Selection and validation of reference genes for real-time qRT-PCR normalization in different tissues of *Eucalyptus tereticornis*

By B. KARPAGA RAJA SUNDARI[1)] and M. GHOSH DASGUPTA[1),*)]

(Received 12th June 2012)

Abstract

Reference genes are generally used as endogenous normalization factor for relative quantification of target genes in quantitative real-time PCR (qRT-PCR). The present work aimed at identifying suitable reference genes for normalization of qRT-PCR data in tissues of *Eucalyptus tereticornis*. The expression levels of housekeeping genes like Actin (*EtAct2*), Isocitrate dehydrogenase (*EtIDH*), ribosomal RNA (*Et18s rRNA*), SAND family protein (*EtSAND*), Histone protein (*EtH2B*), αTubulin (*EtTUB*), and eukaryotic initiation factor (*EteIF4B*) were studied to characterize their normalization stability in different tissues including young leaves, internodes, developing and mature xylem. The expression level of these genes was analyzed using different algorithms like geNorm, NormFinder and BestKeeper. Among the seven reference genes analyzed, *EtAct2* was expressed with less variance and was found to be the most stable reference gene across different tissues using all the three programs, while the least stable gene identified was *EtH2B*. Further, the normalization efficiency of the reference genes were assessed to predict the expression levels of three primary cell wall specific cellulose synthase transcripts (*EtCesAs*) in *E. tereticornis* tissues. The relative expression of *EtCesA4*, *EtCesA5* and *EtCesA6* was determined to be 3-19 fold higher in leaf and internode tissues when compared to developing and mature xylem tissues. This study will allow accurate normalization of qRT-PCR experiments across different tissues in *E. tereticornis* for future genomic research in this tropical eucalypt species.

Key words: Reference gene, selection, normalization, cellulose synthase gene, differential expression, qRT-PCR, *Eucalyptus*.

Introduction

The genus *Eucalyptus* (2n = 22) with more than 800 species is native to Australia and its neighboring islands and is grown across Australia, Asia, South America, Southern Europe and Africa (TURNBULL, 1999). It is one of the widely planted hardwood species in the world because of its superior growth, adaptability and wood properties (FAO, 2005). Eucalypts occupy 19.61 M hectares globally and India ranks second in area under eucalypts plantation (3.943 M ha) after Brazil (4.259 M ha) (IGLESIAS TRABADO and WILSTERMANN, 2008). The major eucalypt species planted for use as raw material for paper and pulp industries include *E. globulus*, *E. grandis*, *E. nitens*, *E. camaldulensis*, *E. urophylla*, *E. tereticornis*, and *E. viminalis* (MAXWELL, 2004).

Eucalyptus tereticornis Sm., commonly known as forest red gum has an extensive natural distribution from southern Papua New Guinea to southern Victoria of Australia (5°20'S–38°08'S). The species ranks among the most extensively planted *Eucalyptus* in the tropics and subtropics (ZOBEL et al., 1987; EVANS, 1992). It has been introduced to several countries to provide wood for fuel, poles, construction and pulp. Its popularity is attributed to rapid growth and production of desirable wood in a wide range of environmental and soil conditions (Zobel et al., 1987). Molecular studies in this species is restricted to the application of molecular markers for genetic diversity analysis in seed orchards, species identification and genetic/QTL mapping (BALASARAVANAN et al., 2005, 2006, CHEZHIAN et al., 2010; GAN et al., 2003; MARQUES et al., 1998). However, gene expression studies in *E. tereticornis* are sparse and genomic resources available in public domain databases are limited to few EST submissions (POKE, 2004).

Reliable quantification of gene expression levels by qRT-PCR requires the standardization and fine-tuning of several parameters, such as amount of initial sample, RNA recovery and integrity, enzymatic efficiencies of cDNA synthesis and PCR amplification, overall transcriptional activity of the tissues or cells analyzed (GINZINGER et al., 2002). Among various methods, internal control genes (reference genes) are most commonly used to normalize qRT-PCR data and reduce possible errors generated during quantification of gene expression (GINZINGER et al., 2002; HUGGETT et al., 2005). Nevertheless, this method relies on the choice of appropriate control genes, which ideally has stable expression under different experimental conditions and in different tissues types.

The reference genes most widely used for qRT-PCR analysis in plant species include 18S rRNA, actin, tubulin, polyubiquitin and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), which have been commonly employed also in traditional methods of gene expression analysis such as northern blot, RNase protection test and conventional semi-quantitative RT-PCR (HUGGETT et al., 2005). However, several reports have shown that the most widely used housekeeping genes (HKG) are not reliable controls, since their expression level varies with tissues and experimental conditions, making them unsuitable for normalization of gene expression data (SELVEY et al., 2001; CZECHOWSKI et al., 2005; GUTIERREZ et al., 2008; BUSTIN, 2002; BUSTIN and NOLAN, 2004; LEE et al., 2001; SUZUKI et al., 2000) as they participate in cellular functions (ISHITANI et al., 1996). Thus, it is nec-

[1)] Division of Plant Biotechnology, Institute of Forest Genetics and Tree Breeding, P.B. No. 1061, R.S. Puram P.O., Coimbatore-641002, India.

