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Identification and characterization of conserved microRNAs and their target genes in wheat (*Triticum aestivum*)

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ABSTRACT. MicroRNAs (miRNAs) are non-coding small RNAs that regulate gene expression by translational repression or transcript degradation. A large number of miRNAs have been identified from model plant species; however, the character of conserved miRNAs is poorly understood. We studied 42 miRNA families that are conserved within the plant kingdom, using the miRBase database. Some conserved miRNA families were found to be preferentially expressed in dicots relative to monocots, especially miR403, miR472 and miR479. Using an improved homology search-based approach and the conserved miRNAs as the query set, 34 conserved miRNAs and the miR482 family were identified in wheat. Forty-six wheat mRNAs were predicted as their putative target genes. Most conserved wheat miRNAs were found to retain homologous target interactions and have analogous molecular functions. The miR172 displayed a wheat-specific function and was found to have an additional target interaction with succinyl-CoA ligase.

We concluded that although miRNAs are conserved, the expression and function of some have drifted during long periods of plant evolution.

Key words: MicroRNAs; Wheat; Target genes; Conservation

INTRODUCTION

MicroRNAs (miRNAs) are endogenous, non-coding small RNAs that regulate the flow of genetic information by controlling the translation or stability of mRNAs (Carrington and Ambros, 2003). It has been estimated that miRNAs account for ~1% of predicted genes in higher eukaryotic genomes, and that up to 10-30% of genes may be regulated by miRNAs (Cui et al., 2006).

Since the discovery of the first miRNA, *lin-4*, in *Caenorhabditis elegans* (Lee et al., 1993), thousands of miRNAs have been identified by experimental cloning or computational approaches. Some miRNAs are difficult to find using cloning or deep sequencing, owing to their physical properties, including sequence composition, methylation, and post-transcriptional modifications (Berezikov et al., 2006). Therefore, computational approaches were widely used as a rapid and affordable method to overcome the limitation of experimental strategy (Zhang et al., 2006a). Many miRNAs are evolutionarily conserved within the same kingdom. Relying on its conservation, Zhang et al. (2005) developed a homology search-based approach for identifying plant miRNAs. Using this approach, more than 700 miRNAs have been identified in plants and viruses (Zhang et al., 2006a; Pan et al., 2007). Since there are a number of non-conserved miRNAs (Lu et al., 2005; Devor et al., 2009), the use of only the conserved miRNA sequences as the query set in the above approach would be more sensitive and specific for the detection of miRNA homologues. Furthermore, analysis of these conserved miRNAs and corresponding families may provide information concerning the phylogenetic distribution, species-specific miRNA family preference and conservation.

With the identification of increasing numbers of miRNAs and their targets, our current knowledge of their regulatory roles has spread over a large spectrum of plant developmental programs, including growth and developmental patterning, metabolic processes, hormone responses, stress defense, and signaling (Jones-Rhoades et al., 2006; Sunkar et al., 2007). Plant miRNAs were found to regulate gene expression by binding to targeted mRNAs in a perfect or near-perfect complementary site (Schwab et al., 2005; Axtell et al., 2007). This suggested that the miRNA-target modules should be conserved in long evolutionary timescales. As expected, earlier studies demonstrated that the target mRNAs of conserved miRNAs have a narrower range of functions than the targets of non-conserved miRNAs (Willmann and Poethig, 2007). Therefore, a comparative analysis of the target genes for ancient miRNA families may broaden our understanding of the conserved regulatory interactions within the plant kingdom.

To date, a total of 2043 miRNAs have been identified in the plant kingdom, and the information has been deposited in the miRBase database (Release 14: September 2009). In this study, we examined 42 conserved miRNA families from the miRBase database in an attempt to increase our knowledge of the phylogenetic distribution and preferential expression of miRNA genes. Furthermore, using an improved computational approach and

the sequences of conserved miRNAs as the query set, 34 conserved miRNAs and 46 target genes were detected in wheat. Most conserved miRNAs have retained homologous target interactions except miR172.

