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Towards computational prediction of microRNA function and activity in turmeric (*Curcuma longa* L.)

R Santhi and T E Sheeja*

Division of Crop Improvement and Biotechnology, Indian Institute of Spices Research
Marikunnu PO, Kozhikode (Calicut) 673 012, India

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MicroRNAs (miRNAs) are recently discovered class of highly conserved, non-coding small RNAs that regulate gene expression in plants. High conservation of miRNAs in plants provides the basis for identification of new miRNAs in other plant species through homology alignment. Expressed sequence tags (ESTs) provide an alternative resource to facilitate identification of miRNAs and their targets. We have identified 8 conserved miRNAs representing two miRNA families from turmeric by *in silico* analysis of ESTs. The computational prediction was based on the conservation of miRNA sequences, the stem-loop hairpin secondary structures of miRNAs and a series of filtering criteria. Parameters like length of mature miRNA and precursor miRNA, nucleotide composition and free energy values were well within the range of other plant miRNAs. Multiple sequence alignment of miR167 precursors revealed high conservation of mature miRNA sequences. It was observed that though miRNAs are highly conserved, some specific sites are more likely to mutate. Most of the predicted targets appeared conserved and were classified as proteins involved in stress response, development and metabolism.

Keywords: Computational prediction, EST, miRNA, targets, turmeric

Introduction

MicroRNAs (miRNAs) are endogenous small, non protein coding, single-stranded RNAs that can negatively regulate gene expression at the post transcriptional level[1]. miRNAs arise from larger precursors that can form self-complementary fold back structures[2], which are further processed by a ribonuclease III. In plants, miRNAs play a significant role in a range of developmental processes including meristem cell identity[3], leaf organ morphogenesis and polarity[4], floral differentiation, and development[5] and stress responses[6]. Plant miRNAs show a greater complementarity to their targets than do animal miRNAs[7].

Comparative genomics across divergent taxa has shown that many miRNAs are evolutionarily conserved from species to species, from moss to high flowering eudicot species in plant kingdom[8] and from worms to humans in animal kingdom[9]. This conservation provides a powerful approach to their identification using comparative genomics[10]. Computational strategy has been proved to be successful for discovery of new and species specific miRNAs[8]. Owing to the virtually perfect base pairing between miRNA and their target sequences in plants, computational predictions have mostly been validated experimentally[11].

The identification of miRNAs through interspecies comparative genomics coupled with computational approaches was mostly focused on genomic sequences[10]. Such predictions have limitations during validation of data since the transcription information for many genomic regions are unknown. Alternatively, prediction of miRNAs from EST sequences can help in functional analysis due to the availability of expression data[12]. Moreover miRNA identification using EST analysis can be conducted without specialized software, using Blastn search algorithm[13] as in radish, worm wood, tomato and cotton[14-17].

Turmeric, also known as the “golden spice”, is one of the most important herbs in tropical and sub-tropical countries. Turmeric rhizome is valued worldwide and has been in use since ancient times as a spice, foodstuff, dye and in traditional medicine[18]. It is a commodity with lot of export potential. There are 12,593 ESTs available in turmeric in the public domain that is not annotated. 37 miRNAs were identified from turmeric by *in silico* method[19]

*Author for correspondence:
Tel: +91-495-2731410; Fax: +91-495-2730294
sheeja@spices.res.in

and 6 miRNAs are also available in miRNEST (http://mirnest.amu.edu.pl). However, the recent developments in computational methods involving homology searches and pre-miRNA (miRNA precursor) secondary structure predictions[20,21] were not taken into consideration. In the present study, previous known plant miRNAs were blasted against turmeric ESTs to identify a total of 8 conserved miRNAs belonging to two families. We have predicted potential targets for these miRNAs that are mostly involved in stress response, development and metabolism. This information is expected to provide a better insight into the regulatory mechanism involving development and physiology besides providing a basis for RNAi based strategy in this medicinally important plant.

