Cell. Mol. Life Sci. 63 (2006) 246–254
1420-682X/06/020246-9
DOI 10.1007/s00018-005-5467-7

Cellular and Molecular Life Sciences

Research Article

Evidence that miRNAs are different from other RNAs

B. H. Zhang, X. P. Pan, S. B. Cox, G. P. Cobb and T. A. Anderson*

The Institute of Environmental and Human Health (TIEHH), and Department of Environmental Toxicology, Texas Tech University, Lubbock, Texas 79409-1163 (USA), Fax: +1 806 885 4577, e-mail: todd.anderson@tiehh.ttu.edu

Received 5 October 2005; received after revision 4 November 2005; accepted 16 November 2005
Online First 6 January 2006

Abstract. An examination of 513 known pre-miRNAs and 237 other RNAs (tRNA, rRNA, and mRNA) revealed that miRNAs were significantly different from other RNAs ($p < 0.001$). miRNA genes were less conserved than other RNA genes, although their mature miRNA sequences were highly conserved. The A + U content of pre-miRNAs was higher than non-coding RNA ($p < 0.001$), but lower than mRNAs. The nucleotides in pre-miRNAs formed more hydrogen bonds and base pairs than in other RNAs. miRNAs had higher negative adjusted minimal folding free energies than other RNAs except tRNAs ($p < 0.001$). The MFE index (MFEI) was a sufficient criterion to distinguish miRNAs from all coding and non-coding RNAs ($p < 0.001$). The MFEI for miRNAs was 0.97, significantly higher than tRNAs (0.64), rRNAs (0.59), or mRNAs (0.65). Our findings should facilitate the prediction and identification of new miRNAs using computational and experimental strategies.

Key words. MicroRNA; plant; tRNA; rRNA; mRNA; bioinformatics; minimal folding free energy index; MFEI.

MicroRNAs (miRNAs) represent an abundant class of newly identified non-protein-coding small RNAs that negatively regulate gene expression at the posttranscriptional level by targeting mRNAs for cleavage or repressing translation [1–3]. The first miRNA (lin-4) was discovered by the Ambros laboratory in 1993 [4], and is now recognized as the founding member of the large miRNA family [4–6]. In the past 4 years, great progress has been made in this field. miRNAs have been identified that play important roles in many biological processes, including cell identity fate, developmental timing, apoptosis, carcinogenesis, and response to different environmental stresses including disease [7–14]. To date, thousands of miRNAs have been identified (from plants, animals, and viruses) and deposited in miRNA databases [15]. Although the founding members of miRNAs, lin-4 and let-7, were identified by a genetic screening approach, the majority of miRNAs have been identified by computational approaches [15, 16]. One of the core principles of computational approaches for identifying miRNAs is based on the fact that all pre-miRNAs have a stem-loop hairpin in their secondary structure predicted by RNA software such as MFOLD [17, 18]. However, a stem-loop structure is not an unique characteristic of miRNAs [19]. A majority of other RNAs, such as rRNAs, tRNAs, and mRNAs also can fold into hairpin secondary structures. In addition, the secondary structure of plant pre-miRNAs is usually more complex than a simple hairpin structure; most have branched structures [20]. This has made it more difficult and insufficient to predict plant miRNAs using current computational approaches. Recently, Bonnet and colleagues [21] found that miRNA precursors have lower folding free energies than their shuffled sequences. This indicated that pre-miRNAs can form a stable secondary structure for processing by a Dicer-like enzyme. They also concluded that pre-miRNAs possess lower folding free energies than random sequences of other non-coding RNAs based on analysis of miRNAs mostly obtained from animals [21]. However, there were not enough plant miRNAs available when they conducted their comparison;

* Corresponding author.

only 43 plant miRNAs were used in their analysis. In addition, folding free energy was affected not only by secondary structure, but also by the length of the predicted RNA [22]. For plant miRNAs, precursor lengths vary, and the length of other non-coding RNAs also differs from one another. This made the original folding free energies incomparable to each other. To better serve the computational approaches for predicting plant miRNAs, and to better understand plant miRNAs and their function, accuracy guidelines or criteria should be developed.

