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ORIGINAL ARTICLE

Transcriptome deep sequencing, identification of novel microRNAs and validation under drought stress in turmeric (*Curcuma longa* L.)

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Abstract microRNAs (miRNAs) are small non-coding RNAs that execute regulation of gene expression at the post-transcriptional level. Information on turmeric miRNAs is very rare. In the present study, we have identified 27 conserved and 40 non-conserved miRNA families by high-throughput Illumina sequencing and downstream in silico analysis. We could also identify 97 novel candidate miRNAs specific to turmeric. Selected miRNAs were validated by stem-loop RT-PCR and northern blotting. The targets were predicted for identified miRNAs and were found to be involved in plant growth and development, stress response and metabolism. Expression pattern of selected conserved miRNAs in response to drought stress was analyzed by stem-loop qRT-PCR. This is the first report on miRNA content of turmeric transcriptome, which might serve as a foundation for more future work on miRNA based gene regulation in this medicinally important spice crop.

Keywords microRNA · Illumina · Gene expression · Targets · Gene regulation · Turmeric

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Introduction

Regulatory small RNAs (sRNAs) are reported as universal components of endogenous plant transcriptome (Axtell 2013). sRNAs belong to two major classes: microRNAs (miRNAs) and small interfering RNAs (siRNAs), and miRNAs have been reported as the best characterized among them (Jones-Rhoades et al. 2006). Plant miRNAs are endogenous small non-coding 19–24 nt RNAs with high complementarity to target transcripts and mediate endogenous gene silencing at transcriptional and post-transcriptional levels (Voinnet 2009). miRNA genes are distinct non-coding transcriptional units transcribed by RNA polymerase II as primary miRNAs and subsequently cleaved into precursor miRNAs (pre-miRNAs) in the nucleus by RNase III-type enzyme Dicer-like 1 or DCL1 (Lee et al. 2004). Pre-miRNA processing results in miRNA/miRNA* duplexes (Chen 2005) which are further stabilized by 2′-O-methylation, catalyzed by HEN1 (Yu et al. 2005) and exported to cytoplasm by HASTY (Park et al. 2005). One of the strands of each miRNA/miRNA* duplex is selectively incorporated into RNA-induced silencing complex (RISC) (Chen 2005) to inhibit target gene expression (Hammond et al. 2000) and play significant roles in plant growth and development (Chen, 2005), hormone signaling (Liu et al. 2009) small RNA metabolism (Yoshikawa et al. 2005), stress responses (Zhang et al. 2011; Fujii et al. 2005) and maintenance of genome integrity (Khraiwesh et al. 2012).

miRNA discovery from plants is mainly achieved by three major approaches such as forward genetics, bioinformatic prediction and direct cloning and sequencing. miRNA has been identified by forward genetics in *Arabidopsis* (Aukerman and Sakai 2003) and EST-based in silico prediction identified only conserved miRNAs from

plant species including *Arabidopsis*, rice and lettuce (Zhang et al. 2005, Han et al. 2010). Cloning-based methods failed to detect under-expressed miRNAs (Ong and Wickneswari 2011). High-throughput sequencing allows the discovery of novel classes of small RNAs as well as their quantitative profiling (Hsieh et al. 2009), and a large number of miRNAs have been identified by high-throughput sequencing and are available in the miRNA database miRBase (http://microrna.sanger.ac.uk). miRNAs deposited in miRBase disclose the complexity and abundance of miRNAs, (Zhang et al. 2009) which helps in detection and characterization of miRNAs and their corresponding targets in various species (Yanik et al. 2013).

Turmeric belonging to the family Zingiberaceae is an important medicinal spice widely used as food preservative, natural dye in food industry and in cosmetics and drugs (Krup et al. 2013). Besides this, turmeric has also been traditionally used in Ayurvedic medicine and against various malignant diseases, diabetes, allergies, arthritis, Alzheimer's disease and other chronic illnesses. Medicinal properties associated with turmeric are due to one of the important secondary metabolites curcumin (Aggarwal et al. 2007). Considering the importance of *Curcuma* species, it is important to find good practices to increase their growth parameters and major components (Mohamed et al. 2014). Effect of abiotic stress factors including salt and drought were found to adversely affect growth and productivity of the plant (Mohamed et al. 2014; Mostajeran et al. 2014). Dry weight of leaf and pseudo-stem of turmeric compared to rhizome and root was found to be reduced under salt stress. Curcumin synthesis was found to be increased at low salt concentration while salinity higher than 60 mM resulted in 24 % reduction in the production of curcumin (Mostajeran et al. 2014). The productivity of *Curcuma* species in response to different irrigation intervals have also been reported (Mohamed et al. 2014). For example, weekly irrigation of turmeric was found to improve vegetative growth of the plant including fresh and dry weight of rhizomes as well as number and width of the leaves. Continuous irrigation of every 1 week improved the yield of volatile oil and curcumin content. Curcumin and volatile oil contribute to its medicinal properties. Drought is one of the major abiotic stress factors and unfavorable environmental conditions that causes major decline in agricultural production. Drought also affects plant growth and development. Microarray and deep sequencing studies have revealed almost hundreds of protein coding genes associated with abiotic stress conditions (Baxter et al. 2014; Golldack et al. 2014). Even though a lot of researches have been conducted to elucidate gene expression during drought stress, the basic regulatory mechanisms of such gene expression remain unknown (Frazier et al. 2011). As significant regulatory molecules, miRNAs are known to play major role in drought tolerance.

Drought responsive miRNAs have been reported from model plants *Arabidopsis* and rice (Li et al. 2008; Liu et al. 2008; Zhao et al. 2007; Zhou et al. 2010). In rice 19 miRNAs have been differentially expressed in rice under drought condition (Zhou et al. 2010). miR169-mediated drought resistance has also been reported in *Arabidopsis* (Li et al. 2008). By regulating the expression of mitogen-activated protein kinase (MAPK), phospholipase (PLD), praline dehydrogenase (PDH) and peroxidase (POD) miRNAs have been reported to control drought tolerance in maize (Wei et al. 2009). miR398- and miR408-mediated downregulation of COX5b, CSD and plantacyanin was observed during water deficit condition in *Medicago* (Trindade et al. 2010).

Although a number of miRNAs have been identified from other plant species, information on identification and characterization of miRNAs from the family Zingiberaceae is limited. The study of miRNAs in turmeric has been reported previously using in silico approach (Rameshwari et al. 2013; Santhi and Sheeja 2015). The study conducted by us (Santhi and Sheeja 2015) that identified eight conserved miRNAs from turmeric suggests bioinformatics as a powerful strategy for identification of homologs or orthologs of miRNA genes in other plant species. Twenty-five conserved and three novel miRNAs in response to *Pythium aphanidermatum* infection have already been identified and validated from turmeric (Chand et al. 2016). However, this is the first report on identification miRNAs in large scale through high-throughput Illumina sequencing from turmeric.

