Planta (2020) 251:79
https://doi.org/10.1007/s00425-020-03368-4

ORIGINAL ARTICLE

Dynamic changes in the expression pattern of miRNAs and associated target genes during coconut somatic embryogenesis

Abdulla Abdulla Sabana[1,2] · Muliyar Krishna Rajesh[2] · Ginny Antony[1]

Received: 16 November 2019 / Accepted: 24 February 2020

Abstract

Main conclusion **Genome-wide analysis of small RNAs identifies somatic embryogenesis- specific miRNAs and their targets and provides novel insights into the mechanisms governing somatic embryogenesis in coconut, a highly in vitro recalcitrant species.**

Abstract Coconut, a major plantation crop of the tropics is recalcitrant to in vitro culture with a very low rate of somatic embryo turnover. Clonal propagation to enhance the production of high yielding, disease-free planting material in coconut has remained a distant reality. To better understand the molecular basis of this recalcitrance and to throw light on the complex regulatory network involved in the transition of coconut somatic cells to embryogenic calli, genome-wide profiling of small RNAs from embryogenic (EC) and non-embryogenic calli (NEC) was undertaken using Illumina Hiseq 2000 platform. We have identified a total of 110 conserved miRNAs (representing 46 known miRNA families) in both types of calli. In addition, 97 novel miRNAs (48 specific to EC, 21 specific to NEC and 28 common to both the libraries) were also identified. Among the conserved miRNAs, 10 were found to be differentially expressed between NEC and EC libraries with a log2 fold change > 2 following RPM-based normalization. miR156f, miR167c, miR169a, miR319a, miR535a, and miR5179 are upregulated and miR160a, miR166a, miR171a, and miR319b are down-regulated in NEC. To confirm the differential expression pattern and their regulatory role in SE, the expression patterns of miRNAs and their putative targets were analyzed using qRT- PCR and most of the analyzed miRNA-target pairs showed inverse correlation during somatic embryogenesis. Selected targets were further validated by RNA ligase mediated rapid amplification of 5′ cDNA ends (5′RLM-RACE). Our data suggest that a few conserved miRNAs and species-specific miRNAs act in concert to regulate the process of somatic embryogenesis in coconut. The results of this study provide the first overview into the regulatory landscape of somatic embryogenesis in coconut and possible strategies for fine-tuning or reprogramming to enhance somatic embryo turn over in coconut.

Keywords *Cocos nucifera* · High-throughput sequencing · microRNAs · Somatic embryogenesis

Abbreviations

5′ RLM–RACE	5′ RNA ligase-mediated rapid amplification of cDNA ends
EC	Embryogenic calli
NEC	Non-embryogenic calli
SE	Somatic embryo
SEG	Somatic embryogenesis

Introduction

Coconut (*Cocos nucifera* L.), the only known species of the genus *Cocos*, supports the livelihood of millions of people in the coastal regions of the tropics. This perennial palm is popularly called the 'tree of life' and provides a wide array of products ranging from coir, oil, coconut milk, inflorescence sap, and sugar. One of the major constraints in coconut cropping is the short supply of elite planting materials. Breeding in coconut for high yielding, disease-resistant,

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00425-020-03368-4) contains supplementary material, which is available to authorized users.

✉ Ginny Antony
ginnyantony@cukerala.edu.in

[1] Central University of Kerala, Periya, Kasaragod, Kerala 671320, India

[2] ICAR-Central Plantation Crops Research Institute, Kasaragod, Kerala 671124, India

Published online: 12 March 2020

genetically uniform palm is a cumbersome, long and expensive process due to the lack of suitable vegetative propagation method (Arunachalam and Rajesh 2008). Coconut palms are mainly propagated through nuts and nuts from selected mother palms do not meet the requirements for large scale planting. Moreover, nut to nut variation results in many poor-performing palms which are identified only at the fruit-bearing stage. Improved disease resistant and high yielding planting materials are rare and therefore in vitro propagation or micro-propagation via somatic embryogenesis (hereafter referred to as SEG) is considered as a suitable alternative method for mass propagation of elite planting materials (Fernando et al. 2010). Though many labs around the world had initiated research on coconut in vitro propagation, limited success has been reported (Pérez-Núñez et al. 2006; Nguyen et al. 2015). The absence of reliable SEG and regeneration protocol for coconut is an impediment to crop improvement and functional analysis of its recently decoded genome (Xiao et al. 2017). Some of the major bottlenecks in clonal propagation of coconut via SEG include the heterogeneous response of different explants, low rate of formation of somatic embryos and poor establishment of in vitro generated plants (Nguyen et al. 2015).

Somatic embryos (hereafter referred to as SE) are formed when somatic cells first dedifferentiate and then re-differentiate to produce viable embryos that develop into plants. The embryos may be formed directly from somatic cells, a process called direct SEG or indirectly through an intermediate callus phase (Horstman et al. 2017). A variety of tissues namely immature embryos, immature inflorescence, shoot tip, tender leaf, plumule, and unfertilized ovaries have been used as explants in the clonal propagation research of coconut (Nguyen et al. 2015). SE are induced either by stress or hormones or both and depend on the plant genotype and the type of explant used (Dantu and Tomar 2010). SE is used for clonal propagation for industrial and academic applications like scaling up of plant materials for testing and for reducing the breeding span of plants with a long life cycle. The initiation of SE involves the stimulation of a complex signaling network and reprogramming of cell's transcriptome and a number of transcription factors are believed to play a crucial role in this process (Méndez-Hernández et al. 2019). Global analytical profiling like microarray and RNA- Seq of SE induced cells in a number of studies suggest the involvement of multiple transcription factors in SE induction (Che et al. 2006; Rajesh et al. 2016). In *Arabidopsis,* transcription factors like *LEC* genes (*LEC1, LEC2, FUSCA3, FUS3*), AGAMOUS-LIKE 15 (*AGL15*), BABYBOOM (*BBM*) and WUSCHEL RELATED HOMEOBOX (*WOX*) have been found to promote SE induction (Guan et al. 2016). Transcription factors, the key regulators of plant growth and development, are in turn regulated by micro RNAs (Samad et al. 2017). Till date, detailed studies of only four genes with potential roles in coconut SEG have been reported: somatic embryogenesis receptor kinase (*SERK*) (Pérez-Núñez et al. 2009; Rajesh et al. 2016), cyclin-dependent kinase (*CDKA*) (Montero-Cortés et al. 2010a) and KNOTTED-like homeobox (*KNOX*) (Montero-Cortés et al. 2010b) and AINTEGUMENTA-like (*ANT*) (Bandupriya et al. 2013). In a recent study, Rajesh et al. (2016) identified 14 genes that could be potentially involved in the regulation of coconut SEG, based on RNA-Seq data of coconut embryogenic calli, and experimentally validated their expression pattern in different stages of SE through quantitative real-time PCR (qRT-PCR).

Increasing studies are attributing key roles to micro RNAs (miRNAs) in the control of meristem development, cell proliferation and embryogenesis in plants (Lin et al. 2015; Long et al. 2018). miRNAs are one class of endogenous non-coding RNAs of size 19–24 nt which act as critical post-transcriptional regulators of gene expression through target mRNA degradation or translation inhibition. Several miRNA families have been identified to have a role in SEG in different plants (Siddiqui et al. 2019). Key understanding into miRNAs regulation of different stages of SEG have been obtained in rice (Luo et al. 2006), poplar (Tingting et al. 2011), larch (Zhang et al. 2012), longan (Lin and Lai 2013), radish (Zhai et al. 2014), maize (Chávez-Hernández et al. 2015), wheat (Chu et al. 2016), *Lilium* (Zhang et al. 2017), and Tuxpeno maize (Alejandri-Ramírez et al. 2018). The conserved miRNA family miR156, which targets the squamosa promoter-binding-like protein (*SPL*) gene, has been reported to regulate embryogenic callus differentiation, cotyledon embryo development and globular embryo development in rice, maize, longan and cotton, respectively (Luo et al. 2006; Lin and Lai 2013; Shen et al. 2013; Yang et al. 2013). Also, miR166 has been reported to participate in the transcriptional and post-transcriptional regulation of SEG in *Lilium* and *Dimocarpus longan*, by binding to *LMBR1*, *IDD1* (INDETERMINATE DOMAIN 1) and *PHB* (PHABULOSA) in the former (Yang et al. 2020), and *HD-ZIP III* in the latter (Zhang et al. 2020).

Additionally, miR159, miR167, and miR171 families have been identified to play a major role in reproductive development in citrus (Wu et al. 2011). Comparison of expression levels of miRNA between embryogenic callus (EC) and non-embryogenic callus (NEC) have been undertaken in cotton (Yang et al. 2013), citrus (Wu et al. 2015) and *Larix leptolepis* (Zhang et al. 2010). In *Arabidopsis* SEG, a set of candidate miRNAs regulates the transition from vegetative to embryogenic state (Szyrajew et al. 2017). Knowledge of miRNAs and other small RNAs of coconut is very limited. According to miRBase 22.1 (Griffiths-Jones et al. 2007; accessed on 01 January 2020), a total of 2422 mature miRNAs from 14 monocotyledons have been registered. Of these, only six miRNAs have been reported from the Arecaceae family (Mehrpooyan et al.

2012). In our earlier study, we had predicted a total of 27 mature miRNA sequences, belonging to 15 miRNA families along with their target genes, from the RNA-Seq data of coconut embryogenic callus utilizing computational approaches (Sabana et al. 2018). To gain deeper insights into the regulatory roles of miRNA in SEG, small RNA libraries derived from embryogenic and non-embryogenic calli obtained from plumular explants were sequenced and analyzed. In this study, we report the identification of conserved and novel coconut miRNAs and their expression patterns during SEG. We also identified miRNA targets and analyzed their expression during SEG. The miRNAs identified in this study, and their targets, provide the basic framework for deeper analysis of miRNA mediated regulatory network of SEG induction in coconut.

Materials and methods

Plant material and in vitro culture

The procedure of Bhavyashree et al. (2016) was followed for in vitro plumule culture. In brief, the mature nuts of West Coast Tall (WCT) cultivar were harvested from 30 year old palms maintained in the plantation at ICAR-CPCRI (Kasaragod, Kerala, India), dehusked, cut open and embryos with endosperm were excised using a cork borer and thoroughly washed with distilled water. In a laminar air-flow chamber, the endosperm plugs containing the embryos were washed with 0.01% mercuric chloride ($HgCl_2$) for 5 min and rinsed 3–4 times with sterile distilled water to remove the traces of mercuric chloride. Embryos were then excised from endosperm plug, and surface sterilized with 20% sodium hypochlorite NaClO solution for 20 min and subsequently washed 5–6 times with sterile distilled water. Plumular tissues were then excised from the sterilized embryos by using sterile blade and scalpel and inoculated into full strength Y3 basal medium (Eeuwens 1976), supplemented with 2,4-dicholorophenoxyacetic acid (2,4-D, 74.6 µM), thidiazuron (TDZ, 4.5 µM), sucrose (87.6 mM) and charcoal (1 g l^{-1}) with pH 5.8, for callus induction. The cultures were incubated in dark at 27 ± 2 °C. The initial calli obtained were subcultured into media supplemented with gradually reducing concentrations of 2,4-D (45.24 µM and then to 22.6 µM) in the later stages. A polyamine (spermine, 50 µM) and a cytokinin (TDZ, 4.5 µM) were also incorporated in this medium for better multiplication of callus in the embryogenic stage (Rajesh et al. 2014a). Embryogenic (EC) and non-embryogenic calli (NEC) were selected visually and immediately frozen in liquid nitrogen.

