## RESEARCH ARTICLES

# A Family of MicroRNAs Present in Plants and Animals [W][OA]

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Although many miRNAs are deeply conserved within each kingdom, none are known to be conserved between plants and animals. We identified *Arabidopsis thaliana* miR854 and miR855, two microRNAs (miRNAs) with multiple binding sites in the 3′ untranslated region (3′UTR) of *OLIGOURIDYLATE binding PROTEIN1b* (At *UBP1b*), forming miRNA:mRNA interactions similar to those that cause translational repression/mRNA cleavage in animals. At *UBP1b* encodes a member of a heterogeneous nuclear RNA binding protein (hnRNP) family. The 3′UTR of At *UBP1b* is sufficient to repress reporter protein expression in tissues expressing miR854 or miR855 (rosette leaves and flowers, respectively) but not where both miRNAs are absent (cauline leaves). Intergenic regions containing sequences closely resembling miR854 are predicted to fold into stable miRNA precursors in animals, and members of the miR854 family are expressed in *Caenorhabditis elegans*, *Mus musculus*, and *Homo sapiens*, all with imperfect binding sites in the 3′UTR of genes encoding the T cell Intracellular Antigen-Related protein, an hnRNP of the UBP1 family. Potential binding sites for miR854 are absent from *UBP1*-like genes in fungi lacking the miRNA biogenetic machinery. Our results indicate that plants and animals share miRNAs of the miR854 family, suggesting a common origin of these miRNAs as regulators of basal transcriptional mechanisms.

## INTRODUCTION

Several classes of small RNAs have emerged as key regulators of eukaryotic gene expression (Ambros, 2004; Bartel, 2004; Baulcombe, 2004). MicroRNAs (miRNAs) are endogenously expressed 21- to 24-nucleotide RNAs that regulate specific mRNA targets at the posttranscriptional level. These short RNA molecules are derived from longer imperfect foldback (stem-loop) precursors that are processed by ribonuclease III–like nucleases of the DICER family (Bernstein et al., 2001; Hutvagner et al., 2001; Kurihara and Watanabe, 2004; Kidner and Martienssen, 2005). In recent years, the overall importance of miRNAs as key molecular components of development has been demonstrated by the identification of hundreds of miRNAs in plants and animals through cloning strategies or computational analysis combined with the identification of the corresponding target mRNAs (Griffiths-Jones et al., 2006; Jones-Rhoades et al., 2006).

Animal miRNAs typically anneal with imperfect sequence complementarity to the 3′ untranslated region (3′UTR) of the target mRNAs and reduce protein levels through miRNA-mediated transational repression and eventually mRNA cleavage (Carrington and Ambros, 2003). The founding member of the miRNA family, *lin-4*, was initially discovered as a small temporal RNA that negatively regulates gene expression to control developmental transitions in *Caenorhabditis elegans* (Lee et al., 1993; Wightman et al., 1993; Ha et al., 1996). *lin-4* was shown to anneal imprecisely at multiple sites of the 3′UTR of a mRNA encoding the nuclear protein LIN-14, causing its translational repression without affecting mRNA levels (Feinbaum and Ambros, 1999; Olsen and Ambros, 1999). A second small temporal RNA, *let-7*, was later demonstrated to encode a miRNA that repressed the translation of *lin-41* by imprecisely annealing at two different sites of its 3′UTR (Reinhart et al., 2000). Recently, the regulation of *lin-4* and *let-7* was shown to cause partial degradation of their corresponding target mRNAs, suggesting that in animals miRNA-mediated transcript cleavage is more common than previously expected (Bagga et al., 2005; Lim et al., 2005). Animal miRNAs artificially directed against a perfectly complementary 3′UTR target site result in mRNA cleavage, indicating that the presence of nucleotide mismatches in the miRNA:mRNA duplex is essential for avoiding complete mRNA degradation (Hutvagner and Zamore, 2002; Doench et al., 2003; Saxena et al., 2003). Extensive genomic analysis showed that *let-7* is conserved throughout bilateral animals (including molluscs, insects, and mammals) but not in sponges or cnidarians, a discovery suggesting that the *let-7* regulatory circuit appeared late in metazoan evolution (Pasquinelli et al., 2000, 2003)

The evolutionary relationship between animal and plant miRNAs remains poorly understood. In contrast with animal miRNAs,

most plant miRNAs cause mRNA degradation by interacting with their target sequence through prefect or near-perfect complementarity at a single location, usually in the protein coding region, with only a few examples of miRNAs having binding sites within the UTRs of the target mRNA (Rhoades et al., 2002; Jones-Rhoades et al., 2006). A unique miRNA (miR172), initially thought to act through translational repression despite almost perfect complementarity to several target mRNAs, was later shown to also guide mRNA degradation of at least two of its targets (Aukerman and Sakai, 2003; Chen, 2004, 2005; Schwab et al., 2005). The molecular features of plant miRNA stem-loop precursors are also different from those of animal miRNA precursors, having longer and more variable predicted secondary structures (Llave et al., 2002; Reinhart et al., 2002). Global expression profiling has been used as a phylogenetic survey to demonstrate that many plant miRNA circuits are strictly conserved since before the emergence of flowering plants (Axtell and Bartel, 2005; Zhang et al., 2006). In addition, several transcription factors of the *Arabidopsis thaliana* class III homeodomain-leucine zipper gene family are regulated by conserved miRNAs in bryophytes, lycopods, ferns, and seed plants (Floyd and Bowman, 2004). These findings indicate that plant miRNAs are at least 400 million years old (Millar and Waterhouse, 2005); however, and although many miRNAs are now known to be deeply conserved within each kingdom (Pasquinelli et al., 2000; Axtell and Bartel, 2005), no miRNA circuits are known to be structurally or functionally conserved between plants and animals. As a consequence, it has been suggested that miRNAs originated independently in both kingdoms after their divergence and not from a common ancestor (Millar and Waterhouse, 2005; Jones-Rhoades et al., 2006).

Here, we use a bioinfomatic approach in *Arabidopsis* to perform a genome-wide analysis in search of miRNAs that could act by imperfectly binding to the 3′UTR of their target mRNA at multiple sites. We identify nine candidate miRNAs, four of which have potential target sites in the 3′UTR of genes encoding RNA binding proteins containing RNA recognition motifs (RRMs). We show that miR854 and miR855 are two miRNAs with multiple target sites in the 3′UTR of *OLIGOURIDYLATE binding PROTEIN1b* (*UBP1b*), a gene encoding a heterogeneous nuclear RNA binding protein (hnRNP) involved in the regulation of pre-mRNA maturation at different levels, including pre-mRNA splicing (Lambermon et al., 2000). Both miR854 and miR855 are within ATHILA nonautonomous retrotransposons, and the expression of miR854 depends on the miRNA biogenetic pathway. The 3′UTR of At *UBP1b* is sufficient to repress reporter protein expression in tissues where miR854 and miR855 are active, such as developing leaves and flowers, suggesting that At *UBP1b* is negatively regulated at the posttranscriptional level. Intergenic regions containing sequences closely resembling miR854 are predicted to fold into stable miRNA precursors in animal organisms, and members of the miR854 family are expressed in *C. elegans*, *Mus musculus*, and *Homo sapiens*. The animal miR854 family members have imperfect binding sites in the 3′UTR of genes encoding the T cell Intracellular Antigen-Related (TIAR) protein, an hnRNP of the UBP1 family (Kawakami et al., 1992). Our results indicate that the miR854 family is shared by plant and animal organisms, opening the possibility for an

ancient origin of these miRNAs as regulators of basal transcriptional mechanisms.