Currently, 513 plant miRNAs have been deposited in the miRNA database [15]. These miRNAs were obtained from seven different plant species, and belong to 42 miRNA gene families. These data are robust enough to statistically determine their common and unique characteristics. Here, we present a study of 513 plant miRNAs and 237 other RNAs (tRNA, rRNA, and mRNA). We analyzed not only folding free energies, but also base pairs, nucleotide composition, and other characteristics in an effort to find more comprehensive evidence that miRNAs differ from other RNAs. We also analyzed target mRNAs and random mRNAs separately to try to find some evidence for predicting miRNA targets. We also employed statistical tools to analyze the difference between miRNAs and other RNAs. Our results show that miRNAs are significanly different from other RNAs.

Materials and methods

miRNA sequences. Plant miRNAs and their precursor sequences were downloaded from the miRNA database [15]. A total of 513 miRNAs belonging to 42 miRNA families and seven plant species were obtained. The 7 plant species were *Arabidopsis thaliana*, rice (*Oryza sativa*), soybean (*Glycine max*), *Medicago truncatula*, *Saccharum officinarum*, sorghum (*Sorghum bicolor*), and maize (*Zea mays*). A majority of *Arabidopsis* and rice miRNAs have been validated by experimental approaches including direct cloning technologies, Northern blotting, PCR, and/or 5′ rapid amplification of cDNA ends (5′RACE) [15]. A majority of miRNAs in other plant species were homologous to *Arabidopsis* and/or rice miRNAs [15], which were predicted by computational approaches [23–25] and/or expressed sequence tag (EST) analysis [26].

mRNA sequences. Two sets of mRNAs were studied in this research. One was miRNA-targeted genes, the other was miRNA non-targeted genes. A total of 45 target genes were obtained from previous reports [23, 27–39]. The nucleotide sequences of these targeted genes were downloaded from http://www.arabidopsis.org; detailed information about these target genes can be found on this website. Fifty non-targeted mRNA sequences were selected at random from the GenBank DNA database.

tRNA sequences. All tRNAs were obtained from the web-searchable tRNA database: 'compilation of tRNA sequences and sequences of tRNA genes (September 2004 edition)' (http://www.uni-bayreuth.de/departments/biochemie/sprinzl/trna/) [40]. A total of 111 tRNA gene sequences were selected from three plant species, namely *Arabidopsis*, rice, and soybean.

rRNA sequences. Ribosomal RNA (rRNA) sequences were selected at random from the European ribosomal RNA database (http://www.psb.ugent.be/rRNA/) [41]. A total of 30 5S and 31 5.8S small subunits of rRNA were selected at random from different plant species. In this study, only 5S and 5.8S RNAs were chosen due to their small size (similar to pre-miRNAs).

RNA secondary structure. The secondary structure and minimal folding free energies (MFEs) of each RNA gene sequence were predicted by publicly available web-based computer software MFOLD (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/) [17, 18]. MFE, length of sequence, number of nucleotides (A, C, G, or U), and number of base pairs were determined and exported into an Excel file for each type of studied RNA.

The MFOLD software predicts the secondary structure based on minimizing the free energy of the RNA or DNA sequence by maximizing the number of favorable base-pairing interactions [17, 18]. However, the biologically correct structure for RNAs is not often the calculated optimal structure [21, 42]. Therefore, the predicted structures of tRNAs and/or rRNAs by MFOLD may differ slightly from the biologically active structure. To better compare pre-miRNAs with other RNAs, only the optimal folding structure was used in this study.

Multiple sequence alignments. A group of the same gene from different plant species was aligned with the MULTALIN program (http://prodes.toulouse.inra.fr/multalin/multalin.html) [43].

