J Struct Funct Genomics (2010) 11:233–240
DOI 10.1007/s10969-010-9098-3

Computational identification of microRNAs and their targets from the expressed sequence tags of horsegram (*Macrotyloma uniflorum* (Lam.) Verdc.)

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Received: 29 June 2010 / Accepted: 14 October 2010 / Published online: 27 October 2010

Abstract MicroRNAs (miRNAs) are a class of naturally occurring and small non-coding RNA molecules of about 21–25 nucleotides in length. Their main function is to downregulate gene expression in different manners like translational repression, mRNA cleavage and epigenetic modification. To predict new miRNAs in plants different computational approaches have been developed. In the present study, an EST based approach has been used to identify novel miRNAs in horsegram. Identification of miRNAs was initiated by mining the EST database available at NCBI. Total of 989 ESTs were obtained for the identification of miRNAs. These ESTs were subjected to CAP3 assembly to remove the redundancy. This resulted in an output of 72 contigs and 606 singletons as non redundant datasets. The miRNAs were then predicted by using miRNA-finder. A total of eight potential miRNAs were predicted and named as hor-miR1 to hor-miR8. None of identified miRNAs showed significant homology with the previously reported in plants and therefore should be considered novel. These miRNAs were inputted to miRU2 program to predict their targets. The target mRNAs for these miRNAs mainly belong to zinc finger, chromosome condensation, protein kinase, abscisic acid-responsive, calcineurin-like phosphoesterase, disease resistance and transcriptional factor family proteins. These targets appeared to be involved in plant growth and development and environmental stress responses.

Keywords in silico biology · microRNA · Horsegram · Targets · Structure

Introduction

For years, RNA molecules have been thought to bear just two major functions in cells. The coding RNAs (messenger RNAs) are essential intermediaries in gene expression and non-coding RNAs (ribosomal and transfer RNAs) have structural, catalytic and information decoding roles in protein synthesis. The path breaking discovery of silencing of genes by non-coding RNAs known as RNA interference (RNAi) has changed the insight of people in this field [1]. Non-coding RNAs are abundant in eukaryotic cells. These small RNAs play central roles in important regulatory mechanisms mediating many biological processes in plants and animals.

The microRNAs (miRNAs) and small interfering RNAs (siRNAs) represent two major classes of small RNAs that regulate gene expression at the post-transcriptional level in plants [2, 3]. siRNAs are processed from long, double-stranded RNA precursors and direct gene silencing through both mRNA degradation and chromatin modification [4]. Though miRNAs are chemically and functionally similar to siRNAs they are derived from local stem-loop structures in the genome. The miRNAs should have following characteristic features: (a) miRNA should consist of 20–24 nt [2, 5], (b) all miRNA precursors should have a well predicted stem-loop hairpin structure with low free energy [6, 7], (c) usually mature miRNAs for specific functions are conserved in plants [2].

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The miRNAs are classified into families. The miRNA family classification is based on the Rfam database. The basic idea behind family classification is that each family represents sequences that have evolved from a common ancestor. The biogenesis and the function of many miRNAs in various systems including plants have been worked out [8–11]. In plants, miRNAs originate mostly from independent transcriptional units and are transcribed by RNA polymerase II into long primary transcripts (pri-miRNAs). Subsequently, the pri-miRNA is cut into miRNA precursors (pre-miRNAs) with stem-loop (hairpin) structures. The loop region of the hairpin is removed by ribonuclease III-like enzyme Dicer (DCL1) and the remainder (miRNA-miRNA duplex) is exported to the cytoplasm by Hasty (plant ortholog of exportin). Further plant miRNA is methylated at 3′ end by HEN1 factor. One strand of the duplex becomes mature miRNA and gets incorporated into the RNA-induced silencing complex (RISC) and guides RISC to complementary mRNA targets. Eventually, the RISC inhibits translation elongation or triggers the degradation of target mRNA [12].

miRNAs are implicated in diverse aspects of plant growth and development, including leaf morphology and polarity, lateral root formation, hormone signaling, transition from juvenile to adult vegetative phase and vegetative to flowering phase, flowering time, floral organ identity and reproduction [13, 14]. Several miRNAs are regulated in response to diverse stress conditions, suggesting important role in plants to cope with the stresses. Identification of miRNAs in large number of diverse plant species is important to understand the evolution of miRNAs and miRNA-targeted gene regulations. The low abundance of some miRNAs and their time- and tissue-specific expression patterns make experimental miRNA identification difficult. Now-a-days, publicly available databases play central role in the *in silico* biology [13, 15–21].