[*)] Corresponding author: MODHUMITA GHOSH DASGUPTA (Scientist E). Tel: +91 422 2484123, Fax: +91 422 2430549. E-Mail: ghoshm@icfre.org; modhumitaghosh@hotmail.com

DOI:10.1515/sg-2012-0035
edited by Thünen Institute of Forest Genetics

essary to conduct preliminary evaluation for identifying the most stable reference gene in each species and under specific experimental conditions (different type of tissues and types of treatment etc.). The importance of reference genes for qRT-PCR analysis in plants has been emphasized in several reports even though the identification of these genes is quite laborious (REID et al., 2006; BRUNNER et al., 2004).

Hence, the present study was undertaken to identify suitable reference gene(s) for normalization of qRT-PCR data. Further, the differential expression of primary cell wall specific cellulose synthase transcripts were analyzed in different tissues using the most stable reference gene combination for data normalization. This study will provide a reference in future for conducting qRT-PCR analysis in *E. tereticornis* tissues.

Material and Methods

One gram tissue was harvested from fifteen year old *E. tereticornis* tree (South of Helenvale Provenance, CSIRO seed lot no. 12944) maintained at the seed orchard at Karunya Research Station, Coimbatore, India. The tissues used for this study included leaves, internodes, developing xylem (1.5 cm of the soft wood) and mature xylem tissues (3.0 cm of the soft wood). All tissues were sourced from the same tree. Fresh tissues were harvested and immediately frozen in liquid nitrogen and stored at -80°C until RNA isolation.

Total RNA isolation and cDNA synthesis

RNA was extracted from all the tissue samples (100 mg) using an in-house protocol (Patent Pending). The integrity of RNA samples were assessed in 1% (w/v) agarose gel electrophoresis followed by purification using Nucleospin RNA Cleanup kit (Machery Nagel, Germany) following the manufacturer's protocol. The quantity and purity of RNA was quantified in NanoDrop-ND1000 spectrophotometer (Thermo Fisher Scientific Inc., USA). Subsequently, total RNA was treated with RNase free DNase I (Fermentas, USA) according to the manufacturer's protocol and first strand cDNA was synthesized from all tissues using cDNA synthesis kit (Fermentas, USA) with 10 µm oligo dT primer and random hexamer (used only for18s rRNA) and 500ng of purified total RNA followed by incubation at 37 °C for 60 min and inactivation at 65 °C for 5 min. The concentration of cDNA was also quantified using Nanodrop-ND 1000 (Thermo Fisher Scientific Inc, USA).

Selection of endogenous reference gene for qRT-PCR

A set of seven reference genes were selected for the study and primer pairs were synthesized for Actin (*EtAct2*) α-Tubulin (*EtTUB*) and eukaryotic initiation factor (*EteIF4B*) (BAOVA et al., 2010), 18srRNA (*Et18s*) (OLBRICH et al., 2008) and NADP Isocitrate dehydrogenase (*EtIDH*), SAND protein (*EtSAND*) and Histone protein (*EtH2B*) (DE ALMEIDA et al., 2010) for qRT-PCR analysis (*Table 1*). Initially, PCR amplification was conducted in the first strand cDNA derived from different tissues to determine the cross amplification of primer pairs in *E. tereticornis*. The PCR reaction included 200ng of cDNA, 10 µM forward and reverse gene specific primers, 1 µl of 10x Taq buffer, 1 unit of Prime Taq polymerase (Genetbio, Korea), 0.4 µl of 2.5mM dNTP mix (Sigma, USA) in a 10 µl reaction and amplified with the following program: 94 °C for 5min, 30 cycles of 94 °C for 1 min, 58 °C for 30 seconds, 72 °C for 2 min and final extension at 72 °C for 7 minutes in Veriti 96 well Thermal cycler (Applied Biosystems, USA). The PCR products were checked for amplification using 1% (w/v) agarose gel electrophoresis and sequenced.

Amplification of primary cell wall specific cellulose synthase (EtCesA) transcripts in E. tereticornis tissues

Total RNA was isolated from the internode tissues using the in-house protocol and cDNA was synthesized as described above. Primer pairs targeting the CSR II region of *EtCesA* genes expressed in primary cell wall (*EtCesA4*, *CesA5* and *EtCesA6*) were synthesized (Sigma Aldrich Ltd, USA) based on the earlier report from *E. grandis* by RANIK and Myburg (2006) (*Table 1*). Approximately, 200 ng of cDNA was used as template in 10 µl reaction mixture and products were amplified using the program: 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 58 °C for 30 seconds, 72 °C for 2 min and final extension at 72 °C for 7 minutes in Veriti 96 well Thermal cycler (Applied Biosystem, USA). The PCR products were analyzed on 1% (w/v) Agarose gel and sequenced.

qRT-PCR of endogenous reference genes and EtCesA transcripts

The qRT-PCR reactions were performed in fast optical reaction tube (Microamp- Applied Biosystems,USA) and 25 µl reaction included 11.5 µl Milli-q water, 200 ng of cDNA, 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems, USA), 0.5 µl each of 200 nM forward and reverse primer. A negative control with water (instead of template cDNA) was also included. All reactions were conducted in three independent technical replicates in ABI PRISM 7500 Step one plus Sequence Detection System (Applied Biosystems, USA) using the following program: one cycle of 95 °C for 10 min; 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. The melting curve was determined for each primer pair to confirm the specificity of the amplified product.

