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# Identification of conserved microRNAs and their targets in chickpea (*Cicer arietinum L.*)

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**Abbreviations:** MFE, minimal folding free energy; MFEI, minimal folding free energy index

The microRNAs (miRNAs) are a new class of non-protein coding small RNAs that regulate gene expression at the post-transcriptional level in plants. Although thousands of miRNAs have been identified in many plant species, little studies have been reported about chickpea microRNAs. In this study, 28 potential miRNA candidates belonging to 20 families were identified from 16 ESTs and 12 GSSs in chickpea using a comparative genome-based computational analysis. A total of 664 miRNA targets were predicted and some of them encoded transcription factors as well as genes that function in stress response, signal transduction, methylation and a variety of other metabolic processes. These findings lay the foundation for further understanding of miRNA function in the development of chickpea.

MicroRNAs (MiRNAs) are a class of non-coding small RNAs that average 21 nucleotides (nt) in length, play an important role in plant biological processes such as development, biotic and abiotic stresses response, signal transduction and protein degradation.[1,2] Since a large number of mature miRNAs are evolutionarily conserved in different plant species, miRNAs can be identified using homology-based computational approaches. Using publicly available expressed sequence tag (EST) and genomic survey sequence (GSS) data sets in NCBI, conserved miRNAs were identified in many plant species, such as Chinese cabbage,[3] sorghum,[4] *Brassica oleracea*[5] and so on.

Chickpea (*Cicer arietinum* L.) is a self-pollinated, diploid (2n = 2x = 16), grain legume crop with a genome size of 740 Mb. It is the third most important cool season food legume, cultivated in arid and semi-arid regions of the world.[6] Recently, thousands of miRNAs have been identified in many plant species; however, to our knowledge, no chickpea microRNAs have been reported. Therefore, in this study, we employed a comparative genome-based homolog search to identify chickpea miRNAs using the known plant miRNAs.

Totally, 5236 miRNA homolog sequences from the first BLASTN alignment were collected for secondary alignment by WATER. After removing protein-coding sequences and checking the sequences secondary structures by MFOLD 3.2, a total of 28 miRNA candidates were identified in chickpea from 16 EST and 12 GSS sequences, belonging to 20 miRNA families (**Fig. 1A**; **Fig. S1** and **Table S1**). This suggested that ~0.0287% of chickpea ESTs and GSSs contained potential miRNAs.

In the 28 identified miRNAs, miR156 family had the largest number (5) of miRNAs with the other families having one or two miRNAs. The length of mature miRNAs was between 18 and 23 nt (**Fig. 1B**). The majority of miRNAs have lengths of approximately 20 and 21 nt, which account for 25.0% and 39.3%, respectively. The length of pre-miRNAs ranged from 45 to 182 nt, with an average of 89 ± 37 nt. The majority of pre-miRNAs were 50–150 nt in length (**Fig. 1C**).

Minimal folding free energy (MFE) is an important parameter for determining the stability of pre-miRNAs. Generally, a lower MFE value indicates a more stable secondary structure of a RNA sequence. Our results showed that the average value of MFE was -26.31 kcal/mol with a range of -7.30 kcal/mol to -60.5 kcal/mol. Previous studies have shown that MFEI (Minimal folding free energy index) value higher than 0.85 is used as a criterion for distinguishing miRNA from other types of RNAs.[7] In the present study, the average MFEI of the predicted chickpea pre-miRNAs was 0.85 ± 0.40.

The newly identified chickpea miRNA169 showed conservation across other nine plant species mature miRNAs (**Fig. 2A**). The miRNA sequence of miRNA169 was more conserved than its miRNA* site and pre-miRNA sequence (**Fig. 2B**). This conservation made them strong candidates of miRNAs in chickpea and suggested their similar physiological function.

