Identification of new microRNA-regulated genes by conserved targeting in plant species

Uciel Chorostecki, Valeria A. Crosa, Anabella F. Lodeyro, Nicolás G. Bologna, Ana P. Martin, Néstor Carrillo, Carla Schommer and Javier F. Palatnik*

IBR (Instituto de Biología Molecular y Celular de Rosario), Facultad de Ciencias Bioquímicas y Farmacéuticas, UNR, Suipacha 531, 2000 Rosario, Argentina

Received April 6, 2012; Revised June 2, 2012; Accepted June 4, 2012

ABSTRACT

MicroRNAs (miRNAs) are major regulators of gene expression in multicellular organisms. They recognize their targets by sequence complementarity and guide them to cleavage or translational arrest. It is generally accepted that plant miRNAs have extensive complementarity to their targets and their prediction usually relies on the use of empirical parameters deduced from known miRNA–target interactions. Here, we developed a strategy to identify miRNA targets which is mainly based on the conservation of the potential regulation in different species. We applied the approach to expressed sequence tags datasets from angiosperms. Using this strategy, we predicted many new interactions and experimentally validated previously unknown miRNA targets in Arabidopsis thaliana. Newly identified targets that are broadly conserved include auxin regulators, transcription factors and transporters. Some of them might participate in the same pathways as the targets known before, suggesting that some miRNAs might control different aspects of a biological process. Furthermore, this approach can be used to identify targets present in a specific group of species, and, as a proof of principle, we analyzed Solanaceae-specific targets. The presented strategy can be used alone or in combination with other approaches to find miRNA targets in plants.

INTRODUCTION

MicroRNAs (miRNAs) are ~21 nt small RNAs that are key regulators of gene expression in animals and plants [reviewed in (1)]. They are processed from larger precursors by ribonuclease type III enzymes that release the miRNA (2–5), which is subsequently assembled into ARGONAUTE (AGO)-containing complexes (1,6,7). MiRNAs recognize target mRNAs by base complementarity and control their translation and stability. Animal miRNAs have only limited complementarity to their targets, and the pairing of a seed region between nt 2 and 7 at the 5'-end of the small RNA is a key feature of their interaction [reviewed in (8)]. In plants, miRNAs have an extended homology to their targets and frequently guide them to cleavage, although they can also inhibit their translation (9).

The first miRNA targets in plants were identified by allowing a maximum of three mismatches along the miRNA–target pair (10). Further strategies refined the requirements for miRNA–target interaction in plants considering larger numbers of mismatches, their position, the existence of G-U wobbles and the minimum free energy (MFE) (11–17).

The experimental validation of a plant miRNA target usually relies on the detection of the endonucleolytic cleavage guided by the small RNA. Many AGO proteins, such as Arabidopsis AGO1 (18), cleave the target RNA between positions 10 and 11 of the miRNA 5'-end (19,20). A modified rapid amplification of cDNA ends (RACE)-polymerase chain reaction (PCR) has been designed to identify mRNA fragments that are remnant of this activity in vivo (20,21). Recently, this modified RACE-PCR has been combined with next-generation sequencing techniques allowing the systematic identification of miRNA targets in plants (22,23). Bioinformatic methods have also been developed to identify potential miRNAs guiding the cleavage of these RNAs (24,25).

Many plant miRNAs are young small RNAs that have appeared recently during evolution, although their biological role is unclear (14,26–29). In contrast, ancient plant miRNAs play relevant functions in plant biology and regulate targets whose miRNA-binding sites are also conserved during evolution (30,31). This conservation, specially between Arabidopsis and rice, has been used to...
support the prediction of targets based on empirical approaches (10,13,17); however, it has not been fully exploited to identify new targets. Furthermore, it has been recently shown that the regulation of a target present only in species related to Arabidopsis thaliana by an ancient miRNA has biological significance (32).

In this study, we present an alternative approach to identify miRNA-regulated transcripts in plants based on conserved targeting of homologous genes present in large expressed sequence tags (EST) datasets of different species. Using this strategy, we found many potential miRNA–target interactions and experimentally validated new targets in A. thaliana. Furthermore, we were able to identify potential miRNA–target interactions that are specific to a group of related species and validated two of them. This approach represents a novel strategy to search for miRNA targets in plants.

