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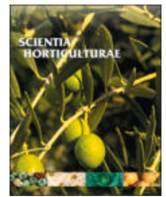

Profiling of miRNAs in Bhut Jolokia (*Capsicum chinense*) and Kon Jolokia (*C. frutescens*) of Northeast India

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A B S T R A C T

MicroRNAs (miRNAs) are crucial elements of convoluted gene regulatory network that governs plant development and stress responses. The role of small RNAs (sRNA) especially in *C. chinense* (Bhut Jolokia) and *C. frutescens* (Kon Jolokia) of Capsicum genus are still unexplored. Therefore, to identify miRNAs small RNA sequencing was performed from leaf, flower, stem and fruit tissues of *C. chinense* and *C. frutescens*. The profiling of small RNAs identified a total of 279 and 254 conserved; along with 490 and 155 novel miRNAs in *C. chinense* and *C. frutescens*, respectively. The genomic distribution of miRNAs revealed the extensive genome wide distribution as well as clustering of miRNAs across the twelve *Capsicum* chromosomes. Synteny analysis provided insights into conservation of high confidence *Capsicum* miRNAs in Solanaceae family. Furthermore, the expression pattern of 5 differentially expressed and 16 tissue-specific miRNAs was validated through stem-loop qRT-PCR. The target prediction and gene ontology enrichment analysis revealed the possible role of miRNAs in carotenoid biosynthesis, fruit ripening, aroma formation, transcription regulation, signal transduction, and cellular metabolism. About 51 putative Solanaceae–specific miRNAs were predicted which targets diverse range of developmental and disease resistance proteins. The expression analysis demonstrated that several miRNAs were expressed in a tissue-specific/preferential manner indicating their involvement in tissue/organ development. This is the most comprehensive study of miRNA identification in Bhut jolokia and Kon jolokia which would contribute in strengthening the study of miRNA mediated gene regulation involved in growth and development in *Capsicum* species.

1. Introduction

Several studies during the last two decades have given evidences that small RNAs regulate important developmental and biological processes in both plants and animals (Bartel and Bartel, 2003; Carrington and Ambros, 2003; Chen, 2009; Rubio-Somoza and Weigel, 2011; Djami-Tchatchou et al., 2017). In plants, among several non-coding RNA (ncRNA) families, short-interfering (si) RNAs and miRNAs are known to repress gene expression at transcriptional and post-transcriptional levels (Chen, 2009). The miRNAs are short [20–24 nucleotide (nt) long], conserved, single-stranded RNAs that control gene expression either by transcript cleavage or translational inhibition of targeted mRNAs (Bartel and Bartel, 2003; Chen, 2009). An RNA polymerase II transcribed *MIR* gene forms capped and polyadenylated primary-miRNA which conforms to imperfectly folded internal stem-loop structure known as precursor miRNA (pre-miRNA) (Chen, 2005; Rogers and Chen, 2013). Later, the pre-miRNA is recognized by *Dicer-like 1* (*DCL1*) protein for sequential cleavage, to produce pre-miRNAs and ultimately produces miRNA: miRNA* duplex (Zhu, 2008). Further, after dissociating from the duplex, the mature miRNA integrates with RNA-induced silencing complex

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(RISC) that leads to either target mRNA degradation and/or its translation inhibition (Jones-Rhoades et al., 2006).

Several studies provided evidence affirming the imperative role of miRNAs in diverse developmental and biological processes such as leaf morphogenesis, formation and sustenance of floral and shoot apical meristems, flower and root development, transition of vegetative to reproductive phase, vascular development, pathogen invasion, hormone signaling pathways, response to various stresses and regulation of own biogenesis (Rubio-Somoza and Weigel, 2011; Djami-Tchatchou et al., 2017). Although miRNAs are generally conserved in plants but a handful of them are observed to be species-specific (Allen et al., 2004), while the majority of them remain unexplored and uncharacterized.

The high-throughput Next Generation Sequencing (NGS) technology and computational prediction tools have encouraged miRNAs discovery in numerous plants (Fahlgren et al., 2007; Moxon et al., 2008; Morin et al., 2008; Song et al., 2010; Martínez et al., 2011; Chi et al., 2011; Li et al., 2013; Hwang et al., 2013; Yang et al., 2013; Jain et al., 2014; Lakhotia et al., 2014; Chhapekar et al., 2016; Seo et al., 2018; miRBase; http://www.mirbase.org/). Therefore, the knowledge of the entire repertoire of small RNAs (microRNA) is becoming essential to understand the convoluted gene regulatory pathways in plants.

Solanaceae family contains the third most economically important crop plants after Poaceae and Fabaceae. Among the Solanaceae plants, the identification and characterization of several microRNAs have been reported in *Solanum lycopersicum*, *S. tuberosum*, and *S. melongena* (Moxon et al., 2008; Zhang et al., 2008; Kim et al., 2011; Zuo et al., 2011; Xie et al., 2011; Hwang et al., 2013; Yang et al., 2013; Lakhotia et al., 2014; Seo et al., 2018). Few of those identified miRNAs has also been experimentally shown to govern economically important traits (Reviewed in Djami-Tchatchou et al., 2017; Beltramino et al., 2018; Zhang et al., 2018; Yang et al., 2020a, 2020b). However, the genome-wide identification of miRNAs are still very few in *Capsicum* species. The whole-genome sequencing and miRNA profiling in *C. annuum* reported the identification of few miRNAs in hot pepper (Hwang et al., 2013; Kim et al., 2014; Qin et al., 2014). Furthermore, in *C. annuum*, Hwang et al. (2013) reported the identification of 29 conserved and 35 novel miRNA families from ten different tissue libraries, followed by identification of 59 known and 310 novel miRNAs by Liu et al. (2017). Further, in sweet pepper (*C. annuum*) 193 conserved and 73 novel miRNAs were identified from fruit (Taller et al., 2018); 18 miRNAs responsive to chilling temperature (Zuo et al., 2018) and 43 miRNAs in fruit ripening of bell pepper were identified (Zuo et al., 2019). Recently, transcriptome profiling of non-coding RNAs in *C. chinense* fruit ripening were reported in which 142 novel miRNAs, 9928 circRNAs and 20,563 lncRNAs were identified (Yang et al., 2020a, 2020b). However, the identified miRNAs in *Capsicum* is very less number compared to other plant species such as rice, Arabidopsis, tomato, etc. and given a large genome size of approx. 3.5 GB in *Capsicum*, the presence of more miRNAs is expected.

The *C. chinense* (Bhut Jolokia), a very high pungent and *C. frutescens* (Kon Jolokia) a moderate pungent, are native chilli peppers of Northeast India with unique fruit morphological and pungency characteristics which are still unexplored for genomic and non-coding RNA studies (Ramchiary et al., 2014; Sarpras et al., 2016; Chhapekar et al., 2020). Therefore, in this study, we performed deep sequencing of small RNAs from four tissues/organs (leaf, flower, fruit, and stem) in *C. chinense* and *C. frutescens*. We reported the identification and characterization of high-confidence miRNAs and predicted their potential targets that appertain to distinct biological and cellular processes. Our differential expression analysis showed spatio-temporal variation in miRNA expression, thereby implying their diverse roles in *Capsicum* development.

2. Materials and methods

2.1. Plant material and growth conditions

Surface sterilized (4% sodium hypochlorite) seeds of Bhut Jolokia and Kon Jolokia were germinated in the Petridish using moist germination paper and transferred to a glass house condition (24−26 °C with 16 h light period) until fruit setting and maturity (Dubey et al., 2019). Various fruit developmental stages such as 5 Day Post Anthesis (DPA), 10DPA, 20DPA, breaker (30–45 DPA), and mature (45–60 DPA) were collected (frozen in liquid nitrogen) and later pooled as unified fruit tissue sample. The fresh young fully expanded leaves along with flower buds of different size and age, and stem were also collected separately from healthy plants.

2.2. RNA extraction, library preparation, and sequencing

The RNA extraction was carried out using the NucleoSpin RNA kit following manufacturer's instruction (Macherey-Nagel, USA). The total RNA (1 μg each) was pooled from three biological replicates from the individual tissue samples. Further, the quality and the quantity of RNAs were estimated with Bioanalyzer (Agilent, USA) plant Nano kit. For small RNA library preparation, RNA samples with a 260/280 ratios of 1.9–2.0 and RNA integrity number (RIN) of ≥ 8 were selected. The sRNA libraries were prepared by using the TruSeq Small RNA sample preparation kit (Illumina, USA). Each sRNA library was sequenced using Illumina Genome Analyser II at Genotypic Technologies Pvt. Ltd, Bangalore, India.