MATERIAL AND METHODS

Sequences of miRNAs, expressed sequence tag, genomic survey sequences, and mRNA

All sequences of mature miRNAs and their precursors (pre-miRNAs) were downloaded from the miRBase database (Release 10.1, http://microrna.sanger.ac.uk/sequences/index.shtml). This set contained 1467 miRNAs from 17 plant species: *Arabidopsis thaliana*, 184; *Oryza sativa*, 243; *Physcomitrella patens*, 220; *Pinus taeda*, 27; *Populus trichocarpa*, 215; *Selaginella moellendorffii*, 58; *Sorghum bicolor*, 72; *Vitis vinifera*, 100; *Zea mays*, 96; etc. On the basis of the conservation of miRNAs, 42 miRNA families present in more than one species were examined. The wheat expressed sequence tag (EST), genomic survey sequence (GSS), and mRNA databases were obtained from the NCBI database site (http://www.ncbi.nlm.nih.gov/).

Prediction of conserved miRNAs in wheat

Minimizing false positives is important for the identification of new miRNAs. The procedure used to search for conserved miRNAs in this study was essentially as described in previous studies (Nasaruddin et al., 2007; Zhang et al., 2007; Yin et al., 2008), but with some modifications (Supplementary Figure S1, A). Herein, to improve the sensitivity and specificity of the prediction, non-conserved miRNA sequences were eliminated. Only the conserved sequences were used as the query to perform BLAST searches against wheat EST and GSS databases. The EST or GSS sequences that have only 0-2 nucleotide (nt) mismatches compared with the query sequences were selected manually, and used to search against a protein database to remove the degradation products of protein-coding sequences. The remaining sequences were used to predict the hairpin structures by Mfold 3.2 (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi). Ultimately, redundant nucleotides were discarded to obtain the mature sequences of new miRNAs in wheat.

Prediction of target genes and the analysis of their functional similarity

The major steps and parameter settings for predicting target genes of miRNAs were performed as described in previous studies (Schwab et al., 2005; Yao et al., 2007; Supplementary Figure S1, B). Briefly, the miRNA sequences identified were used in a BLAST search against the wheat mRNA database. Sequences with only 0-4 nt mismatches compared with the query miRNA sequences were selected manually. The Unigene database contains many transcript sequences that appear to come from the same transcription locus, and information on protein similarities. There are 41,256 entries for wheat in the Unigene database, and closely related wheat mRNAs have been assembled in the Unigene cluster. Thus, data from the Unigene accessions and *Arabidopsis* Small RNA Project (ASRP, http://asrp.cgrb.oregonstate.edu/db/) were used to analyze the functional similarity of target genes.

RESULTS AND DISCUSSION

The conservation of plant miRNAs

It is recognized that many miRNAs are evolutionarily conserved across species boundaries from mosses to eudicots. In this study, 42 conserved miRNA families that are present in more than one plant species were examined from the miRBase database (Table 1). Among them, 8 families (miR156/157, miR159, miR160, miR166, miR167, miR171, miR319, and miR396) are present in more than 10 species; 12 families are present in 6 to 10 species, and the remaining 22 families are present in 2 to 5 species.

Table 1. The number of land plant species that contain a conserved microRNA (miRNA) family and the corresponding conserved miRNA families.

Number of plant species	Conserved miRNA family
11-17	miR156/157 miR159 miR160 miR166 miR167 miR171 miR319 miR396
6-10	miR162 miR164 miR168 miR169 miR172 miR390 miR393 miR394 miR395 miR398 miR399 miR408
2-5	miR161 miR397 miR403 miR413 miR414 miR415 miR416 miR417 miR418 miR419 miR420 miR426 miR444 miR472 miR477 miR479 miR482 miR529 miR535 miR536 miR783 miR824

All families are present in more than one plant species in the miRBase database (Release 10.1).

During screening of conserved miRNA families, we found that the conserved miRNAs mainly exist in dicots and monocots, and are less frequent in ferns and mosses. The proportion of conserved miRNAs is 0.823 and 0.614 in poplar and rice, respectively. However, the proportion in *S. moellendorffii* and *P. patens* is only 0.281 and 0.264, respectively. Since the bryophytes are the most ancient land plants, this observation seems in contrast to the hypothesis that the conservation of miRNAs is the result of shared ancestry or functional convergence from an independent origin during evolution (Maher et al., 2006). One possible explanation is that miRNA genes were subjected to strong purifying selection during evolution, and that some were lost, while others experienced multiple rounds of duplication (Jiang et al., 2006; Fahlgren et al., 2007; Axtell, 2008).

