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ORIGINAL ARTICLE

De novo assembly and characterization of global transcriptome of coconut palm (*Cocos nucifera* L.) embryogenic calli using Illumina paired-end sequencing

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Abstract Production and supply of quality planting material is significant to coconut cultivation but is one of the major constraints in coconut productivity. Rapid multiplication of coconut through in vitro techniques, therefore, is of paramount importance. Although somatic embryogenesis in coconut is a promising technique that will allow for the mass production of high quality palms, coconut is highly recalcitrant to in vitro culture. In order to overcome the bottlenecks in coconut somatic embryogenesis and to develop a repeatable protocol, it is imperative to understand, identify, and characterize molecular events involved in coconut somatic embryogenesis pathway. Transcriptome analysis (RNA-Seq) of coconut embryogenic calli, derived from plumular explants of West Coast Tall cultivar, was undertaken on an Illumina HiSeq 2000 platform. After de novo transcriptome assembly and functional annotation, we have obtained 40,367 transcripts which showed significant BLASTx matches with similarity greater than 40 % and *E* value of $\leq 10^{-5}$. Fourteen genes known to be involved in somatic embryogenesis were identified. Quantitative real-time PCR (qRT-PCR) analyses of these 14 genes were carried in six developmental stages. The result showed that *CLV* was upregulated in the initial stage of callogenesis. Transcripts *GLP*, *GST*, *PKL*, *WUS*, and *WRKY* were expressed more in somatic embryo stage. The expression of *SERK*, *MAPK*, *AP2*, *SAUR*, *ECP*, *AGP*, *LEA*, and *ANT* were higher in the embryogenic callus stage compared to initial culture and somatic embryo stages. This study provides the first insights into the gene expression patterns during somatic embryogenesis in coconut.

Keywords Coconut · Somatic embryogenesis · Transcriptome · RNA-Seq · Recalcitrant

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Introduction

Coconut (*Cocos nucifera* L.), a member of the family Arecaceae, is a perennial palm found throughout the tropical and subtropical areas in the world, covering an extent of about 12.5 million hectares with an annual production of around 73 million nuts (APCC 2013). The palm is quite unique as it provides a wide array of products and is the source of direct livelihood for more than 11 million people in more than 93 countries. In recent times, the coconut sector is being confronted with many challenges, which include low productivity and fluctuating prices, biotic stresses, detrimental effect of climatic vagaries on productivity, imperfect market scenario, and stringent competition from other edible oil sectors. The competitiveness of the coconut sector, therefore, should be enhanced from the production front to the ultimate market node for the sustenance of the sector. In the current context, in order to augment the competitiveness, it is imperative to enhance the production through systematic varietal improvement, which in turn depends on availability of quality planting material of high yielding mother palms. The major propagation method is by using nuts which does not meet the requirement of quality planting materials and since the palms are mainly allogamous, the resulting progenies would be highly heterogeneous. To increase the availability

of quality propagating materials, the only feasible means is rapid multiplication of elite coconut genotypes via tissue culture. Although coconut tissue culture research was initiated about four decades ago in a number of laboratories worldwide, limited success has been achieved so far due to its inherent recalcitrant nature (Fernando et al. 2010).

Plant cells display developmental plasticity which allows their reprogramming and manifestation of cellular totipotency (Costa and Shaw 2007). Somatic embryogenesis, which forms the basis of cellular totipotency, is the process by which already differentiated somatic cells reverse their developmental program and become embryogenic, form somatic embryos, which develop into complete plantlets via characteristic morphological and biochemical changes (Reinert 1959). In coconut, somatic embryogenesis has been reported from different explants (Branton and Blake 1983; Verdeil et al. 1994; Pannetier and Buffard-Morel 1982; Buffard-Morel et al. 1988; Karunaratne and Periyapperuma 1989; Hornung 1995; Chan et al. 1998; Fernando and Gamage 2000; Fernando et al. 2003; Oropeza et al. 2005; Rajesh et al. 2005, 2014; Perez-Nunez et al. 2006). However, the multiplication and regeneration rate has generally remained quite low and an economically viable protocol is yet to be developed, for explants from adult palms, in spite of concerted efforts in this direction (Fernando et al. 2010). Some of the major constraints in coconut tissue culture include low rate and heterogenous response of different explants in vitro, low rate of formation of somatic embryos, formation of abnormal somatic embryos, and poor establishment of in vitro regenerated plantlets (Fernando et al. 2010). In order to overcome these bottlenecks and to develop an economically viable protocol, it is imperative to understand the molecular events involved in coconut somatic embryogenesis pathway to identify possible bottlenecks which could contribute substantially to the improvement of existing protocols and also, to the establishment of new protocols with novel plant growth regulators.

The molecular events underlying cellular totipotency are still to be deciphered completely (Namasivayam 2007). The embryogenic potential of somatic cells is achieved by the reprogramming of gene expression patterns and other physiological changes (Solis-Ramos et al. 2012). It involves different molecular events including differential gene expression and various signal transduction pathways for activating or repressing numerous genes sets (Chugh and Khurana 2002). Several genes have been reported to be involved in somatic embryo induction and formation (Yang and Zhang 2010). Somatic embryogenesis receptor-like kinase (*SERK*) is one of the important genes involved in initiation of somatic embryogenesis in carrot cell suspensions (Schmidt et al. 1997). The role of other genes viz., late embryogenesis abundant (*LEA*) protein genes (Perez-Grau and Goldberg 1989), storage protein genes (Galau et al. 1987; Misra et al. 1993), and leafy cotyledon (*LEC*) genes (Braybrook and Harada 2008; Schellenbaum et al. 2008) in various stages of somatic embryogenesis has also been reported in various plants.

The transcriptome represents the complete transcript or RNA present in the cell including its quantity. Transcriptome analysis, therefore, is a powerful tool for functional validation and evaluation of molecular constituents and its expression level (Wang et al. 2009). It can also be used to identify novel genes and non-coding genes and to determine the structure and base constitution thus providing the information about the complexity of the RNA genome (Mortazavi et al. 2008; Nagalakshmi et al. 2008; Sultan et al. 2008). High-throughput RNA sequencing (RNA-seq) is one of the new-generation methods for identifying differential expression of genes. Genomic or proteomic information on coconut somatic embryogenesis is scarce. With the aim to generate a global transcriptome data to identify genes expressed in coconut embryogenic callus, our study was conducted using embryogenic calli developed from coconut plumular explants, which has so far proven to be the most responsive explant for somatic embryogenesis compared to other explants (Chan et al. 1998; Saenz et al. 2006; Azpeitia et al. 2003; Perez-Nunez et al. 2006; Rajesh et al. 2005, 2014). Higher yields of calli and calli bearing somatic embryos could be obtained from plumular explants compared to inflorescence explants (Chan et al. 1998; Perez-Nunez et al. 2006). Brassinosteriod treatment promoted formation of calli, embryogenic calli, and somatic embryos from plumular explants (Azpeitia et al. 2003).

