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Cloning and characterization of pre-miR159a and pre-miR1123 from Indian hexaploid wheat and their evolutionary linkage analysis

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MicroRNAs (miRNAs) are important post-transcriptional regulator of genes in plants. In spite of their unusual small size (-21-24 nt), the evolutionary history of miRNA gene families appears to be similar to their protein-coding counterparts. Although many miRNA genes are conserved across the plant species, the same gene family varies significantly in size and genomic organization in different species. In the present study, we characterized *Tae* pre-miR159a (*Triticum aestivum* precursor-miR159a) and *Tae* pre-miR1123 from a most popular Indian wheat cultivar Agra local. miR159a is conserved across the species while miR1123 is reported in wheat. Sequence analysis of the pre-miR159a among different plant species suggest conserved motif in duplex miRNAs. Beside mature miRNAs, certain other regions are also found to be conserved across species which might be the site of processing of the precursors by DCL1. Sequence identity matrix suggests 43-82% variation in precursor of *Tae* AL pre-miR159a (*Tae* Agra local pre-miR159a) across the species. On the other hand, *Tae* AL pre-miR1123 (*Tae* Agra local pre-miR1123) showed >83% sequence similarity with the reported sequence of *Tae* pre-miR1123. Sequence similarity of precursor and matured miRNA across the species suggests the common evolutionary point from the same gene family which is essential for the processing and stem loop structure of the precursor miRNAs.

Key words: Cloning, stem-loop structure, sequence comparison, duplex, precursor.

INTRODUCTION

MicroRNAs are generated from larger precursors which usually form a stem-loop structure (Li and Mao, 2007). In plants, a ribonuclease III-like protein in the nucleus called DICER-LIKE 1 (DCL1) is responsible for processing of

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the primary miRNA (pri-miRNA) gene transcript (Papp et al., 2003). The processed pri-miRNA is called pre-miRNA which is eventually processed into mature miRNA: miRNA* duplex (guide stand: passenger strand) (Bartel and Chen, 2004). The mature miRNAs duplex is then transported into the RNA-induced silencing complex (RISC) which either degrade the corresponding target mRNA or repress its translation. In both animal and plant genomes, multiple precursors are present to produce similar mature miRNA (Tanzer et al., 2005; Maher et al., 2006). Animal genomes have a large number of small miRNA gene families while plant genomes have fewer but larger miRNA gene families. Plant miRNAs derived from the same gene family are often highly similar unlike animal miRNA genes where divergence has occurred even on the mature miRNA sequences. The similarities are not only restricted to mature miRNA regions but also throughout the genes suggesting the recent origin of plant miRNA gene families which might still be going on. Recently Zhang et al. (2006) has reported a total of 481 miRNAs from more than 6 million plant EST sequences that belong to 37 miRNA families from 71 different plant species.

In the recent past, efforts have been put forth to identify the different mechanism of evolutionary origin of miRNA genes. Four mechanisms have been proposed so far. First mechanism implies evolutionary origin of miRNA genes *via* duplication of protein coding genes (Fahlgren et al., 2007, 2010). Second mechanism involves the conversion of transposable elements in to miRNA genes (Piriyapongsa and Jordan, 2008). Third and fourth includes duplication of pre-existing miRNA genes with subsequent mutation and natural origin by spontaneous mutation from hairpin structure in the genome respectively (Li and Mao, 2007; De Felippes et al., 2008). Plant miRNAs have been reported to be conserved across wide taxa of gymnosperms, fern, moss, and liverwort (Floyd and Bowman, 2004; Cuperus et al., 2011). Conserved nature of miRNAs across the species might be due to the specific mechanism of origin over the years.

MicroRNAs are a gene family united not by a common sequence, nor by a common phenotypic output, but by a unique mode of biogenesis and molecular mechanism (Axtell, 2008). Most of the predicted targets of these microRNAs are members of transcription factor “gene families” involved in developmental patterning or cell differentiation (Rhoades et al., 2002) that is, miRNA genes also form gene families. Since the evolution of animal miRNA gene families has been a subject of many paper (Tanzer and Stadler, 2006; Bompfunewerer et al., 2005), in this paper we cloned and characterized the *Tae* pre-miR159a and *Tae* pre-miR1123 from Indian hexaploid wheat cultivar Agra local and compared the obtained sequences with the sequences of same miRNA reported in the other plant species to study the evolutionary linkages of miRNA gene families in wheat genomes.

