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In silico identification of microRNAs and their targets associated with coconut embryogenic calli

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ABSTRACT

Coconut palms are propagated mainly through nuts, which does not meet the requirement of quality planting materials for large scale planting. *In vitro* propagation to enhance production of high yielding, disease-free planting material in coconut has remained a distant reality because of its *in vitro* recalcitrance. MicroRNAs (miRNAs) have been implicated in the regulation of a plethora of cellular, physiological and developmental processes which include developmental regulation, hormone response and adaptation to stresses. In this study, computational methods were utilized to identify conserved miRNA from transcriptome data of coconut embryogenic calli. A total of 117,790 unigenes from coconut embryogenic calli were compared against monocot mature miRNA sequences. A total of 27 mature miRNA sequences, belonging to 15 miRNA families, *viz.* miR156, miR164, miR166, miR167, miR169, miR171, miR172, miR394, miR397, miR408, miR444, miR535, miR827, miR1134 and miR2118, were identified. Many of these have well defined and crucial roles in developmental pathways and hormone signalling in other plant species. Each of the identified miRNA had its own predicted targets. This is the first *in silico* study describing miRNAs and their role in the regulation of *in vitro* embryogenesis in coconut. The results obtained in this study would provide a base for future studies to address molecular mechanisms that govern *in vitro* recalcitrance in coconut and the role of miRNAs in the process.

1. Introduction

MicroRNAs (miRNAs) are a class of endogenous non-coding single stranded small RNAs (Bartel, 2004). They are of ~20–22 nucleotides (nt) length in animals and ~20–24 nt in plants and are usually formed from stem-loop hairpin structures of ~80 nt called miRNA precursors (pre-miRNAs) (Lee et al., 2002). All miRNA precursors have a well-predicted stem loop hairpin structure (Krol et al., 2004). Many studies on different species have led to the identification of conserved and species specific miRNAs revealing the complexities of gene regulation since the discovery of first miRNA (*lin-4*) in *Caenorhabditis elegans* (Lee and Ambros, 2001).

miRNAs have been reported to cause post-transcriptional gene silencing in plants by inhibiting gene expression through complimentarily binding to mRNA (Lanet et al., 2009). miRNAs have been implicated to play significant roles in numerous physiological processes including growth, development, metabolism, behaviour and apoptosis through mRNA cleavage or translational repression (Carrington and Ambros, 2003). A single miRNA can target the mRNA of several genes or several miRNAs may be required to regulate a single mRNA, permitting miRNAs to simultaneously regulate multiple genes within a single physiological pathway (Webster et al., 2009). The miRNA-mediated repression of target genes have been shown to play a significant role in plant embryogenesis (Willmann et al., 2011; Wu et al., 2015) and possess vital roles which include regulation of leaf, stem and root development (Palatnik et al., 2003; Mallory et al., 2004; Guo et al., 2005).

Genetic suppression screens, gene cloning and high throughput sequencing techniques, combined with bioinformatics tools, are common methods utilized for identification of miRNAs. Gene cloning is a one of the conventional and accurate methods to detect new miRNAs. Effort in finding miRNAs which express at low levels, difficulty in cloning and degradation of RNA during sample separation are some of the major drawbacks of this method (Zhang et al., 2006). Recently, many computational programs, both web-based or stand alone, have been developed for successful identification/prediction of miRNAs and their targets (Ekimler and Sahin, 2014).

Coconut (*Cocos nucifera* L.) is one of the important palms grown

Abbreviations: ARF, Auxin Response Factors; BR, Brassinosteroids; CUC2, Cup-shaped cotyledon 2; MAPK, Mitogen-activated protein kinase; MFE, Mean Forecast Error; miRNA, micro RNA; NJ, Neighbor-joining; pre-miRNA, precursors micro RNA

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both as a homestead and plantation crop in countries and most island territories of tropical regions. Nearly every part of the coconut tree can be used in either making commercial products or meeting the food requirements of rural communities (Arunachalam and Rajesh, 2008, 2017). Improved disease resistant planting materials are rare and seed propagation does not yield adequate material to satisfy the rapidly growing demand. Therefore, alternative methods to overcome these bottlenecks need to be developed. *In vitro* propagation or micropropagation *via* somatic embryogenesis is seen as a suitable alternative due to its potential for mass propagation.