Materials and Methods

Reference Set of miRNAs

All mature miRNA sequences previously identified in other 9 plant species including those from *Arabidopsis thaliana*, *Oryza sativa*, *Physcomitrella patens*, *Populus tricocarpa*, *Zea mays*, *Sorghum bicolour*, *Vitis vinifera*, *Brachypodium distachyon* and *Glycine max* were downloaded from miRNA sequence database, miRBase 19 (http://microrna-sanger.ac.uk/). In view of high conservation of miRNAs in plants, all repeat sequences were removed from different plant species to avoid the repeat search of redundant miRNAs. And 1864 unique sequences, defined as reference set, were retrieved from a total of 3135 sequences.

Identification of Turmeric miRNAs Using EST Based Comparative Genomics

Fig. 1 summarises the general procedure for identifying conserved miRNAs using EST based comparative genomics. A total of 12,593 turmeric EST sequences were obtained from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). All these ESTs were subjected to Blastn against reference set of

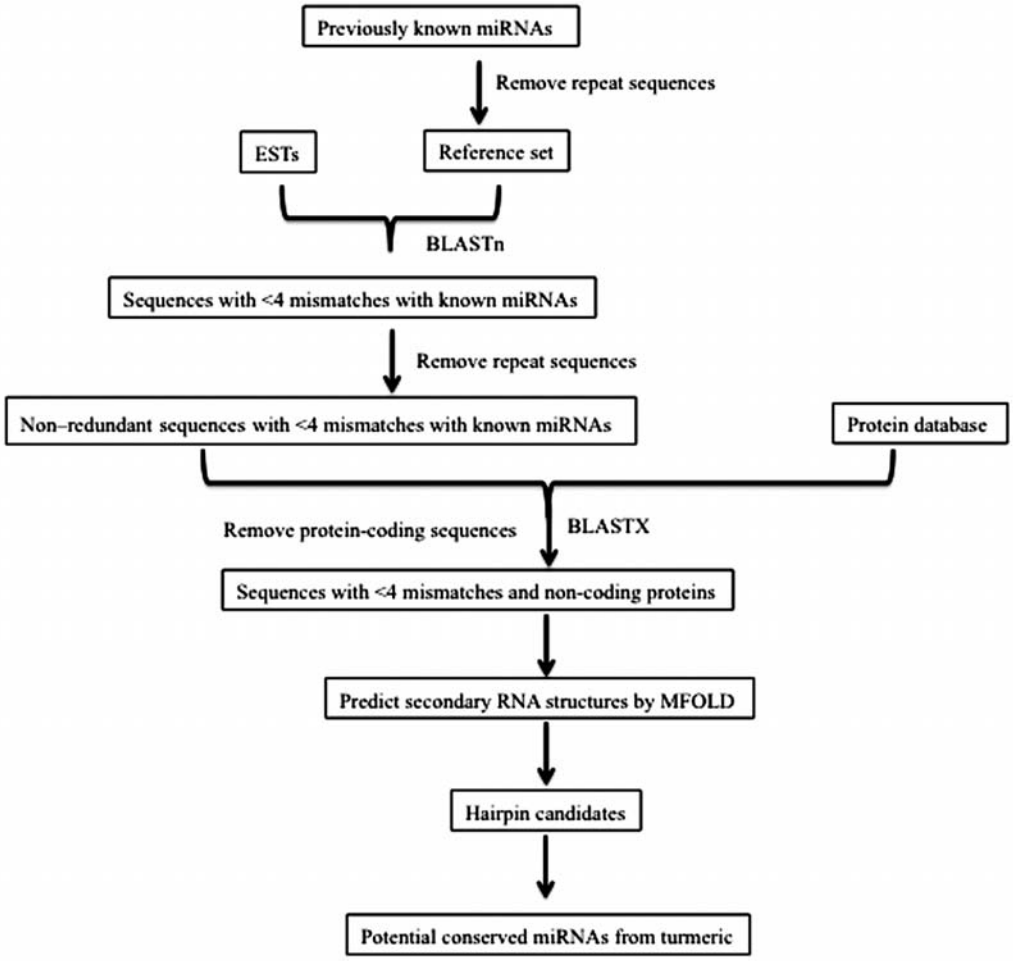

Fig. 1—Flow diagram illustrating the protocol used to identify conserved miRNAs from turmeric.