## RESULTS

### Simultaneous Identification of Candidate miRNAs and Their Potential 3′UTR Targets

To identify plant miRNAs that could act by a mechanism of translational repression analogous to animal miRNAs, we developed a bioinformatic strategy that relies on a Perl script allowing simultaneous prediction of candidate miRNAs and their potential 3′UTR targets. The strategy is illustrated in Figure 1. The script
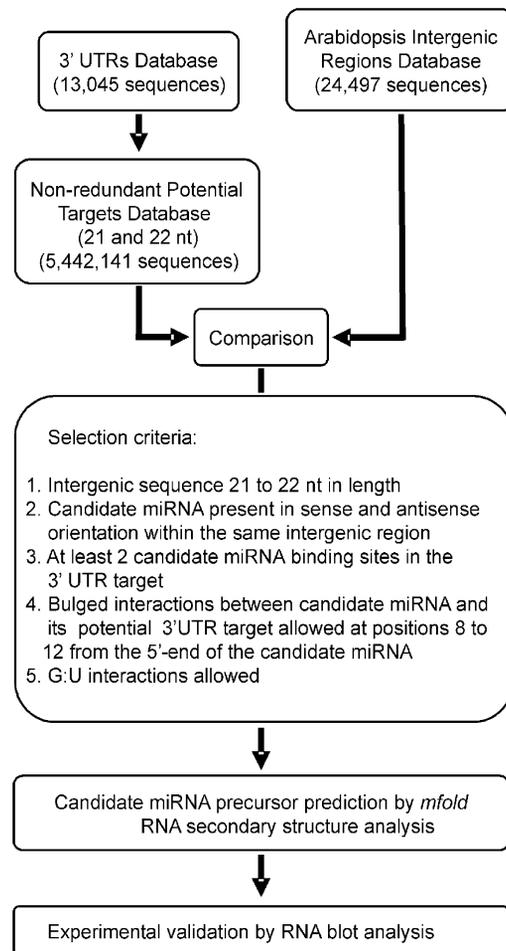


**Figure 1.** Strategy for the Prediction of Candidate miRNAs and Their Target 3′UTRs in *Arabidopsis*.

A Perl script was designed to compare an intergenic regions database built from the *Arabidopsis* genome against a database containing non-redundant 21- and 22-nucleotide sequences derived from a 3′UTR database. Larger genomic regions containing sequences that respected the established criteria were analyzed for subsequent prediction of potential RNA secondary structure using MFOLD (Zuker, 2003). Experimental validation for all candidate miRNAs was performed by RNA gel blot analysis (see main text for details). nt, nucleotides.

compares a database containing all known intergenic regions of the *Arabidopsis* genome (24,497 sequences) to a nonredundant database containing a large collection of publicly available 3′UTRs experimentally supported by cDNA or EST information (13,045 sequences) (Pesole et al., 2002; http://mips.gsf.de/proj/thal/db/). This 3′UTR database was used to generate the potential target database by extracting and storing all possible 21- to 22-nucleotide sequences present in the available 3′UTRs. Each of 5,442,141 different sequences contained in the potential target database was compared with sequences contained in the intergenic region database. Candidate miRNAs and their potential 3′UTR targets were selected on the basis of the following criteria: (1) a candidate miRNA sequence had to be 21 or 22 nucleotides in length, (2) a candidate miRNA was contained within at least one intergenic region in both sense and antisense orientations, (3) a 3′UTR target had at least two potential binding sites for the candidate miRNA, (4) bulged interactions between a candidate miRNA and its potential 3′UTR target were allowed at positions of nucleotides 8 to 12 from the 5′-end of the candidate miRNA sequence, (5) noncanonical G:U pairing was allowed along the miRNA:mRNA interaction. Whereas criterion (2) was

introduced to increase the probability of having a predicted mature miRNA included in the stem region of a potential stem-loop precursor identified using MFOLD (Zuker, 2003), criteria (3) to (5) were established following current knowledge on the functional characteristics of the *let-7:lin-41* circuit in *C. elegans* (Pasquinelli et al., 2000; Reinhart et al., 2000).

Nine candidate miRNAs and their corresponding potential 3′UTR targets were identified. These candidates and their predicted target mRNAs are shown in Table 1. Strikingly, our script predicted that four of the nine candidates had potential targets in the 3′UTR of two mRNAs encoding RNA binding proteins containing RRMs. Candidates miR854 and miR855 were predicted to bind specific sequences in the 3′UTR of At *UBP1b* (At1g17370), a gene encoding an hnRNP involved in the regulation of pre-mRNA maturation at different levels, including pre-mRNA splicing (Lambermon et al., 2000). Candidates MAV3 and MAV5 were predicted to target specific sequences in the 3′UTR of At3g07810, a different hnRNP that contains two RRMs. Additional candidates were predicted to target the 3′UTR of an mRNA encoding a protease inhibitor lipid transfer protein (At2g45180), the disease resistance protein NDR1 (At3g20600),

**Table 1.** Predicted Candidate miRNAs and Potential 3′UTR Targets

| miRNA | Sequence (5′→3′) | RNA Blot[a] | Size (nt) | No. of Loci | Chromosome[b] | Genomic Context | Locus 3′UTR Target | Predicted Function |
|---|---|---|---|---|---|---|---|---|
| MAV1 | CAAGCAUUAUAAUCAAGUUUGA | − | 22 | 4 | I (2) | Intergenic | At2g45180 | Protease inhibitor LTP family protein |
| | | | | | III (1) | Intergenic | | |
| | | | | | V (1) | Intergenic | | |
| miR855 | AGCAAAAGCUAAGGAAAAGGAA | + | 22 | 45 | I (9) | Retrotransposon[c] | At1g17370 | RNA binding protein[d] |
| | | | | | II (7) | Retrotransposon | | |
| | | | | | III (7) | Retrotransposon | | |
| | | | | | IV (10) | Retrotransposon | | |
| | | | | | V (12) | Retrotransposon | | |
| MAV3 | AAGAAGAAAAAAAAAUAGGAA | − | 21 | 1 | II (1) | Intergenic | At3g07810 | RNA binding protein |
| MAV4 | UGAAAAAUCUUAAAAUUAAUA | + | 21 | 1 | II (1) | Intergenic | At3g20600 | NDR1 disease resistance protein |
| MAV5 | GAGGAGACGACUGAGAUGAAGA | + | 22 | 4 | I (3) | Hypothetical protein | At3g07810 | RNA binding protein |
| | | | | | IV (1) | Hypothetical protein | | |
| MAV6 | UUUAAUGAUAUUGCUGAAUUCC | − | 22 | 13 | I (3) | Hypothetical protein | At1g52010 | Mutator-like transposase protein |
| | | | | | II (2) | Hypothetical protein | | |
| | | | | | III (3) | Hypothetical protein | | |
| | | | | | IV (3) | Hypothetical protein | | |
| | | | | | V (2) | Hypothetical protein | | |
| MAV7 | AGAUGGUCGAAGAAGAAGAAGA | + | 22 | 1 | V (1) | Intergenic | At2g18090 | PHD finger family protein |
| MAV8 | GAGAAAGCUAAAGAGAUAGAGA | − | 22 | 1 | V (1) | Intergenic | At5g60980 | Nuclear transport factor 2 (NTF2) family protein |
| miR854 | GAUGAGGAUAGGGAGGAGGAG | + | 21 | 12 | II (2) | Retrotransposon[e] | At1g17370 | RNA binding protein |
| | | | | | III (1) | Retrotransposon | | |
| | | | | | IV (1) | Retrotransposon | | |
| | | | | | V (8) | Retrotransposon | | |

[a] Detection (+) or nondetection (−) of candidate miRNA by RNA blot analysis is indicated.
[b] Chromosomal position; the number in parentheses indicates the number of miRNA origins in each chromosome.
[c] Within the LTR of the Athila_4B retrotransposon family.
[d] All RNA binding proteins contain RRMs.
[e] Within the open reading frame of Athila_6A retrotransposon family.

a PHD finger family protein (At2g18090), a nuclear transport factor 2 family protein (At5g60980), and a *Mutator*-like transposase protein (At1g52010).