In the present study, we have generated sRNA sequences from the rhizome of 4-month-old plants of a high curcumin variety Mega Turmeric. The downstream bioinformatic analyses of sRNA data generated by Illumina sequencing led to the identification of 67 known miRNA families. We could also identify 97 putative novel miRNA candidates and their precursor sequences. We have also used stem-loop RT-PCR and northern blotting approaches to validate some of the representative miRNAs. Selected miRNAs were also validated by qPCR under drought condition. The present study also identified putative miRNA targets with significant roles in plant growth and development, stress response and metabolism.

Materials and methods

Plant material and preparation of sRNA cDNA library

Turmeric plants were grown and maintained in the experimental farm of ICAR—Indian Institute of Spices

Research. Rhizomes were collected from 4-month-old rhizome and stored in RNA later (Ambion). Total RNA was isolated using CTAB method (Ghawana et al. 2011). Integrity of the total RNA was analyzed using RNA 6000 Nano kit (Bioanalyzer) and those with RIN value above 8 (8.2) was selected for library preparation using sRNA sample preparation kit (Illumina) according to the manufacturer's protocol and subjected to Illumina sequencing. Preparation of cDNA library as well as sequencing was carried out at Sandor Proteomics, Hyderabad, India.

Data preprocessing and miRNA identification

The quality of the bases was evaluated by measuring the quality of reads according to the percentage of bases with quality greater than or equal to 30 (Q30) followed by removal of low quality reads and adaptors. Adaptor sequences were removed by cutadapt. After this redundancy in reads were removed to make the reads unique. Sequences with 18–30 nts were used for downstream bioinformatic analysis. Following this step, coding RNAs including t/rRNAs were removed using Rfam database (http://www.sanger.ac.uk/Software/Rfam), and the remaining sequences were mapped to unique mature plant miRNAs from miRBase using Bowtie alignment tool. A maximum of two mismatches were allowed for each alignment. After the discovery of conserved miRNAs, remaining unaligned sRNA sequences were used for novel candidate miRNA prediction. Due to the lack of whole-genome data, novel miRNAs were predicted from ESTs (National Center for Biotechnology Information, NCBI, http://ncbi.nlm.nih.gov/; http://www.plantrhizome.org), nucleotides (NCBI, http://ncbi.nlm.nih.gov) and transcriptome sequences (NCBI under Bioproject ID PRJNA270561) from turmeric using the tool miRCat (http://srna-tools.cmp.uea.ac.uk/;) with default parameters. We have also used *C. aromatica* transcriptome sequences (NCBI under Bioproject ID PRJNA277549) to identify novel miRNAs. The generated novel pre-miRNA sequences were further filtered according to the previously established criteria (Zhang et al. 2005, 2006b). Furthermore, minimal folding free energy index (MFEI) was manually calculated according to Zhang et al. (2006b).

Identification of potential miRNA targets

Putative targets for identified miRNAs were predicted by aligning the miRNA sequences with ESTs, transcriptome and nucleotides of turmeric using psRNATarget (http://plantgrn.noble.org/psRNATarget/) with default parameters using stringent expectation value cutoff of 1 and GO annotation for known and novel miRNA targets were carried out using Blast 2GO.

Validation of miRNAs by stem-loop RT-PCR

Total RNA was isolated from different tissues of turmeric using SDS-based method (Deepa et al. 2014). Primer design, cDNA preparation and end-point PCR were performed as described previously (Varkonyi-Gasic et al. 2007). All primers used in the study were listed in additional Table 1. The PCR products were analyzed on 4 % agarose gel and specificity of some of the amplifications were checked. For this, PCR amplicons were extracted from the gel using GenElute^{TM} Gel Extraction Kit (Sigma) and cloned into pGEM-T Easy vector (Promega) and sequenced.

Validation of miRNAs by northern blotting

sRNA of <200 nts were isolated using *mir*Vana^{TM} miRNA Isolation Kit (Ambion). Around 100 ng of sRNA was resolved on 15 % PAGE containing 7 M urea at 120 V for 2 h along with microRNA marker (NEB). The gel was stained with SYBR gold nucleic acid gel stain (Invitrogen) and was further transferred into positively charged nylon membrane (Roche) using TransBlot SD semiDry transfer apparatus (Bio-Rad) at 20 V for 1.5 h. The 5′ digoxigenin-labeled locked nuclear acid (LNA) probes were used for miRNA detection. Prehybridization and hybridization steps were performed as per DIG Northern Starter kit (Roche). The membrane was hybridized with 2.5 pmol/µl probe at 37 °C overnight. Washing and detection were carried out by using the DIG Northern Starter kit (Roche).

qPCR analysis of miRNAs under drought conditions

Two-month-old in vitro plant cultures were transferred to Murashige and Skoog medium (MS medium) with 0, 5, 10 and 15 % PEG to induce varying degrees of drought stress. The plants were placed under 16 h day/8 h night cycle for 15 days and leaves were collected and immediately stored in RNA later (Ambion).

Total RNA was isolated from the collected samples using the protocol developed by Deepa et al. (2014). The quality and quantity of total RNA was checked using Biophotometer plus and agarose gel electrophoresis and further digested with DNase (Turbo DNA-free kit, Ambion) to remove genomic DNA contamination. To check the expression fold change in miRNAs, about 100 ng of DNA-free total RNA from three biological replicates was hybridized with miRNA specific stem-loop RT primer. The hybridized miRNA molecules were then reverse transcribed into cDNA as previously reported (Varkonyi-Gasic et al. 2007). Real-time PCR was

performed using QuantiFast SYBR® Green PCR Kit (Qiagen) on Rotor Gene-Q real-time PCR (Qiagen). All reactions were performed three times. qPCR parameters were 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s. Melting curve analysis was performed at 70–95 °C to check the specificity of PCR products. Relative gene expression was calculated using $2^{-\Delta\Delta CT}$ method. 18S rRNA was used as the endogenous control. The statistical analysis was done by one-way analysis of variance (ANOVA) using GaphPad Prism, and the *P* values less than 0.05 were considered to be statistically significant.