Somatic embryogenesis is one of asexual reproduction starting from isolated somatic cells wherein these cells under experimental conditions are induced to form a somatic embryo *in vitro*. This is remarkable phenomenon unique to plants only (Zimmerman, 1993). The process is feasible because plants possess cellular totipotency whereby individual somatic cells can regenerate into a whole plant (Reinert, 1959). In coconut, various tissues including shoot tips (Weerakoon, 2004), roots (Justin, 1978; Fulford et al., 1981), shoot apical meristem (Apavatjrut and Blake, 1977), endosperm (Kumar et al., 1985), leaves (Karunaratne et al., 1991), zygotic embryos (Karunaratne et al., 1991) and immature inflorescence (Branton and Blake, 1983; Verdeil et al., 1994) have been used for *in vitro* culture, but the success achieved has been limited. Plumular explants have shown better response in terms of callus formation and embryogenic capacity (Hornung, 1995; Chan et al., 1998; Rajesh et al., 2005, 2014). However, the highly recalcitrant nature of coconut tissue to *in vitro* conditions, has limited the success and somatic embryo turnover from various explants is poor (Fernando and Gamage, 2000).

Somatic embryogenesis involves different molecular events including differential gene expression and various signal transduction pathways for activating or repressing numerous genes sets (Chugh and Khurana, 2002). It is intensely associated with plant cell differentiation and embryo development and therefore, may be subjected to regulation by miRNA. Somatic embryogenesis related miRNAs have been studied in various plant species. Lin and Lai (2013) profiled novel and specific miRNA during longan somatic embryogenesis by large scale cloning and deep sequencing; a total of 24 miRNAs (20 conserved and four novel) were identified with possible roles in longan somatic embryogenesis. Conserved and novel miRNAs and their targets in non-embryogenic callus and embryogenic callus have also been identified during somatic embryogenesis process in 'Valencia' sweet orange (*Citrus sinensis*) and cotton (Wu et al., 2011, 2015; Yang et al., 2013). Similarly, miRNA expression has been also characterized during somatic embryogenesis in rice (Luo et al., 2006), poplar (Tingting et al., 2011), maize (Chávez-Hernández et al., 2015) and larch (Zhang et al., 2012). But there are no reports available on miRNA regulation during coconut somatic embryogenesis. Therefore, a study of miRNA's expressed during coconut somatic embryogenesis would allow not only an deeper understanding of the process, but it might lead to deciphering the basis of *in vitro* recalcitrance in coconut and provide leads for means to overcome it. With this background, the aim of this study was to predict miRNA's and their targets using callus transcriptome data of coconut embryogenic calli, generated in an Illumina HiSeq 2000 platform.

2. Materials and methods

2.1. Computational prediction of conserved miRNAs

A total of 117,790 unigenes, assembled from RNA-Seq data of coconut embryogenic calli transcriptome data of the West Coast Tall cultivar (SRX 472157) generated in an Illumina HiSeq 2000 platform (Rajesh et al., 2016), was utilized for *in silico* prediction of miRNA. Published monocot mature miRNA sequences were retrieved from miRBase database Release 21 (Kozomara and Griffiths-Jones, 2014). A stand-alone BLASTN (Altschul et al., 1990) search was performed to identify mature miRNA by setting the unigene sequences as query against non-redundant miRNA reference dataset. The parameters *viz.*, (i) e-value < 0.001, (ii) percent identity of 100 and (iii) a word match of 7, were selected. A custom PERL program was developed to extract sequence 100 nucleotides both upstream and downstream of the unigene sequences matching with known miRNAs. A sliding window program in BioPYTHON script was used to obtain probable pre-miRNA sequences. A sliding window of a given size sufficiently long to contain a pre-miRNA was considered, in which pre-miRNA hairpins were searched. The sliding window was shifted by 10 nt in each step, since plant precursor miRNA length have been reported to range between 55 and 930 nt with an average of ~146 nt (Thakur et al., 2011).