A total of 664 potential targets were identified for 20 miRNA families, involving in stress response, metabolism, methylation, transport, signal transduction and so on. After aligning with the NR protein database using BLASTx, 164 EST sequences had been annotated (**Table S2**). Totally, 13 predicted miRNA targets

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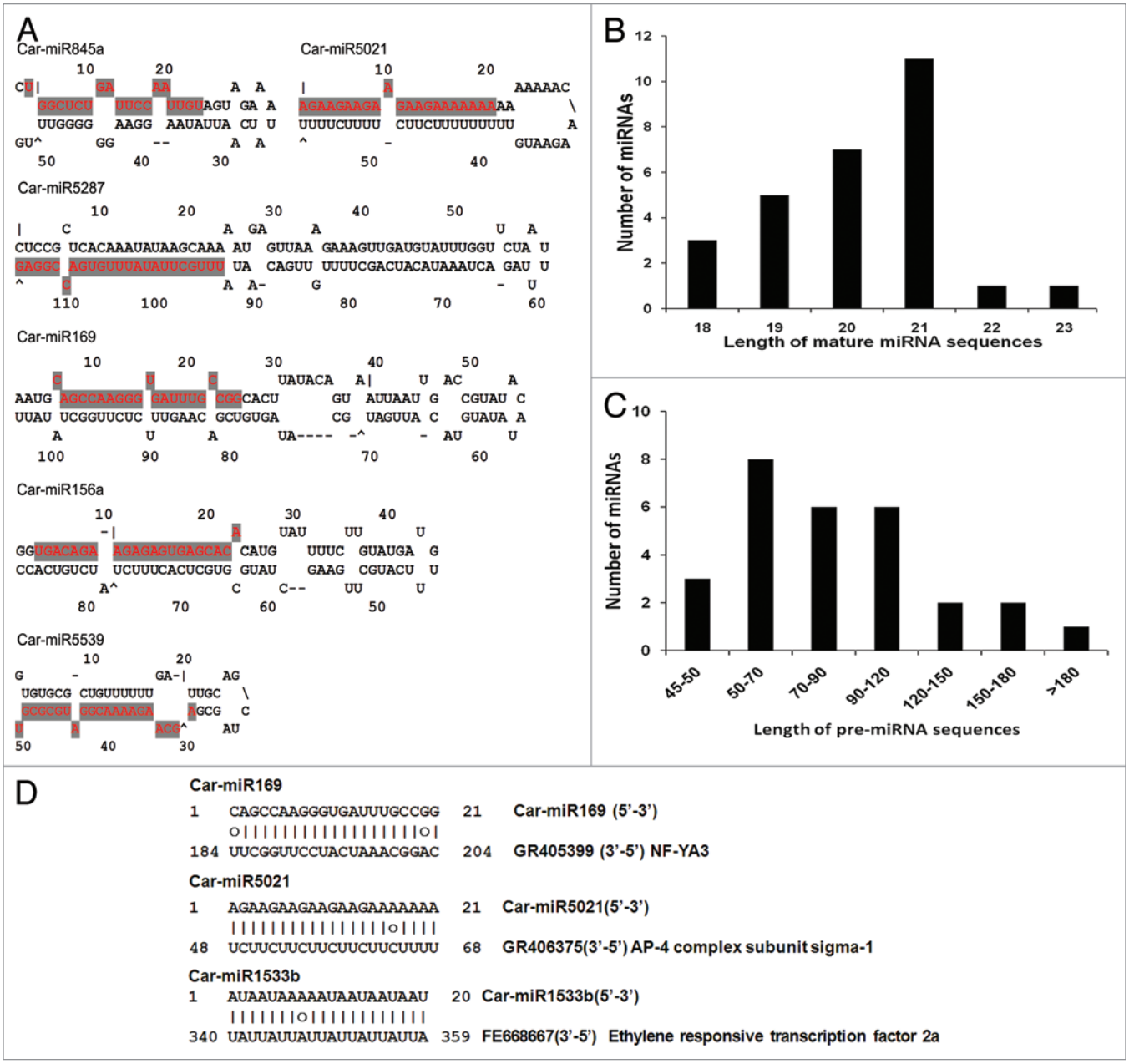


