Unraveling MicroRNA Mediated Gene Regulation in Centella Asiatica (L.) Urb. by High-Throughput Sequencing based Small-RNA Profiling.

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Research Article

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Abstract

Centella asiatica is a widely spread herb mostly found in the tropics having extensive medicinal values. Here, we report for the first time, transcriptome-wide characterization of miRNA profile from the leaves of *C. asiatica* using high-throughput Illumina sequencing. We identified 227 conserved and 109 putative novel miRNAs. Computational screening revealed potential mRNA targets for both the conserved and novel miRNAs encoding diverse transcription factors and enzymes involved in plant development, disease resistance, metabolic and signaling pathways. Gene ontology annotation and KEGG analysis revealed the miRNA targets to be involved in a wide range of metabolomic and regulatory pathways. The differential expression of the miRNA encoding genes in diverse tissues was determined by real-time PCR analysis. We also found that gene expression levels of miR156, 159 and 1171 was reduced in salicylic acid treated axenic shoot cultures of *C. asiatica* compared to its control. Furthermore, RLM-RACE experiments mapped miRNA-mediated cleavage at two of the mRNA targets. The present study represents the large-scale identification of microRNAs from *C. asiatica* and contributes to the base for the up-coming studies on miRNA-mediated gene regulation of plant secondary metabolite pathways in particular.

1. Introduction

Centella asiatica (L.) Urb. is a perennial herbaceous medicinal plant mostly found in the tropical and sub-tropical regions of the world. The plant contains bioactive ursane-type tritepenoid saponins, such as madecassoside and asiaticoside and its sapogenins asiatic acid and madecassic acid commonly known as centellosides [1]. They are found to be responsible for activities and uses such as protection against amyloid-beta-induced neurotoxicity [2], Alzheimer type of dementia [3], increasing intelligence, longevity, and memory [4], wound healing potential [5, 6], attenuating inflammatory response [7, 8], protective effects against nephrotoxicity [9], treatment of excoriations, burns, hypertrophic scars or eczema [10] and uses in cosmetology [11].

Depending on the plant source, geographical location and environmental conditions, the relative proportion of these compounds vary (Prasad et al., 2019; Roy et al., 2018). The exploitation of the wild population and increase in industrial demand of the medicinal plant has led to the development of several biotechnological avenues for the increased production of centellosides [14–21]. Moreover, the complex structures of the pentacyclic triterpenoids make chemical synthesis of the compounds an infeasible option economically [22]. The restricted understanding of the determined steps involved in centelloside biosynthesis and their regulation makes them unsuitable for industrial production or commercialization [23]. Better insights regarding the molecular mechanisms behind the rate limiting steps that govern the production of centellosides is central for the maximum exploitation and increase in production of the much-valued bioactive metabolites.

Small RNAs (sRNAs) are essential regulatory molecules for gene expression in majority of eukaryotic biological processes. They have distinct characteristics and precursors and can be separated into two big

families, siRNA (small interfering RNA) and miRNA (microRNA) although they are similar in size and perform interchangeable biochemical functions [24]. MicroRNAs gained most of the attention owing to their functional importance in controlling significant biological processes [25]. They are small nucleotide RNA products of non-protein-coding genes which are capable of silencing gene expression by repressing translation and accelerating target mRNA degradation [26]. First significant effort to characterize plant sRNAs by sequencing revealed a large set of endogenous small RNAs of predominantly 21 to 24 nucleotides in *Arabidopsis* [27, 28]. From then on regulatory activities of miRNAs have been extensively studied through target identification, physiological and phenotypic assays [29]. Many miRNAs and their targets have been identified by several experimental approaches such as genetic screening (Aukerman, 2003), cloning [31], and splinted-ligation mediation [32]. These methods are considerably costly, time-consuming and labor-intensive; thus, they are not suitable for comprehensive studies [33]. The onset of Next Generation Sequencing (NGS) technology and computational approach greatly accelerated miRNA discovery and profiling in non-model plants [34]. The latest release of miRBase (v22), a database of published miRNA sequences and annotation contains miRNA sequences from 271 organisms [35] with no representative miRNA entries from *C. asiatica*. As well-known molecules for their role in regulating various plant processes under biotic and abiotic stress, the possible involvement of miRNAs in regulating the biosynthesis and accumulation of secondary metabolites in plants is under rigorous exploration amongst plant biotechnologists recently [36, 37]. Various experiments have been carried out in-order to understand miRNA based regulation of secondary metabolites in plants such as *Papaver somniferum* [38], *Catharanthus roseus* [39], *Xanthium strumarium* [40], *Ferula gummosa* (Najafabadi and Naghavi, 2018), *Lonicera japonica* [42], *Medicago truncatula* [43], *Salvia miltiorrhiza* [44], *Withania somnifera* [45] etc.

In this study, sRNA sequencing was used to identify miRNAs and their corresponding targets that are potentially involved in important biological processes in *C. asiatica*. Expression levels of eleven miRNAs in leaf, petiole and root tissues were studied using qRT-PCR. In addition, axenic shoot cultures of *C. asiatica* were raised to study the effect of salicylic acid on the abundant miRNAs present in *C. asiatica* and genes involved in the biosynthetic pathway of centelloside production. The predicted cleavage sites in target gene of two *C. asiatica* miRNAs were verified through 5′ RLM-RACE. These results establish the foundation for comprehending the regulation of miRNAs and their respective target genes in *C. asiatica*.

2. Materials And Methods

2.1. Plant materials

Centella asiatica (L.) Urb. plant (accession number CA301) grown and maintained in the experimental plot (N 08°45'28.7" E 77°01'41.3" altitude 148m) of Jawaharlal Nehru Tropical Botanical Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram, India was used for the experiments. The tender leaves *Centella* were collected and used for the isolation of small RNA.

2. 2. Small Rna Library Preparation And Sequencing

Extraction of total RNA enriched with small RNA from leaves of the plant with its replica was carried out using miRNeasy Kit (Qiagen, Germany). Small RNA sequencing was performed using Illumina HiSeq 2500 Platform (Illumina, CA, USA) at AgriGenome Labs Private Limited, Kerala, India (http://www.aggenome.com/). Illumina TruSeq miRNA Sample Preparation protocol was followed for small RNA library construction and sequencing. Libraries were quantified using the Agilent 4200 TapeStation (Agilent Technologies, USA). Small RNA reads of 50 bp length were generated using the Illumina platform. Low quality reads were removed on the basis of average base quality, GC content, and phred score. The sequencing data were deposited in the NCBI Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra/PRJNA553029) as accession numbers SRX6411842 and SRX6411843.

2. 3. Data Pre-processing And Mirna Identification

Cutadapt was used to remove adaptors from the raw sequencing reads using with error rate (-e) set to 0.1 (https://cutadapt.readthedocs.io/en/stable/). The remaining reads were checked against rRNAs, tRNAs, snRNAs and snoRNAs, from NCBI database and the perfect matches were trimmed the using Bowtie alignment tool [46]. PERL scripts were used to remove the perfect matches with other RNAs and reads shorter than 17 and longer than 24 nucleotides. FASTQ/A Collapser tool available in the FASTX-Toolkit was used to collapse the identical sequences into a single sequence. To identify conserved miRNAs in *C. asiatica* small RNA libraries, a Blastn search was performed against plant mature miRNAs from miRBase database [35] by allowing a maximum of three mismatches. To identify novel miRNAs, the remaining reads were mapped onto the transcriptome sequence of *C. asiatica* (SRA acc. no. ERX2099703).The aligned reads were used as input to miRDeep-P software to predict novel miRNAs [47]. The secondary structures of all novel miRNA precursors were obtained by RNA fold web server http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi.

2. 4. Microrna Target Prediction And Functional Annotation

Targets of all the *Centella* miRNAs determined in this study were predicted using the psRNATarget [48] (http://plantgrn.noble.org/psRNATarget/) software with default parameters with a score or maximum expectation of 3. ‘User-submitted small RNAs/preloaded transcripts’ option was fixed and selected *Arabidopsis thaliana* and *Daucus carota* as the reference genomes for this analysis. Besides, the transcription factor family distribution prediction among the predicted miRNA targets was analyzed using Plant Transcription Factor Database (PlantTFDB v5.0) (http://plantregmap.gao-lab.org/). Gene Ontology (GO) analysis was carried out using PANTHER (Protein Analysis Through Evolutionary Relationships, (http://pantherdb.org) [49]. Supported species was selected as *A. thaliana*. KEGG pathway analysis for target genes involved in secondary metabolism was performed by using BlastKOALA tool

(https://www.kegg.jp/blastkoala/). The biological network of the miRNA and their targets were visualized by Cytoscape version 3.7.1 software (https://cytoscape.org/).