Statistic analysis. All MFEs were expressed as negative kcal/mol. Adjusted MFE (AMFE) represented the MFE of 100 nucleotides. It was calculated by (MFE ÷ length of RNA sequence)×100. The minimal folding free energy index (MFEI) was calculated by the equation:

$$\text{MFEI} = \text{AMFE}/(\text{G}+\text{C})\%$$

All data on MFE, AMFE, MFEI, G+C content, A+U content, and ratios of base pair formation were processed using standard statistical software (SigmaPlot, Version 8.0; SPSS, Chicago, Ill.). Analysis of variance (ANOVA) was chosen for comparing the means of different RNAs on base pairs, U content, A+U content, MFE, AMFE, and MFEI. If there was a significant difference among

groups, LSD multiple comparisons were conducted to compare the mean of each RNA group.

Results and discussion

miRNA precursor genes are less conserved than other RNA genes. Plant miRNAs were only conserved in their mature miRNA sequences, rather than the complete precursor sequences. In addition, the conservation of plant miRNA precursors was lower than that of other RNA sequences, especially for rRNA and tRNA. Figure 1B shows that 5.8S rRNA from different plant species had more than 80% nucleotide sequence identity. Although *ap2* gene transcripts only had 70% identity, this is still significantly higher than the similarities of miRNA precursors (fig. 1A, C).

miRNA precursors contain more A+U, and form more base pairs in their predicted secondary structures than other RNAs. miRNA precursor sequences formed significantly more hydrogen bonds and base pairs than other RNAs (table 1, fig. 2A) ($p < 0.001$). In miRNA precursors, more than 72% of the nucleotides formed hydrogen bonds and base pairs with other nucleotides. In contrast, less than 60% of nucleotides formed hydrogen bonds and base pairs in other coding or non-coding RNAs. More hydrogen bonds and base pairs make miRNA hairpin precursors more stable. This may benefit the transport of miRNA precursors from the nucleus to the cytoplasm. This also possibly allows Dicer-like enzymes to recognize the stem-loop structures and cut miRNA precursors to miRNA : miRNA* complexes and further form mature miRNAs.

miRNA precursors and mature miRNAs contained more A + U nucleotides, especially U, than G + C (table 1, fig. 2B, C) ($p < 0.001$). More than 28% of the nucleotides in pre-miRNAs were U; this was significantly higher than in other RNAs except the random gene transcripts. Given that A-U and G-C form two and three hydrogen bonds, respectively, a higher A-U content may make the pre-miRNA secondary structure less stable and thus easier to be processed into mature miRNA by the RISC complex. In addition, more A + U, especially U content, may serve as a signal for miRNA biogenesis.