2.3. Data analysis

After the sequencing of sRNAs, the quality of FASTQ files was evaluated by SeqQCv2.2 software (http://genotypic.co.in/SeqQC.html). The low-quality sequence reads with <30 Phred scores and sequences shorter than 18 nucleotides long were eliminated. The remaining high-quality reads were trimmed for adapter sequences using the UEA srnaWorkbench programme (Stocks et al., 2012). After this, redundant reads were filtered and unique reads were retained. The high-quality reads (18–30 nt long) were mapped to the *C. annuum* reference genome v1.2 (Qin et al., 2014) using Bowtie program allowing a maximum of two base pair mismatches. About 100 bp flanking regions of each aligned miRNA reads were extracted from the *C. annuum* reference genome. From the extracted aligned reads, non-coding RNAs such as tRNA, rRNA, snRNA, and snoRNA were removed. The remaining reads were used to predict conserved and novel miRNAs. For the identification of conserved miRNAs from *Capsicum* sRNA libraries, the filtered and extracted read populations from individual tissues were aligned against all reported plant miRNAs (including from *C. annuum*) sequences from miRBase 21.0 database (Griffiths-Jones et al., 2007) using NCBI-BLAST-2.2.30. For identification of precursor sequences of putative miRNAs, criteria described by Axtell and Meyers (2018) were followed. The sequences with 0–2 mismatches with known plant miRNAs were described as conserved miRNAs.

To identify novel miRNAs, the remaining unaligned reads (against the already known plant miRNAs), were further aligned against the *C. annuum* reference genome to extract their putative precursor sequences as mentioned by Yang et al. (2011). These potential miRNA precursor sequences were used for the identification of novel miRNAs, their secondary structure, minimum free energy (MFE), and Dicer cleavage site were predicted using MIREAP 0.2. (http://sourceforge.net/projects/mireap) programme. The predicted small RNA sequences were recognized as potential candidate miRNA genes following recommendations by Axtell and Meyers (2018). RNAfold programme of Vienna RNA software package was used with default parameters to predict the secondary hairpin structures (Hofacker, 2003). The minimum free energy (MFE), the most important criteria which indicates the

strength of hairpin structure (Llave et al., 2002; Adai et al., 2005) was calculated and potential miRNAs with standard accepted MFE value of less than -18 kcal mol^{-1} were retained. The complete sRNA sequence data produced in this study is available with accession number GSE112738 at the Gene Expression Omnibus database of NCBI.

2.4. Differential expression analysis of miRNAs

To investigate the amount of expression of individual miRNAs, the DESeq programme was used to normalize the expression in every tissue (Anders and Huber 2010). The identification of tissue-specific expression of miRNAs was done following the method described by Breakfield et al. (2012). The miRNAs expression heatmaps were produced using the Multiple Experiment Viewer tool (Howe et al., 2010).

2.5. miRNA target prediction and functional annotation

The prediction of potential targets of the conserved and novel miRNAs was performed with the psRNATarget tool (Dai and Zhao, 2011). The miRNA sequences were used as an input with *C. annuum* mRNA sequences using stringent criteria (strict alignment in the seed region, i.e., offset positions 2–8) in the programme. This stringent option precludes the detection of target sites that lacks perfect complementarity in the seed region such as gaps or non-canonical base pairing. The miRNA hits having minimum free energy <= -25 are assumed to be targets for reported miRNA. The *C. annuum* genome annotation (Qin et al., 2014 and Kim et al., 2014) was used for the identification of putative functions of the predicted targets. Subsequently, the gene ontology (GO) terms were assigned to the target genes using Blast2GO (Götz et al., 2008). The significantly enriched GO terms were identified using an agriGO tool with *P*-value ≤0.05 (Tian et al., 2017).

2.6. Distribution and mapping of Capsicum miRNA genes with tomato and potato

The whole-genome sequences of *Capsicum* (accession GCF_000710875.1; Qin et al., 2014), tomato (accession GCF_000188115.3; The Tomato Genome Consortium, 2012) and potato (PGSC DM assembly version 3; The Potato Genome Sequencing Consortium, 2011) were retrieved from respective resource databases. The ncbi-blast-2.5.0 was used to map the 22 high confidence *Capsicum* miRNA families in tomato and potato genome. The circos plot was used to depict the synteny relationships of *C. chinense* and *C. frutescens* miRNAs in *C. annuum* reference genome with that of tomato and potato genomes (Krzywinski et al., 2009).

2.7. Validation of miRNA and target gene expression using quantitative real-time polymerase chain reaction (qRT-PCR)

To validate the expression of miRNAs, we performed the stem-loop qRT-PCR (SL-qRT). Here, the aliquot of same RNA samples, i.e. from stem, leaf, flower, and three developmental stages of the fruit (20 DPA, 40 DPA, and 60 DPA) of Bhut Jolokia and Kon Jolokia used for miRNA sequencing was used for expression analysis. The forward and reverse stem-loop primers for each miRNAs were designed and synthesized from Sigma (Sigma-Aldrich, USA, Table S1). The synthesis of the first-strand cDNA was performed with 1 μg of template RNA by using SuperScript III first-strand synthesis system (Invitrogen, USA) as per manufacturer's instruction with some modifications. To increase reverse transcription efficiency, a pulsed RT reaction was carried out with following conditions: a single step of 30 min at 16 °C, followed by pulsed RT of 60 cycles of the 30 s at 30 °C, 30 s at 42 °C, 1 s at 50 °C (Varkonyi-Gasic et al., 2007). After this, for the inactivation of the reverse transcriptase, the reaction was incubated at 85 °C for 5 min. The qRT-PCR was done with SYBR Premix Ex Taq (Clontech USA) as per the manufacturer's instruction. The qRT-PCR was conducted in ABI7500 Fast system (Applied Biosystems) with the following thermal protocol: 95 °C for 2 min followed by 40 cycles of amplification of 15 s at 95 °C and 1 min at 60 °C. Immediately after the final PCR cycle, a melt curve analysis was executed via 60–95 °C in increments of 0.5 °C to check the PCR product specificity. The reactions were carried out in triplicate, and the experiment was repeated at least twice. Also, the control reaction with the absence of template and reverse transcription were included for individual miRNA. In this study as an internal reference, U6 snRNA gene was used.

The control reactions without template and reverse transcriptase enzyme were included for individual mRNA. Actin gene was used as an internal reference. The primer sequences of all the genes, including the actin gene are listed in Table S1. After the completion of the reaction, the comparative Ct method $2^{-[\Delta\Delta Ct]}$ was used to quantify the relative expression of individual miRNA and target genes (Livak and Schmittgen, 2001). The analysis of variance (ANOVA) test determined the significance level. The correlation coefficient between qRT-PCR and RNA-sequencing (RNA-seq) data were calculated with the R program and displayed in the form of a scatter plot.

3. Results

3.1. Small RNA sequencing in Capsicum species

A total of 66.5 and 61.7 million raw reads were generated from four tissues [leaf, flower, fruit and stem (Fig. S1)] belonging *C. chinense and C. frutescens* using small RNA sequencing, respectively. Adaptor sequences were trimmed and reads with <18 and >30 nucleotide length along with low quality score were filtered out. Subsequently, removal of redundant sequences ultimately gave us 3.02 and 6.61 million cleaned unique reads from *C. chinense and C. frutescens*, respectively, which were further used to identify conserved and novel miRNAs (Table S2). The length distribution analysis of these sRNA sequences displayed considerably identical pattern in all the tissue libraries (Fig. S2). The significant fractions of small RNA reads were of 21–24 nt, indicating the characteristics of *DCL* processed sRNAs. Length distribution analysis showed that among the 21−24 nt sRNA class, the 24 nt sRNAs class was highly abundant in Bhut Jolokia (55 %) and Kon Jolokia (59 %), while 21 nt sRNAs class was least abundant (6% in Bhut jolokia and 6.6 % in Kon jolokia, respectively). The 23 nt sRNAs were 7.1 % and 8.2 %, while 22 nt sRNAs were 6.2 % and 7.6 % in Bhut jolokia and Kon jolokia respectively (Fig. S2). Of total sRNA population, the 21–24 nucleotide sRNAs comprised of about 74 % of Bhut jolokia and 82 % of Kon jolokia confirming that they are exclusive cleavage products of DCL proteins.

3.2. Identification of conserved and novel miRNAs in Capsicum species

For the generation of high-confidence miRNA dataset, miRNAs with less than ten read count in tissue samples were excluded (Table S2). Overall, a total of 279 and 254 conserved miRNAs from four tissues of Bhut jolokia and Kon jolokia, respectively were identified (Tables S3 and S4). Several of these miRNAs were found to express in multiple tissues. Also, we observed that majority of miRNAs from Bhut jolokia were conserved among the reported miRNAs of potato (34) followed by soybean (25), *Medicago* (23), rice (22) and *Arabidopsis* (17), whereas miRNA belonging to Kon jolokia were found conserved with that of soybean (29) followed by potato (28), rice (26), *Arabidopsis* (22) and tomato (16), (Fig. S3a & b). Further, potential precursor sequences related to the remaining unaligned distinct reads against the already known plant miRNAs were screened for potential novel miRNAs sequences (see in method Section 2.3). A total of 490 high confidence non-redundant novel miRNAs in Bhut jolokia and 155 novel miRNAs in Kon jolokia were identified (Tables S3 and S4). Altogether, a total of 769 and 409 non-redundant miRNAs were identified in Bhut jolokia and Kon jolokia, respectively (Tables S3 and S4).