Results

Analysis of sequences from sRNA library

A total of 39,505,308 raw reads were generated by Illumina-based high-throughput sequencing of sRNA cDNA library constructed from 4-month-old rhizome of variety Mega Turmeric maintained at the Experimental Farm of ICAR—Indian Institute of Spices Research. Reads were quality filtered, and adaptors and small sequences of <16 nts were removed to obtain 35,508,108 clean sequences. Among these, 11,489,882 sequences ranging from 16 to 30 nts corresponded to 987,309 unique reads. Around 23.34 % of unique reads matched with non-coding RNAs including tRNA (4.08 %) and rRNA (19.26 %). Thus, 460,310 sequences in the range of 18–24 nts excluding other non-coding RNAs were used for downstream analysis to identify miRNAs (Additional Table 2).

The abundance of sRNAs in our dataset varied considerably. While some were present only a few times, others were present thousands of times. For example, 987,309 sequences out of 11,489,882 were sequenced only once. This observation suggests that (1) considerable differences exist in sRNA expression from rhizome and (2) sRNA survey in rhizome is far from being exhausted (Mandhan et al. 2012).

In turmeric, size of sRNA was unevenly distributed. Among these, sequences of 21 nt were the most abundant followed by 22 nt and 18 nt. Sequences of 28 nt length could be the degradation products formed during sRNA library construction (Mandhan et al. 2012).

Identification of conserved, non-conserved and novel miRNAs

To identify known miRNAs, unique mature already reported miRNA sequences were extracted from miRBase version 21 and aligned against sRNA dataset from turmeric using bowtie, which is more suitable for the alignment of short reads. A maximum of two mismatches were allowed for each alignment. After performing bowtie, 67 miRNA families (Additional Table 3) were found to be similar to known miRNAs among which 27 families were highly conserved (Fig. 1). Homologs of miR156, miR166, miR319 and miR396 were identified in 41, 34, 32, 43 different species including *Arabidopsis thaliana* and *Oryza sativa*, respectively. Among the 27 conserved miRNA families, miR166 had the highest number of reads (17, 405) while miR159, 396, 319 and 156 were also abundant in the library with 16388, 4398, 2678 and 1683 reads, respectively. There are also miRNAs that were infrequently sequenced (<100). It was also noted that isoforms of miRNAs within a family showed significant variations in their expression. For example, sequencing frequency of miR159 varied from 1 to 16,388. Diversity of conserved miRNAs could also be found in number of members in each family, which ranged between 1 and 322. The largest families were miR166 and miR159 with 322 and 150 members, respectively, while miRNA families such as miR394, miR530 had only one member. The sizes of mature miRNA were also found in the range of 18–22 nts.

Recently, in addition to conserved miRNAs, other known miRNAs were also reported which are not conserved and present only in one or a few plant species (Jones-Rhoades et al. 2006). Such 40 non-conserved miRNA families were also identified from our dataset (Additional Table 3) with 20–24 nts. The expression level of these miRNAs was found to be less compared to the conserved miRNAs. The number of members in each family varied from 1 to 36.

Since the whole-genome data of turmeric is not available, unique unannotated sRNAs of 458,751 reads were mapped to turmeric ESTs, transcriptome and nucleotide sequences. Mapping the sRNAs against these sequences and predicting the secondary structure of surrounding sequences, 97 novel miRNAs that satisfied the secondary structure criteria as established by Zhang et al. (2005) could be identified (Additional Table 4). Representative structure of novel miRNA precursors is given in Fig. 2. All these precursors had a minimal folding free energy in the range of −149.5 to −17.9 kcal/mol with an average of −40.12 kcal/mol. The lengths of pre-miRNAs were found to vary from 60 to 210 nts with an average of 95 nts while the length of mature miRNAs was found to be 21 (except clo-miR86). For a better measurement of RNA/DNA molecule strand stability, adjusted minimal folding free energy (AMFE) value was calculated. AMFE of novel pre-miRNA sequences ranged from −64.09 to −25.58 kcal/-mol with an average of −40.18 kcal/mol. Minimal folding free energy index (MFEI) is a unique criterion to distinguish miRNAs from other coding and non-coding RNAs. Reports suggest that RNA sequence with MFEI greater

