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Identification of novel micro RNAs and their targets in *Cocos nucifera*–A Bioinformatics approach

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ABSTRACT

MicroRNAs are endogenous, non-translated, small RNAs of ~21 nucleotides that are processed from stem-loop regions of long transcripts. MicroRNA is responsible for regulation of gene expression of diverse aspects of plant development at the post-transcriptional level. Coconut is one of the major perennial crop, that has wide commercial importance. The identification of novel miRNAs from non-sequenced genome of plants can be done by comparative genomics approach using expressed sequence tags (ESTs) following filtering criteria based on structural features.In an attempt to identify potential miRNAs from *Cocos nucifera*,1008 ESTs were employed for homology based search to reported viridiplantae miRNAs. The candidate miRNAs were used for secondary structure prediction that led to the identification of one novel miRNA from mir2673 family. The gene targets predicted miRNA shows crucial role in regulation of auxin signaling pathway, transcription factors, abiotic stress, retrotransposons etc. The outcomes of this study will considerably enhance the scope to understand the role of miRNAs in *C.nucifera*.

KEY WORDS: *COCOS NUCIFERA L.* ,EXPRESSED SEQUENCE TAGS, MICRORNA, MICRORNA TARGETS, RNA SECONDARY STRUCTURE.

INTRODUCTION

MicroRNAs are a set of endogenous non-coding RNAs of ~21 nucleotides in length, which has vital role in the transcriptional and post-transcriptional regulation of gene expression (Zhang *et al.*, 2006). These act as an important regulator in various processes of development, cell signaling and stress conditions (Millar *et al.*, 2005;Sunkar *et al.*, 2006; Khraiwesh *et al.*, 2012). The expression of single gene may be controlled by multiple miRNAs and single miRNA can have multiple gene targets (Dehury *et al.*, 2013).

The plant miRNA genes are present in both introns and exons which are transcribed to pre-miRNA(primary miRNA) transcripts and there exists capped structures as well polyA tail that are processed to form stem loop structures. The mature miRNAs are produced from these pre-miRNA transcripts by DCL1 (Dicer like-1) and endo-

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nuclease III-like enzyme,is exported via HASTY 5 to the cytosol. The target gene expression is regulated by RNA-induced silencing complex (RISC) containing mature miRNA incorporated into it (Bartel., 2004). In plants, the first miRNA was reported in *Arabidopsis thaliana* ,following which several thousands of novel miRNAs has been discovered in plants by computational and experimental techniques(Reinhart *et al.,* 2002; Griffiths-Jones and Sam., 2006; Zhang *et al.,* 2016; Akter *et al.,* 2014).

The miRNAs in plants represent a high degree of perfect or nearly perfect complementary to their targets(Rhoades *et al.,* 2002). This highly conservative character of plant miRNAs promises in screening of homolog sequences of miRNAs from nucleotide archives of different plant kingdoms using *in silico* techniques based on homology (Das *et al.,* 2010). Many conserved miRNAs have been screened in various model and non-model plants by using the genomic data and homology based computational techniques, as exemplified in the study which resulted in identification of 682 miRNAs in 155 diverse plant species (Sunkar *et al.,* 2008). Computational predictions of miRNAs has also been reported in many of the non-model crops such as garlic, ginger, cannabis and basil (Panda *et al*., 2014; Singh *et al*., 2014; Das *et al*., 2015 and Singh *et al*., 2016).

Cocos nucifera.L. (2n=2x=32), a member of Arecaceae family , is one of the commercially important palms. Being a perennial tree, it is cultivated widely across the world for its versatile utilities uses from food to cosmetics (Govaerts., 2003). Since the available genetic resources are less, comprehensive analysis on the available data in online repositories has to be exercised. At present, no miRNA has been predicted using the available ESTs for *C.nucifera*. Hence, in this study, an *in silico* approach for screening of potential miRNAs was performed using homolog search of Viridiplantae miRNAs (miRBase) against the expressed sequence tags. The elimination of coding sequences and use of non-coding ESTs for secondary structure of the precursor miRNA was predicted using MFOLD which led to the identification of novel miRNA. Finally, the predicted novel miRNA was used to find the target genes from *Arabidopsis thaliana* transcripts.