3.3. Characteristics of *Capsicum* miRNAs

The entire set of miRNAs from different tissues displayed broader distribution which varied from a minimum of 85 (in leaf) to maximum 509 (in flower) of Bhut Jolokia. However, in the case of Kon Jolokia the minimum 116 miRNAs in flower tissue to a maximum of 228 miRNAs in fruit tissue were identified (Table S2). The higher number of novel miRNAs in Bhut Jolokia was identified in flower (334) followed by fruit (111) and stem (88). However, in Kon Jolokia the highest number was identified in flower tissue (52) followed by leaf (44) and fruit (40) and (Table S2). The number of tissue-specific, as well as expression in more than one tissue but not common to all tissues, and common in all tissues, are depicted in Fig. 1a and b.

The sRNA size distribution analysis indicated that 24 nucleotides represent the major fractions of miRNAs in both Bhut jolokia and Kon jolokia (Fig. 1c and d). Furthermore, substantial variation in the proportion of 24 nt class miRNAs in different tissues was observed. The distribution analysis revealed that in the stem (21 %) and leaf (20 %) tissues their proportion is similar, however, in flower (29–31 %) and fruit tissues (32–34 %) the proportion was quite high for both Bhut

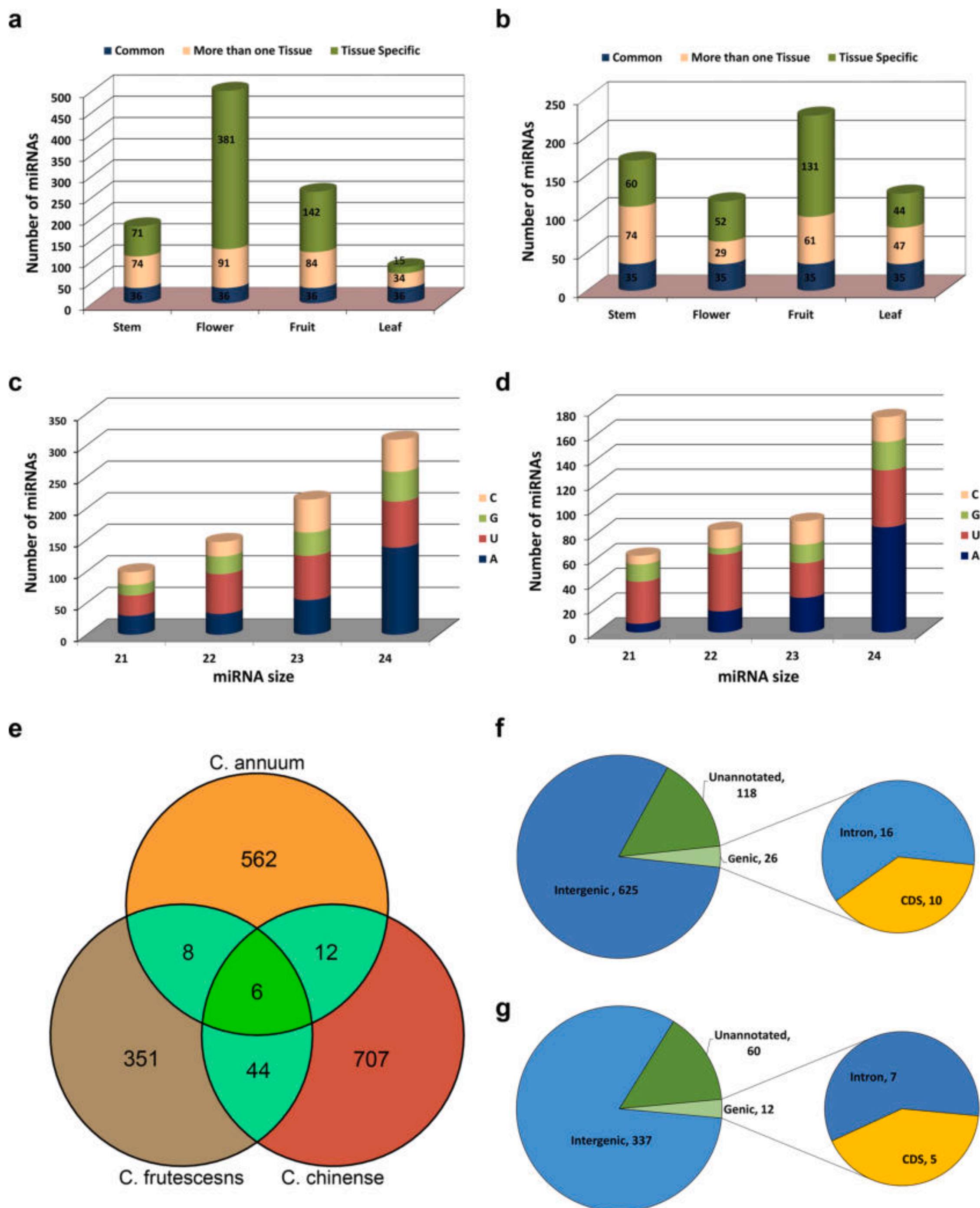

Fig. 1. Characteristics of *Capsicum* miRNAs. The number of tissue specific, found in more than one tissue but not in all, and common miRNAs observed in **a** *C. chinense* and **b** *C. frutescens*. Size distribution and base specificity analysis of miRNAs in **c** *C. chinense* and **d** *C. frutescens*. **e** Venn diagram of common and species specific miRNAs in *Capsicum* species. The distribution of **f** *C. chinense* and **g** *C. frutescens* miRNAs in different genomic locations of *Capsicum* genome are shown in the Venn diagram.

jolokia and Kon jolokia. The base specificity analysis revealed that the more substantial fraction of *Capsicum* miRNAs showed a typical uracil/adenine (U/A) at 5′ base of miRNA structure (Fig. 1c and d).

Additionally, we have investigated the nucleotide constitution of mature miRNAs in *C. chinense* and *C. frutescens*. As per Watson–Crick base pairing rule, GC composition is a crucial factor for the strength of RNA secondary structure, and they might have significant control on sRNA biology, especially Dicer intervened dsRNA cleavage. The majority of the *Capsicum* miRNAs has a GC content in the range of 35–70 %. The analysis revealed the average GC composition of miRNAs in *C. chinense* (44 %) and *C. frutescens* (46 %) and their detailed information is provided in Tables S3 and S4.

Further, we performed a comparative analysis against *C. annuum* data sources from previous sRNA studies (Hwang et al., 2013; Liu et al., 2017; Taller et al., 2018) showing specificity to *C. chinense* (707; 40 %), *C. annuum* (562; 31.8 %) and *C. frutescens* (351; 19.8 %; Fig. 1e) (Table S5). Remaining comparisons which involved, overall commons (6; 0.3 %), *C. annuum* vs *C. chinense* (12; 0.7 %) and *C. annuum* vs *C. frutescens* (8; 0.4 %), meanwhile 44 i. e. 2.5 % were found to be common among *C. chinense* and *C. frutescens* (Table S5).

The precursor sequences (pre-miRNAs) of miRNAs were investigated to identify miRNA distribution across genic and intergenic regions of the genome. It was observed that in *C. chinense* about 625 miRNAs (81 %) were located in intergenic regions while at genic regions only 26 (16 intronics and 10 exonic) miRNAs were identified (Fig. 1f). Similarly, in *C. frutescens* 337 miRNAs (82 %) were originated from intergenic regions whereas only 12 (7 intronic and 5 exonic) were originated from the genic regions (Fig. 1g). Our results suggested that the major proportion of miRNAs in *C. chinense* and *C. frutescens* are derived from non-coding regions of the genome.