**Figure 1.** Characterization of potential miRNAs and targets in chickpea. (**A**) Potential hairpin secondary structures of some selected chickpea miRNAs identified in this study. Mature miRNA sequences are labeled in red. (**B**) Distribution of chickpea mature miRNAs lengths. (**C**) Distribution of chickpea pre-miRNAs lengths. (**D**) Predicted miRNA targets and their complementary sites within defined EST mRNAs in chickpea. Complementary base pairing is indicated by solid lines while mismatch base pairing is indicated by circle.

in chickpea are transcription factors, including auxin response factor (ERF), MYB, NF-YA3, AP4, bHLH and leucine zipper protein (**Fig. 1D**; **Table S3**). In plant, MYB transcription factors are a superfamily of a protein with MYB domain that plays regulatory roles in developmental processes and defense responses.[8] Previous studies reported that MYB transcription factors were targeted by miR414 or miR1533 in Chinese cabbage.[3] In this study, our results also showed that miR414 and miR1533 might target MYB proteins in chickpea. Furthermore, the potential chickpea miR169 was predicted to target NF-YA3 transcription factor. NF-YA3 encodes a subunit of the Nuclear Factor Y (NF-Y) complex that was previously reported to be downregulated in drought-affected wheat leaves.[9]

As a leguminous crop, chickpea provides a rich source of nitrogen due to the symbiotic nitrogen fixation with rhizobia and miRNAs have been reported to be associated with soybean-rhizobial interaction. Previous studies demonstrated that cationic peroxidase played specific roles in root nodule development.[10] In our study, miR1533 was predicted to target cationic peroxidase in *Cicer arietinum*, suggesting miR1533 may involve in nodule development and chickpea-rhizobial interaction.

