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Research Paper

Evaluation of reference genes for gene expression studies in senna (*Cassia angustifolia* Vahl.) using quantitative real-time polymerase chain reaction

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ABSTRACT

Quantitative real-time polymerase chain reaction (RT-qPCR) is one of the most common methods used for quantification of gene expression due to its high sensitivity, specificity, accuracy and reproducibility. However, a selection of stable and suitable reference genes is crucial for reliable estimation of target gene expression under varied experimental conditions. The aim of the present study was to identify reference gene for gene expression studies in Senna (*Cassia angustifolia* Vahl.), a medicinal plant with potent laxative properties. Nine genes commonly used as internal controls in plants were selected in this study: Actin (*caActin2-7*), 18srRNA (*ca18s*), Glyceraldehyde-3-phosphate dehydrogenase (*caGAPDH*), Actin11 (*caActin11*), Alcohol dehydrogenase (*caADH*), ATP synthase (*caATPSyn*), β-Tubulin (*caβ-TUB*), Eukaryotic elongation factor 1-alpha (*caEEF1-α*) and Eukaryotic elongation factor 1-beta (*caEEF1-β*) for their suitability as internal controls in RT-qPCR to understand sennoside biosynthesis as well as abiotic stress. The expression stability of reference genes was studied using qRT-PCR in leaf (young and mature), pod (young and mature) and flower tissue of three (Sona, DCA-80 and DCA-149) genotypes of senna differing with sennoside content. All genes studied showed low variability (6.0-15.93% CV of Cq). Gene stability was assessed using the statistical algorithm GeNorm. The reference genes, *caActin2-7*, *caGAPDH*, *caADH*, *caATPsyn*, *caEEF1-α* and *caEEF1- β* are stable (M= <1.5) across all the tissues and genotypes studied thus enabling their wider use as internal controls. The study provides the foundation for reference gene(s) selection and will contribute towards more accurate target gene expression estimates which will help to elucidate the biosynthetic pathways that underlie sennoside production and abiotic stress tolerance in senna.

Key words: Senna, *Cassia angustifolia,* Reference genes, Expression stability, RT-qPCR, Sennoside biosynthesis.

INTRODUCTION

Expression pattern of genes involved in various metabolic pathways are required for understanding biological processes in plants. Among the various methods to estimate gene expression, fluorescence based RT-qPCR has shown significant advantage due to its high sensitivity, specificity, accuracy and reproducibility. Therefore, it has become the method of choice for quantification of gene expression in plants and animals (Heid et al., 1996). Besides, it is the best method for the confirmation of global gene expression based on microarrays (Argyropoulos et al., 2005; Schmittgen et al., 2000). Measuring the kinetics of the reaction in the early phases of PCR provides a distinct

advantage over traditional PCR detection. RT-qPCR is the most accurate and sensitive method to study gene expression with low concentrations of mRNA in any tissues (Bustin and Nolan, 2004; Kumar et al., 2011). Although it is widely used for quantification of gene expression, RT-qPCR suffers certain drawbacks such as differences in initial sample amount, RNA integrity issues, differences in the efficiency of cDNA synthesis, and differences in the overall transcriptional activity of the tissues or cells analyzed (Bustin, 2002), beside variation such as biological and technical variation during the procedure of RNA extraction, cDNA synthesis, real time PCR, and sample loading (Vandesompele et al., 2002; Andersen et al., 2004). Therefore, selection of an appropriate normalization method becomes imperative for obtaining reliable quantitative gene expression results. Reference genes or endogenous controls are used for data normalization during RT-qPCR (Andersen et al., 2004). Normalization of gene expression eliminate non-specific template variations between samples due to mRNA integrity, purity and reverse transcription efficiency, as well as pipetting errors (Nolan et al., 2006). Ideally, a reference gene should be uniformly transcribed and exhibit a similar transcription abundance to that of the target gene under different experimental conditions (Goossens et al., 2005; Nolan et al., 2006; Schmittgen and Zakrajsek, 2000; Wan et al., 2010). Although it is aptly sound to use reference genes which have been used in other near related plant species, it has been shown that reference genes have different stabilities depending on the type of tissue or experimental conditions (Ong et al., 2016). Further, commonly used reference genes in plants and animal systems such as 18s and 28s rRNA are affected by many biological factors (Warner, 1999). So, identification of the most stable reference gene(s) for specific experimental conditions is important and imperative for unbiased estimation of gene expression pattern.

Senna (*Cassia angustifolia* Vahl.) is used as a natural laxative and is widely used as a purgative, laxative, expectorant, wound dresser, antidysentric, and carminative (Dave and Ledwani, 2012; Reddy et al., 2015). The medicinal properties of senna are due to production of sennosides in leaves and pods. Senna is drought tolerant annual shrub (Mehta et al., 2017) and a rich source of galactomannan gum (Rajput et al., 2015). To date, improvements in transcriptome sequencing have provided an unprecedented opportunity to enhance our understanding on biosynthetic pathway of sennosides (Reddy et al., 2015) and abiotic stress tolerance (Mehta et al., 2017) in senna. Therefore, senna is regarded as a plant model for research of the biosynthesis of sennoside anthraquinones, galactomannan gums and drought in higher plants.

The statistical models, geNorm, NormFinder and BestKeeper are used for estimating variance reference genes for normalization (Vandesompele et al., 2002; Pfaffl et al., 2004). The models, geNorm and NormFinder, use raw data as input and allow easy comparison, whereas, Bestkeeper calculates the geometric mean of the best suited genes by raw Cq values of each gene and employs a pair-wise correlation analysis to determine the optimal reference genes (Pfaffl et al., 2004).