In this study, we have generated a global transcriptome from embryogenic calli, derived from plumular explants of West Coast Tall cultivar, using high-throughput Illumina RNA sequencing and analyzed functions, classification, and metabolic pathways of the resulting transcripts. We have also analyzed the comparative expression patterns of selected transcripts participating in coconut somatic embryogenesis in different stages of the regeneration pathway. We hope that the transcriptomic resources generated in the study would serve as an invaluable resource for the identification of genes involved in coconut somatic embryogenesis.

Materials and methods

Plant materials

Matured nuts (11–12 months old) were harvested from palms of West Coast Tall cultivar. Zygotic embryos, along with endosperm, were excised using a cork borer from the split-opened mature nuts (12 months old) and placed in distilled water. The endosperm plugs, enclosing the embryos, were sterilized with 0.01 % $HgCl_2$ for 5 min and then rinsed thrice with sterile distilled water to remove the traces of $HgCl_2$.

Embryos were extracted from endosperm plugs, surface sterilized with 20 % sodium hypochlorite solution for 20 min and subsequently rinsed with sterile distilled water for 5–6 times. Plumule portions were directly scooped from the surface sterilized matured embryos in aseptic condition by using sterile blade and scalpel. The scooped plumular explants were inoculated to initial callus induction medium viz., Y3 basal medium (Eeuwens 1976) supplemented with 3 % (*w*/*v*) sucrose, 0.1 % (*w*/*v*) activated charcoal with 2,4-D (74.6 μM), spermine (50 μM), and thidiazuron (TDZ) (4.54 μM) and kept for incubation in dark condition. Cultures were transferred to a media with gradual decrease of 2,4-D (74.6 μM→45.24 μM→22.62 μM) at monthly intervals, while the concentrations of spermine (50 μM) and TDZ (4.54 μM) were maintained at a constant rate. Embryogenic calli were obtained after 90 days of culture incubation. Formation of somatic embryos was noticed when the cultures were placed in hormone-free Y3 basal media. Germination of the somatic embryos occurred in Y3 medium supplemented with BAP (22.2 μM), glutamine (34.2 μM), GA_3 (2.8 μM), and 2,4-D (0.045 μM).

RNA extraction

Five independent embryogenic callus samples (after 90 days of initial explant inoculation) were snap-frozen immediately in liquid nitrogen. Total RNA was extracted from each of these five samples using the Trizol reagent (SIGMA) and treated with DNase I (Fermentas, USA) according to the manufacturer's instructions. The quality and purity of the extracted RNA were assessed with OD 260/280 ratio. Also, RNA integrity number (RIN) was analyzed using Agilent Technologies 2100 Bioanalyzer using the Agilent RNA chip.

Transcriptome sequencing

RNA sequencing was performed on an Illumina HiSeq 2000 platform (SciGenom, India). RNA obtained from the five samples were pooled and approximately 5–10 μg of the pooled RNA was then utilized to prepare the RNA-seq library using TruSeq RNA Sample Prep Kits (Illumina). The poly $(A)^+$ containing messenger RNA (mRNA) molecules were purified using poly-T oligo-attached magnetic beads. After purification, the mRNA was fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were then used to synthesize the first-strand complementary DNA (cDNA) using reverse transcriptase and random primers. This was followed by synthesis of the second-strand cDNA using DNA polymerase I and RNase H. The cDNA fragments thus obtained were then subjected to an end repair process, the addition of a single "A" base, and then ligation of the adapters. The products were subsequently purified and enriched with PCR to generate the final cDNA library. At every step, to assess mRNA quality, enrichment success, fragmentation sizes, and final library sizes, bioanalyzer plots were generated. The size distribution of the sequencing library was verified by gel electrophoresis. Assessment of the quantity of the library was carried out by qPCR before sequencing. After construction of the libraries, paired-end run was performed on Illumina HiSeq 2000 platform and 2×100 bp reads were obtained.

De novo transcriptome assembly and evaluation

The raw reads (fastq) were trimmed before performing assembly using a Perl script program. Initially, 15 bases were removed from all reads to avoid specific sequence bias and low quality bases. Further, reads with average base quality score <Q20 Phred score were discarded from the analysis. The trimmed reads were then assembled using SOAPdenovo v1.05 (http://soap.genomics.org.cn/soapdenovo.html) with default options. The contigs obtained were then assembled into scaffolds and finally into transcripts.

Gene expression estimation

The trimmed reads were aligned to the assembled transcriptome (length≥50 bp) using Bowtie program, allowing up to three mismatches in the seed region (length=36 bp) and all multiple mapped positions were reported. RPKM (reads per kilobase of exon model per million mapped reads) were used for evaluation of the expressed value and quantification of transcripts (Mortazavi et al. 2008). For downstream annotation and differential expression analysis, we focused only on those transcripts with length of ≥150 bp and expression of ≥1 RPKM.

Gene annotation, classification, and metabolic pathway analysis

Annotations of the assembled transcripts were performed in the following manner. The assembled transcripts were compared with NCBI non-redundant protein database using BLASTx program. Matches with *E* value of $\leq 1\times10^{-5}$ and similarity score of ≥40 % were retained for further annotation. The predicted proteins from BLASTx were annotated against UniProt databases. We have also used Blast2GO (Conesa et al. 2005), in conjunction with non-redundant annotations, to retrieve gene ontology (GO) annotations of assembled transcripts from coconut embryogenic calli. Pathway annotations were performed using Kyoto Encyclopedia of Genes and Genomes—KEGG Automation Annotation Server (KEGG-KAAS) program (Kanehisa et al. 2010).

Gene validation and expression analysis by real-time quantitative PCR

Primers were designed using Primer 3.0 software (http://primer3.ut.ee/) for selected genes with potential roles in coconut somatic embryogenesis. Three stages of development viz., initial calli (30 and 60 days after inoculation of plumular explants), embryogenic calli (90 and 120 days after inoculation) and somatic embryos (150 and 180 days after inoculation), were selected (Fig. 1). Total RNA was isolated from these samples using QIAGEN plant RNA mini kit and DNase 1 (QIAGEN) treatment were performed. The integrity, concentration, and quality check of the extracted RNA were carried out. Synthesis of cDNA was performed using High Capacity RNA-to-cDNA kit (Applied Biosystems). Quantitative RT-PCR (qRT-PCR) reactions were carried out in a total volume of 25 μL containing 12.5 μL of 2× SYBRGreen PCR Master Mix (Applied Biosystems), 1 μL of each primer (Sigma), 1 μL of template (10× diluted cDNA from samples), and 9. 5 μL of sterile distilled water. The thermal conditions were as follows: initial holding stage 52 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and a final step at 60 °C for 1 min. All reactions were performed in triplicate in 48-well reaction plates using Step One Real-Time PCR machine (Applied Biosystems). Alpha-tubulin was used as endogenous control (forward primer: 5′-CTGGTGTCCTACTGGCTTC-3′; reverse primer: 5′- GACCATGATTACGCCAAG -3′) (Rajesh et al. 2014a). Two biological replicates and three technical replicates were included for each sample. No-template controls were also included for each gene to detect any spurious signals arising from amplification of any DNA contamination or primer dimer formed during the reaction. The results (Cq values) were generated using sequence detection software SDS version 1.1 (Applied Biosystems). The Cq values were imported to qBASE software (Biogazelle) and relative fold change in transcript levels were generated using comparative Cq method. Normalization was done using α-tubulin gene. Finally, calibrated normalized relative quantity (CNRQ) values were exported from the qbase software and statistically investigated using Student's *t* test. Significance was accepted at $p<0.05$. These CNRQ values with the corresponding standard errors were then plotted.