Horsegram is an important legume crop and source of proteins in vegetarian diet of many developing countries. It is known to be drought tolerant and possesses many neutraceutical properties [22]. The grain is used as human food and also as a concentrated feed for cattle. The US National Academy of Sciences has identified this legume as a potential food source for the future [23]. Till date no miRNA from this pulse crop has been reported. In this study, *in silico* approach has been used to identify potential miRNAs from the ESTs of horsegram. For this, we searched the EST databases to find ESTs matched with the previously known *Arabidopsis* miRNAs. Then we predicted the secondary structures of the identified ESTs in the first step using RNA MFOLD software. Finally, we identified new miRNAs. Further, the newly identified miRNAs have been used to find out targets that improve our understanding towards their possible regulatory roles in horsegram.

Materials and methods

EST database mining and processing

The ESTs of horsegram were retrieved from dbEST available at http://www.ncbi.nih.gov/dbEST/site. The redundancy of EST sequences was removed using the sequence assembly program CAP3 (http://pbil.univlyon1.fr/cap3.php). The overlapping sequences were clustered by CAP3 program as contigs and non-overlapping sequences as singleton.

Prediction of potential miRNA

The processed ESTs were used for the prediction of potential miRNAs with miRNAFinder (http://bioinfo3.noble.org/mirna/). miRNAFinder can accept three kinds of input sequences such as EST/cDNA, genomic sequence and small RNA. Therefore, miRNAFinder predicted potential intronic miRNA in intron regions of expressed genes (ESTs/cDNAs), find possible miRNA in genomic sequence or predict if the input small RNA is mature miRNA. The sequences are needed to be submitted in FASTA format. miRNAFinder execute back-end prediction pipeline and output a list of putative pri-miRNAs, their position information, and potential target genes. In this study, only ESTs data of horsegram was used. The processed ESTs of horsegram were submitted to miRNAFinder to produce output after comparative analysis with target ESTs library of *Arabidopsis thaliana*. False positive prediction of miRNAs was also removed using *Oryza sativa* ESTs library as a reference.

Prediction of targets for identified miRNAs

It has been documented that most of the known plant miRNAs bind to the protein coding region of mRNA targets with perfect or nearly perfect sequence complementarities [24, 25]. The targets were predicted with a plant miRNA potential target finder miRU2 available at http://bioinfo3.noble.org/miRNA/miRU.htm [26]. The *Arabidopsis thaliana* genome sequences were used as a base to predict the targets. Targets were predicted with potential complementarities in sequences against the submitted miRNAs with no gaps and <4 mismatches.

Prediction of secondary structures of miRNA precursor sequences

The secondary structures of miRNA precursor sequences were predicted with MFOLD software [6]. The parameters selected for predicting the secondary structures were as a fixed folding temperature of 37°C, 1 M NaCl ionic

conditions with no divalent ions and rest of the parameters kept as default. For selecting the potential miRNAs or pre-miRNAs, various criteria have been considered as used in the previous studies [27–30]. Predicted mature miRNAs were allowed to have only 0–3 nucleotide mismatches in sequence with all previously known plant mature miRNAs. The pre-miRNAs sequence should be folded into an appropriate hairpin secondary structure. No loop or break in miRNA sequences was allowed. The MFEI was calculated using the following equation:

$$\text{MFEI} = [(\text{MFE}/\text{length of the pre-miRNA sequence}) \times 100]/(\text{G} + \text{C})\%$$

where MFE denotes the negative folding free energies (ΔG Kcal/mol).

