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Identification of candidate reference genes in tropical bamboos stable across species, tissues, and developmental stages

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Abstract

Bamboo possesses many unique physiological characteristics, but the molecular understanding of many of these processes remains poorly understood till to date. One major reason is unavailability of sufficient sequence and expression data. Selection of suitable reference genes is pivotal to initiate any gene expression analyses. Although, suitable reference genes have been identified in the temperate bamboo *Phyllostachys edulis*, it has not been done for tropical bamboo. In this study, expression stability of 10 candidate reference genes were investigated in 4 widely grown tropical bamboo species (*Bambusa tulda*, *B. balcooa*, *B. bambos*, and *B. vulgaris*), different organs (young leaves from flowering and non-flowering culms, flag leaf (leaf just below the mature inflorescence), possible flag leaf (leaf covering the immature inflorescence), culm sheath, internode, root, rhizome, and inflorescence bud), different parts (basal, middle, and tip regions of leaf; internodes located in the basal, middle, and tip region of the branch, and developmental stages early, middle, and late inflorescence buds) by using 3 reliable computational tools (*geNorm*, *NormFinder*, and *RefFinder*). A universal single reference gene for normalization of gene expression data was not identified. However, the eukaryotic initiation factor 4 α (*eIF4 α*), clathrin adaptor complexes medium subunit (*CAC*), and nucleotide tract-binding protein (*NTB*) were found stable in the selected organs across different bamboo species. On the other hand, *eIF4 α* ranked top when different organs and *peptidyl prolyl cis-trans isomerase/cyclophilin* (*CYP*), *eukaryotic elongation factor 1 α* (*eEF1 α*) and *ubiquitin 5* (*UBQ5*) ranked top when different developmental stages of *B. tulda* were analyzed. Taken together, this study not only identifies reference gene/s that are stable across species, organs, and developmental stages of bamboo, but it also assesses the impacts of major contributing factors regulating expression stability of the reference genes.

Additional key words: *Bambusa balcooa*, *B. bambos*, *B. tulda*, *B. vulgaris*, *CAC*, *CYP*, *eEF1 α* , *eIF4 α* , *NTB*, *UBQ5*.

Introduction

Bamboos are members of the subfamily *Bambusoideae*, family *Poaceae*. They are profusely growing in different regions of Asia, America, and Africa (Clark *et al.* 2015). A well-developed bamboo culm offers a multiple usage, such as in construction purposes, charcoal production, and a raw material for paper and pulp production, whereas young shoots are used as vegetable (Lobovikov

et al. 2007, Owen 2015). Research on bamboo has traditionally been restricted to phylogenetic studies (Zhang 2000, Clark *et al.* 2007, Das *et al.* 2008), standardization of *in vitro* regeneration methods (Das and Pal 2005, Ramanayake *et al.* 2006, Yuan *et al.* 2017), ecological studies on the unusually long flowering cycle (Janzen 1976, Marchesini *et al.* 2009, Sertse *et al.* 2011),

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Abbreviations: 18S - 18S ribosomal RNA; ACT7 - actin 7; CAC - clathrin adaptor complexes medium subunit; CS - culm sheath; CYP - peptidyl prolyl *cis-trans* isomerase/cyclophilin; E - early staged inflorescence bud; eEF1 α - eukaryotic elongation factor 1 α ; eIF4 α - eukaryotic initiation factor 4 α ; FL - flag leaf; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; I - internode; L - late staged inflorescence bud; M - middle staged inflorescence bud; NTB - nucleotide tract-binding protein; PFL - possible flag leaf; R - root; RH - rhizome; RPKM - reads per kilobase million; TOC1 - timing of CAB expression1; UBQ - ubiquitin; YLF - young leaf from flowering culm; YLN - young leaf from non-flowering culm.