3.4. Genomic distribution of miRNAs

Our analysis revealed that 87 % of the *C. chinense* and 89 % of *C. frutescens* miRNA were successfully mapped to twelve *Capsicum* chromosomes while the remaining miRNAs were mapped on scaffolds (unassigned contig sequences, Qin et al., 2014; Figs. S4 & S5). While majority of miRNAs were found to be distributed in different chromosomes, few miRNA clusters were also observed on same chromosomes (Figs. S4 & S5). The members of the same miRNA families were observed to form miRNA clusters. For example, potential clusters of miR168, miR482, miR396 were detected on chromosome 1, 6 and 7 of *C. chinense* respectively. We also found two clusters of different miRNA family such as miR159 and miR5303 on chromosome 3 of *C. chinense*. In the case of *C. frutescens*, clusters of members of miR393, miR482, miR396 and miR408 families were detected on chromosome 5, 6, 7, and 8 respectively. Interestingly two different miRNA family clusters; miR159 and miR162 were observed on chromosome 3. In few cases we observed novel miRNAs clustered together with conserved miRNAs, for instance, Cch-NovmiR024 was clustered with miR482 on chromosome 6 of *C. chinense* indicating miRNA rich region at the clustered chromosomal region of *Capsicum* genome.

3.5. Synteny analysis of Capsicum miRNAs with other Solanaceae plants

The precursor sequences of 22 high confidence miRNAs families were used to study the shared synteny among homologous *Capsicum*, tomato, and potato miRNA genes (Fig. 2a and b). As shown in figures, conserved synteny between miRNAs was observed in majority chromosomes of *Capsicum* (both for *C. chinense*, and *C. frutescens* genome), tomato and potato genomes. For example, the miR398 family found on chromosome 12 of *Capsicum* genome was also mapped to corresponding orthologous chromosome 12 of tomato and potato. The miR169 family located on chromosome 1, 2, and 7 of *C. frutescens* showed homologous sequence hit in the tomato and potato genome on the same chromosomes. In our study, a considerable expansion of several miRNA families

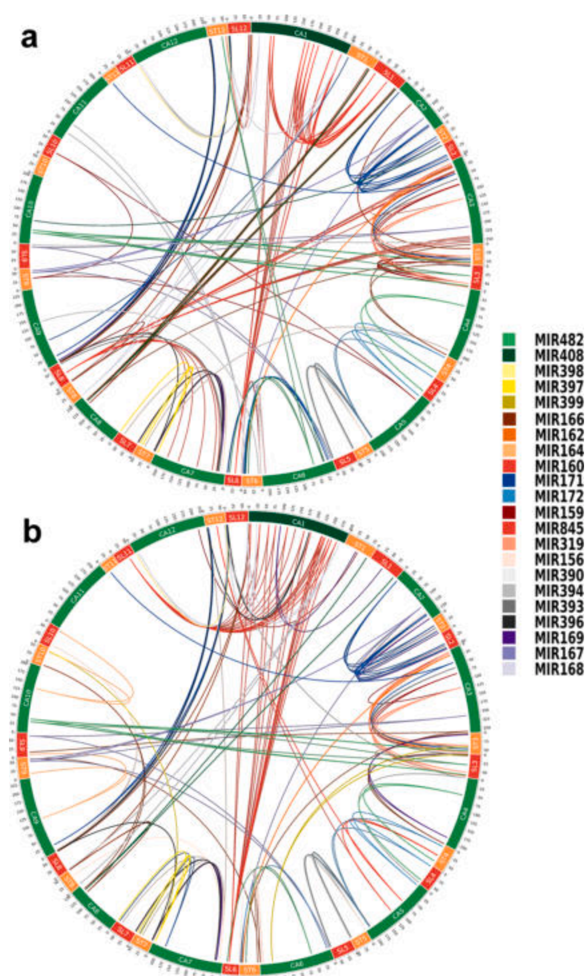

Fig. 2. Comparative map of **a** *C. chinense* and **b** *C. frutescens* with tomato and potato genomes shown by links between syntenic miRNA genes. The links connect the locations of miRNA homologs between genomes which is based on the comparison of sequence information of mapped miRNAs of *C. chinense* and *C. frutescens* with genome sequence of tomato and potato. Each miRNA family is coloured uniquely.

across chromosomes in the genome such as miR159 and miR172 was observed. These miRNA families were detected on more than one chromosomes including miR159 found on chromosome 3, 5 and 6 of *C. chinense*, and chromosome 3 and 12 of *C. frutescens* and their corresponding orthologous tomato and potato chromosomes.

3.6. Identification of potential targets of Capsicum miRNAs

In our study, we predicted the targets of all the identified miRNAs in both *C. chinense* and *C. frutescens* using *C. annuum* reference genome (Qin et al., 2014). It was observed that approximately 80 % of *Capsicum* miRNA targets were predicted to be governed by the cleavage mechanism and the remaining through translational inhibition. The target prediction analysis showed variation in the number of identified targets for each miRNAs, i.e. the targets differs from minimum one to maximum twenty (Tables S6–S9), the highest fractions (25 %) of targets genes are involved in the transcription (Tables S6–S9). Genes/mRNAs targets with diverse functions such as those associated with inorganic ion, metal, and amino acid transport, response to stresses, RNA processing and modification, post-translational modification, signal transduction pathways, carbohydrate, and secondary metabolite biosynthesis were identified.

Specifically, to cite few examples, Cch-NovmiR0421 (*C. chinense* miRNAs) as well as Cfr-miR5812 and Cfr-miRNov00061 (of

C. frutescens) targets 9-cis-epoxycarotenoid dioxygenase (*NCED*) (Capana00g003114) gene involved in carotenoid biosynthesis (Tables S6–S9). We identified both Cch-NovmiR0270 and Cfr-miR528-5p targets beta-galactosidase gene (Capana12g001292) involved in fruit development. In this study we observed that, miR156 family members targets *SBP* transcription factors; miR160 family members targets *ARF* transcription factors, miR164 targets *NAC*, miR165/166 targets LRR (leucine rich repeat) receptor-like serine/threonine-protein kinase, and miR172 targets *AP2* in *Capsicum* (Tables S6–S9). The family members of miR167, miR168, miR169, miR172, and miR390 targets genes transcribing F-box proteins harboring distinct conserved domains, like, *WD40* and *LRR*. These F-box proteins are involved in proteasome-guided degradation pathway (Lechner et al., 2006) and hormone signaling pathways. Moreover, we identified potential targets of some conserved miRNA family members and these targets were not reported earlier. For instance, besides SBP proteins, several other transcription factor genes such as *MYB,* and *bHLH,* were found to be targeted by miR496 family. Like conserved targets, the novel pepper-specific targets were also found to be enriched in transcription factors, but along with this the targets comprised of mRNAs encoding the helicase protein, gypsy/Ty-3 retroelement polyprotein, DNA methyltransferase, splicing factor, and copper/ferrous transporter, suggesting that the analogous novel targets of conserved miRNAs might be associated with particular biological/cellular processes in pepper. For example, miRNAs such as miR164 and miR396 possibly targets flowering time control protein indicating the vital role of miRNAs in flower development. We observed that miR396 potentially targets DNA methyltransferase gene in both the *C. chinense* and *C. frutescens*. The *Domains Rearranged Methylase* (DRM) methyltransferase gene has high sequence complementarity with miR396 in *Capsicum* and tomato as compared to tobacco and potato. The miR396 associated with methyltransferase may be involved in cytosine methylation process, thereby influencing transcriptional silencing of repetitive elements in complex *Capsicum* genome.

In *C. chinense* GO enrichment analysis, the terms like cellular responses to stress and stimulus along with transcription and their regulation, the processes linked to serine/threonine and protein kinase activity, ATP binding, DNA binding, and adenyl ribonucleotide binding activity, were abundantly represented (Fig. 3a). Similarly, in *C. frutescens* GO analysis amino acid phosphorylation process and hormone-mediated signaling pathway (Fig. 3b) were key annotations. The similar enriched processes from cellular component and molecular function category were observed in both *C. chinense and C. frutescens* and no noticeable difference could be found in the enrichment analysis of these two *Capsicum* species except the frequency of enrichment was higher in *C. frutescens* as compared to *C. chinense* as shown in Fig. 3b. The top thirty most abundant gene ontology processes of *C. chinense* and *C. frutescens* is given in Fig. 3c and d.

A total of 41 diverse transcription factor families were identified to be the potential targets of the miRNAs in *C. chinense*. It comprises majorly the members of *MYB* (15 %) and *NAC* (11 %) gene family followed by several other members like *AP2* (10 %), *bHLH* (10 %), *ARF* (9%), *C3H* (8%), *WRKY* (4%), *GRAS* (4%), *TCP* (3%), etc. (Fig. S6a). In our analysis, at least five members from about 25 transcription factor families was identified as putative targets of *C. chinense* and *C. frutescens* miRNAs. Similarly, in *C. frutescens* the identified major targets were *MYB* (15 %), ARF (13 %), *NAC* (11 %), *AP2* (11 %), *TCP* (9%), *bHLH* (7%) transcription factors (Fig. S6b). The transcription factor abundance analysis signifies that *MYB* and *NAC* play a very crucial role in the overall development of *Capsicum* plant. The remaining target transcription factor family including *Homeobox-leucine* (*HB*) *Leucine zipper, DOF, Bromodomain, bZIP* constitutes 2–4% of the total transcription factor families. The diverse function of *Capsicum* miRNAs targets has been mentioned in Table S10.