Results and discussion

Identification of potential miRNAs from horsegram

The computational approaches based on the software which are used in this study have already been used for such miRNA analysis in various plant and animal systemts [13, 15–21]. In this study, a computational approach was used for searching the miRNAs from horsegram ESTs database following strict filtering criteria. From the available 989 ESTs, 72 contigs and 606 singletons were achieved as non redundant data using CAP3 program. In the first phase of CAP3 program, 5′ and 3′ poor regions of each read were identified and removed. Overlaps between reads were computed. False overlaps were identified and removed. In the second phase of CAP3 program, reads were joined to form contigs in decreasing order of overlap scores. Then, forward–reverse constraints were used to make corrections to contigs. In the third phase, a multiple sequence alignment of reads was constructed. During multiple sequence alignments a consensus sequence along with a quality value for each base was computed for each contig. A total of eight potential miRNAs were predicted from the processed data using miRNA-finder program and named as hor-miR1 to hor-miR8 (Table 1). The predicted miRNAs were either 20 or 21 nt in size. Majority of known miRNAs in other plants are of same size [2, 5, 27, 31]. The A + U content of predicted miRNAs ranged from 45 to 53%. The predicted miRNAs show higher negative minimum fold energies (MFEs). The MFEI is another useful criterion for distinguishing miRNAs from other types of coding and non-coding RNAs. The miRNA precursors with secondary structures had minimal free energy index (MFEIs) than other different types of RNAs. The newly identified miRNAs show MFEI in range of 0.45–0.75. The length of horsegram pre-miRNA varies from 97 to 110. These parameters are in agreement with the previously reported results for *in silico* predicted miRNAs [7, 28, 32].

Generally, miRNAs are distinguished from other RNAs on the basis of their surrounding sequences ability to adopt the hair-pin structure [5]. Therefore, secondary structures of all the identified miRNAs were predicted (Fig. 1). The identified miRNAs are found to vary in their locations in precursor sequences. The hor-miR1, hor-miR3, hor-miR5 and hor-miR7 are located at the 5′ end of their precursor sequences, whereas hor-miR2, hor-miR4, hor-miR6 and hor-miR8 are located at the 3′ end of their precursor sequences.

Prediction of targets for newly identified miRNAs and their putative role

The functional importance of miRNAs can de understood or described well by gaining insight into the miRNA targets. The predicted targets for the identified miRNAs are shown in Table 2. Targets were predicted for miRNAs sequences by using miRU2 software. Most of the predicted targets are involved in the regulation of plant growth and development and are functionally crucial for the plant

Table 1 List of miRNAs predicted from ESTs of horsegram by using miRNA-finder and their characteristic features

Sr. no.	EST ID (Gene source)	Predicted miRNA (5′–3′)	LM (nt)	LP (nt)	A + U (%)	PN	MFEs	MFEIs
hor-miR1	CO751629.1	CAGCCACCCUUGAAAGAGUGC	21	107	47	5′	28.2	0.49
hor-miR2	CO751629.1	GCGAUCUGCCGAAGCUGUGGG	21	107	47	3′	28.2	0.49
hor-miR3	CO751629.1	GUUUGCCUAGAAGCAGCCACC	21	110	45	5′	27.68	0.45
hor-miR4	CO751629.1	GGGGCUAAGCGAUCUGCCGAA	21	110	45	3′	27.68	0.45
hor-miR5	DR988686.1	AGAUCUGGUUGUGUCUGUUAU	21	97	53	5′	34.2	0.75
hor-miR6	DR988686.1	AUGGGAGAGGAGCAGAUUUGU	21	97	53	3′	34.2	0.75
hor-miR7	DR989012.1	AAGUCUGUUGAGAUGCACCAC	21	110	50	5′	24.52	0.44
hor-miR8	DR989012.1	GUGAAGGAUCUCAAGCGUGG	20	110	50	3′	24.52	0.44

LP Length of precursor, *LM* Length of mature miRNA, *PN* Position of miRNA at pre-miRNA, *MFE* Minimum free energy, *MFEIs* minimum free energy indexes