## MAV4, MAV5, MAV7, miR854, and miR855 Are Expressed as 21-Nucleotide RNA Species

To determine if our bioinformatic strategy had identified expressed candidate miRNAs, we conducted RNA gel blot analysis by hybridizing low molecular weight RNA from rosette leaves, stems, cauline leaves, and developing inflorescences of adult *Arabidopsis* plants (Hamilton and Baulcombe, 1999; Mette et al., 2000) with radiolabeled probes complementary to each candidate miRNA. Figure 2 shows that five out of nine candidates were detected as small 21-nucleotide RNA species, indicating that our bionformatic approach was successful in identifying expressed small RNAs. Whereas miR855 and MAV4 showed specific expression in rosette leaves, MAV5 was expressed in both cauline and rosette leaves. MAV7 was expressed in all tissues tested, with abundant expression in developing inflorescences. miR854 was weakly expressed in stems and developing inflorescences. The remaining four candidates (MAV1, MAV3, MAV6, and MAV8)
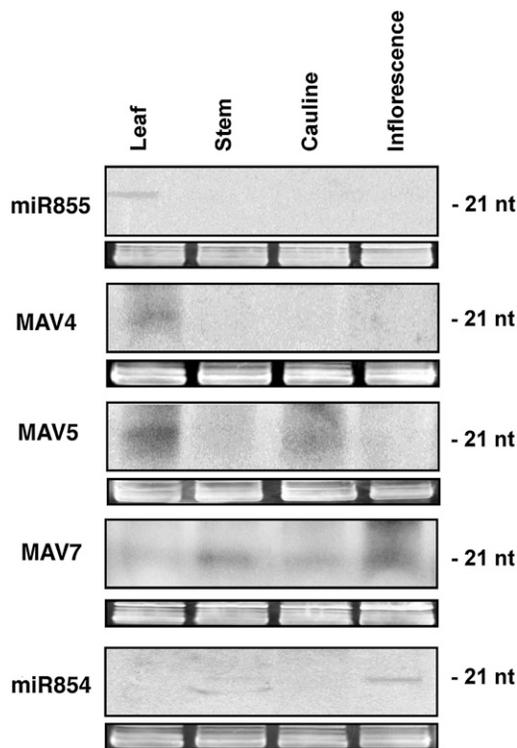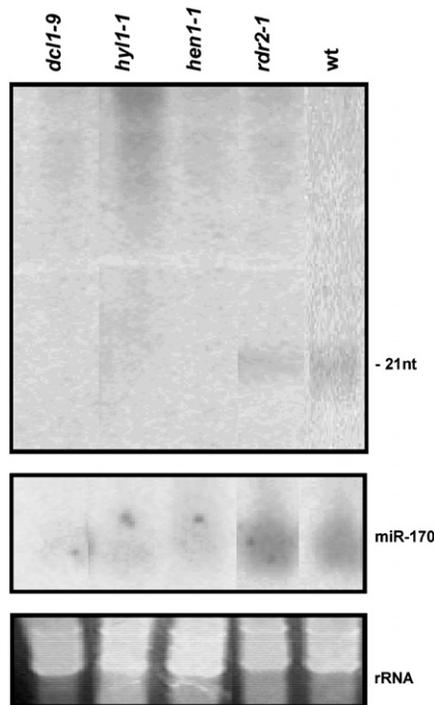
were not detected by our RNA gel blot experiments, suggesting that they represent false positive sequences, that they are active during different temporal or spatial patterns of expression, or that their expression is below the level of detection of our experimental conditions.

## miR854 and miR855 Are Contained within Predicted Stem-Loop Precursors

Previous results have shown that miRNAs in plants or animals can originate from genomic regions annotated as encoding proteins, hypothetical proteins, processed pseudogenes, transposable elements, or genomic repeats (Wang et al., 2004; Smalheiser and Torvik, 2005; Sunkar et al., 2005b; Devor, 2006). We identified the location of all predicted candidate miRNAs by following the genomic annotation of *Arabidopsis* (The Arabidopsis Information Resource [TAIR] release 6.0). As shown in Table 1, while four candidates are present at a single genomic location (MAV3, MAV4, MAV7, and MAV8), five candidates are located multiple times and are present in all chromosomes of the *Arabidopsis* genome (MAV1, MAV5, MAV6, miR854, and miR855).

We focused our structural analysis in miR854 and miR855, two candidates predicted to target the same 3′UTR of a mRNA encoding an hnRNP with three RRMs. These results are shown in Figure 3. Whereas miR855 is present throughout the genome at 45 different locations, all 12 sequences corresponding to miR854 are within retrotransposons of the ATHILA family (Pelissier et al., 1995). The presence of multiple genomic sequences corresponding to a candidate miRNA sequence is not exceptional, as both plant and animal species have been found to contain known miRNAs families represented by 14 to 80 loci (Bentwich, 2005; Giraldez et al., 2005; Jones-Rhoades et al., 2006). Several regions adjacent to both mature candidate miRNAs are predicted to fold into stable hairpin structures using MFOLD (Figures 3A and 3B). Whereas four intergenic regions containing miR854 were predicted to fold into stable miRNA precursors, a single potential miR855 precursor was identified in a region of chromosome II (see Supplemental Table 1 online for details). Interestingly, whereas all miR855 sequences are within the long terminal repeat (LTR) of the ATHILA_4A nonautonomous retrotransposable element, four of the regions adjacent to a candidate miR854 sequence are within an ATHILA_6A_I retrotransposon located in the pericentromeric region of chromosome V (Figure 3B). ATHILA members belong to the *Ty3/Gypsy* family LTR retrotransposons (Kumar and Bennetzen, 1999; Jurka et al., 2005). An in-depth analysis of miRBase entries (http://microrna.sanger.ac.uk/sequences/ftp.shtml) showed that free energies are within the range of previously reported plant miRNA precursors (−53.75 to −59.89 Kcal/mol for miR854 and −82.39 Kcal/mol for miR855). A search of the *Oryza sativa* genome identified a sequence with 20 out of 21 nucleotides identical to miR854, located in chromosome IV. The rice miR854 sequence has adjacent regions that form stem-loop precursors included in a Rim2-M101 transposon, with the corresponding mature miRNA sequence on the same arm of the precursor in both *Arabidopsis* and rice (Figure 3C). In poplar (*Populus trichocarpa*), we also identified a sequence with 19 out 21 nucleotides homologous to miR854; although we could not identify a stable



**Figure 2.** Expression Patterns of Candidate miRNAs.

RNA gel blot analysis was conducted by hybridizing low molecular weight RNA from rosette leaves, stems, cauline leaves, and developing inflorescences of adult *Arabidopsis* plants with radiolabeled probes complementary to each candidate miRNA. rRNA bands were visualized by ethidium bromide staining of polyacrylamide gels and served as loading controls. A labeled RNA oligonucleotide was used as a size marker.

**Figure 3.** Predicted Precursors for miR854, miR855, and Os miR854 and Their Potential Binding Sites in the 3′UTR of At *UBP1b*.

**(A)** Foldback secondary structures of the predicted precursor for miR855 as determined by MFOLD; the intergenic region corresponds to coordinates 4,681,515 to 4,681,536 in chromosome II.

**(B)** Foldback secondary structures of the predicted precursor for miR854 as determined by MFOLD and localization of miR854 predicted precursor in the ATHILA6A_I retroelement; the intergenic region corresponds to coordinates 11,855,326 to 11,855,546 in chromosome V.

**(C)** Foldback secondary structures of the predicted precursor for Os miR854 as determined by MFOLD; the intergenic region corresponds to coordinates 7,974,551 to 7,974,531 in chromosome IV. For *Arabidopsis*, coordinates follow TAIR release 6.0 annotation.

potential precursor associated with it, the sequence is part of a HERVIP10FH retrotransposon also present in the human genome (Jurka et al., 2005). In combination with data presented in previous paragraphs, these results demonstrate that miR854 and miR855 encode intergenic RNAs of 21 nucleotides in length that can fold into stable hairpin precursors characteristic of plant miRNAs. In the case of miR854, the mature miRNA is conserved in rice, indicating that it represents a new *Arabidopsis* miRNA conserved in monocotyledonous species.

