCUCUCUCUGUCAGUCCUCUA ---3'
zma-MIR156b     5'--- UGUGCGUGCUCACC-CUCUAUCUGUCAGUCACUCA ---3'
zma-MIR156c     5'--- UAUGCGUGCUCACUUCUCUCUUUGUCAGCCAUUAG ---3'
zma-MIR156d     5'--- GCCGCGUGCUCACUUCUCUUUCUGUCAGCCUCUUU ---3'
zma-MIR156e     5'--- GCCGCGUGCUCACUGCUCUCUCUGUCAUCCGCUGG ---3'
zma-MIR156f     5'--- GUUGCGUGCUCACUUCUCUUUCUGUCAGCUCUCUC ---3'
zma-MIR156g     5'--- GUUGCGUGCUCACUUCUCUUUCUGUCAGCU----- ---3'
zma-MIR156h     5'--- GCCGCGUGCUCACUGCUCUUUCUGUCAUCCGCUGG ---3'
zma-MIR156i     5'--- GACGCGUGCUCACUGCUCUAUCUGUCAUCCACUCU ---3'
zma-MIR156j     5'--- GCCGUGUGCUCUCUGCUCUCACUGUCAUCGCCCAC ---3'
zma-MIR156k     5'--- GCCGCGUGCUCGCUUCUCUUUCUGUCAGCCUCUCC ---3'
zma-MIR156l     5'--- GCCGCGUGCUCACUGCUCUAUCUGUCACCC----- ---3'
```

3. Hairpin structure of *MIR156*

The hairpin structure of *MIR156* below (e.g., *ath-MIR156c*) shows the relative position of the signatures mentioned above.

```
C       AAC   AA-   A        -      -           A     ---       UU     UA
 AAGAGA   GCA   GAA CUGACAG AAGAG AGUGAGCAC  CAA    AGGCAA  UGCA   U
 UUCUCU   CGU   CUU GACUGUC UUCUC UCACUCGUG  GUU    UUCGUU  ACGU   C
U       AGU   GGC   A        U      G           C     CUC       C-     UA
```

F. Target genes of 20- and 22-nt miRNAs

1. 20nt miRNA targets

	Development Regulation	Target Gene
miR156 miR157	juvenile-to-adult stage transition[1]	***SPL/SPB***
	prevention of precocious flowering[2]	
	plastochron length and organ size[3]	
miR158	cell wall biogenesis, xyloglucan biosynthetic process	*At2g03210*, *At2g03220*
	DNA binding, RNA editing	***PPR***
miR319	leaf senescence via jasmonic acid[4]	***TCP***
	leaf morphogenetics[5]	
miR394	meristem identity (embryonic, floral and vegetative development)[6]	F-box family (*At1g27340*)

※ Information of miR158 is predicted data from MPSS (http://mpss.udel.edu/).

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2. 22nt miRNA targets

	Environmental Response	Validated Target Gene
miR167d	salinity and drought[1]	***ARF*** (Auxin Response Factor)
	nitrate response[2]	
	antibacterial resistance[3]	
miR173		***TAS1***, ***TAS2*** (targets PPR)[4]
miR393ab	antibacterial resistance[3,5]	***TIR1, AFB2, AFB3*** (antibacterial resistance by repressing auxin signaling)
	nitrate[6], salinity[1,7] and alkaline[7]	
	cold stress[1]	
miR402	salt stress[8]	***DML3*** (DNA methylation)
miR447abc	phosphate deficiency[9]	***2PGK*** (2-phosphoglycerate kinase-related)
miR472	UV-B[10]	**CC-NBS-LRR** class (disease resistance)
miR828	phosphate deficiency[11]	***TAS4*** (targets MYB, biosynthesis of anthocyanin)
miR840	antibacterial resistance[12]	***WHY3*** (whirly3) defense response
	phosphate deficiency[13]	***PIP, TIP*** (AM symbiosis in tomato)
miR856		***CHX18*** (cation/hydrogen antiporter)[14] ***ZAT1*** (Zinc transporter)[15]
miR773	antibacterial resistance[16]	***DMT2*** (DNA Methyltransferase, reducing agrobacterium-mediated tumor formation.)

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評語

這是一件在完整性，及創新性上都有傑出表現的作品。更難得的是作者對自己研究主題及相關的背景知識都瞭解得很深入。已具有大學生，甚至研究生的研究能力。是一件讓人激賞的好作品。