PRINSEQ tool (v0.20.4) (http://edwards.sdsu.edu/cgi-bin/prinseq/prinseq.cgi) was used to remove those sequences with GC content between 30%–60%, any duplicate sequences or those with a length < 80 nucleotides from the selected sequences and the remaining sequences were used for further analysis. The miPred tool (http://www.bioinf.seu.edu.cn/miRNA/) (Jiang et al., 2007) was utilized to identify if the miRNAs formed were authentic or possessed pseudo hairpin loops. The real hairpin loop pre-miRNAs with a high negative minimum folding free energy (MFE $\leq$ 40 kcal/mol) were selected for further analysis. BLASTX (Altschul et al., 1990) was done using the selected sequences to remove protein coding sequences. Rfam database (http://rfam.xfam.org/) search was also carried out to detect other small RNAs.

2.2. Secondary structure prediction

The hairpin loop structures of the selected pre-miRNAs were constructed using mfold software (http://unafold.rna.albany.edu/) (Zuker, 2003) with default parameters. The structures were tested for less than four mismatches in the base-pairing between the miRNA and the other arm of the hairpin (miRNA*).

2.3. Target prediction of selected miRNAs

Target prediction of the identified miRNA was carried out using psRNATarget tool (http://plantgrn.noble.org/psRNATarget/) (Dai and Zhao, 2011), with default settings, by searching the miRNA against submitted coconut embryogenic calli transcriptome data (SRX 472157) and Circos plot was constructed to visualize the interaction between miRNAs and their corresponding targets using online tool Circoletto (Darzentas, 2010).

2.4. Phylogenetic analysis

Two of the miRNAs identified were selected for phylogenetic analysis. Precursor sequences of same miRNAs families of other monocots were randomly selected for each predicted miRNAs from miRBase (Griffiths-Jones et al., 2008). MEGA 6.0 (Tamura et al., 2013) was used to construct Neighbor-joining (NJ) tree based on Kimura 2-parameter substitution model with 1000 bootstrap replications. Conservation of predicted miRNAs was analyzed using WebLogo, a sequence logo generator (Crooks et al., 2004).

3. Results

Monocot mature miRNA sequences, retrieved from mirBASE, were compared against 117,790 coconut callus unigenes by BLASTN and 558 matches of miRNA sequences were obtained (identity 100%, *E*- value 0.001). miPred analysis of selected precursor sequences gave the real miRNA precursor satisfying the indicators of GC content and minimum free energy value (MFE), and these were used for secondary structure prediction. This resulted in 27 mature miRNA sequences belonging to 15 miRNA families (Table 1). The distribution of coconut miRNA into consistent families is provided in Fig. 1. The length of the miRNAs ranged between 20 and 23 nt and most of the miRNAs were 21 nt in length. MFE value for the precursor miRNAs ranged from -39.8 to

Table 1
Predicted coconut miRNA families.

miRNA family	Name	miRNA sequence	Length	MFE value	Coconut unigene id
miR156	cnu-miR156a	UGCUCUCUAUCUUCUGUCAAC	21	− 49.2	535042
	cnu-miR156b	CUCACUUCUCUUUCUGUCAGCU	22	− 65.00	475213
miR164	cnu-miR164	GGAGAAGCAGGGAACUUGCUC	21	− 45.3	525922
miR166	cnu-miR166a-5p	GAAUGUUGUCUGGUUCGAGGC	21	− 41.7	547854
	cnu-miR166a-3p	CUCGGACCAGGCUUCAUUCCC	21	− 41.7	547854
	cnu-miR166b	CGGAUCAGGCUUCAUUCCUCA	21	− 50.2	533194
miR167	cnu-miR167a	GAAGCUGCCAGCAUGAUCUGAU	22	− 58.2	510186
	cnu-miR167b-5p	GUGAGGCUGUCACAGCAUGAC	21	− 57.00	540352
	cnu-miR167b-3p	AGAUCAUGCUGGCAGCUUCAC	21	− 57.00	540352
miR169	cnu-miR169-5p	AGCCAACGAGACUGCCUACGA	21	− 47.7	567634
	cnu-miR169-3p	AGGCAAGUCAUCCUUGGCUAU	21	− 47.7	567634
miR171	cnu-miR171-5p	AUUGGUGAGGUUCAAUCCGAU	21	− 45.2	550504
	cnu-miR171-3p	AUUGAGCCGCGCCAAUAUCA	20	− 45.2	550504
miR172	cnu-miR172-5p	UGCAGCAUCAUCAAGAUUCUC	21	− 39.8	568806
	cnu-miR172-3p	GUGAAUCUUGAUGAUGCCACA	21	− 39.8	568806
miR394	cnu-miR394	GAGGUGGACAGAAUGCCAAU	20	− 41.6	432682
miR397	cnu-miR397-5p	AUUGAGUGCAGCGCCGAUGAA	21	− 42.1	512640
	cnu-miR397-3p	UCAUCAACGCUGCACUCAAUG	21	− 42.1	512640
miR408	cnu-miR408a	AGGGAUGGAGCAGAGCAAGGA	21	− 40.2	454823
	cnu-miR408b	AGGGACAAGGCAGAGCAUGGG	21	− 45.50	491355
miR444	cnu-miR444	GCAGUUGCUGCCUCAAGCUUG	21	− 71.8	460390
miR535	cnu-miR535-5p	GACAACGAGAAAGAGCACGCC	21	− 58.70	562616
	cnu-miR535-3p	CGUGCUCUCUCUCGUUGUCAA	21	− 58.70	562616
miR827	cnu-miR827	UAGAUGACCAUCAGCAAACG	20	− 51.8	469947
miR1134	cnu-miR1134	CUUCUUCUUCUUGUAGUUCUUGC	23	− 49.3	511506
miR2118	cnu-miR2118a	AGGAAUGGGAGGCAUCGGCAAAU	23	− 52.9	537704
	cnu-miR2118b	GCAUGGGAGGUAUCGGGAAA	20	− 49	484417