miRNAs to find the turmeric miRNA homologs. To improve blast search, expect values were set at 1000 to increase the number of potential hits; the word match size between query and database sequences was set at 16; the number of descriptions and alignments was raised to 1000. All Blastn results were saved. If the searches reveal partial sequence similarity to the queried miRNA sequences, non-aligned regions were manually inspected and compared to determine the number of matching nucleotides to assess their potential as miRNA candidates. Only EST sequences with four mismatches or less to the reference set of miRNAs were manually chosen for further consideration, compared with each other and all non-redundant protein databases from NCBI, to remove repeated sequences and protein coding sequences. Then, the secondary structures of the remaining sequences were generated using the Zuker folding algorithm with web based computational software MFOLD, which was publicly available at http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi. The software default parameters were used to predict the secondary structure of the selected EST sequences. All MFOLD outputs including free energy (ΔG kcal/mol), the number of nucleotides (A, G, C & U), location of the matched region and the number of arms per structure were recorded.

In the present study, following criteria were used to designate a RNA sequence as potential miRNA precursor or pre-miRNA. Briefly, (1) Predicted mature miRNAs should have only 0-4 mismatches in sequence with all previously known plant mature miRNAs. (2) Sequences of miRNA precursors should fold into a typical stem loop secondary structure that contain mature miRNA sequence within one arm of the hairpin. (3) No more than six mismatches between potential miRNA sequence and the miRNA* (opposite miRNA sequence) sequence should be allowed but a loop or break in this field will not be permitted. (4) miRNA precursors with secondary structure should have high minimal free energies (MFEs) and minimal free energy index (MFEI) than other different types of RNAs. The adjusted MFE (AMFE) is defined as the MFE of a 100 nucleotide length sequence. AMFE=(MFE/sequence) 100. MFEI=AMFE/(G+C)%. (5) A+U content of precursor miRNA should be 30-70%.

Identification of Potential Targets of EST-Predicted miRNAs

The target prediction was done based on perfect or near perfect complementarity of miRNAs and their target mRNAs. All mature miRNA sequences identified in turmeric were used for searching against mRNA sequences from other plants listed in web based servers, psRNATarget (http://plantgrn.noble.org/psRNATarget/), MicroPC (http://www.biotec.or.th/isl/micropc) and Target Align (http://www.lenoxie.com/targetAlign.php) with default parameters. To avoid false positives, authentic targets consistently identified by atleast two tools were only considered. The criteria used in determining miRNA targets were based on a previous study[22]. Briefly, (1) The total number of allowed mismatches at complementary site between the mature miRNA and its potential target was no more than four and no gaps were allowed at the complementary sites. (2) There was no more than one mismatch between nucleotide positions 2 and 9 from 5′ end of the miRNA, but not at the cleavage site (10 &11 nucleotides) and up to three mismatches between 12 and 21/24 nucleotide positions, but no more than two continuous mismatches in this region.

Results and Discussion

Identification of Potential Turmeric miRNAs by EST Analysis

Conservation of mature miRNA sequence from species to species has greatly simplified the identification of conserved miRNAs by EST analysis. ESTs are partial cDNA sequences of expressed genes cloned into a plasmid and this strategy has been used to predict a lot of conserved miRNAs in divergent plant species including *Arabidopsis*[20], *Gossypium hirsutum*[23], tomato[16], *Vigna unguiculata*[24], wormwood[15] and *Raphanus sativus*[14]. Identifying miRNAs within turmeric ESTs was possible due to this conserved nature. miRNAs can occur in intergenic regions, in introns of protein coding regions, or in exons and introns of noncoding genes[25].