To analyze the preferential expression of miRNAs, we plotted the number of miRNA members in each conserved family that are found in the sequenced (or almost fully sequenced) genomes for dicots and monocots. We used *Arabidopsis*, poplar and grape for the dicots, and rice, sorghum and maize for the monocots. We found that most miRNA families have an equal number of members between dicots and monocots. In a few species, miRNA families were preferentially expressed in dicots relative to monocots, especially miR403, miR472 and miR479 (Figure 1). This specific distribution may be indicative of their specific function in miRNA-mediated gene regulation in dicots, although the details of this process are unknown.

Identification of conserved miRNAs in wheat

In order to improve the sensitivity and specificity of miRNA prediction, we eliminated non-conserved plant miRNA sequences, and used only the sequences of 42 conserved miRNA families described above as the query set. Following a set of strict filtering criteria, a total of 34 conserved miRNAs were detected in wheat. They were classified into 18 families on the

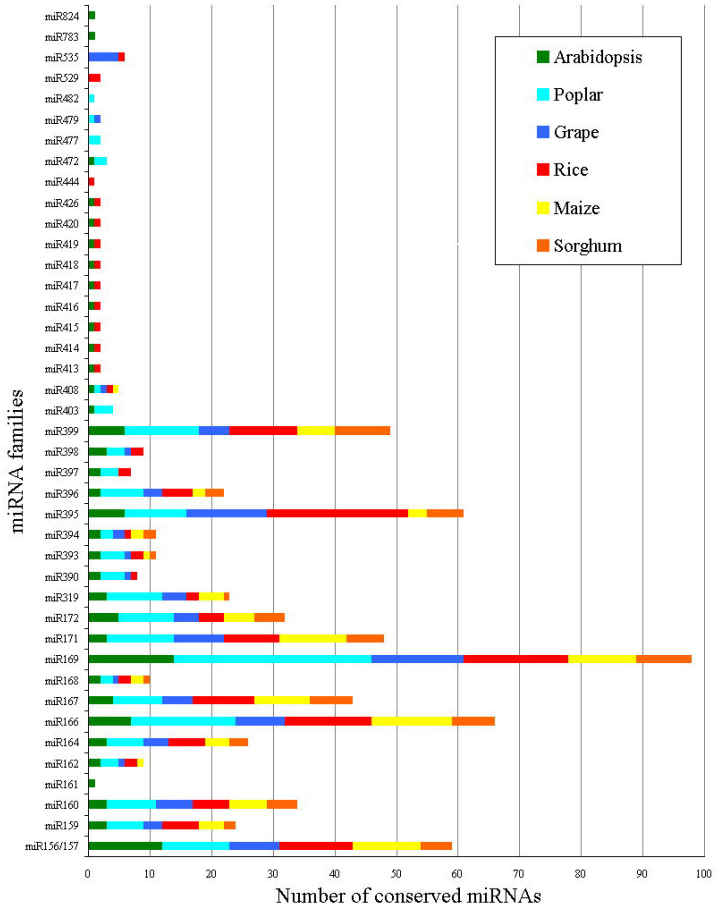

Figure 1. The number of miRNAs in each conserved family (dicots, green/greenish/blue; monocots, red/yellow/reddish).

basis of sequence similarity (Table 2). The mature sequences of 25 miRNAs begin with 5'-uridine, a characteristic feature of plant miRNAs. Among the 34 wheat miRNAs identified, 14 have the same nucleotide sequences as the query miRNAs. This suggests that these miRNAs are more highly conserved than others.

Considering that computational approaches or high-throughput sequencing have identified many wheat miRNAs, the mature sequences of the 34 identified miRNAs were compared with published data for miRNAs (Yao et al., 2007; Dryanova et al., 2008; Jin et al., 2008; Han et al., 2009; Wei et al., 2009). BLAST results show that 27 miRNAs have identical sequence with previously reported miRNAs in wheat. The miR482 family and the other miRNAs, including miR172b, miR319b, miR414c, miR419, and miR444b, were newly identified. The precursors of newly identified miRNAs had a high minimal folding free energy

Table 2. MicroRNAs (miRNAs) in wheat.