miRNA precursors have significantly higher negative minimal free energies and higher minimal free energy index than other RNAs. Formation of the stem-loop hairpin secondary structure is a critical step in miRNA maturation and one of the most important characteristics of pre-miRNAs. However, a stem-loop hairpin structure is not a unique characteristic of miRNA [19]. Other RNAs (mRNA, rRNA, and tRNA) can also form similar hairpin structures (fig. 3). Thus, a potential stem-loop hairpin structure containing the ~22-nt mature miRNA sequence within one arm of the hairpin is a fundamental precondition for predicting and annotating new miRNAs or miRNA homologs, but it cannot be considered a unique criterion for designating a new miRNA.

To avoid designating other RNAs or RNA fragments as new miRNAs, Ambros and colleagues [19] established a uniform system for annotating a new miRNA. In this system, they recommend combining both expression and biogenesis information for identifying new miRNAs. One of these criteria is that the predicted hairpin structure has the lowest folding free energy. However, they did not give any evidence on folding free energies. Recently, Bonnet and co-workers [21] compared the MFEs of miRNAs that were available at that time (a majority of which were animal miRNAs) and other small non-coding RNAs. They found that some miRNA precursors have lower folding free energies than certain tRNAs. However, for most of the miRNA precursors, there was no significant difference. In our study, we also found the same pattern. The folding free energy was not always lower than in other RNAs. For example, the MFEs of *M. truncatula* miRNAs and *Arabidopsis* miRNAs were –57.38 and –58.93 kcal/mol, respectively. These were higher or close to the MFE of 5.8S rRNA (–58.05 kcal/mol). In addition, all folding free energies of all miRNAs from seven plant species were higher than the free energies of mRNA (table 1). Seffens and Digby [22] demonstrated that the MFEs were related to RNA length. The longer the RNA sequence, the lower the MFEs will be. The length of plant miRNA precursors ranges from 60 to more than 400 nucleotides. Thus, a comparision based only on their original MFEs is not appropriate. However, the MFEs are still a good criterion to compare different types of RNA if the sequence length remains constant. Therefore, we developed a new term called the adjusted MFE (AMFE) to make the MFEs comparable. AMFE is defined as the MFE of a 100-nucleotide length of sequence, which is calculated by the equation: AMFE = (MFE/sequence length)×100. After this adjustment, all nucleotide sequences are comparable based on their MFEs. The average AMFE of 513 plant miRNA precursors was –45.93 ± 9.43 kcal/mol, and plant miRNA precursors had significantly higher negative AMFEs than other types of RNA, including tRNAs (–32.67 ± 6.47 kcal/mol), rRNAs (–33.10 ± 2.56 kcal/mol), and mRNAs (–28.53 ± 3.04 or –30.44 ± 2.08 kcal/mol) (table 1).