After the first round of search by using reference set of miRNAs against 12,593 turmeric ESTs a total of 165 homologs were identified. After eliminating the repeat sequences 112 ESTs were subjected to secondary structure prediction by MFOLD. Finally after the removal of protein-coding sequences and applying the filtering criteria 8 turmeric miRNAs could be identified (Fig. 2; Table 1), which is higher than the estimated number one. In plants, it is estimated that 10,000 EST sequences contain one mature miRNA[8]. The 8 turmeric miRNAs that represented two miRNA families were designated as clo-miR1-8. clo-miR1-7 belong to miR167 family, while clo-miR8 is included

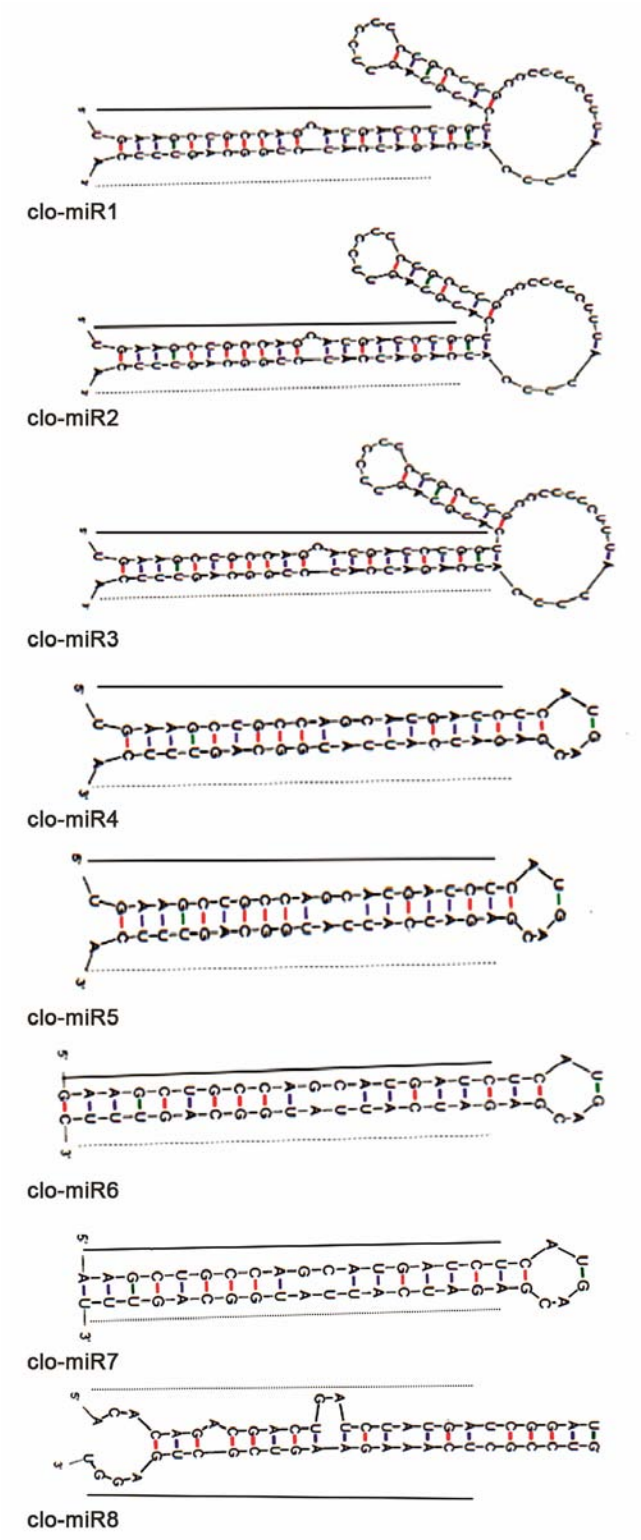

Fig. 2—Predicted hairpin secondary structures of the turmeric miRNAs identified in the present study. [Mature miRNA sequences are indicated by plane line and miRNA* by dashed line. The length of actual miRNA precursors is longer than what is presented here.]

in miR4401 family. In an earlier *in silico* approach, 37 miRNAs have been predicted in turmeric[19], but the study did not take into consideration the filtering criteria. Hence, the results of the present study were compared with those that employed a similar method of prediction[26].