Statistical analysis for stability of gene expression

The expression level and stability of seven endogenous genes in different tissues were evaluated with three statistical programs including geNorm (VANDESOMPELE, 2002) downloaded from GenEX standard software (http://GenEx.gene-quantification.info), Normfinder Version 0.953 (ANDERSEN, 2004) downloaded from (http://www.mdl.dk/publicationsnormfinder.html) and BestKeeper version1 (PFAFFL et al., 2004) downloaded from (http://www.gene-quantification.de/bestkeeper.html). Expression levels were assessed based on the number of amplification cycles needed to reach a fixed threshold (Cycle threshold-Ct) in the exponential phase of PCR.

In the first approach, Ct values were converted to relative quantity (log transformed) using sample with low-

DOI:10.1515/sg-2012-0035
edited by Thünen Institute of Forest Genetics

Table 1. – Primer sequences and amplicon size of reference genes and *EtCesA* transcripts.

Reference gene	Primer ID	Primer sequence (5' - 3') Forward/Reverse	Amplicon size (in bp)	Accession number
EtAct2	EgACTFP	TAAGCATGACAAGGAACCAG	150	JX080713
	EgACTRP	TCAGGTCCAAGAAATCGT		
Et18S rRNA	Fs18SrRNAFP	AAACGGCTACCACATCCAAG	200	JX080715
	Fs18SrRNARP	CCTCCAATGGATCCTCGTTA		
EtIDH	EgIIDHFP	CTGTTGAGTCTGGAAGATGAC	250	JX080719
	EgIIDHRP	CATTTAATTCCTCCCCAACAAA		
EtSAND	EgISANDFP	CCATTCAACACTCTCCGACA	250	JX080717
	EgISANDRP	TGTGTGACCCAGCAGAGTAAT		
EtH2B	EgIH_2BFP	GAAGAAGCGGGTGAAGAAGA	200	JX080718
	EgIH_2BRP	GGCGAGTTTCTCGAAGATGT		
EtTUB	EgITUBFP	GTGACATTCCTCCGACTG	150	JX080714
	EgITUBRP	GCAAGAAAGCCTTCCTG		
EteIF4	EgIEIF4BFP	CCCAAATATGAACCGTCCA	200	JX080716
	EgIEIF4BRP	GTTCGATCCATAGCGTCC		
EtCesA4	EgCesA4FP	AGCCAAAGCAGAGAAAGTCA	350	JM175073
	EgCesA4RP	ATAAGCGTAGAGGCCACAAA		
EtCesA5	EgCesA5FP	GGGAAGGGTGGCAATAAGAA	400	HR182513
	EgCesA5RP	AACAGGAGACTGACCGAATC		
EtCesA6	EgCesA6FP	CCACCACCTTTGGAAGGTAT	400	HR182514
	EgCesA6RP	GGCTCGTGCCTTTCAGTGTT		

est Ct as calibrator and then imported to geNorm V.35 software. The gene with lowest M (stability value) value was considered to have most stable expression (VANDESOMPELE, 2002). In the second evaluation algorithm, Ct values were log transformed and the values were imported into NormFinder program which calculates M value based on inter and intra- specific variation between samples (ANDERSEN, 2004). In BestKeeper, the average Cp (Crossing point) values of each triplicate reaction was used to analyze the stability value of studied genes. BestKeeper creates a pairwise correlation coefficient between each gene and generates the BestKeeper index (BI). The gene with the highest coefficient of variance with BI indicates the highest stability (PFAFFL et al., 2004).

Determination of relative expression of primary cell wall specific cellulose synthase transcripts

The relative expression profiles of *EtCesA4*, *EtCesA5* and *EtCesA6* were analyzed in different tissues of *E. tereticornis* (leaf, internodes, developing xylem and mature xylem). The relative expression analysis was obtained through the delta-Ct method using *EtAct2* as endogenous control and *EtSAND* as the calibrator. Analysis was also conducted using *EtAct2* in combination with other reference genes to determine the most stable gene combination.

Results and Discussion

Cross amplification of reference genes in E. tereticornis

PCR amplification was conducted for all the seven reference genes in the first strand cDNA derived from all tissues to determine the cross amplification of primer pairs in *E. tereticornis*. The amplicon size ranged from 150bp to 250 bp (*Table 1*). The amplicon size of *EtAct*, *EtTUB*, and *EteIF4B* were comparable to their orthologs from *E. grandis* hybrids as reported by BAOVA et al. (2010), while the amplicons size of *EtSAND*, *EtIDH* and *EtH2B* were comparable to *E. globulus* (DE ALMEIDA et al., 2010). The amplicon sequences showed significant similarity to their respective orthologs (actin, tubulin, *eIF4B*, *SAND*, *IDH*, *18s rRNA* and *H2B*) from other plant species and the sequences were submitted to NCBI (GenBank accession numbers are given in *Table 1*).