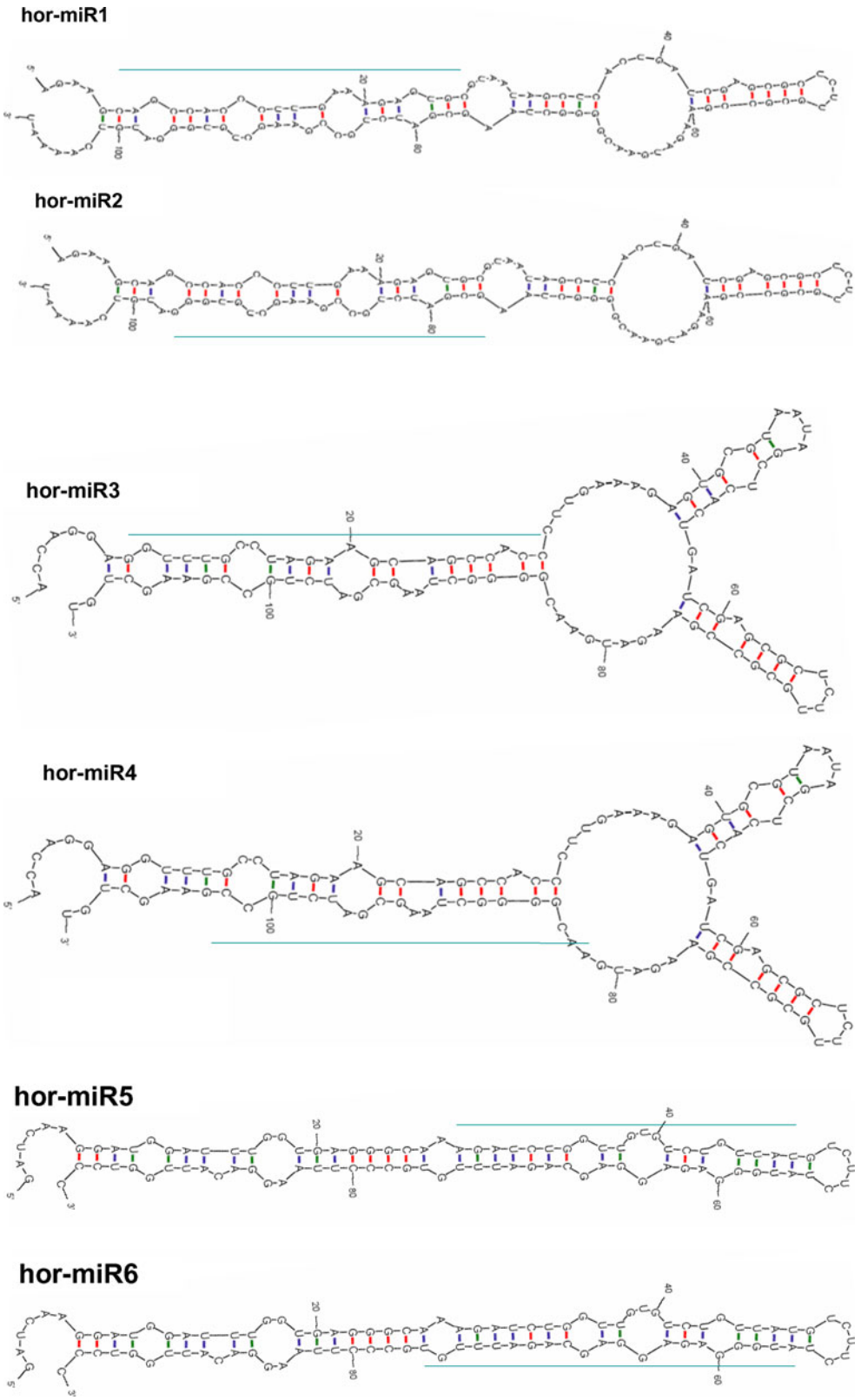

Fig. 1 Predicted secondary structures of identified precursor miRNAs in horsegram. These structures were produced using MFOLD program. The mature miRNAs sequences are marked with *line*. The actual size of the precursors may be slightly shorter or longer than the presented here

