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Full Length Research Paper

Rubisco-bis-phosphate oxygenase (*RuBP*)- A potential housekeeping gene for qPCR assays in tea

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The present experiment is an effort to find a stable reference gene in *Camellia sinensis* and *Camellia assamica* under different biotic and abiotic stresses. This study evaluate the variation in gene expression across tea leaf tissues in nine experiments. The suitability of *18S rRNA, 26S rRNA*, *rubisco-bis-phosphatase oxygenase* (*RuBP*) and *Camellia tubulin* (*CaT)* as reference genes were validated by geNorm and BestKeeper programs. The finding reveals *18S rRNA* and *RuBP* to be the most stably expressed housekeeping genes, the latter being the first report of its kind in tea. The finding paves the way for their application in accurate quantification of trait specific gene expression and other genomic studies in tea.

Key words: *Camellia sinensis, Camellia assamica, qPCR*, BestKeeper, geNorm, housekeeping gene.

INTRODUCTION

With the improvement of PCR techniques, the PCR amplification can now be estimated in real time. The amplification data can be analysed with bioinformatics aids to calculate the relative expression across several tissues, different experimental types etc. (Pfaffl, 2001). The most essential component for measurement and analysis of gene expression data in the determination of a stable reference/ internal control genes remains unaffected throughout biological samples (Andersen et al., 2004; Huggett et al., 2005; Bustin and Nolan, 2002). We estimated the gene expression stability of four candidate house-keeping genes *viz*. *18S, 26S, RuBP* and *CaT* in leaf tissues of *Camellia sinensis* and *Camellia assamica*. Two software packages *viz*. geNorm and BestKeeper have been used in the study which is also reported to be used in coffee, chicory, and peach (Cavallari et al., 2009).

Tea, *Camellia sinensis*, L (O. Kuntze), belongs to the family Theaceae (Lupton, 1984). The study undertaken involves a range of experimental conditions encompassing different tea clones, treatments and time course experiments in the design viz. *C. sinensis, C. assamica*, garden series clones, tocklai vegetative clones, tea suspension cultures, infested tea leaves [*Empoasca flavescens* Fabricius, *Scirtothrips dorsalis* and *Helopeltis theivora* infested], blister [*Exobasidium vexans*] infected leaf samples and drought induced tea clones. Moreover, determination of the stability of these genes in tea clones grown at two different climates viz. Darjeeling and Assam was also considered. Darjeeling tea is grown on tea estates located on the foothills of the Himalayas, at about 7000 ft. above sea level. The Darjeeling tea gardens experience a comparatively lower temperature than the gardens in Assam. Gene expression profiling of tea clones from these two locations will allow identification of the most stably expressing genes among the four genes considered. Moreover, huge crop loss due to *Exobasidium vexans* and *Helopeltis theivora* attack in Darjeeling and Assam respectively (Muraleedharan et al.,

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Table 1. Experimental conditions.

S/N	Experimental condition	Clone considered	Tissue
1	Darjeeling clones in 2 climates	5 Darjeeling type clones	Leaves
2	Assam clones in 2 climates	4 Assam type clones	Leaves
3	Blister blight infected time course exp.	4 blister tolerant and susceptible clone	Leaves
4	Experiment on resistance for blister blight	2 Blister tolerant and 2 susceptible clone	Leaves
5	Helopeltis infested tea samples	1 tolerant, 2 susceptible	Leaves
6	Greenfly and thrips infestation	3 Darjeeling clones	Leaves
7	Drought induced 2 leaves and a bud	2 Assam type clones	Leaves
8	Drought induced tea progeny	1 Assam type clones' progenies	Leaves
9	MeJA, SA elicited tea suspension cultures	Suspension cultures	Leaves
10	Assam quality clones	4 Assam Quality clones	Leaves

Experimental conditions, types of tea clones and tissue types considered in the different experiments.