3.7. Expression profiling and specificity of miRNAs

The expression profiles of all the identified miRNAs from *C. chinense* (769) and *C. frutescens* (409) were investigated to understand the potential roles of miRNAs in plant development. We found significant diversity in the expression profiles of *Capsicum* miRNAs categorizing into

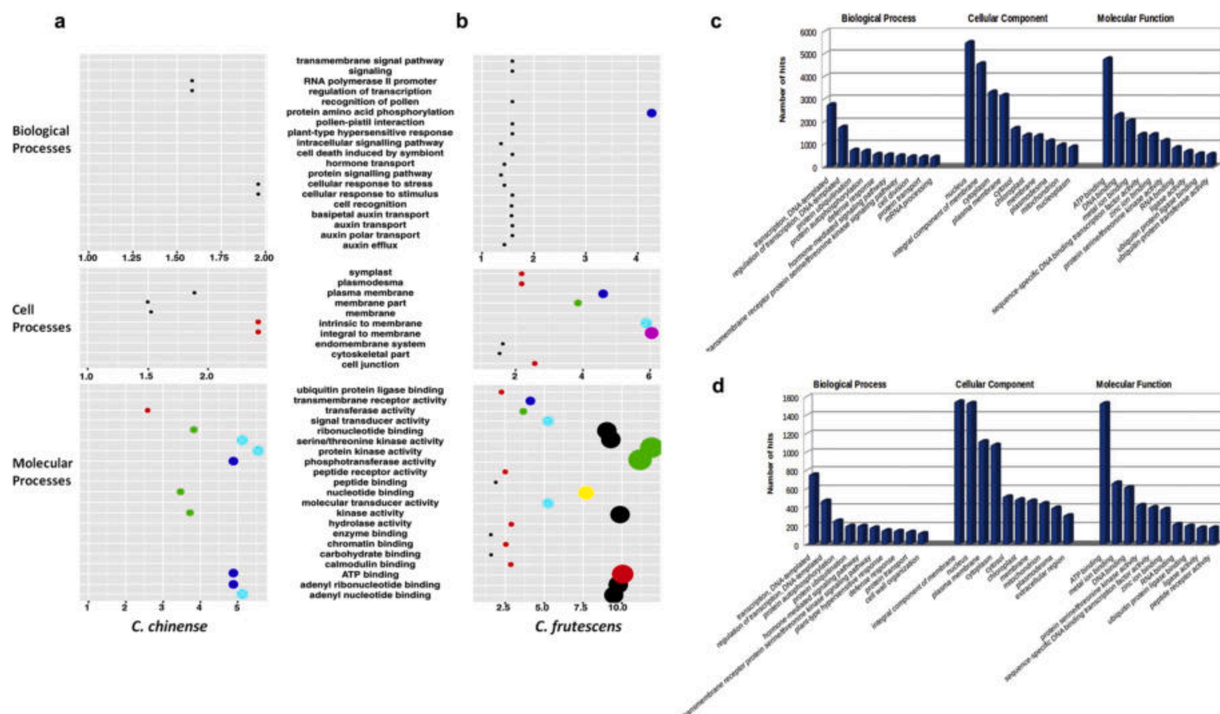

Fig. 3. Gene ontology analysis of target genes of *Capsicum* miRNA. The enrichment analysis of the target genes of identified miRNAs in **a** *C. chinense*, and **b** *C. frutescens.* The significantly enriched terms obtained using agriGO were summarized and visualized as a scatter plot using R programme. Most abundant (top 30) miRNA targets involved in biological process, molecular function, and cellular component GO terms in **c** *C. chinense*, and **d** *C. frutescens* are shown.

five classes based on normalized expression values:- 1) very low (<10 RPKM), 2) low (>10 < 50 RPKM), 3) moderate (>50 < 100 RPKM), 4) high (>100 < 1000 RPKM), and 5) very high (>1000 RPKM). Among these, the largest proportion (43–54 %) of miRNAs displayed very low expression abundance in all the tissues of both *Capsicum* species followed by low expression category (18–29 %). The moderate expression varied between 8–13 %, followed by high (7–13 %) and very high expressed (5–11 %) miRNAs (Fig. 4a).

Further, we observed variations in miRNA expression from ubiquitous to tissue or organ specific, more in novel miRNAs among the analyzed tissues compared to the conserved miRNAs. To identify tissue-specific miRNAs, the low expressed miRNAs were eliminated from the analysis leading to the selection of high-confidence miRNAs. The tissue preferential categories were assigned to individual miRNA as demonstrated by Breakfield et al. (2012). Our analysis showed a large population of miRNAs specifically expressed in one/several other analyzed tissue types of *Capsicum* species. In *C. chinense*, about 44 % miRNAs were preferentially expressed in flower tissue followed by 20 % in fruit, 10 % in the stem and 8% in leaf (Table S3). In contrast, in *C. frutescens* 17 % miRNAs were preferentially expressed in fruit tissue followed by 8% in the stem, 7% in leaf and 6% in flower (Table S4). The tissue specificity analysis revealed that in *C. chinense*, flower-specific miRNAs were most abundant followed by fruit specific, while it was the least in leaf. In *C. frutescens* the tissue-specific miRNAs were most abundant in fruit and were least in flower and leaf (Figs. 1a, b, and 4 b). About 18 % and 13 % of miRNAs were expressed in more than one tissue, while around 8% and 7.7 % of miRNAs were expressed in all the tissues of *C. chinense* and *C. frutescens,* respectively (Tables S3 and S4). This specificity analysis implies that the proportions of tissue-specific/developmental stage--specific expression of miRNAs are significantly higher compared to other types. Further, we detected differential expression of several miRNAs between flower and fruit tissues in both the *Capsicum* species, indicating a rigid control on the miRNA-mediated regulation of flower and fruit development in *Capsicum* species.

It was observed that members of miRNA from the same family do not always display identical expression profiling, as shown by miRNA families with four or more miRNAs members. Further, miRNA families showing differential expression throughout the *Capsicum* tissues were also observed (Fig. 5). For example, miR166e majorly expressed in flower and stem of *C. chinense* compared to the *C. frutescens*; in contrast, miR166d was expressed in all the tissues except in the flower of *C. frutescens*. Significantly high expression of miR166b was detected all tissues of *C. frutescens* as compared to *C. chinense* tissues. The high expression of miR166 g was observed in leaf tissues of both *Capsicum* species but found low expression in fruit tissues. In the case of miR171 family members, miR171d was found to be expressed at a high level throughout the tissues of *C. chinense* and *C. frutescens*, whereas expression of miR171b was limited to flower and fruit tissues of *C. frutescens.* A miR396a and miR396e expressed in all tissues of both species while remaining miR396b, miR396c and miR396d expressed particularly in one or two tissues. High expression of most of the miR482 family members was observed in all the tissues of both *Capsicum* species suggesting their major role in *Capsicum* plant development.

3.8. Validation of miRNAs by qRT-PCR analysis

The expression profiles of miRNAs were validated by randomly selecting 21 miRNAs (5, differentially expressed; 16, tissue-specific) from both *Capsicum* species and was analyzed using the qRT PCR. Moderately high expression levels of novel miRNAs in the flower and fruit tissues (Fig. 6a), indicated their potential role in respective tissue development and differentiation. Besides this, we observed a strong correlation between miRNA expression patterns by qRT-PCR analysis and RNA-seq data with the Pearson correlation coefficient of 0.76 with p-value <2.2e-16 (Fig. 6b). Additionally, we detected significant fruit preferential expression of novel miRNAs such as Cch-NovmiR0137 and Cch-NovmiR0191 in *C. chinense*; and Cfr-NovmiR0026 and Cfr-NovmiR0087 in *C. frutescens* compared to other tissues (Fig. 6e). This result indicates that these novel miRNAs and their corresponding target genes may play a significant role in fruit development of *Capsicum*