Small RNA library construction, sequencing and data analysis

Small RNAs were extracted from five each of EC and NEC samples using the mirPremier microRNA isolation kit (Cat# SNC50, Sigma Aldrich) following the manufacturer's instructions. The quantity and quality of the isolated miRNAs were analyzed in 4% agarose gel and using Bioanalyser 2100 using specialized small RNA analysis kit (Cat# 5067–1548, Agilent). Equal quantities (500 ng) of small RNA from five samples were pooled together to constitute the EC and NEC bulks. Small RNA libraries for the EC and NEC samples were constructed using TruSeq Small RNA Library Prep Kit (Cat# RS-200-0024, Illumina) and deep sequencing of each sample was carried out on an Illumina HiSeq 2000 platform (Bionivid, Bengaluru, India). In brief, the small RNAs were ligated first to 5′ RNA adaptor and then to 3′ RNA adapter. Adapter-ligated fragments were then reverse transcribed, amplified with PCR, gel purified and finally subjected to Illumina high-throughput sequencing.

Raw reads generated by the Illumina sequencer were subjected to quality statistics using NGSQC toolkit (v2.3.3) (Patel and Jain 2012) without adapter trimming. High-quality reads were then subjected to adapter trimming using Cutadapt (v1.10) and filtering of reads smaller than 18 nt. Adapter trimmed files were subjected to trimming based on the quality score using NGSQC toolkit (v2.3.3). The number of unique tags and their read count, fasta sequences and tag length distribution were fetched using Perl and awk scripts. The final filtered reads were subjected to BLASTn (Altschul et al. 1990) against Rfam database (https://rfam.xfam.org/) to identify and eliminate other small RNAs like ribosomal RNA (rRNA), transfer RNA (tRNA), small nucleolar RNA (snoRNA) and small nuclear RNA (snRNA), with filtering criteria 100% query coverage, 2 mismatches and 0 gaps best hits.

Information on coconut miRNAs is not available in miRBase. To identify conserved miRNAs in coconut, clean reads of length 18–22 bases from each sample, were analyzed against plant mature miRNA sequences in miRBase Database (22.1 release) (https://www.mirbase.org) using srna-workbench (v 3.2) (https://srna-workbench.cmp.uea.ac.uk/), miRProf is the tool used for known miRNA analysis (https://srna-workbench.cmp.uea.ac.uk/mirprof/). Only the perfectly matched sequences were considered to be conserved miRNAs. Novel miRNA candidates were identified by using miRcat (https://srna-workbench.cmp.uea.ac.uk/tools/mircat/), with the following parameters: minimum free energy: − 25 kcal/mol; minimum number of paired bases in miRNA region is 17; the number of mismatches between miRNA and miRNA* not more than 3 nt; minimum GC content: 30%; length of a miRNA is 18–22 nt; maximum percentage of unpaired bases in hairpin region is 40; minimum

hairpin length is 55 nt and *P*-value: 0.1. Novel miRNAs with *P*-value ≤ 0.05 were taken as significant miRNAs. Secondary structure prediction for novel miRNAs was done using the mfold web server (https://unafold.rna.albany.edu/).

Differential expression analysis of miRNAs in the two coconut libraries

Reads with abundance ≥ 10 in both the libraries were taken for differential analysis, RPM (Reads Per Million) based normalization was followed. RPM normalization was calculated as the actual number of miRNA reads/total number of clean reads × 1,000,000. The differential expression of miRNAs between EC and NEC libraries was calculated as fold change = log2 (miRNA normalized reads in NEC vs miRNA normalized reads in EC). Reads with fold changes ≥ 2 and *P*-values ≤ 0.05 were taken as differentially regulated miRNAs.

miRNA target prediction and annotation

Target prediction of the identified conserved and novel miRNAs was carried out using Plant Small RNA Target Analysis online Server (psRNATarget) tool (https://plantgrn.noble.org/psRNATarget/) with default settings as maximum expectation (ME) at 3, length for complementarity scoring (hsp size) at 19, target accessibility allowed maximum energy to unpair the target site (UPE) at 25, flanking length around the target site for target accessibility analysis at 17 bp in upstream and 13 bp in downstream and the range of central mismatch that leads to translational inhibition between 10 and 11 nt. It was done by searching the miRNA against preloaded coconut embryogenic calli transcriptome data (SRX 472157). Annotation of the target was done using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). miRNA targeted scaffolds from each sample (fasta format input) were subjected to BLASTn against NRDB (https://pubmlst.org/analysis/nrdb.shtml) and GO annotation was carried out using BLAST2GO (v3.3.5) program (https://www.blast2go.com/).

Validation of miRNAs and their target mRNAs using qRT-PCR

Expression profiling of selected conserved and novel miRNAs was verified using stem-loop qRT-PCR (Kramer 2011). Small RNA was extracted from plumular explants, EC and NEC using a mirVana miRNA isolation kit (Cat# AM1560, Ambion). The forward primers were designed based on the mature miRNA sequences and the reverse primers were universal, complementary to the regions of stem-loop RT primer (details of primers are provided in Suppl. Table S1a). Isolated miRNA was first reverse transcribed with the stem-loop RT primer using the TaqMan microRNA reverse transcription kit (Cat#4366596, Applied Biosystems) and qRT-PCR was performed using miScript SYBR Green PCR Kit (Cat# 218073, Qiagen). Each qRT-PCR reaction was conducted in a volume of 10 µl containing 5 µl of SYBR green, 5 pmol each of forward and reverse primers and 1 µl of cDNA (50 ng). The reactions were first incubated at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 64 °C for 30 s and 70 °C for 34 s. All the PCR reactions were performed using the Step One Real-Time PCR System (Applied Biosystems). All reactions were carried out using three biological replicates each with three technical replicates. Primer pair specificity was verified by automated gel electrophoresis (MultiNA, Shimadzu) and melt curve analysis. For the selection of the reference gene, the expression pattern of three non-coding small nuclear RNA viz., U1, U2 and U6 genes present in coconut transcriptome data were evaluated. Primers were designed based on the stem-loop RT procedure (Suppl. Table S1b). Expression stability of all these primers was checked in plumule, EC and NEC tissues by qRT-PCR. Among these primers, U6 snRNA had the lowest Ct value and constant expression in all the tissues and therefore was selected as an endogenous control. The relative changes in expression was calculated by a comparative CT method (ΔΔCT) using the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001). The initial plumular explant sample CT value was selected as the reference, with the expression level set as 1.0.

The expression profile of the selected target genes was validated by qRT-PCR. RNA was isolated from plumule, EC and NEC by using the Nucleospin plant RNA kit (Cat#740949.50, Macherey–Nagel). Primescript RT reagent kit (Cat# RR037B, Takara) was used for the RT reactions and the qRT-PCR was conducted on One-step Real-Time PCR System (Applied Biosystems) with Power SYBR™ Green PCR Master Mix (Cat# 4367659, Applied Biosystems). Each reaction included 1 µl of product from the diluted cDNA (50 ng), 1 µl of forward and reverse primer (2 µM) and 5 µl of SYBR Green (2X). The reactions conditions were 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 30 s and 72 °C for 30 s. The β-tubulin gene was used as the endogenous control (Rajesh et al. 2014b) and all reactions were performed in triplicate. The relative expression was calculated by a comparative CT method (ΔΔCT) using the formula $2^{-\Delta\Delta CT}$. The primers used for the target validation are listed in Suppl. Table S1c. The one-tailed Student's *t* test ($P_{0.05}$) was used to determine the significant difference of relative expression of miRNA and their targets between EC and NEC.

Cleavage site validation of miRNA targets

The cleavage site of two selected miRNA targets, auxin responsive factor (*ARF*) targeted by miR160 and

Scarecrow-like (*SCL*) targeted by miR171, were validated by 5′ RNA Ligase-Mediated Rapid Amplification of cDNA Ends (5′RLM–RACE) using Gene Racer kit (Cat# L1502-01, Invitrogen Life Technologies). Total RNA was isolated from coconut calli tissue and then ligated with an RNA adaptor using T4 RNA ligase. This product was reverse transcribed to cDNA using M-MLV reverse transcriptase and PCR amplification was carried out by using GeneRacer 5′ primers and gene-specific primers (Suppl. Table S1d). The PCR products were then cloned into TA cloning vector pTZ57R/T (Cat# K1214, Thermo Scientific) and sequenced (AgriGenome, Kochi).

Results

Callus morphology of embryogenic and non-embryogenic tissues

Excised shoot meristem showed initial callus induction after 25 days of incubation in dark. The initiated calli were chopped and subcultured to a medium incorporated with 2,4-D (45.24 µM), TDZ (4.5 µM) and spermine (50 µM). Embryogenic calli were formed after 55–60 days of incubation in this media. Non-embryogenic calli were also formed in some of the cultures in this media. Morphologically, embryogenic calli are translucent and smooth structures that appeared after 50 days which later transforms into globular and elongated structures. The non-embryogenic calli are sponge-like structures and do not undergo much change in morphology.

Increased accumulation of miRNAs in non-embryogenic calli

Sequencing of EC and NEC small RNA libraries on Illumina Hiseq 2000 platform resulted in 30,791,710 raw reads, comprising 30,682,755 high-quality reads and 10,8955 low-quality reads from EC, and 27,556,676 raw reads comprising 27,481,895 high-quality reads and 74,781 low-quality reads from NEC. After removing adaptor contamination and low-quality reads, a total of 27,990,086 high quality reads from EC and 27,137,845 from NEC were obtained (Table 1). The clean reads constituted 92,078,24 and 11,797,511 unique tags in EC and NEC respectively and were distributed between 11 to 41 nt in length. The length distributions of small RNA from unique reads are summarized in Fig. 1. The read length was not uniformly distributed in each library, but they showed a high abundance of 21–24 nt lengths. In both libraries, 24 nt length reads were most abundant with 44.93% and 50.23% in EC and NEC, respectively. The next largest fraction was that of the 21 nt sequences, which represented 19.16% in EC and 9.25% in the NEC. The small

Table 1 Summary of reads of the EC and NEC small RNA library from coconut

Type	NEC	EC
Total reads	27,556,676	30,791,710
Total HQ reads	27,481,895 (99.7%)	30,682,755 (99.6%)
Adapter contaminant	9058	9233
Smaller than 11 nt	3,20,133	2,668,058
Non ATGC	14,859	15,378
Clean reads	27,137,845	27,990,086
Total unique reads	92,07,824	11,797,511

RNA-sequence data of coconut was submitted at the NCBI with the SRA accession number PRJNA546491.

Further, by performing a BLASTN search against the Rfam database, we identified small RNA corresponding to rRNA, tRNA, snRNA and snoRNA from unique sequences in both EC and NEC small RNA libraries (Table 2). The total reads of length 18–22 bases were compared against all plant mature miRNAs listed in miRBase (miRBase 22.1). Only 10, 46, 997 (11.4%) and 6, 52, 157 (5.5%) of the unique sequences in NEC and EC, respectively, were found to be similar to known miRNAs with perfect matches to known miRNAs from Viridiplantae. The data suggests that there is a considerable increase in the miRNA population in non-embryogenic tissues compared to embryogenic calli.