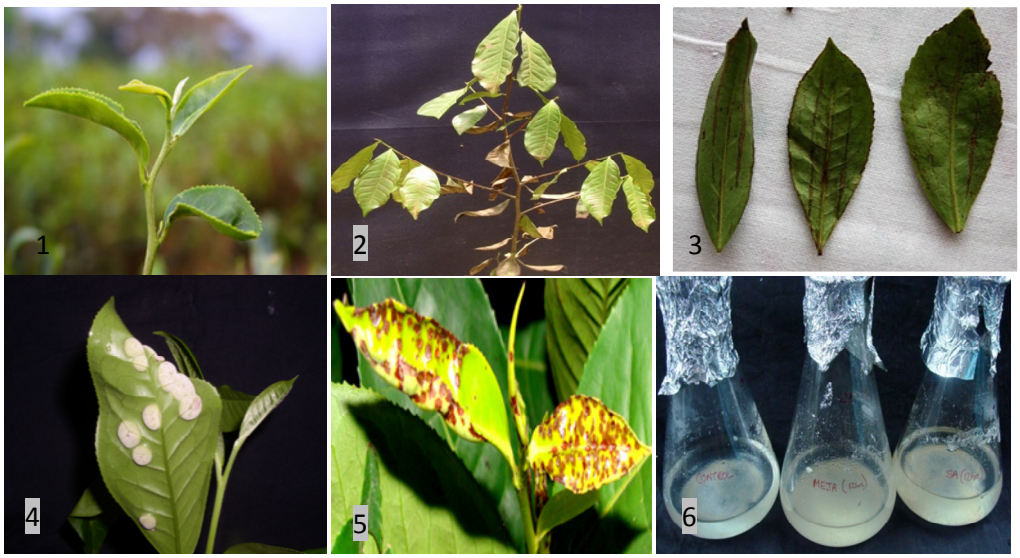

Figure 1. Different leaf samples considered for the study depicting the severity of the stress, both abiotic and biotic are: **1.** Greenfly infested leaf. **2.** Drought induced leaf samples. **3.** Thrips infested leaf samples. **4.** Blister blight infected tea leaf. **5.** Helopeltis infested tea leaf. **6.** MeJA and SA elicited cell suspension cultures.

1988) necessitate investigations on gene expression patterns underlining defense pathways in the plant. Interestingly, certain plant-insect (*Empoasca flavescens* Fabricius, *Scirtothrips dorsalis*) interactions have shown to enhance the emission of volatile compounds and eventually increase the worth of Darjeeling tea in terms of aroma and flavour attributes (Bornali Gohain, PhD thesis, 2011) and hence needs to be studied. These apart, abiotic stresses like drought are also an important hindrance to tea cultivation in certain tea growing regions of Eastern India (www.climatesignals.org/2011).

In view of the above, stress induced gene expression profiling in tea is expected to reveal interesting facets of plant-herbivore interactions, plant defense strategies and their role in influencing tea quality. Moreover, studying transcriptome changes in tea suspension cell culture after MeJA and salicylic acid treatments will help corroborate and interpret the above findings. Considering all the above molecular needs, it is necessary to identify suitable internal control/housekeeping genes that will help accurate quantification of changes in expression of target genes in the studied sample.

MATERIALS AND METHODS

Plant materials and conditions

The leaf samples were collected from a diverse range of conditions (Table 1 and Figure 1) and the same kind of tissues, that is, the leaves, since this is the economically important tissue in tea. The

Table 2. BestKeeper analysis.

Parameter	18S rRNA	26S rRNA	*RuBP*	*Camellia tubulin*
BestKeeper vs.	HKG 1	HKG 2	HKG 3	HKG 4
coefficient of corr. [r]	0.663	0.23	0.82	0.711
p-value	0.001	0.237165	0.001	0.001

BestKeeper analysis of all experimental conditions stated in Table 1.

following conditions were studied.

1. Tea clones grown at Darjeeling and Assam climatic condition; four Assam type quality clones and five Darjeeling type tea clones were grown at both the locations.
2. Blight induced tea leaf samples; Blister blight tolerant and susceptible Darjeeling clones were considered.
3. Herbivory induced leaf samples (*Scirothrips dorsalis* infested, *Empoasca flavescens* Fabricius and *Helopeltis theivora* infested); *Camellia irrawadiensis*, *Camellia sinensis* and garden series clones were analysed for plant herbivory interactions.
4. Drought induced tea leaf samples; transcriptome changes due to drought stress in few popular garden series clones were considered.
5. Methyl jasmonate induced, salicyclic acid induced suspension culture of tea; cell culture of tea quality clone developed at TRA, Jorhat were subjected to Jasmonic acid and salicyclic acid treatment at 12 and 24 h interval.