MATERIALS AND METHODS

Plant materials

Hexaploid wheat cultivar Agra Local was grown at 30 seeds per 10 cm pot at 15-25°C temperature under natural lighting conditions for ten days in glass house at Division of Plant Pathology, IARI, New Delhi. This cultivar is characterized by its larger area of production as well as susceptibility to stem rust as reported by our previous publication (Gupta et al., 2012). Leaf samples were collected and quickly frozen in liquid nitrogen and stored at -800°C until used for total RNA isolation and subsequent cloning.

Isolation of total RNA from leaf sample

Leaf samples were harvested from healthy plants of 10 days old seedlings and quickly frozen in liquid nitrogen and stored at -80°C prior to total RNA isolation. Total RNA was extracted from 100 mg of leaf tissues using RNeasy Plant Kit (Qiagen) according to manufacturer’s instruction. The purity and concentration of RNA was determined by spectrophotometer NanoDrop, ND-1000 (NanoDrop technologies).

cDNA synthesis and PCR amplification

Specific primers were synthesized from Sigma Pvt Ltd. Primer sequences of pre-miR159a were designed from chromosome no. 1 of *Arabidopsis* collected from NCBI and pre-miR1123 were designed from wheat miR1123 sequences available in Sanger miRBase http://miRNA.sanger.ac.ck. For cDNA synthesis, Reverse transcriptase (RT) (NEB) was used. cDNA was synthesized using the reverse primer of both the miRNAs (pre-miR159a reverse primer: 5’TGA GTC GAC ATG TAG AGC TCC CTT CAA TCC3’ Tm:90°C; pre-miRNA 1123 reverse primer: 5’TTC TAT GAG ACC AGG TCT CAC3’ Tm:62°C) and the reaction mixture was prepared in 200 µl microfuge tube.

The reaction was carried out at 42°C for 60 min followed by purification by cDNA purification kit (Qiagen). A ~175 bp and ~220 bp fragment containing the entire sequence of the wheat pre-miR159a and miR1123 respectively were amplified by PCR amplification using complementary DNA (cDNA). 20 µl PCR reaction volume was prepared using 2 µl of cDNA (25 ng/µl) as template, 1 µl (10 mM) reverse primer, 1 µl (10 mM) forward primer (pre-miR159a forward primer: 5’CAC CAC AGT TTG CTT ATG TCG GAT CC3’ Tm:78°C; pre-miRNA 1123 forward primer: 5’AAA ATT ATA TGA GAC CAG GCT C3’ Tm:60°C), 1 µl (25mM) $MgCl_2$, 0.25 U Taq DNA polymerase (NEB) and 11.5 µl sterile distilled water (SDW) using PCR programme (94 °C for 4 min followed by 30 cycles of 94°C for 40 s, 58°C for 30 s and 72 °C for 90 s for pre-miR159a and 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 58°C for 1 min and 72°C for 1 min for pre-miR1123). PCR amplification was performed in a thermo cycler separately (ERICOMP Power Block II System). The amplified product was separated on 1% agarose gel run in 1X TAE buffer and detected by ethidium bromide staining.