Conserved and novel miRNAs of coconut

To identify conserved miRNAs in coconut, unique small RNAs from coconut were compared against mature plant miRNAs listed in miRBase 22.1. A total of 110 conserved miRNAs from 46 miRNA families, with perfect matches to known mature miRNAs, could be identified in both types of calli (Table 3 and Suppl. Table S2). Two miRNAs viz., cnu-miR4995 and cnu-miR5368 were present in only in EC library, while cnu-miR828 was present only in NEC library. These miRNAs varied in length from 18 to 22 nt and the most abundant miRNAs were 21 nt long. Length distributions of miRNAs are summarized in Fig. 2. Some of the miRNA families were represented by multiple members viz., miR166 (14 members); miR319 and miR159 (10 members each); miR168 (four members); miR396, miR167 and miR2118 (five members each); miR156 (seven members); miR157, miR171, miR408 and miR169 (three members each) and miR160, miR535, miR390 and miR399 (two members each). The rest of miRNAs were represented by only one member. miR166 family was found to have the highest read count in both libraries (852,935 in NEC library and 369,645 in EC library). The second abundant miRNA family was miR159, which had a read count of 101,830 in the NEC library and 136,954 in the EC library. Members

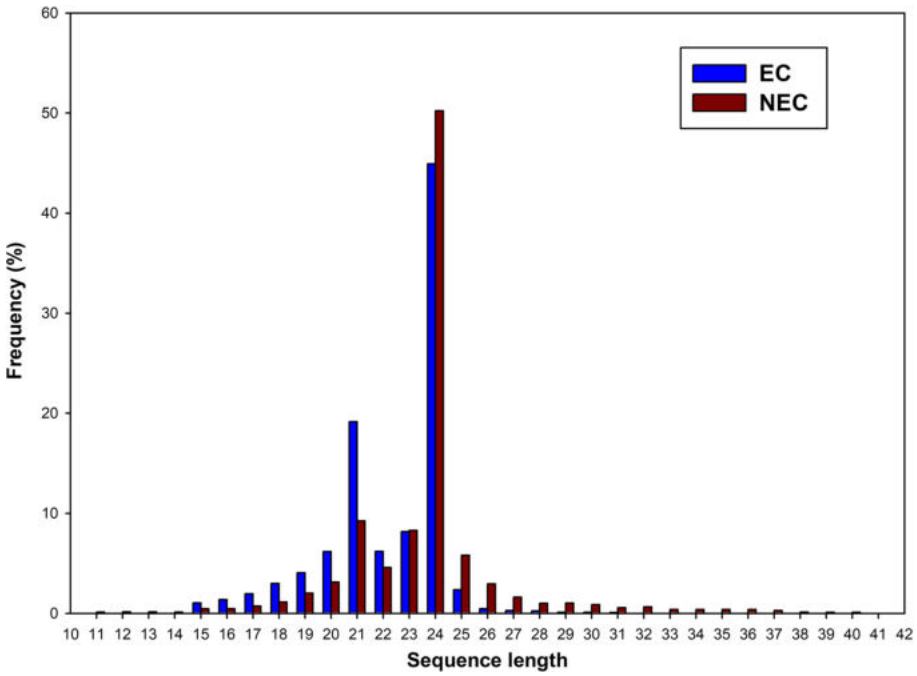

Fig. 1 Length distributions of small RNAs in coconut

Table 2 Distribution of different categories of small RNAs

Category	Read count in NEC	Read count in EC
Total small RNAs	9,207,824	11,797,511
miRNA	1,046,997 (11.4%)	652,157 (5.5%)
rRNA	71,754 (0.78%)	71,754 (0.61%)
tRNA	30,238 (0.33%)	30,238 (0.26%)
SnRNA	13,355 (0.15%)	13,355 (0.11%)
SnoRNA	54,889 (0.61%)	54,889 (0.47%)
Unannotated	8,003,696 (87.2%)	10,982,203 (93.1%)

within a family showed considerable difference in read count in both libraries (Suppl. Table S2).

In addition to conserved miRNAs, we also identified a total of 97 novel miRNAs using miRCat program, of which 48 were specific to EC, 21 were specific to NEC and 28 were common to both the libraries. These novel miRNAs were named as cnu-miRn_number and using a, b and c to differentiate miRNAs from the same precursor sequence and some of them are represented as 5p or 3p to distinguish miRNA from 5′ or 3′ arm of the same precursor (Suppl. Table S3). The length of the novel miRNAs varied from 18–21 nt with a majority of them being 21 nt length. These novel miRNAs exhibited a strong bias towards U at the 5′ terminal nucleotides (Fig. 3a), a property which was shown by conserved miRNAs too (Fig. 3b). Precursors of miRNAs were also identified and the length varied from 55 to 100 nt with an average length of 79 nt. Secondary stem-loop structures were predicted by mfold with default parameters (Suppl. Fig. S1). The minimum folding free energy (MFE) for the hairpin precursors for novel miRNAs ranged from − 15.1 to − 76.3 kcal/mol and an average of − 34.08 kcal/mol. Four of the novel miRNAs contain star sequences (cnu-miRn8, cnu-miRn13, cnu-miRn15 and cnu-miRn17) (Fig. 4).

miRNAs are differentially regulated during SEG induction in coconut

To identify differentially expressed miRNAs between EC and NEC, RPM (Reads Per Million) based normalization was followed and fold changes were calculated. Out of 46 miRNA families, 10 miRNA families were found to be differentially expressed between NEC and EC libraries with a log2 fold change > 2. Six conserved miRNA family members viz., miR156f, miR167c, miR169a, miR535a, miR319a and miR5179 were upregulated, while four conserved miRNA family members viz., miR171a, miR319b, miR160a, and miR166a were down-regulated in NEC/EC comparative analysis (Fig. 5a). Interestingly, multiple members of the same family displayed differential expression patterns (e.g. miR319a is upregulated, while miR319b is downregulated in NEC) (Fig. 5a).

Among the novel miRNAs, six were differentially expressed with more than two fold changes between EC and NEC libraries (Suppl. Table S4). Four miRNAs, that were present in both libraries (viz., cnu-miRn17, cnu-miRn7, cnu-miRn11, and cnu-miRn6), were upregulated in NEC library. Two of the miRNAs viz., cnu-miRn15, and cnu-miRn8 were down-regulated in NEC (Fig. 5b).

Table 3 Number of miRNA families identified in coconut SE

Sl. no.	miRNA family	miRNA name	Sequence (5′–3′)
1	MIR156	cnu-miR156a	UUGACAGAAGAUAGAGAGC
		cnu-miR156b	CUGACAGAAGAGAGUGAGCAC
		cnu-miR156c	UGACAGAAGAGAGUGAGCAC
		cnu-miR156d	CUGACAGAAGAGAGUGAGCA
		cnu-miR156e	UGACAGAAGAGAGUGAGCACU
		cnu-miR156f	UGACAGAAGAGAGUGAGCACA
		cnu-miR156g	UUGACAGAAGAGAGUGAGCAC
2	MIR157	cnu-miR157a	UGACAGAAGAUAGAGAGCAC
		cnu-miR157b	UUGACAGAAGAUAGAGAGCAC
		cnu-miR157c	GCUCUCUAUGCUUCUGUCAUC
3	MIR159	cnu-miR159a	UUUGGAUUGAAGGGAGCUCUA
		cnu-miR159b	UUUGGAUUGAAGGGAGCUCUU
		cnu-miR159c	CUUGGAUUGAAGGGAGCUCCU
		cnu-miR159d	CUUGGAUUGAAGGGAGCUCC
		cnu-miR159e	UUUGGUUUGAAGGGAGCUCUA
		cnu-miR159f	CUUGGAUUGAAGGGAGCUCU
		cnu-miR159g	UUUGGAUUGAAGGGAGCUCCU
		cnu-miR159h	UUUGGAUUGAAGGGAGCUCUG
		cnu-miR159i	UUUGGAUUGAAGGGAGCUCUC
		cnu-miR159j	CUUGGAUUGAAGGGAGCUCUA
4	MIR160	cnu-miR160a	UGCCUGGCUCCCUGUAUGCCA
		cnu-miR160b	GCGUGCAAGGAGCCAAGCAUG
5	MIR162	cn-miR162	UCGAUAAACCUCUGCAUCCGG
6	MIR164	cn-miR164	UGGAGAAGCAGGGCACGUGCA
7	MIR165	cnu-miR165	UCGGACCAGGCUUCAUCCCCC
8	MIR166	cnu-miR166a	UCGGACCAGGCUUCAUUCCUC
		cnu-miR166b	UCGAACCAGGCUUCAUUCCCC
		cnu-miR166c	UCGGACCAGGCUUCAUUCCCC
		cnu-miR166d	UCGGACCAGGCUUCAUUCCCG
		cnu-miR166e	GGACCAGGCUUCAUUCCCC
		cnu-miR166f	UCUCGGACCAGGCUUCAUUC
		cnu-miR166g	UCGGACCAGGCUUCAUUCC
		cnu-miR166h	CUCGGACCAGGCUUCAUUCCC
		cnu-miR166i	UUGGACCAGGCUUCAUUCCCC
		cnu-miR166j	UCGGACCAGGCUUCAUUCCC
		cnu-miR166k	UCUCGGACCAGGCUUCAUUCC
		cnu-miR166l	GGAAUGUUGUCUGGCUCGAGG
		cnu-miR166m	CGGACCAGGCUUCAUUCCCC
		cnu-miR166n	UCGGACCAGGCUUCAUUCCCU
9	MIR167	cnu-miR167a	UGAAGCUGCCAGCAUGAUCU
		cnu-miR167b	UGAAGCUGCCAGCAUGAUCUA
		cnu-miR167c	UGAAGCUGCCAGCAUGAUCUG
		cnu-miR167d	UGAAGCUGCCAGCAUGAUCUC
		cnu-miR167e	UGAAGCUGCCAGCAUGAUCUU
10	MIR168	cnu-miR168a	UCGCUUGGUGCAGGUCGGGA
		cnu-miR168b	CCCGCCUUGCAUCAACUGAAU
		cnu-miR168c	UCGCUUGGUGCAGGUCGGGAC
		cnu-miR168d	UCGCUUGGUGCAGGUCGGGAA
11	MIR169	cnu-miR169a	CAGCCAAGGAUGACUUGCCGA
		cnu-miR169b	CAGCCAAGGAUGACUUGCCGG
		cnu-miR169c	CAGCCAAGGAUGACUUGCC

Table 3 (continued)