Results

Illumina sequencing and read quality check and de novo transcriptome assembly

The transcriptome data was obtained from the total RNA isolated from the coconut embryogenic calli, using Illumina HiSeq 2000 sequencing platform. Paired-end method generated higher quality sequence with highly precise alignment of reads. About 50,839,994 paired-end reads (comprising 7.73 Gb of nucleotide data) were obtained after sequencing (SRX472157). The raw data sequences were obtained as fastq file with an average GC content of 47.38 %. The quality of the sequences was checked by base quality distribution analysis, base composition distribution analysis, and GC distribution analysis. The base quality of the left end of paired-end reads was above Q20 (error probability≥0.01), whereas the quality of 10 bases were low in the right end reads. Before performing the transcriptome assembly, the raw data sequences were trimmed. First 15 bases were removed from all left end reads, and first 14 bases and last 10 bases were trimmed from right end reads to avoid specific sequence bias and low quality bases. Reads whose average quality score was <20 were filtered off. After performing the trimming process, the total reads were reduced to 46.2 million, with approximately 5.42-Gb bases.

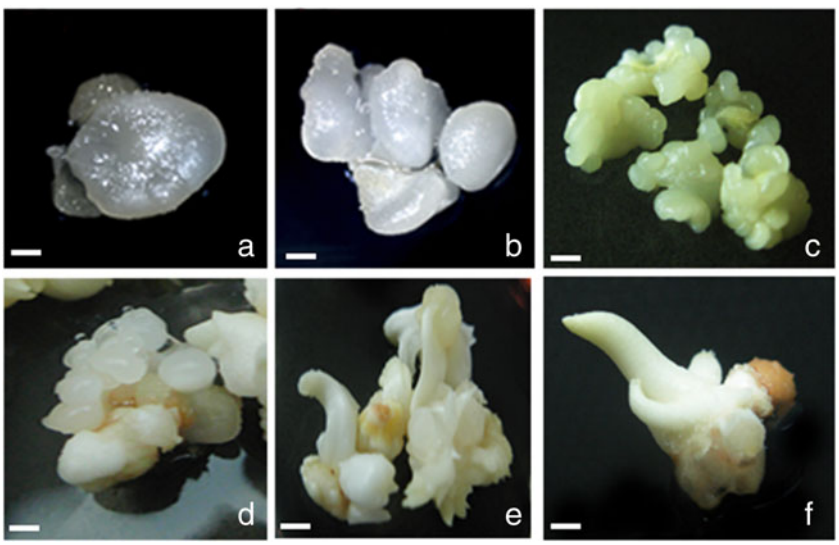

Fig. 1 Stages in coconut somatic embryogenesis developmental pathway from plumular explants: Initial calli 30 days (**a**) and 60 days (**b**), embryogenic calli 90 days (**c**) and 120 days (**d**) and somatic embryos 150 days (**e**) and 180 days (**f**) after inoculation of plumular explants (*Bar*=1 mm)

De novo assembly was conducted because of the unavailability of reference genome for coconut. The trimmed reads were assembled using SOAPdenovo-31mer algorithm. After conducting the assembling program, a total of 2,90,936 contigs were obtained with an average length of 230 bp and N50 value of 369 bp. The contigs were then assembled into scaffolds, which were trimmed to remove contaminations using SeqClean. CAP3 was then used to reduce the redundancy of scaffolds and to extend the lengths of scaffolds. A total of 161,426 transcripts were finally obtained with an average length of 436 bp and a N50 of 561 bp. Overall, we found 73,607 transcripts with a length of more than 150 bp and expression of ≥1 RPKM. For downstream annotation, we focused only on these transcripts. The expression value (RPKM) distribution is shown in Table 1.

Functional annotation

Functional annotation was done by performing different steps like comparison with NCBI database using BLASTx program, gene, and protein annotation to the matched transcript, gene ontology annotation, and pathway annotation. A total of 73,607 transcripts that were above 150 bp and RPKM≥1.0 were utilized for functional annotation and we have obtained 40,367 (54.84 %) transcripts which showed significant matches in the database with similarity greater than 40 % and *E* value≤10–5 (Supplementary Table 1). From the annotated transcripts (40,367), 8 % of the transcripts showed 100 % similarity through BLASTx, about 43 % of the transcripts showed similarity values of 80–99 % and around 23 % of the transcripts found had confidence level of at least 1E-50, which indicates low protein conservation. Nearly 86 % of the transcripts showed 60 % similarity at protein level with the existing proteins at NCBI database using BLASTx program. Among the 40,367 transcriptome sequences, 13,031 transcripts (32.28 %) could be annotated with UniProt database.

The 15 most highly expressed transcripts based on RPKM values are listed in Table 2. The transcripts coded for proteins with varied functions. From the data obtained, we have also identified the presence of 14 genes known to be involved in somatic embryogenesis by comparing the transcripts with the available sequences in NCBI. The list of genes identified, length, source, etc. are detailed in Table 3. These transcripts included protein kinases like receptor-like kinases [somatic embryogenesis receptor kinase (*SERK*) and CLAVATA 1 (*CLV1*)], mitogen-activated protein kinase (*MAPK*), transcription factors [*WUSCHEL* (*WUS*), APETALA2/Ethylene-responsive factor (*AP2*/*ERF*), *PICKLE* (*PKL*), *AINTEGUMENTA* (*ANT*), and *WRKY*], extracellular proteins [arabinogalactan protein (*AGP*), Germin-like protein (*GLP*), embryogenic cell protein (*ECP*), and late embryogenesis-abundant protein (*LEA*)] and glutathione S-transferase (*GST*).

Table 1 Expression value (RPKM) of transcripts above 150 bp

RPKM value	No. of transcripts
1.0–2.0	20,530
2.0–5.0	19,653
5.0–10.0	14,224
10.0–20.0	10,609
20.0–50.0	6363
50.0–100.0	1468
≥100.0	760
Total	73,607

GO functional annotation and classification of coconut embryogenic calli transcriptome

Gene ontology (GO) items were assigned to the assembled transcripts to functionally categorize expressed genes in coconut embryogenic calli. GO annotations and classification were obtained according to molecular function, biological process, and cellular component ontologies. The GO classification was done based on the matching of sequence with the known proteins available in the database. In this study, the number of gene ontology terms identified in each category was 8300 (biological processes) (Fig. 2a); 13,193 (molecular functions) (Fig. 2b); and 6076 (cellular components) (Fig. 2c). The majority of sequences in the biological process category were involved in the process of transcription and its regulation. In the molecular function category, the sequences mostly expressed were those that were involved in ATP binding followed by those involved in zinc ion and metal ion binding process. In the third category, viz., cellular components, the highly expressed sequences were components integral to membrane followed by nucleus.