MATERIAL AND METHODS

The reported 8,496 miRNAs belonging to Viridiplantae were downloaded from miRBase (Released 21: June, 2014) and clustered by CD- HIT-EST, with threshold value of 100 (Li *et al.,* 2006). From the clustered sequences of 3777, non-redundant miRNAs were selected which in turn used as reference miRNAs for finding the homologs in *C. nucifera* to create a local nucleotide sequence database. Publically available, 1008 ESTs were downloaded from EST database, NCBI (*www.ncbi.nlm.nih.gov/dbEST/*) to represent query sequences for miRNA prediction.

The alignment tool NCBI Blast+ was used for conserved miRNA prediction of *Cocos nucifera* ESTs by making local database of miRNA after retrieving the representative sequences of miRNA (Cock *et al.,* 2015). The secondary structures of pre-miRNAs were performed through online version of MFOLD (Zuker and Michael., 2003). The putative target genes for the predicted miRNA of coconut were identified using plant small RNA psRNATarget Web Server (Dai *et al.,* 2011). The circos plot was drawn using online Circoletto tool(Darzentas *et al.,* 2010).

The EST sequences were taken to perform blastn search against locally setup Viridiplantae miRNA database downloaded from miRBase. The blast search was performed with following parameters: (a) e-value threshold<0.01 (b) mismatches=0-2. The obtained hits with less than 3 nucleotide mismatches and without gap were selected to extract the precursor sequences (pre-miRNA). The method of a sliding window of 100 nt in size from ~80nt upstream and ~80nt nucleotide downstream of the mature miRNA were set to pick the best miRNA precursors (Singh and Nagaraju., 2008).

The secondary structures were predicted using web based MFOLD tool with following criteria:

(a) linear RNA sequence
(b) folding temperature fixed at 37 ^{o} C
(c) ionic conditions of 1 M NaCl without divalent ions
(d) percent sub-optimality number of 5
(e) maximum interior/bulge loop size of 30
(f) energy dot plot was turned on and the other parameters were set as default.

After prediction of the secondary structure of the precursor sequence of potential miRNA homologs, the following criteria were setup to select potential miRNA as described by Zhang in 2005 (Zhang *et al.,* 2005): (a) A minimal length of the pre-miRNA: 60 nt. (b) The pre-miRNA must be into appropriate stem loop hairpin secondary structure. (c) The position of mature miRNA sequence should be in one arm of the hairpin structure. (d) The mature miRNA sequence and its opposite miRNA strand should not have more than 6 nt mismatches. (e) The total A+U % should be in the range of 30-70%. (f) should have higher minimal folding free energy index (MFEI) and negative minimal folding free energy (MFE) to distinguish the miRNA secondary from other small RNAs. (f) The MFEI and adjusted MFE(AMFE) was calculated using the following equations:

$$MFEI = [(AMFE/(G+C)\%]$$

$$AMFE = [MFE \div \text{length of precursor miRNA}] \times 100$$

Table 1: List of coding and non-coding ESTs

ID	Coding/ Non-coding	Coding potential score
573332413	coding	2.81173
573332205	noncoding	-1.34675
573332140	coding	0.197992
573332096	noncoding	-1.39801
573332024	noncoding	-0.82414
573332021	noncoding	-1.21493
573331999	noncoding	-1.05615
573329619	noncoding	-1.26066
573329529	noncoding	-1.23343
573329506	coding	0.746432
573329506	coding	0.746432
323150074	noncoding	-1.08997
323150034	noncoding	-1.39945

The perfect complementarity or near complementarity permits the identification of miRNA targets. The *C. nucifera* miRNA targets were identified through homolog search by subjecting mature miRNA sequence as query against *Arabidopsis thaliana* transcript library with removed miRNA gene set(ftp://ftp.arabidopsis.org/home/tair/Genes/TAIR10_genome_release/TAIR10_blastsets/TAIR10_cdna_20101214_updated) using psRNAtarget webserver. The following parameters were employed in prediction of miRNA targets :

(a) No.of target genes for each small RNA:10
(b) Maximum mismatch at complementary site:≤2 without any gaps
(c) Maximum exception of 2.0
(d) Target accessibility-allowed maximum energy to un-pair the target site (UPE): 25
(e) Range of central mismatch leading to translation inhibition: 9–11 nt
(f) Flanking length around the target accessibility analysis: 17 bp upstream and 13 bp in downstream length of complementarity score: 20