Sl. no.	miRNA family	miRNA name	Sequence (5′–3′)
12	MIR171	cnu-miR171a	UGAUUGAGCCGUGCCAAUAUC
		cnu-miR171b	UUGAGCCGCGUCAAUAUCUCC
		cnu-miR171c	UUGAGCCGCGCCAAUAUCACU
13	MIR172	cnu-miR172	AGAAUCUUGAUGAUGCUGCAU
14	MIR319	cnu-miR319a	AGAGCUUCCUUCAGUCCACUC
		cnu-miR319b	UUGGACUGAAGGGAGCUCC
		cnu-miR319c	AUUGGACUGAAGGGAGCUCC
		cnu-miR319d	UUGGACUGAAGGGAGCUCCC
		cnu-miR319e	UUUGGACUGAAGGGAGCUCCU
		cnu-miR319f	UUGGACUGAAGGGAGCUCCU
		cnu-miR319g	UUGGACUGAAGGGAGCUCCCA
		cnu-miR319h	UUGGACUGAAGGGAGCUCCCU
		cnu-miR319i	CUUGGACUGAAGGGAGCUCC
		cnu-miR319j	CUUGGACUGAAGGGAGCUCCC
15	MIR390	cnu-miR390a	AAGCUCAGGAGGGAUAGCGCC
		cnu-miR390b	AGCUCAGGAGGGAUAGCGCC
16	MIR393	cnu-miR393	UCCAAAGGGAUCGCAUUGAUC
17	MIR394	cnu-miR394	UUGGCAUUCUGUCCACCUCC
18	MIR395	cnu-miR395	CUGAAGUGUUUGGGGGAACUC
19	MIR396	cnu-miR396a	UUCCACAGCUUUCUUGAACUG
		cnu-miR396b	UUCCACAGCUUUCUUGAACU
		cnu-miR396c	UUCCACAGCUUUCUUGAACUU
		cnu-miR396d	UCCACAGGCUUUCUUGAACUG
		cnu-miR396e	GUUCAAUAAAGCUGUGGGAAA
20	MIR397	cnu-miR397	UCAUUGAGUGCAGCGUUGAUG
21	MIR398	cnu-miR398	UGUGUUCUCAGGUCGCCCCUG
22	MIR399	cnu-miR399a	UGCCAAAGGAGAAUUGCCCUG
		cnu-miR399b	UGCCAAAGGAGAGUUGCCCUG
23	MIR408	cnu-miR408a	UGCACUGCCUCUUCCCUGGC
		cnu-miR408b	UGCACUGCCUCUUCCCUGGCU
		cnu-miR408c	AUGCACUGCCUCUUCCCUGGC
24	MIR444	cnu-miR444	UGCAGUUGCUGCCUCAAGCUU
25	MIR477	cnu-miR477	ACUCUCCCUCAAGGGCUUCUC
26	MIR479	cnu-miR479	UGAGCCGAACCAAUAUCACUC
27	MIR528	cnu-miR528	UGGAAGGGGCAUGCAGAGGAG
28	MIR529	cnu-miR529	AGAAGAGAGAGAGUACAGCCU
29	MIR535	cnu-miR535a	GUGCUUUCUCCCGUUGUCACU
		cnu-miR535b	UGACAACGAGAGAGAGCACGC
30	MIR536	cnu-miR536	UCGUGCCACGCUGUGUGCGUC
31	MIR827	cnu-miR827	UUAGAUGACCAUCAGCAAAC
32	MIR828	cnu-miR828	UCUUGCUCAAAUGAGUAUUCCA
33	MIR894	cnu-miR894	CGUUUCACGUCGGGUUCACC
34	MIR1432	cnu-miR1432	UCAGGAGAGAUGACACCGAC
35	MIR2118	cnu-miR2118a	UUCUCGAUGCCUCCCAUUCCUA
		cnu-miR2118b	UUCCCGAUGCCUCCCAUUCCUA
		cnu-miR2118c	UUCCCGAUGCCUCCUAUUCCUA
		cnu-miR2118d	UUCCUGAUGCCUCCCAUUCCUA
		cnu-miR2118e	UUUCCGAUGCCUCCCAUUCCUA
36	MIR4995	cnu-miR4995	AGGCAGUGGCUUGGUUAAGGG
37	MIR5139	cnu-miR5139	AAACCUGGCUCUGAUACCA

Table 3 (continued)

Sl. no.	miRNA family	miRNA name	Sequence (5′–3′)
38	MIR5141	cnu-miR5141	AGACCCGACGCGACUGACAGA
39	MIR5179	cnu-miR5179	UUUUGCUCAAGACCGCGCAAC
40	MIR5368	cnu-miR5368	GGACAGUCUCAGGUAGACA
41	MIR5538	cnu-miR5538	ACUGAACUCAAUCACUUGCUGC
42	MIR6173	cnu-miR6173	AGCCGUAAACGAUGGAUACU
43	MIR6300	cnu-miR6300	GUCGUUGUAGUAUAGUGG
44	MIR6478	cnu-miR6478	CCGACCUUAGCUCAGUUGGU
45	MIR8175	cnu-miR8175	GAUCCCCGGCAACGGCGCCA
46	MIR11602	cnu-miR11602	UCUAACGGAACGCUAUUGGAUC

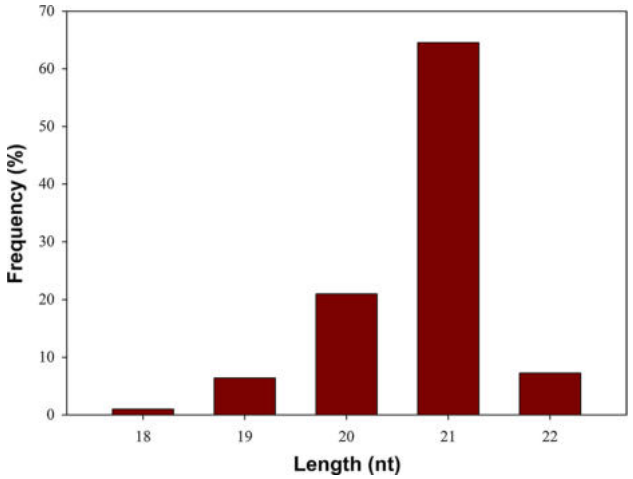

Fig. 2 The length distribution of conserved miRNAs

Expression dynamics of miRNAs and their targets during SEG

We have identified 520 putative targets for 110 conserved miRNA families and a total of 994 targets were identified for 97 novel miRNAs from the transcripts of coconut EC library (Suppl. Table S5 and Suppl. Table S6). BLASTx results of the identified targets revealed that most of the targets of conserved miRNAs are consistent with the miRNA targets of other plant species. The majority of the targets comprises transcription factors including squamosa promoter-binding protein—*SPL* family (targeted by miR156 and miR157), auxin response factor—*ARF* family (controlled by miR 160 and miR159), homeobox-leucine zipper protein (targeted by miR165 and miR166), *APETALA 2* (targeted by miR172), laccase—*LAC* family (regulated by miR397), nuclear transcription factor Y subunit A-7 (targeted by miR169), *WRKY* transcription factor (regulated by miR529 and miR390), *MYB* transcription factor (targeted by miR156, miR157, miR529 and miR408) etc. In addition, some of the targets were kinases and transporters. All the conserved miRNAs regulate multiple targets and also the same target is regulated by more than one miRNAs. Similarly, novel miRNAs also target a broad range of proteins including transcription factors, kinases, etc. Several genes with unknown functions were also identified as targets of both conserved and novel miRNAs (Suppl. Table S5 and Suppl. Table S6).

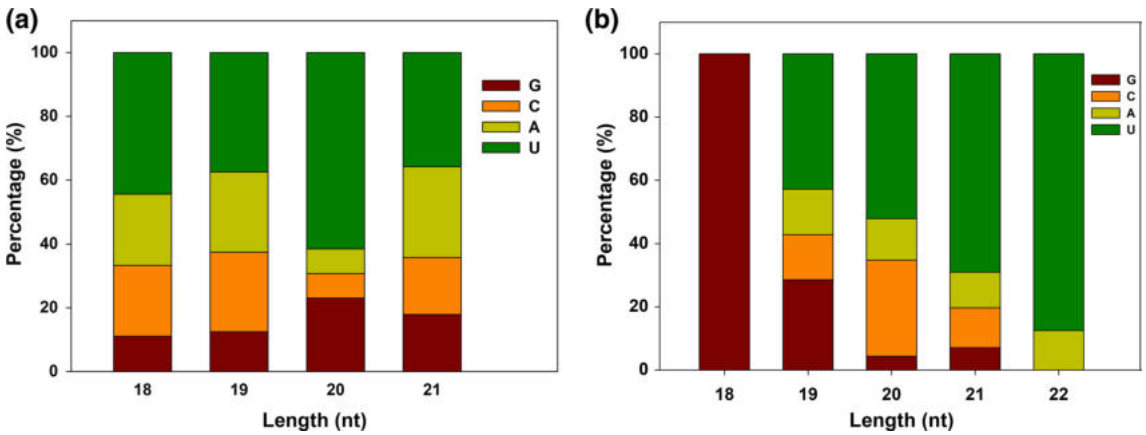

Fig. 3 First nucleotide bias of identified miRNAs in coconut. **a** Novel miRNAs. **b** Conserved miRNA candidates. Only a single miRNA, of length 18 nt was found among the conserved miRNAs

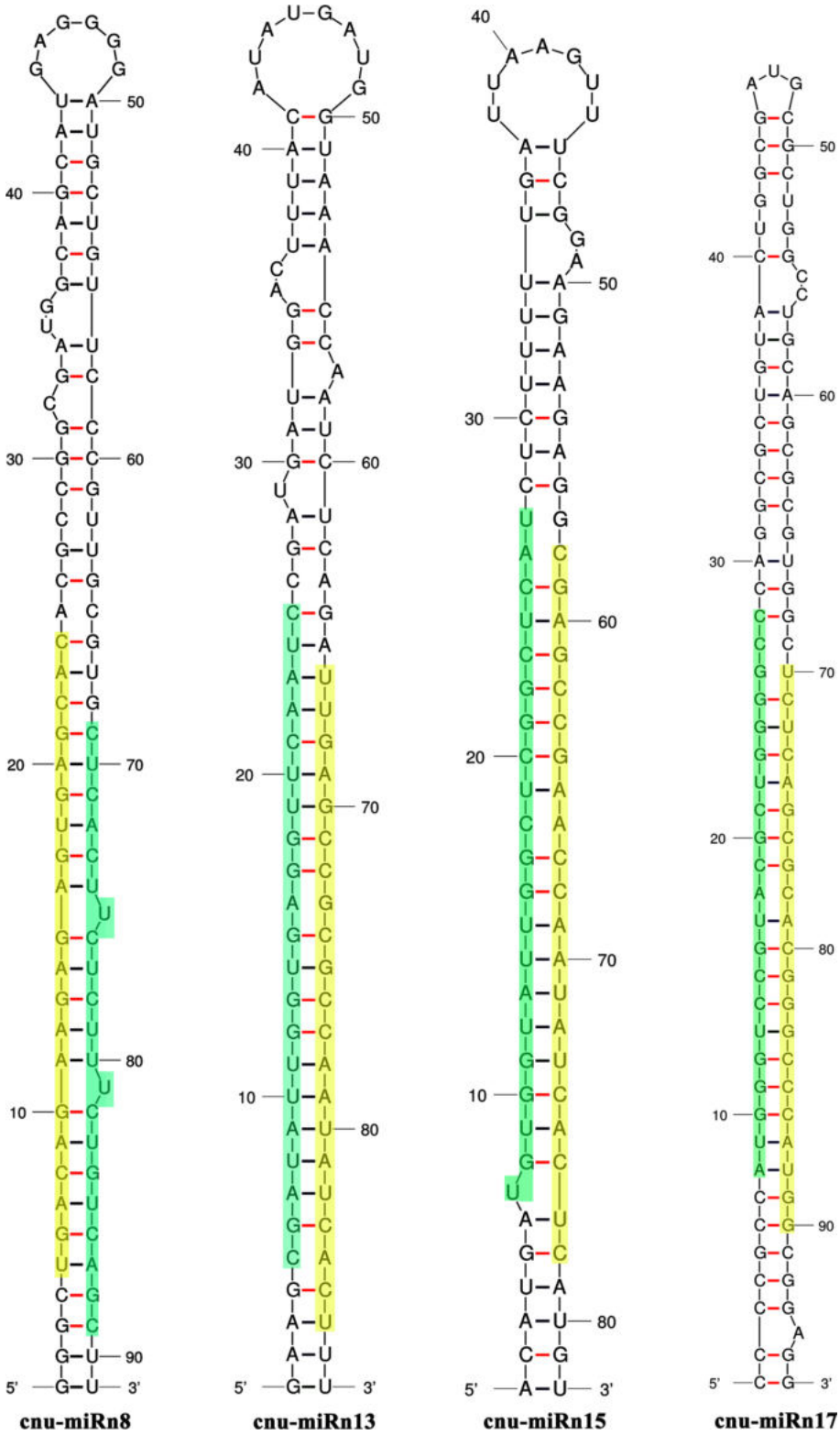

Fig. 4 Secondary stem loop structure of novel miRNAs (cnu-miRn8, cnu-miRn13, cnu-miRn15 and cnu-miRn17). Green and yellow color represents mature miRNA and miRNA* sequence, respectively