2. 5. In vitro multiple shoot propagation followed by elicitor treatment by salicylic acid

Healthy young intact shoots of *C. asiatica* collected from greenhouse maintained germplasm at (JNTBGRI) was surface-sterilized and the nodal segments were cultured in culture tubes containing phytagel (1.3%) impregnated MS medium [50] supplemented with a combination of 1.0 mg/L BAP and 0.2 mg/L NAA for culture initiation. The shoot initials obtained after 4 weeks were transferred to the same hormonal medium at a regular interval of 4 weeks to induce multiple shoots. Established shoot cultures were elicited with filter sterilized salicylic acid (SA) at a concentration of 150 µM over a period of 3 weeks.

2. 6. Quantitative real time PCR (qRT-PCR) of C. asiatica miRNAs

Total RNA was isolated from leaves, petiole and root of *C. asiatica* plant (CA301) and from multiple shoot cultures elicited with and without SA (150 µM) using RNeasy Mini Kit (Qiagen, Germany). The complementary DNA (cDNA) was prepared using miScript II RT Kit (Qiagen, Germany). Real time PCR primers for selected miRNAs genes were designed using miRprimer2 software (Supplementary Table: 5). Primer sequences for genes involved in triterpenoid biosynthesis pathway was procured from the published data of *C. asiatica* transcriptome [51].

5.8S rRNA was selected as the internal control. Each reaction was performed on CFX96 Real-Time system (Bio-Rad, USA) using TB Green *Premix Ex Taq* (Tli RNaseH Plus) (TaKaRa, Japan) containing 10 µL of TB Green *Premix Ex Taq II* (2X), 2 µL of each forward and reverse primers (5 µmol), 2 µL of diluted cDNA, made up to 20 µl using sterile double-distilled water. Ampli□cation of target genes in PCR reactions was as follows: Initial denaturation for 30s at 95°C; 40 cycles of 5s at 95°C, 30s at 55°C followed by one cycle of melting. The relative expression level of miRNAs were calculated using CT and $2^{-\Delta\Delta CT}$ method [52]. The average of the fold change values from the three experiments was finally taken.

2. 7. Validation Of Mirna-mediated Cleavage Of Mirna

The modified 5□ RNA Ligase-Mediated Rapid Amplification of cDNA Ends (5□ RLM RACE) was done by using FirstChoice™ RLM-RACE Kit (ThermoFisher Scientific, USA) to validate the miRNA-mediated cleavage in the predicted mRNA targets. An RNA oligo adapter was ligated to the total RNA isolated from *C. asiatica* plants without calf intestinal and alkaline phosphatase treatment. Gene specific reverse primer and universal forward primer were used for the amplification. The DNA bands of expected size were eluted from the gel (NucleoSpin® Gel and PCR Clean-up Kit, Macherey-Nagel™, Germany) cloned into pGEM®-T Vector System I (Promega, USA) and sequenced. The list of all the primers used in the study is provided in Supplementary Table 6.

3. Results

3. 1. Overview of small RNA sequencing

To identify miRNAs, two sRNA libraries were constructed from leaves of *C. asiatica* and sequenced by Illumina HiSeq 2500 analyzer with 50 single end reads. A total of 58,861,370 raw reads were generated from the sRNA libraries. After removing adapter contaminations and low-quality reads, clean reads of 12,112,674 were obtained. Subsequently selection of size and removal of other non-coding RNA sequences yielded 8,121,083 reads. The size distribution pattern of small RNAs (Fig. 1) showed higher abundance of 21 nt. 24 nt sequences was the next largest fraction followed by 20 and 22 nt. After mapping with the *Centella* transcriptome (accession no: SRX2572577), 8,123,381 unique reads were obtained. The small RNA sequence data was submitted to the NCBI SRA archive under the accession numbers SRX6411842 and SRX6411843. Among the total unique reads 2,182 unique sequences were identified as potential miRNAs, showing perfect or near perfect matches to the known miRNAs from viridiplantae.

3. 2. Identification Of Conserved And Novel Mirnas

Thirty-seven conserved miRNA families having high sequence similarity to the currently known plant mature miRNAs have been identified from *C. asiatica*. Comparing with miRNAs from miRBase database, miRNAs present in *C. asiatica* which are conserved in other plant species were identified. Mapping of filtered reads against miRBase 22.1 identified 227 miRNAs matching against conserved miRNAs. Apparent expression level of miRNA in *C. asiatica* leaf tissue was observed by analyzing the read count for each miRNA. The largest number of members were identified from miR156 family containing 20 members while 18 miRNA families (miR2118, miR482, miR3630, miR1436, miR827, miR165, miR2673, miR6167, miR2655, miR408, miR174, miR1171, miR530, miR1128, miR6138, miR5174, miR845, miR5653) contained one member each (Fig. 2). The most abundantly expressed miRNAs in both the libraries were miR159, miR396, miR156, miR160, miR172 and miR398. It was possible to detect 25 3p and 22 5p miRNA sequences in the libraries. Every member showed different expression levels within each miRNA family as evident from their reads which further clarifies the fact that different miRNAs play distinct role in plant growth and development. The conserved miRNA population of *C. asiatica* had high, moderate and lowly conserved miRNAs (Supplementary Table: 1) which can be classified according to the number of plant families containing identified miRNAs [53].

The remaining 7,373,800 reads were retained for novel miRNA prediction and mapped to *C. asiatica* transcriptome sequence (SRX2572577) using software package miRDeep-P which is specifically designed to characterize miRNA transcriptome in plants. We could identify 109 miRNAs with their precursor sequences (pre-miRNAs) (Supplementary Table: 2) which formed canonical stem-loop hairpin secondary structures as predicted by RNAfold (Fig. 3). The length of the novel mature miRNAs varied from 21 to 25 nt, with the majority being 23 nt. Length of these novel miRNA precursors ranged from 45-200, with an average of 83, which was in compliance with the commonly observed length of plant miRNA precursors [54]. The minimal folding energy (MFE) values of these novel miRNA precursors ranged from

-38 to -84.3 with an average of -56.71 Kcal mol^{-1} which was similar to plant miRNAs [55]. Among the novel miRNAs predicted, all the candidates were found to have complementary miRNA* in the sRNA sequence data.

3. 3. Identification Of Mirna Targets

To comprehend the functions of the miRNA families identified in *C. asiatica*, we predicted the possible targets of them by psRNATarget. The total numbers of potential target genes supported by psRNATarget were 3313 and 3635 for *A. thaliana* and *D. carota* respectively (Supplementary Table: 3). Majority of the target genes (95.51%) were predicted to be regulated through transcript cleavage whereas the remaining targets are regulated by translational repression. Most of the predicted targets were orthologs of known conserved miRNAs targets which are involved in wide range of biological processes including metabolism, plant reproduction, and regulation of development. Moreover, miRNA 396 family had highest number of target genes targeting GRF TFs such as GRF3, GRF4 and GRF12, TATA-binding protein-associated factor BTAF1 etc. The major targets of the miRNA156 included genes encoding squamosa promoter binding like (SPL) proteins such as SPL2, SPL3, SPL6, SPL7, SPL9 and SPL13 as well as F-box and LRR family proteins and NAC domain containing proteins, all of which are transcription factor (TFs) families. A less conserved miRNA such as miR2673 was found to have a variety of targets such as chloroplastic heat shock protein 90-5, nucleosome assembly protein 1, kinase-interacting family proteins, bHLH30, DNA topoisomerase 3-beta isoform etc. In addition, auxin response factor - like TFs, homeobox-leucine zipper protein, scarecrow-like protein, AP2-like ethylene-responsive transcription factor mostly by miR160, miR166, miR171 and miR172 respectively. Thus, target genes were functionally diverse which included enzymes, transcription factors and functional as well as regulatory proteins. A transcription factor-based classification of the targets performed using PlantTFDB illustrated distribution of various TFs including families which were over-represented such as C2H2, WD40-like, HB-PHD, Myb, NAC etc. (Fig. 4).

Among 109 of novel miRNAs detected from *C. asiatica*, gene targets were predicted for 92 of them (Supplementary Table: 4). C.as_miR91 was found to have maximum number of targets including various types of mRNAs encoding heavy metal transport/detoxification superfamily, AP2/B3-like transcriptional factor family, homeobox 16, cytochrome p450, drought-responsive family, leucine-rich receptor-like protein kinase family etc. On the other hand, phytochrome interacting factor 3-like protein family was predicted to be targeted by C.as_miR106 while C.as_miR16, C.as_miR100, and C.as_miR107 were found to target F-box family proteins, which are considered to be broadly regulated by microRNA-mediated gene silencing [56]. The miRNA-target network of genes encoding secondary metabolites was created using Cytoscape 3.8.1 is depicted in Supplementary Figure 1. Further experiments that elucidate miRNA-target interaction of the predicted targets and their regulatory mechanisms in biological processes are essential.