In plants, gene expression studies include precise quantification of mRNAs expressed in various situations, developmental stages and tissues. The techniques such as northern blotting and ribonuclease protection assay (RPA) were used for quantification of mRNAs (Suzuki et al., 2000) in the past which are less precise than RT-qPCR. Reference genes are used for normalization of gene expression data in RT-qPCR. Use of reference genes without prior verification of their expression stability can lead to an inaccurate interpretation of the data and generate incorrect results. The most reliable reference genes are those constitutively expressed and involved in basic cellular processes and maintenance of cell structure (Cruz et al., 2009). Most commonly used reference genes in plants include those encoding actin, rRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), elongation factor (EF), α-tubulin, and ubiquitin, which are required for maintaining basic cellular processes and thus assumed not to be differently regulated under different experimental conditions. The major difference between a reference gene and a housekeeping gene is that a housekeeping gene should stably expressed whatever the conditions, at every stage of the plant life and in every tissue. However, such a gene can be used for normalization of gene expression data in RT-qPCR. Many studies have shown that reference genes are not sufficiently stable under some treatments (Thellin et al., 1999; Barsalobres-Cavallari, 2009), during development (Bustin, 2002), or even in different tissues (Czechowski et al., 2005) due to the variable nature of plant gene regulation and functions. Because universal reference genes expressed at a constant level are not available, the selection of appropriate reference genes in specific plant species under different conditions is fundamental for the normalization of target gene expression levels. Thus, there is an urgent need to systematically evaluate the stability of potential reference genes for particular experimental systems prior to adopting them for use in RT-qPCR. Identification of stable reference genes facilitate quick and accurate estimation of gene expression thus enabling identification of candidate genes for genetic manipulation for the improvement of sennosides and to facilitate future genomic research in Senna. At present, no study been conducted on the stability of reference genes in Senna, a model system to study sennoside biosynthesis. Our aim was to identify stable reference genes across a range of tissues and genotypes of Senna. This study evaluates the expression stability of nine reference genes in five different tissues of three genotypes of Senna. Optimal reference genes should be considered stable and expressed at constant levels in various tissues and genotypes.

MATERIALS AND METHODS

Plant materials and tissues

Three genotypes, Sona, DCA-149 and DCA-80 of Senna (*Cassia angustifolia*) were used to identify stable reference genes. The genotype Sona is a cultivar developed from CSIR-Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India. While the genotypes, DCA-149 and DCA-80 are the germplasm accessions maintained at ICAR-Directorate of Medicinal and Aromatic Plants Research (DMAPR), Anand, Gujarat, India. Pure seed of all the genotypes were raised in the research block of ICAR-DMAPR, Anand, Gujarat, India during 2015-2016. Leaves (young and mature), pods (young and mature) and flower tissues were collected at the time of flowering. The tissues were dipped in RNA later (Sigma-Aldrich, St. Louis, Missouri, United States) and were immediately frozen in liquid nitrogen, and then stored at -80°C for isolation of total RNA.

RNA extraction and cDNA synthesis

Total RNA from the leaves (young and mature), pods (young and mature) and flowers of Sona, DCA-149 and DCA-80 tissues was isolated using Trizol (Invitrogen) as per manufacturer's instruction using the standard protocol described by the manufacturer (Thermo Fisher Scientific168 Third Avenue Waltham, MA USA 02451). The quality of the isolated RNA was checked on 1% denatured Agarose gel for the presence of 28S and 18S bands. cDNA was synthesized using oligo (dT) as primers by M-MLV reverse transcriptase according to the manufacturer's instructions (Thermo scientific, Waltham, Massachusetts, United States). After the reverse transcription, synthesized cDNA was stored at -20°C until use.

Primers and real-time PCR

Nine constitutively expressing genes in plants were selected based on previous studies (Stolf-Moreira et al., 2011; Hu et al., 2009; Mehta et al., 2017). Orthologs of the reference genes were picked from the senna leaf transcriptome library (Reddy et al., 2015). The primers were designed using primer3 with default parameters[http://frodo.wi.mit.edu/primer3/]. The expected allele size range given was 100-200 bp while picking the primers. The specificity of the primers was further confirmed by BLAST search. Annealing temperatures were optimized according to individual primers by testing several annealing temperatures ranging from 50 to 65°C around the respective primer T^m, and the annealing temperature with the best efficiency was chosen.

Real-time PCR was performed in a 25 µl volume using SYBR premix (Genetix, Delhi, India) on the CFX Connect Real-time PCR detection system (Bio-Rad, Hercules, USA) by following a program: 5 min at 95°C; followed by 45 cycles of amplification with denaturation for 5 s at 95°C, annealing for 30 s at 55°C, and extension for 20 s at 72°C. A melting step was performed to confirm a single gene-specific peak by a stepwise temperature increase ranging from 60 to 95°C at ramp rate of 1°C /s with continuous monitoring of fluorescence. No-template controls (NTCs) were included for each primer pair. Further analysis of amplicon specificity and size were also evaluated by running qPCR products in a standard 2% agarose gel electrophoresis. Triplicates under identical conditions were synchronously performed for all genes. Standard curves were made to calculate the amplification efficiency during real-time PCR using five-fold serial dilutions of cDNA for each tissue and each reference gene. The quantification cycle (Cq) was automatically determined for each reaction by the CFX Maestro™ Software (Bio-Rad, Hercules, USA). Finally, the specificity of the qRT-PCR reactions was determined by melt curve analysis of the amplified products.