Cloning of amplified product and restriction analysis

Amplified products were gel eluted and purified by Genei PCR

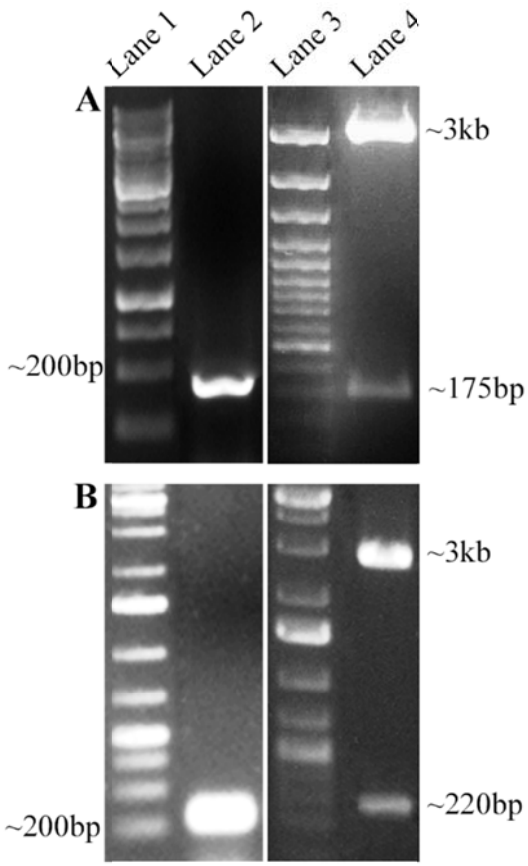

Figure 1. Amplification and confirmation of pre-miR159a and pre-miR1123 from Agra local wheat cultivar. **(A)** Lane 1 and 3, molecular weight marker (1 kb and100 bp respectively); lane 2, PCR amplicon of pre-miR159a; lane 4, restriction digestion of pre-miR159a to release ~175 bp amplicon from pGEMT. **(B)** Lane 1 and 3, molecular weight marker (1 kb); lane 2, PCR amplicon of pre-miR1123; lane 4, restriction digestion of pre-miR1123 to release ~220 bp amplicon from pGEMT.

purification kit following the manufacturer's protocol. Purified PCR product was used for T-A cloning in p-GEMT Easy vector (3015 bp) provided by Promega according to Sambrook and Russell (2001). Positives clones were selected based on blue white screening followed by colony PCR and *Not I* restriction digestion. After restriction analysis, positive clones were sequenced and obtained sequences were analysed *in silico* using BioEdit Sequence Alignment Editor (version 5.0.9).

Bioinformatic analysis

Raw sequences of both the pre-miRNAs obtained after sequencing were used to analyse the evolutionary linkage with the sequence of same pre-miRNAs across the different plant species. Pre-miRNA159a sequences from different plant species were collected from Sanger miRBase (http://miRNA.sanger.ac.ck). The sequence of wheat specific Pre-miR1123 from Agra local cultivar was compared with the reported sequence of wheat. BioEdit Sequence Alignment Editor (version 5.0.9) was used for the sequence and multiple sequence alignment analysis. Secondary stem loop structure of both the pre-miRNA sequences was done using MFOLDROOT secondary structure prediction software (http://www.bioinfo.rpi.edu/applications/mfold).

RESULTS

Amplification, cloning and sequencing of pre-miR159a

An amplicon of ~175 bp corresponding to *Tae* AL pre-miR159a was obtained by PCR from wheat cultivar Agra local (AL). The PCR purified amplicon (~175 bp) was then cloned in p-GEMT Easy vector (Figure 1A, lane 2). Fifty white colonies were found on X-gal, IPTG, ampicillin plate. All these colonies were streaked on master plate separately. From the master plates, ten colonies were subjected to colony PCR using the specific primer described earlier. On the basis of the colony PCR results, five clones were selected for recombinant plasmid isolation. Final confirmation was carried out by releasing ~175 bp fragment using *Not I* restriction digestion (Figure 1A). Two clones (p-GEMT- *Tae* AL pre-miR159a) was sequenced to get the nucleotide sequence of AL pre-miR159a. Similar approach was used to amplify and clone the ~220 bp amplicon of AL pre-miR1123 (Figure 1B, lane 4). The raw sequences obtained were analysed using BioEdit Sequence Alignment Editor (version 5.0.9) to get the precursor sequence of *Tae* AL pre-miR159a and *Tae* AL pre-miR1123.

Sequence analysis of pre-miR159a and pre-miR1123

Sequence analysis of pre-miR159a from 11 different reported plant species indicates the presence of exact 21 nt long mature miRNA towards the 3' end. The passenger strand of mature miRNA residing at 5' end of gene showed 5-6 mismatches. On close comparison, it was found that mismatches in miRNA duplex are conserved in the species (Figure 2). Although the overall sequence similarity of *Tae* AL pre-miR159a was 82% with *At* pre-miR159a (*At*: *A. thaliana*) and only 64% with reported *Tae* line3338 pre-miR159a, the pattern of mismatches of duplex miRNA (miRNA:miRNA*) within species were found to be exactly same (Figure 3A). Sequence identity matrix suggests 43-82% variation in precursor of miR159a (Table 1). Multiple sequence alignment showed conserved motif at 3' and 5' end of miRNA gene representing mature miRNA and its complementary sequence respectively. Besides this, certain motif were found to be conserved which might be essential for processing of precursor sequences by DCL1.