3. 4. Go And Kegg Analysis Of Targets

Gene ontology (GO) classification annotation analysis of the miRNA targets reflected diverse biological characteristics (Fig. 5). GO analysis of both conserved and novel miRNAs targets could be classified into 14 biological processes, 8 molecular functions and 6 cellular components. For biological process, “metabolic process” (GO: 0008152) and “cellular process” (GO: 0009987) were the two most represented GO categories. Considering molecular functions, the most dominant GO terms were catalytic activity (GO: 0003824) and binding (GO: 0005488), and the two most dominant GO terms in cellular compartments were cell (GO: 0005623) and organelle (GO: 0043226).

There were several conserved miRNA target genes (14.29%) that had no functional annotation. The most frequently represented protein class among the miRNA targets was nucleic acid binding (PC00171) (17%) followed by hydrolase (PC00121) (15.2%) and transferase (PC00220) (13.9%). KEGG analysis using BlastKOALA which is an internal annotation tool for KEGG genes was performed wherein 44.1% of the targets were assigned with KO (KEGG ORTHOLOGY) IDs. Seventeen different pathways related to metabolism were found (Fig. 6), and the most frequently represented pathways were related to “genetic information processing” and “carbohydrate metabolism” after screening out unannotated and repeated sequences. As seen in Fig. 6, 50 genes were related to metabolism of “terpenoids and polyketides” and 58 genes related to “biosynthesis of other secondary metabolites” were found.

3. 5. Expression profiles of conserved miRNAs in leaf, petiole and root

To investigate the gene expression pattern of conserved miRNAs in different tissues of the plant, differential expression of eleven conserved miRNAs were validated by qRT-PCR in the leaf blade, petiole and root. We selected eleven highly abundant conserved miRNAs according to the high-throughput sequencing data namely miR171b, miR171g, miR171f, miR398b, miR396a, miR399b, miR159e, miR159a, miR156d, miR156k and miR2673.

Leaf tissue was considered as reference to calculate fold change expression. In the study most of the miRNAs were up-regulated in leaf tissue compared to both petiole and root such as miR171g, miR156k, miR159a, miR159e, miR398b, miR399b and miR2673. Other miRNAs such as miR156d, miR171a, miR171f and miR396a were found to be up-regulated in both leaf and root compared to petiole. The results (Fig. 7) suggest that the miRNAs profiled from leaves of *C. asiatica* through high throughput sequencing were considerably abundant in leaf compared to the other tissues, petiole and root.

3. 6. SA responsive triterpenoid pathway genes and miRNAs in in-vitro grown multiple shoot cultures of C. asiatica

To uncover the expression profiles of mRNAs involved in triterpenoid pathway and miRNAs in a controlled condition, we established an *in-vitro* multiple shoot culture system of the plant. To set up an environment where the triterpenoid pathway genes are essentially up-regulated which will further lead on to increased production of the centellosides, the multiple shoot cultures were subjected to SA acid (150 μM) treatment over a period of 3 weeks with its control (devoid of SA). Genes encoding enzymes which participate in the production of centellosides such as β-amyrin synthase (βAS), squalene epoxidase (SQE), squalene

synthase (SQS), farnesyl diphosphate synthase (FPS), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), hydroxyl methyl glutaryl-CoA reductase (HMGR), and glucosyl transferase (GT) were used for the study (Fig. 8). βas, the enzyme which catalyze synthesis of β-amyrin which acts as direct precursor for the production of centellosides was found to increase up-to 14-fold within 3 weeks of SA (150 µM) treatment compared to its control. Other enzymes such as SQE, SQS, FPS, DXR, HMGR and GT were also found to get up-regulated approximately 2.5 to 5-fold, reinforcing the fact that SA has the potential to induce expression of triterpenoid pathway genes. Similarly, twelve miRNAs belonging to eight miRNA families such as miR171 (miR171b, miR171g, miR171f), miR398b, miR399b, miR396g, miR2673, miR156 (miR156i, miR156k, miR156d), miR159a and miR1171 were analyzed for their expression in SA (150 µM) elicited as well as non-elicited multiple shoots *in vitro*-grown cultures (Fig. 9). All members of the miR156 family viz. miR156i, miR156k and miR156d as well as miR159a and miR1171 showed down regulation in SA elicited samples compared to its control. MiR156i was down regulated approximately up to 5.5-fold while miR156k, miR156d, miR159a and miR1171 were down-regulated up to 0.5-fold. No considerable change was observed for miRNA family miR171, miR398, miR399 and miR396g with SA treatment compared to its control while miR2673 was up-regulated up to 2-fold compared to its control.

3. 7. Validation Of Mirna-guided Cleavage On Mrnas

One of the distinctive features of miRNA mediated mRNA cleavage as compared to other mRNA degradation method is that cleavage of mRNA takes place between the 10th and 11th nucleotides from the 5′ end of the complimentary miRNA. [57]. In this study, two *C. asiatica* miRNA target gene sequences were verified as targets of miRNAs through 5′ RLM-RACE. Sequencing of the 5′ ends revealed that the gene SPL6 and MYB54 was cleaved between 10 and 11th nucleotide complementary region to miR156 and miR398 respectively (Fig. 10). The results confirmed the predicted cleavage sites of these miRNA targets.

4. Discussion

The use of high-throughput sequencing (HTS) technology provides unparalleled efficient way for genome-wide and transcriptome-wide studies [58]. The identification and functional annotation of plant miRNAs through HTS mostly in non-model plants have advanced dramatically over the recent years [59]. MicroRNAs are considered as vital players in regulating gene expression in any organism [60]. The plausible effect of miRNAs in regulating metabolic pathways especially secondary metabolite production has convoked immense attention due to its utilities in eliminating human ailments.

In our study, sRNA profile of leaves of field grown *C. asiatica* was obtained, which represents first major assessment of miRNAs in the plant. The raw sequence obtained after Illumina HiSeq 2500 sequencing was subjected to in-depth analysis to identify conserved and novel miRNAs in the plant. Observing the size distribution of the total reads, the 21 nt was the most abundant followed by 24 nt. Similar pattern of nucleotide distribution was found in miRNA profiling of cotton [61], radish [62] and flax [63]. This might

be due to the selective cleavage of pre-miRNA sequences preferred by DCL1 which produce 21 nt and DCL3 as well as DCL4 which produce 24 nt miRNAs [64]. We could identify 227 conserved miRNA sequences belonging to 37 miRNA families in which number of members of each family varied significantly. MicroRNA profiling of leaves in Celery (*Apium graveolens*) which belongs to same family, Apiaceae resulted in the discovery 35 miRNA families with major representation of families such as miR156, miR159, miR171, miR396 etc. (Jia et al., 2015). Similarly, the above-mentioned miRNA families were also found most abundant in *C. asiatica*. Further, our study revealed 109 putative novel miRNAs using the available transcriptome data of this plant as reference. The predicted novel miRNAs were confirmed by forming secondary structure of their precursor. All novel miRNA precursors have stem loop hairpin structure and miRNA*, both of which further proved the authenticity of the identified novel miRNAs [66]. The validity of the predicted novel miRNAs further needs to be confirmed and their functions are to be experimentally validated. To understand the functional implications of miRNAs, identification of their target genes is vital. Besides, plant miRNAs are found to possess near-perfect complementarities to their targets, which make the bioinformatic prediction of target sequences lucrative [53]. Majority of the identified miRNAs were predicted to target transcription factors indicating their roles in transcription regulatory networks. The most abundant miRNA family found in *C. asiatica*, miR396 was found to target multiple members of the GRF family, plant specific TFs which mostly regulate growth of multiple tissues and organs in a variety of species [67, 68]. Adding to it, TATA-binding protein-associated factor BTAF1, a TF involved in meristem development was also predicted to be target of the family especially by miR396a and miR396b. Likewise, miR156 family was found to target the SPL family which is also known to play roles in plant development such as leaf development, vegetative phase change, phytohormone signaling etc. [69]. In *Arabidopsis*, anthocyanin accumulation was negatively regulated by SPL9. Expression of anthocyanin biosynthetic genes was prevented through destabilization of a MYB-bHLH-WD40 transcriptional activation complex [70]. The miR156 targeted SPL promoted biosynthesis of sesquiterpenes in *Arabidopsis* by upregulating patchoulol synthase (PatPTS) gene expression [71]. GAMYB, identified as a positive regulator of gibberellin (GA) signaling was predicted to be targeted by miR159 family. Another work reported involvement of the miRNA family in the regulation of GAMYB expression in cereal aleurone cells and flower development in rice [72]. The miRNA families, miR171 and miR172 targeted scarecrow-like protein and AP2-like ethylene-responsive transcription factors respectively which was in line with studies in plants such as *Arabidopsis* [73, 74], barley [75] and maize [76].