Data analysis

We calculated the cycle threshold (Cq) values and real-time PCR efficiencies (E) for each reference gene to know the gene expression pattern. Relative standard curve was constructed using a 5-fold dilution series of cDNA from each tissue. PCR efficiency was determined by converting quantification cycles (Cq values) into raw data (relative quantities). The real-time E value was calculated from the given slopes in the SAS software according to the equation: (E = 10(−1/slope)−1) (Hellemans et al., 2007). Only Cq values <35 were used for calculating E values. Cq and E values were then analyzed in geNorm on the SAS 9.5 software (SAS, 2010) using SAS Proc qPCR program (Ling, 2012), which ranks the reference genes based on the M values. Meanwhile, geNorm software was used to calculate the pair-wise variation (V_n/V_{n+1}) between the sequential normalization factors NF_n and NF_{n+1}. A one-way ANOVA was also performed on Cq values obtained from the expression in genotypes and tissues. We used Microsoft Excel 2010 to calculate other statistical parameters.

RESULTS

Selection of reference genes

Housekeeping genes are widely used as the reference genes for plant gene expression studies using RT-qPCR in plants and animal systems. Based on the reference genes

Table 1: Reference genes evaluated in this study.

Gene	Gene name	Forward (5' -3')	Reverse (5'-3')	Annealing temp.(◦C)	Amplicon size (bp)	Efficiency (E %)
caActin2-7	*Actin 2/7*	ACACCCTCCTTCAAACTTGC	ACGATGAGTCTGGTCCATCC	55	166	90.40
ca18s	*18S ribosomal RNA*	TTGGCAAGGGTGTGTTTGTA	GCCACGATCCACTGAGATTC	55	199	91.40
caGAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*	TGTAACCCCACTCGTTGTCA	GAAGGGGATTCTGGAGGTGT	55	152	92.90
caActin11	*Actin11*	AACCAGCCTTGATCGGTATG	TCCGGAGAGGACAATGTTTC	55	116	93.10
caADH	*Alcohol dehydrogenase*	TGGATCGTGCTGTTGAATGT	ACTCGGATGGGTTTTGAATG	55	134	90.40
caATPsyn	*ATP synthase*	ACAGATGGCTGTCATTTGGA	GGACCGAGGGAAGTTTCAA	55	169	87.60
caβ -Tubulin	*Beta tubulin*	TGCGCTTAGTCTTGATGGTG	AGCGCATTTGAACCTTCATC	55	139	83.80
caEEF1-α	*Eukaryotic elongation factor 1-alpha*	GCTGAGCGTGAAAGAGGAAT	ACCAGCTTCAAAACCACCAG	55	189	83.50
caEEF1-β	*Eukaryotic elongation factor 1-beta*	AGCATCCAAACTGGTTCCTG	TACTCGTTGCATGGCTCAAC	55	129	88.90

in soya bean (Stolf-Moreira et al., 2011; Hu et al., 2009), a close relative of Senna (both belong to Fabaceae family) and other plant species (Crismani et al., 2006; Expósito-Rodríguez et al., 2008; Liu et al., 2013; Liu et al., 2015; Wu et al., 2015), we selected Actin (*caActin2-7*), 18srRNA (*ca18s*), Glyceraldehyde-3-phosphate dehydrogenase (*caGAPDH*), Actin11 (*caActin11*), Alcohol dehydrogenase (*caADH*), ATP synthase (*caATPSyn*), β-Tubulin (*caβ-TUB*), Eukaryotic elongation factor 1-alpha (*caEEF1-α*) and Eukaryotic elongation factor 1-beta (*caEEF1-β*) reference genes for the study (Table 1). These genes encode proteins with a wide variety of biological functions such as transcription factors, ubiquitous enzymes or cytoskeleton elements and others. Variability of RNA samples was evaluated by RT-PCR for18srRNA gene. All the RT-PCR reactions produced a single 18srRNA specific band with a predicted molecular weight on agarose gel, confirming that the extracted RNA samples were suitable for transcript level analysis. RT-PCR with all the reference genes produced a single band in agarose gel electrophoresis, indicating specificity of amplification.

Expression profile of reference genes

Nine reference genes were amplified in young leaf, mature leaf, young pod, mature pod and flower tissues of three genotypes viz. Sona, DCA-129 and DCA-80 of senna using cDNA as a template in a Real-time PCR detection system (Figure 1). To check the specificity of the primers, agarose gel electrophoresis was performed following completion of the qRT-PCR assays. The primer pairs amplified a single band of the expected respective size and formed no primer dimers or other non specific amplification products. All the real-time PCR assays produced a single bond indicating the specificity of amplification (Supplementary Figure S1 and S2).

Efficiency (E) value of reference genes, mean, standard deviation (SD) and Coefficient of variation (CV) of Cq values for each tissues are given in Table 2. All reference genes had a Cq values below 31.19 cycles across tissues, indicating an abundance of cDNA in the samples. The reference gene *caEEF1-α* showed the most abundant expression level with an average Cq value of 17.81, whereas *caADH* gene showed the least abundant expression level with an average Cq value of 25.10. The PCR efficiency of the nine candidate reference genes ranged from 83.50% for *caEEF1-α* to 93.10% for *caActin11* (Table 1). There was no significant variation in Cq values of all the tissues of sona, DCA-129 and DCA-80 genotypes in senna.

Expression stability of reference genes

We used SAS Proc qPCR to calculate expression stability (M value) using Cq and E values. M values were used to rank reference genes based on the stability, depending up on the type of tissue and genotype (Figures 2 and 3). M value below 1.5 indicates stable gene expression (Nygard et al., 2007). M-values ranged from 0.09 to 7.55 across all the tissues in the present study. In young leaf, all the nine genes except Actin 11 recorded M-value less than 1.5, indicating stability in the gene expression (Figure 3a). *caGAPDH* (rank 1), *caActin2-7* (rank 2), *caATPSYN* (rank 3), *caβ-TUB* (rank4*), caELF1-α* (rank 5), *caELF1-β* (rank 6), c*aADH* (rank 7) and *ca18s* (rank 8) are the most stable reference genes