To analyze the biological functions, the predicted targets of EC and NEC libraries were subjected to gene annotation and ontology (GO) analysis, based on which the targets could be classified into three GO categories viz., molecular function, cellular component and biological process level (Fig. 6). More number of genes were involved in biological process such as primary metabolic process and organic substance metabolic process in the EC

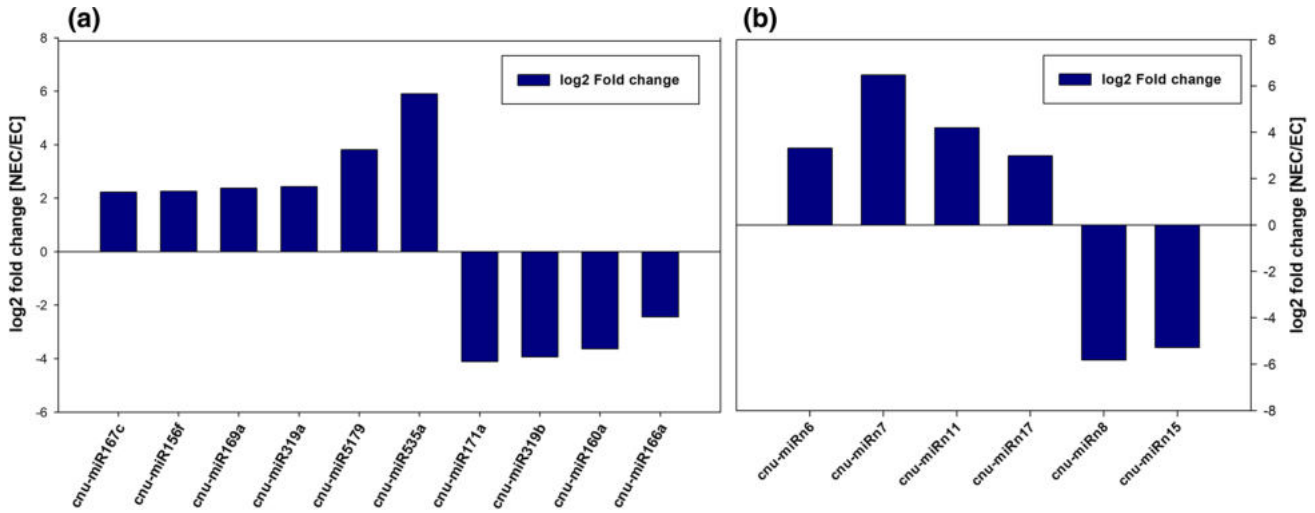

Fig. 5 Differentially expressed miRNAs obtained by high throughput sequencing. **a** Conserved miRNAs. **b** Novel miRNAs

library, whereas the NEC library was enriched with cellular metabolic process. In the case of molecular function, a large number of genes were grouped into heterocyclic compound binding and organic cyclic compound binding activity in both EC and NEC libraries.

The expression pattern of 16 miRNAs (10 conserved and six novel miRNAs) and 30 of their putative corresponding targets, which were randomly selected, were observed in three stages of SEG (initial plumular explant, EC and NEC) by stem-loop qRT-PCR. The expression level of each of the miRNAs and their targets was compared in EC and NEC tissues against the plumular explant, which was taken as the reference sample. Among 10 conserved miRNAs, five miRNAs (cnu-miR164, cnu-miR166a, cnu-miR167c, cnu-miR397, and cnu-miR535a) showed the highest expression in the NEC tissue. Two miRNAs viz., cnu-miR169a and cnu-miR171a displayed the highest expression in EC in comparison to plumule and NEC. Expression of cnu-miR156f, cnu-miR160a, and cnu-miR319b was abundant in the plumular explant. Among the miRNAs studied, cnu-miR397 showed the highest expression in NEC followed by EC. Out of the six novel miRNAs analyzed, four miRNAs (cnu-miRn7, cnu-miRn28, cnu-miRn9-5p, and cnu-miRn9a) were expressed at high levels in NEC in comparison to EC and plumular tissue. Only one miRNA, cnu-miRn39b displayed the highest expression in EC, whereas cnu-miRn22 showed abundant expression in plumule.

The expression level of a total of 30 targets, corresponding to both conserved and novel miRNAs, were also analyzed in three stages of SEG (initial plumular explant, EC and NEC) by qRT-PCR. We found that the expression levels of all the targets showed an expected inverse correlation with the expression level of their corresponding miRNA (Fig. 7).

Validation of miRNA directed target cleavage

The cleavage of mRNA takes place between the 10th and 11th nucleotides from the 5′ end of the complimentary miRNA. This is one of the distinctive features of miRNA-mediated mRNA cleavage as compared to other mRNA degradation method. So, this feature was used to predict the targets of miRNAs. In this study, two coconut miRNA target gene sequences were verified as targets of miRNAs through 5′RLM-RACE. Sequencing of the 5′ ends revealed that the unigene C536610 (*ARF*17) and C422012 (*SCL*22) was cleaved between 10 and 11th nucleotide complementary region to cnu-160a and cnu-171a, respectively (Fig. 8). The results confirmed the predicted cleavage sites of these miRNA targets.

Discussion

Deep sequencing of small RNAs has revealed that the miRNA expression is significantly modulated during SEG induction. Prior to this study, only 27 mature miRNAs from coconut had been reported (Sabana et al. 2018). In the current study, we identified 110 conserved miRNAs and 97 novel miRNAs via small RNA sequencing of embryogenic and non-embryogenic calli derived from the plumular explant. A larger fraction of the conserved miRNAs (64.5%) and 41% of the novel miRNAs started with 5′ uridine, which is a significant feature of miRNAs (Yao et al. 2007; Yang et al. 2013). All the novel miRNAs were identified using universal rules for miRNA annotation (Meyers et al. 2008) and all of them are coconut specific with lower abundance than that of conserved miRNAs. These results are consistent with the reports from other plants (Liu et al. 2014). In

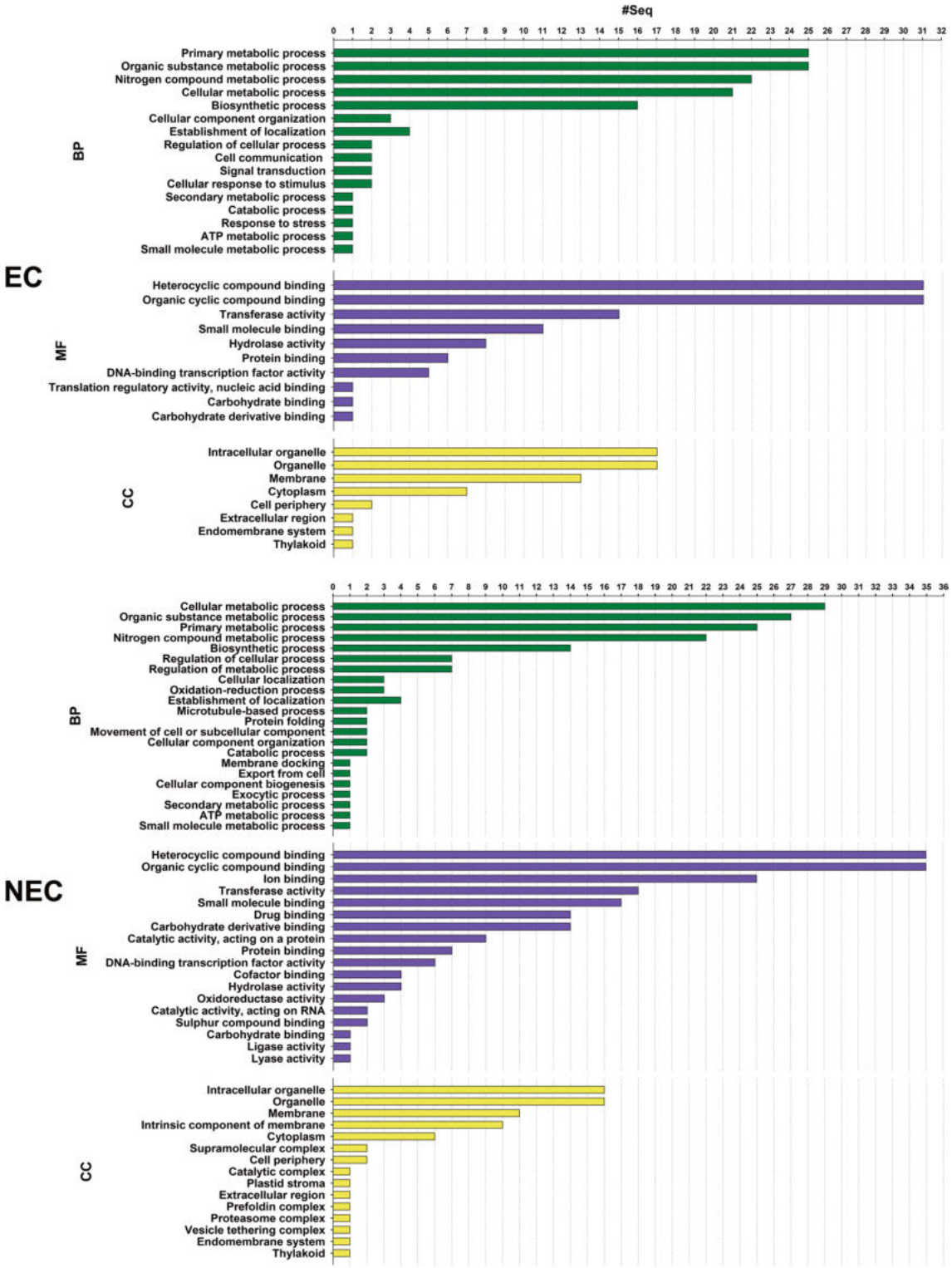

Fig. 6 Gene ontology classification of predicted targets genes for miRNAs identified in coconut. BP, Biological Process; MF, Molecular Function; CC, Cellular Component

our study, we observed that the percentage of miRNA of the total small RNA was 11.4% in the NEC sample compared to the percentage count in the EC sample which was only 5.5%. A lower count of miRNA was also reported in the embryogenic sample compared to control sample, (explant) in a similar study in cotton (Yang et al. 2013) and a similar