We used qRT-PCR to detect expression trends of some selected miRNAs in different tissues of centella miRNAs with leaf having the maximum number of miRNAs up-regulated compared to petiole and root. Furthermore, this adds to the fact that miRNA expression differs between tissues of the same plant [77–79]. Variation in plant secondary metabolites in response to external stimuli including biotic and abiotic stress is been subjected to a great deal of research. Furthermore, most of the researchers emphasize on the need to completely understand the genes and regulatory mechanisms involved in their biosynthesis [80–82]. In spite of the accumulated knowledge of the miRNA-mediated regulation of diverse functions, the likely representation of miRNAs participating in secondary metabolite pathways in plants is less

explored. Due to the immense utility of the biochemical compounds, recent works are coming up with details of miRNAs regulating the rate limiting enzymes as well as other regulators such as TFs involved in the plant biosynthetic pathways. It was observed that miR5021 and miR5293 targeted HMGS, the first committed enzyme MVA pathway in *Panax notoginseng* roots [83]. In *Ferula gummosa* miRNA families including miR2919, miR5251, miR838, miR5021, and miR5658 were computationally predicted to target genes involved in the pathway of terpene biosynthesis [84]. Similarly, enzymes participating in the terpenoid biosynthesis were predicted as targets for miRNA families such as miR156, miR159, miR166, miR167, miR396, miR171 and miR172 in *Chlorophytum borivilianum* [85]. Besides their roles in growth and development, miRNAs participate in diverse stress responses [86]. We investigated the differential expression of genes involved in triterpenoid pathway as well as of miRNAs in SA augmented, *in vitro* grown cultures of *C. asiatica.* SA is a potent elicitor for increasing triterpenoid content in *C. asiatica* as many workers have experimentally proved it multiple times [87–90]. This could provide an indication whether effects of SA on centelloside biosynthesis could be mediated by changes in miRNAs. As predicted, qRT-PCR showed an upregulation of the entire triterpenoid pathway genes used in this study in the SA augmented samples compared to its control counterpart, reaffirming the positive effect of SA on the increased production of centellosides. We found that the expression levels of three miRNAs from family miR156 (miR156d, i, and k), miR159a and miR1171 were down regulated in SA elicited samples compared to its control. Four members of miR156 family (mir156a, b, c and d) were all significantly down regulated by the addition of methyl jasmonate in *Taxus chinensis* cell lines [91]. Reduced activity of miR156 resulted in high levels of flavonols in *Arabidopsis* [70]. Thus, SA acid responsive differential expression shown by these miRNAs (miR156, miR159 and miR1171) suggest that they can potentially affect triterpenoid pathway genes directly or indirectly (probably through transcription factors) and requires further experimental validation to explore the underlying mechanism. Thus, this study provides new insights into the miRNA-directed molecular processes involved in *C. asiatica* with special reference to the regulation of these miRNAs on triterpenoid biosynthetic pathway of the plant which has got wide range of medicinal properties. In agreement with previous findings, RLM RACE experiments could detect the predominant cleavage sites on two of the target mRNAs between 10 and 11th base from 5′ end of the miRNA aligned position (Asha and Sreekumar, 2015; Sabana et al., 2018; Zhang et al., 2018). Thus, the miRNA-mediated cleavage on the conserved targets encoding SPL and MYB domain class transcription factor was validated in *C. asiatica.*

5. Conclusion

In this study, high-throughput sequencing of smallRNA population identified conserved and novel miRNAs participating in complex biological processes in the leaves of *C. asiatica.* By computational prediction, the conserved miRNAs were found to be associated with triterpenoid biosynthetic pathway. Additionally, we also detected miRNAs targeting mRNAs encoding transcription factors, which implicated miRNA's role in regulating various biological processes, including plant growth and development. Results obtained through qRT-PCR proved that the miRNAs showed tissue specific expression. The differential expression of miRNAs in SA augmented multiple shoot *in vitro* cultures of *C. asiatica* showed interesting results in

which miRNA family miR156, miR159 and miR1171 was predicted to be involved in the production of centellosides. The present study provides a comprehensive database of miRNA for *C. asiatica* as well as a theoretical basis for further examinations of the function of miRNA in regulating the biological features especially the production of its much-valued biosynthetic compounds.

Abbreviations

bp, base pair(s); KEGG, The Kyoto Encyclopedia of Genes and Genome; BLAST, basic local alignment search tool; TF, transcription factor; GO, Gene ontology; E-value, expectation value; SRA, Sequence Read Archive; HMGR, hydroxymethylglutaryl-CoA reductase; DXR, deoxylulosephosphate reductoisomerase; FPS, farnesyl diphosphate synthase; βas, beta amyrin synthase; SE, squalene epoxidase; SS, squalene synthase; GT'S: glycosyltransferase; MVA: mevalonate; MEP: methylerythritol 4-phosphate.

Declarations

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Ethical approval

This chapter does not contain any studies with human or animals performed by any of the authors

Consent of participation

As our study involves plants and microorganisms, the consent of participation does not apply to our work.

Consent to publish

All the authors read the final version of the manuscript and agree to publish.

Authors Contributions

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and materials

The sequencing data were deposited in the NCBI Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra/PRJNA553029) as accession numbers SRX6411842 and SRX6411843.