KEGG classification of coconut embryogenic calli transcriptome

Kyoto Encyclopedia of Genes and Genomes (KEGG) is an online database for connecting the genomic information with higher order functional pathways to reveal the functional profile of genes. A total of 73,607 transcripts (those above 150 bp and RPKM≥1.0) were utilized for KEGG-KAAS analysis. Among these, 14,287 transcripts were involved in KEGG pathways related to the plants. The transcripts encoded a total of 2705 enzymes which could be mapped to 105 KEGG pathways. About 7164 (50.14 %) sequences were found to be involved in metabolic pathways, mainly starch and sucrose metabolism (423, 5.9 %), glycolysis/gluconeogenesis (329, 4.6 %), amino sugar, and nucleotide sugar metabolism (328, 4.57 %). Among the remaining sequences, 3688 (26 %), 1521 (10.6 %), 786 (5.5 %), 664 (4.64 %), and 464 (3.25 %) were sorted to genetic information processing, cellular process, environmental information

Table 2 List of 15 highly expressed transcripts based on RPKM values obtained from RNA-Seq data of coconut embryogenic calli

Sl. no.	Transcript ID	RPKM value	*E* value	Gene	Source
1.	580790	4830	2.00E-76	Cytochrome P450-like TBP	*Medicago truncatula*
2.	567460	3970	2.00E-152	1-cys peroxiredoxin	*Elaeis guineensis*
3.	561596	3380	2.00E-70	Metallothionein	*Phoenix dactylifera*
4.	555002	1917	2.00E-22	Lipid-transfer protein	*Zea mays*
5.	556012	1808	2.00E-27	Defensin	*Elaeis guineensis*
6.	386055	1647	2.00E-39	Translationally controlled tumor protein	*Phoenix dactylifera*
7.	526906	1505	8.00E-81	Auxin-repressed protein	*Phoenix dactylifera*
8.	580464	1404	0	Yt521-b-like family expressed	*Phoenix dactylifera*
9.	491343	1180	9.00E-157	Elongation factor 1	*Ricinus communis*
10.	553184	1142	4.00E-99	Metallothionein-like protein type 2	*Phoenix dactylifera*
11.	574586	1097	9.00E-107	Thaumatin-like protein	*Citrus jambhiri*
12.	445236	911	3.00E-88	Phosphoenolpyruvate carboxykinase	*Phoenix dactylifera*
13.	576336	818	4.00E-163	Polyamine oxidase	*Phoenix dactylifera*
14.	570776	769	1E-102	Peptidyl-prolyl cis-trans isomerase	*Elaeis guineensis*
15.	581192	693	0	Pentatricopeptide repeat-containing protein	*Medicago truncatula*

processing, biosynthesis of secondary metabolites, and organismal systems, respectively. The most important pathways and its mapped enzyme number are mentioned in Supplementary Table 2. The matched sequences were involved in various processes like metabolism, synthesis of secondary metabolites, genetic information-related process, signal transduction, and cellular process like cell cycle and cell communication. Out of the 105 pathways, 66 were involved in metabolism. About 471 (3.3 %) transcripts were involved in plant hormone signal transduction pathway.

Gene validation and expression analysis using quantitative real-time PCR

The analysis of 14 transcripts expressed during somatic embryogenesis of coconut was performed using comparative C_t analysis method. Primers were designed to amplify fragments of less than 150 bp and the details of the primer sequences are given in Table 4. Three stages of developmental pathway of somatic embryogenesis [initial calli (30 and 60 days after inoculation of plumular explants), embryogenic calli (90 and 120 days after inoculation), and somatic embryos (150 and

Table 3 List of 14 transcripts related to somatic embryogenesis obtained from RNA-Seq data of coconut embryogenic calli

Sl. No.	Transcript id	Transcript length (bp)	*E* value	Gene	Source
1.	578404	2255	0	Somatic embryogenesis receptor kinase (*SERK*)	*Oryza sativa*
2.	567344	1170	0	Glutathione S-transferase (*GST*)	*Phoenix dactylifera*
3.	439278	231	5E-15	Wuschel (*WUS*)	*Medicago truncatula*
4.	556118	804	6E-25	Embryogenic cell protein (*ECP*)	*Daucus carota*
5.	555772	797	0	Germin-like protein (*GLP*)	*Phoenix dactylifera*
6.	581560	4694	0	PICKLE (*PKL*)	*Vitis vinifera*
7.	560808	921	0	WRKY transcription factor (*WRKY*)	*Phoenix dactylifera*
8.	577164	2021	0	CLAVATA1 (*CLV*)	*Phoenix dactylifera*
9.	564618	1047	0	Mitogen-activated protein kinase (*MAPK*)	*Phoenix dactylifera*
10.	530086	500	1E-22	AP2/ERF domain-containing transcription factor (*AP2*)	*Populus trichocarpa*
11.	539062	570	0	SAUR family protein (*SAUR*)	*Phoenix dactylifera*
12.	562916	986	2.00E-63	Arabinogalactan protein (*AGP*)	*Vitis vinifera*
13.	533908	527	3.00E-179	Late embryogenesis-abundant protein (*LEA*)	*Phoenix dactylifera*
14.	553982	765	3.00E-25	Aintegumenta (*ANT*)	*Populus canescens*