miRNA	ID	Sequence	End/Source	A+U (%)	MFEI
miR156a	51666269	ugacagaagagagugagcac	5′/GSS	46.43	0.925
miR156b	20120267	cugacagaagaGagagagcaU	5′/EST	50.12	0.712
miR156c	20122061	ugacagaagaGagagagcac	5′/EST	50.12	0.754
miR159	93066835	uuggaAugaagggagcucca	3′/EST	45.82	0.744
miR160a	93162853	ugccuggcucccuguaugcca	5′/EST	36.67	0.909
miR160b	93237563	ugccuggcucccuguaugccU	5′/EST	39.69	0.738
miR164	25426214	uggagaagcagggcacgugca	5′/EST	30.26	0.898
miR167a	143399055	ugaagcugccagcaugaucua	5′/EST	48.68	0.921
miR169a	19956307	uagccCaggGugacuugccca	5′/EST	39.39	0.889
miR169b	93186556	aagccaaggaugaGuugccug	5′/EST	40.37	0.701
miR171	32685227	ugauugagccgugccaauauc	3′/EST	38.30	0.816
miR172a	9696528	aAaauccugaugaugcugcaG	5′/EST	40.75	0.726
miR172b	**143362423**	**AgGaucuugaugaugcugcag**	**5′/EST**	**61.11**	**0.781**
miR319a	24978824	uuuggaUugaagggagcucU	3′/EST	52.80	0.927
miR319b	**24977949**	**GuggacugaagUgagcucccu**	**3′/EST**	**48.73**	**0.922**
miR319c	25440767	cuuggacugaagUgagcuAc	3′/EST	32.33	0.740
miR319d	75684012	uuggacugaagggagcucccu	3′/GSS	48.99	0.965
miR395a	39556435	augaaguguuuggggaacuc	3′/EST	56.75	0.909
miR395b	55605069	ugaaguguuuggggaacuc	3′/EST	54.55	1.162
miR395c	39556094	uugaaguguuuggggaacuc	5′/EST	57.41	0.942
miR396	141574195	uccacaggcuuucuugaacug	5′/EST	45.26	0.808
miR398	93224516	uguguucucaggucgccccCgC	3′/EST	44.19	0.923
miR399a	93255560	ugccaaaggagaguugccc	3′/EST	41.33	0.925
miR399b	93234499	ugccaaaggagaauugccc	3′/EST	40.35	0.899
miR399c	93057089	ugccaaaggagaguugcccug	3′/EST	41.77	0.976
miR414a	93058127	ucaucAucaucaucaucgucG	3′/EST	41.01	0.727
miR414b	93270953	ucaucAucaucaucGucguca	5′/EST	40.42	0.741
miR414c	**93236205**	**ucauccucaucCucaucgucc**	**3′/EST**	**47.62**	**0.719**
miR415	92237256	GCcagagcagaaacagaacau	3′/EST	52.17	0.832
miR419	**39002573**	**AuaugaaugcugaggUuguug**	**5′/EST**	**61.54**	**0.975**
miR444a	39562974	uugcugccucaagcuugcugc	3′/EST	50.33	1.021
miR444b	**70966362**	**uugcugUcucaagcuugcugA**	**3′/EST**	**46.02**	**0.919**
miR482a	**29168987**	**ucuucccAacuccCcccauucc**	**3′/EST**	**52.60**	**0.727**
miR482b	**22099570**	**ucuucccCacuccCcccauucc**	**3′/EST**	**45.24**	**0.722**

The miRNAs newly identified in wheat are shown in bold. The capital letters in sequences represent the mismatched nucleotides. MFEI = minimal folding free energy index; GSS = genomic survey sequence; EST = expressed sequence tag.

index of 0.719-0.975 (Table 2), which is significantly higher than those reported for tRNAs (0.64), rRNAs (0.59), and mRNAs (0.62-0.66) (Zhang et al., 2006b). The diverse fold-back of pre-miRNAs required a length of 41-462 nt (Supplementary Figure S2), which is similar to findings in other plant species.

Conserved miRNA-target interactions in wheat

Gaining insight into miRNA target genes can shed light on the range of miRNA regulation and can lead to a detailed description of miRNA-target interactions. Herein, a total of 46 target genes were detected in the wheat mRNA database (Table 3). Subsequently, the entries in the Unigene database and ASRP were selected to examine the functional similarity of predicted target mRNAs.