### Expression of miR854 Depends on miRNA Biogenesis

Biogenesis of miRNAs in *Arabidopsis* is a stepwise process that requires the activity of three main proteins: DICER LIKE-1 (DCL1), a double-stranded RNA binding protein with RNase-type III activity required for miRNA biogenesis (Schwartz et al., 1994; Ray et al., 1996; Jacobsen et al., 1999; Golden et al., 2002; Schauer et al., 2002; Finnegan et al., 2003); HYPONASTIC LEAVES1 (HYL1), a nuclear double-stranded RNA protein that assists efficient and precise cleavage of primary miRNAs required for miRNA but not short interfering RNA (siRNA) biogenesis (Lu and Fedoroff, 2000; Han et al., 2004; Kurihara et al., 2006); and HUA Enhancer1 (HEN1), a methyltransferase essential for the activity of miRNAs that adds a methyl group to their 3′-most nucleotide (Chen et al., 2002; Chen, 2005; Yu et al.,

2005). In *dcl1-9*, *hyl1-1*, and *hen1-1* alleles, the abundance of most miRNAs is severely reduced (Park et al., 2002; Reinhart et al., 2002; Han et al., 2004). To determine if the expression of miR854 was dependent on the miRNA biogenetic pathway, we conducted RNA gel blot analysis by hybridizing low molecular weight RNA from *dcl1-9*, *hyl1-1*, *hen1-1*, and *RNA-dependent RNA polymerase2* (*rdr2*) mutant individuals with radiolabeled probes complementary to miR854. Figure 4 shows that miR854 is absent in flowers of *dcl1-9*, *hyl1-1*, and *hen1-1* mutants, indicating that miR854 expression depends on the activity of *DCL1*, *HYL*, and *HEN1* (Figure 4). By contrast, the expression of miR854 does not require the activity of *RDR2*, a gene necessary for the generation of endogenous siRNAs (Xie et al., 2004). These results demonstrate that miR854 is generated through the miRNA biosynthetic pathway, confirming that miR854 is processed as a plant miRNA.

### The 3′UTR of At *UBP1b* Confers Posttranscriptional Regulation of Gene Expression

To this date, a single *Arabidopsis* insertional T-DNA line altering the wild-type sequence of At *UBP1b* has been recovered from freely available collections (Alonso et al., 2003). Although the insertional element is inserted in the 3′UTR of *UBP1b*, the insertion does not alter the structure of a potential miR854 or

**Figure 4.** miR854 Is Produced by the miRNA Biogenetic Pathway.

RNA gel blot analysis was conducted by hybridizing low molecular weight flower RNA from *dcl1-9*, *hyl1-1*, *hen1-*1, and *rdr2-1* mutant individuals and wild-type plants with radiolabeled probes complementary to miR854. Whereas *dcl1-9*, *hyl1-1*, and *hen1-1* mutants are affected in the miRNA biogenesis pathway, the *rdr2-1* mutant is affected in siRNA production. The membrane was subsequently reprobed with an ath-miR170 complementary end-labeled oligonucleotide as a positive control. rRNA bands were visualized by ethidium bromide staining of polyacrylamide gels and served as loading controls. A labeled RNA oligonucleotide was used as a size marker.

miR855 target site, and individuals homozygous for the T-DNA insertion did not exhibit an obvious mutant phenotype (data not shown). To determine if the presence of the 3′UTR of *UBP1b* is sufficient to cause posttranscriptional gene silencing, we transformed wild-type *Arabidopsis* plants with a construct in which the *uidA* (β-glucuronidase [*GUS*]) reporter gene was fused to the 3′UTR of At *UBP1b* under the control of the constitutive cauliflower mosaic virus *35S* promoter (*Pro$_{35S}$*). We examined GUS expression in transgenic individuals harboring either the *Pro$_{35S}$:GUS:UBP1-3′UTR* construct or a *Pro$_{35S}$:GUS* construct used as a control. These results are shown in Figure 5. In the T1 generation, *Pro$_{35S}$:GUS* transgenic plants showed GUS expression in all tissues examined, including seedlings, roots, rosette leaves, and inflorescences. By contrast, transgenic *CaMV35S:GUS: UBP1b-3′UTR* seedlings showed GUS expression in the emerging cotyledons at stage 0.7 (Boyes et al., 2001) (Figures 5A and 5B). During stage 1.0, before the emergence of the first leaves, GUS expression was absent in the cotyledons and the hypocotyl but present in roots (Figure 5C). During rosette leaf formation, in contrast with transgenic *Pro$_{35S}$:GUS* individuals that showed

constitutive GUS expression at all developmental stages (Figure 5F), transgenic *Pro$_{35S}$:GUS:UBP1b-3′UTR* plants lost GUS activity early during rosette leaf development and flower formation (Figures 5D, 5E, and 5G). Loss of GUS expression initiated at variable stages of growth but always occurred early during blade expansion, progressing from the center midportion to the margins. Mature leaves showed weak GUS activity in the vasculature and eventually in some blade margins. GUS expression persisted in trichomes, hydathodes, hypocotyls, roots, and cauline leaves (Figures 5D and 5E). These results indicate that the 3′UTR of At *UBP1b* is sufficient to cause the inhibition of reporter expression in tissues where miR854 and miR855 are active (rosette leaves, stems, and flowers) but not in tissues where both are absent (cauline leaves).

We also examined *GUS* expression by RNA gel blot and immunoblot analysis using RNA and proteins extracted from *Pro$_{35S}$:GUS:UBP1b-3′UTR* transgenic individuals of the T2 and T3 generations. These results are shown in Figure 6. Steady levels of reporter mRNA expression could be detected by RNA gel blot analysis in leaves where miR855 is expressed (Figure 6D); however, GUS protein expression was not detected using both histochemical and immunodetection assays (Figures 6A and 6B), indicating that reporter protein expression is attenuated at the translational level in leaves of *Pro$_{35S}$:GUS:UBP1b-3′UTR* lines. Histochemical analysis of additional tissues, such as immature flowers, mature flowers, and seedlings, showed that GUS expression remained absent in tissues where miR854 and miR855 are active (rosette leaves, stems, and flowers; Figure 6F). Partial base pairing between a miRNA and its target usually results in at least some degree of downregulation at the target mRNA level (Bagga et al., 2005; Lim et al., 2005). Therefore, and although our results indicate that At *UBP1b* is regulated predominantly at the translational level in tissues where miR854 and miR855 are active, we do not eliminate the possibility that the absence of reporter protein expression could be partially due to undetected levels of mRNA degradation.