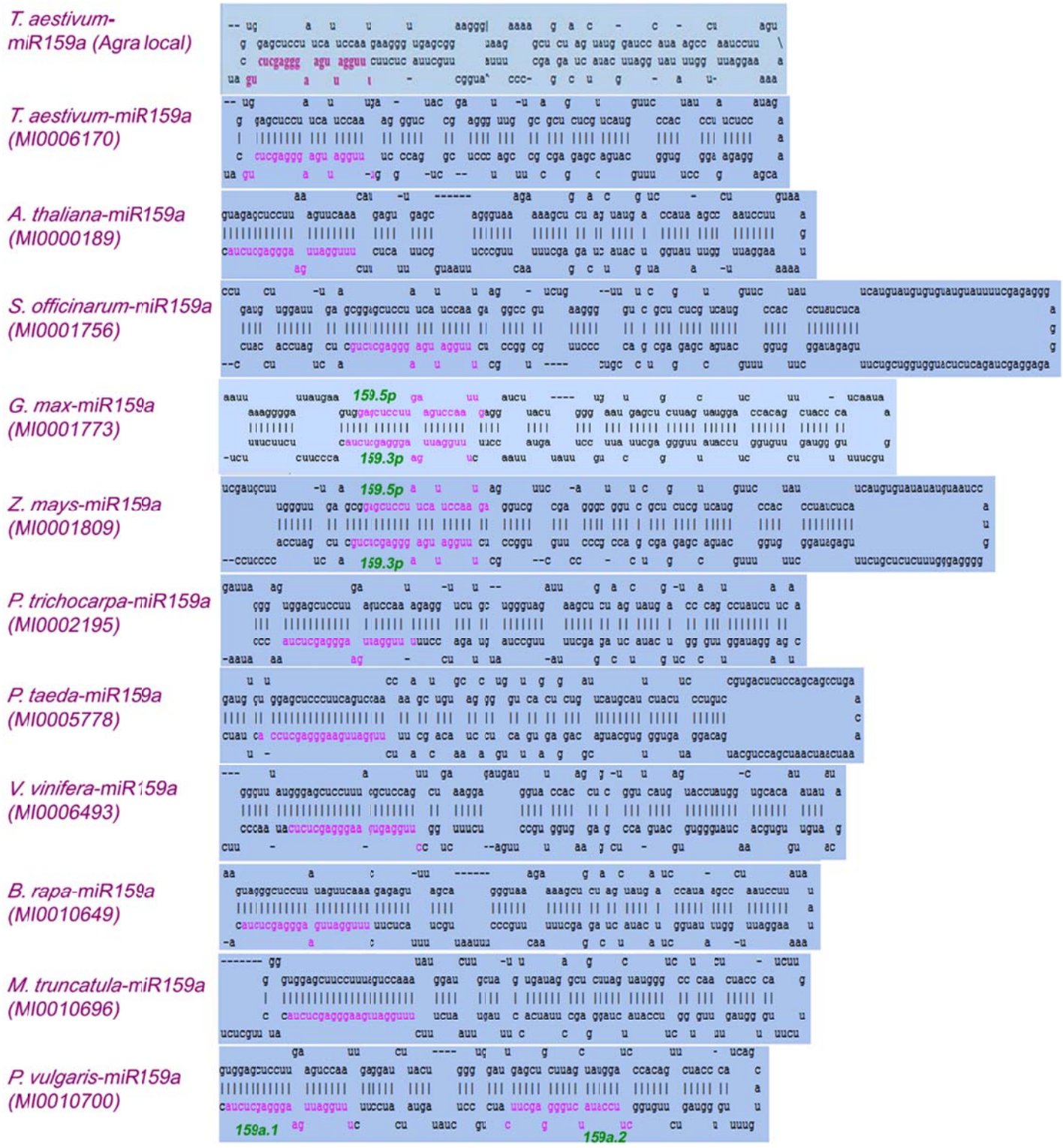

Figure 2. Comparison of matches and mismatches in pre-miR159a duplex across the species. Extreme left represents the name of species along with miRNA with accession number while mature miRNAs are represented in between the loop structure (red color). Pre-miR159a in three species namely *G. max, Z. mays* and *P. vulgaris* have two mature miRNA sequences in the respective backbone which is denoted by green colour. Mature miR159a.1 of *P. vulgaris* and miR159a.3p of *G. max* and *Z. mays* were used to compare with mature miRNAs of other species