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and fiber extraction processes for the benefit of paper and pulp industry (Wai *et al.* 1985, Sood *et al.* 2005). Compared to that, molecular studies are very limited and the only bamboo genome that has been sequenced till to date is the temperate bamboo *Phyllostachys edulis* (Peng *et al.* 2013, Biswas *et al.* 2016). However, in order to harvest the full economic potential of this plant group, molecular studies on *Bambusa* that hosts approximately 100 species (Kellogg 2015) are very important. In spite of enormous economic potential, one major reason for lack of sufficient molecular studies on *Bambusa* is the unavailability of sufficient gene sequences and their expression data. The first step towards this effort is the identification of suitable reference genes.

Real time quantitative (q) PCR method is one of the most reliable and commonly used techniques to study expression of a limited number of genes. One major advantage of this technique over large scale analyses, such as microarray or the reads per kilobase million (RPKM) method employed in transcriptome sequencing, is its high accuracy and specificity, which enables it to measure the transcriptional abundance of even lowly expressed genes (Ginzinger 2002, Bustin *et al.* 2005, Fedick *et al.* 2012). However, in order to avoid obtaining differential expression due to technical discrepancies, proper data normalization using a set of stable reference genes is mandatory. Therefore, extensive studies have been performed over the years to identify suitable

reference genes that are stable across species (Iskandar *et al.* 2004, Morgante *et al.* 2011), developmental stages (Jain *et al.* 2006, Fan *et al.* 2013, Li *et al.* 2016), tissues (Jian *et al.* 2008, Fan *et al.* 2013, Reddy *et al.* 2016), different seasons (Kim *et al.* 2015), and even diverse environmental conditions (Janská *et al.* 2013, Guo *et al.* 2014, Silva *et al.* 2014, De Andrade *et al.* 2017).

Although standardization of a suitable reference gene has been perfected in the temperate bamboo *P. edulis* (Fan *et al.* 2013), it is not known yet whether these identified reference genes can be useful for gene expression analyses in the phylogenetically diverged tropical bamboo group (Clark *et al.* 1995, Gielis *et al.* 1997). In particular, that study was limited to only six tissues and two developmental stages (Fan *et al.* 2013). The main objective of this study was to identify suitable reference genes that are stable across species, organs, and developmental stages, and therefore, could be utilized for an accurate normalization of gene expression data for *Bambusa*. Ten candidate genes were selected for identification of suitable reference genes in bamboo. These genes were previously found effective for normalization in *Oryza sativa* (Jain *et al.* 2006), *Saccharum officinarum* (Ling *et al.* 2014), *P. edulis* (Fan *et al.* 2013), *Camellia sinensis* (Wang *et al.* 2017), *Sapium sebiferum* (Chen *et al.* 2017), *Fortunella crassifolia* (Hu *et al.* 2014,) and *Capsicum annuum* (Wan *et al.* 2011).

Materials and methods

Collection of plant tissues for gene expression analyses: In order to avoid any possible variability due to seasonal effects, plant tissues from eight different organs, three different regions, and four *Bambusa* species were obtained over a short collection period between May and June in 2017. However, since flowering incidence was rare, flowering tissues of different developmental stages had to be collected over a longer period of time starting from April 2013 to July 2017 from plants located at Shyamnagar (22.83° N, 88.40° E) and Bandel (22.93° N, 88.38° E), West Bengal, India. Three biological replicates for each tissue stage were snap frozen in liquid nitrogen and stored in at -80 °C. To check the effects of organ variability on the expression stability of the selected reference genes, young leaves from flowering (YLF) and non-flowering (YLN) culms, flag leaves (FL leaf just below the mature inflorescence), possible flag leaves (PFL, leaf covering the immature inflorescence), possible flag leaves (PFL), culm sheaths (CS), internodes (I), roots (R), rhizomes (RH), and early staged inflorescence buds (E) were selected, whereas for analyzing variability due to tissue age, different regions of leaves (basal, middle, and tip), internodes (located in the basal, middle, and tip regions of the branch), and inflorescence buds of varying

developmental stages (early, middle, and late) were collected from *Bambusa tulda* Roxb. In order to assess the impact of species variability, three representative organs (rhizomes, leaves, and internodes) were collected from four widely grown bamboo species *B. tulda* Roxb., *B. balcooa* Roxb., *B. bambos* L., and *B. vulgaris* Schrad. ex Wendl.