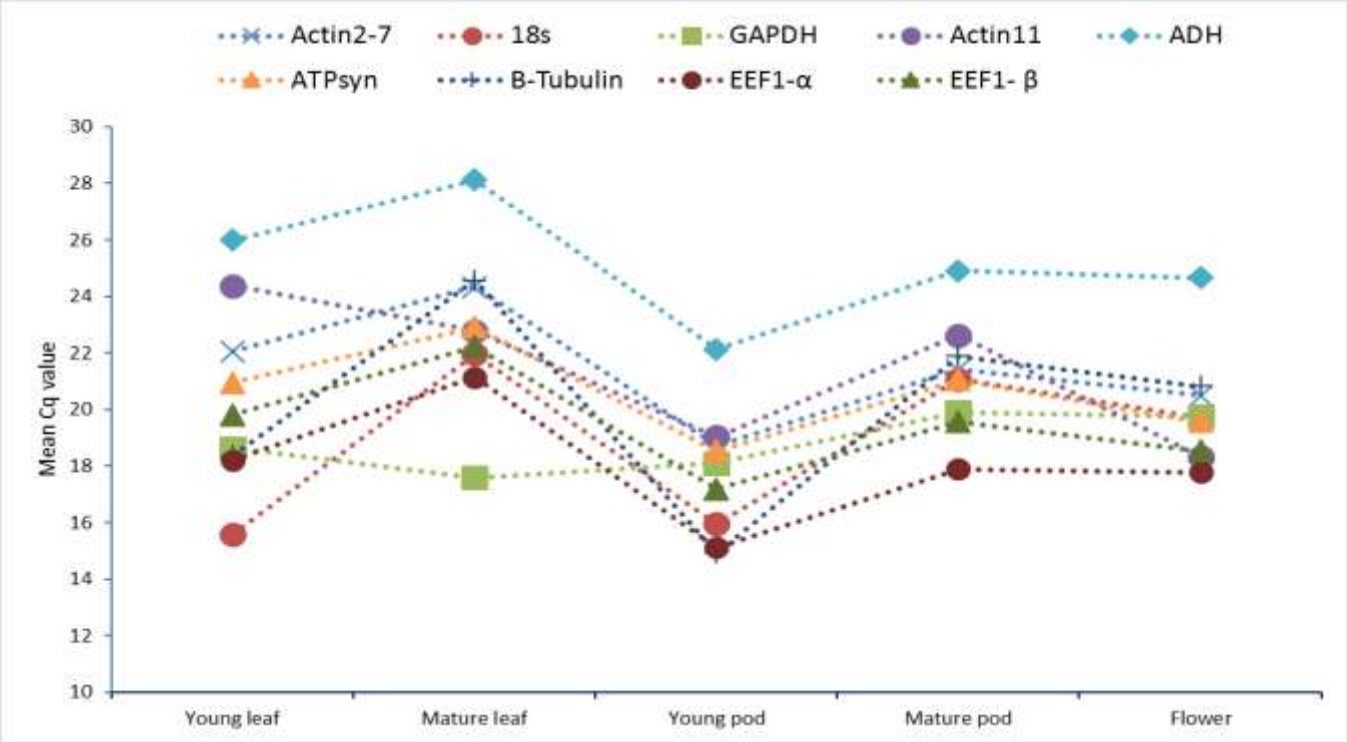

Figure 1: RNA transcription levels of reference genes presented as absolute Cq values in different tissues of Senna (*Cassia angustifolia*). The reference genes are: Actin (*caActin2-7*), 18srRNA (*ca18s*), Glyceraldehyde-3-phosphate dehydrogenase (*caGAPDH*), Actin11 (*caActin11*), Alcohol dehydrogenase (*caADH*), ATP synthase (*caATPSyn*), β-Tubulin (*caβ-TUB*), Eukaryotic elongation factor 1-alpha (*caEEF1-α*) and Eukaryotic elongation factor 1-beta (*caEEF1-β*) tissue samples.

Table 2: Mean, standard deviations (SD) and coefficient of variation (CV) of Cycle threshold (Cq), and reaction efficiency (E) values for individual genes in examined tissues of Senna (*Cassia angustifolia*).

Tissue	Genotype	*ca*Actin2-7	*ca*18s	*ca*GAPDH	*ca*Actin11	*ca*ADH	*ca*ATPsyn	*caβ*-Tubulin	*ca*EEF1-α	*ca*EEF1-β
Young leaf	Sona	21.39	15.86	17.92	19.68	25.97	20.08	17.24	16.90	18.54
	DCA-149	22.74	15.35	19.37	29.08	26.01	21.83	19.50	19.52	21.10
	Mean	**22.06**	**15.60**	**18.65**	**24.38**	**25.99**	**20.96**	**18.37**	**18.21**	**19.82**
	SD	**0.76**	**0.44**	**0.84**	**5.43**	**0.40**	**1.12**	**1.31**	**1.53**	**1.48**
	CV	**3.47**	**2.82**	**4.49**	**22.26**	**1.53**	**5.32**	**7.12**	**8.41**	**7.46**
	E	**0.92**	**0.93**	**0.91**	**0.91**	**0.88**	**0.85**	**0.81**	**0.81**	**0.87**
Mature leaf	Sona	23.42	22.04	16.21	21.04	26.56	21.69	20.25	18.75	21.24
	DCA-149	26.00	24.11	19.41	24.85	29.07	23.87	24.11	22.77	23.57
	DCA-80	23.57	19.71	17.13	22.44	28.73	23.18	29.33	21.92	21.84
	Mean	**24.33**	**21.95**	**17.21**	**22.10**	**27.88**	**22.29**	**22.73**	**20.07**	**21.48**
	SD	**1.26**	**1.91**	**1.42**	**1.93**	**1.12**	**1.46**	**4.84**	**2.56**	**1.65**
	CV	**5.18**	**8.70**	**8.26**	**8.72**	**4.02**	**6.57**	**21.31**	**12.78**	**7.68**
	E	**0.92**	**0.93**	**0.91**	**0.91**	**0.88**	**0.85**	**0.81**	**0.81**	**0.87**
Young pod	Sona	19.07	15.99	17.99	18.43	22.16	18.26	15.16	14.71	17.06
	DCA-149	18.39	15.92	18.23	19.66	22.11	18.83	14.71	15.51	17.32
	Mean	18.73	15.96	18.11	19.05	22.13	18.54	14.94	15.11	17.19
	SD	**0.41**	**0.13**	**0.14**	**0.71**	**0.12**	**0.67**	**0.33**	**0.47**	**0.15**

Table 2: Continued.