## 3′UTR Target Sites for miR854 Are Conserved in hnRNP Proteins of the At *UBP1b* Family

Figure 7 shows that candidates miR854 and miR855 are predicted to bind specific sequences in the 3′UTR of At *UBP1b* (At1g17370), a gene encoding the closest *Arabidopsis* homolog of UBP1, a constitutively expressed oligourydilate RNA binding protein previously characterized in *Nicotiana plumbaginifolia* (Lambermon et al., 2000). Whereas the 3′UTR of At *UBP1b* contains two potential target sites for miR855 (Figure 7), a detailed sequence analysis showed that miR854 has at least four potential imperfect target sites in the same 3′UTR, two of which could not have been predicted by our Perl script based on the value of our parameter settings for mismatch positions. Only specific motifs are prone to strictly pair with the sequence of miR854, indicating that specific U-rich regions in this 3′UTR constitute potential target sites. Other miRNA circuits containing noncanonical G:U pairing in the 5′-seed have been shown to be fully functional in *Populus* (Lu et al., 2005b). The computational analysis of 100,000 permutated sequences indicated that the
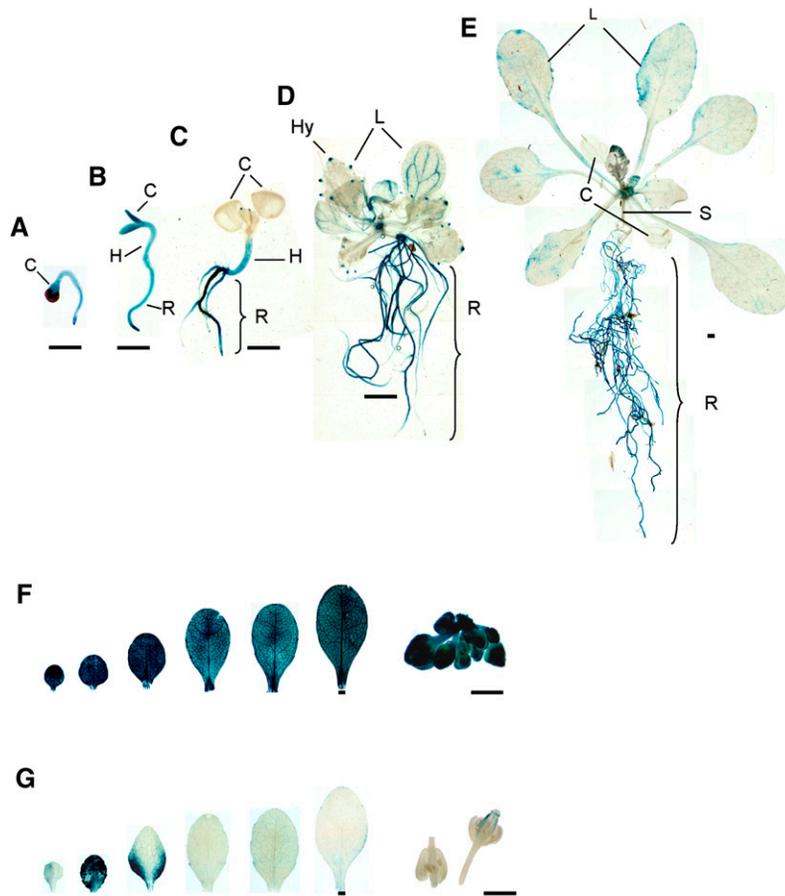
**Figure 5.** Patterns of GUS Expression in *Pro₃₅ₛ:GUS:3′UTR-UBP1b* Transgenic Plants.

**(A)** to **(E)** *Pro₃₅ₛ:GUS:3′UTR-UBP1b* transgenic *Arabidopsis* plants.
**(A)** Germinating seedling at growth stage 0.5. Bar = 2.5 mm.
**(B)** Seedling at growth stage 0.7. Bar = 2.5 mm.
**(C)** Seedling at growth stage 1.0. Bar = 2.5 mm.
**(D)** Seedling at growth stage 1.05. Bar = 2.5 mm.
**(E)** Plant at growth stage 3.20. Bar = 2.5 mm.
**(F)** Individual leaves from developing rosettes at growth stage 1.10 and inforescences at growth stage 6.1 of *Pro₃₅ₛ:GUS* transgenic plants. Bar = 2.5 mm
**(G)** Individual leaves from developing rosettes at growth stage 1.10 and inforescences at growth stage 6.1 of *Pro₃₅ₛ:GUS:3′UTR-UBP1b* transgenic plants. Bar = 2.5 mm
Growth stages are as previously described in Boyes et al. (2001). C, cotyledon; H, hypocotyl; Hy, hydathodes; L, rosette leaves; R, root; S, stem.

number of BLAST hits to genomic sequences occurs at a lower frequency in miR854 than in several previously identified *Arabidopsis* miRNAs (see Supplemental Table 2 online).

To explore additional conservation of the miR854 circuit in the plant kingdom, we conducted a bioinformatical analysis of 3′UTR sequences corresponding to *UBP1* and At *UBP1b* homologs. Using the sequence of miR854 as a reference, we looked for short 3′UTR regions that could represent imperfect binding sites for this miRNA, paying particular attention to the stability of a potential 5′-seed pairing. The results are shown in Supplemental Figure 1 online. In all cases analyzed, the 3′UTRs of At *UBP1b*–like genes include CT-rich regions that are imperfectly complementary to the sequence of miR854. Whereas in

*N. plumbaginifolia* the 3′UTR of *UBP1* (protein accession number AJ272011) contains three potential binding sites for miR854, in *Zea mays* the At *UBP1b* homolog (Zmrs072.5) contains two potential binding sites in its 3′UTR. In the case of *O. sativa* (XM_479160), we found two potential binding sites for the miR854 homolog Os miR854. Finally, in the At *UBP1b* homolog of *Physcomitrella patens* (PPP_CL_7771), we identified at least one CT-rich region complementary to miR854. These results indicate that the miR854 family of miRNAs is conserved across the plant kingdom. In the case of miR855, we could identify a potential binding site in the 3′UTR of the At *UBP1b* homolog in rice but not in homologs of the other species analyzed (see Supplemental Figure 1 online).
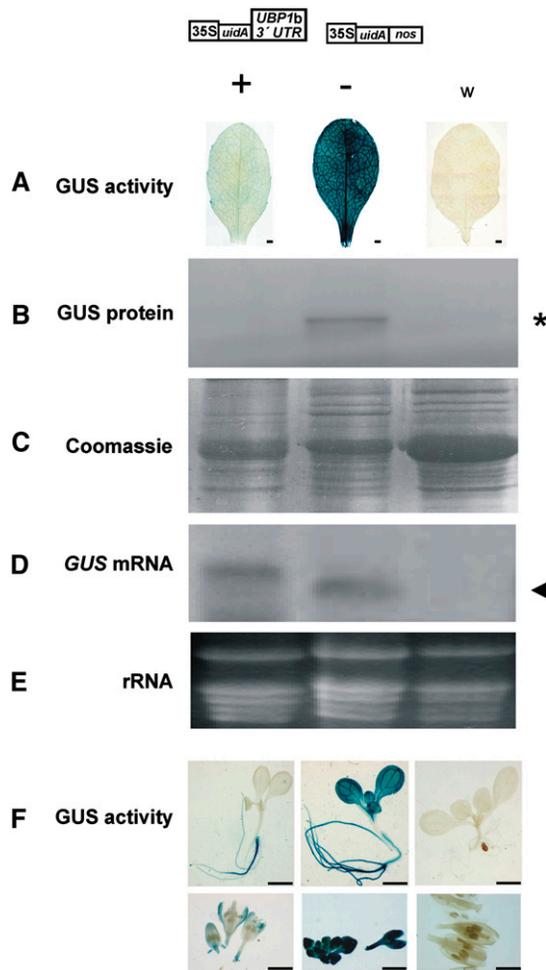
**Figure 6.** Posttranscriptional Gene Silencing Conferred by the 3′UTR of At *UBP1b*.

**(A)** GUS activity in rosette leaves from *Pro35S:GUS:3′UTR-UBP1b* (+) and *Pro3S:GUS* (−) transgenic and wild-type *Arabidopsis* plants (w) at growth stages 1.10 and 6.1.

**(B)** Immunoblot analysis of GUS protein in rosette leaves from *Pro35S: GUS:3′UTR-UBP1b* (+), *Pro35S:GUS* (−) transgenic, and wild-type plants at 1.10 growth stage. The 68.4-kD GUS protein is indicated by an asterisk. **(C)** Coomassie blue staining shown as a protein loading control.

**(D)** RNA gel blot analysis of *GUS* mRNA in rosette leaves from *Pro35S: GUS:3′UTR-UBP1b* (+), *Pro35S:GUS* (−), and wild-type plants at 1.10 growth stage. The arrowhead indicates the 1802-nucleotide GUS transcript for (−); transcript for *GUS:3′UTR-UBP1b* (+) is 2048 nucleotides.

**(E)** rRNA was visualized by ethidium bromide staining of polyacrylamide gels and is shown as a loading control.

**(F)** GUS activiy in T2 seedlings and developing flowers from *Pro35S: GUS:3′UTR-UBP1b* (+) and *Pro35S:GUS* (−) transgenic and wild-type plants at 1.0 growth stage. Bars = 2.5 mm.