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Figures

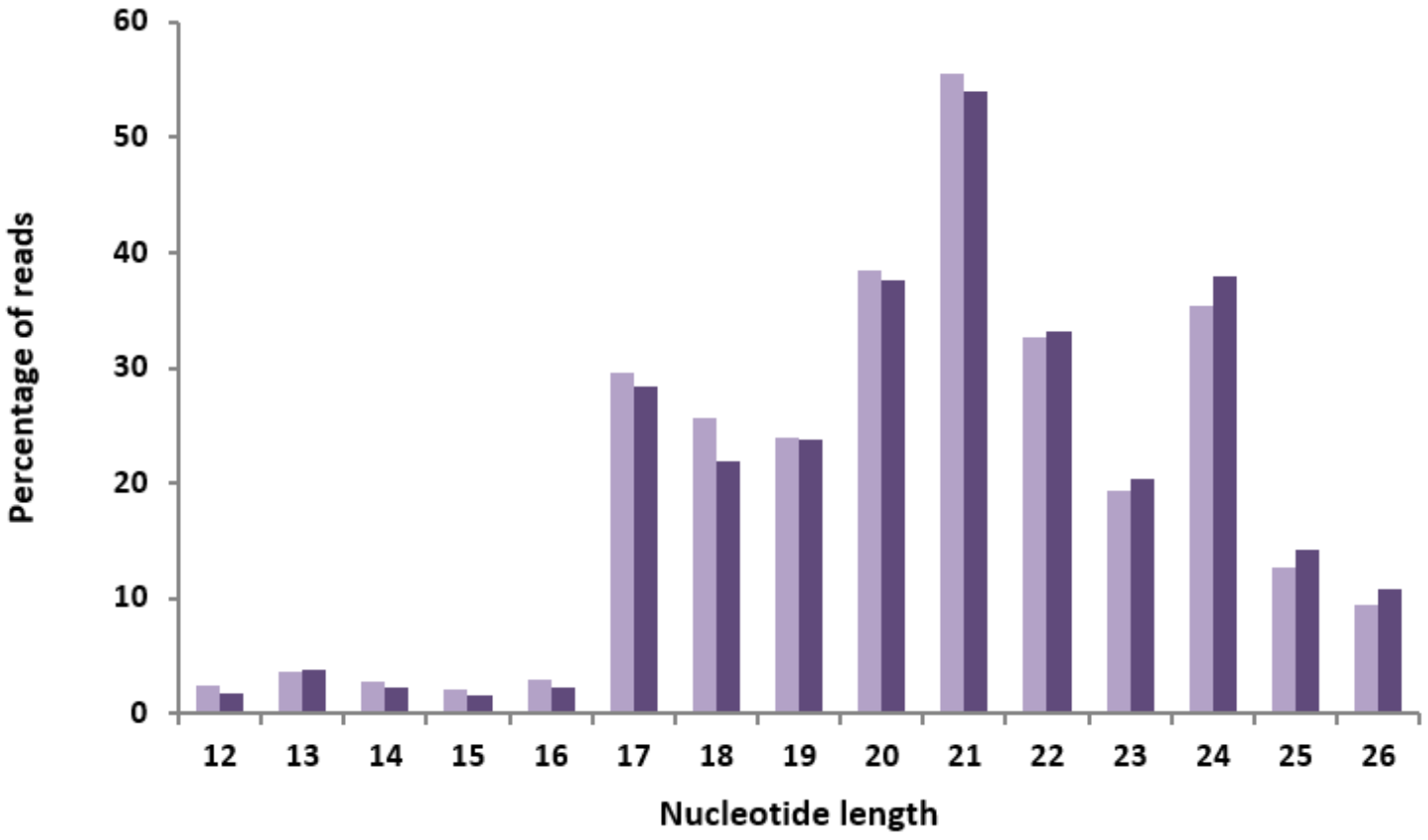

Figure 1

Length distribution of small RNAs in C. asiatica leaf library

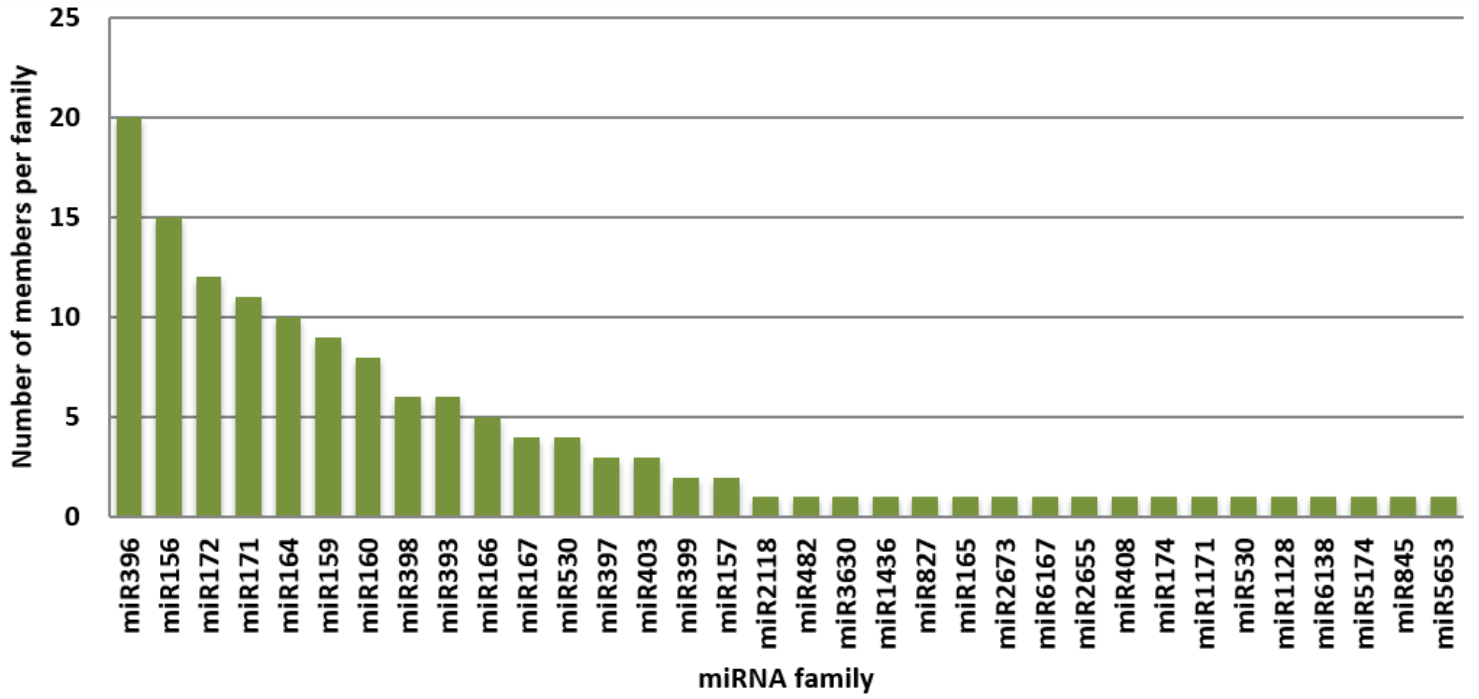

Figure 2

Member numbers of known miRNA families identified in C. asiatica

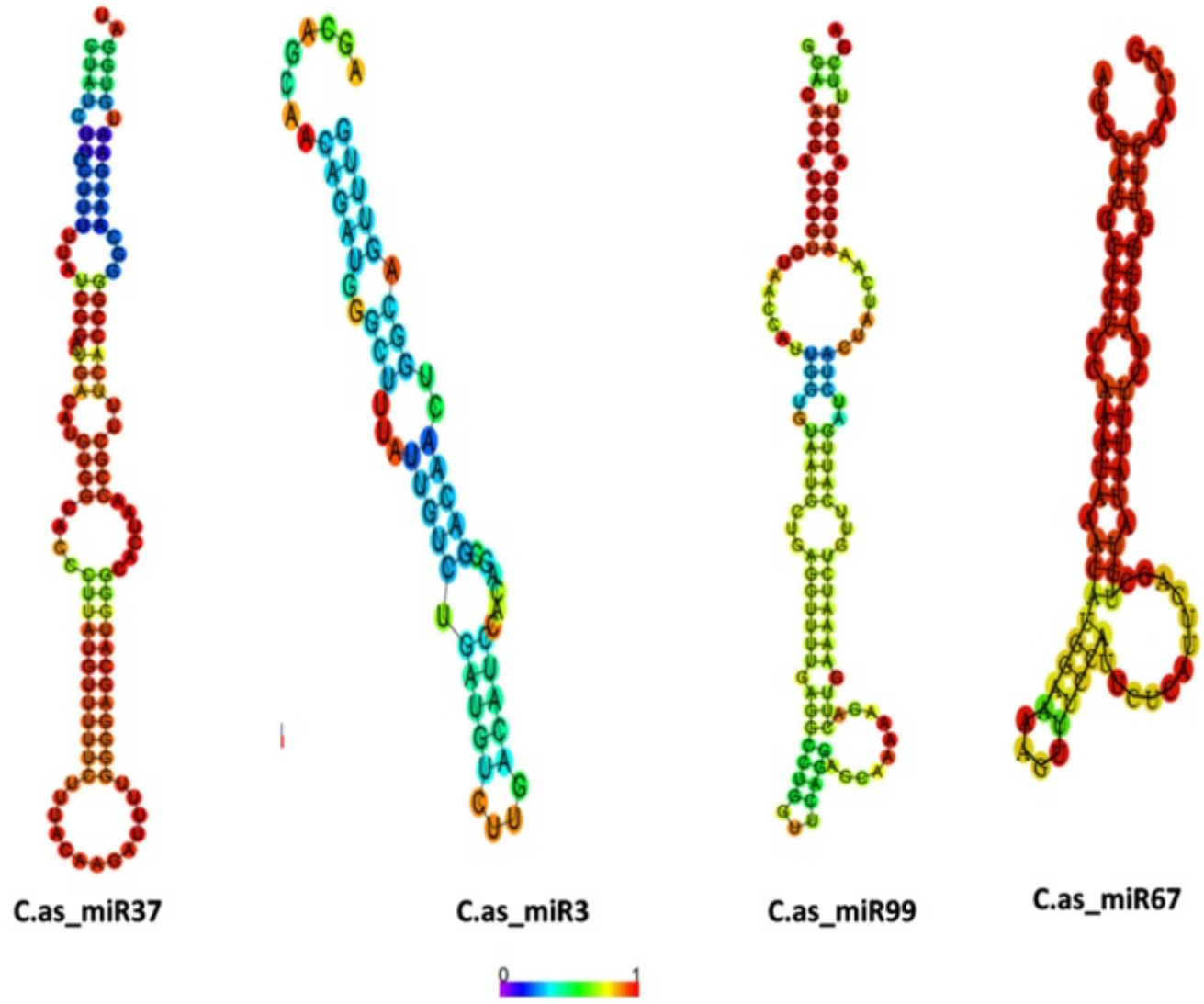

Figure 3

Secondary stem loop structure of novel miRNAs (C.as_miR37, C.as_miR3, C.as_miR99, C.as_miR67).