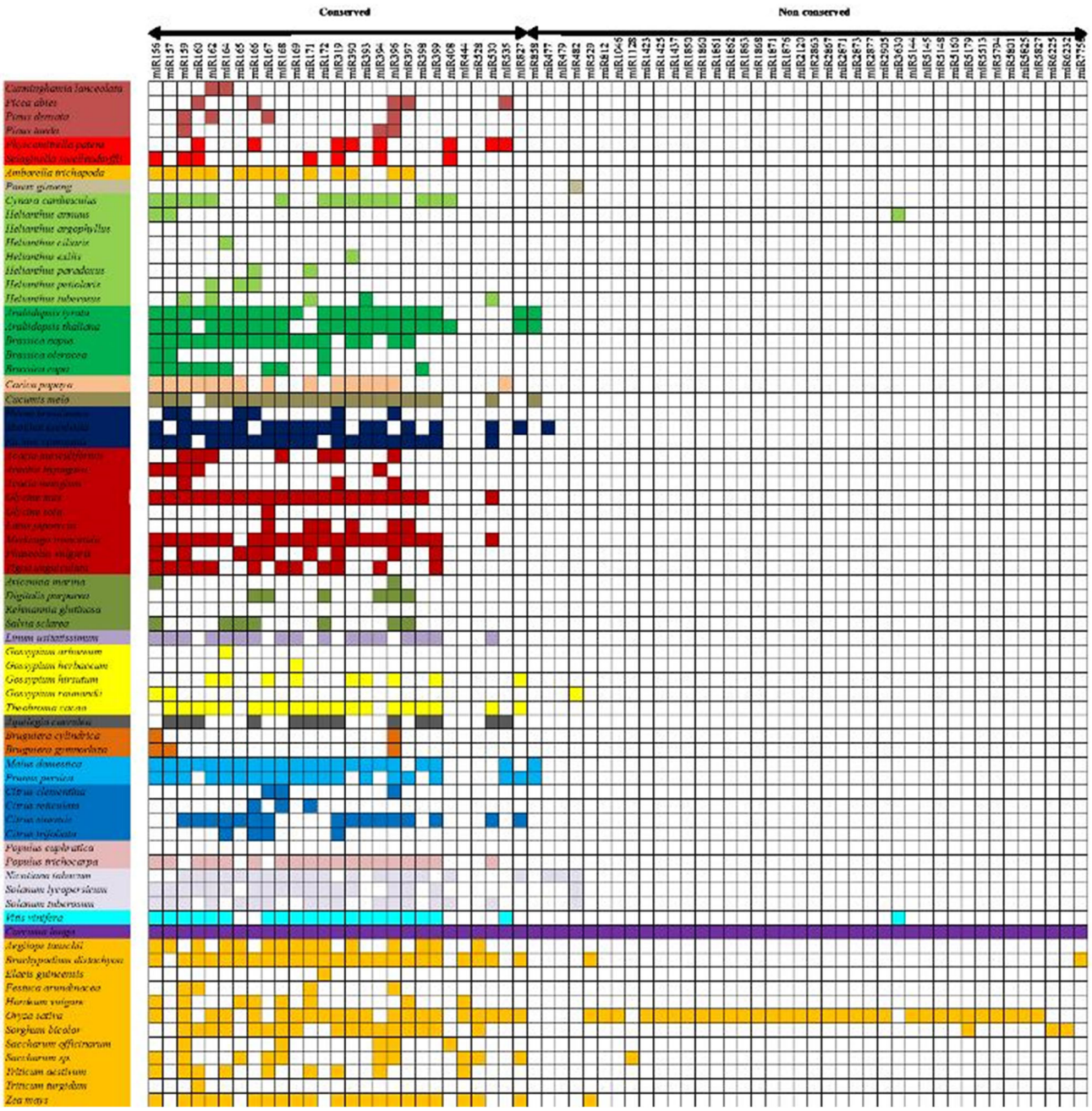

Fig. 1 Conserved and non-conserved miRNA families in turmeric and across species. Closely related species are shown in the same color. *Colored boxes* indicate the presence of a particular miRNA family

than 0.85 is most likely to be miRNAs (Zhang et al. 2006b). The precursors of these novel miRNA candidates had minimum folding free energy index (MFEI) value with an average of approximately 0.85, which is greater than other RNAs such as tRNA, rRNA and mRNA.

Of 97 novel miRNA precursors, 20 were found to have miRNA* sequences, which is regarded as an additional criteria for the annotation of novel miRNAs. According to the criteria established by Zhang et al. 2006b and Meyers et al. 2008, class I miRNA precursors have mature miRNA and miRNA* sequences found in sRNA libraries. Accordingly present study could identify 20 *bonafide* precursors with miRNA/miRNA* precursors. 32 of the novel miRNAs started with 5′ uridine while 10 miRNAs have adenine in the same position, which is in congruence with statistical characteristics of mature miRNAs. Expression profile of novel miRNAs was also found to vary from 2 to 16,118 reads, and majority of the novel miRNAs were

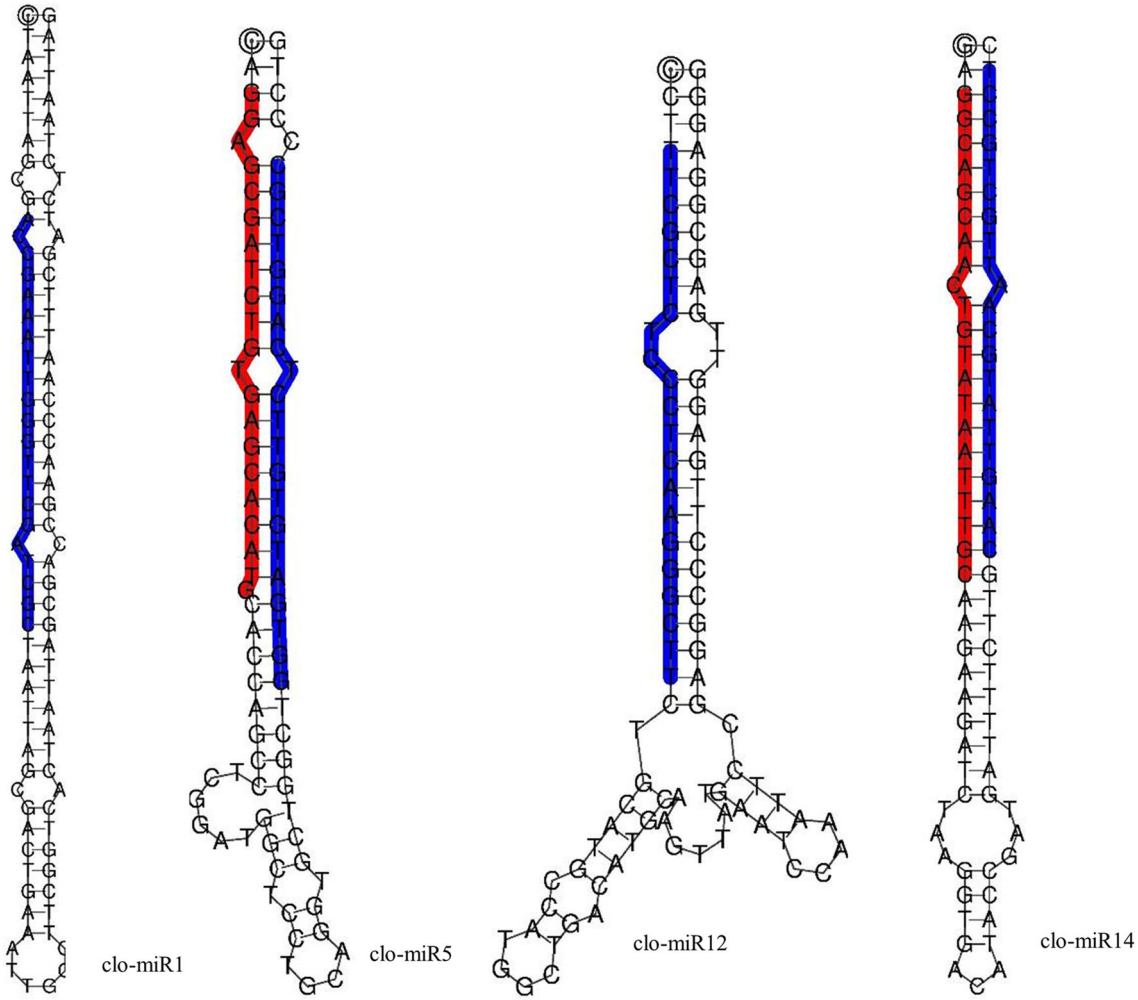

Fig. 2 Predicted hairpin stem-loop secondary structure of novel miRNAs. Mature miRNA sequences are represented in *blue*, and miRNA* sequences are given in *red color*

mapped to a single locus in the EST sequences of turmeric and represented unique sequences.

Validation of miRNAs by stem-loop RT-PCR and northern blotting

In order to validate some of the predicted miRNAs, we have randomly selected 13 conserved miRNAs and 8 novel miRNAs for stem-loop RT-PCR-based analysis. Thus, 13 conserved miRNAs (representing the families miR156, miR157, miR159, miR160, miR166, miR167, miR169, miR172, miR319, miR396, miR398) and 8 novel miRNAs (miR3, miR8, miR14, miR45, miR65, miR93, miR94 and miR95) showed amplification (Figs. 3, 4) with bands corresponding to 60 bp. Moreover, we have also cloned and sequenced some of the PCR amplicons and all the miRNAs cloned were found to be 100 % identical to the miRNAs from miRBase.