Isolation of total RNA and preparation of cDNA libraries: Total RNA was isolated from the selected tissues by using a combination of *Trizol* (Invitrogen, Carlsbad, USA) and *RNAeasy* plant mini kits (Qiagen, Hilden, Germany; Das *et al.* 2010, 2016). In order to avoid any genomic DNA contamination, the isolated RNA samples were treated with DNase I (*Thermo Fisher Scientific*, San Jose, USA). The quality and quantity of RNA was assessed with a *BioSpectrometer* (Eppendorf, Hamburg, Germany) and agarose (1 %, m/v) formamide gel electrophoresis (Table 1 Suppl., Fig. 1 Suppl.). Approximately 1 µg of total RNA was used for first strand cDNA synthesis using a *Verso cDNA* synthesis kit (*Thermo Fisher Scientific*) following the manufacturer's protocol, and 2 mm³ of the 1/20 diluted cDNA sample was used for real-time qPCR analyses.

Primer designing for real-time qPCR analyses: Ten candidate reference genes *eukaryotic initiation factor 4a* (*eIF4a*), *eukaryotic elongation factor 1a* (*eEF1a*), *clathrin adaptor complexes medium subunit* (*CAC*), *nucleotide tract-binding protein* (*NTB*) *peptidyl prolyl cis-trans isomerase/cyclophilin* (*CYP*), *ubiquitin 5* (*UBQ5*), *UBQ3*, *18S RIBOSOMAL RNA* (*18S*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), and *actin* (*ACT7*) were selected for this study. Homologous sequences of these genes were identified from *P. edulis*, *Oryza sativa*, *Brachypodium distachyon*, *Zea mays*, *Triticum aestivum*, and *Setaria italica* employing the *BLASTN* program of the *PLAZA 4.0* database using a default parameter. These sequences were multiple aligned using the *MUSCLE* program (<https://www.ebi.ac.uk/Tools/msa/muscle/>) and primers were designed using the *Primer3* program by targeting the conserved regions (Table 2 Suppl., Fig. 3 Suppl.). For the purpose of the validation of the housekeeping genes, the coding sequences of *B. tulda MADS14* (KX186751) and *TIMING OF CAB EXPRESSION1* (*TOC1*, KY249524) were obtained to design gene specific primers. In order to avoid amplification from contaminating genomic DNA, if any, primers were designed from either intron flanking or intron spanning regions.

Real-time qPCR analysis was performed using *SsoAdvanced Universal SYBR Green Supermix* (Bio-Rad, Hercules, USA) in a *CFX Connect* real-time PCR detection system (Bio Rad). Amplification cycles used were: 95 °C for 30 s, 40 cycles at 94 °C for 10 s and at 55 °C for 40 s. A dissociation curve analysis was

performed at the end of each amplification reaction to confirm the absence of any primer-dimer formation. No template control (NTC) was also included where nuclease free water was used as a template. The efficiency of the qPCR reaction was determined from a standard curve based on 1:5 dilution of cDNA templates using a formula $10^{(-1/\text{slope})} - 1$ (Ginzinger 2002). In order to investigate the validity of the putative reference genes, relative expressions of *BtMADS14* and *BtTOC1* were measured in 7 different tissues using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). In this analysis, rhizomes were used as calibrator tissues since they are neutral to flowering, whereas the two tested genes are involved in flower induction and organ differentiation, respectively.

In silico analyses to study the stability of reference genes: To identify the most stable reference genes within the studied experimental sets, three different computational tools were used: *geNorm* (<https://genorm.cmgg.be/>, Vandesompele *et al.* 2002), *NormFinder* (<https://moma.dk/normfinder-software>, Andersen *et al.* 2004), and comprehensive ranking analysis of *RefFinder* (<http://150.216.56.64/referencegene.php>, Silver *et al.* 2006). To perform *geNorm* and *NormFinder* analyses, the C_t values were converted to relative quantities by using the $2^{-\Delta C_t}$ method (Vandesompele *et al.* 2002, Andersen *et al.* 2004), whereas in the case of *RefFinder*, the C_t values were used as input data for further analyses (Silver *et al.* 2006). To estimate the optimum number of reference genes, pairwise variation analysis in the *geNorm* program was performed by assessing the pairwise variations observed between normalization factors.