In order to distinguish miRNAs from other small RNAs, the characteristics of conserved miRNAs were further analysed. The length of miRNA precursors in turmeric varied from 151 to 445 with an average precursor length of 273 (Fig. 3). It is reported that lengths of miRNA precursors varied significantly in plants from 60 to more than 400 nucleotides[21]. The diversity of identified miRNAs can also be found in the location of mature miRNA sequences. The sequences of miRNA clo-miR1-7 were located at the 5′ end of the precursors, while the other was found at the 3′ end. It was also observed that members belonging to the same miRNA family are located at the same end of precursors. Though the length of miRNA precursors varied significantly, lengths of mature miRNAs was mainly confined to 18 to 22 nucleotides, which is in agreement with a majority of miRNAs already identified in other plants[24].

It has been reported that the strong bias of uracil in the first 5′ nucleotide position is due to its important role in the recognition of miRNA by ARGONAUTE1[27]. Consistent with this, 6 of the turmeric miRNAs have uracil as the dominant nucleotide at their first position. Conclusions on the importance of position-specific nucleotide preference at sites other than the first nucleotide of mature miRNA have varied. In soybean, 61% cytosine preference at position 19 has been reported[28] and suggested that this may be important for RNA induced silencing complex (RISC) or Dicer cleavage sites on precursor miRNA, while other study reported[27] that apart from the first position, no other position specific nucleotide preference could be detected. Similar to earlier reports[28], 5 miRNAs containing cytosine at their 19th position were detected in turmeric. The A+U content of the pre-miRNAs ranged from 48.53-67.41%, with an average of 64.89, which is well within the range reported in previous studies.

To bring in more accuracy, we have adopted a recently reviewed computational approach classified not only as homology based but also structure similarity based search[20]. Previous studies have reported that miRNA precursors have MFE in the

Table 1—List of miRNAs predicted from ESTs of turmeric using MFOLD and their characteristic features[a]

miRNAs	Sequence	EST length (nt)	Gene ID	Location	NM (nt)	LM (nt)	LP (nt)	(A+U) %	MFE (kcal/mol)	AMFE	MFEI
Clo-miR1	UGAAGCUGCCAGCAUGAUCU	887	DY392188	5’	1	20	445	67.41	91.28	20.51	0.62
Clo-miR2	UGAAGCUGCCAGCAUGAUCUG	887	DY392188	5’	0	21	445	67.41	91.28	20.51	0.62
Clo-miR3	UGAAGCUGCCAGCAUGAUCUGG	887	DY392188	5’	0	22	445	67.41	91.28	20.51	0.62
Clo-miR4	UGAAGCUGCCAGCAUGAUCUC	710	DY390333	5’	0	21	155	67.09	41.01	26.45	0.80
Clo-miR5	UGAAGCUGCCAGCAUGAUCU	710	DY390333	5’	1	20	155	67.09	41.01	26.45	0.80
Clo-miR6	GAAGCUGCCAGCAUGAUC	710	DY3930333	5’	3	18	153	66.66	39.31	25.69	0..77
Clo-miR7	AAGCUGCCAGCAUGAUCU	710	DY390333	5’	3	18	151	67.54	36.91	24.44	0.75
Clo-miR8	UCAAAGAAGUCGCUGAGGU	593	DY388098	3’	4	19	239	48.53	65.49	27.40	0.53

[a]Number of mismatches (NM), length of precursors (LP), minimal folding free energy (MFE), adjusted minimal folding free energy (AMFE), minimal folding free energy index (MFEI)