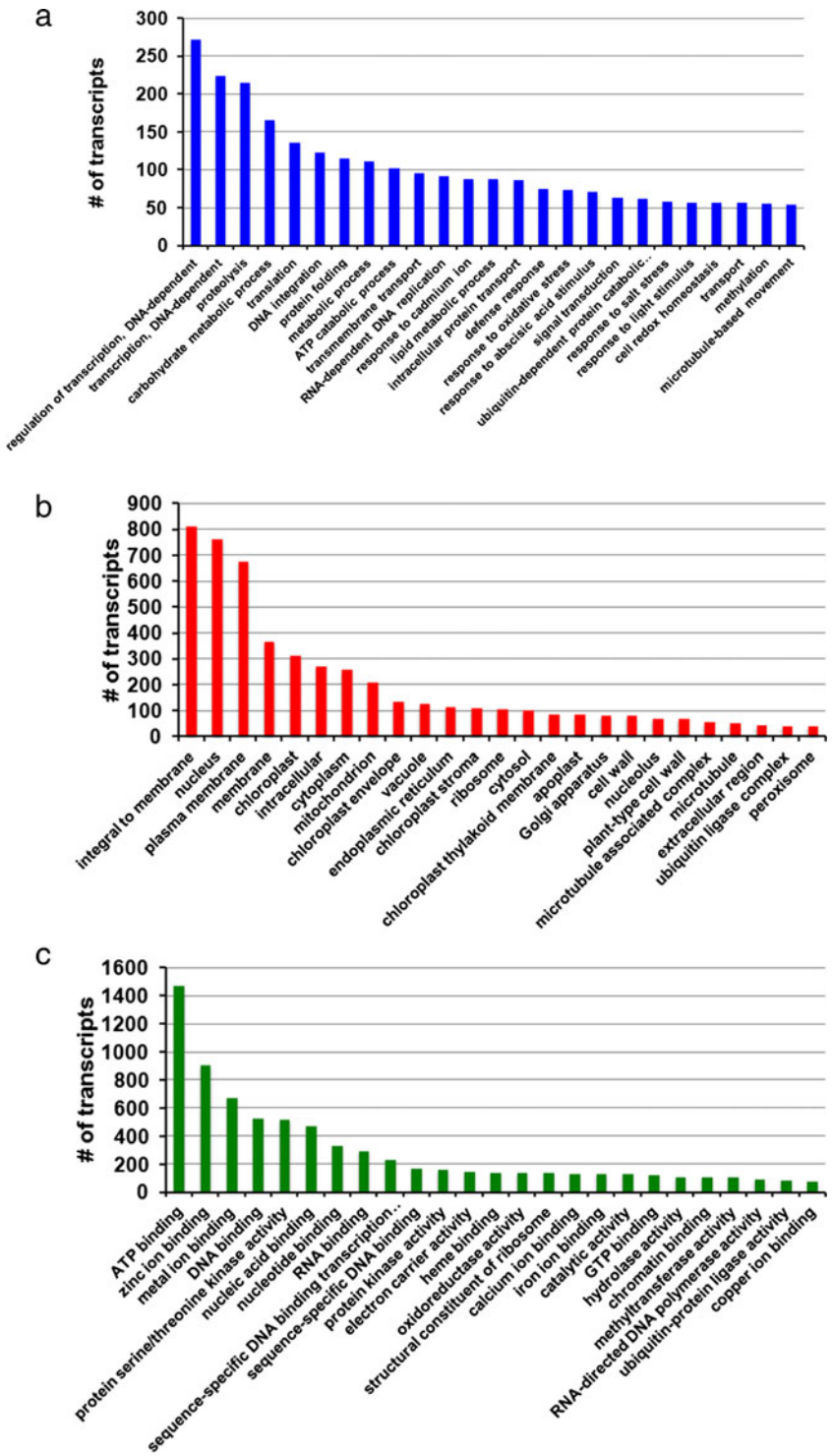

Fig. 2 Gene ontology (GO) classification of coconut plumular-tissue derived embryogenic calli. GO term assignments to coconut transcripts based on significant plant species hits against the NR database were summarized into three main GO categories: biological process (**a**), cellular component (**b**), and molecular function (**c**)

180 days after inoculation] were selected (Fig. 1). Two biological and three technical replications were performed for each gene. Melt curve analysis indicated that the 14 primer pairs amplified a specific PCR product. The analyses of the results indicated that *CLV* was upregulated in the initial stage of callogenesis. Transcripts GLP, *GST*, *PKL*, *WUS*, and *WRKY* were expressed more in somatic embryo stage. The expression of *SERK*, *MAPK*, *AP2*, *SAUR*, *ECP*, *AGP*, *LEA*, and *ANT* were higher in the embryogenic callus stage compared to initial culture and somatic embryo stages (Fig. 3).

Table 4 Gene-specific primers used in qRT-PCR experiments

Sl. No.	Gene	Sequence (5′–3′)
1.	*SERK*	F: GCCGTACATCGAAACTTGCT R: GGATCACAATGATCATGCAAA
2.	*GST*	F: CCTGCCTTTGGTGATGTCATA R: GAGGCATGGGTGATGAAGTT
3.	*WUS*	F: TGTACTGCCTGCCTGGTTC R: CCACCTGATAAGAGGGACGA
4.	*GLP*	F: CCTGGTTAACACCCACAACC R: CAGGGTTCGAAGTCACGAAG
5.	*PKL*	F: CCAAGACACATCCTCGACTG R: TCCCAGGTGCATTCATCATA
6.	*WRKY*	F: GGATGATTGTGTTCACCCTTG R: GCAACCGACAACTAGTGGAGA
7.	*CLV*	F: GTGAATTCCAGCGGTAGCTC R: ATAACCTCACCGTGCTCGAC
8.	*ECP*	F: TATAGGCGAGGCTTTGGAGA R: CTGGCAACATTCATGGTACG
9.	*MAPK*	F: CAAGCATTCTCTCCAGCAAATC R: TGAGACAGCTACCGCAATATC
10.	*AP2*	F: CCCTGATCTCCTTTGAAACCC R: TCACCCTTTCCCTCTCTTATCT
11.	*SAUR*	F: GGATCACGAACCTCTTCATCTC R: AGAGAACCGTCTCCTTCTCTAA
12.	*AGP*	F: ACAAAGTCCTGCTGCCTTAT R: CTGGAGCCTTCTTAGGTTTCTT
13.	*LEA*	F: CCATCTGCGAGATCTCTTACTC R: ATGTCCAACTTGGTCGTCTC
14.	*ANT*	F: ATCTCCAAAGGTGGAGGATTTC R: GGCTCATGAGTGGTGTTGTA

Discussion

Coconut is one of the major economic crops cultivated in more than 93 countries. It is a perennial crop that meets almost all requisites of life, such as food, drink, oil, shelter, etc. The conventional method of propagation through nuts is unable to meet the requirement of quality planting material to replace diseased and senile palms and this has hindered productivity increase. Vegetative propagation by means of tissue culture can be a promising alternative for clonal multiplication of superior quality planting material in coconut. Over the past four decades, even though many endeavors for the clonal propagation of coconut through in vitro techniques were initiated, the success has been limited because of its inherent recalcitrant nature (George and Sherrington 1984). Out of the various explants tried in coconut, plumular explants were found to be better responsive to in vitro interventions (Hornung 1995; Chan et al. 1998; Oropeza et al. 2005; Rajesh et al. 2005, 2014). However, the number of somatic embryos formed and the conversion of somatic embryos into plantlets are the major bottlenecks which are yet to be overcome.

Somatic embryogenesis constitutes a complex developmental program wherein competent somatic cells embark on restructuring through a series of morphological, biochemical, and molecular changes to form embryogenic cells. The molecular events underlying somatic embryogenesis, which involve a differential gene expression pattern triggered by a sequence of signal transduction cascades (Chugh and Khurana 2002), are yet to be decoded precisely. Therefore, identification of genes which play important roles in the molecular regulation of somatic embryogenesis has drawn substantial attention from researchers.