	CV	**2.17**	**0.83**	**0.77**	**3.73**	**0.52**	**3.64**	**2.19**	**3.10**	**0.88**
	E	**0.92**	**0.94**	**0.90**	**0.90**	**0.87**	**0.84**	**0.80**	**0.80**	**0.86**
Mature pod	Sona	21.7	20.72	20.15	21.67	25.68	21.01	17.63	17.81	19.61
	DCA-149	21.9	22.47	20.28	25.37	24.35	21.35	17.35	18.47	20.04
	DCA-80	20.64	20.01	19.31	20.82	24.67	20.79	30.65	17.41	19.02
	Mean	**21.42**	**21.07**	**19.91**	**22.62**	**24.90**	**21.05**	**21.88**	**17.90**	**19.56**
	SD	**0.59**	**1.10**	**0.47**	**2.16**	**0.84**	**0.38**	**6.82**	**0.48**	**0.46**
	CV	**2.77**	**5.22**	**2.36**	**9.57**	**3.37**	**1.81**	**31.19**	**2.68**	**2.37**
	E	**0.88**	**0.89**	**0.97**	**0.97**	**0.94**	**0.92**	**0.88**	**0.88**	**0.93**
Flower	Sona	20.55	20.23	18.95	19.22	24.35	19.52	16.07	16.97	18.21
	DCA-149	19.93	19.77	19.54	16.7	24.33	19.61	29.14	17.18	18.36
	DCA-80	21.01	19.13	20.77	19.04	25.28	19.57	17.24	19.2	19.06
	Mean	**20.50**	**19.71**	**19.75**	**18.32**	**24.65**	**19.57**	**20.82**	**17.78**	**18.54**
	SD	**0.47**	**0.48**	**0.83**	**1.26**	**0.50**	**0.47**	**6.47**	**1.11**	**0.41**
	CV	**2.30**	**2.45**	**4.20**	**6.86**	**2.03**	**2.39**	**31.06**	**6.23**	**2.23**
	E	**0.88**	**0.89**	**0.96**	**0.96**	**0.94**	**0.91**	**0.88**	**0.87**	**0.92**
Over all	**Mean**	**21.41**	**18.86**	**18.73**	**21.29**	**25.11**	**20.48**	**19.75**	**17.81**	**19.32**
	SD	**2.06**	**2.92**	**1.13**	**2.54**	**2.10**	**1.45**	**3.15**	**1.77**	**1.59**
	CV	**9.62**	**15.50**	**6.05**	**11.93**	**8.35**	**7.08**	**15.93**	**9.95**	**8.23**

in the order of increasing M-values (Table 3). The M-value of 5.30 was recorded with *caActin 11* reference gene in young leaf. While in mature leaf, all the nine genes, except *caβ-TUB* and *ca18s*, had E-value less than 1.5 and were ranked as *caATPSYN* (rank 1), *caADH* (rank 2), *caELF1-β* (rank 3), *caGAPDH* (rank 4), *caActin2-7* (rank 5), *caActin11* (rank 6) and *caELF1-α* (rank 7) as the most stable reference genes in the order of increasing M-values. The M-values in the young pod ranged from 0.09 to 0.76, indicating high stability of genes studied. *caGAPDH* (rank 1), *caELF1-β* (rank 2), *caADH* (rank 3), *ca18s* (rank 4), *caATPSYN* (rank 5), *caELF1-α* (rank 6), *caβ-TUB* (rank 7), *caActin2-7* (rank 8) and *caActin11* (rank 9), respectively were ranked in the order of increasing M-values. In contrast, the M-values in the mature pod ranged between 0.33 (*caGAPDH*) and 7.55 (*caβ-TUB*). The genes, *caGAPDH* (rank 1), *caActin2-7* (rank 2), *caELF1-β* (rank 3), *caELF1-α* (rank 4), *caATPSYN* (rank 5), *ca18s* (rank 6) and *caADH* (rank 7) recoded M-value less than 1.5 and were respectively ranked in the order of increasing M-values. In flower, all the reference genes, except *caβ-TUB* recorded M-value less than 1.5, indicating the stability in the gene expression (Figure 3F). The reference genes, *caADH* (rank 1), *caELF1-β* (rank 2), *caActin2-7* (rank 3), *caATPSYN* (rank 4), *caGAPDH* (rank 5), *caELF1-α* (rank 6), *ca18s* (rank 7) and *caActin11* (rank 8) were ranked in the increasing order of their expression stabilities. When all samples in different tissues were of 0.30, while the *caβ-TUB* was the least stable gene with M value of 3.90.