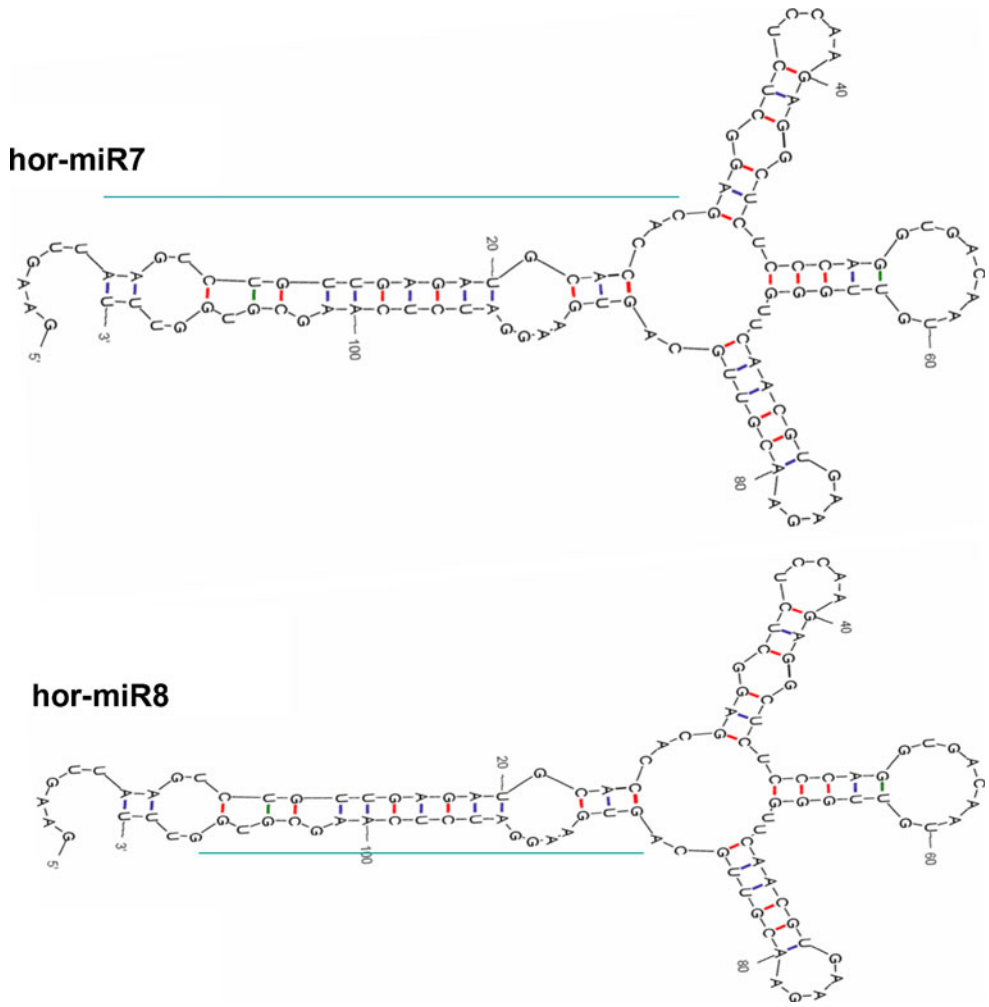

Fig. 1 continued

physiology. It has been observed that one miRNA can target more than one regulatory gene [7, 28, 33]. In this study, hor-miR5, hor-miR6 and hor-miR7 are found to target 13, 22 and 6 sequences, respectively.

Earlier studies have documented that most of the miRNAs largely target transcription factors, signal transduction factors and metabolic transporters [28–30, 32]. In complementation with earlier studies, hor-miR1 and hor-miR6 are found to target zinc finger family protein. Such proteins are involved in numerous cellular processes including transcription, signal transduction, and recombination [34]. Most zinc finger proteins are E3 ubiquitin ligases [35] that mediate the transfer of ubiquitin to target proteins and play important roles in diverse aspects of cellular regulations in plants [36].

In an attempt to delve into the functional importance of the newly identified miRNAs, their targets were studied extensively. The hor-miR2 is found to target chromosome condensation proteins which play an important role in transcriptional gene silencing during cell cycle [37]. Furthermore, the hor-miR5 targets RNA recognition motif which are apparently known to control the post transcriptional gene expression. These RNA binding proteins either directly bind or indirectly control the expression by modulating other regulatory factors. The post-transcriptional regulatory events are pretty crucial in plant development [38]. The transcription factor B3 family protein targeted by the hor-miR7, has been very well characterized and found to have significant functional and evolutionary roles in plant development [39]. The hor-miR3 targets coatomer protein complex which are involving in trafficking of secretory proteins between the endoplasmic reticulum (ER) and the Golgi apparatus [40]. The hor-miR3 also targets WD-domain containing proteins which are essentially involved in plant growth and development [41, 42].