A repertoire of genes have been implicated in regulation of the somatic embryogenesis pathway (Yang and Zhang 2010; Mahdavi-Darvari et al. 2014). Transcriptome analysis of somatic embryogenesis have been undertaken using microarray analyses in soybean (Thibaud-Nissen et al. 2003), maize (Che et al. 2006), and rape seed (Joosen et al. 2007) and suppression subtractive hybridization in oil palm (Lin et al. 2009). With the advent of next-generation sequencing (NGS) technologies, which possess high-throughput and accuracy and are cost-effective, RNA-Seq technique has been implemented to study the gene expression patterns during somatic embryogenesis in cotton (Yang et al. 2012), longan (Lai and Lin 2013), cocoa (Maximova et al. 2014), and maize (Salvo et al. 2014). These studies have provided a fillip to the understanding of genomic factors which are involved in somatic embryogenesis in these crops. Studies on differential expression of genes during somatic embryogenesis in a recalcitrant species like coconut are limited; expressions studies till date have focused on three genes viz., somatic embryogenesis receptor kinase gene (Perez-Nunez et al. 2009), cyclin-dependent kinase (*CDKA*) gene (Montero-Cortes et al. 2010a) and KNOTTED-like homeobox gene (Montero-Cortes et al. 2010b). In coconut, RNA-Seq analyses have been undertaken to characterize genes involved in fatty acid biosynthesis (Fan et al. 2013) and DNA methylation (Huang et al. 2014). In order to obtain better insights into the global gene expression patterns during somatic embryogenesis of coconut, we have performed a RNA-Seq analysis of plumule-derived embryogenic calli on an Illumina HiSeq 2000 sequencing platform.

RNA-Seq analysis generated 50.83 million reads (7.73 Gb bases), and after trimming, 46.2 million reads (5.42 Gb bases) were obtained. The de novo assembly of these reads was conducted because of the unavailability of reference sequence for coconut. In this study, after assembly, we obtained 161,426 unigenes, of which 73,607 transcripts with a length of ≥150 bp and expression of ≥1 RPKM were used for subsequent annotation. Among them, 40,367 (54.84 %) transcripts possessed significant similarity with the NCBI database (BLASTx) and 13,031 transcripts (32.28 %) could be annotated with UniProt database.

A survey of the 15 most highly expressed transcripts in coconut embryogenic calli reflects transcripts with diverse

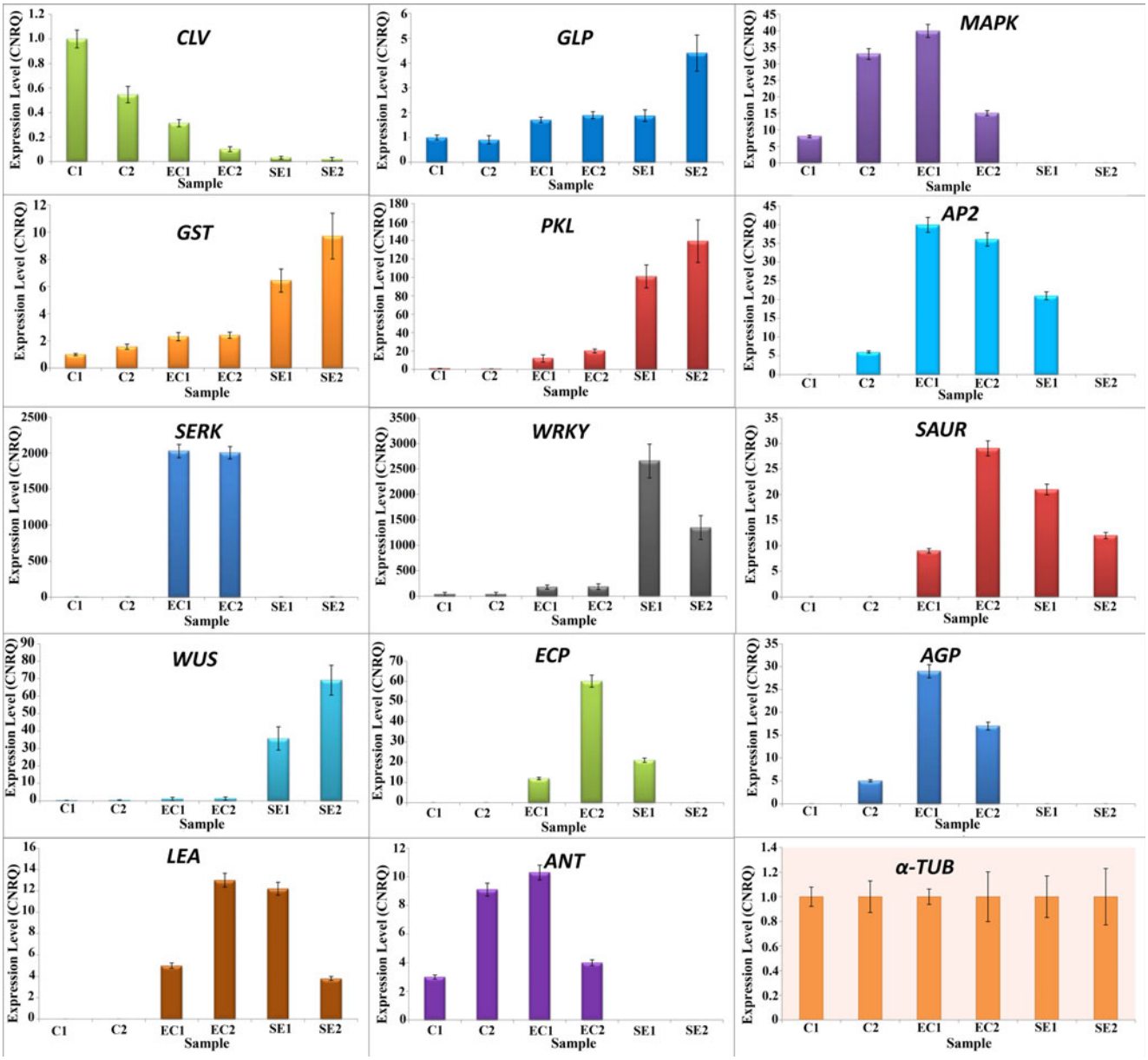

Fig. 3 Calibrated and normalized relative gene expression (CNRQ) levels of 14 transcripts of coconut plumular-derived callus, along with the endogenous control, alpha-tubulin (*α-TUB*). The developmental stages taken are initial calli [30 days (C1) and 60 days (C2)], embryogenic calli [90 days (EC1) and 120 days (EC2)] and somatic embryos [150 days (SE1) and 180 days (SE2)] after inoculation of plumular explants [*x*-axis: samples (C1, C2, EC1, EC2, SE1, SE2); *y*-axis: expression level (CNRQ)]

functions. One among the highly expressed transcripts in coconut embryogenic calli, coding for lipid-transfer protein, has been reported as an early marker for somatic embryogenesis in many plants (Blanckaert et al. 2002; Poulsen et al. 1996; Sabala et al. 2000; Sterk et al. 1991) and might possibly be involved in cell expansion process during embryo development (Dodeman et al. 1997) and cell differentiation (Potocka et al. 2012). Another highly expressed transcript, coding for translationally controlled tumor protein, has been previously implicated for its role in embryo formation in plants, playing an essential role in cell growth and cell division through stabilization of microtubules (Zhang et al. 2013; Qin et al. 2011; Nogueira et al. 2007). The expression of housekeeping protein, elongation factor 1, has been reported early in the process of somatic embryogenesis of carrot cells (Kawahara et al. 1992). Similarly, the expression of 1-cys peroxiredoxin was mainly found to be associated with somatic embryo development in *Medicago truncatula* (Imin et al. 2005) and buck wheat (Akulov et al. 2010). Transcripts encoding metallothionein were found to be expressed during somatic embryogenesis in white spruce (Dong and Dunstan 1996) and Douglas fir (Chatthai et al. 1997). Changes in levels of the polyamine

oxidase, an enzyme involved in polyamine catabolism, has been observed in developing somatic embryos of Norway spruce (Santanen 2000). Changes in expression pattern of thaumatin-like protein were reported in early somatic embryogenesis in white spruce (Lippert et al. 2005) and soybean (Thibaud-Nissen et al. 2003). A change in activity of phosphoenolpyruvate carboxykinase was observed during soybean somatic embryogenesis (Thibaud-Nissen et al. 2003). Expression of pentatricopeptide repeat-containing protein (*PPR*) was detected in embryogenic culture of *Carica papaya* (de Moura et al. 2014). *PPR*s have been reported to be essential for normal morphogenesis of embryos and mutations in *PPR* were found to cause morphological arrest of embryos before the globular-heart transition (Cushing et al. 2005).