For a total of 513 known plant miRNA precursors, the minimal negative folding free energies were 8.5–180.8 kcal/mol with an average of 65.05 kcal/mol. A majority of them have a negative MFE of 40–100 kcal/mol. Although this can be easily distinguished from mRNA and tRNA, it is very hard to distinguish miRNAs from 5.8S rRNA because rRNA had a similar MFE range and the

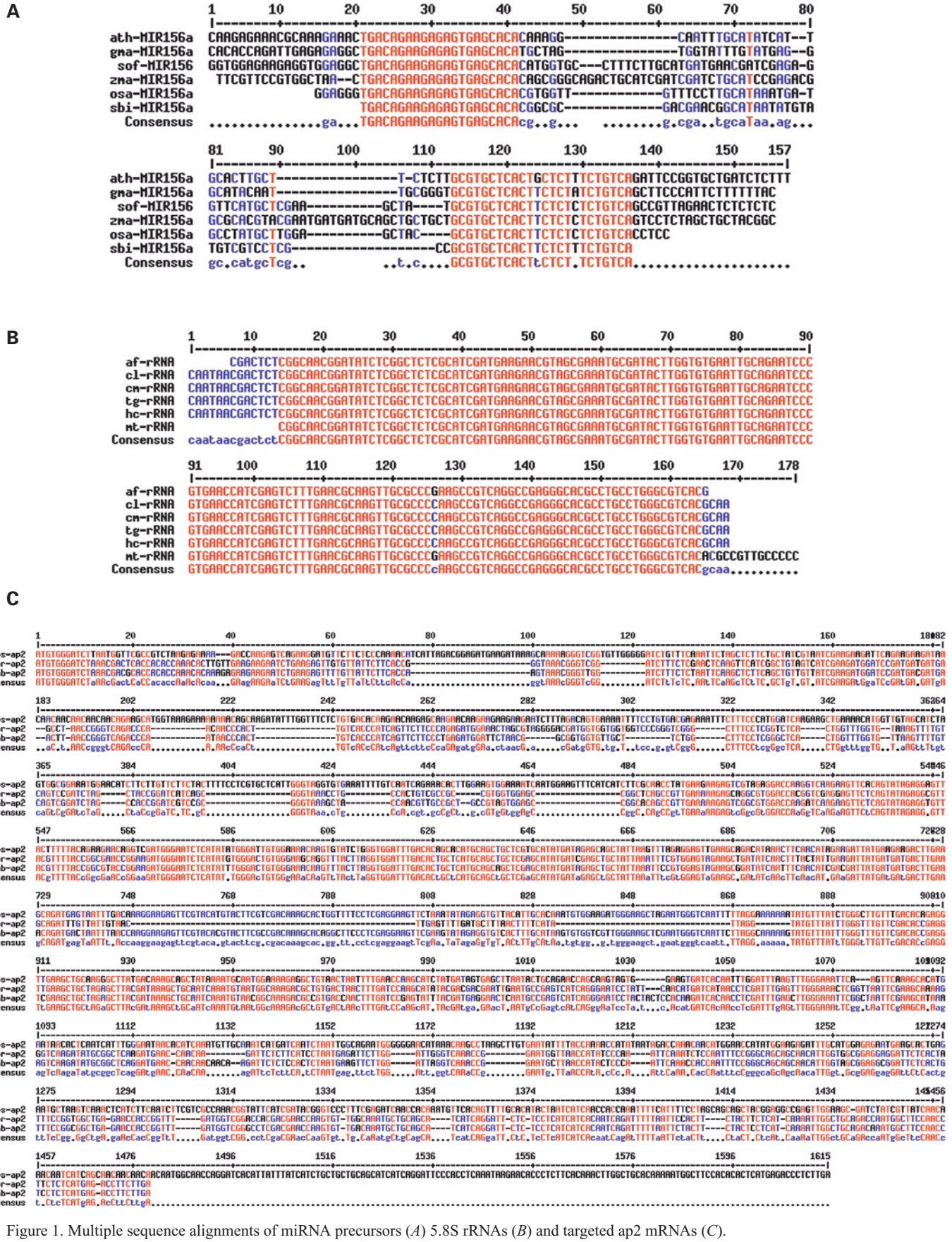

Figure 1. Multiple sequence alignments of miRNA precursors (*A*) 5.8S rRNAs (*B*) and targeted ap2 mRNAs (*C*).

Table 1 Comparison of miRNAs and other RNAs.

	n	Length	MFE	AMFE	bp %	A%	C%	G%	U%	(G+C)%	(A+U)%	MFE
miRNA												
Arabidopsis	117	149.05±63.41	58.93±24.18	40.11±8.54	35.94±3.21	27.19±4.60	19.47±3.82	20.71±3.52	32.56±4.19	40.18±6.18	59.75±6.26	1.00±0.20
Oryza sativa	173	139.34±52.46	65.35±23.82	40.70±10.12	36.84±3.00	23.48±6.13	22.46±5.26	25.58±5.08	28.48±5.50	48.04±9.43	51.96±9.37	1.01±0.24
Glycine max	22	150.05±35.13	62.82±15.41	42.18±5.08	34.93±1.98	24.95±3.26	21.40±2.23	23.96±3.80	29.69±3.99	45.36±4.98	54.64±4.98	0.93±0.11
Medicago truncatula	16	151.06±85.73	57.38±16.01	40.53±6.39	34.06±3.11	27.16±3.74	18.83±3.64	21.58±3.57	32.48±3.78	40.42±5.12	59.64±5.03	1.01±0.14
Saccharum officinarum	16	219.06±67.61	95.09±21.72	45.15±7.14	33.53±2.51	20.46±4.02	25.68±6.29	29.23±2.59	24.64±5.91	54.90±8.53	45.10±8.53	0.83±0.10
Sorghum bicolor	72	132.39±39.59	63.01±18.75	48.32±8.24	35.90±3.25	20.90±4.26	25.14±4.50	26.94±4.26	26.79±5.09	52.08±7.24	47.69±7.30	0.93±0.13