− 71.8 (kcal/mol) and the length of the pre-miRNAs ranged from 92 to 130 nt. Stem-loop secondary structures of miRNA are shown in Supplementary Fig. S1.

Target prediction of these miRNA's was carried out using psRNATarget tool. All the 27 miRNA's were found to possess particular roles in plant development. Predicted targets could be mainly classified into transcription factors, comprising of auxin response factor (miR167), nuclear transcription factor Y subunit (miR169), transcription factor bHLH118-like (miR172), and transcription factor AS1-like (miR408). Some of the miRNAs were observed to possess tendencies to regulate kinases such a calcium-dependent protein kinase (miR408), mitogen-activated protein kinase (miR164 and miR1134) and serine/threonine-protein kinase-like protein (miR156 and miR166). Multiple targets were found for all the predicted miRNAs. The details of target information are provided in Supplementary Table S1. The miRNA targets plotted against coconut embryogenic callus transcriptome data, represented in the Circos network, is provided in Fig. 2.

In this study, most of the identified miRNAs have been reported in both monocots and dicots. Few miRNAs, like miR444 and miR2118 are specific to monocots and have been reported to be involved in plant embryogenesis (Lin and Lai, 2013; Zhai et al., 2014). For showing conservation between miRNAs, we have selected two miRNAs *viz.*, miR172 and miR444. While miR172 has been reported from both monocots and dicots, miR444 is specific to monocots. To detect the coconut-specific nucleotide variations in mature miRNAs, we compared coconut miRNA families with corresponding miRNA families in other plant species. In the case of mir172-3p, it was found to have mismatches at the 2nd, 18th and 19th nucleotide positions with other plant species, such as oil palm, rice, wheat, *Arabidopsis* and cocoa. When it was compared with grapes mature miRNA, it was shown to contain only one mismatch *i.e.*, cytosine at the 18th nucleotide position of the mature coconut miRNA instead of the conserved uracil observed in grapes and other plant species mentioned above (Fig. 3A 1). In the case of miR444, there were mismatches at the 8th and 11th nucleotide positions with other aligned mature miRNA (Fig. 3B 1). Phylogenetic analysis was also carried out using miR172 and miR444. Phylogenetic analysis of identified coconut miR172 precursor and other selected monocots and dicots were carried out using MEGA 6 (Fig. 3A 2). Coconut miR172 clustered with *Elaeis guineensis* miR-172e and *Vitis vinifera* miR-172a. Similarly, phylogenetic analysis of miR444 precursor and other selected monocots revealed that miR444 was closely related to bdi miR444a (*Brachypodium distachyon*) and also osa-miR444a (*Oryza sativa)* and anzma-444a (*Zea mays*) (Fig. 3B 2). The precursor sequences clearly aligned in the mature miRNA sequence region (Fig. 3A 3 and B 3). The conserved nature of the sequences was analyzed and presented using WebLogo. The WebLogo consists of stacks of letters and the height of the stack at the region of mature miRNA indicates the sequence conservation at this region (Fig. 3A 4 and B 4).