The study comprised of different experimental setups to subsequently identify a set of housekeeping genes which can be used in gene expression profiling experiments in tea across a range of biotic and abiotic stresses.

RNA extraction

RNA was extracted from each of the biological sample to yield six RNA sample replicates from leaves. Total RNA was extracted using guanidine-HCl RNA extraction protocol with slight modification (Sambrook and Russell, 2003). Following extraction the isolated total RNA was gel electrophoresed in 1% agarose gel to roughly estimate its quality, integrity and concentration.

Reverse transcription (RT)

Moloney Murine Leukaemia virus (MMLV) reverse transcriptase with RNase H activity was used to generate cDNA strands following supplier's protocol. Briefly, RT was carried out in a 20 µl mixture including primer (OligodT) and 1 µg total RNA. The reaction was performed at 50-60°C using transcriptor single strand synthesis kit (Roche diagnostics, Germany), according to manufacturer's instruction.

Housekeeping genes and corresponding primers in the study

Sequences for the house keeping genes were obtained from the public databases viz. the DDBJ and NCBI. The four genes considered in the study were *18S rRNA*, *26S rRNA*, *RuBP,* and *Camellia tubulin*. The corresponding GenBank accession number and primer pair sequence for the gene are *18S rRNA* (GenBank: AY563528) (5' GGCCGGCTCCGTTACTTTG 3'/ 5' GTTTCAG-CCTTGCGACCATACTC 3'), *26S rRNA* (GenBank: AY283368) (5' TCAAATTCCGAAGGTCTAAAG 3'/5' CGGAAACGGCAAAAGTG 3'), *Ribulose-1, 5-bisphosphate carboxylase/oxygenase* (GenBank: EF011075.1) (5' AAGCACAATTGGGAAAAGAAG 3' /5' AAAGTG-AAAATGAAAAGCGACAAT 3') and *Camellia tubulin* (GenBank: DQ444294) (5'AGCGTGCGGTTTGCATGA 3'/5' GCCCAAA-GGTTTGGCATCA 3').

Housekeeping genes amplicons and size validation and PCR efficiency

Melting curve analysis, PCR efficiency checks and gel electrophoresis of amplified products do not guarantee that non target genes are not amplifying along with the target genes. In order to rule out such a possibility, genes were initially amplified from cDNA templates of concerned tissues using designed primers (http://frodo.wi.mit.edu/primer3/). Such amplicons were gel eluted ((Himedia gel extraction kit) and sequenced (ABI 3130xl genetic analyser). The sequences thus obtained, were aligned against the public database BLASTx (http://blast.ncbi.nlm.nih.gov/Blastx) and results documented.

Quantitative real time PCR

Light Cycler 480 SYBR Green I Master (Roche Diagnostics, Germany) was used to carry the expression assays. PCR primers at a final concentration of 0.4 µM were used; both forward and reverse in the reaction. Standard procedures were optimized for use with the Light Cycler 480 II (Roche Diagnostics, Germany), employing the 'second derivative maximum' method (Rasmussen, 2001).

Data analysis

The BestKeeper software and the geNorm VBA applet for Microsoft Excel were used for gene expression normalisation.

RESULTS

BestKeeper analysis in all experimental condition

Descriptive statistics of the derived crossing points (assays run in a LightCycler 480 Ver. II) was performed based on BestKeeper program, and were calculated to investigate the variation of each candidate gene following Pfaffl (2004). The expression stability can be calculated as a measure of the coefficient of correlation which can range from -1.00 to +1.00. The estimates of expression stability for the different experimental setup using BestKeeper are presented in Table 2, which describes the pair-wise correlation analysis between all the experimental conditions taken together and the housekeeping genes. It is an estimate where all the possible combinations of the genes and samples are drawn and a

relative comparison between them is calculated. The data analysed for four housekeeping genes in six experimental types and twenty eight sample with three replications each (the experimental setup and treatments comprised of two tea species namely *Camellia sinensis, Camellia assamica*) proved *RuBP* to be the most stable gene with a coefficient of correlation of 0.82. The results obtained showed *26S rRNA* as the most unstably expressing housekeeping gene.