The entire workflow for the prediction of coconut miRNAs is illustrated in Fig.1

RESULTS AND DISCUSSION

The conserved microRNAs in plants among different species including monocot and dicot are involved in various biological activities (Yang *et al.*,2007). The novel miRNA in *C.nucifera* was predicted using homology based *in silico* approach. The known miRNA from mirBase and the available *C.nucifera* ESTs were performed BLAST to obtain a total of 13 homologs after elimination of repeated sequences. Thereafter, 13 ESTs were

Table 2: Details of the predicted miRNA

Length of EST sequence	1008
Length of precursor miRNA	99nt
Length of mature miRNA	19nt
Precursor miRNA coordinates	74-172
Mature miRNA coordinates	135-153
Family of miRNA	mir 2673
MFE	49.70 -Kcal/mol
MFEI	0.80
AMFE	50.2020 -Kcal/mol
Nucleotide mismatch	1
Number of each nucleotides in pre-miRNA sequences	A-14 T/U-23 G-36 C-26
(A+T/U)%	37.38%
(G+C)%	62.62 %
Precursor miRNA sequence	GGGUUUUUGGGUCUCGCUCCCUUCUU CUGCCCUUCGCCUCUCGGCGUAGGUCA GGUGAGGCGAAGACGAAGAGGAAGAG GAGGAGCCCUCGCCGUGUGG
Mature miRNA sequence	GAAGACGAAGAGGAAGAGG
Star miRNA sequence	CUUCCCGUCCUUCUUCCC

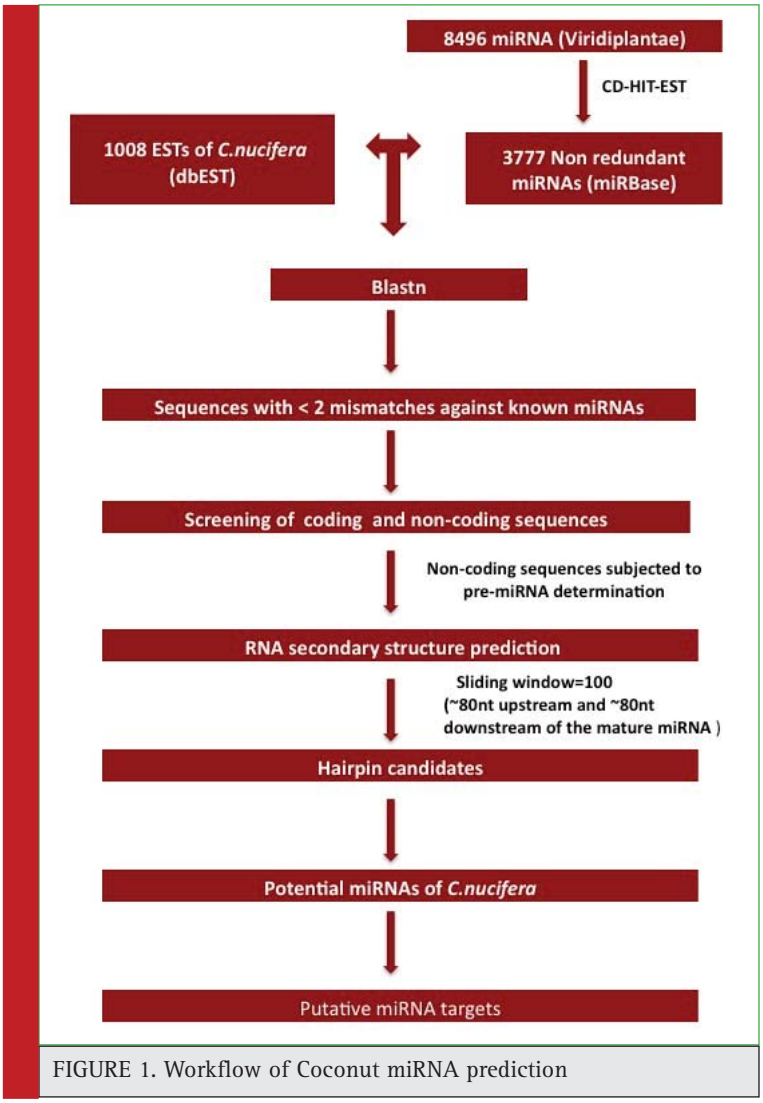

FIGURE 1. Workflow of Coconut miRNA prediction

screened for coding and non-coding sequences, which resulted in 9 non-coding miRNAs, out of which strong non-coding sequences were taken based on the basis of coding potential score (Ref. Table 1).