Optimization of reference genes for normalization

Optimal number of reference genes required for accurate normalization was calculated using geNorm which determines the pairwise variation V_n/V_{n+1} that measures the effect of measuring additional reference genes on the normalization factor. A cut-off value of 0.15 has been widely accepted as the criterion for selecting a suitable number of reference genes, below which the inclusion of additional reference genes are not needed (Vandersompele, 2002). The pair-wise variation $V_{1/2}$ was lower than 0.15 in leaf (young and mature), pod (young and mature) and flowers, indicating that the single or two most stable reference genes were sufficient for gene normalization in various tissues of senna (Figure 4). The best combinations among the nine reference genes were *caGAPDH* and *caActin2-7* for young leaf ($V_{1/2}$=0.029), *caATPSYN* and *caADH* for mature leaf ($V_{1/2}$=0.126), *caGAPDH* and *caELF1-β* for young pod ($V_{1/2}$=0.007), *caGAPDH* and *caActin2-7* for mature pod ($V_{1/2}$=0.052) and *caADH* and *caEEF1-β*in flower ($V_{1/2}$=0.063) for target gene normalization in Senna.

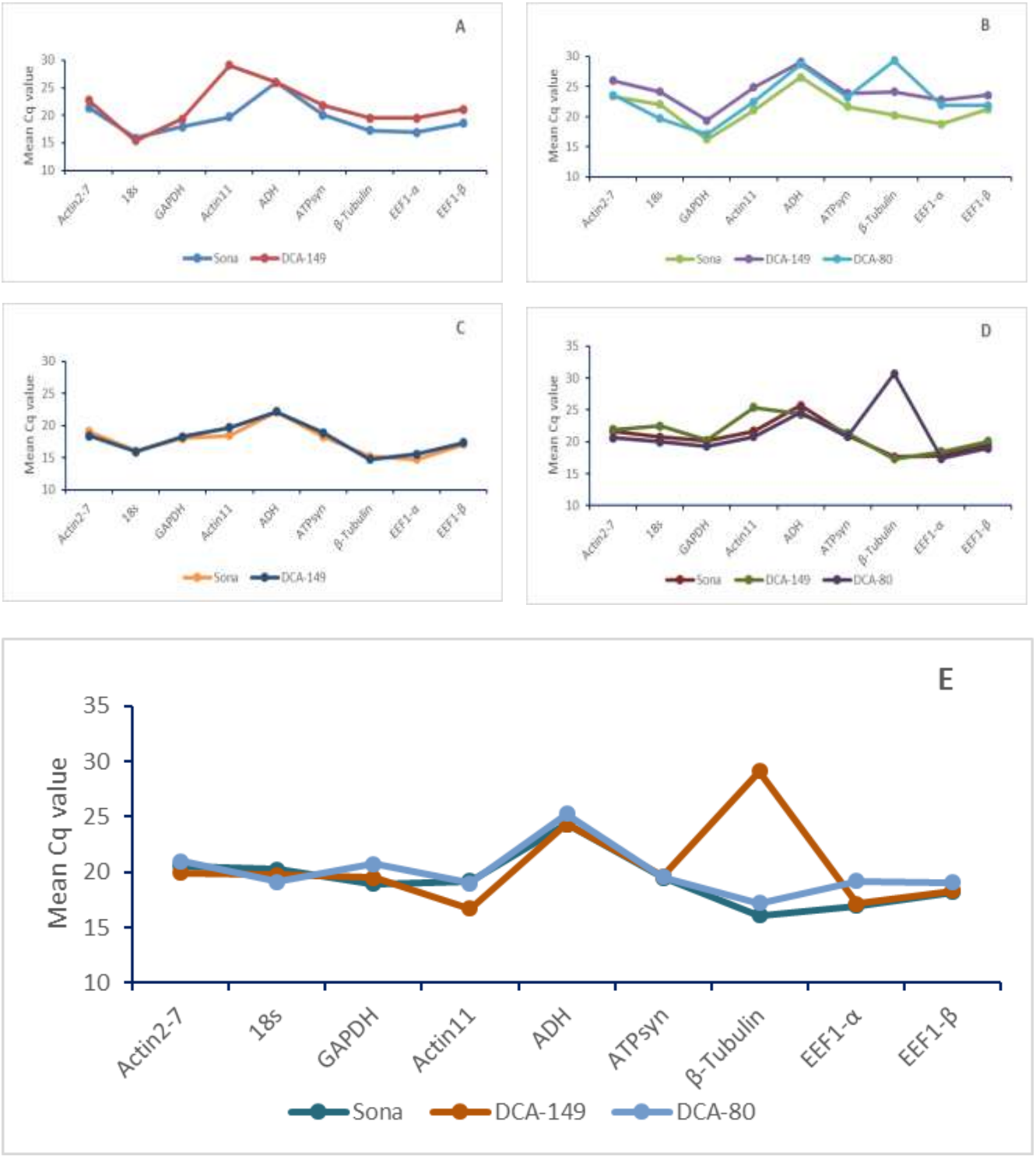

Figure 2: Average RNA transcription levels in different tissues presented as absolute Cq values in Senna (*Cassia angustifolia*). The tissues: Young leaf (A), Mature leaf (B), Young pod (C), Mature pod (D) and Flower (E).

DISCUSSION

Gene expression is heterogeneous and complex, and depends on the tissue, developmental stage, and condition of the experiment (Tenea et al., 2011; Chapman and Waldenström, 2015). RT-qPCR has become a routine technique for gene expression studies, due to its high sensitivity, specificity, accuracy and reproducibility. Normalization of target gene expression data from qRT-PCR is essential for obtaining accurate gene expression results