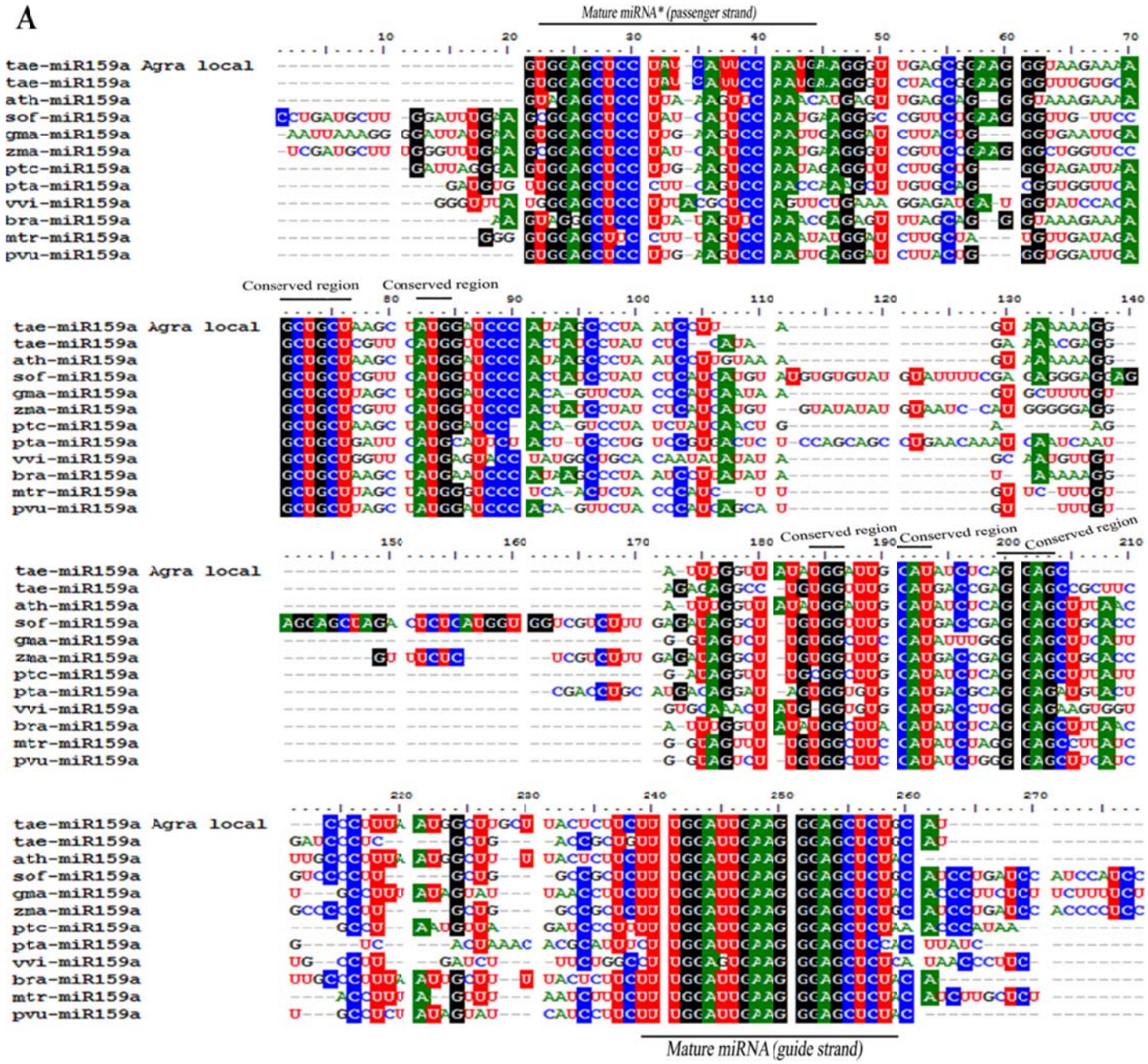

Figure 3. A. Comparision of pre-miR159a sequence isolated from Agra local wheat cultivar with different species. Mature miRNAs are conserved in nature across the species while the sequences flanking to the mature miRNA are not conserved across the backbone length. In between the backbone conserved nucleotides are highlighted. (tae, *T. aestivum*; ath, *A. thaliana*; sof, *S. officinarum*; gma, *G. max*; zma, *Z. mays*; ptc, *P. trichocarpa*; pta, *P. taeda*; vvi, *V. vinifera*; bra, *B. rapa*; mtr, *M. truncatula*; pvu, *P. vulgaris*). **B.** Comparison of pre-miRNA1123 sequence isolated from Agra local (*Tae* AL) wheat cultivar with reported sequences of line *Tae* 3338.

Phylogenetic relationship suggests that *Tae* AL pre-miR159a is showing close relationship with backbone (pre-miRNA) of *Arabidopsis, Brassica, and Triticum*. The *Tae* line 3338 pre-miR1123 sequence was downloaded from miRNAs Registry database (http://miRNA.sanger.ac.uk) and used to compare with the *Tae* AL pre-miR1123. On comparing, *Tae* AL pre-miR1123 showed >83% sequence similarity with the reported sequence of pre-miR1123 from *Tae* line 3338 (Figure 3B).