Two miRNAs viz miR156b and miR167c were also validated by northern blotting (Fig. 5).

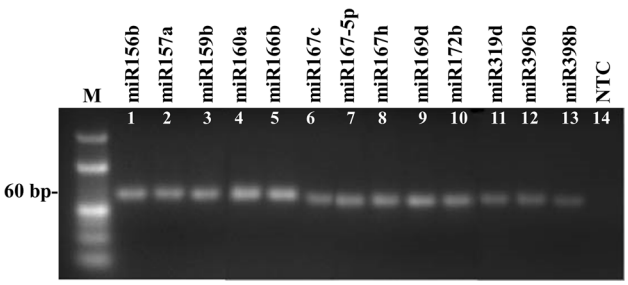

Fig. 3 Stem-loop RT-PCR for identified conserved miRNAs. Thirteen conserved miRNAs were validated by stem-loop RT-PCR. The sizes of PCR products were ~60 bp. *M* marker, *NTC* non-template control

Prediction of miRNA targets

An essential step to understand the functions of miRNAs is the prediction of their target mRNA. In plants, miRNAs identify their targets through perfect complementarity, which facilitates successful target prediction by computation (Rhoades et al. 2002). A total of 322 putative targets were predicted for 27 known miRNA families by computational algorithm psRNATarget against turmeric transcript library (EST, nucleotide and transcriptome) as reference set (Additional Table 5). No targets could be identified for 51 miRNA families which might be due to insufficient turmeric mRNA sequences. Number of predicted targets for known miRNA families also varied from 1 to 69.

Among the targets of known miRNAs, seven were found to be conserved among various plant species, which reveals the conserved regulatory roles of these miRNAs (Additional Table 5). All these conserved targets were transcription factors such as squamosa promoter binding-like protein genes (SPLs), growth-regulating factors (GRFs), NAC domain-containing proteins, F-box family proteins, GAMYB transcription factor such as proteins, floral homeotic protein APETALA 2-like isoform X1, homeobox leucine zipper proteins and three auxin response factors targeted by miR156, miR396, miR164, miR394, miR319, miR172, miR166 and miR160 families, respectively. We could also detect some proteins with unknown functions and hypothetical or uncharacterized proteins as targets of miRNAs.

For novel miRNAs, targets were predicted successfully for 57 out of 97 miRNAs (Additional Table 6). Their targets included ap2-like ethylene-responsive transcription factor toe3, transcription factor gamyb-like isoform × 3, heat stress transcription factor a-1-like, cytochrome P450-like TBP, GDSL esterase/lipase, 40 s ribosomal protein s3-2-like isoform x1, metallothionein-like protein and tubulin alpha-1 chain. Results suggest that these novel miRNAs might be involved in some specific developmental as well as essential biological processes in turmeric. Out of 104 turmeric specific targets, 39 transcripts coded for unannotated mRNAs.

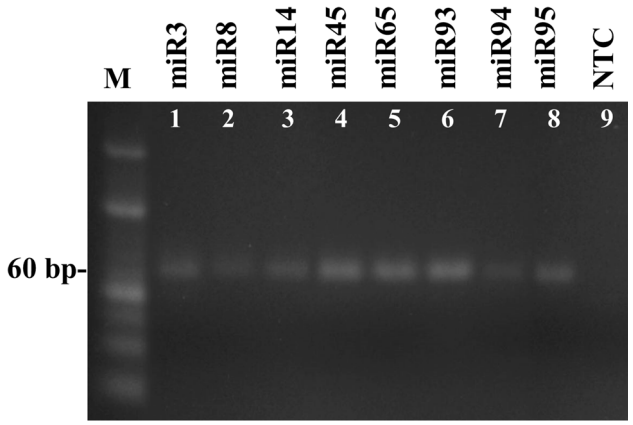

Fig. 4 Stem-loop RT-PCR for identified novel miRNAs. Eight randomly selected novel miRNAs were validated by stem-loop RT-PCR. The sizes of PCR products were ~60 bp. *M* marker, *NTC* non-template control

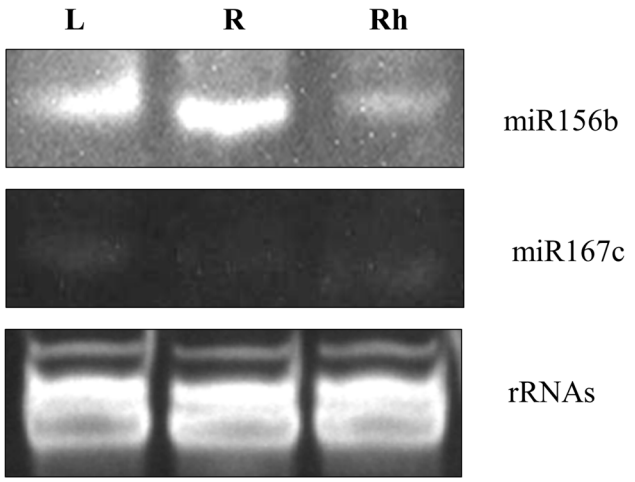

Fig. 5 Northern blotting detection of miR156b and miR167c. Small RNA was extracted from different tissues including leaf (L), root (R) and rhizome (Rh), separated and transferred to nylon membranes. LNA probes were used to detect miR156b and miR167c. Ethidium bromide-stained rRNAs were used as gel loading controls

Further, all the predicted targets of known and novel miRNAs were subjected to GO analysis using Blast 2GO in order to better understand their functions. GO terms were categorized into biological process, cellular component and molecular function. Thirty-nine genes were categorized as cellular components, and within this category itself, most of the genes are localized in the nucleus. On the basis of molecular function, 74 genes were categorized and most of them were involved in binding and catalytic activities. On the basis of biological process, most of the genes participated in metabolic; GTP catabolic and oxidation reduction processes (Fig. 6).

qRT-PCR analysis of miRNAs following drought stress

Selected conserved miRNAs were validated by qPCR under drought conditions induced in vitro. The changes in expression of these miRNAs are shown (Fig. 7). miR156, miR166, miR167 and miR172 showed differential expression with increasing PEG concentration. miR156 showed a sudden increase in 5 % PEG followed by an abrupt decrease in the expression level at 10 % PEG compared to control. miR167 was upregulated in leaves exposed to 5 %