MATERIALS AND METHODS

MiRNA consensus

The 22 conserved miRNA families in angiosperms were considered for our studies (14,30). MiR319 and miR159, which encode similar miRNAs, were considered as different families because they regulate different targets (33). We considered all members of these families, obtained from miRBASE 18.0 (http://mirbase.org/) of A. thaliana, Populus trichocarpa and Oryza sativa. Variations at positions 1, 20, and 21 are quite common among miRNA family members (32). A consensus was then defined as the most common sequence (positions 2–19) of the different members of each family. Note that the same results were obtained in the three species.

MiRNA target prediction

Plant datasets

Sequence data were extracted from libraries from the Gene Index project (http://compbio.dfci.harvard.edu/tgi/), which consist of assemblies of ESTs. We selected datasets belonging to angiosperms (see Supplementary Table S2). We also used the miRNA sequences of A. thaliana (http://arabidopsis.org) and O. sativa (http://rice.plantbiology.msu.edu/).

Target search was performed using PatMatch (34), which allows ambiguous characters, mismatches, insertions and deletions. We searched for potential targets with three mismatches to the miRNA consensus, while G:U wobbles and bulges were considered as mismatches. To perform the alignment of the miRNA–target pair, we developed an implementation of the Needleman–Wunsch dynamic programming algorithm (35) in Perl (http://www.perl.org/). Modules using BLASTX (36) against the Arabidopsis proteome and RNA hybrid (37) were integrated by developing in-house scripts.

Filters

Candidate sequences were labeled with the locus ID of the best hit in Arabidopsis using the BLASTX module (E-value cutoff of $10^{-5}$) as a tag. Genes from different species having the same tag were grouped together as they have the same homolog gene in A. thaliana. The evolutionary conservation filter referred to the minimum number of species where the same tag was present for a particular miRNA. The empirical filter was based on previous work (38) and referred to the energy of the interaction (MFE at least 72% of the perfect match) and that only one mismatch is allowed between positions 2 and 12 of the miRNA (1–11 of our modified search with the consensus sequences).

Controls

As a control, we performed the same search using shuffled miRNA sequences. For each miRNA, we generated 20 random sequences shuffling the dinucleotide composition as described previously (13). From these 20 random sequences, we chose 10 with the most similar number of total targets to the real miRNA. The signal-to-noise ratio was calculated as the relation between the number of targets for the miRNAs and the average number obtained for the shuffled sequences. As another source of control, we selected two miRNAs not conserved during evolution, miR158 and miR173.

Plant material

Arabidopsis ecotype Col-0 was used for all experiments. Plants were grown in long days (16 h light/8 h dark) at 23°C. Nicotiana tabacum (cv Petit Havana) plants were grown in long days during 8 weeks and the second leaf was used for RNA analysis.

Cleavage site mapping of target mRNA and expression analysis

Poly(A)+ RNA was extracted from 50 μg of total RNA of Col-0 seedlings using PolyAT tract kit (Promega). Ligation of an RNA adaptor, reverse transcription and 5′ RACE were performed as described before (33). Two nested gene-specific reverse oligonucleotides were used for 5′ RACE. The PCR products were resolved on 2% agarose gels and detected by ethidium bromide staining. Real-Time quantitative PCR (RT–qPCR) for miR396 and miR159 targets was performed as described before (33,39). Lists of primers used for these assays are described in Supplementary Tables S7 and S8. Plants overexpressing miR396 and miR159 have been described previously (33,39).

RESULTS AND DISCUSSION

Design of an approach to identify plant miRNA targets by sequence conservation

We focused our analysis on 22 miRNAs that are conserved in angiosperms (27,30,40,41) (Table 1). In general, these miRNAs are encoded by small gene families of up to 32 members. In the fully sequenced genomes of Arabidopsis, poplar and rice, it is common to find variations in the sequence of miRNAs belonging to the same family, especially in the first, 20th and 21st positions (Supplementary Table S1) (32).

However, we observed that the region between positions 2 and 19 is quite conserved and we could find a consensus...
The search could also be performed in combination with empirical rules of miRNA targeting, which take into account the energy of interaction and the position of the mismatches (see Materials and Methods). Of the initial 38,597 targets, 9375 passed this filter (Figure 1, bin 4). Combination of the empirical and evolutionary filter selected 1563 genes corresponding to 146 tags (Figure 1, bin 5).