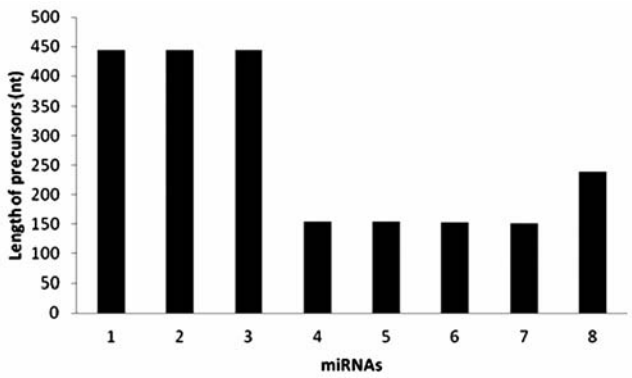

Fig. 3—Length of precursors of miRNAs in turmeric.

range -135.33 kcal mol^{-1} to -5.2 kcal mol^{-1} [26]. In the present study, MFE values ranged from -91.28 to -39.31 kcal mol^{-1} with an average of -62.19 kcal mol^{-1}. MFE values are strongly related to the RNA length. AMFE of turmeric miRNAs ranged from -27.40 to -20.51 kcal mol^{-1} with an average of -23.99 kcal mol^{-1}. In fact, the AMFE of more than 50% of tRNAs falls in the range reported for miRNAs[21]. It was also reported that the MFEI of miRNA precursor sequences was significantly higher compared to that of RNAs, including other small RNAs, and is being adopted as an important criterion for distinguishing miRNAs from other RNAs[21]. MFEI of turmeric miRNA precursors varied from 0.53 to 0.80 and all mature sequences were in the stem portion of the hairpin structures. RNA sequence with a calculated MFEI of >0.67 is more likely a pre-miRNA[16]. However, the lower values do not rule out a sequence being true miRNA, provided the number of nucleotide substitutions in the particular miRNA does not exceed three when compared to other species[29]. miRNAs with low MFEI values of 0.41 was reported in cotton[17]. In soybean miRNAs with low MFEI values of 0.34 were validated by RT-PCR[28]. In the present case, the mismatch with other plant miRNAs was 0-3 in all cases except for one miRNA that showed four (Table 1). This is possibly due to partial identification of miRNAs identified in other plants. Maybe as and when more ESTs are available it would be possible to identify miRNAs identical to the currently known ones. It is also possible that though the miRNAs are highly conserved, there may be mutations in the evolution of miRNA[26].

The predicted secondary structure indicated that there are at least 14 nucleotides engaged in canonical base pairings between the mature miRNA and opposite arm of miRNA* in the hairpin structure and the stem-loop precursor did not contain large internal loops or bulges (Fig. 2).

The current results confirmed that the approach of EST analysis is a relatively efficient way to identify miRNAs. It was also evident that the turmeric miRNAs have similar characteristics to the miRNAs from other plants[26,28]. This also implied that turmeric miRNAs have a common ancestor with other plant species and shared similar regulatory mechanisms.

Recently, through illumina sequencing of small RNA cDNA from rhizomes of variety Mega turmeric, we have validated two of these *in silico* predicted miRNAs, *viz.*, clo-miR2 and clo-miR4 (unpublished results).

Conservation and Divergence of miRNAs in Turmeric

EST analysis can also be employed as a powerful strategy to investigate the conservation, divergence and evolution of miRNAs in the plant kingdom. One of the newly identified turmeric miRNAs was used to compare conservation with their counterparts in other plant species (Fig. 4). The results indicated that mature miR167 is highly conserved among plant species, though it is more likely to mutate at some specific sites. Conservation of miRNAs assessed by bioinformatics or experimentation is a