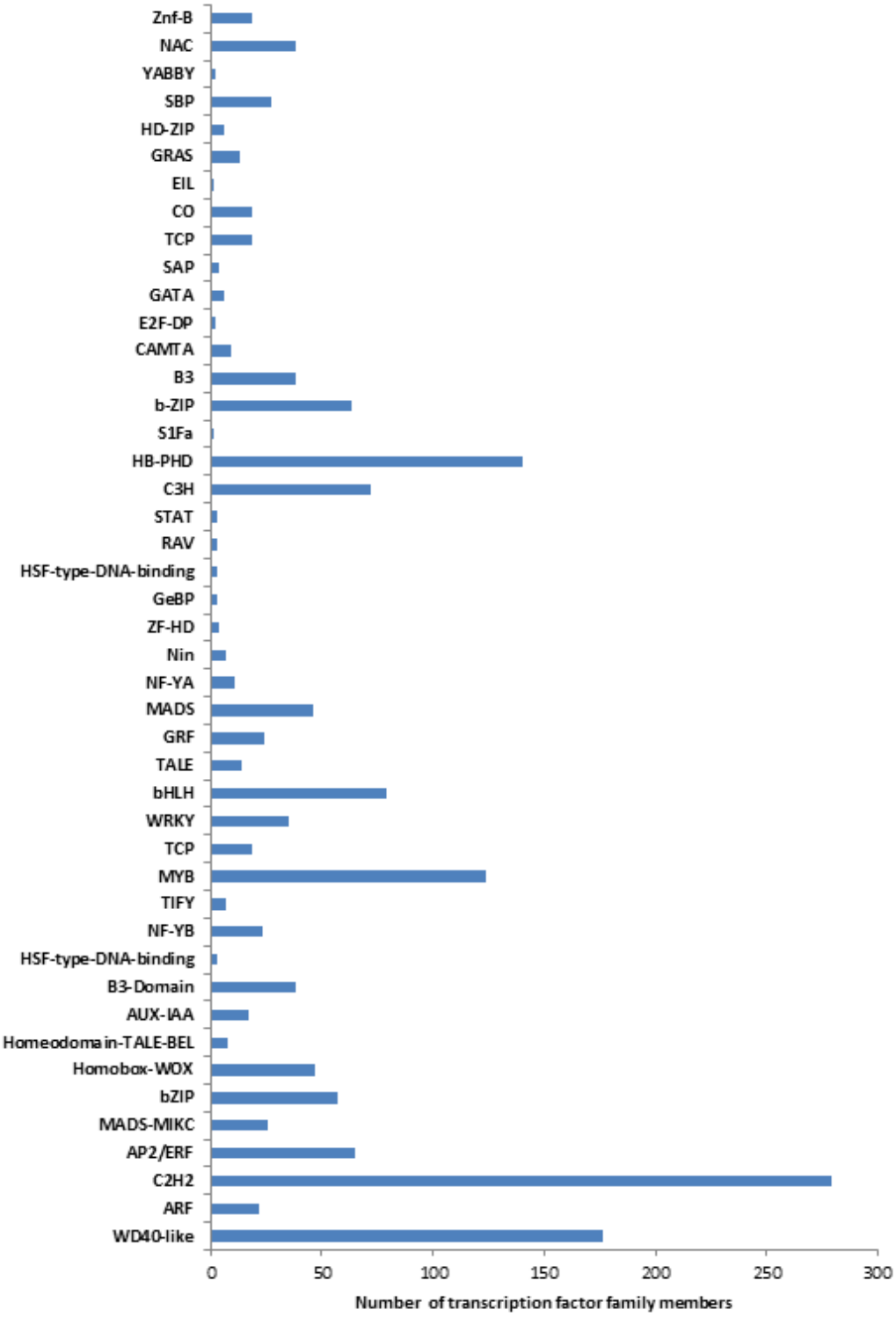

Figure 4

Member numbers of TF families identified as miRNA targets in C. asiatica

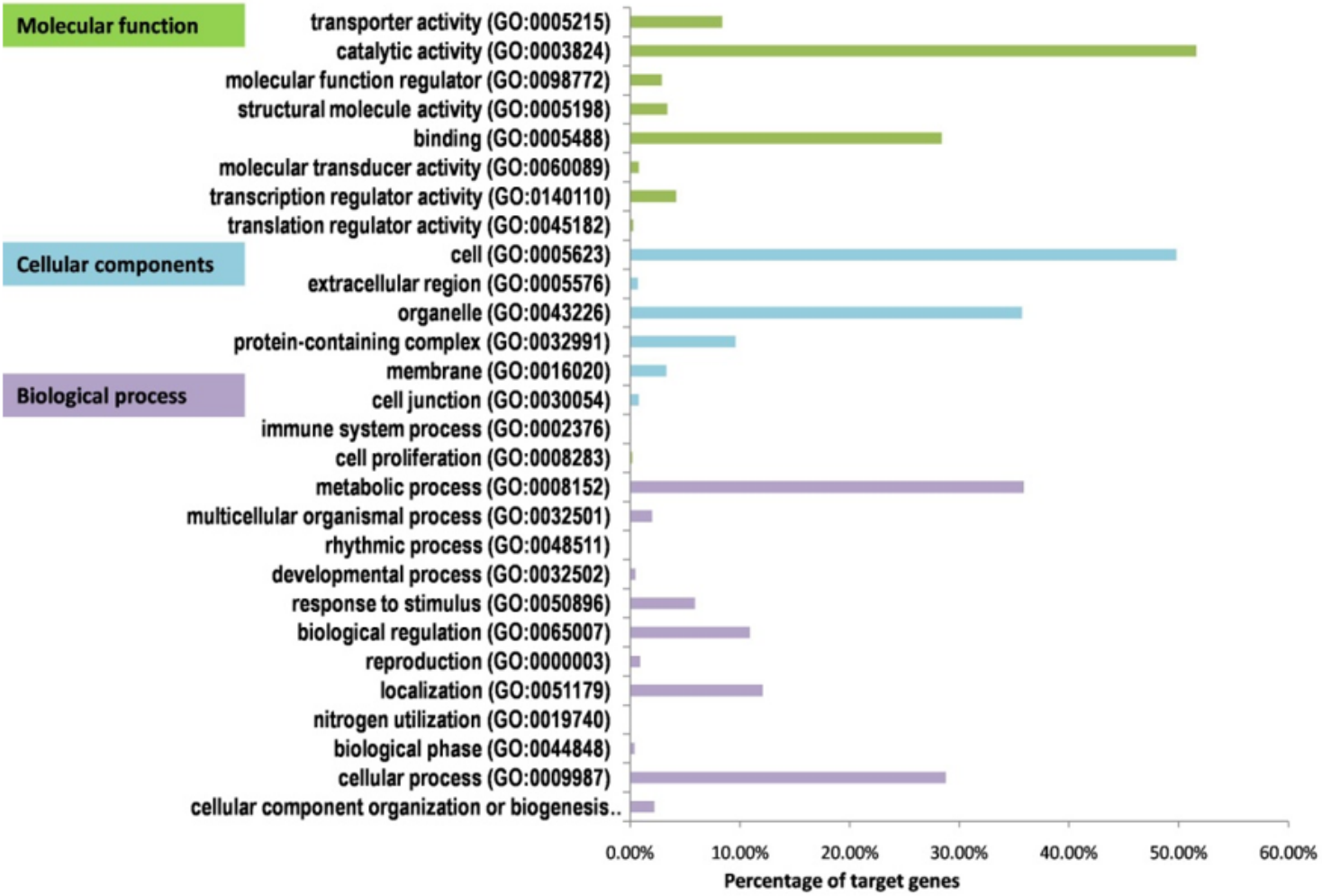

Figure 5

GO categories and distribution of miRNA targets in C.asiatica

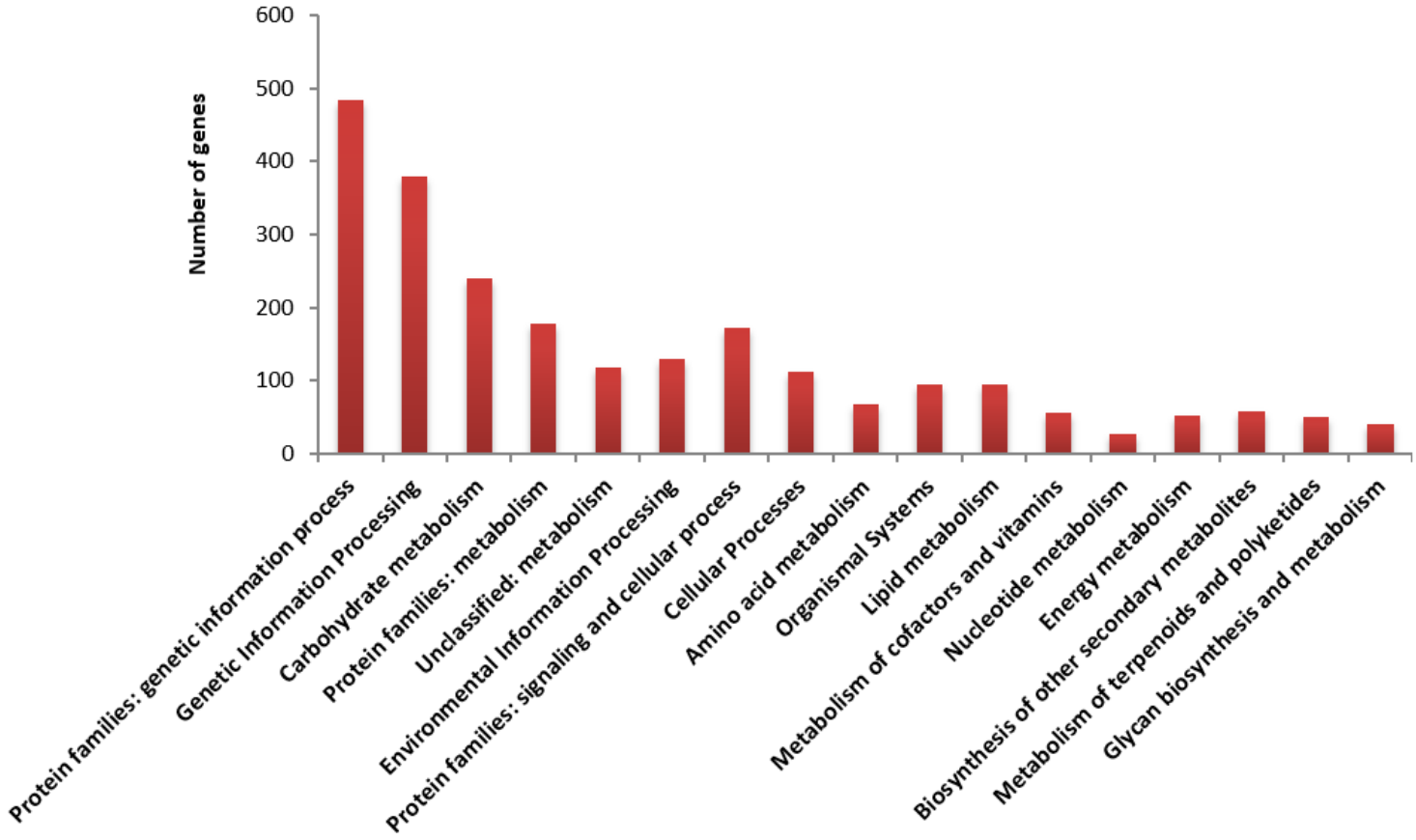

Figure 6

KEGG categories and distribution of miRNA targets in C. asiatica

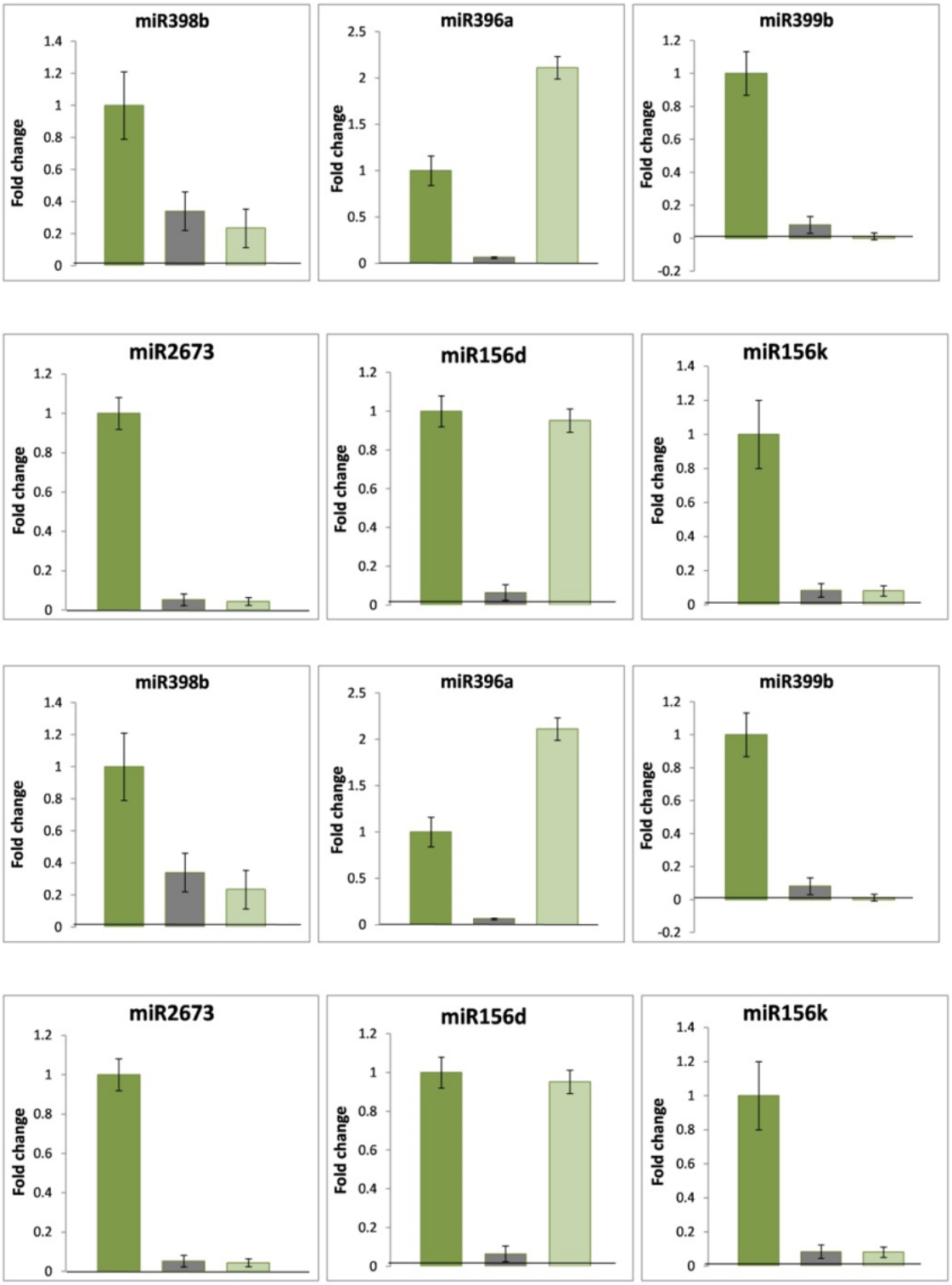

Figure 7

qRT-PCR expression profiling of conserved miRNAs in leaf, petiole and root.