Zea mays	97	142.95±55.37	70.26±24.43	49.88±7.58	35.93±2.60	20.17±4.12	26.62±4.52	28.01±3.77	24.94±5.28	54.63±7.03	45.11±7.25	0.92±0.11
Total	513	144.57±56.91	65.06±23.65^{bc}	45.93±9.43^{a}	36.06±3.05^{a}	23.42±5.61	22.88±5.28	25.04±5.03	28.56±5.71^{a}	47.92±9.34	51.98±9.41^{b}	0.97±0.19^{a}
mRNA												
Random gene	50	1349.84±688.03	392.24±212.06^{d}	28.51±3.04^{d}	29.94±1.82^{b}	28.33±2.40	20.32±3.22	23.35±2.73	28.00±2.59^{ab}	43.67±2.77	56.33±2.77^{a}	0.65±0.06^{b}
Targeted gene	45	1644.31±590.00	504.19±195.31^{e}	30.44±2.08^{cd}	29.70±1.27^{b}	27.34±2.30	23.08±3.09	22.79±1.98	26.76±1.84^{bc}	45.87±2.93	54.10±2.96^{ab}	0.66±0.04^{b}
tRNA												
Arabidopsis	34	74.79±5.46	25.76±3.96	34.48±4.97	30.51±3.39	20.26±3.83	24.00±3.21	30.75±3.56	24.99±3.38	54.76±5.46	45.24±5.46	0.63±0.07
Glycine max	10	73.00±1.05	25.71±3.21	35.20±4.16	29.46±2.42	21.51±4.60	24.10±3.64	29.44±4.22	24.95±5.05	53.54±7.55	46.46±7.55	0.66±0.04
Oryza sativa	67	73.01±5.77	23.10±6.16	31.37±7.12	29.89±3.46	25.11±6.58	22.44±4.40	26.10±6.03	26.35±4.44	48.54±9.19	51.46±9.19	0.65±0.08
Total	111	73.56±5.45	24.15±5.48^{a}	32.67±6.47^{bc}	30.04±3.35^{b}	23.30±6.10	23.07±4.05	27.83±5.64	25.81±4.22^{c}	50.89±8.54	49.11±8.54^{c}	0.64±0.07^{b}
rRNA												
5.8S rRNA	31	175.42±29.37	58.05±10.69^{b}	33.10±2.56^{bc}	28.95±1.30^{b}	22.45±2.26	28.07±2.31	28.10±2.06	21.38±3.59^{d}	56.17±3.75	43.83±3.75^{e}	0.59±0.04^{b}
5S rRNA	30	111.43±28.10	34.60±7.20^{a}	31.60±5.03^{bc}	31.06±5.03^{b}	23.21±4.76	25.06±4.55	28.61±3.05	23.05±2.66^{cd}	53.68±7.03	46.26±7.05^{d}	0.59±0.06^{b}