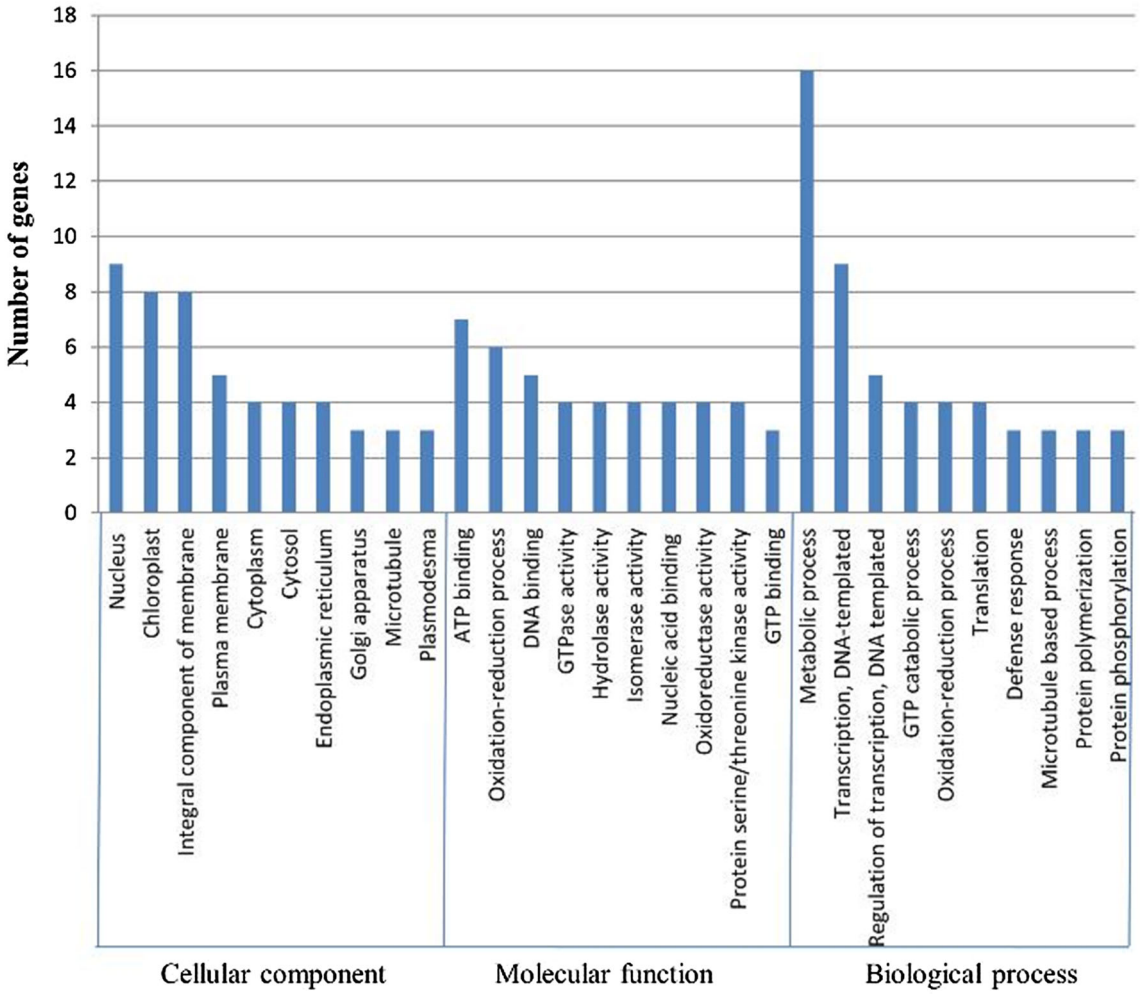

Fig. 6 GO analysis and categorization of putative targets of miRNAs. According to GO analysis putative miRNA targets were categorized into cellular component, molecular function and biological processes

PEG while miR166 was found to be downregulated. However, in 10 % PEG miR156 and miR166 were downregulated while miR167 and miR172 were found to be upregulated compared to control. In 15 % PEG, miR166 and miR172 were downregulated while miR156 and miR167 were upregulated in comparison with control.

Discussion

Plant miRNA studies from spice crops are very limited. But, several studies on miRNAs have revealed their roles in developmental as well as other morphogenetic processes in plants (Yanik et al. 2013; Jagadeeswaran et al. 2012). In spite of its great medicinal value, systematic study of turmeric miRNAs is scarce. Identification of complete set of miRNAs and corresponding targets will lay foundation for understanding miRNA-mediated regulatory systems controlling development and other physiological process (Jones-Rhoades et al. 2006). Present study identified known and novel miRNAs from turmeric using Illumina-based deep sequencing and downstream bioinformatic predictions. Compared to in silico method, high-throughput sequencing technology provides more reliable predictions of miRNAs and also helps us to discover species specific novel miRNAs. Lack of whole-genome data made miRNA identification by deep sequencing from turmeric difficult. However, here we have taken advantage of the recently sequenced rhizome transcriptomes from *C. longa* and a related species *C. aromatic*a (Sheeja et al. 2015) for prediction of miRNAs and targets in this important medicinal spice.

In total 35,508,108 high-quality reads were generated from 39,505,308 raw sequences representing 89 % of the total reads. sRNA of 21, 18 and 22 nts dominated in the dataset with 21 nt being unusually the most abundant. Previous reports from other plants including *M. trunculata and peanut* were shown to contain more 24 nts than 21 nt