Most target mRNAs in wheat were similar or related to previously validated miRNA targets in *Arabidopsis*, rice, and popular. For instance, squamosa-promoter binding protein-like (SPL) genes were predicted to be targeted by miR156. This consists of the recent demonstration that miR156 target members of the SBP-box gene family in both mono- and di-

Table 3. The target genes of wheat microRNAs (miRNAs).

miRNA	Targeted protein	Target genes	Conserved with *Arabidopsis* or other plant species
tae-miR156	SPL2	Ta.3711	Yes
	SPL3	Ta.6374	Yes
	SPL11	Ta.7021 Ta.67187	Yes
	Unknown	AL810223 CJ653710	
tae-miR 159/319	MYB domain protein	Ta.24098 Ta.49649 Ta.58485	Yes
	Unknown protein	Ta.2427 Ta.58485 Ta.29557 DR737225	
tae-miR160	Auxin response factor 10	Ta.13246	Yes
	Auxin response factor 16	Ta.35774 Ta.44707	Yes
tae-miR164	NAC domain containing protein	Ta.33080	Yes
tae-miR167	Auxin response factor 6	Ta.9550 Ta.38988 Ta.63221	Yes
	Auxin response factor 8	Ta.6394	Yes
tae-miR169	CBF-B/NF-YA family protein	Ta.10047 Ta.48680 Ta.57252 Ta.49366 Ta.38584	Yes
tae-miR171	Scarecrow-like transcription factor (SCL)	Ta.39354	Yes
	Hypothetical protein	Ta.36668	
tae-miR172	TARGET OF EAT (Apetala2-like protein)	Ta.13336 Ta.24445	Yes
	Succinyl-CoA ligase (GDP-forming) beta-chain	Ta.14585	No
	Unknown protein	Ta.11232	
tae-miR395	ATP sulfurylase	Ta.9352	Yes
	Unknown protein	Ta.39519 CV779320	
tae-miR396	Growth-regulating factor 3	Ta.36890	Yes
tae-miR399	Unknown protein	Ta.43848 CK210056	
tae-miR414	Unknown protein	Ta.23809 Ta.38775 Ta.6362	
tae-miR415	Aminoacylase, putative/N-acyl-L-amino-acid amidohydrolase	Ta.9380	
tae-miR482	Quinolinate synthetase	Ta.35819	
	Unknown protein	Ta.26024 Ta.13326 Ta.24321	

cots, and have a conserved role as positive regulators of the vegetative to reproductive phase transition (Schwarz et al., 2008). Auxin response factors were predicted as targets of miR160 and miR167. In addition, other miRNA-target interactions were also conserved in wheat as expected, such as miR171 - SCL (scarecrow-like transcription factor), miR395 - ATP sulfurylase, miR396 - growth-regulating factor, etc. (Table 3). Among the targets, the additional targeting of succinyl-CoA ligase by miR172 was of particular interest. In the plant kingdom, miR172 have been well known to TARGET OF EAT (TOE) transcription factors (Mlotshwa et al., 2006). Herein, the mRNAs of succinyl-CoA ligase have a complementary site similar to that of TOE, as illustrated in Figure 2. This novel phenomenon suggested that species-specific regulations for miR172 may exist in wheat. In popular, Lu et al. (2005) found that ptr-miR156 target not only SPL genes but also nitrate transporter, exhibiting a popular-specific function. These observations suggest that the function of some well-conserved miRNAs have drifted during long periods of plant evolution. The recognition of target genes by miRNAs requires a high degree of base-pairing between miRNA sequence and target sites. Although miRNAs are well conserved in long evolutionary timescales, some of their sequences have changed and display variations in a few nucleotide positions (Axtell and Bowman, 2008). It provides the chance for some miRNAs to base pair with other target mRNAs, exhibiting species-specific regulatory pattern.

In summary, 42 conserved miRNA families were examined from the miRBase database. The distribution analysis showed that some conserved miRNA families have a preferential expression in dicots relative to monocots, especially miR403, miR472 and miR479. Subsequently, 34 conserved miRNAs were identified in wheat. Five miRNAs (miR172b,