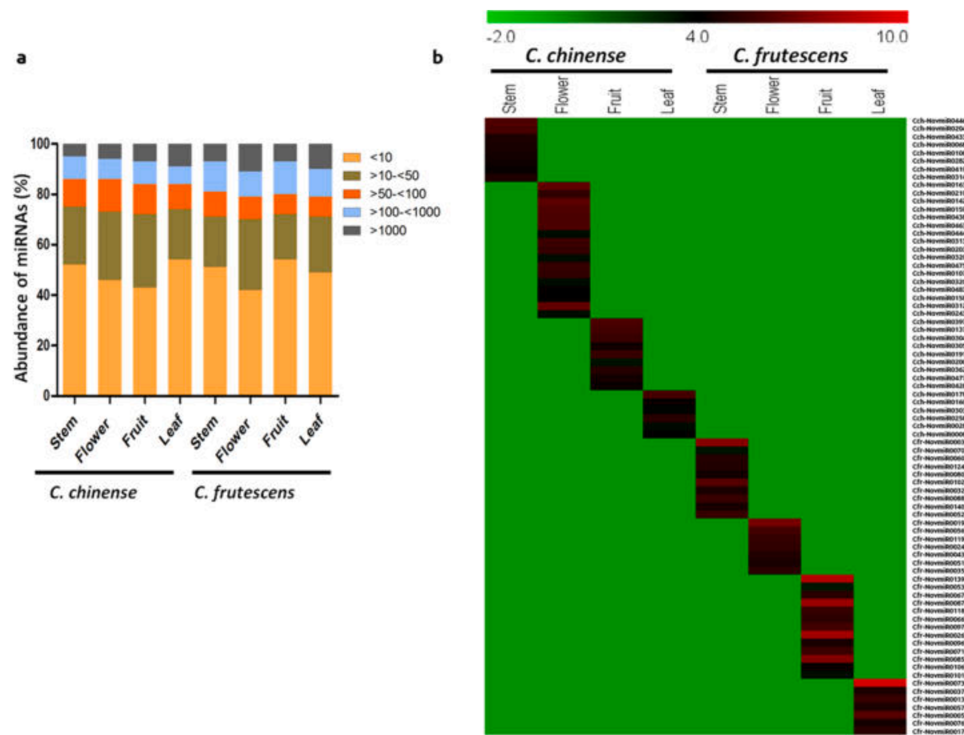

Fig. 4. Expression analysis of *Capsicum* miRNAs. **a** Percentage of miRNAs with different level of expression abundances among tissues of *C. chinense* and *C. frutescens*. **b** Representative diagram of tissue specific novel miRNAs in *Capsicum* Species. Heat map showing expression profiling of tissue specific novel miRNAs in $\log_2$ transformed normalized expression values. The bar correspond to the degree of the $\log_2$ transformed expression levels for individual miRNA.

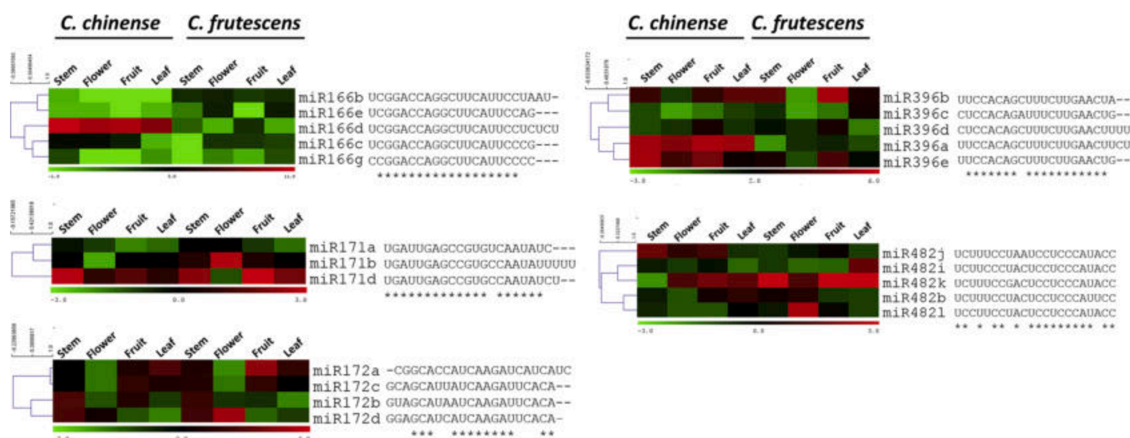

Fig. 5. Heat map showing differential expression of miRNA members from the same family. Heat map showing miRNA expression data from leaf, flower, fruit and stem from *C. chinense* and *C. frutescens*. The name of each miRNA family is mentioned on the left side while its sequence is indicated on the right side. The scales represent Log_2 transformed values.

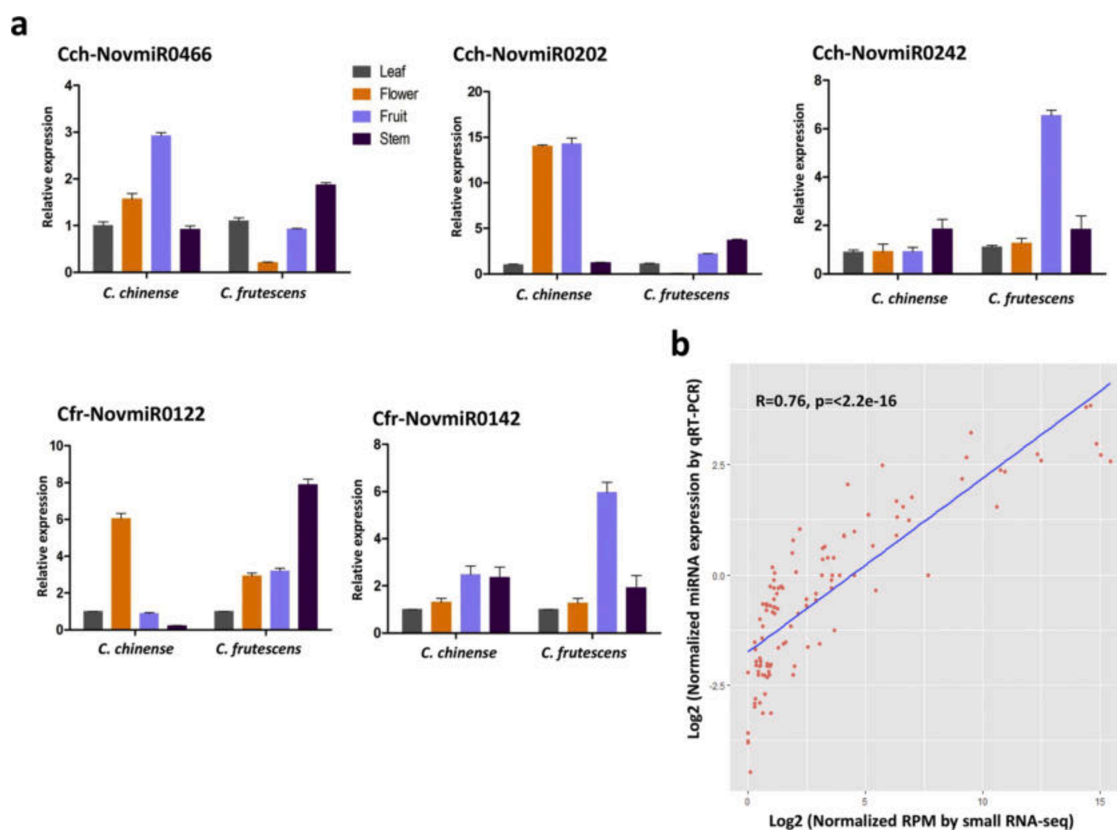

Fig. 6. qRT-PCR and correlation analysis of differentially expressed and tissue-specific miRNAs in *C. chinense* and *C. frutescens*. **a** The histograms demonstrate the relative expression levels of miRNAs in leaf, flower, fruit and stem tissues of both the *Capsicum* species. **b** Correlation analysis of expression revealed by qRT-PCR and RNA-seq data of selected twenty one miRNAs in four tissues each from *C. chinense* and *C. frutescens*. The tissue specificity of miRNAs is shown in **c** stem, **d** leaf, **e** fruit, and **f** flower tissue. The expression level of each miRNA was normalized with U6 snRNA expression. The error bars signifies standard deviation among biological replicates of tissue samples. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

species.

Similarly, Cch-NovmiR0302 and Cch-NovmiR0159 (in *C. chinense*), and Cfr-NovmiR0019 and Cfr-NovmiR0024 (in *C. frutescens*) were significantly enriched in flower tissue in contrast to other tissues (Fig. 6f). This implies a probable role of these miRNAs in flower development. Also qRT-PCR analysis revealed tissue preferential expressions of miRNAs in stem and leaf tissues of *Capsicum* species (Fig. 6c and d). Overall, we observed considerable consonance in the expression patterns of miRNAs derived through small RNA-seq and qRT-PCR. Several of these miRNAs displayed major differences in the level of expression throughout various tissues (Fig. 6a–f). In general, the differential and tissue-specific expression profile observed here will be useful to explore the specific function/biological roles of miRNAs in *Capsicum* fruit and plant development (Jaiswal et al., 2020).