To check the validity of our observations, we also considered analysing the data from individual experimental setups. There was a variation in stability of housekeeping genes in experiments when analysed in isolation, as five out of the ten experimental setups/condition depicted *18S rRNA* gene as a stable housekeeping gene. Two experimental conditions observed *RuBP* as the most stably expressing gene; two experimental conditions reported the expression of *26S rRNA* gene to be stable. However, no experimental condition depicted *Camellia tubulin* genes' expression to be stable after BestKeeper analysis (Table 4).

Analysis using BestKeeper and geNorm in different samples

Clones grown at two different climates

The experimental setup comprised of two conditions based on the genotype of the clones under study. In first experimental condition, Assam clones were taken for study while the second comprised of Darjeeling clones. The constant factor in both the analysis was the climate in which they were grown, both set of clones (Assam clones and Darjeeling clones) were grown in Assam and Darjeeling climate (Table 4).

i. Assam clones: Lowest expression levels based on CP values was observed in *Camellia tubulin* with about 22-25 cycles and the highest expression for *26S rRNA* gene with cycle number 8. The most stable gene expression measured on the basis of standard deviation and coefficient of correlation was for *26S rRNA* gene (r=0.959) using BestKeeper gene expression analysis software. However, the analysis using geNorm depicted that *RuBP* and *Camellia tubulin* were the two most stable housekeeping genes in Assam clones. Supplementary data sheets depict the corresponding values of expression for housekeeping genes across Assam clones grown in two different climatic condition and geographical location.

ii. Darjeeling clones: Lowest expression level of expression was observed for the housekeeping *Camellia tubulin* gene whose CP value range was from 23-36 cycles and the highest expression was observed for *26S rRNA* at cycle 5. According to the calculated coefficient of correlation values it was observed that *Ribulose-1, 5-bisphosphate carboxylase/oxygenase* (r = 0.988) was the most stable housekeeping gene in the study followed by *Camellia tubulin* and *26 S rRNA*. The expression analysis f in Darjeeling clones grown under both the climatic conditions using geNorm showed that *RuBP* had the most stable expression as depicted by the M (expression stability measure) value of 2.2.

Blight induced tea leaf samples

Two experimental designs were performed for blight infected samples; first experiment dealt with the time course infection of blister blight and the other was designed to derive with the expression of the most tolerant and susceptible clones upon subjection to blister blight infection (Table 4).

i. Time course experiment: The study of expression stability of 4 housekeeping genes in blight infected and control leaf tissues showed that a gradient in gene expression exists. Based on CP values, 26s rRNA and *Camellia tubulin* genes showed the highest (8-10 cycles) and the lowest (31-35 cycles) levels of expression respectively. Upon descriptive analysis of the data in Best keeper software the SD and (r) values for the four genes led us to conclude that the most stably expressing housekeeping genes in the blight infected sample was *Ribulose-1, 5-bisphosphate carboxylase/oxygenase* (r = 0.803) followed by *18S rRNA, 26S rRNA* (SD = 0.47125) and *Camellia tubulin*, which showed a (r) value of 0.376, 0.248 and 0.134 respectively. The analysis in geNorm showed *18S rRNA* gene expression to be the most stable with M (expression stability measure) value of 0.971 which was a good stability far less than the accepted threshold of 1.5.

ii. Tolerant and susceptible clones for blister blight resistance in tea: Both BestKeeper (r = 0.996) and geNorm software's (M=0.275) showed 18S rRNA to be the most stable and valid gene for normalising gene expression essays. The M value in the latter was found to be well within the recommended threshold limit of M=1.5.

Herbivory infested leaf samples

i. *Helopeltis* infestation: The highest and lowest level of gene expression based on CP values was observed for *26S rRNA* (5-9 cycles) and *Camellia tubulin* (22-25 cycles) respectively. Like the other samples, the SD and CV values for the different samples in the experiment showed that the most stably expressing housekeeping gene for this kind of infested tissue was the *18S rRNA* with a (r) value of 0.831. However, geNorm analysis showed that among the four genes considered, *RuBP* was the most stably expressing gene with a stability measure of 1.144 (Table 3).

Table 3. GeNorm analysis.