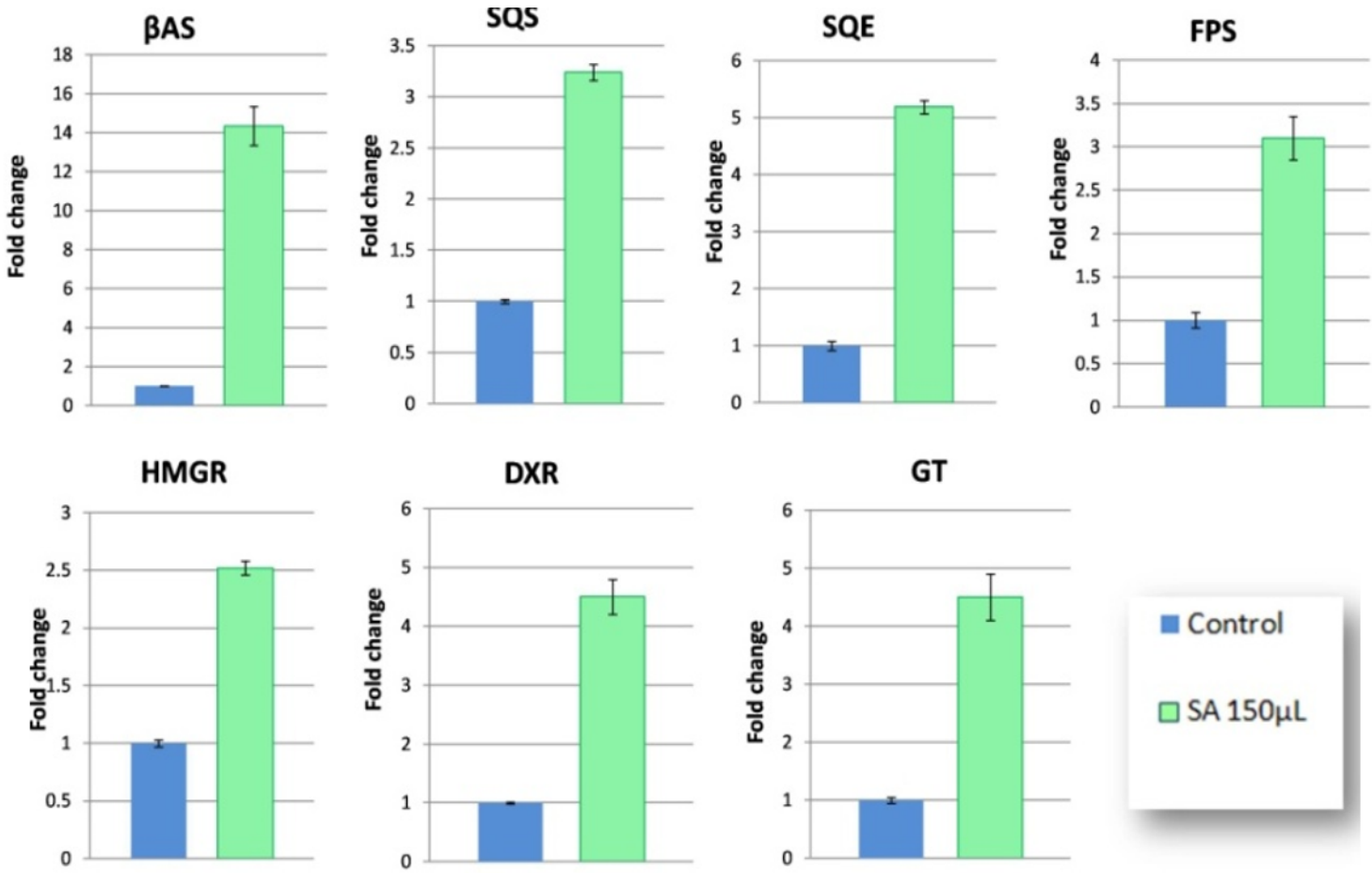

Figure 8

qRT-PCR expression profiling of genes involved in triterpenoid biosynthesis pathway under SA (150 µM) treatment with its control. Abbreviation: HMGR: hydroxymethylglutaryl-CoA reductase; DXR: deoxylulosephosphate reductoisomerase; FPS: farnesyl diphosphate synthase; βAS: beta amyrin synthase; SE: squalene epoxidase; SS: squalene synthase; GT: glycosyltransferase.