Sequence conservation and empirical parameters can act synergistically to identify miRNA targets

Potential miRNA targets were classified according to the minimum number of species in which they were detected (Figure 2A–E). As a control, we also generated random miRNAs shuffling the 18 nt miRNA consensus sequences (10 random sequences per miRNA). These randomized miRNAs were used to search for potential targets as was performed for the bona fide small RNAs (Figure 2A–E). The signal-to-noise ratio was calculated as the relation between the number of targets for the miRNAs and the average number obtained for the shuffled sequences (Figure 2, insets). The ratio was 1.2 for all miRNA targets without requesting any conservation and steadily increased with the number of species in which they were detected (Figure 2, insets). The ratio was 1.2 for all miRNA targets without requesting any conservation and steadily increased with the number of species in which they were detected (Figure 2, insets). The ratio was 1.2 for all miRNA targets without requesting any conservation and steadily increased with the number of species in which they were detected (Figure 2, insets).
their potential targets conserved in at least four species are included in Table 2.

Next, we studied the selection of target candidates by empirical parameters. To do this, we applied a modified version of the filters described before and requested (i) a minimum free energy (MFE) of at least 72% of the perfect match of each 18 nt consensus and (ii) that only one mismatch was present between positions 1 and 11 of the consensus (2–12 of the miRNA). Of the initial search, 9375 genes passed this filter containing 97% of the validated Arabidopsis targets (Figure 1, bin 4).

The application of this empirical filter alone gave a signal-to-noise ratio of 1.7 when grouping all miRNAs together (Figure 2A). We observed that the simultaneous application of the empirical and conservation filters significantly increased the signal-to-noise ratio for the group of all miRNAs (Figure 2A, inset) and in individual miRNAs as well (Figure 2B–E, insets) (see also Table 2). In many cases, this ratio reached above 10 when it was requested that the targets were present in more than five species and that they pass the empirical filters (Figure 2A–D). These synergistic effects indicate that the evolutionary conservation filter and the empirical parameters might be selecting different aspects of a miRNA target interaction.

We observed that the number of target candidates and the signal-to-noise ratio varied among the different miRNAs. MiR396 had the highest number of potential targets, 92 of them being present in at least four species and 26 of them also passed the empirical filter (Table 2; Figure 2B). MiR408 and miR398 also had high numbers of potential targets and favorable signal-to-noise ratios (Figure 2C–D).

In contrast, certain miRNAs such as miR162, miR168 and miR399 had only one potential target conserved in at least four species according to our search (Table 2; Figure 2E). At least in the case of miR162 and miR168, this result might reflect their specific roles in the feedback regulation of miRNA biogenesis, as they control DCL1 and AGO1 expression levels, respectively (43,44).

As an additional control to our strategies, we searched for targets of miR158 and miR173, which are miRNAs present only in A. thaliana and closely related species (27). As expected, these miRNAs did not generate more candidate targets than their shuffled versions (Table 2; Figure 2F).

Then, we tested whether conserved miRNA–target pairs have a stronger interaction than those present in few species. To do this we calculated the MFE for each interaction detected in our assay. We observed that miRNA–target pairs present in many species tend to have stronger interaction energy than those present in only few (Figure 3A). However, the correlation was not striking and some conserved miRNA–target interactions had a low MFE (Figure 3A). These results show that a high conservation might not necessarily be equivalent to a strong interaction which might provide an explanation for the synergistic effects caused by the evolutionary and empirical filters on the signal-to-noise ratios.

Identification of new miRNA targets in A. thaliana by sequence conservation

To search for new targets, we focused on the potential targets selected only by sequence conservation, as the empirical parameters have been extensively used before [e.g., (11,13,38)]. First, we analyzed the detection of targets previously validated in A. thaliana [based on (14)] using our strategy and found that 84% of them were still present in at least four species (Figure 3B). We considered these results a good outcome as not all Arabidopsis targets might be evolutionarily conserved.