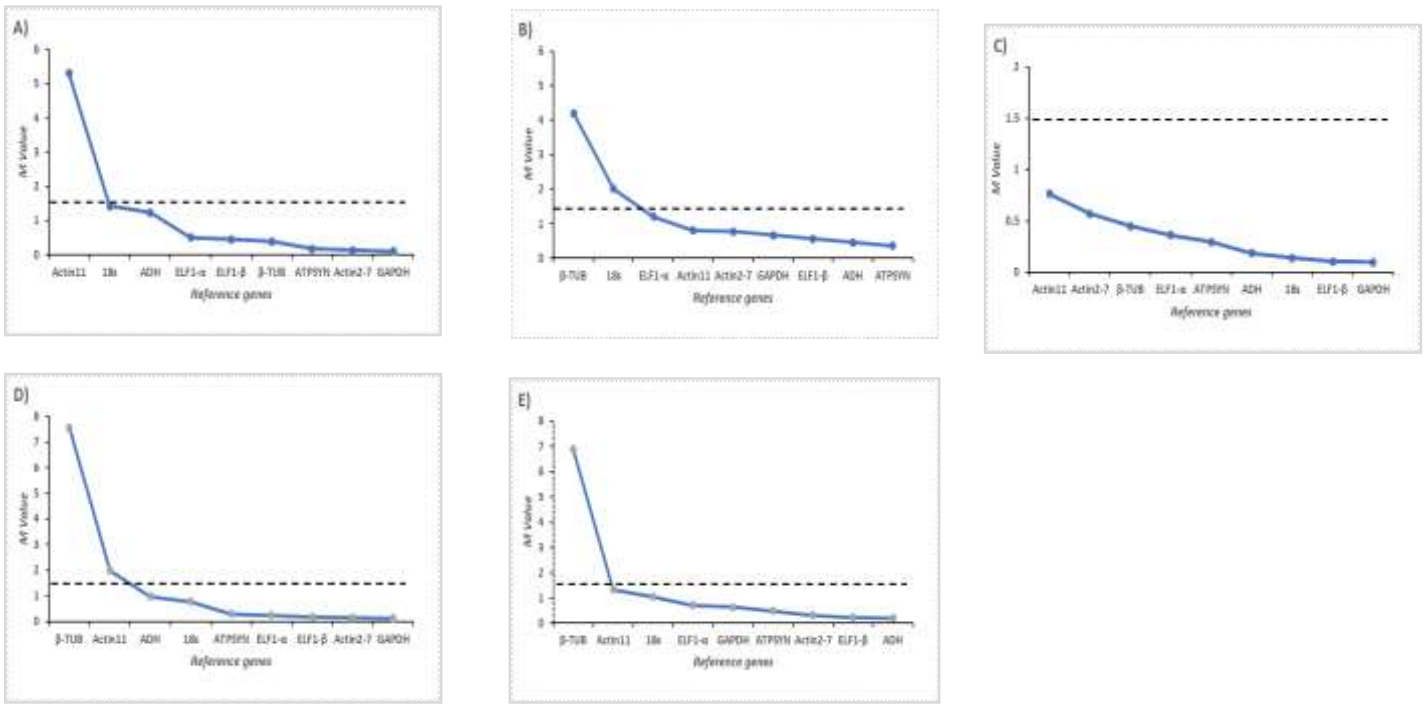

Figure 3: Expression stability values (M) of reference genes in tissues of Senna (*Cassia angustifolia*). The reference genes are: Actin (*caActin2-7*), 18srRNA (*ca18s*), Glyceraldehyde-3-phosphate dehydrogenase (*caGAPDH*), Actin11 (*caActin11*), Alcohol dehydrogenase (*caADH*), ATP synthase (*caATPSyn*), β-Tubulin (*caβ-TUB*), Eukaryotic elongation factor 1-alpha (*caEEF1-α*) and Eukaryotic elongation factor 1-beta (*caEEF1-β*) tissue samples. A) Young leaf, B) mature leaf, C) young pod, D)Mature pod, and E) flowers.

Table 3: Control genes ranked in the order of their expression stability.

Rank[a]	Young leaf	Mature leaf	Young pod	Mature pod	Flower
1	*ca*GAPDH	*ca*ATPSYN	*ca*GAPDH	*ca*GAPDH	*ca*ADH
2	*ca*Actin2-7	*ca*ADH	*ca*ELF1-β	*ca*Actin2-7	*ca*ELF1-β
3	*ca*ATPSYN	*ca*ELF1-β	*ca*ADH	*ca*ELF1-β	*ca*Actin2-7
4	*ca*β-TUB	*ca*GAPDH	*ca*18s	*ca*ELF1-α	*ca*ATPSYN
5	*ca*ELF1-α	*ca*Actin2-7	*ca*ATPSYN	*ca*ATPSYN	*ca*GAPDH
6	*ca*ELF1-β	*ca*Actin11	*ca*ELF1-α	*ca*18s	*ca*ELF1-α
7	*ca*ADH	*ca*ELF1-α	*ca*β-TUB	*ca*ADH	*ca*18s
8	*ca*18s	*ca*18s	*ca*Actin2-7	*ca*Actin11	*ca*Actin11
9	*ca*Actin11	*ca*β-TUB	*ca*Actin11	*ca*β-TUB	*ca*β-TUB

Reference genes with M values below 1.5 are considered as suitable reference genes for a particular tissue. [a]Increasing expression stability from top to bottom.

with biological relevance. Reference genes are used for normalization in gene expression studies; however, choosing the best reference gene is the key for normalization. An ideal reference gene must be transcribed ubiquitously in the tissues being examined and remains is unchanged by any experimental treatment (Bustin, 2002; Czechowski et al., 2005). Reference genes/Internal control genes must be selected with caution. However, it must be noted that there are no universal reference genes that are stably expressed under all biological materials and/or experimental conditions. Several studies have shown that the use of unstable reference genes may result in significant biases and misinterpretations of target gene expression (Gutierrez et al., 2008; Ferguson et al., 2010; Mafra et al., 2012; Ling, 2011). Therefore, it is necessary to validate the expression stability of the selected reference gene for various plant species under different conditions prior to its use for target gene expression quantification. To our knowledge, this study represents the first effort to comprehensively identify reference genes with highly uniform gene expression in senna. We provided a detailed analysis of the stability and expression levels of nine

reference genes, in five different tissues and three genotypes of senna which would help to assay the expression of genes involved in sennoside biosynthesis and abiotic stress tolerance. The identification of diagnostic reference genes was required to elucidate the biosynthetic pathways that underlie sennoside production and abiotic stress tolerance in senna, and the dynamics of gene expression are a valuable resource to achieve this goal.