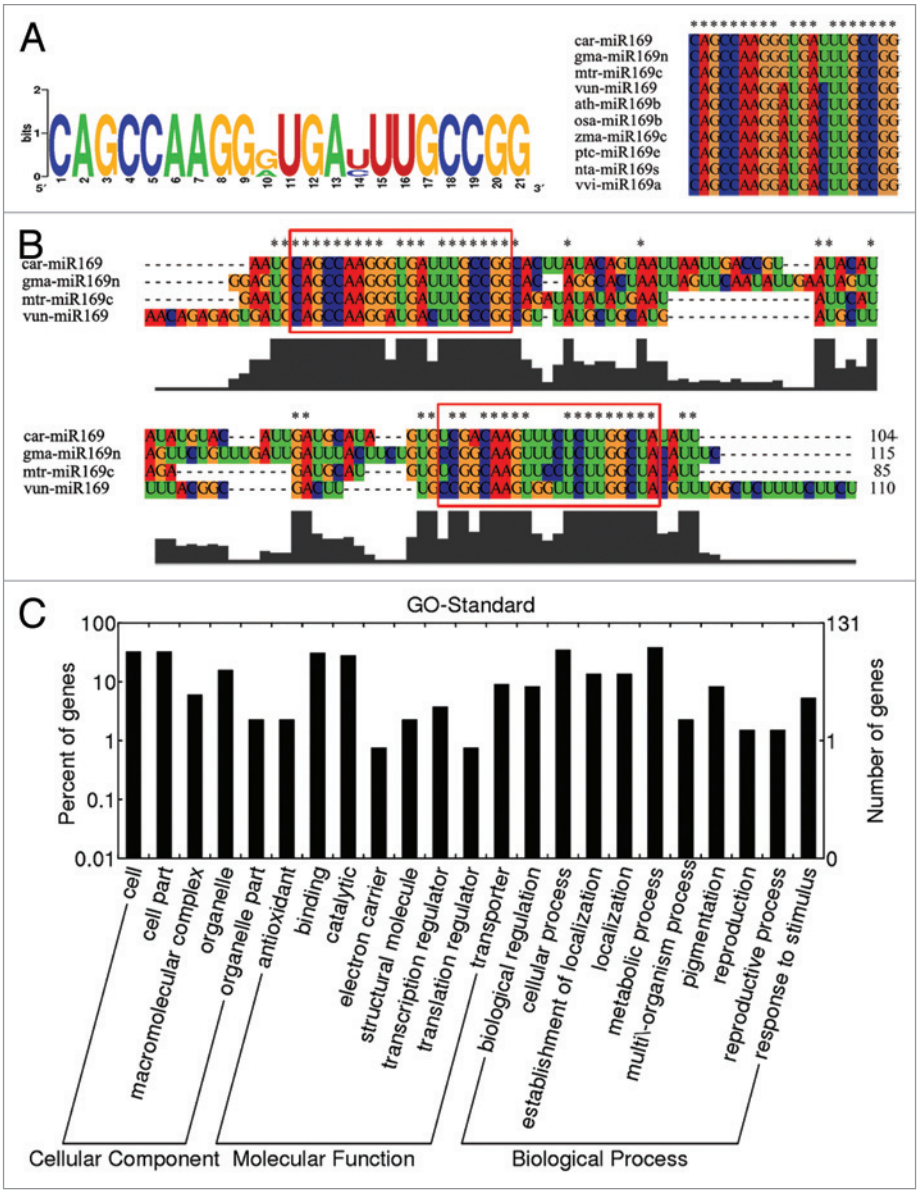


**Figure 2.** Conservation of chickpea miRNA169 and gene categories of miRNA targets in chickpea. (**A**) Left, sequence logo showing conserved nucleotide composition of ten mature miRNA169 sequences using Weblogo. Right, potential car-miR169 and nine mature miRNA169 sequences from miRbase (www.mirbase.org/) were aligned by CLUSTAL X. *C. arietinum* (car), *G. max* (gma), *M. truncatula* (mtr), *V. unguiculata* (vun), *A. thaliana* (ath), *O. sativa* (osa), *Z. mays* (zma), *P. trichocarpa* (ptc), *N. tabacum* (nta), *V. vinifera* (vvi). (**B**) Chickpea pre-miRNA169 was aligned with three other pre-miRNA sequences from fabaceae family (*G. max*, *M. truncatula*, *V. unguiculata*). (**C**) Gene categories and distribution of miRNA targets in chickpea.

To better understand miRNA function, the predicted target genes were analyzed by GO. In the present study, 71 target genes taken part in 107 biological processes, 71 target genes involved in 151 different molecular functions and 32 target genes participated in 61 cellular component functions (**Fig. 2C**). The GO biological process demonstrated that ten miRNA families may involve 107 different biological processes, such as defense response, signal transduction, metabolic process, protein phosphorylation, oxidation-reduction process and so on (**Table S4**). KEGG was used to enrich the pathway of the predicted miRNA target genes. Totally, 75 involved metabolism networks were found (**Table S5**). These pathways included sorting and degradation, lipid metabolism, nitrogen metabolism, amino acid metabolism, signal transduction and others.

## Materials and Methods

After removing the repeats using a Perl script, 2073 unique known plant mature miRNA sequences were selected as the reference set from miRBase database[11] (www.mirbase.org; release 18, November 2011). In order to identify potential chickpea miRNAs, 46,064 EST and 51,513 GSS sequences were downloaded from NCBI (www.ncbi.nlm.nih.gov) in April 2012. The conservations of newly identified miRNA169 and pre-miRNA169 sequence in chickpea were aligned with other nine miRNAs sequences from the miRBase database using weblogo (http://weblogo.berkeley.edu/) and Clustal X. Predictions of putative miRNAs and their targets in chickpea were performed as described previously.[3,5] Because only a small number of protein-coding genes were reported in chickpea, detected EST targets of chickpea were BLASTX searched against the NR protein databases among *M. truncatula* and other plant species to identify potential protein-coding homolog genes in chickpea. All the predicted target proteins were checked against the Interpro and KEGG databases. The biological process, molecular function and cellular component of the targets were obtained using the Interpro database; and were found the same as those in the Gene Ontology database.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.


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### Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/psb/article/23604

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