As plant miRNAs usually regulate genes coding for proteins of the same family, we evaluated whether at least one member of each family was detected in our approach. We found targets belonging to nearly all conserved protein-coding gene families present in at least four species (Figure 3B, Supplementary Table S4), with the exception of the miR390-regulated TAS3, which, being a non-coding RNA, is not detected by BLASTX.
To search for new miRNA-regulated genes, we focused on potential targets with miRNA-binding sites conserved in at least four species, *A. thaliana* being one of them (Figure 1, bin 3). MiRNA targets not present in *A. thaliana* might include genes that have lost their regulation during evolution or genes that gained control by a conserved miRNA more recently in other species. Conservation in four species was chosen as an evolutionary filter because it provided a good sensitivity for known targets.

We identified 114 potential targets that fulfill these criteria (Supplementary Table S4). That included 76 previously described targets or closely related genes (Figure 3C, Supplementary Table S3 and S4). Interestingly, there were 38 genes unrelated to known miRNA targets (Supplementary Table S4), and we decided to study this group in more detail. We focused first on genes present in a large number of species, as we would have a better specificity (Figure 2), and tried to validate the predicted miRNA-guided cleavage using the modified 5’ RACE PCR (20, 21).

MiR408 was potentially targeting At5g21930, encoding P-TYPE ATPase OF ARABIDOPSIS 2 (PAA2), and was found in 22 different species including dicots and monocots (Supplementary Table S4). MiR408 is unusual as it has a 5’-A; however, >30% of the mature miR408...
sequences correspond to a shifted variant starting with 5'-U (45) (Figure 4A). The experimental validation revealed miRNA fragments compatible with this latter cleavage site (Figure 4A). PAA2 is necessary for the transport of copper ions to PLASTOCYANIN (46), and its regulation by miR408 is related to the role of this protein in copper homeostasis (47).

Another miR408 target candidate was At3g22110 that encodes PROTEASOME ALPHA SUBUNIT C1 (PAC1), present in 20 species. PAC1 encodes PROTEASOME ALPHA SUBUNIT C1, which participates in plastocyanin (46), and its regulation by miR408 is related to the role of this protein in copper homeostasis (47).

Table 2. Detection of miRNA targets using different filters

| miRNA  | RND | Ratio | miRNA  | RND | Ratio | miRNA  | RND | Ratio | miRNA  | RND | Ratio |
|--------|-----|-------|--------|-----|-------|--------|-----|-------|--------|-----|-------|
| mir136 | 3915 | ±150  | 1.0    | 890 | 705   | ±45    | 1.3  | 34    | 40    | ±3.1 | 0.9   |
| mir159 | 1663 | ±148  | 1.3    | 472 | 255   | ±22    | 1.9  | 20    | 10    | ±1.1 | 2.0   |
| mir160 | 793  | ±30   | 1.1    | 277 | 158   | ±29    | 1.8  | 5     | 4     | ±0.9 | 1.1   |
| mir162 | 1191 | 930   | ±140  | 108 | 165   | ±24    | 0.7  | 18    | 14    | ±3.5 | 1.3   |
| mir164 | 2486 | 1480  | ±60   | 1.7  | 678  | 333    | ±32  | 2.0   | 39    | 12    | ±1.9  |
| mir166 | 879  | 816   | ±45   | 1.1  | 231  | 129    | ±14  | 1.8   | 16    | 11    | ±1.4  |
| mir167 | 1777 | 1364  | ±147  | 1.3  | 478  | 215    | ±28  | 2.2   | 22    | 20    | ±3.6  |
| mir168 | 962  | 798   | ±48   | 1.2  | 209  | 185    | ±14  | 1.1   | 6     | 4     | ±0.8  |
| mir169 | 1540 | 1477  | ±70   | 1.5  | 464  | 181    | ±16  | 2.6   | 26    | 11    | ±2.1  |
| mir171 | 884  | 732   | ±12   | 1.8  | 202  | 114    | ±13  | 2.2   | 7     | 7     | ±1.4  |
| mir172 | 3007 | 1694  | ±125  | 1.8  | 540  | 288    | ±40  | 1.9   | 34    | 18    | ±1.7  |
| mir173 | 1367 | 1274  | ±114  | 1.1  | 324  | 249    | ±22  | 1.3   | 18    | 15    | ±2.8  |
| mir183 | 397  | 814   | ±64   | 1.1  | 335  | 173    | ±22  | 1.9   | 8     | 5     | ±1.2  |
| mir193 | 988  | 845   | ±59   | 1.2  | 276  | 125    | ±11  | 2.2   | 14    | 7     | ±1.2  |
| mir194 | 1569 | 1531  | ±57   | 1.0  | 188  | 237    | ±25  | 0.8   | 26    | 21    | ±2.2  |
| mir195 | 1472 | 1227  | ±67   | 1.2  | 426  | 218    | ±16  | 2.0   | 11    | 9     | ±1.3  |
| mir196 | 4641 | 2979  | ±247  | 1.6  | 1246 | 391    | ±39  | 3.2   | 92    | 51    | ±5.9  |
| mir197 | 1426 | 1051  | ±28   | 1.4  | 368  | 237    | ±23  | 1.6   | 26    | 10    | ±0.8  |
| mir198 | 935  | 834   | ±35   | 1.1  | 376  | 144    | ±18  | 2.6   | 11    | 8     | ±1.6  |
| mir199 | 1192 | 1138  | ±72   | 1.0  | 275  | 208    | ±25  | 1.3   | 5     | 14    | ±1.7  |
| mir200 | 2782 | 2503  | ±104  | 1.1  | 695  | 469    | ±51  | 1.5   | 51    | 35    | ±3.0  |
| mir201 | 2261 | 2000  | ±120  | 1.1  | 317  | 297    | ±45  | 1.1   | 44    | 22    | ±1.9  |
| mir202 | 3197 | 3012  | ±1860 | 1.2  | 9375 | 5473   | ±576 | 1.7   | 533   | 348   | ±47.0 |
| Control |      |       |       |     |       |       |       |       |       |       |       |
| mir158 | 1364 | 1463  | ±69   | 0.9  | 170  | 208    | ±16  | 0.8   | 15    | 16    | ±1.7  |
| mir173 | 1386 | 1232  | ±101  | 1.1  | 243  | 216    | ±23  | 1.1   | 11    | 12    | ±2.4  |