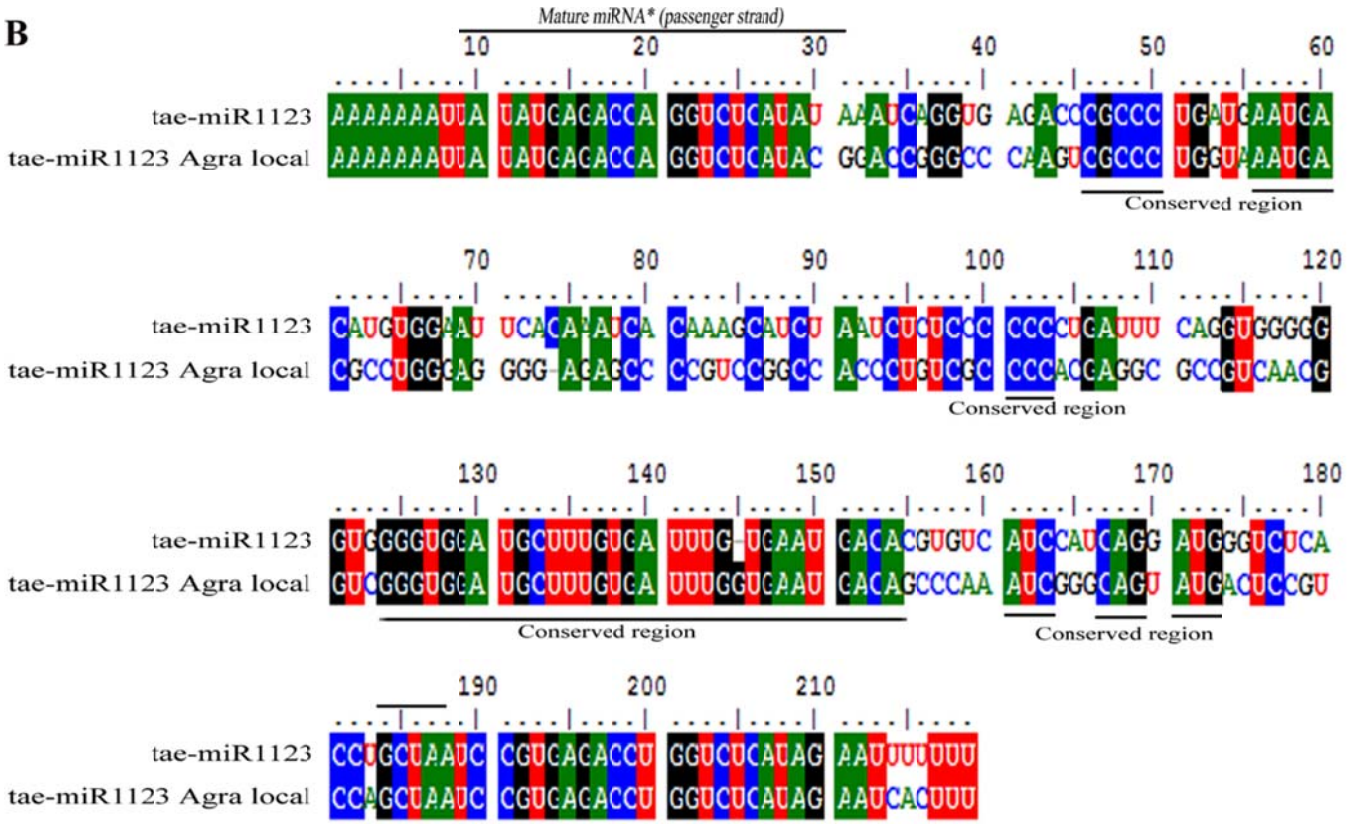

Figure 3. Contd.

Table 1. Sequence identity matrix of pre-miR159a.

Parameter	tae	tae Agra local	ath	sof	gma	zma	ptc	pta	vvi	bra	mtr	pvu
tae	ID											
tae Agra local	0.642	ID										
ath	0.566	0.826	ID									
sof	0.575	0.434	0.394	ID								
gma	0.454	0.515	0.524	0.443	ID							
zma	0.613	0.463	0.419	0.846	0.474	ID						
ptc	0.5	0.559	0.562	0.418	0.672	0.45	ID					
pta	0.479	0.43	0.43	0.418	0.395	0.472	0.466	ID				
vvi	0.479	0.434	0.433	0.382	0.434	0.396	0.42	0.415	ID			
bra	0.549	0.776	0.909	0.394	0.533	0.419	0.592	0.417	0.418	ID		
mtr	0.505	0.564	0.563	0.403	0.686	0.43	0.639	0.45	0.465	0.558	ID	
pvu	0.545	0.59	0.619	0.397	0.771	0.422	0.701	0.433	0.45	0.607	0.751	ID

In silico prediction of secondary loop structure of pre-miR159a and pre-miR1123

The sequences of *Tae* line3338 pre-miR159a and *Tae* line 3338 pre-miR1123 were downloaded from miRNAs Registry database (http://miRNA.sanger.ac.uk) and used to predict the secondary stem loop structure using MFOLDROOT secondary structure prediction software