Results

The specificity of the individual primer pairs used for real time qPCR analysis was confirmed by obtaining a single peak in each case when melting curve analysis was performed (Fig. 2 Suppl.). This was reaffirmed by observing single amplified bands in agarose gel electrophoresis (Fig. 4 Suppl.). The estimated PCR efficiency of the primers ranged from 89 to 109 % (Table 2 Suppl.). The mean C_t values of all the reference genes studied under diverse experimental settings varied (Table 2 Suppl.). The highest C_t value was observed for *ACT7* (29.55 ± 0.03), while the lowest one found was *UBQ3* (16.14 ± 0.24 , Fig. 1). In terms of the observed variations among C_t values of a particular gene under different experimental settings, the lowest variation was observed for *CAC* and *18S*, whereas it was highest in *GAPDH* and *eIF4a*.

Four members of the genus *Bambusa* were selected to study the impact of species variability on the expression stability of the candidate reference genes. Tissues were collected from three different organs (young leaf,

internode, and rhizome) from each of the four species. In order to understand the effect of species only, data from each tissue were separately compared among species so that other factors, such as organ or developmental stage, may not confound the interpretation. The obtained expression data were analyzed in *geNorm*, *NormFinder*, and *RefFinder*.

Out of 10 candidate reference genes, the lowest M value in *geNorm* analysis (0.21) was obtained for *eIF4a* and *UBQ3* when stability of the reference genes was studied in young leaf tissue across 4 different species (Fig. 2A), whereas *CAC* was predicted as the most stable gene by *NormFinder* and *RefFinder* (Table 3 Suppl.). Similarly, *CAC* was found most stable by these two tools for internode tissue, whereas in *geNorm* analysis, these were *eEF1a* and *eIF4a* (Fig. 2B). However, when data from rhizome tissue were compared across species, *NTB* was predicted as the most stable reference gene in all three computational tools (Fig. 2C, Table 3 Suppl.).

The expression of a gene may significantly vary

Fig. 1. The Ct values obtained from different developmental stages, organs, and species of *Bambusa*. The 25th and 75th percentile are indicated in *each box*. The median is depicted by the straight line crossing the *box*. The *whisker caps* represent maximum and minimum values. *eEF1a* - eukaryotic elongation factor 1 α , *eIF4a* - eukaryotic initiation factor 4 α , *NTB* - nucleotide tract-binding protein, *CAC* - clathrin adaptor complexes medium subunit, *CYP* - peptidyl prolyl cis-trans isomerase/cyclophilin, *18S* - 18S ribosomal RNA, *UBQ3* - ubiquitin 3, *ACT7*- actin 7, *GAPDH* - glyceraldehyde-3-phosphate dehydrogenase, and *UBQ5* - ubiquitin 5.

Fig. 2. Gene expression stability and ranking 10 selected candidate reference genes as predicted by *geNorm* analyses. The M value refers to average expression stability and the lower M value signifies a higher stability of expression. The least stable reference genes are *on the left* and the most stable reference genes are *on the right*. Leaf tissue across different species (A), internode tissue across different species (B), rhizome tissue across different species (C), nine different tissues from six different organs (D), three regions of leaf (E), internodes collected from top, middle, and base of a branch (F), three developmental stages of inflorescence buds (early, middle, and late) (G).

across plant organs. In order to understand the influence of organ variability on the expression stability of candidate reference genes, the expression data were assessed in nine tissue samples of *B. tulda*. Out of the 10 candidate reference genes investigated, *UBQ5* and *eIF4α* were identified as ideal reference genes in *geNorm* analysis having the lowest M value 0.55 (Fig. 2D). The

NormFinder and *RefFinder* analyses also predicted *eIF4α* as the most stable reference gene having a stability value of 0.545 and 1.41, respectively (Table 3 Suppl.). However, disagreements among computational tools were noticed in terms of the predicted least stable gene. The *18S* was found to be least stable in *NormFinder* and *geNorm* analyses, whereas it was *GAPDH* in *RefFinder*.