aNo filter, initial search using an 18 nt miRNA consensus sequence and three mismatches.
bEmpirical filter, an interaction energy of at least 72% of the perfect interaction and 1 mismatch in the 2–12 miRNA-target region.
cConservation of the ID tag in at least four species. Note that different genes with the same ID tag give only one hit, so that the total numbers of hits is reduced by this filter.
dAll filters, combination of the empirical and conservation filters in at least four species.

These two targets had stronger interaction energies than SVP and SU1. In these two cases, we successfully detected miR396-guided cleavage (Figure 4C and D). Determination of MMG4.7 and FLU transcript levels in 35S:miR396 plants revealed a significant decrease of FLU and a minor effect on MMG4.7 (Supplementary Figure S1).

In contrast to miR408 and miR396, which had several potential targets, miR159 hits were all but one MYB transcription factors, which regulate stamen and pollen development (48). The additional target was At4g27330, also known as NOZZLE/SPOROCYTELESS. This transcription factor, which participates in stamen and ovule development (49,50), was also validated by 5'RACE-PCR (Figure 4E). In good agreement, 35S:miR159 caused a reduction of both MYB and NOZZLE transcript levels (Supplementary Figure S2). A miR159 target with a NOZZLE-like domain has been also recently validated in tomato (51), which together with our results point toward a general role of miR159 in the regulation of NOZZLE-like genes. Interestingly, at least the functions of already described targets of miR159 and miR408, SHORT VEGETATIVE PHASE (SVP) and the eukaryotic translation initiation factor SUI1, were present in 29 and 19 species, respectively, as potential targets of miR396 (Supplementary Table S4). In both cases, however, we failed to obtain a PCR product using the modified 5'RACE (not shown). The lack of regulation by miR396 might be related to the weak MFE of these miRNA–target pairs, although we cannot rule out that miR396 is controlling their translation.

Two other potential miR396 targets were At5g43060 and At3g14110 that encode the protease MMG4.7 and FLUORESCENT IN BLUE LIGHT (FLU), respectively.
present only in dicots (Figure 4A–E). Positions in the miRNA-binding sites were highly conserved, and many of the variable positions corresponded to mismatches in the interaction with the miRNA or alternating G-C/G-U wobbles. Moreover, this method does not require that the sequence of the target site is conserved, but rather that there is a predicted interaction with the miRNA in different species. This way, the target site of NOZZLE, which changes in sequence among species (Figure 4E), could be found by this approach.