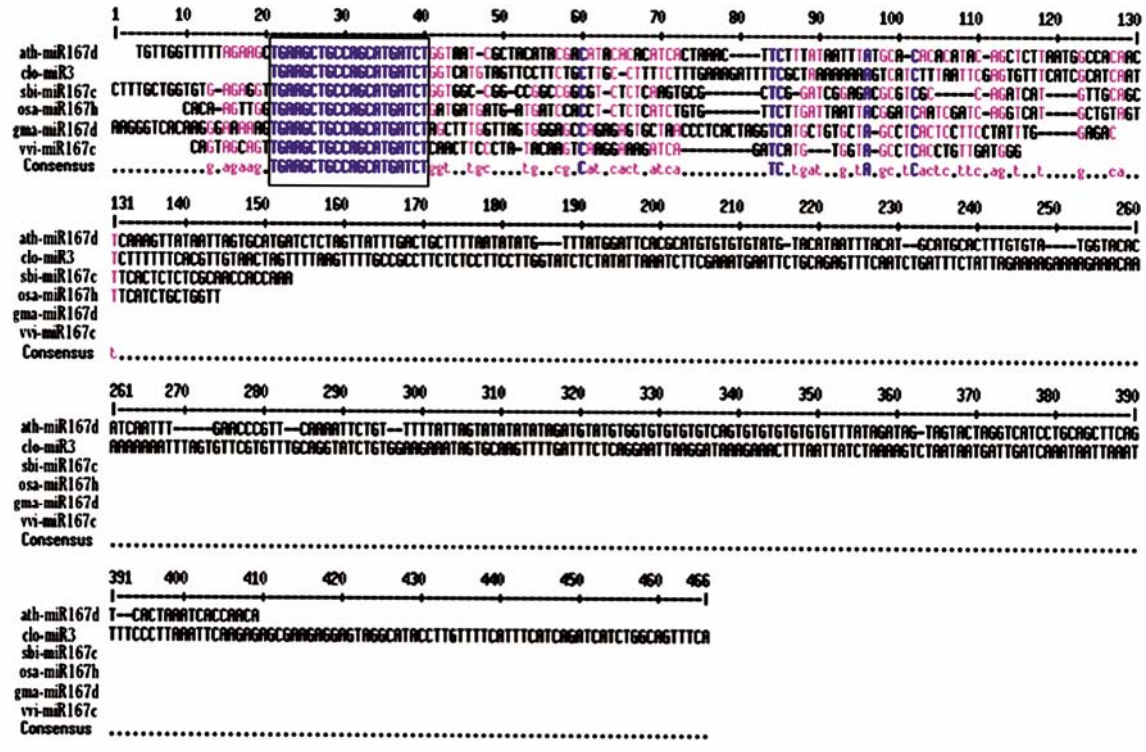

Fig. 4—Multiple sequence alignment of miR167 precursor revealing conservation (as shown in the box) of mature miRNA sequences.

powerful indicator of their functional relevance and ancient origin. The last two sites in the miR167 are more prone to mutation, which indicated that evolution of miRNAs is initiated by mutations at some specific points.

Targets of Turmeric miRNAs

Identification of targets is necessary to understand the biological function of miRNAs in plant development. The basis for this prediction is the perfect or near perfect complementarity of miRNAs and their targets and direct mRNA cleavage. We have predicted potential targets for 7 members of miR167 family (Table 2). No target could be identified for one miRNA (clo-miR8). Almost all the predicted target genes included were reported as miRNA targeted proteins in various plants[16,26]. It has been observed that one miRNA can target more than one regulatory gene. In the resent study, clo-miR1, clo-miR2, clo-miR4, clo-miR5 and clo-miR7 were found to target more than one protein.

MiR167 family members (clo-miR2, clo-miR3 & clo-miR4) from turmeric were found to target auxin response transcription factors, which are an important class of transcription factors functioning in auxin signal transduction during plant growth and development. Complementarity of miR167 to auxin response factors have been already reported in other plants like *Arabidopsis*[30].