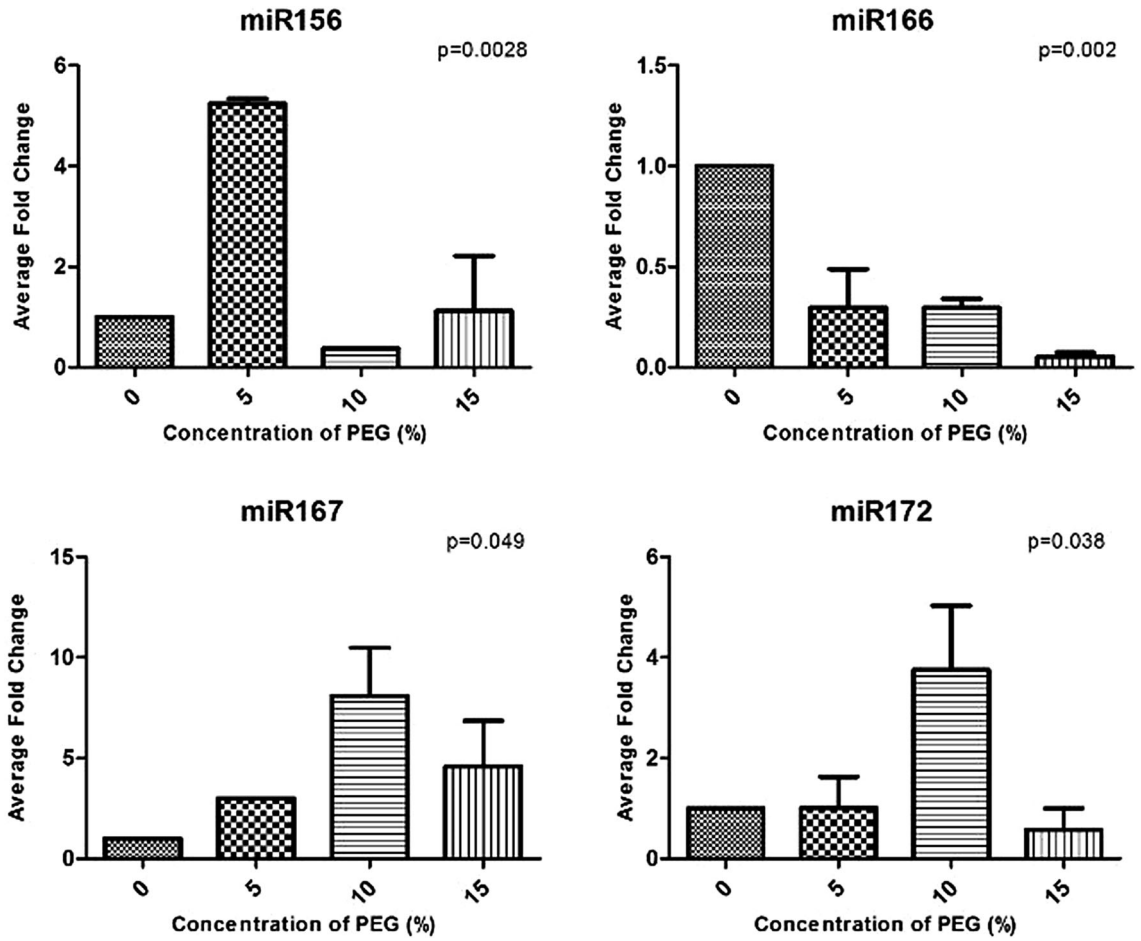

Fig. 7 Relative expression levels of four miRNAs in leaves under drought stress treatments

sRNAs (Szittya et al. 2008; Zhao et al. 2010) which are known to guide DNA methylation and heterochromatin formation (Cuperus et al. 2011). Abundance of 21 nt sRNA sequences has been reported from gymnosperms and other conifer species (Dolgosheina et al. 2008; Morin et al. 2008). Reports suggests that only very small fraction of 24 nts (2.6 %) miRNAs in conifers is due to the lack of DCL3, the enzyme that matures 24 nt RNAs in angiosperms (Dolgosheina et al. 2008; Morin et al. 2008). Our results were also in agreement with that from grapevine (Pantaleo et al. 2010) and tree species (Barakat et al. 2007).

Conserved miRNAs identified in our study were found to be orthologous to miRNAs identified from other plants including *Arabidopsis* (Jones-Rhoades and Bartel 2004; Rajagopalan et al. 2006) and rice (Jones-Rhoades and Bartel 2004). Earlier reports suggest that evolutionary conserved miRNAs exist as gene families (Rajagopalan et al. 2006). In our case, most of the conserved miRNAs viz miR166, miR159, miR160, miR162, etc. existed as gene families. While miR169 was reported as the most abundant miRNA in rice (Sunkar et al. 2008), high number of sequencing reads of miR166 is observed in our case, which is also reported in *Vigna mungo* (Paul et al. 2014). Significant differences were observed in the number and abundance of members in each family as found in other plants (Martinez et al. 2011; Lertpanyasampatha et al. 2012).

Non-conserved miRNAs are not conserved, evolutionarily recent and represented by single copy *MIR* genes (Jones-Rhoades et al. 2006; Pantaleo et al. 2010), and such non-conserved miRNAs have been reported from other studies also (Paul et al. 2014; Martinez et al. 2011). Our studies could identify 40 non-conserved miRNA families in turmeric, and most of them showed low levels of expression, which is in agreement with previous reports (Pantaleo et al. 2010).

Reports on miRNAs from other plants have revealed that each species has a set of unique species specific novel miRNAs (Zhao et al. 2010; Yi et al. 2013). In agreement with this 97 novel species, specific miRNAs could be identified from turmeric of which majority (88 miRNAs) showed low levels of expression which is in conformity

with earlier studies (Zhao et al. 2010; Yi et al. 2013). The low level of expression of these miRNAs is the result of their highly specific expression patterns in tissues or cells as well as the lack of regulatory elements that confer robust expression (Cuperus et al. 2011). It has also been reported that majority of plant miRNAs begin with 5′ U (Reinhart et al. 2002) which in turn might affect the incorporation by AGO 1 (Mi et al. 2008) or stability (Rajagopalan et al. 2006). Deep sequencing detects miRNA* or passenger strand being the counterparts of mature miRNA in the duplex, that are generally degraded during miRNA biogenesis. Thus, the presence of miRNA* is reported as a strong evidence supporting proper biogenesis of miRNA (Meyers et al. 2008). Recent reports also show the importance of miRNA* in various cellular functions (Devers et al. 2011). Relevant levels of miRNA* were observed in Drosophila with regulatory activity (Okamura et al. 2008). Increased level of miR399* accumulation is also reported in *Arabidopsis* in response to phosphate deficiency (Hsieh et al. 2009). In turmeric, 31 such miRNA* sequences were detected with low level of sequencing frequency, which is in line with the idea that miRNA*strands are usually degraded during miRNA biogenesis (Rajagopalan et al. 2006). The presence of miRNA* indicates its importance in the developmental stages of turmeric.