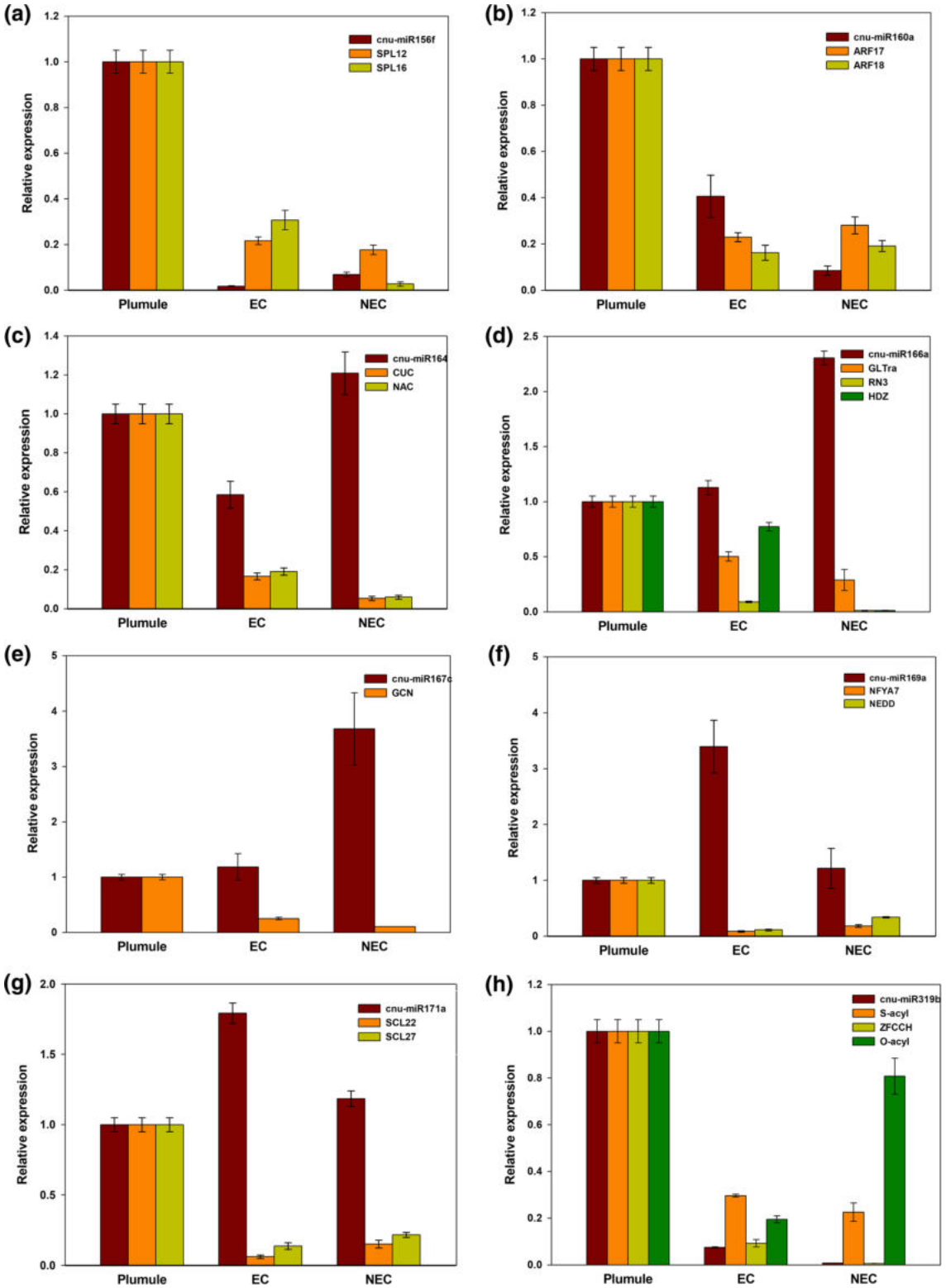

Fig. 7 qRT-PCR expression profiling of conserved and novel miRNAs and their corresponding targets in plumular explants, EC and NEC tissues of coconut. **a** cnu-miR156f. **b** cnu-miR160a. **c** cnu-miR164. **d** cnu-miR166a. **e** cnu-miR167c. **f** cnu-miR169a. **g** cnu-miR171a. **h** cnu-miR319b. **i** cnu-miR397. **j** cnu-miR535a. **k** cnu-miRn28. **l** cnu-miRn9-5p. **m** cnu-miRn7. **n** cnu-miRn22. **o** cnu-miRn9a. **p** cnu-miRn39b. Each bar represents the mean ± SE of triplicate experiment. Significant differences were calculated using one tailed student *t*-test with corrected *P*-value ≤ 0.05

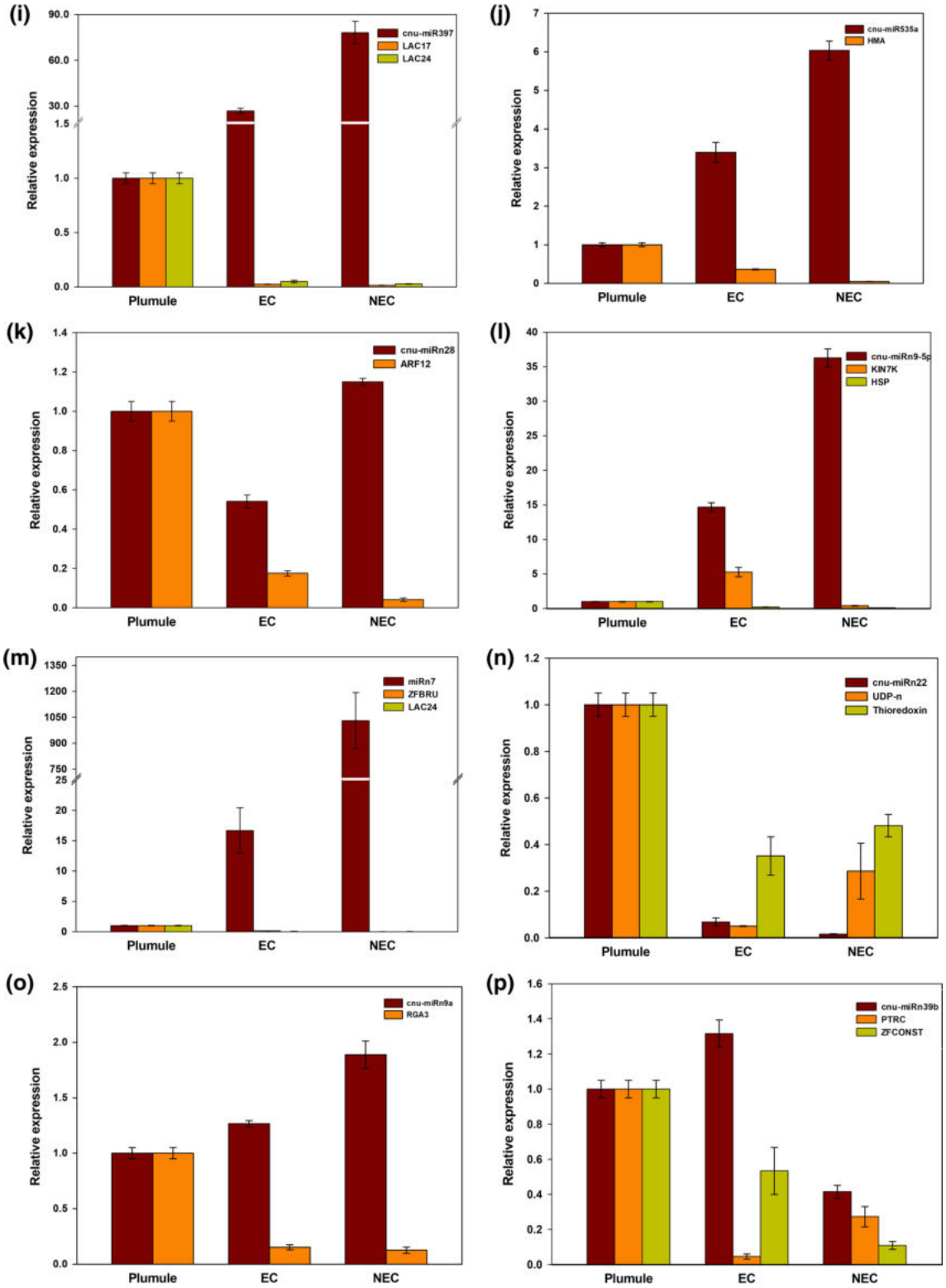

Fig. 7 (continued)

observation was also reported in the comparative analysis of miRNAs in NEC versus EC samples in citrus (Wu et al. 2015). Increased accumulation of miRNA in NEC suggests greater repression of target genes and on the other hand in EC relatively low accumulation of miRNAs suggests more target genes are de-repressed or activated. The gene ontology

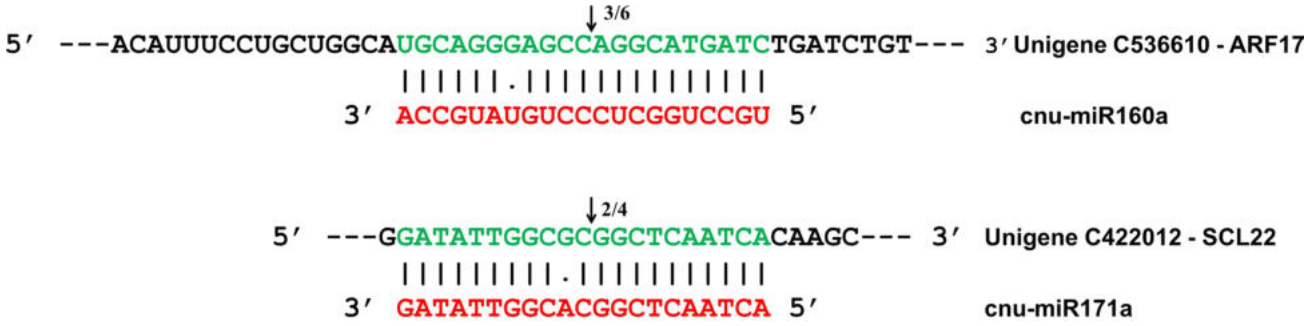

Fig. 8 Cleavage site mapping of coconut miRNA and its target gene. The arrows indicate the cleavage sites on miRNA aligned position on the corresponding mRNA in coconut. The numbers indicate the fraction of the cloned PCR product

of predicted targets of miRNAs upregulated in NEC reveals a more regulatory role i.e., there is a greater number of target genes with transcription factor activity, protein binding, and catalytic activity compared to the predicted targets of EC (Fig. 6). In NEC, the targets involved in primary metabolic processes are over-represented suggesting general repression of these processes relative to EC. The most abundant class of miRNAs were 21 nucleotides long and this is again consistent with the observation reported in date palm (Xin et al. 2015; Yaish et al. 2015).