Values in the same column with different letter(s) are significantly different at the 0.05% level.

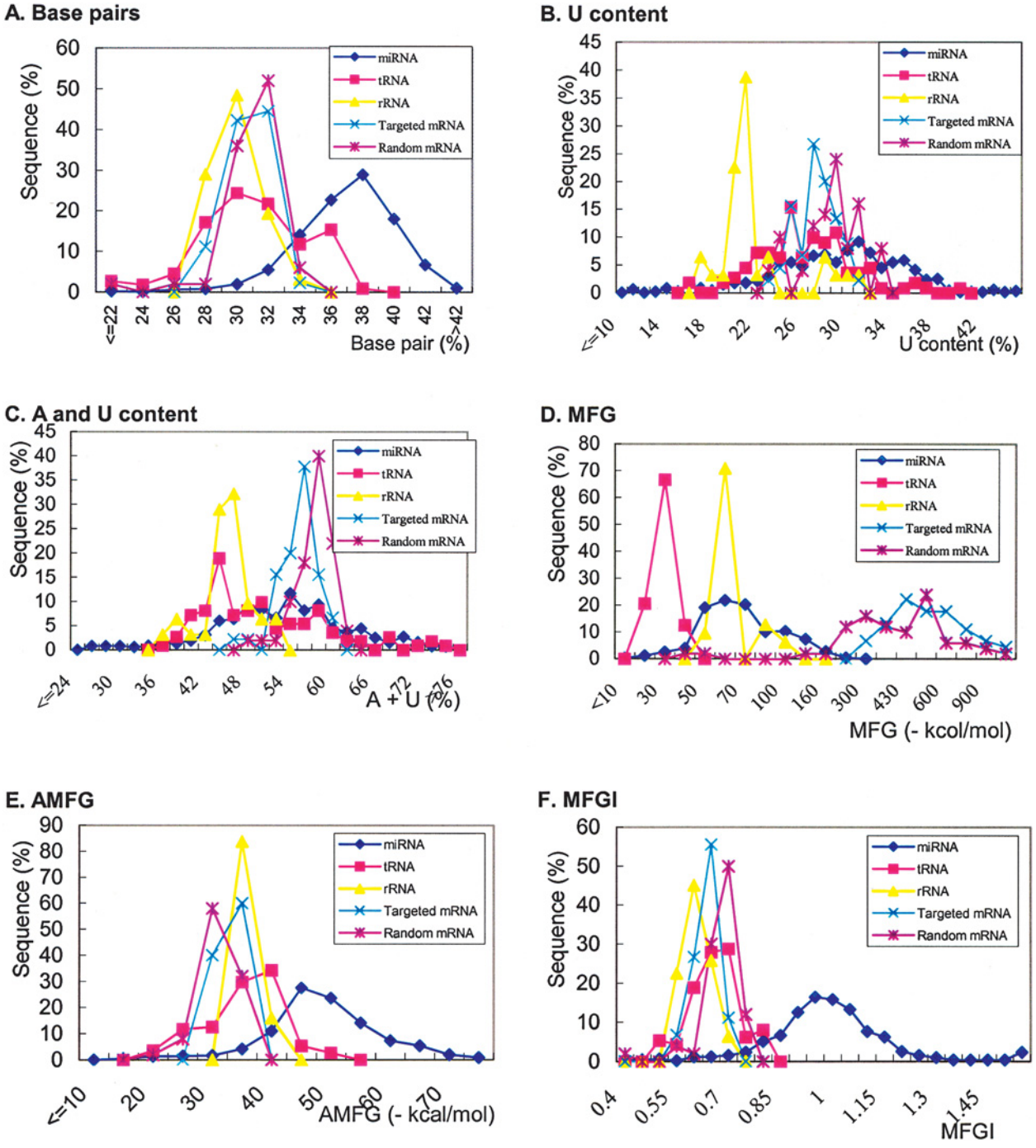

Figure 2. Comparison of miRNA precursors with other RNAs. (*A*) Base bairs. (*B*) U content. (*C*) A+U content. (*D*) Original minimum folding free energy (MFE). (*E*) Adjusted minimum folding free energy (AMFE). (*F*) Minimum folding free energy index (MFEI).

same average MFE (fig. 2D). After accounting for the effect of nucleotide sequence length, AMFE rather than MFE was a better parameter to distinguish miRNAs from rRNA and mRNA. However, the AMFE of more than 50% of tRNAs falls into the range of miRNAs (fig. 2E). To overcome the interference of tRNA, the MFEI gave the best prediction of miRNAs. The average MFEI of 513 known miRNA precursors was 0.97. It was significantly higher than tRNAs (0.64), rRNAs (0.59), and mRNAs (0.62–0.66) ($p < 0.001$). More than 90% of miRNA precursors had an MFEI greater than 0.85, and no mRNAs, tRNAs, or rRNAs had more MFEI higher than 0.85. This suggests that the MFEI can be easily used to distinguish miRNA from other non-coding and coding RNAs. The