Parameter	18S	26S	*Tubulin*	rbcl	Experimental condition
M < 1.5	-----	------	------	2.2	Darjeeling clone 2 climates
M < 1.5	------	------	------	1.95	Assam clones 2 climates
M < 1.5	0.971	------	------	------	Blister blight infection time course experiment
M <1.5	0.275	------	------	------	Blister blight infection tolerance study
M < 1.5	------	------	------	1.144	Helopeltis infestation time course experiment
M < 1.5	------	------	------	0.074	Greenfly and thrips infested tea samples
M < 1.5	1.140	------	------	------	Drought induced 2 leaves and a bud
M < 1.5	------	------	0.394	------	Drought induced progeny screening
M < 1.5	2.689	------	------	------	MeJA and SA elicited suspension culture
M < 1.5	------		1.863	------	Assam quality clones

GeNorm analysis for stable genes in different experimental samples. The values corresponding to the most stable values for M are only documented in the table above. All the stable genes under the various experimental conditions are considered within a threshold value of M < 2.7 for 1 experiment and M< 1.5 for 8 experimental types and M < 2.3 for 1 experimental condition.

Table 4. Ranking of stable genes.

S/N	Experimental condition	BestKeeper gene expression stability	GeNorm gene expression stability
1	Darjeeling clones in 2 climates	*RuBP (r=0.977)*	*RuBP(M=2.2)*
2	Assam clones in 2 climates	*26S rRNA (r=0.959)*	*RuBP and Camellia tubulin (M=1.94)*
3	Blister blight infected time course experiment	*RuBP(r=0.803)*	*18S rRNA(M=0.971)*
4	Experiment on resistance for blister blight in tea	*18S rRNA(r=0.996)*	*18S rRNA(M=0.275)*
5	Helopeltis infested tea samples	*18S rRNA(r=0.831)*	*RuBP (M=1.144)*
6	Greenfly and thrips infestation	*18S rRNA(r=0.916)*	*RuBP (M=0.074)*
7	Drought induced 2 leaves and a bud	*18S rRNA(r=0.998)*	*18S rRNA(M=1.140)*
8	Drought induced tea progeny	*18S rRNA(r=0.98)*	*Camellia sinensis tubulin (M=0.394)*
9	MeJA and SA elicited tea suspension cultures	*26S rRNA(r=0.986)*	*18S rRNA(M=2.689)*
10	Assam quality clones	*26S rRNA (r=0.935)*	*Camellia sinensis tubulin (M=1.863)*

Ranks and represents the best valid and stably expressing housekeeping gene across different *Camellia sinensis* and *Camellia assamica* leaf samples considered for gene expression studies.

ii. Greenfly and thrips infested tea leaf samples: The expression profile for the housekeeping genes in greenfly and thrips infested Darjeeling tea samples depicted the highest expression for *RuBP* with around 19-22 cycle, and lowest expression of *Camellia tubulin* of around 28-37 cycles. Coefficient of correlation (r=0.916) values depicted *18S rRNA* to be the most stable housekeeping gene in greenfly and thrip infested Darjeeling tea leaf. The most stable gene as depicted by geNorm for greenfly and thrips infested tea leaf samples was *RuBP* (Table 4).

Drought induced tea leaf samples

i. Drought induced two leaves and a bud of tea: The analysis showed that highest expression was recorded for 26 S genes in drought induced two leaves and a bud of tea and the lowest expression was observed in *18S rRNA* genes. The most stable housekeeping gene for the drought induced tea leaf samples was *18S rRNA* (r=0.998) using BestKeeper analysis software. The analysis using geNorm established *18S rRNA* gene as the most stable gene with a stability of 1.140. This experimental design verified *18S rRNA* gene to be the most stable upon analysis by both softwares (Table 4).

ii. Drought tolerant and susceptible progenies of tea clones in an artificial drought induction experiment: We found that *18S rRNA* gene was the most stable gene with a coefficient of correlation of 0.98 upon analysis in BestKeeper software. The application of geNorm analysis in progenies of drought induced experiment showed *Camellia sinensis tubulin* gene to be the most stable housekeeping gene with an expression stability of 0.394 which is within the permissible stability value range and in fact it is a value under the most stable expression scale regime (Table 4).

iii. Methyl jasmonate and salicyclic acid elicited tea suspension culture: The highest levels of gene expression was observed for 26S gene in the elicited tea

suspension cultures while the lowest was recorded for *Camellia tubulin* gene expression; this estimate was based on crossing point values. The most stable gene expression upon elicitation was observed for Ribulose-1, 5-bisphosphate carboxylase/oxygenase which calculated a SD=2.12, followed by *Camellia tubulin*, *18S rRNA* and *26S rRNA* which had a SD of 2.42, 4.78 and 5.93 respectively using BestKeeper (Table 4).