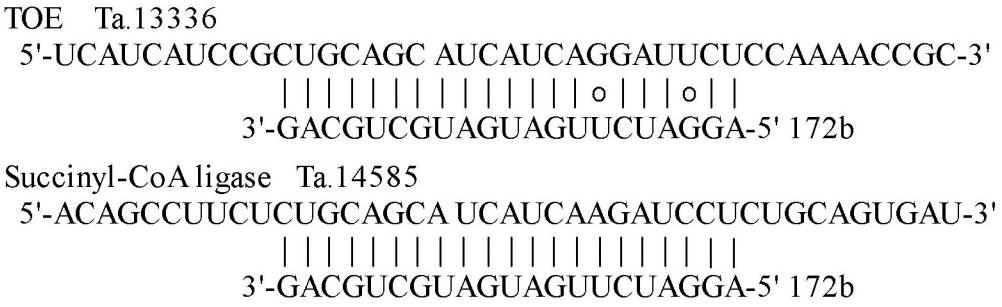

Figure 2. Two predicted target mRNAs of miR172. Succinyl-CoA ligase mRNAs has a complementary site similar to that of TOE mRNAs, which are targeted by miR172.

miR319b, miR414c, miR419, and miR444b) and the miR482 family were newly identified. Most of the conserved wheat miRNAs have retained homologous target interactions throughout plant evolution. However, miR172 display a wheat-specific function and have an additional target interaction with succinyl-CoA ligase. These results suggest that the expression and function of some conserved miRNAs have drifted during long evolutionary timescales. The next major step will be to experimentally verify the above observations. The identification of these miRNAs and target genes reveal the future path leading to the understanding of the core regulatory interactions in wheat.