The hor-miR5 targets abscisic acid-responsive (ABA) family protein which is another example of transcriptional control under abiotic stress conditions in plants. The MADS-box proteins targeted by hor-miR5 are found to be a diverse class of transcription factors in the seed plants,

Table 2 Predicted targets for newly identified miRNAs in horsegram

Sr. no.	Target acc. no.	Target sequence (5′ → 3′)	Predicted targets
hor-miR1	At5g25560.1	GUCGGUGUGAACAUUGUCACU	Zinc finger (C3HC4-type RING finger) family protein
hor-miR2	At1g29790.1	CGCUAGACCUCUUCGACGCUC	Chromosome condensation family protein
	At1g49245.1	CGCUAGGCUGCUUCAACAUCA	
	At5g37630.1	AGCUAGACGACUUCGGUACCG	
hor-miR3	At1g62020.1	GAAACGGAACAUCGUCUGUGG	Coatomer protein complex, subunit alpha, putative proteins containing WD domain, G-beta repeat
	At3g27930.1	CGGACGGUUCUGCGUCGGUGG	
hor-miR4	At1g07650.1	CUUCGAUUCGCUAGACGAGUC	Leucine-rich repeat transmembrane protein kinase
hor-miR5	At1g19950.1	UCUAGGCCAACAAAGACAAGA	RNA recognition motif (RRM)-containing protein, S-locus protein kinase, Abscisic acid-responsive (ABA) HVA22 family protein, MADS-box family protein
	At1g67950.1	UCUAGUUCAACACAGACAGUA	
	At1g67950.2	UCUAGUUCAACACAGACAGUA	
	At1g67950.3	UCUAGUUCAACACAGACAGUA	
	At1g67950.4	UCUAGUUCAACACAGACAGUA	
	At4g02150.1	UUUCGACCAACACAGACAAGU	
	At4g19090.1	CCUAGAAUAACACAGACAUUA	
	At1g11280.1	UAUAGAUUAACACAGAUAAUC	
	At1g11280.2	UAUAGAUUAACACAGAUAAUC	
	At1g11280.3	UAUAGAUUAACACAGAUAAUC	
	At1g54850.1	UCUAGACUAACACAAACGUUU	
	At3g19120.1	UCUAGACAAGUACAGAAAAUG	
	At5g40120.1	ACUAGGCCAAAACAGACAAAG	
hor-miR6	At1g26640.1	UACCCUCUCCUCGUCUAGACA	(Suppressor-of-White-Apricot)/surp domain-containing protein, Preprotein translocase secY subunit, chloroplast (CpSecY), Calcineurin-like phosphoesterase family protein, S-locus protein, Disease resistance family protein contains leucine rich-repeat (LRR) domains, Fasciclin-like arabinogalactan family protein, RNA recognition motif (RRM), Syntaxin, putative (SYP32), Pseudouridine synthase family protein, RNA pseudouridylate synthase, Zinc finger (C2H2-type) family protein, Transmembrane amino acid transporter protein, Short-chain dehydrogenase/reductase (SDR) family protein
	At4g20420.1	AACCCUUUCCUCGUAUGAACA	
	At3g24350.1	UACCUUCUCCUUGUCUAAGUG	
	At1g23720.1	UACCCCCUCCUCCUCUAAACA	
	At4g23420.1	CACCUUCUGGUCGUCUAAACA	
	At4g23420.2	CACCUUCUGGUCGUCUAAACA	
	At2g21050.1	AACCCUCUUCUCGUUUACGCA	
	At3g05650.1	AACUUUCUUCUCGUCAAAACA	
	At4g21770.1	UAUCCUUUCCUCGUCUACAUU	
	At1g21310.1	UACCUCCUCCUCCUCUAAACA	
	At1g66500.1	UACCUUCUCCUUGUUAAACC	
	At2g18710.1	UACCUUCUAUUCGUCUAAAGA	
	At5g43620.1	UACCUUCUCCUUGUUAAACC	
	At1g13750.1	GGCCCUUUCCUCUUCUAAACU	
	At2g15440.1	CACCUUCUCUUAGUCUAAUCA	
	At3g21100.1	UACUCUUUCCUAGUCAAAACG	
	At3g46550.1	UACCUUCGCCUCCUCUAGACG	
	At4g24890.1	UACCCUCUUCUCGUAUACAUU	
	At4g31200.1	GACUCUCUUCUAGUGUAAACA	
	At4g31200.2	GACUCUCUUCUAGUGUAAACA	
	At4g31200.3	GACUCUCUUCUAGUGUAAACA	
	At3g57560.1	UACUCUUUCUUCUUCUAAAUG	
hor-miR7	At1g26140.1	GUCAGACGACGUUUACGUGGUG	Lipase, Transcriptional factor B3 family protein, octicosapeptide/Phox/Bem1p (PB1) domain-containing protein,
	At1g28600.1	UUCAGACAACUCAAGGUGGAA	
	At1g43130.1	UUCAGACAACUCUCCUUGGGA	
	At3g26510.3	UUGAGACAAUUUUGCGUGGUG	
	At1g26680.1	CUUACACAACUCUGUGUGGUG	
	At2g36190.1	UUUAGACUACUUUACGUAGUU	
hor-miR8			Unknown target