DOI:10.1515/sg-2012-0035
edited by Thünen Institute of Forest Genetics

Normalization of reference gene in qRT-PCR

In the present study, the geNorm software predicted that the M value was lowest for *EtAct2* (1.10147) and *EtSAND* (1.10147) indicating that they were the most stably expressed gene pair across four different tissues, while the least stable gene was *EtH2B* with highest M value of 3.33819 (*Table 1*; *Fig. 1*). Similarly, Normfinder analysis revealed *EtAct2* (1.0875) as the best reference gene with lowest stability value (*Table 2*) and *EtAct2* and *EtTUB* was predicted as the best combination of genes with a stability value of 1.1207 (data not shown). BestKeeper program calculated the gene with the lowest variation as *EtAct2* with standard deviation (±CP) of 0.60, while the highest variation was observed using *EtH2B* with standard deviation of 3.98 (data not shown). All the three programs revealed *EtAct2* as the best reference gene for normalization experiments in tissues of *E. tereticornis* (*Table 2*).

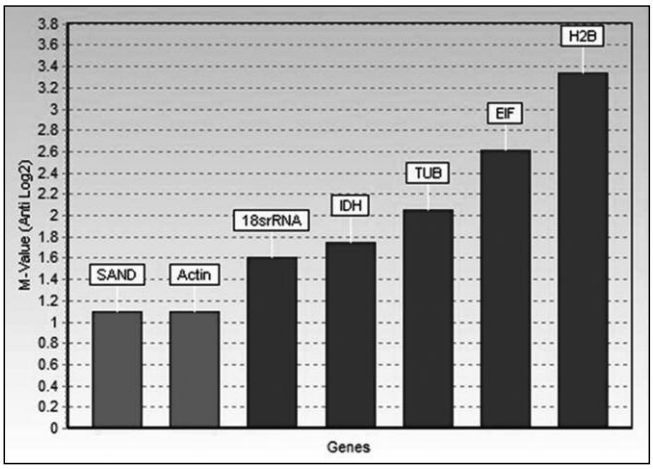

Figure 1. – Stability value of the reference genes calculated using geNorm. The figure indicates the average expression stability values of the reference genes, obtained by geNorm analysis where M value indicates the stability value of the gene.

In *Populus* hardwood cuttings, *18srRNA* along with *Ef1a* gene were found to be the most appropriate reference gene for expression studies during adventitious rooting (Xu et al., 2011) while in Peach (*Prunus persica*), polyubiquitin, translation elongation factor (*TEF2*) and RNA polymerase II (*RPII*) were selected as reliable reference gene across different developmental stages and tissues (Tong et al., 2009). However, in European Beech (*Fagus sylvatica*), *Act2* was identified as the most stable housekeeping gene during biotic and abiotic stress condition (Olbrich et al., 2008). In *Quercus suber*, selection and validation of ten candidate reference genes were carried out in different tissues and experimental conditions and *Act* and *CACs* (Clathrin adaptor complexes medium subunit family) were reported as the most stable genes (lowest M value of 0.462), followed by *EF-1a* (M value of 0.525) using geNorm and NormFinder algorithm (Marum et al., 2012). In all the above reports, a set of five to seven reference genes were evaluated for their suitability in gene expression studies.

In eucalypts, several studies on differential expression of genes using qRT-PCR are reported, but most of the studies have adopted only one endogenous reference gene for normalization, without screening for suitable reference genes. In *E. globulus*, *EgIDH* (Isocitrate Dehydrogenase) gene was used as candidate reference gene for differential expression of target gene expression in secondary xylem and root (Goicoechea et al., 2005; Legay et al., 2007), while in *E. grandis*, *Arf* (ADP ribosylation factor) was used for normalizing the differential expression of primary and secondary cell wall specific cellulose synthase genes (Ranik and Myburg, 2006). However, selection of reference genes was performed for normalization studies in *E. globulus*, where Histone (H_2B) and tubulin (*Tub*) were reported to be the best reference genes to understand the gene expression during adventitious rooting (De Almeida et al., 2010), while polyubiquitin C (*UBC*), α-tubulin (α-*TUB*) and elongation factor 1-α (*EF1-α*) were reported as the most stable genes across cold acclimation and de-acclimation treat-

Table 2. – Ranking of the candidate reference genes according to their order of stability calculated by geNorm, NormFinder and BestKeeper.