Fig. 3. Estimation of the optimum number of reference genes necessary for normalization of gene expression data. The *geNorm* software calculated a pairwise variation ($V_n/n+1$) between normalization factors (NF_n and NF_{n+1}). The pairwise variation value < 0.15 suggests that addition of one more reference gene was not necessary for normalization of gene expression data. Leaf, internode, and rhizome tissues across species (A), nine different tissues from six different organs (B), and three developmental regions of leaf, internode, and three developmental stages of inflorescence bud (C).

In order to study the effects of developmental stages on the expression stability of the studied reference genes, *B. tulda* tissues were obtained from basal, middle, and tip regions of leaf and also from internodes located in lower, middle, and upper parts of a branch, as well as from early, middle, and late stages of inflorescence buds. When the developmental stages of leaf and internode

were considered, the predictions made by *NormFinder* and *RefFinder* overlapped. For different regions of leaf, *ACT7* was predicted as the most stable reference gene (Table 3 Suppl.). On the contrary, *CAC* and *CYP* were the best reference genes identified in *geNorm* (Fig. 2E). Similarly, for different regions of internode, *eIF4α* was identified as the most stable reference gene by

NormFinder (a stability value of 0.143) and *RefFinder* (a stability value of 2.00, Table 3 Suppl.) analyses, which was contradicted by the *geNorm* analysis suggesting *eIF4a* and *CYP* as the most stable ones (an M value of 0.190, Fig. 2F). When inflorescence buds of three different developmental stages were analyzed, both *geNorm* and *RefFinder* predicted *UBQ5* as a suitable reference gene (Fig. 2G, Table 3 Suppl.), but it was *CYP* in *NormFinder* analysis (Table 3 Suppl.).

The pairwise variation analysis in the *geNorm* program was used to estimate the optimum number of reference genes that can be used for data normalization. A pairwise variation value > 0.15 indicates that addition of another gene is necessary for data normalization. This analysis suggests that use of two genes were sufficient for normalization of data in the case of young leaves and internodes across the selected bamboo species and also across three developmental tissue stages of *B. tulda* (Fig. 3). However, more than three genes were necessary for rhizome tissue across species and also across selected

organs of *B. tulda*.

To confirm the suitability of the identified reference genes for normalization of gene expression data, tissue specific expression patterns of a flower inductive circadian clock gene *BtTOC1* and a floral organ identity gene *BtMADS14* were analyzed in seven different tissues of *B. tulda*. Expression data of these two genes were normalized using the two most stable (*eIF4a* and *UBQ5*) and one least stable (*18S*) reference genes (Fig. 4). When normalization was performed using *eIF4a* and *UBQ5*, the transcript abundance of both these genes was higher in young leaves collected from the flowering culm (YLF) than in young leaves from the non-flowering culm (YLN). This is in line with their established involvement in flower induction (Fig. 4A,B). However, when data were normalized using the predicted least stable reference gene *18S*, the gene expression of *BtTOC1* was higher in YLN than in YLF, whereas no difference in expression between YLF and YLN could be observed in the case of *BtMADS14* (Fig. 4C).

Fig. 4. Relative quantification of the floral inductive circadian clock gene *BtTOC1* and the floral organ identity gene *BtMADS14* using two most stable (*eIF4a* and *UBQ5*) and one least stable (*18S*) reference genes in different organs of *Bambusa tulda*. A relative fold change was calculated by the $2^{-\Delta\Delta C_t}$ method using the expression data in rhizome as calibrator. Means of three biological replicates $\pm$ SEs. Critical differences in expression pattern between young leaves from flowering culm (YLF) and young leaves from non-flowering culm (YLN) could be detected when *eIF4a* and *UBQ5* were used for data normalization but not in case of *18S* (the arrow). Relative quantification of expressions of *BtTOC1* and *BtMADS14* genes using the one of the most stable reference genes *eIF4a* (A) and *UBQ5* (B), or using the least stable reference gene *18S* (C). E - early staged inflorescence bud, I - internode, CS - culm sheath, R - root.