Several miRNA families showed a differential expression pattern in EC and NEC samples following RPM-based normalization and fold change calculation. miRNA molecules with differential accumulation in EC and NEC samples were further validated by qRT-PCR. We analyzed the qRT-PCR based expression pattern of both conserved and novel miRNAs and their corresponding targets in three tissues including plumule (explant), EC and NEC and found the expected inverse relationship between the miRNA and its predicted targets barring two anomalies. miRNAs 166a and 169a showed inconsistent expression patterns between sequencing and qRT-PCR analysis. Such contradictions between the two analyses have been reported earlier in *Arabidopsis,* grapevine, and banana (Rajagopalan et al. 2006; Pantaleo et al. 2010; Zhu et al. 2019). The expression pattern of most of the miRNAs is consistent with the other previous reports but few of them showed deviation from previous reports. SEG related miRNAs like miRNA 156 and miRNA 167 were found to be up-regulated in NEC samples. miR156 is one of the largest miRNA families in plants and plays pivotal regulatory roles in plant SEG and has significant regulatory roles during juvenile to adult phase transitions (Cardon et al. 1997; Xie et al. 2006) and flowering (Schwab et al. 2005) by targeting different members of the *SPL* gene. Expressions of the miR156 were significantly higher in embryogenic callus than in non-embryogenic callus in citrus (Wu et al. 2015) and also higher in differentiated callus than in the undifferentiated tissues (Luo et al. 2006). In the present study, miR156f showed higher accumulation in NEC and the putative targets *SPL*12 and *SPL*16 were down-regulated in NEC. By qRT-PCR analysis, we observed that miR156f was highest in the plumule stage and lowest in EC (Fig. 7a). *SPL* genes encode transcription factors that regulate plant growth and development and their down-regulation in NEC might suppress the process of SEG. On the contrary in EC, miR156f is repressed and its targets *SPL*12 and *SPL*16 showed relatively higher expression levels (Fig. 7a). Another conserved miRNA that is upregulated in NEC is miR167c and its predicted target, General Control Non de-repressible 1 (GCN1), was down-regulated compared to EC (Suppl. Table S2; Fig. 7e). In eukaryotes, GCN1 regulates GCN2 kinase activity resulting in phosphorylation of translation initiation factor eIF2α during a repertoire of stresses in *Arabidopsis* (Wang et al. 2017). During stress, the phosphorylation of eIF2α inhibits global protein synthesis. miR167 was previously reported to be highly expressed in later stages of SEG in larch (Zhang et al. 2012) and longan (Lin and Lai 2013). The increased accumulation of miRNAs, the upregulation of miR156f and miR167c in non-embryogenic callus suggest that there is an active mechanism in place to repress translation in general in NEC. Another miRNA with significantly high expression in NEC is miR535a validated by qRT-PCR. The target of miR535a, cadmium/zinc transporting ATPase HMA2 like is down-regulated in NEC (Fig. 7j) with the highest expression in plumule followed by EC. This miRNA is not previously implicated in the process of SEG (Fig. 7j). In EC, conserved miRNAs 160a, 171a and 319b are up-regulated. This is consistent with earlier reports in *Larix leptolepis,* Valencia sweet orange and *Lilium* (Zhang et al. 2010, 2017; Wu et al. 2011). The expression levels of cnu-miRNAs 160a and 319b are highest in the plumule and lowest in NEC. The targets of cnu-miRNA 160a, auxin responsive factors, *ARF* 17 and 18 showed lower expression levels in EC and NEC compared to plumule (Fig. 7b). In *Arabidopsis*, miR160a is expressed highly in advanced stages of SEG induction and regulates embryonic development

by targeting *ARF*16 and *ARF*17 (Liu et al. 2010) while in longan it is highly expressed during heart- and torpedo-shaped embryonic stages (Lin and Lai 2013). The putative targets of cnu-miR171a, scarecrow-like proteins (*SCL*) 21 and 27 are expressed at low levels in EC compared to NEC (Fig. 7g). A similar observation was made in citrus, where miR171c is highly expressed in EC and its cognate *SCL* targets are expressed at low levels in EC (Wu et al. 2015). *SCL*s are promoters of gibberellin signaling by regulating the GA signaling repressor DELLA in *Arabidopsis* (Zhang et al. 2011). It is hypothesized that the low levels of SCL in EC inhibit GA signaling and promote SEG. The negative regulation of GA metabolism through over-expression of the *AGAMOUS-like15* (*AGL-15*) gene is found to promote SEG in *Arabidopsis* (Zheng et al. 2013). These findings suggest that SEG capability at least in some plants is negatively related to GA metabolism. A recent study of *Arabidopsis* miRNA responses during a wide range of abiotic stress identified miR319b as a multi-stress responsive miRNA with higher expression levels during metal stress and lower levels during drought, heat, and salinity (Barciszewska-Pacak et al. 2015). miR319b is expressed highest in plumule and lowest in NEC. The putative target of miR319b, diacylglycerol *O*-acyltransferase 1 like, is expressed at high levels in NEC relative to EC whereas the alternate target zinc finger CCCH domain-containing protein-like is undetectable in NEC and down-regulated in EC compared to plumule. Stress and hormones are regarded as major stimuli for SEG induction and relatively high levels of 319b in EC compared to NEC is suggestive of its role in SEG competence (Zavattieri et al. 2010). miR319b is also one of the miRNAs identified as highly expressed in early and advanced stages of *Arabidopsis* SEG (Szyrajew et al. 2017).

Of the 97 novel miRNAs identified, 10 showed a differential expression pattern during SEG (Suppl. Table S4). Among these cnu-miRn7 present in both EC and NEC showed very high-level expression in NEC (more than 1000 fold change), which was higher than that of all conserved miRNAs. Zinc finger protein BRUTUS-like and laccase 24-like are the putative targets of cnu-miRn7 and their transcripts were barely detectable in both EC and NEC (Fig. 7m). In multiple cases, the sequence of mature miRNA derived from the same pre-miRNA differed in EC and NEC (Suppl. Table S4). Six novel miRNAs were up-regulated and four down-regulated in the NEC library. Of these cnu-miRn28, cnu-miRn9-5p, cnu-miRn9a, cnu-miRn7 were validated as up-regulated and cnu-miRn22 and cnu-miRn39b as down-regulated in NEC by qRT-PCR. The putative targets of all these novel miRNAs tested also showed an inverse relationship in expression levels with that of the respective miRNA (Fig. 7k–p). cnu-miRn28 is expressed at low levels in EC and high levels in NEC relative to plumule. The predicted target of cnu-miRn28 is auxin response factor 12 (*ARF*12) and it is highly down-regulated in NEC indicating a possible role for ARF12 in SEG induction in coconut.

In summary, genetic reprogramming of the cells leading to the induction of SEG involves dynamic changes in the expression level of the regulatory molecules miRNAs and their targets mostly, transcription factors in coconut. Our data suggest that there is a higher accumulation of miRNA in NEC and therefore there may be general repression of translation. Lower miRNA levels in EC suggest, de-repression of transcription factors. As reported in similar studies earlier, a few conserved miRNAs like miR156f, miR167c, miR160a, miR171a, and miR319b likely play important role in determining SEG capability in coconut. Apart from this, our data indicate that some novel miRNAs also have key roles in this transition. The upregulation or downregulation of a few conserved miRNAs along with some species-specific miRNAs during the process of SEG enables the fine-tuning of the expression of certain transcription factors and stress-responsive genes. It is important to note that, studies so far suggest that there is no universal pattern in the expression of miRNAs in plants during SEG and the pattern is highly species-specific. This study opens up the possibility of enhancing the SEG competence of coconut calli by manipulating the expression level of the miRNAs or its targets. We hope that the data presented in this study would form the foundation for deeper insights into the recalcitrant nature of coconut to SEG.

Author contribution statement The three authors contributed to the design of experiments, execution of the experiments, analysis of data and writing of the manuscript.

Acknowledgements This work was financially supported by Kerala State Council for Science, Technology and Environment (KSCSTE) No. 042/FSHP-LESS/2014/KSCSTE and Department of Biotechnology (Distributed Information Sub-Centre), Government of India.

Compliance with ethical standards

Conflict of interest Authors declare that there are no competing interests.

References

Alejandri-Ramírez ND, Chavez-Hernandez EC, Contreras-Guerra JL, Reyes JL, Dinkova TD (2018) Small RNA differential expression and regulation in Tuxpeno maize embryogenic callus induction and establishment. Plant Physiol Biochem 122:78–89

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Arunachalam V, Rajesh MK (2008) Breeding of coconut palm (*Cocos nucifera* L.). CAB Rev 3(053):1–12

Bandupriya HD, Gibbings JG, Dunwell JM (2013) Isolation and characterization of an AINTEGUMENTA-like gene in different coconut (*Cocos nucifera* L.) varieties from Sri Lanka. Tree Genet Genomes 9(3):813–827

Barciszewska-Pacak M, Milanowska K, Knop K, Bielewicz D, Nuc P, Plewka P, Pacak AM, Vazquez F, Karlowski W, Jarmolowski A, Szweykowska-Kulinska Z (2015) *Arabidopsis* microRNA expression regulation in a wide range of abiotic stress responses. Front Plant Sci 6:410

Bhavyashree U, Fayas TP, Muralikrishna KS, Sajini KK, Rajesh MK, Karun A (2016) A comparative study of three different methods of shoot meristem excision for induction of *Embryogenic calli* in coconut. Res Biotech. https://doi.org/10.19071/rib.2016.v7.2953

Cardon GH, Höhmann S, Nettesheim K, Saedler H, Huijser P (1997) Functional analysis of the *Arabidopsis thaliana* SBP-box gene *SPL3*: a novel gene involved in the floral transition. Plant J 12(2):367–377

Chávez-Hernández EC, Alejandri-Ramírez ND, Juárez-González VT, Dinkova TD (2015) Maize miRNA and target regulation in response to hormone depletion and light exposure during somatic embryogenesis. Front Plant Sci 6:555

Che P, Love TM, Frame BR, Wang K, Carriquiry AL, Howell SH (2006) Gene expression patterns during somatic embryo development and germination in maize Hi II callus cultures. Plant Mol Biol 62(1–2):1–14

Chu Z, Chen J, Xu H, Dong Z, Chen F, Cui D (2016) Identification and comparative analysis of microRNA in wheat (Triticum aestivum L.) callus derived from mature and immature embryos during in vitro culture. Front Plant Sci 7:1302

Dantu PK, Tomar PK (2010) Somatic embryogenesis. In: Tripathi G (ed) Cellular and biochemical science. IK International House Pvt Ltd, New Delhi, pp 892–908

Eeuwens CJ (1976) Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut (*Cocos nucifera*) and date (*Phoenix dactylifera*) palms cultured *in vitro*. Physiol Plant 36:23–28

Fernando SC, Vidhanaarachchi VRM, Weerakoon LK, Santha ES (2010) What makes clonal propagation of coconut difficult? Asia Pac J Mol Biol Biotechnol 18:163–165

Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ (2007) miRBase: tools for microRNA genomics. Nucl Acids Res 36:D154–D158

Guan Y, Li SG, Fan XF, Su ZH (2016) Application of somatic embryogenesis in woody plants. Front Plant Sci 7:938

Horstman A, Bemer M, Boutilier K (2017) A transcriptional view on somatic embryogenesis. Regeneration 4(4):201–216

Kramer MF (2011) Stem-loop RT-qPCR for miRNAs. Curr Protoc Mol Biol 95(1):10–15

Lin Y, Lai Z (2013) Comparative analysis reveals dynamic changes in miRNAs and their targets and expression during somatic embryogenesis in longan (*Dimocarpus longan* Lour.). PLoS ONE 8(4):e60337

Lin Y, Lin L, Lai R, Liu W, Chen Y, Zhang Z, XuHan X, Lai Z (2015) microRNA390-directed TAS3 cleavage leads to the production of tasiRNA-*ARF*3/4 during somatic embryogenesis in *Dimocarpus longan* Lour. Front Plant Sci 6:1119

Liu W, Yu W, Hou L, Wang X, Zheng F, Wang W, Liang D, Yang H, Jin Y, Xie X (2014) Analysis of miRNAs and their targets during adventitious shoot organogenesis of *Acacia crassicarpa*. PLoS ONE 9(4):e93438