Gene ID	Stability value (geNorm)	Ranking order (geNorm)	Stability value (NormFinder)	Ranking order (NormFinder)	Stability value (BestKeeper)	Ranking order (BestKeeper)
EtAct2	1.10147944063115	1	1.0875	1	1.51	1
Et18srRNA	1.60275340653554	2	2.3419	4	1.77	2
EtTUB	2.0453561374345	4	1.1207	2	4.34	5
EtIDH	1.74914148013525	3	1.6805	3	2.46	3
EtSAND	1.10147944063115	1	2.5707	5	1.77	2
EteIF4B	2.61650974761033	5	2.6603	6	4.28	4
EtH_2B	3.33819366894731	6	5.0637	7	15.78	6

DOI:10.1515/sg-2012-0035
edited by Thünen Institute of Forest Genetics

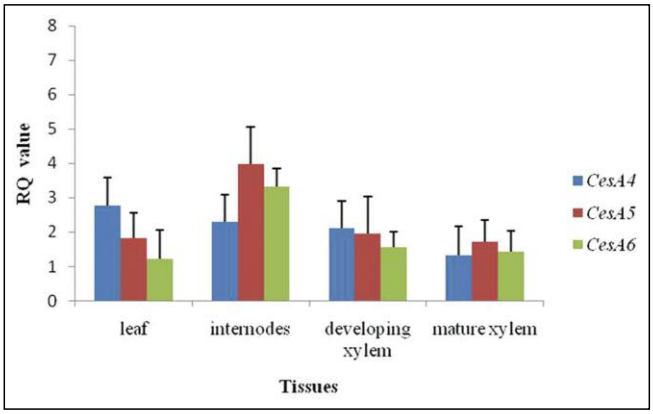

Figure 2. – Relative quantification of *EtCesA* gene transcript expression using *EtAct* (Stable house keeping gene). The figure indicates the Relative quantification value (RQ) of all the primary cell wall specific *EtCesA* gene expression plotted for different tissues using *Actin* as stable housekeeping gene.

ments (FERNANDEZ et al., 2010). *EgIDH* was identified as the most stably expressed reference gene in *Eucalyptus* hybrid clones (*E. grandis* × *E. urophylla*), subjected to biotic and abiotic stress condition (BOAVA et al., 2010).

In the present study, seven genes were tested for their suitability as reference gene and *EtAct2* was identified as the most stable gene across different tissues and along with *EtSAND* as the best gene combination for qRT-PCR analysis. This is the first report in *Eucalyptus* where actin was found to be the most suitable gene for normalization of qRT-PCR experiments.

Relative expression of primary cell wall specific cellulose synthase transcripts using selected reference genes

All the three *CesA* genes including *EtCesA4*, *EtCesA5* and *EtCesA6* transcripts were amplified in the internode tissues with amplicon size of 350bp, 400bp and 400bp respectively (*Table 1*). The amplicons were sequenced and the sequences showed significant similarity to their orthologs from *E. grandis* with an E-value of 2e-10, 2e-08 and 8e-50 for *EtCesA4*, *EtCesA5* and *EtCesA6* respectively and the sequences were submitted to NCBI (GenBank Accession No. given in *Table 1*).

The relative expression of *EtCesA4*, *EtCesA5* and *EtCesA6* was analyzed using *EtAct2* (most stable reference gene) in combination with other reference genes. When *EtAct2* was used as reference gene in combination with *EtSAND*, the relative expression profile of *EtCesA4* was 8 and 15 fold higher in leaf and internode tissues respectively when compared to developing xylem and mature xylem. Similarly, the expression of *EtCesA5* and *EtCesA6* were 8 to 19 fold and 3 to 7 fold higher in leaf and internode tissues, indicating that all three genes were predominantly expressed in primary cell wall of *E. tereticornis* (*Fig. 2*). Nevertheless, when *EtAct2* was used in combination with *EtH2B* (least stable reference gene), the relative expression of *EtCesA* genes varied from 1.8 to 90 fold in leaf and internode tissues while in developing xylem and mature xylem the relative expression was 0.3 to 15 fold, revealing its unsuitability for normalization of qRT-PCR data in *E. tereticornis* tissues (*Table 3*).

The cellulose synthase (*CesA*) and cellulose synthase like (*Csl*) genes are among the most important players involved in the biosynthesis of plant cell wall, which are mainly composed of biopolymers such as celluloses, hemicelluloses, pectins and lignins (DOBLIN et al., 2002, JOSHI, 2003). Specialized plant cells (e.g., xylem and phloem fibers) sequentially deposit primary and secondary cell wall that differ significantly in the quantity and quality of cellulose. Recent studies in several higher plant species have demonstrated that two groups of *CesA* gene families exist which are associated with either primary or secondary cell wall deposition as reported in *E. grandis* (RANIK and MYBURG, 2006; LU et

Table 3. – Validation of Relative expression level of *EtCesA* transcripts using different reference gene combinations.