**Identification of new potential targets allowing GU wobbles**

The targets identified here had several mismatches and bulges with their cognate miRNAs, which might explain why they were missed by previous approaches. We also noticed that many of the new miRNA–target interactions contained positions which were alternating between G-C and G-U in different species (Supplementary Figure S3). As we considered G-U as a mismatch in our initial search, we decided to perform another search for targets of the 18-nt miRNA consensus allowing four mismatches, with at least one of them being a G-U wobble. This search would allow miRNA–target interactions with only 14 matches.

To compensate the use of these relaxed parameters in terms of mismatches, we requested that the target should appear in at least 10 different species to increase the specificity (Figure 5A). We found 125 potential targets in A. thaliana that fulfill these criteria (Figure 5A, Supplementary Table S5) and 34 of them did not appear in our previous searches. The miR398 target CSD2 that was missing in our first approach was detected with these parameters.

We next screened the latter group for potential miRNA-regulated genes that were performing ancillary functions to the targets already described for each miRNA. We found that miR167 that regulates AUXIN RESPONSE FACTORS (ARFs), was potentially targeting At1g51760, IAA-ALANINE RESISTANT 3 (IAR3) (Figure 5B and C), which is involved in the control of free auxin levels (52,53).

The Arabidopsis IAR3 has three mismatches with respect to miR167, but at position 12 of this miRNA–target interaction there is a G-U wobble in several species (Figure 5B and C). Modified 5′RACE-PCR confirmed that the gene was actually a target of miR167 (Figure 5C).

**Identification of Solanaceae-specific target genes**

We reasoned that the strategy presented here might also be used to find targets present specifically in a group of related species. We therefore tested whether we could find potential miRNA targets specific of the Solanaceae family. We chose this particular family because six species were well represented in the analyzed libraries. The relation between the target of miRNAs and their scrambled sequences was more than two when the empirical or the conservation filters (at least three of the six Solanaceae species) were applied (Figure 6A). Interestingly, the combination of the two filters resulted in a signal-to-noise ratio above 6 (Figure 6A), confirming our previous findings that both filters enhance the detection of miRNA targets.

We found 132 potential target genes present in at least three Solanaceae species. Of this group, 41 targets were not detected in other species (Figure 6B, Supplementary Table S6). The most common target was the metallothionein MT2A, which was present in all six Solanaceae species as a potential target of miR398, while MT2B, a homolog of this gene, was present in five species (Figure 6B–D, Supplementary Table S6).

Then, we took advantage of transgenic tobacco plants harboring a 35S:miR398 transgene (A.F.Lodeyro, N.Carrillo and J.F.Palatnik, unpublished results) and tested the expression of these genes. We found that...
Figure 4. Newly validated miRNA targets in *Arabidopsis thaliana*. The alignments between the miRNAs and their newly identified targets are depicted on the left. The sequence conservation of the miRNA target site in selected species is shown on the right. The figure shows the interaction of miR408 with *PAA2* (A); miR408 with *PAC1* (B); miR396 with *MMG4.7* (C); miR396 with *FLU* (D); miR159 with *NOZZLE* (E). The arrows point the position of cleavage sites as determined by 5’ RACE-PCR and the numbers indicate the cloning frequency of each fragment (21).
CSD2, a broadly conserved target of miR398, decreased its expression >10-fold in 35S:miR398 transgenic plants compared with wild type (Figure 6E). Interestingly, we found that both MT2A and MT2B decreased their transcript levels >5 times in these plants (Figure 6E). These results are in agreement with the regulation of MT2A and MT2B by miR398, although they do not necessarily prove a direct interaction. Altogether, these results show that miRNA targets present in a specific group of species might be found by this strategy.

CONCLUSIONS

Here, we designed a strategy to identify miRNA-regulated genes that is mainly focused on the conservation of the potential targeting. The approach requests that the miRNA targeting should be able to occur in the context of a minimum set of interacting parameters in different species. Therefore, the sequence of the target itself does not need to be conserved. Furthermore, our approach allows adjusting the number of species requested as a filter to search with different sensitivities and signal-to-noise ratios.