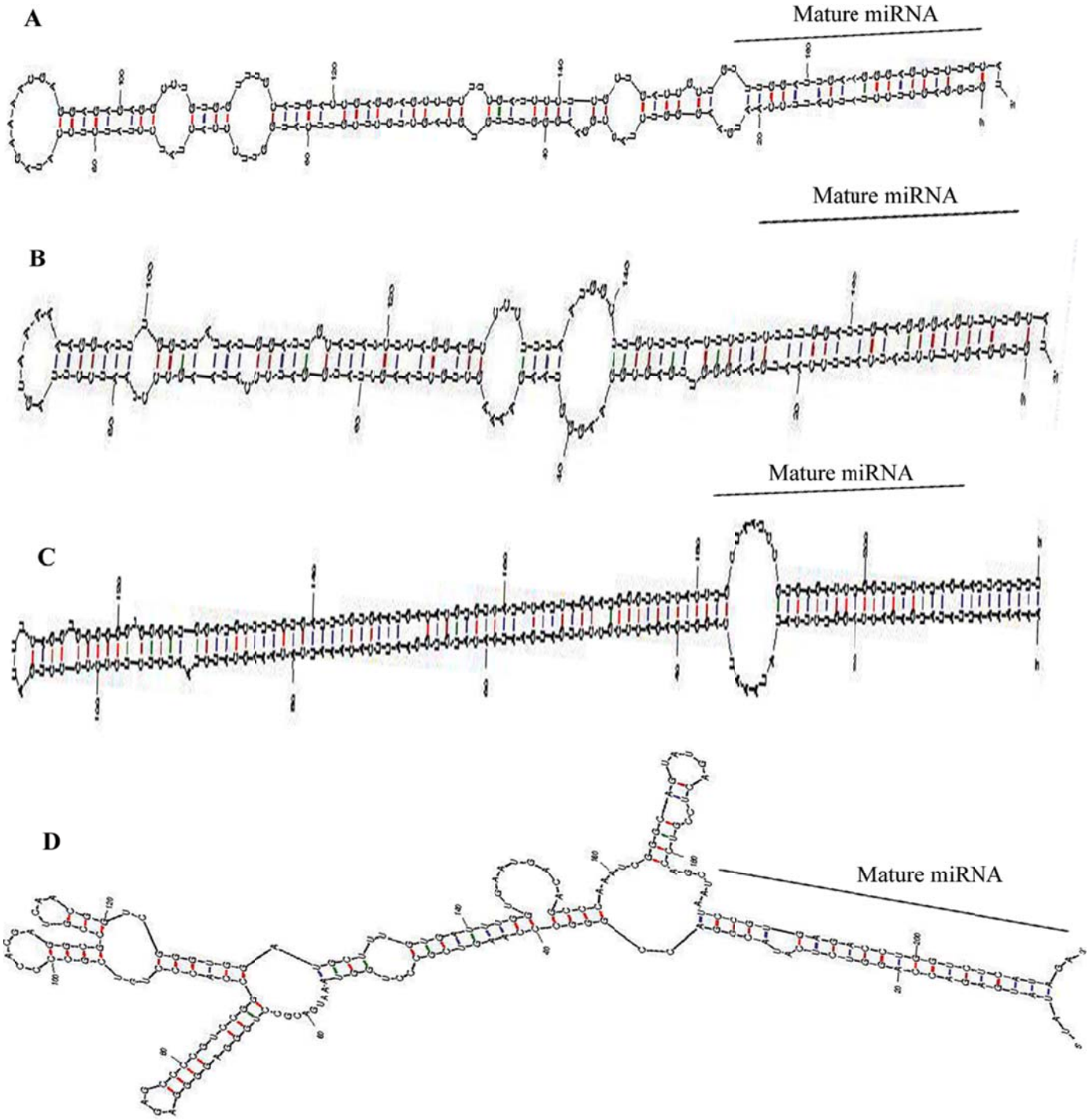

Figure 4. Comparison of secondary stem loop structure of pre-miR159a and pre-miR1123 in wheat. (A) Stem loop structure from reported line *Tae* 3338. (B) Stem loop structure from Agra local wheat cultivar (*Tae* AL). (C) Stem loop structure from reported line *Tae* 3338 (D) Stem loop structure from Agra local wheat cultivar (*Tae* AL).

(http://www.bioinfo.rpi.edu/applications/mfold).

Comparison of secondary structure of *Tae* line 3338 pre-miR159a with *Tae* AL pre-miR159a is shown in Figure 4A ans B and that of *Tae* line 3338 pre-miR1123 with *Tae* AL pre-miR1123 in Figure 4C and D. This secondary loop structure indicates the presence of conserved mature miRNA at 3' end and various other conserved motifs throughout the backbone. These conserved motifs might

be playing important role in processing.

DISCUSSION

To date, limited evidence is available about the origination of miRNA genes in plant genomes. The characteristic stem loop structure and the functional mode by which miRNAs pair with their target genes support the hypothesis that the *de novo* generation of miRNA genes is related to their target genes (Allen et al., 2004). Sequence divergence in miRNA backbone among different plant species maintain with the fold back structure and recognition by DCL1. Among various plant species the sequence divergence continued until the point that the miRNA:miRNA* forming a duplex were maintained. Till date no specific model has been proposed on sequence similarity between miRNA genes. In plants, miRNA gene of same family are often scattered throughout the genome indicating the shuffling since the amplification of these families.

Mature miR159a is found to be nearly conserved across the species although the passenger strand showed 30-35% variability. Mature miR1123 sequences are 100% conserved in two genotypes of the same species. The duplex miRNA strands (guide: passenger) in the stem loop structure of pre-miRNA showed a similar pattern within the species which differs across species. The overall sequence identity matrix showed variation from 43-82% with no such species specific consideration. These results suggest that sequence variation in the backbone maintains two important features: (1) mature miRNA fold back structure and (2) sequence required for its processing.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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