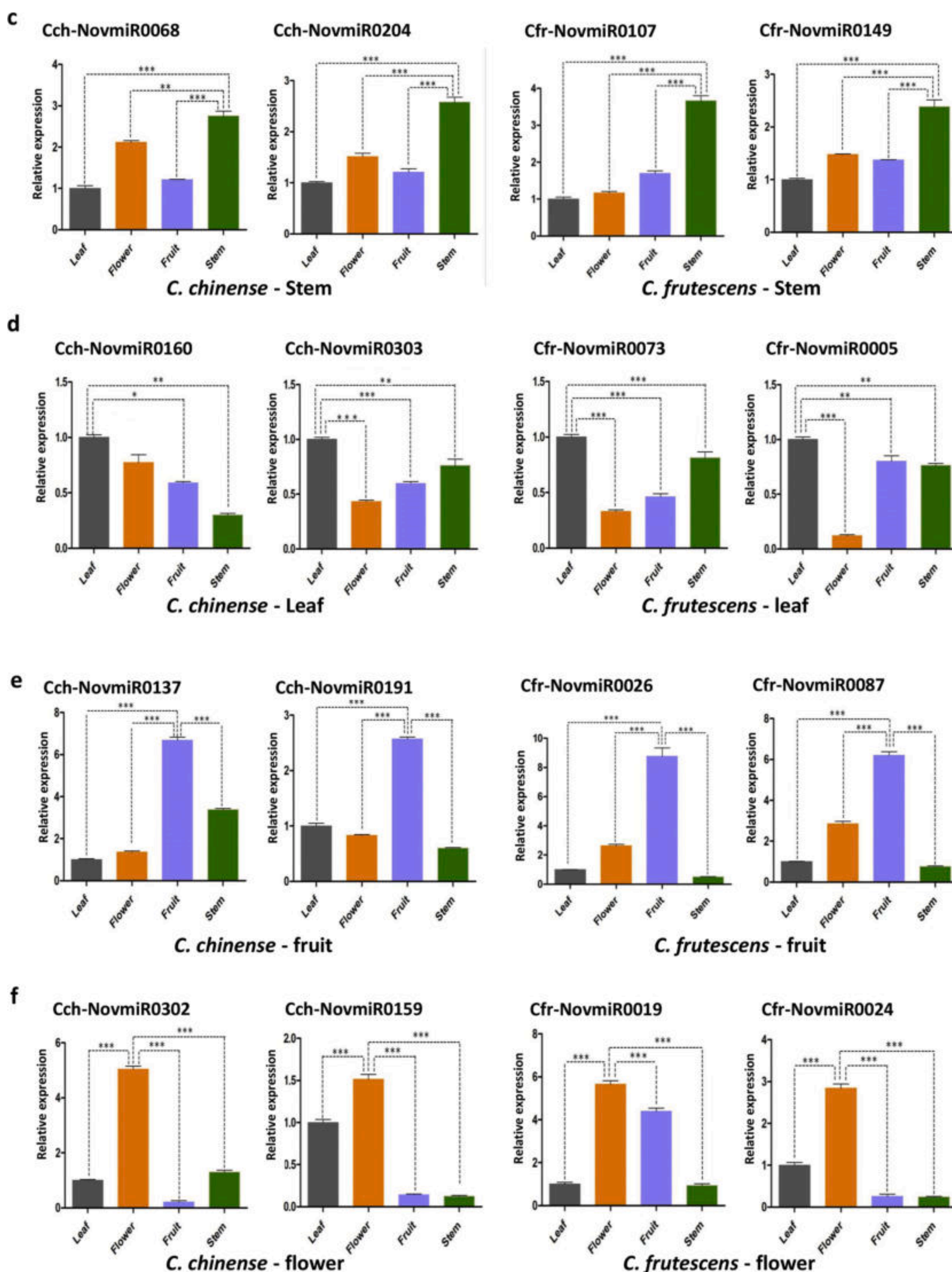

Fig. 6. (*continued*).

3.9. Prediction of putative Solanaceae-specific miRNAs

Several miRNA families are lineage-specific or family-specific or tissue-specific (Allen et al., 2004). The proportion of miRNAs conserved between different families of plants is less than species-specific miRNAs which indicates that many of the known miRNA genes emerged in the recent evolutionary period (Cuperus et al., 2011). These highly conserved miRNA families are the intrinsic component of various functional regulatory pathways of plant development, nutrition, stress and signaling responses (Bartel and Bartel, 2003; Rubio-Somoza and Weigel, 2011). In this study, the prediction of putative Solanaceae-specific miRNAs to explore their role in plant development was done. To achieve this, all plant miRNA (Viridiplantae) sequences from miRBase were retrieved. The tomato and potato miRNAs were further extracted from this miRNA list. Later, the BLASTN was performed with tomato, and potato miRNA sequences as a query against total Viridiplantae plant miRNA sequences, and 104 miRNA sequences (from each potato and tomato) did not show any hit and were inferred as tomato and potato specific miRNAs. Further, alignment with BLASTN by taking these sequences as a query sequence was done against in-house

developed *Capsicum* miRNA sequences which resulted in 51 unique miRNA sequences. The in-house *Capsicum* miRNA dataset was constructed by collecting all the miRNAs from the previous studies (Hwang et al., 2013; Liu et al., 2017; Taller et al., 2018; Yang et al., 2020a, 2020b) and our small RNA dataset of *C. chinense* and *C. frutescens*. Our analysis revealed that several miRNAs were Solanaceae-specific in nature i.e.:-, sly-miR166c, sly-miR167b, sly-miR171c, sly-miR482a, stu-miR167b, stu-miR167c, stu-miR172c, etc. These potential Solanaceae-specific miRNAs target diverse range of proteins such as protein kinase, serine/threonine-protein kinase, late blight resistance proteins and TFs which plays an important role in plant development and defense signaling pathways.

4. Discussion

The present study was designed to identify and develop the non-coding RNAs resources in the two *Capsicum* species i.e. *C. chinense and C. frutescens,* as the miRNAs identification till date were mostly done in *C. annuum*. Furthermore, currently there is very less number of miRNAs identified in *C. annuum* compared to other plant species.

4.1. Characterization of Capsicum miRNAs

Among the identified 18–30 nt long miRNAs, the major fractions of reads were of 21–24 nt (Fig. S2), indicating the characteristics of DCLs processed sRNAs (Axtell, 2013). This observation is in agreement with earlier reports in other plant species like *Arabidopsis thaliana*, tomato, *Citrus trifoliata*, cucumber, peanut, maize, *C. annuum*, rice, potato and chickpea (Fahlgren et al., 2007; Moxon et al., 2008; Song et al., 2010; Martínez et al., 2011; Chi et al., 2011; Li et al., 2013; Hwang et al., 2013; Yang et al., 2013; Lakhotia et al., 2014; Srivastava et al., 2015; Mutum et al., 2016). The variable sizes of miRNAs are known to be responsible for regulating expression of genes involved distinct functions in plants (Xie et al., 2004; Chen, 2009; Cuperus et al., 2011). The observation of predominance of 24 nucleotide miRNA class in both *C. chinense* and *C. frutescens* was similar with a recent study of miRNAs in *C. annuum* (Liu et al., 2017; Taller et al., 2018) and in *C. chinense* (Yang et al., 2020a, 2020b). The richness of 24 nt sRNAs might signify the intricacy of the *Capsicum* genome as mainly long-miRNAs are associated with repeats and heterochromatic (especially transposons) modifications (Axtell, 2013; Taller et al., 2018).

In plants usually, 21 nt sRNA class are predominantly found and processed by association of *DCL1* and *AGO1*, while the 24 nt miRNAs are generated by *DCL3* with *AGO4* (Rajagopalan et al., 2006; Axtell, 2013). Among the analyzed tissues, the representation of 24 nt miRNAs were observed to be high in reproductive tissues (fruits and flower) relative to vegetative tissues (leaf and stem; Tables S3 and S4). This might be because the reproductive tissues require more strict and distinct repression machinery of target genes by these miRNAs (Jeong et al., 2011). The similar findings were observed in *Arabidopsis* and rice in which higher proportion of 24 nt sRNAs was found in reproductive tissues like inflorescences and rice panicles, respectively, as compared to vegetative tissues such as leaves and roots (Kasschau et al., 2007; Jeong et al., 2011). The more substantial fraction of *Capsicum* miRNAs showed a typical uracil/adenine (U/A) at 5′ base of miRNA structure (Fig. 1c and d) as consistent with previous studies in *Capsicum* and other plants (Mi et al., 2008; Czech and Hannon, 2011; Hwang et al., 2013; Liu et al., 2017).