Using this strategy we identified and experimentally validated new targets in A. thaliana, even though this system has already been studied in detail by several different genome-wide approaches (11,13,22,23,28,38). Three new validated targets contain bulged nucleotides. Empirical parameters have usually given a strong penalty to them, which could even be double of regular mismatches (13); however, it is possible that target sites with asymmetric bulges are more frequent than previously thought in plants.

We found that newly validated targets have functions related to those already known. MiR159 regulates MYB transcription factors (33,54,55) and NOZZLE (this work), which are involved in stamen and pollen development (48–50,55). MiR408 regulates the copper transporter PAA2 (this work) as well as copper-binding proteins (13,23,38,56). MiR167 regulates ARFs (10,57) and IAR3 (this work), and both of them participate in the control of auxin levels and activity (52,58). These results confirm the importance of miRNA regulation in plants, further indicating that a miRNA might be regulating different components of a biological pathway.

The approach offers an alternative strategy to other predictions based on empirical parameters of known miRNA–target pairs (11,13,15,38). An advantage of the strategy presented here is that conserved miRNA–target interactions might be likely involved in relevant biological processes. Furthermore, the approach could be easily modified to incorporate data from other expression libraries, and/or search for targets only present in a specific group of plant species.
SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–8 and Supplementary Figures 1–3.

ACKNOWLEDGEMENTS

We thank Edgardo Bresso, Juan Debernardi and other members of JP lab for continuous help and discussions.

FUNDING

Argentinean National Agency of Science and Technology (PICT) and an international grant of HHMI [to J.P.] and fellowships of CONICET [to V.A.C., N.G.B., A.P.M., C.S., A.F.L., N.C. and J.F.P.]. Funding for open access charge: Howard Hughes Medical Institute.

Conflict of interest statement. None declared.

REFERENCES

1. Voinnet, O. (2009) Origin, biogenesis, and activity of plant microRNAs. Cell, 136, 669–687.
2. Mateos, J.L., Bologna, N.G., Chorostecki, U. and Palatnik, J.F. (2010) Identification of microRNA processing determinants by random mutagenesis of Arabidopsis MIR172a precursor. Curr. Biol., 20, 49–54.
3. Bologna, N.G., Mateos, J.L., Bresso, E.G. and Palatnik, J.F. (2009) A loop-to-base processing mechanism underlies the biogenesis of plant microRNAs miR319 and miR159. Enso. J., 28, 3646–3656.