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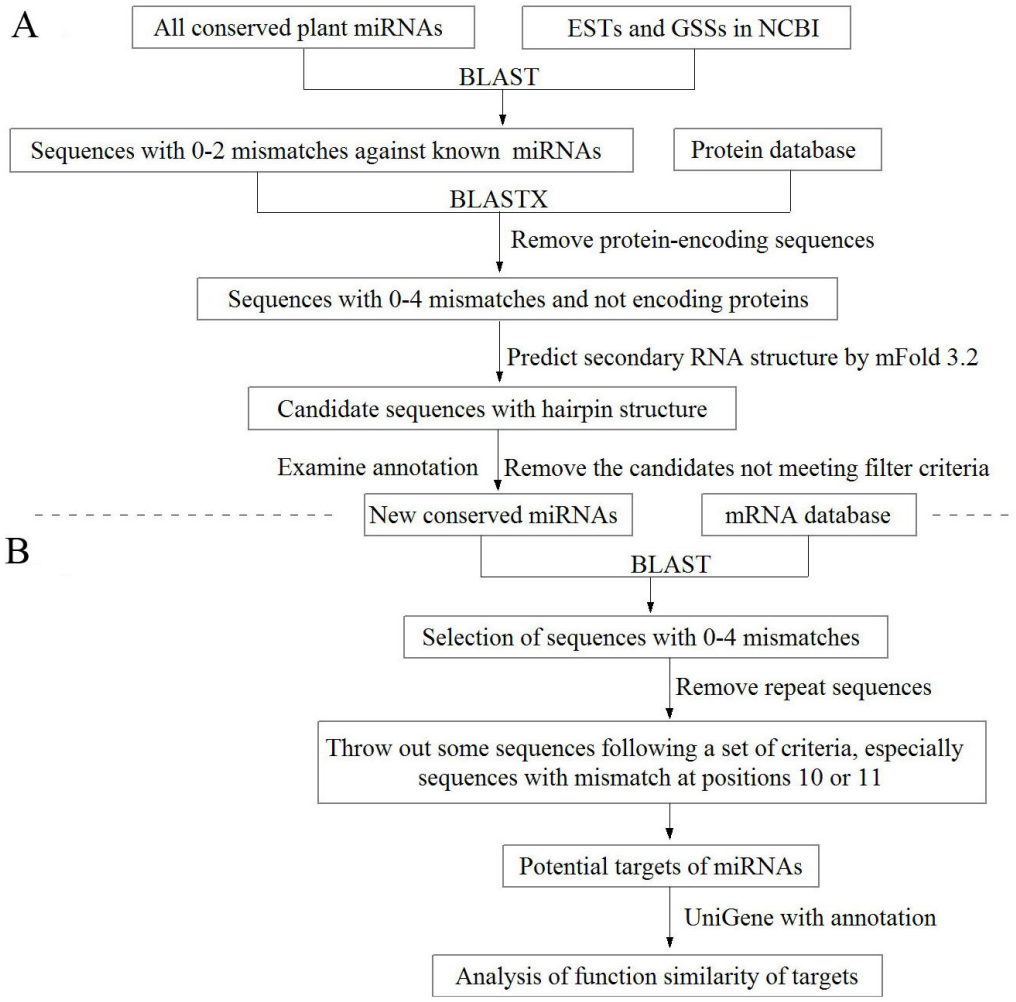
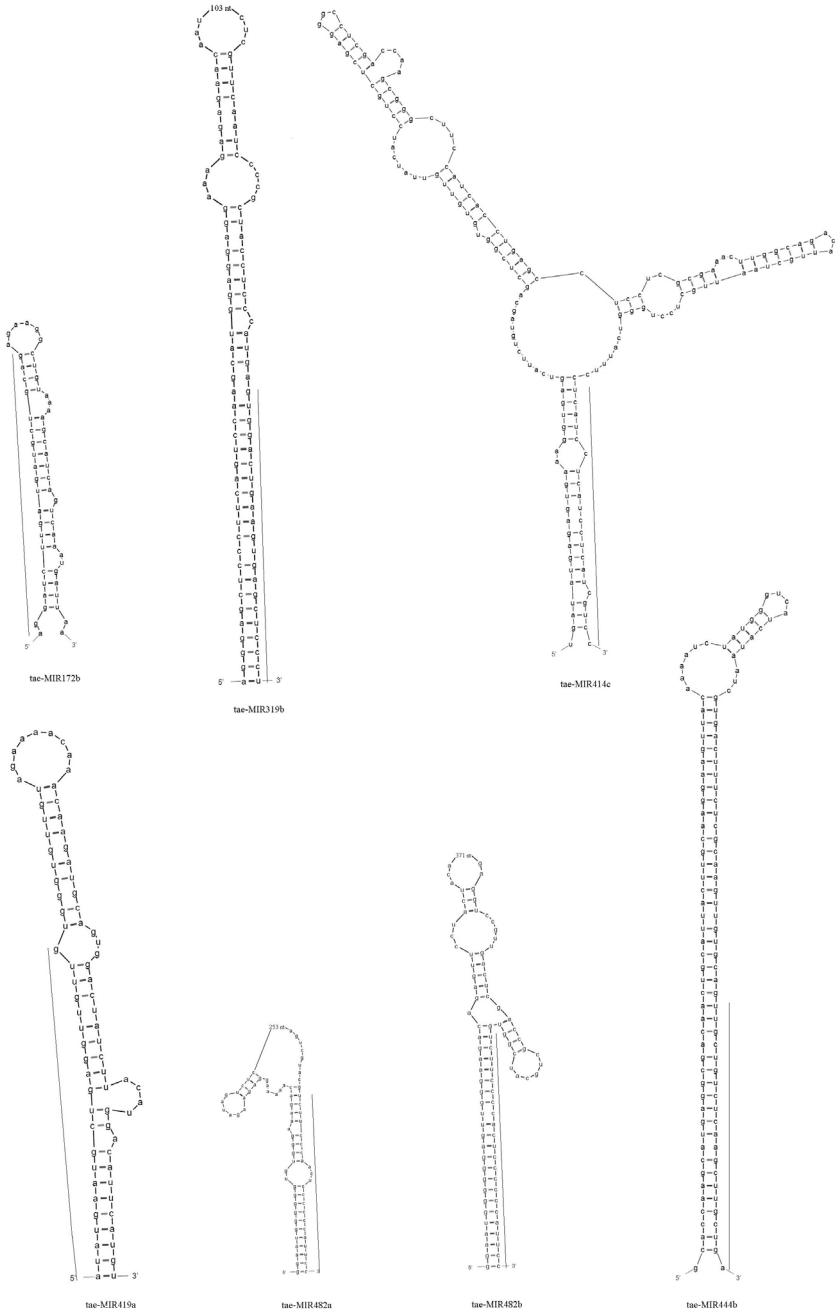


Supplementary Figure S1. Flowchart for the prediction of conserved miRNAs and their target genes in wheat. **A.** Schematic representation for the prediction of conserved miRNAs. **B.** Schematic representation for the prediction of target genes of the miRNAs identified.



Supplementary Figure S2. Predicted fold-back structures of the newly identified 7 miRNA precursors in wheat. Sequences of mature miRNAs are underlined.