Transcripts	Tissues types	Relative expression level using reference gene combinations					
		EtAct2* & *EtSAND (Best reference gene combination)	***EtAct2* & *Et18srRNA***	***EtAct2* & *EteIF4B***	***EtAct2* & *EtIDH***	***EtAct2* & *EtTUB***	***EtAct2* & *EtH$_2$B*** (Least stable reference gene)
EtCesA4	Leaf	8.71	8.26	0.42	33.75	7.34	90.52
	Internodes	15.76	98.14	47.53	55.05	12.61	1.86
	Developing xylem	1.67	1.20	108.94	110.59	0.97	0.71
	Mature xylem	2.44	3.62	52.53	52.53	0.27	8.97
EtCesA5	Leaf	8.24	5.47	0.79	3.09	5.26	84.89
	Internodes	19.46	13.16	63.21	45.95	14.35	41.24
	Developing xylem	1.65	2.12	103.06	391.88	1.32	0.34
	Mature xylem	3.64	9.35	21.39	26.68	0.52	0.51
EtCesA6	Leaf	7.27	26.41	0.92	17.33	2.74	37.45
	Internodes	3.54	181.70	125.44	281.36	3.78	3.75
	Developing xylem	0.75	2.36	258.68	84.42	1.82	14.62
	Mature xylem	1.70	7.70	1.04	16.79	0.45	0.50

DOI:10.1515/sg-2012-0035
edited by Thünen Institute of Forest Genetics

al., 2008), *Populus tremuloides* (JOSHI et al., 2004), *Arabidopsis thaliana* (ARIOLI et al., 1998; FAGARD et al., 2000), *Pinus taeda* (NAIRN and HASELKORN, 2005), *Gossypium hirsutum* (KIM et al., 2012), *Solanum tuberosum* (OBEMBE et al., 2009) and *Bambusa oldhamii* (CHEN et al., 2010).

In *Eucalyptus grandis*, the differential expression patterns of *CesAs* were analyzed in different tissues and three primary (*CesA4*, *5* and *6*) and three secondary cell wall specific *CesAs* (*CesA1*, *2* and *3*) were reported. The expression of *EgCesA4* was about three fold more abundant in unfolding leaves than in immature xylem while the expression of *EgCesA6* was very low in all tissues (RANIK and MYBURG, 2006). Similarly, in the present study, *EtCesA4* also expressed 8 and 15 fold higher in leaves and internode tissues respectively, while expression of *EtCesA5* was 8 and 19 folds higher in leaf and internode tissues when compared to developing and mature xylem. However, in the present study the relative expression of *EtCesA6* was 7 fold higher in leaf tissues which was in variance with its expression levels in *E. grandis*, where no significant expression was reported (RANIK and MYBURG, 2006). This could be attributed to the age of the tree sampled in both the studies, where in the tissues were sourced from a fifteen year old tree in *E. tereticornis* in comparison to a four year old tree sampled in *E. grandis*. In woody species, the physiological and phenotypic changes associated with age and developmental stages are well documented with precise differential gene expression with advancing age (DAY et al., 2002). Recently, in *Pinus radiata*, the variations in expression of 174 genes were studied to understand the loss of morphogenetic capacity with the aging process (ALVAREZ et al., 2011). However, there are no specific reports on the differential expression of cellulose synthase genes with tree age.

In conclusion, the identification of stable reference genes for normalization of qRT-PCR experiments will enable reliable estimation of gene expression in tissues of *E. tereticornis*, a predominant pulpwood species in tropical and sub tropical countries.

Acknowledgements

The authors acknowledge the research grant from Indian Council of Forestry Research and Education, Government of India. The authors also acknowledge Dr. VISWANATHAN, Head, Division of Plant pathology, Sugarcane breeding institute, Coimbatore for providing Real time PCR facility.

References

ALVAREZ, C., L. VALLEDOR, R. HASBÚN, M. SANCHEZ-OLATE and D. RÍOS (2011): Variation in gene expression profile with aging of *Pinus radiata* D. Don. BMC Proceedings 2011, **5** (Suppl 7): P62.

ANDERSEN, C. L., J. L. JENSEN and T. F. ORNTOFT (2004): Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res **64** (15): 5245–5250.

ARIOLI, T., L. PENG, A. S. BETZNER, J. BURN, W. WITTKE, W. HERTH, C. CAMILLERI, H. HOFTE, J. PLAZINSKI, R. BIRCH, A. CORK, J. GLOVER, J. REDMOND and R. E. WILLIAMSON (1998): Molecular analysis of cellulose biosynthesis in Arabidopsis. Science **279**: 717–720.

BALASARAVANAN, T., P. CHEZHIAN, R. KAMALAKANNAN, M. GHOSH, R. YASODHA, M. VARGHESE and K. GURUMURTHI (2005): Determination of inter- and intra-species genetic relationships among six *Eucalyptus* species based on inter-simple sequence repeats (ISSR). Tree Physiol **25**: 1295–1302.

BALASARAVANAN , T., P. CHEZHIAN, R. KAMALAKANNAN, VARGHESE YASODHA, M. VARGHESE, K. GURUMURTHI and M. GHOSH (2006): Identification of species-diagnostic ISSR markers for six *Eucalyptus* species. Silvae Genet **55**(3): 119–122.

BOAVA, L. P., M. L. LAIA, T. R. JACOB, K. M. DABBAS, J. F. GONÇALVES, J. A. FERRO, M. I. T. FERRO and E. L. FURTADO (2010): Selection of endogenous genes for gene expression studies in *Eucalyptus* under biotic (*Puccinia psidii*) and abiotic (acibenzolar-S-methyl) stresses using QRT-PCR. BMC Res Notes **3**: 43.