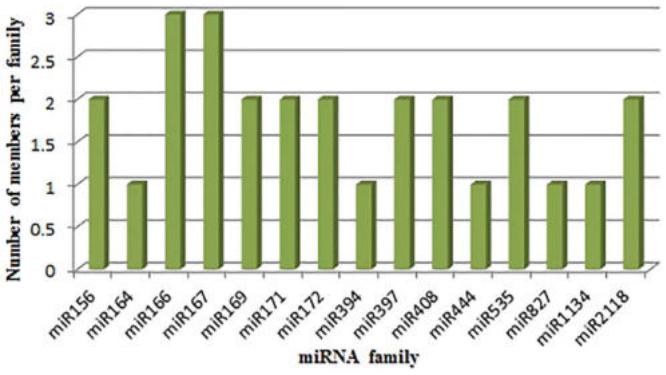

Fig. 1. Distribution of coconut miRNAs in different miRNA families.

4. Discussion

This study was carried out to explore the miRNA regulation of coconut somatic embryogenesis through detailed computational analysis. Plant miRNAs are conserved and they regulate various functions during plant development and differentiation process like zygotic embryogenesis (Nodine and Bartel, 2010; Willmann et al., 2011), hormone signalling (Guo et al., 2005; Reyes and Chua, 2007) and stress responses (Sunkar and Zhu, 2004). In recent years, computational predictions of miRNAs and their involvement in various functional roles in the