After circumspectly considering nucleotide BLAST and CPC tool results, we were able to identify the nine unique EST sequences that showed homology with the known the miRNAs in mirBase.These unique ESTs were subjected for secondary structure prediction and the results attained were investigated for appropriate precursor miRNA and its respective stem-loop structure. On applying the filtering criteria of secondary structure prediction, one novel miRNA was found (Fig. 2 and Fig. 3). The miRNA represented mir 2673 family with

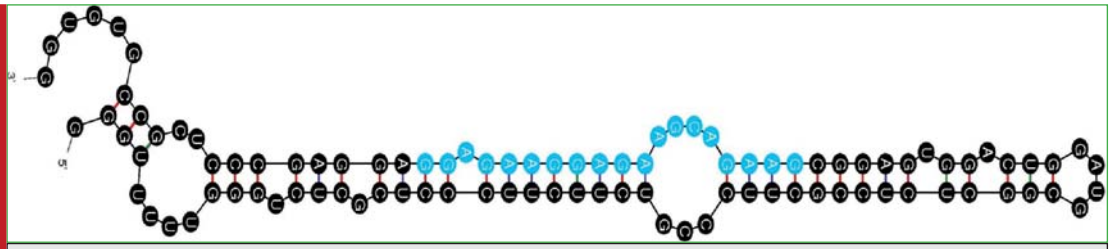

FIGURE 2. The stem loop structure of the predicted potential pre-miRNA miRNA in *C.nucifera* with its mature miRNA sequence (highlighted in blue)

Table 3: List of the potential targets of newly identified miRNA in coconut

Target Acc.	e-Value	Inhibition	Target Desc.	GO Biological Process	GO Molecular Function	GO Cellular Component
AT3G25450.1	0	Cleavage	Transposable element Copia-like retrotransposon family	Not Available	Not Available	Not Available
AT2G37650.1	0.5	Cleavage	GRAS family transcription factor |	regulation of transcription, DNA-templated, transcription, DNA-templated	transcription factor activity, sequence-specific DNA binding	nucleus
AT1G10940.2	1	Cleavage	Protein kinase superfamily, Encodes a plant protein kinase similar to the calcium/ calmodulin-dependent protein kinase subfamily and the SNF1 kinase subfamily (SnRK2) whose activity is activated by ionic (salt) and non-ionic (mannitol) osmotic stress.	intracellular signal transduction, primary root development, protein phosphorylation, response to abscisic acid, response to osmotic stress, response to salt stress	ATP binding, kinase activity, protein binding, protein kinase activity, protein serine/threonine kinase activity	cytosol, membrane, nucleus
AT1G10940.1	1	Cleavage	Protein kinase superfamily, Encodes a plant protein kinase similar to the calcium/ calmodulin-dependent protein kinase subfamily and the SNF1 kinase subfamily (SnRK2) whose activity is activated by ionic (salt) and non-ionic (mannitol) osmotic stress.	intracellular signal transduction, primary root development, protein phosphorylation, response to abscisic acid, response to osmotic stress, response to salt stress	ATP binding, kinase activity, protein binding, protein kinase activity, protein serine/threonine kinase activity	cytosol, membrane, nucleus
AT5G06710.2	1	Cleavage	homeobox from Arabidopsis thaliana / Homeobox-leucine zipper protein	regulation of transcription, DNA-templated, transcription, DNA-templated	sequence-specific DNA binding, transcription factor activity, sequence-specific DNA binding	nucleus
AT5G06710.1	1	Cleavage	homeobox from Arabidopsis thaliana / Homeobox-leucine zipper protein	regulation of transcription, DNA-templated, transcription, DNA-templated	sequence-specific DNA binding, transcription factor activity, sequence-specific DNA binding	nucleus
AT1G21590.1	1.5	Cleavage	Protein kinase protein with adenine nucleotide alpha hydrolases-like domain	Protein phosphorylation, response to stress	ATP binding, hydrolase activity, kinase activity, protein serine/ threonine kinase activity	nucleus

a precursor length of 99nt, which justifies the previous reports that miRNA precursors length varies from 60-400 nucleotides (Zhang *et al.,* 2006). If the calculated MFEI is greater than 0.67, then there is a possibility for precursor miRNA (Yin *et al.,* 2008). MFEI of the predicted coconut miRNA was found to be 0.80, which supports this criterion. The AMFE calculated for the coconut miRNA was 50.2 -kcalmol^{-1} which follows the reported average AMFE (45.93±9.43 -kcalmol^{-1}) of 513 precursor miRNA of plants (Zhang *et al.,* 2006) (Ref. Table 2).