The GC content of miRNA plays a vital role in the prediction of the putative targets (Adai et al., 2005). The GC content of the majority of the *Capsicum* miRNAs in this study varied between 35–70 % (Tables S3 and S4) which is similar to the observation made in other plants (Ho et al., 2007; Mishra et al., 2009; Jain et al., 2014). In *C. chinense* and *C. frutescens*, the average GC composition of miRNAs was found to be 44 % and 46 %, respectively (Tables S3 and S4) and was identical with *Arabidopsis*, chickpea, *Medicago* (all have 44 %), rice, and soybean (46 %) but lower than grapevine (50 %), sorghum and maize (both 52 %) (Adai et al., 2005; Ho et al., 2007; Lelandais-Brière et al., 2009; Turner et al., 2012; Jain et al., 2014).

4.2. Distribution, mapping and target prediction of Capsicum miRNAs

We observed that each miRNA family comprised of a variable number of miRNA members ranging from two to twenty (Tables S3 and S4). In *Arabidopsis,* such a massive expansion of miRNA families was observed due to several rounds of genome duplication events i.e. families like miR156, miR159, miR166 and miR395 (Maher et al., 2006; Li and Mao, 2007). In *Capsicum* also, owing to large genome size (3.5 GB), several duplication regions of the genome including the expansion of coding and non-coding gene (miRNAs) families are expected.

The mapping of miRNAs revealed that members of few miRNA family clustered together on the different chromosome of *Capsicum* species i.e. members of miR159 family on chromosome 3, 6 and 7 of *C. frutescens* and miR482 family members on chromosome 6 and 10 of *C. chinense* (Figs. S4 and S5). This observation is similar to observation made in *C. annuum* (Seo et al., 2018). These findings were consistent with chickpea in which four clusters of miR166 family detected on multiple linkage groups (Jain et al., 2014), and with potato in which the tandem arrays of miRNAs was observed on chromosome 1, 3, 6 and 8 of the genome (Lakhotia et al., 2014). The comparable miRNA clustering pattern was detected in other studies like *Arabidopsis*, rice, *Medicago*, potato and chickpea (Jones-Rhoades and Bartel, 2004; Cui et al., 2009; Lelandais-Brière et al., 2009; Jain et al., 2014; Lakhotia et al., 2014). The distribution analysis suggested that major miRNA proportion s in *C. chinense* (81 %) and *C. frutescens* (82 %) are derived from non-coding regions of the genome (Fig. 1f and g) and are consistent with other reports (Kim, 2005; Jain et al., 2014; Lakhotia et al., 2014).

We identified targets for all the *C. chinense* and *C. frutescens* miRNAs (Tables S6–S9). However, in other sRNA studies about 85%–90% miRNAs target could be predicted (Hwang et al., 2013; Jain et al., 2014; Lakhotia et al., 2014). Furthermore, our finding is in accordance with the previous studies reported in plants indicates that the principal mechanism of miRNA-mediated gene regulation is mRNA cleavage (Schwab et al., 2005; Pasquinelli, 2012; Rogers and Chen, 2013; Jain et al., 2014). Transcription factors (TFs) involved in various processes of plant development are primary targets of miRNAs (Mallory and Vaucheret, 2006; Chen, 2009; Hwang et al., 2013; Jain et al., 2014; Zhang et al., 2017; Seo et al., 2018). We observed that the novel miRNA targets multiple genes which encode for a diverse series of proteins, such as transcription factors like *MYB*, *NAC*, *AP2-EREBP*, *ARF*, *SBP*, *HB*, *GRAS*, kinases, *Fbox* and defense and signaling-related proteins. Overall, putative targets of novel *Capsicum* miRNAs are more distinct compared to conserved miRNAs, which is similar to the previous studies (Jeong et al., 2011; Hwang et al., 2013). Majority of the plant transcription factors targeted by conserved miRNAs were found to be analogous with the targets identified in *Arabidopsis* and other plant species (Yanhui et al., 2006; Zhang et al., 2006; Moxon et al., 2008; Hwang et al., 2013; Jain et al., 2014; Srivastava et al., 2015) indicating the vital role of miRNAs in fundamental biological and cellular processes. We identified 3 miRNA's which are participated in the pigment biosynthetic process by targeting 9-cis-epoxycarotenoid dioxygenase gene of carotenoid biosynthesis (Nisar et al., 2015) and also involved in fruit ripening (Sun et al., 2012). Cch-NovmiR0270 and Cfr-miR528-5p targets beta-galactosidase gene (Capana12g001292) involved in fruit ripening process (Biles et al., 1997). Similarly, beta-galactoside gene was also found to be targeted by other *C. chinense* miRNA (miR396 h) (Yang et al., 2020a, 2020b). We observed Cch-NovmiRN0011 targets alanine-glyoxylate aminotransferase 2 (Capana00g005022) which is associated in aroma formation in pepper fruits (D'Auria, 2006). Recently a circular RNA was identified targeting same Capana00g005022 gene for aroma development in *C. chinense* (Yang et al., 2020a, 2020b) indicating miRNA mediated regulation of fruit ripening and aroma

formation in Capsicum fruits

4.3. Expression pattern of Capsicum miRNAs

Furthermore, our present study revealed that members from the same miRNA family do not always display similar expression pattern (Fig. 5). This was further confirmed by investigating the expression profiling of the miRNA families with at least four miRNA members. Similarly, in *Arabidopsis*, rice and chickpea, the differential expression of members of miRNA families such as miR156, 159, 164, 166, 169, 319, 171, and 172 have been observed (Jeong et al., 2011; Jain et al., 2014). As demonstrated in earlier studies (Palatnik et al., 2007; Sieber et al., 2007; Jeong et al., 2011), different isoforms of miRNAs are found to target a diverse set of genes that control spatio-temporal gene regulation. The tissue specificity analysis implies that the proportions of tissue-specific/developmental stage-specific expression of miRNAs are significantly higher compared to other types (Tables S3 and S4). The similar observation was reported earlier in *C. annuum* (Hwang et al., 2013) and chickpea (Jain et al., 2014).

The expression analysis using qRT-PCR showed overall similarity in the expression patterns of miRNAs derived through small RNA-seq and qRT-PCR (Fig. 6a–f). The prediction of putative Solanaceae-specific miRNAs provided insights into the highly conserved miRNA families that are an intrinsic component of various functional regulatory pathways of plant development, nutrition, stress, and signaling response (Bartel and Bartel, 2003; Rubio-Somoza and Weigel, 2011). Several miRNA families are lineage-specific or family-specific or tissue-specific (Allen et al., 2004). The proportion of miRNAs conserved between plant families is less than that of species or family-specific miRNAs indicating that many of the known miRNA genes emerged in the recent evolutionary period (Cuperus et al., 2011). Synteny analysis illustrates that the majority of high confidence *Capsicum* miRNA families originated from identical loci of the chromosomes of *Capsicum*, potato, and tomato. This indicates higher-level conservation (miRNA genes) between *C. chinense*, *C. frutescens*, potato, and tomato genome (Fig. 2a and b). Our approach for the prediction of Solanaceae specific miRNAs resulted in the identification of 51 putative unique miRNAs that are specific to Solanaceae species comprising *Capsicum*, potato, and tomato (Fig. S7). Similarly, in wheat small RNA analysis, two monocot-specific miRNAs such as tae-miR3075 and tae-miR3014b were found to be conserved in all the studied monocots (Sun et al., 2014). In legume plants, the sequence conservation and secondary structure analysis of small RNAs revealed four novel small RNAs namely miR1507, miR2118, miR2119 and miR2199 to be legume-specific miRNAs (Jagadeeswaran et al., 2009). These predicted Solanaceae specific miRNAs belongs to various different miRNA families and targets a diverse range of disease resistance proteins such as protein kinaseas well as multifarious TFs playing essential roles in plant development and defense signaling pathways (Jagadeeswaran et al., 2009; Seo et al., 2018).

5. Conclusion

In conclusion, our study provides the comprehensive small RNA analysis in Bhut Jolokia (*C. chinense*) and Kon Jolokia (*C. frutescens*) to explore the conserved and novel miRNAs that regulate the *Capsicum* plant development. Using NGS technology we identified 279 conserved and 490 high confidence novel miRNAs in *C. chinense* and 254 conserved and 155 novel miRNAs in *C. frutescens* from four tissues. Our target prediction analysis reveals the crucial role of miRNAs in fundamental biological and cellular processes. The expression analysis discovered the differential and tissue-specific/preferential expression of miRNA, indicating their involvement in tissue/organ development. Overall, our combinatorial efforts of computational analysis and experimental validation offer high-confidence small RNA populations which would to strengthen miRNA related gene regulation studies in pepper and Solanaceae in future.

Author contributions

NR conceived and designed the experiment, SSC collected the samples, generated data, performed biological experiments and wrote draft. NK, AR, and VB performed computational analysis. SM, VJ and AK contributed to analysis. BKS provided inputs during the experiments and involved in manuscript writing. SSC and NR wrote the manuscript. All authors revised and approved the final manuscript.

Declaration of Competing Interest

The authors declare no competing interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2021.109952.

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