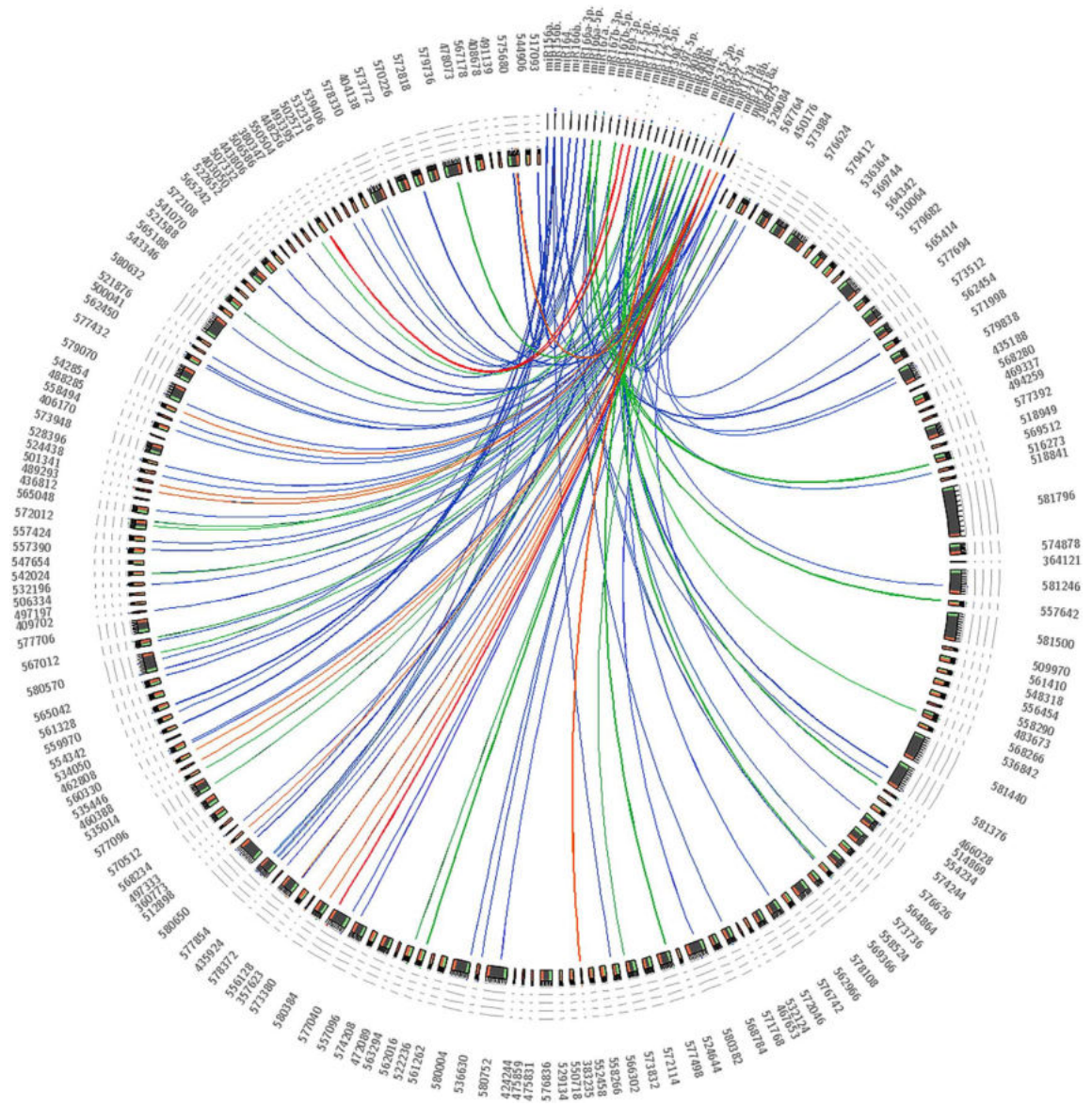

Fig. 2. Coconut miRNAs and their targets based on coconut transcript data plotted using Circos. The outer layer shows the predicted miRNAs and their target unigene id.

development of plants have been extensively studied (Adai et al., 2005; Naganeeswaran et al., 2015; da Silva et al., 2016; Gharat and Shaw, 2016).

In this study, 15 classes of conserved miRNA families were identified in coconut based on previously reported miRNA sequence similarity by computational methods. It was observed that most of the predicted miRNAs such as miR156, miR164, miR166, miR167, miR169, miR171, miR172 and miR397 have been earlier identified during somatic embryogenesis in radish (Zhai et al., 2014), larch (Zhang et al., 2012), 'Valencia' sweet orange (*Citrus sinensis*) (Wu et al., 2015), cotton (Yang et al., 2013) and longan (Lin and Lai, 2013) and have been reported to play important regulatory roles during embryonic development. These miRNAs have been reported to be highly conserved in both monocots and dicots (Cuperus et al., 2011). Phylogenetic analysis revealed that some of the miRNAs are highly conserved in both monocots and dicots, but others are not. Phylogenetic analysis of miR172 revealed that coconut miR172 was clustered with monocots and dicots. However miRNAs like miR444 and miR2118 were highly specific to monocots (Qin et al., 2014). So, in the case of miR444 in coconut, phylogenetic and conservation analyses support its close relation with other monocots.

miR156 was found to target transcript of squamosa promoter-binding-like protein, allene oxide synthase, GPI ethanolamine phosphate transferase 2, peptidyl-prolyl cis-trans isomerase PASTICCINO1, pentatricopeptide repeat-containing protein At5g66520-like, serine/threonine-protein kinase-like protein CCR4 and glutamate receptor. In cotton, Yang et al. (2013) observed that during the dedifferentiation stage and embryogenic calli stage, miR156 displayed low expression levels; the expression level progressively increased throughout somatic embryo development and reached a moderately high expression level at cotyledon stage somatic embryo. Similarly, in rice, miR156 was highly expressed in differentiated callus than in dedifferentiated tissues (Luo et al., 2006). By analysing the role of miR156 in all these plants, it is proposed that miR156 might be also involved in early embryogenic patterning during coconut somatic embryogenesis. Zhang et al. (2010) studied the expression pattern of the abiotic stress induced miRNAs *viz.*, miR166, miR171 and miR172 in embryogenic and non-embryogenic callus tissues of *Larix leptolepis* and it was reported that miR171 was upregulated during embryogenic callus, while the rest of them were downregulated. These miRNAs were also identified in the present study.