Liu X, Huang J, Wang Y, Khanna K, Xie Z, Owen HA, Zhao D (2010) The role of floral organs in carpels, an *Arabidopsis* loss-of-function mutation in microRNA160a, in organogenesis and the mechanism regulating its expression. Plant J 62(3):416–428

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408

Long JM, Liu CY, Feng MQ, Liu Y, Wu XM, Guo WW (2018) miR156-*SPL* modules regulate induction of somatic embryogenesis in citrus callus. J Exp Bot 69(12):2979–2993

Luo YC, Zhou H, Li Y, Chen JY, Yang JH, Chen YQ, Qu LH (2006) Rice embryogenic calli express a unique set of miRNAs, suggesting regulatory roles of miRNAs in plant post-embryogenic development. FEBS Lett 580:5111–5116

Mehrpooyan F, Othman RY, Harikrishna JA (2012) Tissue and temporal expression of miR172 paralogs and the AP2-like target in oil palm (*Elaeis guineensis* Jacq.). Tree Genet Genomes 8(6):1331–1343

Méndez-Hernández HA, Ledezma-Rodríguez M, Avilez-Montalvo RN, Juárez-Gómez YL, Skeete A, Avilez-Montalvo J, De-la-Peña C, Loyola-Vargas VM (2019) Signaling overview of plant somatic embryogenesis. Front Plant Sci 10:77

Meyers BC, Axtell MJ, Bartel B, Bartel DP, Baulcombe D, Bowman JL, Cao X, Carrington JC, Chen X, Green PJ, Griffiths-Jones S (2008) Criteria for annotation of plant microRNAs. Plant Cell 20(12):3186–3190

Montero-Cortés M, Rodríguez-Paredes F, Burgeff C, Pérez-Nuñez T, Córdova I, Oropeza C, Verdeil JL, Sáenz L (2010a) Characterization of a cyclin-dependent kinase (*CDKA*) gene expressed during somatic embryogenesis of coconut palm. Plant Cell Tiss Organ Cult 102:251–258

Montero-Cortés M, Sáenz L, Córdova I, Quiroz A, Verdeil JL, Oropeza C (2010b) GA3 stimulates the formation and germination of somatic embryos and the expression of a *KNOTTED*-like homeobox gene *Cocos nucifera* L. Plant Cell Rep 29:1049–1059

Nguyen QT, Bandupriya HD, López-Villalobos A, Sisunandar S, Foale M, Adkins SW (2015) Tissue culture and associated biotechnological interventions for the improvement of coconut (*Cocos nucifera* L.): a review. Planta 242(5):1059–1076

Pantaleo V, Szittya G, Moxon S, Miozzi L, Moulton V, Dalmay T, Burgyan J (2010) Identification of grapevine microRNAs and their targets using high-throughput sequencing and degradome analysis. Plant J 62(6):960–976

Patel RK, Jain M (2012) NGS QC Toolkit: a toolkit for quality control of next generation sequencing data. PLoS ONE 7:e30619

Pérez-Núñez MT, Chan JL, Saenz L, González T, Verdeil JL, Oropeza C (2006) Improved somatic embryogenesis from *Cocos nucifera* (L.) plumule explants. Vitro Cell Dev Biol Plant 42(1):37–43

Pérez-Núñez MT, Souza R, Sáenz L, Chan JL, Zuniga-Aguilar JJ, Oropeza C (2009) Detection of a *SERK*-like gene in coconut and analysis of its expression during the formation of embryogenic callus and somatic embryos. Plant Cell Rep 28(1):11–19

Rajagopalan R, Vaucheret H, Trejo J, Bartel DP (2006) A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. Genes Dev 20(24):3407–3425

Rajesh MK, Fayas TP, Naganeeswaran S, Rachana KE, Bhavyashree U, Sajini KK, Karun A (2016) De novo assembly and characterization of global transcriptome of coconut palm (*Cocos nucifera* L.) embryogenic calli using Illumina paired-end sequencing. Protoplasma 253(3):913–928

Rajesh MK, Rachana KE, Fayas TP, Merin B, Kiran AG, Anitha K (2014a) Selection and validation of reference genes for quantitative gene expression studies by real-time PCR in coconut. In: Muralidharan K, Rajesh MK, Muralikrishna KS, Jesmi V, Jayasekhar S (eds) Book of abstracts of national seminar on sustainability of coconut, arecanut and cocoa farming-technological advances and way forward. CPCRI, Kasaragod, p 34

Rajesh MK, Sajini K, Karun A (2014b) Polyamine-induced somatic embryogenesis and plantlet regeneration *in vitro* from plumular

explants of dwarf cultivars of coconut (*Cocos nucifera*). Indian J Agric Sci 84(4):527–530

Sabana AA, Antony G, Rahul CU, Rajesh MK (2018) *In silico* identification of microRNAs and their targets associated with coconut embryogenic calli. Agric Gene 7:59–65

Samad AF, Sajad M, Nazaruddin N, Fauzi IA, Murad A, Zainal Z, Ismail I (2017) MicroRNA and transcription factor: key players in plant regulatory network. Front Plant Sci 8:565

Schwab R, Palatnik JF, Riester M, Schommer C, Schmid M, Weigel D (2005) Specific effects of microRNAs on the plant transcriptome. Dev Cell 8(4):517–527

Shen Y, Jiang Z, Lu S, Lin H, Gao S, Peng H, Yuan G, Liu L, Zhang Z, Zhao M, Rong T (2013) Combined small RNA and degradome sequencing reveals microRNA regulation during immature maize embryo dedifferentiation. Biochem Biophys Res Commun 441(2):425–430

Siddiqui ZH, Abbas ZK, Ansari MW, Khan MN (2019) The role of miRNA in somatic embryogenesis. Genomics 111(5):1026–1033

Szyrajew K, Bielewicz D, Dolata J, Wójcik AM, Nowak K, Szczygieł-Sommer A, Szweykowska-Kulinska Z, Jarmolowski A, Gaj MD (2017) MicroRNAs are intensively regulated during induction of somatic embryogenesis in *Arabidopsis*. Front Plant Sci 8:18

Tingting L, Jinhui C, Jisen S, Jin X (2011) Deep sequencing combined with microarray hybridization to identify novel and conserved microRNAs during somatic embryogenesis of hybrid yellow-poplar (*Liriodendron chinense* (Hemsl.) Sarg. x *L. tulipifera* Linn.). BMC Proc 5 (7):P74

Wang L, Li H, Zhao C, Li S, Kong L, Wu W, Kong W, Liu Y, Wei Y, Zhu JK, Zhang H (2017) The inhibition of protein translation mediated by AtGCN1 is essential for cold tolerance in *Arabidopsis thaliana*. Plant Cell Environ 40(1):56–68

Wu XM, Kou SJ, Liu YL, Fang YN, Xu Q, Guo WW (2015) Genome wide analysis of small RNAs in non-embryogenic and embryogenic tissues of citrus: microRNAand siRNAmediated transcript cleavage involved in somatic embryogenesis. Plant Biotechnol J 13(3):383–394

Wu XM, Liu MY, Ge XX, Xu Q, Guo WW (2011) Stage and tissue-specific modulation of ten conserved miRNAs and their targets during somatic embryogenesis of Valencia sweet orange. Planta 233(3):495–505

Xiao Y, Xu P, Fan H, Baudouin L, Xia W, Bocs S, Xu J, Li Q, Guo A, Zhou L, Li J (2017) The genome draft of coconut (*Cocos nucifera*). GigaScience 6(11):gix095

Xie K, Wu C, Xiong L (2006) Genomic organization, differential expression, and interaction of SQUAMOSA promoter-binding-like transcription factors and microRNA156 in rice. Plant Physiol 142(1):280–293

Xin C, Liu W, Lin Q, Zhang X, Cui P, Li F, Zhang G, Pan L, Al-Amer A, Mei H, Al-Mssallem IS (2015) Profiling microRNA expression during multi-staged date palm (*Phoenix dactylifera* L.) fruit development. Genomics 105(4):242–251

Yaish MW, Sunkar R, Zheng Y, Ji B, Al-Yahyai R, Farooq SA (2015) A genome-wide identification of the miRNAome in response to salinity stress in date palm (*Phoenix dactylifera* L.). Front Plant Sci 6:946

Yang X, Wang L, Yuan D, Lindsey K, Zhang X (2013) Small RNA and degradome sequencing reveal complex miRNA regulation during cotton somatic embryogenesis. J Exp Bot 64:1521–1536

Yang Y, Zhang J, Wang J, Ren Y, Zhu Y, Sun H (2020) Dynamic changes of miR166s at both the transcriptional and post-transcriptional levels during somatic embryogenesis in *Lilium*. Sci Hort 261:108928

Yao Y, Guo G, Ni Z, Sunkar R, Du J, Zhu JK, Sun Q (2007) Cloning and characterization of microRNAs from wheat (*Triticum aestivum* L.). Genome Biol 8(6):R96

Zavattieri MA, Frederico AM, Lima M, Sabino R, Arnholdt-Schmitt B (2010) Induction of somatic embryogenesis as an example of stress-related plant reactions. Electron J Biotechnol 13(1):12–13

Zhai L, Xu L, Wang Y, Huang D, Yu R, Limera C, Gong Y, Liu L (2014) Genome-wide identification of embryogenesis-associated microRNAs in radish (*Raphanus sativus* L.) by high-throughput sequencing. Plant Mol Biol Rep 32(4):900–915

Zhang J, Xue B, Gai M, Song S, Jia N, Sun H (2017) Small RNA and transcriptome sequencing reveal a potential miRNA-mediated interaction network that functions during somatic embryogenesis in *Lilium pumilum* DC. Fisch Front Plant Sci 8:566

Zhang J, Zhang S, Han S, Wu T, Li X, Li W, Qi L (2012) Genome-wide identification of microRNAs in larch and stage-specific modulation of 11 conserved microRNAs and their targets during somatic embryogenesis. Planta 236(2):647–657

Zhang S, Zhou J, Han S, Yang W, Li W, Wei H, Li X, Qi L (2010) Four abiotic stress-induced miRNA families differentially regulated in the embryogenic and non-embryogenic callus tissues of *Larix leptolepis*. Biochem Biophys Res Commun 398:355–360

Zhang ZL, Ogawa M, Fleet CM, Zentella R, Hu J, Heo JO, Lim J, Kamiya Y, Yamaguchi S, Sun TP (2011) Scarecrow-like 3 promotes gibberellin signaling by antagonizing master growth repressor DELLA in *Arabidopsis*. Proc Natl Acad Sci USA 108(5):2160–2165

Zhang QL, Su LY, Zhang ST, Xu XP, Chen XH, Li X, Jiang MQ, Huang SQ, Chen YK, Zhang ZH, Lai ZX (2020) Analyses of microRNA166 gene structure, expression, and function during the early stage of somatic embryogenesis in *Dimocarpus longan* Lour. Plant Physiol Biochem 147:205–214

Zheng Q, Zheng Y, Perry SE (2013) AGAMOUS-Like15 promotes somatic embryogenesis in *Arabidopsis* and soybean in part by the control of ethylene biosynthesis and response. Plant Physiol 161(4):2113–2127

Zhu H, Zhang Y, Tang R, Qu H, Duan X, Jiang Y (2019) Banana sRNAome and degradome identify microRNAs functioning in differential responses to temperature stress. BMC Genom 20(1):33