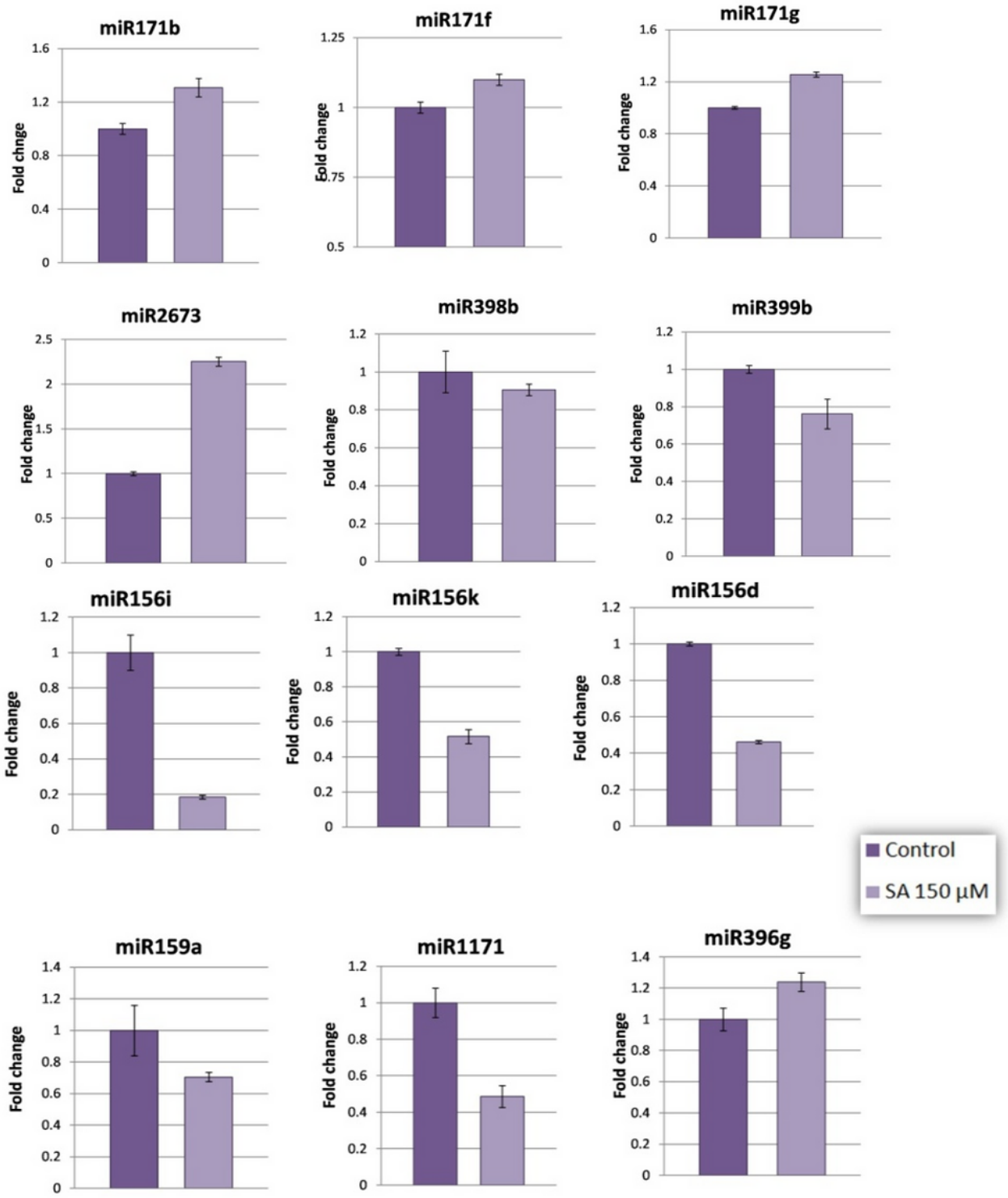

Figure 9

qRT-PCR expression profiling of conserved miRNAs under SA (150 µM) treatment with its control.

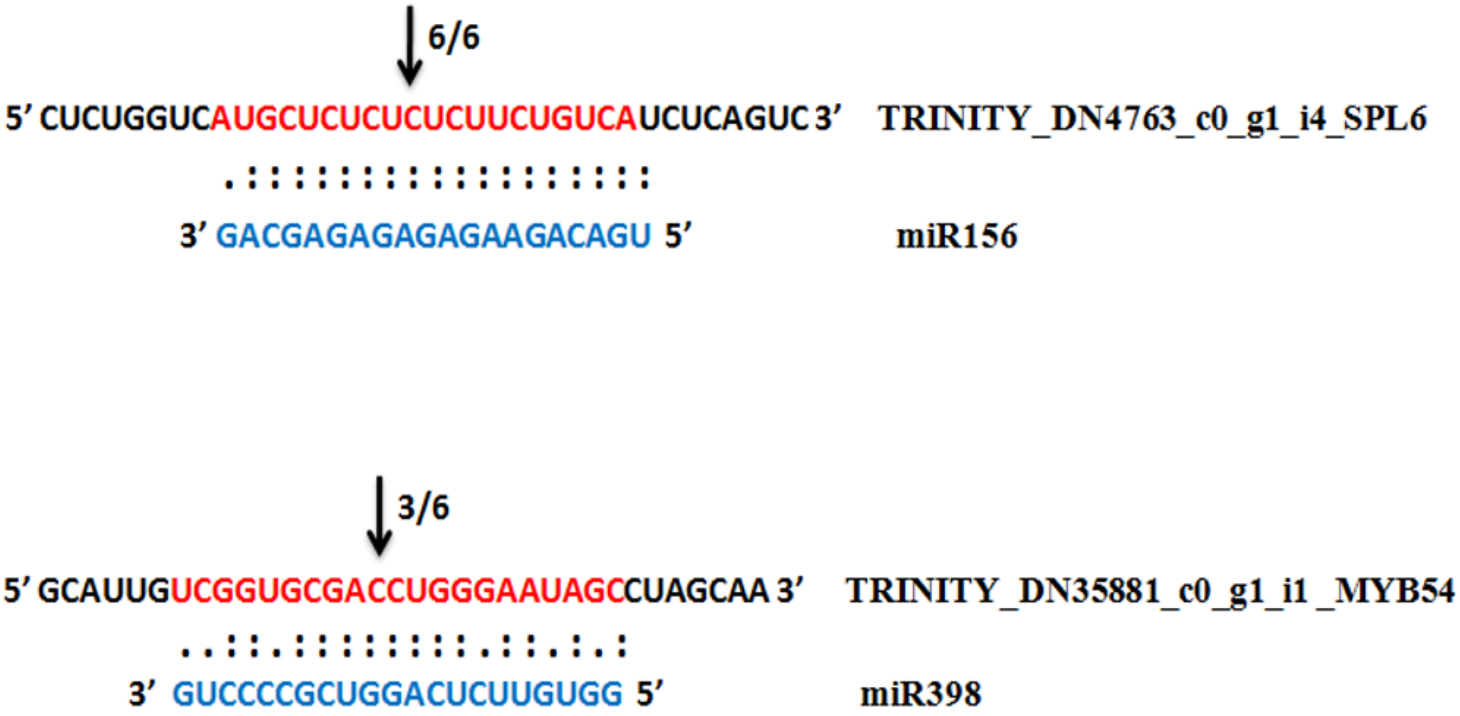

Figure 10

Experimental verification of cleavage mediated by miRNA on the predicted targets using modified 5' RLM RACE. Arrow points to the mapped cleavage sites on miRNA aligned position on the predicted target mRNAs (top) in C. asiatica. The numbers indicate the fraction of cloned PCR product.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supp1miRseq.docx
- Supp2NovelmiRNAsTable.xlsx
- Supp3ConservedmiRNAstarget.xlsx
- Supp4psRNAnovel.xlsx
- Supp5PrimerListmiRNA.docx
- Supp6PrimerListRACE.docx
- suppfig1cytoscape.png