It has been reported that target cleavage is the major mechanism of miRNA-guided gene regulation (Schwab et al. 2005) in plants which was also observed in turmeric. One of the conserved targets identified for miR156 was SPL. In *Arabidopsis*, miR156 targets 11 SPL genes (Wang 2014). SPLs regulate various biological processes such as leaf development, shoot maturation, phase change, flower and fruit development and plant architecture formation (Chen et al. 2010). miR164 also targets NAC domain-containing proteins were found. Previous results from other plants suggest that miR164 is an important regulator in various developmental processes including floral, vegetative organs and lateral root development (Zhang et al. 2006a). miR166 targeted by HD-ZIP transcription factor genes regulates various processes such as root and nodule development (Boualem et al. 2008). It has been reported that balanced activity of miR166 and its targets from class III homeodomain–leucine zipper family regulate root growth in *Arabidopsis* (Singh et al. 2014). GAMYB transcription factor has been predicted as miR319 target in potato (Lakhotia et al. 2014). It has been proved that miR159-mediated regulation of GAMYB affects plant anther development and flowering time in *Arabidopsis* (Zhang et al. 2006a). miR160 was found to target various auxin response factors (ARFs). ARFs are transcription factors that regulate the expression of auxin response genes (Guilfoyle et al. 1998) and ARF 10, ARF 16 and ARF 17 were also reported to target miR160 (Rhoades et al. 2002) and regulate various aspects of plant development (Mallory et al. 2005; Wang et al. 2005). F-box family proteins, conserved targets of miR394 play major role in protein degradation (Schulman et al. 2000) and are also related to signal transduction and cell cycle (Craig and Tyers 1999). Growth-regulating factor (GRF) genes known to play important roles in plant growth, and development and resistance to stress (Rodriguez et al. 2010) were also found to be targeted by miR396 in *Arabidopsis*. Reports also suggest that miR396 targeted AtGRF, is required for leaf development (Wang et al. 2011). Floral homeotic gene APETALA 2 plays role in flower development (Huijser and Schmid 2011) which is predicted as a target of miR172.

In the present study, some of the novel miRNAs were found to target transcription factors and metabolic enzymes. For example, clo-miR37 was found to regulate heat stress transcription factor. Heat stress transcription factor control the accumulation of heat shock proteins and plays a major role in heat stress response (Kotak et al. 2007). clo-miR47 and clo-miR68 were found to target cytochrome p450-like TBP, which plays significant roles in oxidative metabolism (Hackenberg et al. 2013). Similarly clo-miR29 and clo-miR31 targeted GDSL esterase/lipase, which is reported to be involved in plant development, morphogenesis, defense responses and secondary metabolite production (Chepyshko et al. 2012). Previous reports suggest that miRNAs might regulate plant secondary metabolism producing pharmaceutically significant compounds (Prakash et al. 2015). Turmeric is an important medicinal spice with a lot of medicinal importance contributed by various secondary metabolites. Novel turmeric miRNAs might regulate the biosynthesis of secondary metabolite production in turmeric.

Recent reports suggest that miRNAs play very significant role in the regulation of drought responsive genes (Ding et al. 2013) and these reports were mainly on the basis of research on model organisms such as *Arabidopsis* and rice (Frazier et al. 2011). Identification and expression profiling of miRNAs during drought stress in turmeric have not been reported. Thus, in the present study expression profile of miRNAs has been analyzed in turmeric leaves under drought conditions using stem-loop RT-qPCR approach, and four miRNAs were found to be differentially expressed in response to drought in a dose-dependent manner.

For example, miR156 showed an abrupt increase at 5 % PEG followed by a sudden decrease after exposure to 10 % PEG compared to control. Further increase in miRNA expression was observed in 15 % PEG. Elevated level of miR156 has been observed in cotton (Wang et al. 2013) and switchgrass (Sun et al. 2012) under drought condition. miR156 showed 0.7-fold increase in expression under

7.5 % PEG in switchgrass (Sun et al. 2012). 3-4 fold upregulation of miR156 has been observed in drought stressed root of *Triticum dicoccoides* (Kantar et al. 2011). miR156 was found to be upregulated by 0.5-fold in finger millet (Nageshbabu et al. 2013). 2.1-fold downregulation of miR156 was observed in rice during drought stress (Zhao et al. 2009). MiR156 is reported as a highly conserved miRNA that target SPL genes and play significant role in plant development (Spanudakis and Jackson 2014) including vegetative phase change and flowering (Wu and Poethig 2006). Liu et al. 2008 reported the existence of stress-related elements in the upstream region of miRNA genes. For example, stress responsive elements MYB binding site involved in drought inducibility (MBS) have been identified in the promoter sequence of miR156 and miR167 (Liu et al. 2008). The role of miR156 in various biotic and abiotic stress conditions has also been reported (Khraiwesh et al. 2012) including under drought stress (Ding et al. 2013). Dual role of miR156 in both plant development and drought condition indicate that reprogramming of plant development is essential to cope with drought condition (Barrera-Figueroa et al. 2011). Expression level of miR167 was found to be increased in turmeric leaves exposed to different concentrations of PEG compared to control. Liu et al. 2008 reported increased expression of miR167 in *Arabidopsis* during drought stress. miR167 was also found to be increased in cotton leaves treated with 1 and 5 % PEG (Wang et al. 2013). In contrast to this expression, miR167 was found to be downregulated during different dose of PEG concentration in switchgrass (Sun et al. 2012). Downregulation of miR167 by tenfold was observed in finger millet during drought. Inhibited expression of miR167 was observed in maize under drought condition (Wei et al. 2009). Auxin response factors (ARFs) are reported as the conserved targets of miR167 (Wu et al. 2006). Auxins are involved in regulating various aspects of plant development and stress responses (Achard et al. 2006; Liu et al. 2008). Thus, miR167 might contribute to cope drought condition through the regulation of ARF and auxin signaling pathways (Liu et al. 2008). Compared to control the expression of miR172 was found to be downregulated at highest PEG concentration while it was found to be upregulated at 10 % PEG. Differential expression of miR172 was observed in cotton (Wang et al. 2013) where miR172 expression was inhibited as PEG concentration increases while the expression was upregulated at leaves exposed to 5 % PEG. Differential expression of miR172 during drought condition was also observed in tobacco (Frazier et al. 2011). Downregulation of miR172 during drought condition has also been reported in switchgrass (Sun et al. 2012). Significant downregulation of miR172 was also observed during drought condition (Zhou et al. 2010) while it was found to be upregulated in finger millet during drought condition (Nageshbabu et al. 2013). miR166 was found to be downregulated in roots of *Triticum dicoccoides* under drought condition. In agreement with these results, downregulated pattern of miR166 was observed in turmeric in drought condition compared to control. miR166 was also found to be downregulated by tenfold in finger millet root under conditions of drought. miR166 was found to be upregulated during water deficit condition and showed differential expression in root and shoots (Trindade et al. 2010).

In the present study, we have employed high-throughput next-generation sequencing to identify conserved, non-conserved and novel miRNA candidates from medicinally important spice turmeric. Selected miRNAs were validated by stem-loop RT-PCR and northern blotting. We have also validated a few conserved miRNAs by qPCR under conditions of drought. The target prediction of miRNAs and their in silico annotation indicated their diverse roles in plant growth and development as well as stress response. More works on miRNA-mediated gene regulation are required to explore the molecular mechanism of miRNA mediated tolerance to drought condition. Drought responsive miRNAs might be used as a tool in genetic manipulation for the improvement of this medicinally important spice in future.

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