We have also attempted to identify transcripts with well-defined roles in somatic embryogenesis based on previous studies in other plant species. A total of 14 such transcripts were identified from coconut embryogenic calli RNA-Seq data. These transcripts included protein kinases like receptor-like kinases [somatic embryogenesis receptor kinase (*SERK*) and CLAVATA 1 (*CLV1*)] and mitogen-activated protein kinase (*MAPK*). Molecular events accompanying the acquisition of embryogeny and transition from somatic cells to somatic embryos involves various signal transduction cascades which ultimately results in activation/repression of various genes. Various receptor-like kinases, which are generally transmembrane protein kinases possessing an extracellular receptor-like ligand binding domain and an intracellular kinase domain, have been reported to be involved in the signaling pathways (Neelakandan and Wang 2012). Since it was first reported in carrot embryogenic cultures (Schmidt et al. 1997), *SERK*, a leucine-rich repeat receptor-like kinase (LRR-RLK), is one of the most well-studied genes, whose expression marks the transition of somatic cells to embryogenic cells. The key role played by *SERK* in induction of somatic embryogenesis has been experimentally demonstrated in a range of monocots and dicots (Mahdavi-Darvari et al. 2014). *SERK* possibly functions in a signal transduction cascade early in the somatic embryogenesis pathway (Schmidt et al. 1997), possibly as a transmembrane receptor (Yang and Zhang 2010). In an earlier study in coconut, expression of *SERK* could be observed in embryogenic tissues before the development of somatic embryos (Perez-Nunez et al. 2009), similar to the results obtained in this study. *CLAVATA1* (*CLV1*), a receptor-like kinase (Clark et al. 1997), is known for its function of maintenance of balance between undifferentiated cells and cells in the shoot apical meristem, which are destined for differentiation into organs (Fletcher et al. 1999). Linking of oxidative stress response to auxin signaling and cell cycle regulation could be achieved by mitogen activated protein kinase (*MAPK*) phosphorylation cascades (Hirt 2000).

The higher accumulation of glutathione S-transferase (*GST*) gene transcript, as observed in the present study, has been reported earlier during early somatic embryogenesis in different plant species (Galland et al. 2001, 2007; Wilson and Wilson 1993; Zdravkovic-Korac and Neskovic 1999; Malabadi et al. 2013). GST is known to play a major role in plant growth and development (Gong et al. 2005; Moons 2005; Jiang et al. 2010) and can be induced by various phytohormones, including ethylene, auxin, methyl jasmonate, salicylic acid, and abscisic acid (Wagner et al. 2002; Moons 2003; Smith et al. 2003). In addition to its possible role in detoxifying excessive amounts of auxin (Guilfoyle 1999), Karami et al. (2009) had suggested that during acquisition of embryogenic capability, expression of *GST* genes could be associated with protection of cells against the detrimental effects of reactive oxygen species. Feher et al. (2003) had suggested GSTs could possibly be involved in tissue de-differentiation by influencing the cell's redox status by altering the endogenous levels of plant growth regulators, especially auxins.

Transcripts coding for transcription factors like *WUSCHEL* (*WUS*), *APETALA2*/Ethylene-responsive factor (*AP2*/*ERF*), *PICKLE* (*PKL*), and *WRKY* were also found to be expressed during embryogenesis from coconut plumule-derived calli. Transcription factors mainly function as regulatory proteins capable of inducing or repressing the transcription of genes as they can possess the capacity to bind to specific DNA sequences and therefore regulate target gene expression (Neelakandan and Wang 2012). They are therefore considered to be essential mediators of developmental transitions and cellular stress responses (Liu et al. 1999). Many transcription factors have established roles in regulating somatic embryogenesis and meristem induction and development. The expression of *WUS*, a homeobox gene, was identified in our transcripts; *WUS* has been reported in the regulation of stem cells in the shoot apical meristem in an undifferentiated state (Endrizzi et al. 1996; Bhalla and Singh 2006). Ectopic expression of *WUS* has been reported to promote vegetative-to-embryogenic transition in *Arabidopsis* (Zuo et al. 2002). *CLV1* acts upstream of *WUS* and is known to repress *WUS* activity by interacting with *WUS*, in a regulatory loop, to promote callus initiation and maintenance (Schoof et al. 2000). AP2/ERF family of transcription factors, which include *APETALA2* (*AP2*), *BABY BOOM* (*BBM*), PLETHORA (*PLT*), *AINTEGUMENTA* (*ANT*), *AINTEGUMENTA*-like (*AIL*) genes, have established roles in induction of somatic embryos and their subsequent development (El Ouakfaoui et al. 2010; Yang and Zhang 2010; Neelakandan and Wang 2012). Ectopic expression of *BBM* has been shown to induce formation of somatic embryos in *Arabidopsis* and *Brassica napus* (Boutilier et al. 2002). Higher expression of coconut *ANT* gene was recorded in coconut embryogenic callus compared to other stages of somatic embryogenesis (Bandupriya et al. 2014). Ectopic expression of oil palm and coconut *ANT* genes in *Arabidopsis* enhanced regeneration capacity via

somatic embryogenesis pathway (Morcillo et al. 2007; Bandupriya et al. 2014). *PKL*, which encodes a chromosome remodeling factor (CDH3 protein) involved in the repression of transcription, has been reported to be master regulator of somatic embryogenesis (Karami et al. 2009). *PKL* has been proposed to be part of a gibberellin-modulated developmental switch that functions during germination and causes transcriptional repression of genes coding for leafy cotyledon (*LEC*) and seed storage proteins, thus ensuring that traits expressed during embryogenesis are not expressed after germination (Ogas et al. 1999; Nolan et al. 2006). Enhanced expression of *WRKY* has been earlier reported during somatic embryogenesis in cell cultures of *Cyclamen persicum* (Rensing et al. 2005) and leaf tissues of *Dactylis glomerata* (Alexandrova and Conger 2002).