Mitogen-activated protein kinase kinase kinase YODA-like and CUP-

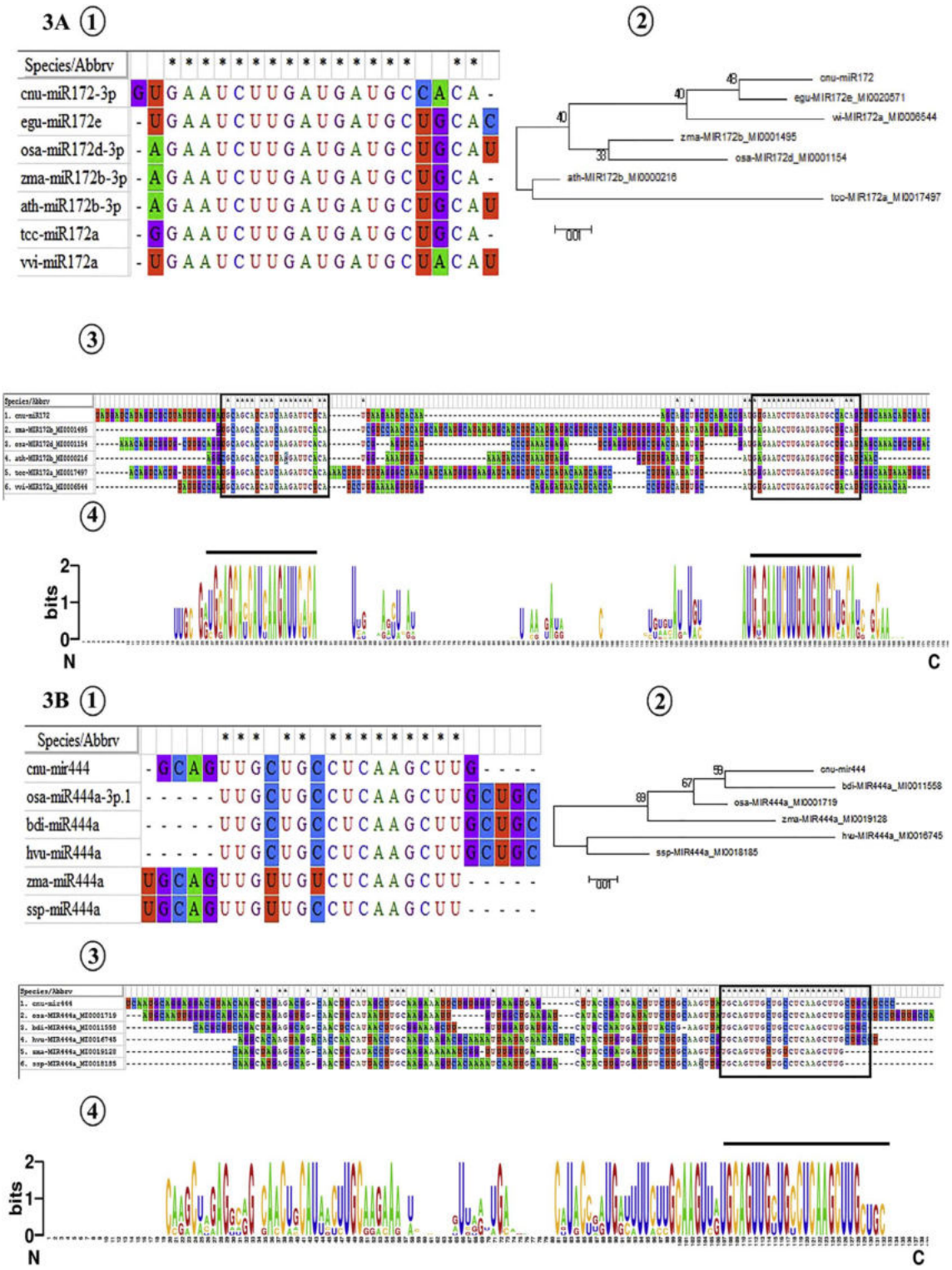

Fig. 3. A & B. Homology, phylogeny and WebLogo analysis of coconut miRNAs with other plant miRNAs.
3A 1 and 3B 1. Alignment of mature coconut miR172 and miR444 with other plant species. 3A 2 and 3B 2. Phylogenetic trees of coconut miR172 and miR444 with precursor miRNA sequences from various members of the plant.
3A 3 and 3B 3. Precursor miRNA sequence alignments of miR172 and miR444 and other plant species. The mature miRNA sites pairing with others are underlined with black boxes. 3A 4 and 3B 4. weblogo analysis of precursor miR172 and miR444.