BRUNNER, A. M., I. A. YAKOVLEV and S. H. STRAUSS (2004): Validating internal controls for quantitative plant gene expression studies. BMC Plant Biol **4**: 14.

BUSTIN, S. A. (2002): Quantification of mRNA using real-time reverse transcription PCR (RT-PCR) trends and problems. J Mol Endocrinol **29**: 23–39.

BUSTIN, S. A. and T. NOLAN (2004): Pitfalls of quantitative real-time reverse transcription polymerase chain reaction. J Biomol Tech **15**(3): 155–166.

CHEN, C. Y., M. H. HSIEH, C. C. YANG, C. S LIN and A. WANG (2010): Analysis of the cellulose synthase genes associated with primary cell wall synthesis in *Bambusa oldhamii*. Phytochemistry **71**: 1270–1279.

CHEZHIAN, P., R. YASODHA and M. GHOSH (2010): Genetic diversity analysis in a seed orchard of *Eucalyptus tereticornis*. New Forest **40**(1): 85–99.

CZECHOWSKI, T., M. STITT, T. ALTMAN, M. K. UDVARDI and W. R. SCHEIBLE (2005): Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol **139**(1): 5–17.

DAY, M. E., M. S. GREENWOOD and C. DIAZ-SALA (2002): Age- and size-related trends in woody plant shoot development: regulatory pathways and evidence for genetic control. Tree Physiol **22**: 507–513.

DE ALMEIDA, M. R., C. M. RUEDELL, F. K. RICACHENEVSKY, R. A. SPEROTTO, G. PASQUALI and A. G. FETT-NETO (2010): Reference gene selection for quantitative reverse transcription-polymerase chain reaction-normalization during in vitro adventitious rooting in *Eucalyptus globulus* Labill. BMC Mol Biol **11**: 73.

DOBLIN, M. S., I. KURECK, D. JACOB-WILK and D. P. DELMER (2002): Cellulose biosynthesis in plants: from genes to rosettes. Plant Cell Physiol **43**: 1407–1420.

EVANS, J. (1992): Plantation forestry in the tropics: *In:* Tree planting for industrial, social, environmental, and agro forestry purposes, (2nd ed.) Oxford Science Publications, Oxford, New York.

FAGARD, M., T. DESONS, D. F. GOUBET, G. REFREGIER, G. MOUILLE, M. MCCANN, C. RAYON, S. VERNHETTES and H. HOFTE (2000): PROCUSTE1 encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of Arabidopsis. Plant Cell **12**: 2409–2423.

DOI:10.1515/sg-2012-0035
edited by Thünen Institute of Forest Genetics

F.A.O. (2005): Global forest resources assessment 2005 – main report. FAO Forestry Paper, 2005; available online: ftp://ftp.fao.org/docrep/fao/008/A0400E/A0400E00.pdf.

FERNANDEZ, M., C. VILLARROEL, C. BALBONTIN and S. VALENZUELA (2010): Validation of reference genes for real-time QRT-PCR normalization during cold acclimation in *Eucalyptus globulus*. Trees **24**: 1109–1116.

GAN, S., J. SHI, M. LI, K. WU, J. WU and J. BAI (2003): Moderate density molecular maps of *Eucalyptus urophylla* S.T. Blake and *E. tereticornis* Smith genomes based on RAPD markers. Genetica **118**: 59–67.

GINZINGER, D. G. (2002): Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp Hematol **30**(6): 503–512.

GOICOECHEA, M., E. LACOMBE, S. LEGAY, S. MIHALJEVIC, P. RECH, A. JAUNEAU, C. LAPIERRE, B. POLLET, D. VERHAEGEN, N. C. GIGOT and J. G. PETTENATI (2005): *EgMYB2*, a new transcriptional activator from *Eucalyptus* xylem, regulates secondary cell wall formation and lignin biosynthesis Plant J **43**: 553–567.

GUTIERREZ, L., M. MAURIAT, J. PELLOUX, C. BELLINI and O. van WUYTSWINKEL (2008): Towards a systematic validation of references in Real-Time RT-PCR. Plant Cell **20**: 1734–1735.

HUGGETT, J., K. DHEDA, S. BUSTIN and A. ZUMLA (2005): Real-time RT-PCR normalization; strategies and considerations. Genes Immun **6**(4): 279–284.

IGLESIAS TRABADO, G. and D. WILSTERMANN (2008): *Eucalyptus* universalis. Global cultivated eucalypt forests map 2008. *In:* GIT Forestry Consulting's Eucalyptologics: Information resources on *Eucalyptus* cultivation worldwide. Retrieved from http://www.git-forestry.com

ISHITANI, R., K. SUNAGA, A. HIRANO, P. SAUNDERS, N. KATSUBE and D. M. CHUANG (1996): Evidence that glyceraldehyde-3-phosphate dehydrogenase is involved in age-induced apoptosis in mature cerebellar neurons in culture. J Neurochem **66**(3): 928–935.

JOSHI, C. P. (2003): Molecular biology of cellulose biosynthesis in plants. *In:* PANDALAI, R. (ed) Recent research developments plant molecular biology. Research Signpost, Kerala, India. p. 19–38.