playing an important role in establishment of certain reproductive structures [43]. Hence, similar to earlier known miRNAs, the newly identified miRNAs in horsegram are mostly targeting transcriptional factors. The hor-miR5 is also found to target S-locus protein kinase. S-locus is responsible for evolutionary transition among flowering plants i.e. the switch from outbreeding to an inbreeding mode of mating [44]. The functional role of hor-miR5 may unwind the intricacies of the above evolutionary transition and could provide a new understanding in the plant growth and development.

In addition, miRNAs have been documented to regulate cell signaling. The hor-miR6 targets mRNA coding for SecY translocase protein. The latter is involved in the insertion of signal transducing and recognizing proteins in the inner cytoplasmic membrane [45]. Interestingly, the disease resistance proteins are also targeted by the newly identified miRNA such as hor-miR6. The leucine-rich-repeat (LRR) domain containing disease resistance proteins have been particularly spotted by the hor-miR4 and hor-miR6. The LRR domain provides the platform for the recognition of pathogen [46] and more so, they are important determinants of specificity [47]. The hor-miR6 also shows complementarities to the sequences encoding fasciclin-like arabinogalactan proteins (FLAs). FLAs are a subclass of arabinogalactan proteins (AGPs) that contain putative cell adhesion domains known as fasciclin domain. These domains are critical for the cell-to-cell interactions and communication as well as for providing key structural, positional, and environmental signals during plant development [48].

Syntaxins are also reported to be targeted by miRNAs. Syntaxins are usually contributing to the plant resistance against bacteria [49]. In this study, hor-miR6 is found to be targeting syntaxin SYP132 transcript. Therefore, hor-miR6 could be an important miRNA to understand the regulation of plant defense system. Similarly, hor-miR7 is targeting some lipases which are involved in the hydrolysis of phospholipids, particularly phospholipases playing an important role in the plant responses to biotic stress [50]. We did not find any targets for hor-miR8. This may be due to incomplete coverage of mRNA in the horsegram database. Possibly, number of targets for miRNAs could not be identified because of their poor expression and stability or because of temporal and location specific expression.

Conclusions

This work presents the prediction of miRNAs and their targets from the available 989 ESTs of horsegram (*Macrotyloma uniflorum* (Lam.) Verdc.). None of the predicted hor-miRNAs showed identity with the previously reported miRNAs in plants. Therefore, these can be considered as novel and grouped in a new family. It is observed that most of the pooled targets predicted are very essential for the plant growth and development. They have been identified to play important role in variety of biological processes including plant defense, transcriptional regulation, stress defense, metabolic processes and structural development of plants.

Acknowledgments Authors are thankful to Dr. P. S. Ahuja, Director, IHBT for his valuable suggestions and guidance to conduct this work. We would like to thank the financial support from Council of Scientific and Industrial Research (CSIR) and Department of Science and Technology (DST), Govt of India. HM is thankful to CSIR for providing research fellowship in the form of JRF.

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