An alternative explanation of *3′UTR* At *UBP1b*–dependent posttranscriptional downregulation of GUS expression could involve different molecular factors that change the stability of the reporter by interacting with the 3′UTR. To determine if conservation of the miR854 binding sites could be associated with non-

miRNA regulatory elements that interact with the 3′UTR region of *UBP1* family members, we looked for regions complementary to miR854 in the 3′UTR of *UBP1*-like genes present in the genome of *Saccharomyces cerevisiae* and *Ustilago maydis*, two organisms in which the absence of encoded miRNAs is correlated with the absence of genes encoding DICER, ARGONAUTE, and RNA-dependent RNA polymerase proteins (Nakayashiki et al., 2006). In both organisms, we identified a single At *UBP1b* homolog: *PUB1* for *S. cereviseae* and *UM1182* for *U. maydis*. Strikingly, we could not find a potential binding site for miR854 in any of the corresponding 3′UTR regions (see Supplemental Figure 2 online), a finding suggesting that the conserved domains in the 3′UTR of At *UBP1b* family members represent binding sites for a miRNA and not binding sites for 3′UTR-interacting regulatory proteins.

## At *UBP1b* Expression Is Mediated by the miRNA Biogenetic Pathway

A review of publicly available expression profiling data generated by microarray experiments (http://www.genevestigator.ethz.ch) or massively parallel signature sequencing (MPSS; http://mpss.udel.edu/at) confirmed that At *UBP1b* is constitutively expressed in *Arabidopsis*, attaining maximum levels in inflorescences, seeds, and rosette leaves (data not shown). Microarray experiments designed to assess global expression profiling in mutants affected in miRNA biogenesis show that, compared with its expression in wild-type plants, At *UBP1b* is significantly upregulated in *dcl1-7* and *hen1-1* but not in other mutants such as *rdr1*, *rdr2*, *dcl2*, and *dcl3*, all involved in the biogenesis of siRNAs (Xie et al., 2004; see Supplemental Table 3 online). The level of upregulation of At *UBP1b* in *dcl1-7* and *hen1-1* is similar to those obtained in these same mutants for previously confirmed targets of known miRNAs, such as *CUP-SHAPED COT-YLEDONS2* (At5g53950) (Laufs et al., 2004; Mallory et al., 2004; Baker et al., 2005) and *TRANSPORT INHIBITOR RESPONSE1* (At3g62980; see Supplemental Table 3 online) (Navarro et al., 2006), suggesting that the activity of At *UBP1b* is mediated by the miRNA biogenetic pathway.

## The miR854 Family Is Conserved in Animal Species

At *UBP1b* is structurally most similar to the mammalian apoptosis-promoting factor TIAR gene (TIAL-1), as both contain three RRMs and bind RNA with specificity to oligouridylates (Kawakami et al., 1992). In animals, TIAR regulates pre-mRNA splicing and is essential for primordial germ cell development (Beck et al., 1998). By conducting a sequence analysis of the genomes of *C. elegans*, *M. musculus*, *Pan troglodytes*, and *H. sapiens*, we identified genomic sequences closely resembling miR854. These results are shown in Figure 8. In comparison with miR854, the four animal candidate miRNAs in *C. elegans* (Ce miR854), *M. musculus* (Mm miR854), *P. troglodytes* (Pt miR854), and *H. sapiens* (Hs miR854) have a single nucleotide substitution (Figure 8A). To determine if miRNAs homologous to miR854 are expressed in animal organisms, we purified total RNA from *C. elegans* adult worms, mouse W9.5 embryonic stem cells, mouse kidney, and the human hepatoma cell line HepG2.

**Figure 7.** Predicted Target Sites of miR854 and miR855 in the 3′UTR of At *UBP1b*.

The distance in nucleotides is indicated between target sites.

High-stringency DNA hybridization with the same ^{32}P end-labeled probe used to detect miR854 in *Arabidopsis* showed that 21-nucleotide small RNA species homologous to miR854 are expressed in all species, albeit at variable levels (Figure 8B). Whereas the expression was weak in adult worms and mouse stem cells, the strongest expression was found in human HepG2 cells and mouse kidney, suggesting that the activity of the miR854 family is also developmentally regulated in animals.

Precursors of miR854 family members predicted to fold into stable hairpin secondary structures were found among genomic regions in *C. elegans* (length: 105 nucleotides), *P. troglodytes* (length: 63 nucleotides), *M. musculus* (length: 105 nucleotides), and *H. sapiens* (length: 80 nucleotides; Figure 8C; see Supplemental Table 4 online). Whereas most of these candidate miRNAs are encoded by intergenic sequences, the candidate in *C. elegans* is predicted to be transcribed from the reverse strand of a predicted gene encoding a hypothetical protein with a short open reading frame. This is not unusual, as other animal miRNAs, such as miR-414, miR220, and miR492, are transcribed from annotated gene regions encoding expressed proteins or pseudogenes (Wang et al., 2004; Devor, 2006). The predicted precursors of these miRNA candidates have a free energy within the range of previously reported animal precursors (Figure 8C). In addition, all four miR854 homologs have at least one potential binding site in the 3′UTR of the corresponding *TIAR* gene, suggesting that these sites are targets of the conserved animal miRNAs (Figure 8D). In the case of Ce miR854, Mm miR854, and Hs miR854, a second potential binding site was identified in the corresponding *TIAR* gene (see Supplemental Figure 3 online). Interestingly, the location and the sequence of one of the binding sites shown in Figure 3D is perfectly conserved in the 3′UTR of the *TIAR* gene in *M. musculus* and *H. sapiens*, 168 nucleotides downstream of the UAG codon. Finally, for at least one site, all four candidates (two in the case of Hs miR854) are predicted to be fully complementary to their mRNA target in the first eight nucleotides of the 5′-seed pair, with two to five G:U noncanonical interactions. No binding sites for miR855 family members were identified in the *TIAR* genes analyzed, indicating that conservation is specific to miR854. These results indicate that miRNA candidates of the miR854 family are expressed in animal orga-

nisms, demonstrating that the miR854 family of miRNAs is present in plants and animals.

## DISCUSSION

We have used a bioinformatic approach to identify *Arabidopsis* candidate miRNAs that could act by posttranscriptional mechanisms analogous to those prevailing in animals. In contrast with previous strategies that take advantage of the high complementarity of plant miRNAs to target mRNAs (Rhoades et al., 2002; Jones-Rhoades and Bartel, 2004; Wang et al., 2004; Adai et al., 2005), our approach used publicly available 3′UTR sequences to identify candidate miRNAs having potential imprecise binding sites similar to those characterizing the *let-7:lin-41* circuit in *C. elegans* (Pasquinelli et al., 2000; Reinhart et al., 2000). The number of 3′UTRs included in this analysis corresponds to ∼50% of the total number of annotated genes in *Arabidopsis*. The level of stringency imposed by the selected criteria resulted in nine candidates with potential target sites in the 3′UTR of protein-encoding genes. This stringency was mainly imposed by criteria (2), as additional searches that relied on the same approach but eliminated the requirement for a candidate miRNA to be present in a sense and antisense orientation within the same intergenic region (a criteria used to increase the probability of selecting for stable stem-loop precursors with a potential mature miRNA present in the stem) increased the amount of candidates to <200, indicating that potential interactions between intergenic transcribed sequences and 3′UTRs could be more frequent that previously expected (M. Arteaga-Vázquez and J.-P. Vielle-Calzada, unpublished data).