Discussion

Bamboo is widely grown plant species across tropical and subtropical regions (Lobovikov *et al.* 2007). on this plant group and the only bamboo genome that has been sequenced is the temperate bamboo *Phyllostachys edulis* (Peng *et al.* 2013). The first step towards identification of stable reference genes in the tropical bamboos is to use real-time qPCR as a sensitive, quick, and reliable tool for gene expression analyses (Subburaj *et al.* 2017, Dutta *et al.* 2018, Santos *et al.* 2018). However, the accuracy of this method is heavily dependent on data normalization done by stably expressed reference genes. It is now well accepted that a single or a pair of reference genes that are useful for one species or experimental settings may not be equally useful for another species or even for the same species but under different experimental conditions (Morgante *et al.* 2011, De Andrade *et al.* 2017, Niu *et al.* 2017).

In this study, 10 candidate reference genes were selected and the stability of their expressions were investigated in 4 widely grown tropical bamboo species, 9 tissues, and 3 developmental stages by using 3 reliable computational tools *geNorm* (Vandesompele *et al.* 2002), *NormFinder* (Andersen *et al.* 2004), and *RefFinder* (Silver *et al.* 2006). When the stability of gene expressions was compared in three individual organs obtained from four different species of *Bambusa*, it was observed that an universal single reference gene could not normalize data in all these tissue stages and species. For instance, *eIF4a* and *CAC* were most suitable in the case of leaf and internode tissues across four different bamboo species, but not for rhizomes (Fig. 2A,B,C; Table 3 Suppl.). Only limited studies have been conducted to identify reference genes that are stable across taxonomic ranks such as species (Iskandar *et al.* 2004, Morgante *et al.* 2011), varieties, and genotypes (Saha and Blumwald 2014, Pabuayon *et al.* 2016).

Analyses conducted in different organs across four bamboo species suggest that gene expression stability can also be seriously affected by organs. All the three computational tools identified *eIF4a* as the most stable reference gene across selected organs of *B. tulda*

(Fig. 2D, Table 3 Suppl.). It is contrary to the findings in *P. edulis*, where *NTB* and *CYP* were identified as the most stable reference genes across different tissues (Fan *et al.* 2013). One possible explanation for such deviation might be the inclusion of additional organs such as rhizome, culm sheath, and different types of leaves used in the present study but not in the previous one (Fan *et al.* 2013). The *eIF4a* and *UBQ5* have been successfully used for normalization of gene expression data in other species as well. For instance, *eIF4a* has been used across different tissues of alfalfa (Guerriero *et al.* 2014) and *UBQ5* for different developmental stages of rice (Lin *et al.* 2016). In addition to organs, also with respect to the developmental stages, this study is much more exhaustive than the previous one. Indeed, the expression stability of the reference genes were affected by developmental stages of the individual organs analyzed. However, a higher variation of the expression stability of a single reference gene was observed across multiple organs than across developmental stages. This heterogeneity in expression stability is confirmatory to the requirement of a higher number of reference genes for data normalization in the case of organ specific analysis. The validity of the selected reference genes was confirmed by studying the relative expression profile of *BtTOC1* and *BtMADS14* in different vegetative and floral tissues. Indeed, an expected flower inductive pattern in YLF and YLN was observed for both *BtTOC1* and *BtMADS14* genes when *eIF4a* and *UBQ5* were used to normalize the gene expression data, whereas it was reversed when *18S* was used. The *TOC1* is an important circadian clock gene known to be involved in the induction of flowering in plants, and its role in the photoperiodic induction of bamboo flowering has also been studied (Dutta *et al.* 2018). The *MADS14* is a floral inductive transcription factor, which determines the identity of floral meristem (Wu *et al.* 2017). Nevertheless, future studies are required to study the impacts of additional factors, such as seasons, on the expression stability of selected genes for normalization (Kim *et al.* 2015).

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