Figure 6. Identification of Solanaceae-specific miRNA targets. (A) Prediction of miRNA targets in five Solanaceae species. The number of targets for all conserved miRNAs is indicated after the application of several filters. Targets obtained by the randomized sequences are also indicated. (B) Scheme showing the strategy to identify miRNA targets specific of Solanaceae species. (C) Conservation of the miR398 target site in MT2A sequences from Solanaceae species. (D) Scheme showing the miR398 binding site in tobacco MT2A and MT2B. (E) Transcript levels of CSD2, MT2A and MT2B in wild-type and transgenic tobacco plants (cv. Petit havana) overexpressing miR398. The data shown are mean ± s.e.m. of three biological replicates.
26. Fahlgren, N., Howell, M.D., Kasschau, K.D., Chapman, E.J., Baumberger, N. and Baulcombe, D.C. (2005) Arabidopsis
18. Baumberger, N. and Baulcombe, D.C. (2005) Arabidopsis
16. Alves, L. Jr, Niemeier, S., Hauenschild, A., Rehmsmeier, M. and Allen, E., Xie, Z., Gustafson, A.M. and Carrington, J.C. (2005) Computational
14. Fahlgren, N., Jogdeo, S., Kasschau, K.D., Sullivan, C.M., Jones-Rhoades, M.W. and Bartel, D.P. (2004) Computational
13. Jones-Rhoades, M.W. and Bartel, D.P. (2004) Computational
11. Allen, E., Xie, Z., Gustafson, A.M. and Carrington, J.C. (2005) microRNA-directed phases during trans-acting siRNA biogenesis in plants. Cell, 121, 207–221.
12. Schwab, R., Ossowski, S., Riester, M., Warthmann, N. and Weigel, D. (2006) Highly specific gene silencing by artificial microRNAs in Arabidopsis. Plant Cell, 18, 1121–1133.
10. Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B. and Bartel, D.P. (2002) Prediction of plant microRNA targets. Cell, 110, 513–520.
9. Brodersen, P. and Voinnet, O. (2009) Revisiting the principles of microRNA target recognition and mode of action. Nat. Rev. Mol. Cell Biol., 10, 141–148.
8. Rajewsky, N. (2006) microRNA target predictions in animals. Nat. Genet., 38(Suppl.), S8–S13.
7. Axtell, M.J., Westholm, J.O. and Lai, E.C. (2011) Vive la difference: comparative analysis of the Arabidopsis degradome. Curr. Biol., 21, 1750–1756.
6. Mallory, A.C., Reinhart, B.J., Jones-Rhoades, M.W., Tang, G., Zamoere, P.D., Barton, M.K. and Bartel, D.P. (2004) MicroRNA control of PHABULOSA in leaf development: importance of regional differences underlie functional specialization of Arabidopsis microRNAs miR159 and miR319. Dev. Cell, 13, 115–125.
5.Needleman, S.B. and Wunsch, C.D. (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins. J. Mol. Biol., 48, 443–453.
4. Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol., 215, 403–410.
3. Mallory, A., Wollmann, H., Schommer, C., Schwab, R., Boisbouvier, J., Rodriguez, R., Warthmann, N., Allen, E., Mallory, A.C., Reinhart, B.J., Jones-Rhoades, M.W., Tang, G., Zamoere, P.D., Barton, M.K. and Bartel, D.P. (2004) Sequence and expression differences underlie functional specialization of Arabidopsis microRNAs miR159 and miR319. Dev. Cell, 13, 115–125.
2. Mallory, A.C., Reinhart, B.J., Jones-Rhoades, M.W., Tang, G., Zamoere, P.D., Barton, M.K. and Bartel, D.P. (2004) MicroRNA control of PHABULOSA in leaf development: importance of regional differences underlie functional specialization of Arabidopsis microRNAs miR159 and miR319. Dev. Cell, 13, 115–125.
1. Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol., 215, 403–410.
49. Schiefthaler, U., Balasubramanian, S., Sieber, P., Chevalier, D., Wisman, E. and Schnitz, K. (1999) Molecular analysis of NOZZLE, a gene involved in pattern formation and early sporogenesis during sex organ development in Arabidopsis thaliana. *Proc. Natl Acad. Sci. USA*, 96, 11664–11669.

50. Yang, W.C., Ye, D., Xu, J. and Sundaresan, V. (1999) The SPOROCYTELESS gene of Arabidopsis is required for initiation of sporogenesis and encodes a novel nuclear protein. *Genes Dev.*, 13, 2108–2117.

51. Buxdorf, K., Hendelman, A., Stav, R., Lapidot, M., Ori, N. and Arazi, T. (2010) Identification and characterization of a novel miR159 target not related to MYB in tomato. *Planta*, 232, 1009–1022.

52. Davies, R.T., Goetz, D.H., Lasswell, J., Anderson, M.N. and Bartel, B. (1999) IAR3 encodes an auxin conjugate hydrolase from Arabidopsis. *Plant Cell*, 11, 365–376.

53. Rampey, R.A., LeClere, S., Kowalczyk, M., Ljung, K., Sandberg, G. and Bartel, B. (2004) A family of auxin-conjugate hydrolases that contributes to free indole-3-acetic acid levels during Arabidopsis germination. *Plant Physiol.*, 135, 978–988.

54. Achard, P., Herr, A., Baulcombe, D.C. and Harberd, N.P. (2004) Modulation of floral development by a gibberellin-regulated microRNA. *Development*, 131, 3357–3365.

55. Allen, R.S., Li, J., Stahl, M.I., Dubroue, A., Gubler, F. and Millar, A.A. (2007) Genetic analysis reveals functional redundancy and the major target genes of the Arabidopsis miR159 family. *Proc. Natl Acad. Sci. USA*, 104, 16371–16376.

56. Sunkar, R. and Zhu, J.K. (2004) Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. *Plant Cell*, 16, 2001–2019.

57. Wu, M.F., Tian, Q. and Reed, J.W. (2006) Arabidopsis microRNA167 controls patterns of ARF6 and ARF8 expression, and regulates both female and male reproduction. *Development*, 133, 4211–4218.

58. Weijers, D. and Jurgens, G. (2004) Funneling auxin action: specificity in signal transduction. *Curr. Opin. Plant Biol.*, 7, 687–693.