So far, the predicted miRNA family is reported only in *Medicago truncatula* and therefore progress has to be made in understanding the role of this novel miRNA (Lelandais-Brière *et al.,* 2009). As proposed by miRBase, the new miRNA of coconut is named as 'cnu-miR2673' following the miRNA nomenclature procedure (Griffiths-Jones and Sam., 2006) .

The understanding of target gene function of miRNA is necessary step to know its regulation. The nature of conserved miRNAs in plants is an important factor that

>573332024
TTTATTTATTTTTTCTCCCAAGAAGAAGTGTGGTTATATATTCTGGAGGAAAGGGAGCCCGAAGCCCTCT
TCCGGGTTTTTGGGTCTCGCTCCCTTCTTCTGCCCTTCGCCTCTCGGCGTAGGTCAGGTGAGGCGAAGAC
GAAGAGGAAGAGGAGGAGCCCTCGCCGTGTGGAGCCCAGCCAGTTGATTTCTCAATCTTTCTTTTTTTTT
GGCTTGCTTCGGAAATTCTCGAATTCTGCTTTTATTTCTGGCGATTTCTTGGTCTCGATCGATCATAGGC
CATGGATGAGAGAATCCTGCCGGGTAGTTACTTCCAGTACATATGTTATCATGGTTACCTGGGGCAGCAT
GGCAGAGCTCCTCGATCCGTCGATCCACCGATCCATTTCTTGGAGTTTTCGATCGAAGGCCTGATCTCGA
TCATGGATCTTGGAGAGTTTCCGACTATAAATAGGAAAGGTCTCTTCTGGAGAAGAGTCATGATCGGAAC
AGGGGCTCTCTGCTCTCTGCTCTCTATTTTTCCTTTCTAGAAGCGACGGATCTGCTGACCTAAGTATGTC
TCCTTGGGATTGGTTCTTGACATCCCGACAGGAGATTTTCCGATCGGACTACCGTCGGAGGCTGTTGCAT
GGCCTGCACCATTTCGGTGAGGCCCCGAACCTGCTACATCAATAGGTTAAACTGCTCCGAATCCACCATT
GTTGCCGGCGAAGAAGGTGTGGTGCCAATGATGGTGTCGAAGCAAGCGGATTTGATTCAGATTTGGCGAC
AGAGGCAGTGGATCTTCGAGTGGTCGTCTTCTTTGGAGACATTTTAAGGTCGAGAGAGAGAATGGAGATG
ACCCTTAAAGGAGAAAGCAAGCAAGATCGATTGAGCACTCCTTTTAGAACAAGGGCTCAGGTGCCCCTCC
TTCTAGCGCCAATCCTATTGGTGCAGAATCTGGTTGACGCCAAATCAAGCTGGAGTTGGGGAAGAAGAAG
TCGCCGTTGGTAACCTACAAAACAAA

FIGURE 3. EST sequence containing pre-miRNA(yellow color higlighted) and mature miRNA(blue color highlighted)

allows the finding of the target genes based on their complementarity or near complementarity of miRNAs to their respective targets(Zhang *et al.,* 2006).We have found the five target genes for this one miRNA family based on our filtering criteria using the psRNAtarget tool using the available transcripts of *Arabidopsis thaliana*. All of the putative target genes seem to inhibit through cleavage mode by the identified miRNA and it has multiple targets as shown in Table 3 and Fig. 4.