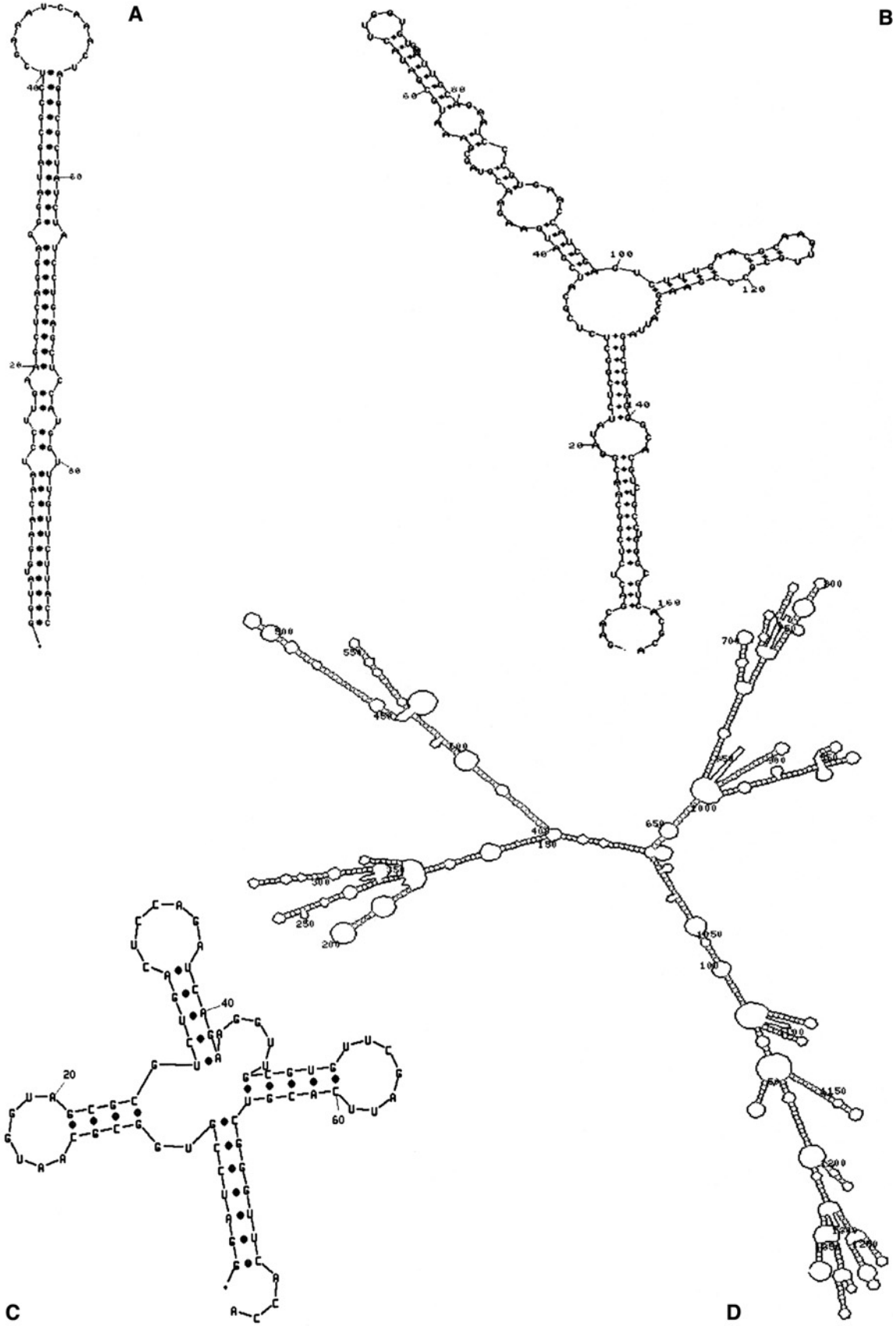

Figure 3. Predicted secondary structures of miRNA 390 (*A*), 5.8S rRNA (*B*), tRNA (*C*), and protein-coding At1g02910 mRNA (*D*). Each type of RNA has a similar hairpin structure for the whole or partial sequence.

MFEI is a unique criterion to designate miRNAs. When the MFEI is more than 0.85, the sequence is most likely to be miRNA.

We also attempted to distinguish targeted mRNAs from random mRNAs. No significant difference was found in this study (table 1).

Conclusion. There are several pieces of evidence that miRNA precursors are significantly different from other non-coding or coding RNAs. miRNA precursors had lower similarity and were less conserved than other RNAs although mature miRNAs are highly conserved. miRNAs had higher AMFEs and a higher base pair rate in their predicted secondary structures than other coding or non-coding RNAs. miRNAs had a lower A + U content than mRNA, but the A + U content in miRNA was higher than in other non-coding RNAs. High A + U content may make mature miRNA easier to separate from the miRNA : miRNA* complex, and become involved in the RISC complex. The MFEI is a sufficient criterion to distinguish miRNA from other types of coding or non-coding RNA. For a majority of miRNA precursors, the MFEI was more than 0.85, with an average of 0.97; it was much higher than in tRNA (0.64), rRNA (0.59), or mRNA (0.65). There was no evidence that miRNA-targeted mRNAs were significantly different from random mRNAs. Our findings should enhance knowledge about miRNAs and allow researchers to predict more new miRNAs using computational strategies and experimental tools.

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