We have also observed expression of genes coding for certain proteins viz., arabinogalactan protein (*AGP*), germin-like protein (GLP), embryogenic cell protein (*ECP*), and late embryogenesis-abundant protein (*LEA*) in coconut embryogenic calli. There are many evidences for synthesis and mobilization of certain proteins during induction of embryogenic competence and somatic embryogenesis (reviews by Yang and Zhang 2010; Mahdavi-Darvari et al. 2014) with possible roles in transduction of signal cascades, as substrates for cell growth and morphogenesis (Lulsdorf et al. 1992) or in the regulation of cell expansion and establishment of biophysical characteristics required for the morphogenesis (Jimenez 2001). During somatic embryogenesis, the embryogenic cells show structural rearrangement in their extracellular matrix, which were considered as structural markers for embryogenic competence in different plant systems (Rumyantseva et al. 2003; Konieczny et al. 2005). AGPs play a major role in these structural changes; additionally, their roles in intracellular and intercellular signaling and also in apoptotic events during embryogenic development has also been suggested (Samaj et al. 2005). AGPs have been reported to stimulate somatic embryogenesis in *Daucus carota* (McCabe et al. 1997), and *Dactylis glomerata* (Zagorchev et al. 2013). Germins and germin-like proteins (GLPs), which are apoplastic proteins of cupin superfamily (Dunwell 1998), have been used as markers for somatic embryogenesis (Tchorbadjieva et al. 2004; Tchorbadjieva 2005). The role of GLPs in enhancing somatic embryogenesis has been reported in *Pinus caribaea* (Domon et al. 1995) and *Pinus radiata* (Bishop-Hurley et al. 2003). Mathieu et al. (2006) has reported that a GLP played a key role in somatic embryo formation in hybrid larch possibly through regulation of cell wall remodeling, which was essential for correct embryo development. Expression of *ECP*s have been reported to be regulated by ABA-inducible genes during acquisition of embryogenic competence (Kiyosue et al. 1992). Similarly, enhanced expression of *LEA* genes, which are also regulated by ABA-inducible genes, have been observed during the later stages of somatic embryo formation (Chugh and Khurana 2002).

To experimentally conform that transcripts obtained from sequencing and computational analyses were indeed expressed, the expression patterns of 14 transcripts were studied through qPCR analysis in six different developmental stages. All these 14 genes were expressed at varying levels during different developmental stages indicating their potential roles in coconut somatic embryogenesis. The role of *SERK* in induction of somatic embryogenesis has been demonstrated in many plants, both monocots and dicots (Mahdavi-Darvari et al. 2014). Zhang et al. (2011) has reported expression of *SERK* in maize was closely related to auxin signaling during initial stages of somatic embryogenesis. The expression of *SERK* has been reported to be reduced in a gradual manner during subsequent development stages of somatic embryogenesis (Hecht et al. 2001; Schmidt et al. 1997), a trend which was observed in the present study too. Increased expression of *GLP* and *WUS* was observed during early stages of formation of somatic embryos in *Pinus radiata* (Bishop-Hurley et al. 2003) and *Ocotea catharinensis* (Santa-Catarina et al. 2012), respectively, and similar results were obtained in coconut.

Utilizing GO and KEGG pathway-based analysis, a global insight into genes expressed in coconut embryogenic calli is provided in this study. The GO study provides annotation concerning molecular function, cellular component, and biological processing by Blast2GO (Conesa et al. 2005). The GO observations revealed that transcripts from coconut embryogenic calli represented all important developmental processes of a cell. Nearly 14,287 of the transcripts were mapped onto 115 pathways using KEGG analysis. Also, a total of 2705 enzymes were identified from the KEGG analysis. Among the 112 KEGG pathways identified, four pathways were associated with the signal transduction process which included 786 transcripts encoding 74 enzymes. The four pathways viz., MAPK signaling pathway, calcium signaling pathway, plant hormone signal pathway, and phosphatidylinositol signaling pathway have been reported earlier for their indispensable role in somatic embryogenesis, as discussed below. Auxin is one of the extensively used plant growth regulator in tissue culture for callus induction and somatic embryogenesis (Jimenez 2005; Joshi and Kumar 2013). The essential role of auxin (2, 4- D) in micropropagation of coconut using plumular tissues has been previously reported (Verdeil and Buffard-Morel 1995; Fernando et al. 2004; Rajesh et al. 2005, 2014). Auxin-mediated signaling has an important role in dedifferentiation and redifferentiation of cells (Yang et al. 2012). We obtained 82 transcript hits in the MAPK signaling pathway, which included nine enzymes of the MAPK pathway. The involvement of MAPKs in hormonal responses, cell cycle regulation, abiotic stress signaling, and defense mechanisms have been well established (Kovtun et al. 2000; Tena et al. 2001). Evidences have been provided through earlier studies on the role of MAPK phosphorylation cascades in linking auxin signaling to oxidative stress response and cell

cycle regulation (Hirt 2000; Neil et al. 2002). Recent studies have revealed the specific role of MAPK pathways in auxin signal transduction and in the control of the cell cycle (Zhao et al. 2013, 2014). Also, 61 of the transcripts, corresponding to seven enzymes, were mapped onto calcium signaling pathways. The potential involvement of calcium concentration and the twofold increase in somatic embryogenesis was reported in carrot embryogenic suspension cultures (Jansen et al. 1990). The crucial role played by calmodulin, a calcium-binding messenger protein mediating the calcium signaling pathway, has been previously reported in sugarcane embryogenesis (Suprasanna et al. 2004). Also, studies in sandalwood (Anil and Rao 2000), *Panax ginseng* (Kiselev et al. 2008) and *Albizia lebbeck* (Kaul et al. 2014) have highlighted the role of Ca^{2+}-mediated signaling in induction and regulation of somatic embryogenesis. A total of 171 transcripts from the present study were identified in phosphatidylinositol pathway, which mapped against 17 enzymes. The role of phosphatidylinositol signaling system has been reported in regulation of somatic embryogenesis in *Coffea arabica* (Ek-Ramos et al. 2003). These annotations provide a valuable resource for investigating specific processes, functions, and pathways and allow for the identification of novel genes involved in somatic embryogenic pathway in coconut.

To conclude, in the present study, we have made available the first large-scale and publicly available EST resource for coconut during somatic embryogenesis. Plant growth regulators are known to play key roles in cell differentiation, vegetative-to-embryogenic transitions, and somatic embryo development and maturation. The transcriptome analysis presented here provides vital information towards augmenting understanding of this complex mechanism, which could assist additional investigations of the comprehensive molecular mechanisms underlying somatic embryogenesis. The identification of these genes offers a possibility for their use as markers for coconut somatic embryogenesis, especially for ascertaining the embryogenic potential of somatic cells before the onset of any morphological changes. We hope that better understanding of the mechanisms that trigger and regulate coconut somatic embryogenesis would guide establishment and optimization of rational regeneration protocols in coconut, which has otherwise remained recalcitrant to in vitro interventions. Future efforts could be directed to induce/enhance expression of specific transcripts during specific developmental stages so as to augment formation of embryogenic calli and somatic embryos by making the explants more competent to respond to growth regulators.

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Conflict of interest The authors declare that they have no competing interests.

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