The predicted targets involves two transcription factors namely Homeobox leucine zipper family and GRAS family of transcription factor. HD-ZIP (Homeobox-leu-

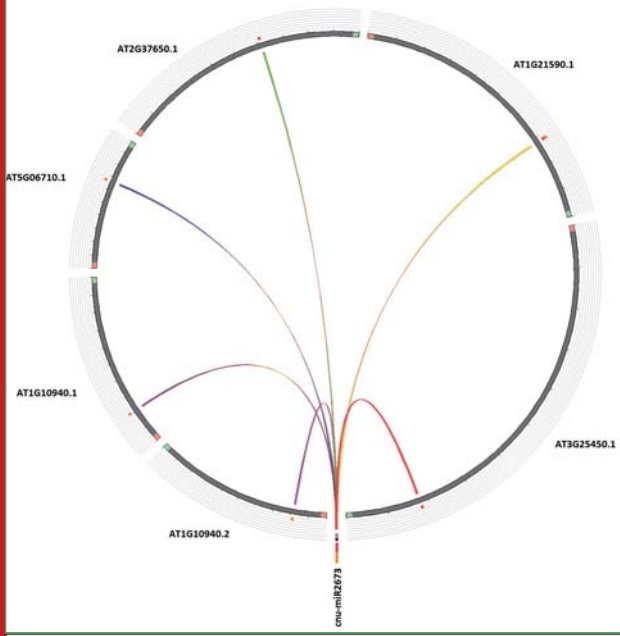

FIGURE 4. Circos plot showing Cnu-miR2673 and its target genes: The coloured ribbons indicate the genes targeted by the coconut microRNA. The labels represent the target genes flanked by green (start of the target sequence) and orange (end of target sequence) blocks.

cine zipper protein) has homeodomain (HD) and linear zipper motif present. This class of transcription factors is unique to plants and are involved in plant growth and development especially in response to abscisic acid, abiotic stress, shade avoidance and auxin signaling (Ariel *et al.,* 2007; Elhiti *et al.,* 2009). GRAS proteins act as transcription factors, a number of which have nuclear localization signal for localization of several other proteins (Tian *et al.,* 2004). It has important functions in GA (Gibberilic acid) and light signaling and regulation of root patterning (Hirsch *et al.,* 2009) .

We have found one of the target gene for Copia retrotransposons. These retrotransposons are flanked by long terminal repeats (LTRs) that certain promoter and downstream controls elements their internal domain usually contains the genes required for reverse transcriptase, integrase and gag (Wilhelm and Wilhelm.,2001). The gene that had Copia like transposons has blast match to gag pol protein from *Glycine max* and previous findings support the existence of SIRI, a copia/Ty1-like retrotransposon element encoding a retroviral envelope like protein assumed to be originated from tomato genome through horizontal gene transfer (Cheng *et al.,* 2009). Two of the target genes showed the exiatence of protein kinase out of which one encodes the calcium/calmodulin mediated kinase super subfamily and SNF1 kinase subfamily activated during ionic and non-ionic omotic stress. As exemplified, CDPKs (Calcium dependent protein kinases) play major roles in development for recycling transcription to hormone levels and are known to function in abiotic stress response and ABA signaling through phosphorylation activity(Ishida *et al.,* 2008; Mori *et al.,* 2006). On the other hand, SnRK(SNF kinases) are expressed in nucleus during seed development and germination in Arabidopsis. Any alteration in these protein induces gene expression change, both, in the up-regulation of ABA repressive genes and down-regulation of ABA inducible genes. The alteration of gene expression leads to loss of dormancy and growth defects during seed development (Nakashima *et al.,* 2009). The other kinase protein with adenine alpha hydrolases phosphorylates proteins fuctions in response to stress with probable interaction with serine/threonine to ATP.

As in plants, for every 10,000 EST sequences is expected to contain one miRNA (Zhang *et al.,* 2006). But, from our study, we propose that even 1000-2000 ESTs could be utilized to mine the conserved potential miRNAs across the non-model crops. The findings from this study indicate that coconut miRNA mir 2673 possibly targets both transcription factors and individual specific genes. Hence, the methodology adopted in this research will enumerate the understanding of regulatory miRNA in coconut in much rapid pace in near future.

CONCLUSION

The quest for potential miRNA is a highlighted research routine in the field of transcriptomics. However, over the recent years the miRNAs from non-model plants have been explored using ESTs. In this research, attempts has been made to screen miRNAs from 1008 ESTs reported in *C.nucifera* by employing, EST based homology search method, which resulted in one novel miRNA. Its putative function of gene targets were also catalogued. The impact of this study will be a starting point in understanding the miRNA biogenesis and its structure in *Cocos nucifera* and to explore repertoire of small RNAs that contribute to different biological mechanisms of the plant system. Since the genetic information is limited, the present *in silico* approach will help to improve the miRNAome in *C.nucifera* through further experimental validation.

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