JOSHI, C. P., S. BHANDARI, P. RANJAN, U. C. KALLURI, X. LIANG, T. FUJINO and A. SAMUGA (2004): Genomics of cellulose biosynthesis in poplars. New Phytol **164**: 53–61.

KIMA, H. J., A. BARBARA, TRIPLETTA, H. B. ZHANGB, M. K. LEEB, D. J. C. HINCHLIFFEA, P. LIA and D. D. FANGA (2012): Cloning and characterization of homeologous cellulose synthase catalytic subunit 2 genes from allotetraploid cotton (*Gossypium hirsutum* L.) Gene **494**(2): 181–189.

LEE, P. D., R. SLADEK, C. M. T. GREENWOOD and T. J. HUDSON (2001): Control genes and variability: absence of ubiquitous reference transcripts in diverse mammalian expression studies. Genome Res **12**: 292–297.

LEGAY, S., E. LACOMBE, M. GOICOECHEA, C. BRIERE, A. SEGUIN, J. MACKAY and J. GRIMA-PETTENATI (2007): Molecular characterization of *EgMYB1*, a putative transcriptional repressor of the lignin biosynthetic pathway. Plant Sci **173**: 542–549.

MARQUES, C. M., J. A. ARAUJO, J. G. FERREIRA, R. WHETTEN, D. M. O'MALLEY, H. LIB and R. SEDEROFF (1998): AFLP genetic maps of *Eucalyptus globulus* and *E. tereticornis*. Theoret Appl Genet **96**: 727–737.

MARUM, L., A. MIGUEL, P. C. RICARDO and C. MIGUEL (2012): Reference Gene Selection for Quantitative Real-time PCR Normalization in *Quercus suber*. PLoS ONE 7(4): e35113. doi:10.1371/journal.pone.0035113.

MAXWELL, A. (2004): The taxonomy, phylogeny and impact of *Mycosphaerella* species on eucalypts in south-western Australia. Dissertation, Perth, Murdoch University.

NAIRN, J. and T. HASELKORN (2005): Three loblolly pine *CesA* genes expressed in developing xylem are orthologous to secondary cell wall *CesA* genes of angiosperms. New Phytol **166**: 907–915.

OBEMBE, O. O., E. JACOBSEN, J. P. VINCKEN and R. G. F. VISSER (2009): Differential expression of cellulose synthase (*CesA*) gene transcripts in potato as revealed by QRT-PCR. Plant Physiol Biochem **47**: 1116–1118.

OLBRICH, M., E. GERSTNER, G. WELZL, F. FLEISCHMANN, W. OSSWALD, G. BAHNWEG and D. Z. Ernst (2008): Quantification of mRNAs and housekeeping gene selection for quantitative real-time RT-PCR normalization in European beech (*Fagus sylvatica* L.) during abiotic and biotic stress. Z. Naturforsch C **63**(7–8): 574–82.

PFAFFL, M. W., A. TICHOPAD, C. PRGOMET and T. P. NEUVIANS (2004): Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotechnol Lett **26**(6): 509–515.

POKE, F. S. (2004): Summary of world-wide research in eucalypt genomics. Prof. R. GRIFFIN, Prof. J. B. REID, Assoc Prof R. Vaillancourt [Consultants Report].

RANIK, M. and A. A. MYBURG (2006): Six new cellulose synthase genes from *Eucalyptus* are associated with primary and secondary cell wall biosynthesis. Tree Physiol **26**: 545–556.

REID, K. E., N. OLSSON, J. SCHLOSSER, F. PENG and S. T. LUND (2006): An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biol **6**: 27.

SELVEY, S., E. W. THOMPSON, K. MATTHAEI, R. A. LEA, M. G. IRVING and L. R. GRIFFITHS (2001): Beta-actin an unsuitable internal control for RT-PCR. Mol Cell Probes **15**(5): 307–311.

SUZUKI, T., P. J. HIGGINS and D. R. CRAWFORD (2000): Control selection for RNA quantitation. Biotechniques **29**: 332–337.

TONG, Z., Z. GAO, F. WANG, J. ZHOU and Z. ZHANG (2009): Selection of reliable reference genes for gene expression studies in peach using real-time PCR. BMC Mol Biol 10:71 doi:10.1186/1471-2199-10-71.

TURNBULL, J. W. (1999): Eucalypt plantations. New Forest **17**: 37–52.

VANDESOMPELE, J., K. DE PRETER, F. PATTYN, B. POPPE, N. VAN ROY, A. de PAEPE and F. SPELEMAN (2002): Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol **3**: 1–11.

XU, M., B. ZHANG, S. X. ZHANG and M. HUANG (2011): Reference gene selection for quantitative real-time polymerase chain reaction in *Populus*. Anal Biochem **408**: 337–339.

ZOBEL, B. J., G. V. WYK and P. STAHL (1987): Growing Exotic Forests, pp. 508 in John Wiley and Sons, New York.

DOI:10.1515/sg-2012-0035
edited by Thünen Institute of Forest Genetics