Although not all candidates were confirmed by RNA gel blot analysis, three of them (MAV5, miR854, and miR855) encode purine-rich sequences expressed as 21- to 22-nucleotide RNA species and are predicted to bind to at least two target sites in the 3′UTR of genes encoding hnRNPs. A genomic analysis of miR854 and miR855 indicated that both are contained within predicted stem-loop precursors typical of plant miRNAs. The low abundance but tissue-specific expression shown by both miRNAs is reminiscent of the expression levels shown by other confirmed miRNAs, such as miR162a, miR437, and miR439, all

**Figure 8.** Conservation of miR854 Family in Animal Species.

**(A)** Alignment of miR854 family members in plants and animals; underlined letters indicate unique nucleotide substitutions compared with *Arabidopsis*.
**(B)** RNA gel blot analysis of animal miRNAs of the miR854 family using RNA from HepG2 human cells (lane 1), adult *C. elegans* individuals (lane 2), mice stem cells (lane 3), and mice kidney tissue (lane 4). rRNA is shown as a loading control.
**(C)** Predicted precursors for animal miRNAs of the miR854 family: Pt miR854 (*P. troglodytes*), Hs miR854 (*H. sapiens*), Mm miR854 (*M. musculus*) and Ce miR854 (*C. elegans*) as predicted by MFOLD (Zuker, 2003).
**(D)** Predicted target sites in the 3′UTR of *TIAR* family members for miR854, Hs miR854, Ce miR854, Mm miR854, and Pt miR854; gray letters indicate single nucleotide substitutions compared with miR854.

detected under similar or more sensitive experimental conditions than those used in this study (Sunkar et al., 2005b, 2005a; Hirsch et al., 2006). The weak level of expression shown by both miR854 and miR855 is in agreement with recent findings demonstrating transcriptional activity of ATHILA retrotransposons in *Arabidop-* *sis* (May et al., 2005). The sequence of ATHILA retrotransposons is known to be highly rich in purine at the polypurine track region (Pelissier et al., 1995). As shown in Supplemental Table 5 online, the purine-rich trinucleotide repeat nature of miR854 is reminiscent of the sequence complexity of other miRNAs, such as

miR156, a plant miRNA family predicted to target the 3′UTR of several genes encoding *SQUAMOSA* promoter binding proteins (Reinhart et al., 2002; Rhoades et al., 2002). As for many other experimentally validated miRNAs, including miR156 h, miR157a to miR157d, miR395d and miR395e, miR397a and miR397b, miR400, miR401, miR404, miR406, miR407, miR413 to miR419, and miR426 (Reinhart et al., 2002; Rhoades et al., 2002; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Wang et al., 2004; Allen et al., 2005), MPSS signatures corresponding to miR854 or miR855 are not included in a report of close to 1.5 million signatures representing transcribed small RNAs (Lu et al., 2005a). Recent results indicate that ∼85% of newly discovered DCL1 small RNA products are not represented in this large collection of MPSS signatures (Henderson et al., 2006). While this could be due to a nonidentified bias specific to the MPSS procedure established, it could also be related to weak expression levels shown by many of these miRNAs.

Both miR854 and miR855 have predicted target sites in the 3′UTR of At *UBP1b*, a member of a hnRNP protein family present in plants and animals and the closest *Arabidopsis* homolog of UBP1. When overexpressed in plant protoplasts of *N. plumbaginifolia*, *UBP1* has been shown to enhance the splicing of inefficiently processed introns and to increase steady state levels of reporter transcripts, possibly by interacting with the 3′UTR and protecting mRNA from exonucleolytic activity (Lambermon et al., 2000). The levels of UBP1 activity appear to be tightly regulated, as UBP1 overexpressor lines have not been recovered presumably due to affected plant viability (Lambermon et al., 2000). In mammals, TIAR associates selectively with pre-mRNAs that contain 5′-splice sites followed by U-rich sequences and binds translational regulatory AU-rich motifs present in the *TUMOR NECROTIC FACTOR*-α mRNA (Gueydan et al., 1999). Consistent with this role, TIAR has been shown to localize in the nucleus; however, in response to environmental challenges, TIAR accumulates in stress granules, cytoplasmic aggregates shown to be functionally equivalent to processing bodies (P-bodies) implicated in miRNA-mediated translational repression (Kedersha and Anderson, 2002; Liu et al., 2005). Our results extend these findings by suggesting that the miR854 family constitutes a higher-order control of the basal transcriptional machinery, probably through a combination of target-directed translational repression and mRNA cleavage.

The identification of small 21-nucleotide RNA species of the miR854 family and their potential stem-loop precursors in *C. elegans*, *M. musculus*, *P. troglodytes*, and *H. sapiens* suggests that we have identified a miRNA shared by plants and animals. Attempts to assess the functional impact of folding free energies using RANDFOLD software (version 2.0) suggest that the distribution for miRNA sequences is biased toward low predicted P values compared with tRNAs or rRNAs (Bonnet et al., 2004). A sequence analysis using RANDFOLD indicates that the P value of plant and animal predicted precursors miR854 family members is lower than the P value of several experimentally confirmed miRNAs in all genomes tested (see Supplemental Table 6 online; Griffiths-Jones et al., 2006). Animal members of the miR854 family are predicted to bind at multiple target sites in the 3′UTR of genes encoding the hnRNP protein TIAR, with several G:U interactions in the 5′-seed of the miRNA:mRNA duplex. Based on the

current evidence, the functional consequence of noncanonical G:U base pairing in the 5′-seed of animal miRNA circuits remains unclear. Although in *Drosophila* the presence of G:U interactions in the 5′-seed has been shown to be detrimental for in vivo miRNA function (Brennecke et al., 2005), recent results show that G:U base pairing is tolerated in the seed region of the *lsy-6* miRNA interaction with its target *cog-1*, demonstrating that perfect seed pairing is not a reliable predictor for miRNA-target interactions (Didiano and Hobert, 2006). Other miRNAs, such as *lin-4*, *let-7*, and *bantam*, generate in vivo functional interactions with G:U pairing or bulged nucleotides in the 5′-seed of experimentally validated 3′UTR target sites (Lee et al., 1993; Wightman et al., 1993; Ha et al., 1996; Feinbaum and Ambros, 1999; Olsen and Ambros, 1999; Pasquinelli et al., 2000; Reinhart et al., 2000; Slack et al., 2000; Ambros, 2003; Brennecke et al., 2003; Stark et al., 2003; Vella et al., 2004; Bagga et al., 2005; Pillai et al., 2005). In plants, G:U base pairing has been shown not to be detrimental for miRNA function (Lu et al., 2005b). Additional experiments will be required to determine if this miRNA circuit is functional in animals.

The conservation of the regulatory circuit in plant species indicates that the miR854 negatively regulates the activity of hnRNP proteins important for mRNA maturation and splicing. This finding shows that some plant miRNAs can imperfectly bind to sequences present within the 3′UTR of their target and generate miRNA:mRNA interactions analogous to those found in animal miRNAs. Furthermore, our results identify miR854 as a member of a family of miRNAs that is shared by flowering plants and animals. Presently it is not possible to determine if this conservation is the result of shared ancestry or of functional convergence from an independent origin, as an explanation of the origins of the miR854 family will require a detailed phylogenetic analysis and molecular characterization. Previous reports indicate that miRNAs but not endogenous siRNAs are conserved among land plants (Axtell and Bartel, 2005), leaving open the possibility that a common ancestor of plants and animals possessed a form of miRNAs. The relationship between miR854 and ATHILA expression remains to be analyzed. Retrotransposons of the ATHILA family are predicted to be among the most ancient insertions in *Arabidopsis* (Pereira, 2004). Interestingly, animal precursors for Mm miR854, Pt miR854, and Hs miR854 are flanked by LTR retrotransposons all located with 2 kb of the mature miRNA (data not shown). The identification of miR854 opens a possibility for a common origin of these miRNAs as regulators of basal transcriptional mechanisms and represents a starting point to investigate functional aspects shared by miRNA regulatory circuits in plants and animals.