Clo-miR4 and clo-miR7 also showed complementarities to LIM domain protein and ring finger protein, respectively. Ring finger family proteins play an important role in plant growth and development in response to biotic and abiotic stresses, while LIM proteins are proposed in cytoskeleton organization. Interestingly, disease resistance protein was also targeted by a turmeric miRNA, such as, clo-miR6. LRR domain provides the platform for recognition of the pathogen and they are important determinants of specificity. NB-LRR resistance protein is also reported as miRNA targets in other plants.

Clo-miR1 and clo-miR5 were found to target transcriptional corepressor SEUSS and carotenoid cleavage dioxygenase (CCD). In *Arabidosis*, SEUSS is involved in the floral organ development. CCD family proteins catalyse the oxidative cleavage of carotenoids, which lead to the production of apocarotenoids[31]. In addition, miRNA has also been documented to regulate cell signalling. Clo-miR2 targets mRNA coding for protein phosphatase 2C. Protein phosphatase 2Cs (PP2Cs) play an important role in signal transduction processes, *viz.*, abscisic acid (ABA) receptor-like kinase (RLK) and mitogen-activated

Table 2—Predicted targets and functions of turmeric miRNAs

miRNAs	Targeted protein	Targeted function
Clo-miR1	SEUSS	Transcriptional corepressor
Clo-miR2, Clo-miR3, Clo-miR4	Auxin response factor	Transcription factor
Clo-miR1, Clo-miR5	Carotenoid cleavage dioxygenase	Metabolism
Clo-miR1, Clo-miR5	P-glycoprotein 8	Development
Clo-miR2	Protein phosphatise 2C	Signal transduction
Clo-miR4	LIM domain protein	Actin cytoskeleton regulation
Clo-miR7	Flavanone 3 hydroxylysae like protein	Metabolism
Clo-miR7	Heat shock factor	Transcription factor
Clo-miR7	ATPase 4	Metabolism
Clo-miR7	Ring H2 finger protein, ATL3	Zn ion binding
Clo-miR7	40S ribosomal protein	-
Clo-miR6	NB-LRR resistance protein RH1 &RH2	Disease resistance
Clo-miR8	Unknown	Nil

protein kinase (MAPK) pathways. Curcumin, the active chemical constituent of turmeric is found to inhibit protein phosphatases leading to rapid induction of MAPKs and apoptosis in tumor cells[32].

Heat shock proteins and 40S ribosomal protein are also reported to be targeted by miRNAs[28,17]. In the present study, clo-miR7 was found to be targeting heat shock proteins and 40S ribosomal protein.

Most of the miR167 members were found to have more than one target and majority of their functions were related to plant growth and development. The results are in agreement with earlier studies. However, no targets could be identified for clo-miR8 probably due to the availability of limited number of turmeric ESTs. It could probably be a turmeric specific miRNA that plays a role in more species-specific characteristics.

In conclusion, EST databases provide a valuable source for identification of conserved miRNAs from plant species where genome sequence is not available. This is the first systematic study that predicted the existence of regulatory miRNAs in non-model plant turmeric by *in silico* analysis of ESTs. The prediction quality was increased by the use of multiple criteria focusing on miRNA sequence quality, stem loop structure and energy requirements. Eight conserved miRNAs were identified, of which 3 are reported for the first time in turmeric (clo-miR6, clo-miR7 & clo-miR8). Due to the comparative sequence analysis adopted in the present study, it is possible that some of the true miRNAs were missed or there could be more miRNAs yet to be identified in the genome. Once increased information on turmeric genomic data is available, more miRNAs can be identified. We have also predicted the potential targets for turmeric miRNAs, which include proteins involved in stress response, development and metabolism. Most of these targets are conserved. The better understanding of the role of miRNAs in post-transcriptional gene silencing in response to various stresses and in relation to biosynthesis of curcumin is very important to develop superior turmeric varieties. The findings of the study will not only strengthen the bioinformatics approach for miRNA identification of non-model crops but will also help in initiating further studies on regulation, mechanism and discovery of more novel miRNAs for improvement of turmeric.

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