SHAPED COTYLEDON 2 were found to be targeted by miR164. CUP-SHAPED COTYLEDON 2 (*CUC2*) has been reported to be involved in shoot apical meristem formation in *Arabidopsis* (Aida et al., 1997). This miRNA was found to be downregulated from embryogenic callus to cotyledon embryo stage in cotton (Yang et al., 2013). In citrus, the expression of miR164 was high in embryogenic calli and it was reported that the miR164-mediated suppression of CUC2 activity is essential to inactivate the postembryonic growth during somatic embryogenesis of citrus (Wu et al., 2015). Mitogen-activated protein kinase was also targeted by miR164. Rajesh et al. (2016) had experimentally validated

the expression of *MAPK* during *in vitro* culture of coconut and it was observed that the expression was higher in the embryogenic callus stage than initial culture and somatic embryo stage. Rajesh et al. (2016) identified 14 transcripts which were involved in somatic embryogenesis and experimentally validated their expression. In this study, we found to be some of these transcripts reported to be targeted by miRNAs. miR172 was found to target the somatic embryogenesis related transcription factor floral homeotic protein APETALA 2-like and extracellular protein arabinogalactan protein. Similar to *MAPK*, expression of both these transcripts were higher in the embryogenic callus stage in comparison to initial stage of callogenesis and somatic embryo stage (Rajesh et al., 2016). Similarly, *CLAVATA* was upregulated in the initial stage of callogenesis whereas *WRKY* was upregulated in somatic embryo stage (Rajesh et al., 2016) and we found that these transcripts were targeted by miR408 and miR1134 respectively.

Scarecrow-like protein and auxin response factor 12-like are important transcription factors targeted by miR171 and miR167 respectively. Scarecrow has been reported to be vital for the asymmetric cell division that gives rise to the cortex and endodermis and to other tissues in aerial organs of *Arabidopsis thaliana* (Di Laurenzio et al., 1996). In *Larix leptolepis*, the expression of miR171 was high in embryogenic callus while it was not expressed in non-embryogenic callus (Zhang et al., 2010). During longan somatic embryogenesis, miR167a was found to target auxin response factors (ARFs) and play a major role during cotyledonary and mature embryonic stages (Lin and Lai, 2013), and similar results have been reported in larch and oranges (Zhang et al., 2012; Wu et al., 2011). miR167 was found to undetectable in 2,4-D containing medium in the longan embryogenic cultures (Lin and Lai, 2013).

From the results of the present study, miR397 was found to target transcript of laccase and serine/threonine-protein kinase EDR1-like. Laccases comprise of a group of polyphenol oxidases and they are associated with lignification and thickening of cell wall during secondary growth (Constabel et al., 2000). They might be involved in maintaining the cells in meristematic state. According to a previous study in rice, Luo et al. (2006) found that laccase gene is down regulated due to high expression of miR397 in rice pro-embryogenic cells, because of which embryogenic cells maintain their meristematic state. On the other hand, low expression causes the accumulation of laccases, leading to the lignification of cell wall in meristematic to mature cell transition. Li et al. (2009) had reported that over expression of miR397 resulted in the inhibition of the expression of laccase genes, and caused coconut endosperm to stay in a meristematic state. In rice, laccase-like protein, is involved in brassinosteroids (BR) signalling and is regulated by miR397. Overexpression of miR397 resulted in the downregulation of laccase which led to grain size enlargement and promoted panicle branching, thus expressively increasing grain production (Zhang et al., 2013).

In conclusion, all the identified miRNA from coconut embryogenic callus transcriptome data using computational approaches, were either expressed or variously regulated in embryogenic callus and different stages of somatic embryogenesis in other plant species. They also regulate some of the hormone signalling pathways. In summary, this study is one small but remarkable step towards the identification of functions of miRNA during coconut somatic embryogenesis and would be helpful for other studies in coconut and related palms.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aggene.2018.01.002.

Author contributions

All the authors were involved in carrying out the computational analysis and writing of the manuscript.

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