## METHODS

### Computational Analysis

The computational identification of miRNA and target mRNA sequences is based on a comparison algorithm (interpreted as a Perl script) between two databases: an intergenic region database built from the *Arabidopsis thaliana* genome (TAIR version 3.0) and a nonredundant 3′UTR database built by joining and curating two publicly available 3′UTR databases supported by cDNA or ESTs (Pesole et al., 2002; http://mips.gsf.de/proj/

thal/db/ publicly downloaded on July 18, 2002). The intergenic region database was built by extracting the sequences located between coding DNA sequences of the *Arabidopsis* genome. The 3′UTR database (13,082 sequences) was used to build a nonredundant oligonucleotide database from the 3′UTR database by extracting a 21- and a 22-nucleotide sliding window from each 3′UTR, moving one nucleotide at a time, and storing each unique sequence. The algorithm compares every sequence from this database (containing 5,442,141 sequences; potential target mRNAs) to every sequence contained in the intergenic region database (potential miRNAs), allowing imprecise pairing or bulged interactions between the 21- or 22-nucleotide oligonucleotide and the intergenic sequence. To consider a site in the 3′UTR sequence as a candidate miRNA target, imprecise pairing and bulged interactions between candidate miRNAs and its potential 3′UTR target were allowed at positions 8 to 12 from the 5′-end of the candidate miRNA sequence. The first seven and the last 10 nucleotides from the 5′-end of the candidate miRNA were required to be perfectly complementary to at least one site in the 3′UTR sequence; G:U pairing was allowed across the predicted duplex. To increase the chances to find a potential miRNA in a stem of a secondary hairpin structure, the algorithm searched for a complementary sequence in the same intergenic sequence where the potential micron mapped. A potential target 3′UTR required two or more binding sites to a particular intergenic sequence (two or more potential miRNA binding sites). Segments of these intergenic regions were subsequently analyzed to determine predicted secondary structures using MFOLD (Zuker, 2003).

### Plant Material and Growth Conditions

Seeds were surface sterilized with 20% bleach and 0.01% Triton X-100 and washed three times with sterile water. Sterilized seeds were plated on Murashige and Skoog medium. Plates were vernalized in darkness for 3 d at 4°C. Plants were grown first in a growth chamber at 22°C under a 16-h-light/8-h-dark photoperiod, transferred to soil, and grown in the greenhouse under long-day conditions.

### RNA Isolation and Analysis

Preparations enriched for low molecular weight RNA were obtained by solubility in polyethylene glycol (Hamilton and Baulcombe, 1999; Mette et al., 2000). Total RNA was extracted from rosette leaves, cauline leaves, stems, and inflorescences of *Arabidopsis* adult plants using Hamilton's homogenization solution (50 mM Tris/HCl, pH 8.5, 10 mM $Na_2EDTA$/NaOH, pH 8.0, 100 mM NaCl, and 2% SDS). Low molecular weight RNA was normalized by spectrophotometry to 60 μg/lane (May et al., 2005), separated by electrophoresis through 15% polyacrylamide, 7 M urea, and 0.5× Tris-borate EDTA gel, and then transferred to Zeta-Probe GT membranes (Bio-Rad). After transfer, membranes were cross-linked with 200 mJ of UV and baked at 80°C for 1 h. Equivalent loading of samples was shown by detection of 5S RNA and tRNA in all gels prior to transfer. DNA oligonucleotide probes for candidate miRNAs were 5′-end labeled using T4 polynucleotide kinase (Invitrogen) and Amersham redivue [γ-$^{32}$P]ATP. In contrast with most miRNA RNA gel blots that are carried at a hybridization temperature ranging from 38 to 50°C, hybridization was performed at high-stringency conditions (55°C) in 7% SDS, 0.5 M $Na_2HPO_4$, pH 7.4, 1 mM EDTA, and 1% BSA overnight. Imaging was performed on a Molecular Dynamics PhosphorImager. A 21-nucleotide RNA oligonucleotide was used as a size marker for electrophoresis and RNA gel blot analysis either by detection with ethidium bromide staining or with a 21-nucleotide DNA radioactive end-labeled probe. To detect *GUS* mRNA in conventional RNA gel blots, a PCR fragment amplified from pBI121 (Clontech) using S1 (5′-AATCTAGATCTCGAGGTGGGAAAGCGCGTT-ACA-3′) and AS1 (5′-AAGGATCCTGGCGCGCCAGCGGGTAGATATCA-CAC-3′) was random-primed using [α-$^{32}$P]dCTP and hybridized at 65°C.

### Plasmid Construction and Plant Transformation

*Arabidopsis* plants of the ecotype Columbia-0 were transformed using the floral dip method (Clough and Bent, 1998) with *Agrobacterium tumefaciens* strain PGV2260 carrying the plasmid pBI121 that harbors the *Pro35S:GUS* or a modified pBI121 plasmid where the 3′UTR of UBP1 was inserted in the *Sac*I site (*Pro35S:GUS:3′ UTR-UBP1b*). Kanamycin-resistant seedlings and wild-type plants were transferred to soil and grown in the greenhouse. *Arabidopsis* T-DNA insertional line SALK_040505 was grown directly on soil, and further phenotypic inspection was performed in T4 and T5 plants.

### Histochemical Analysis of GUS activity

Histochemical localization of GUS activity was performed using a solution containing 10 mM EDTA, 0.1% Triton X-100, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 100 μg mL$^{-1}$ chloramphenicol, and 1 mg mL$^{-1}$ 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid in 50 mM sodium phosphate buffer, pH 7.0. Tissue was incubated in this solution for 24 h at 37°C as previously described (Vielle-Calzada et al., 2000).

### Immunoblot Analysis of Reporter Protein Expression

Samples from the same tissues were used for the estimation of *GUS* mRNA expression and GUS protein abundance. Tissues were ground to powder in liquid nitrogen, and two aliquots were taken either for RNA isolation or for immunoblot analysis. The tissue powder was mixed with 1 volume of diethyl pyrocarbonate–water and shaken for 20 min in an Eppendorf Mixer 5432 at 4°C. Before electrophoresis, extracts were boiled for 5 min in 5× Tris-glycine-SDS loading buffer. The proteins were resolved in 12% SDS/polyacrylamide gels (Laemmli, 1970) and electro-transferred to polyvinylidene difluoride membranes (Bio-Rad Immun-Blot PVDF). Membranes were incubated in 8% milk in TTBS (0.1% Tween-20 in 100 mM Tris-HCl, pH 7.5, 0.9% NaCl) for 1 h at room temperature and then transferred in anti-GUS (Molecular Probes) diluted at 1:5000 in TTBS, overnight at 4°C. After three washes in TTBS, membranes were incubated with the secondary antibody goat anti-rabbit IgG (H+L) conjugated to alkaline phosphatase (Gibco BRL; catalog number 13869-011) at a 1:5000 dilution in TTBS for 2 h at room temperature. Membranes were washed again in TTBS, and signals were resolved using a Bio-Rad alkaline phosphatase conjugate substrate kit (catalog number 170-6432).

### Accession Numbers

Sequence data from this article can be found in the GenBank data library under accession numbers EF190218 and EF190219.

### Supplemental Data

The following materials are available in the online version of this article.

   **Supplemental Figure 1.** miR854 and miR855 Binding Sites in UBP1 Family Members.

   **Supplemental Figure 2.** 3′UTR Region of hnRNP Proteins Homologous to At UBP1b in *Saccharomyces cerevisiae* and *Ustilago maydis*.

   **Supplemental Figure 3.** Mm miR854, Hs miR854, and Ce miR854 Additional Binding sites in the 3′ UTR of TIAR Family Members.

   **Supplemental Table 1.** Genomic Locations of miR854 and miR855.

   **Supplemental Table 2.** Permutation Analysis of Confirmed miRNAs in *Arabidopsis thaliana*.

   **Supplemental Table 3.** Microarray Expression Analysis of At *UBP1b* in Different Genetic Backgrounds.

**Supplemental Table 4.** Genomic Location of Animal miR854 Family Members.

**Supplemental Table 5.** Sequence Composition of Purine-Rich miRNAs.

**Supplemental Table 6.** Bottom-Rank Classification of Confirmed miRNAs According to RANDFOLD.

**Supplemental Text 1.** Perl Script.

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