DISCUSSION

Ranking the most stable gene for consideration in *Camellia sinensis*

Preferably, internal control genes display universal recognition for valid and constant expression levels across all possible tissue types, cells, experimental treatments and design. However, reports till date show that no such housekeeping genes have been found (Schmittgen and Zakrajsek, 2000; Bustin et al., 2000; Suzuki et al., 2000; Thellin et al., 1999; Tricarico et al., 2002; Warrington et al., 2000). Likewise, this study showed a gradation where the same gene is not expressed stably in all experimental samples. If this study considers the descriptive analysis of gene expression stability or the BestKeeper analysis in various tea leaf samples, it was found that out of the 10 experimental conditions studied, five experimental conditions ranked *18S rRNA* as the most stably expressing gene; after comparison with corresponding BestKeeper indices for the experimental conditions (Table 4). In various studies for normalisation of housekeeping genes, it was reported that the 18S rRNA gene expression was stable during the developmental stages, similar is the observation of this study (Al-Bader, 2005). The possible explanation for such an observation is the type and stage of tissue considered in the study was from the same developmental stage. 18S rRNA is therefore recommended as an internal standard for gene expression studies as observed mRNA variations are weak and consequently fail to modify the total RNA level to a great extent. However, the gene with the most stable expression was different under other conditions. It is therefore clear from these reports and observations from different crops, animal system (Vandesompele et al., 2002) and the crop of this study, that is, *Camellia sinensis* that a universally accepted valid housekeeping gene does not exist.

The normalisation factor for the samples was calculated using geNorm for 4 housekeeping genes. The justification for such a design is simple; the variation that is observed while considering multiple genes is less than that observed with single gene. Furthermore, considering multiple control genes also increase the precision of our findings. However, it is expected that if the selection of gene is done with caution an improved normalisation may be obtained. The number of genes would be a switch between practical consideration and also allowing minimization of variation in the normalisation factor.

Experimental condition and tissue types

This study included leaf tissues subjected to various biotic and abiotic stresses, with considerable stable expression. This observation is in accordance with Cavallari et al. (2009), who mentioned that normalisation to a single gene across different tissue types is unwise, since the variation observed between normal tissues of different types may in part be due to the different metabolic demands of those tissues.

The rationale behind considering the four genes was based on available data on use of housekeeping genes for qPCR analysis in tea. Sakata et al. (2007) used *26S rRNA* gene for carrying out qPCR analysis in tea, they found it to be stably expressed in their analysis. This study selected the *rRNA* genes viz. *18S rRNA* and *26S rRNA*, as they demonstrate somewhat constitutive expression across samples as observed in semi quantitative PCR (Gohain, 2011). Singh et al. (2009) also used *26S rRNA* genes as internal control for qPCR assays in *Camellia sinensis*. However, this study showed that *18S rRNA* and *RuBP* genes expressed more stably in *Camellia sinensis* and *Camellia assamica*. Maroufi et al. (2010) used *actin* and *rRNA* genes for validation qPCR data in chicory and found them expressing stably across different chicory samples. A myriad of housekeeping genes have been used and successfully evaluated for normalising real time quantitative PCR data in different organisms and few of them have been evaluated for plant species like rice, poplar, coffee, chicory, Soya bean and *Arabidopsis thaliana* (Kim et al., 2003; Brunner et al., 2004; Volkov et al., 2003). One remarkable observation of this study was that with the herbivory experiments, where geNorm algorithm identified *RuBP* to be the most stably expressing housekeeping gene. This gene has not been reported in tea for use as housekeeping gene, it was observed that the expression of this active component of the photosynthetic apparatus was not altered by leaf feeding insects' attack. However, till date, efficient and robust housekeeping or constitutive gene for carrying qPCR generated gene expression studies have not been defined in *Camellia sinensis* and *Camellia assamica.* This report is a first one of its kind in tea and is expected to serve as a useful and critical reference for all gene expression studies on this medicinally and economically important cash crop.

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