In the present study, the average Cq values for all the reference genes studied in all the tissues were below 28 cycles, indicating strong positive reactions of the target tissue to the reference genes (Figure 1). As expected, the expression stability differs between tissues and genotypes. The study confirms that reference genes are expressed in every cell but are regulated differently in tissues and genotypes. geNorm is more commonly used analysis program for comparing the expression patterns of candidate reference genes and identifying the best suitable reference gene sets under particular conditions.

The *GAPDH* gene, encodes an enzyme in the glycolytic pathway, is present in most cell types and has been used commonly as an endogenous control in RT-qPCR experiments in plants (Expósito-Rodríguez, 2008). In the present study, *caGAPDH* showed an M value lower than the cutoff established by GeNorm (M<1.5) in leaf (young and mature), pod (young and mature) and flowers of senna indicating higher stability in the expression across the tissues. The use of *GAPDH* as a reference gene in RT-qPCR in medicinal plants such as liquarice (*Glycyrrhiza glabra*) has been reported (Maroufi, 2016). The gene for *GAPDH* should be used with caution because its expression can be more abundant in proliferating cells (Bustin, 2002). GADPH is unstably expressed in papaya during storage at different temperature or under nitrogen stress in tomato and actin is unstably expressed under salinity stress in potato or in cucumber (Nicot et al., 2005; Løvdal and Lillo, 2009; Wan et al., 2010; Zhu et al., 2012).

Actin genes encode a family of actin which are globular multi-functional proteins involved in cellular processes, including muscle contraction, cell motility, cell division and cytokinesis, vesicle and organelle movement, cell signaling, and the establishment and maintenance of cell junctions and cell shape. Actin genes are ubiquitously expressed in all eukaryotic cells and are used as reference genes in RT-qPCR experiments in plants (Reid et al., 2006; Maroufi, 2016; Liu et al., 2015). Actin2-7 (ACT2/7) encodes an actin protein that is constitutively expressed in vegetative structures but not in pollen. It is involved in tip growth of root hairs (Berardini et al., 2015). ACT2-7 was extensively used as reference gene in expression studies (Chapman and Waldenström, 2015; Hu et al., 2009). In the present study, *caACT2-7* showed an M value lower than the cutoff established by GeNorm (M<1.5) in all tissues of senna. 18S ribosomal RNA (18srRNA) is a component of the small eukaryotic ribosomal subunit (40S) involved in protein synthesis. 18srRNA is the most commonly used reference gene in expression studies in plants (Stolf-Moreira et al., 2011; Hu et al., 2009) due to its ubiquitous, abundant and stable expression across tissues and treatments. In the present study, *ca18s* showed an M value lower than the cutoff established by GeNorm (M<1.5) in leaf (young), pod (young and mature) and flower indicating higher stability in the expression in the tissues of senna. *ca18s* recorded an M value slightly higher than the cutoff established by GeNorm (M<1.5) in mature pod tissue and ranked last in stability by geNorm analysis. Previous studies have regarded rRNA as an ideal reference gene, given that the regulation of rRNA synthesis is independent of mRNA level (Bustin, 2002). However, an increasing amount of research is revealing the limitations of using 18S as a normalizer in RT-qPCR studies (Zhang et al., 2015a; Shen et al., 2010).

In the present study, the reference genes *caADH*, *caATPsyn*, *caEEF1-α* and *caEEF1-β* recorded an M value lower than GeNorm (M<1.5) in all the tissues and genotypes studied, thus regarded as highly stable reference genes for target gene quantification in Senna. The reference gene *ADH* encodes *Alcohol dehydrogenase* enzyme which facilitates the interconversion between alcohols and aldehydes or ketones with the reduction of nicotinamide adenine dinucleotide (NAD+ to NADH). *ADH* genes are ubiquitously expressed and are used as reference genes in RT-qPCR experiments in plants (Reid et al., 2006; Bas et al., 2012; Maroufi, 2016; Liu et al., 2015). Adenosine triphosphate (ATP) is the most commonly used "energy currency" of cells for most organisms. It is formed from adenosine diphosphate (ADP) and inorganic phosphate (Pi) catalyzed by *ATP synthase* enzyme. The gene *ATPsyn* that encodes *ATP synthase* enzyme is ubiquitous, abundant and has stable expression across tissues and treatments hence, used as endogenous control gene for target gene normalization in plants (Reddy et al., 2013). Eukaryotic elongation factors, *EEF1* mediates the entry of the aminoacyl tRNA into a free site of the ribosome during protein synthesis. *EEF1-α* and *EEF1-β,* the subunits of EEF1 are abundantly expressed in the cytoplasm.

The gene *ACT11* represents a unique and ancient actin subclass in the complex Arabidopsis actin gene family. In the present study, M values of *caActin11* was more than acceptable and established by GeNorm (M<1.5) in young leaf and mature pod hence, did not justify its use as reference gene for expression quantification in senna. *ACT11* gene is strongly expressed in tissues of the emerging inflorescence, pollen, and developing ovules in Arabidopsis (Huang et al., 1997). Similarly, the M values of *caβ-Tubulin* was higher than the cutoff established by GeNorm (M<1.5) in mature leaf, mature pod and flowers indicating poor stability in the tissues of senna. Use of less stable genes in estimating the gene expression leads to variation in the gene expression stability (Brunner et al., 2004; Czechowski et al., 2005). The results of this study confirmed earlier research that demonstrated tissue specific regulation of some reference genes in plants (Zhuang et al., 2015b;

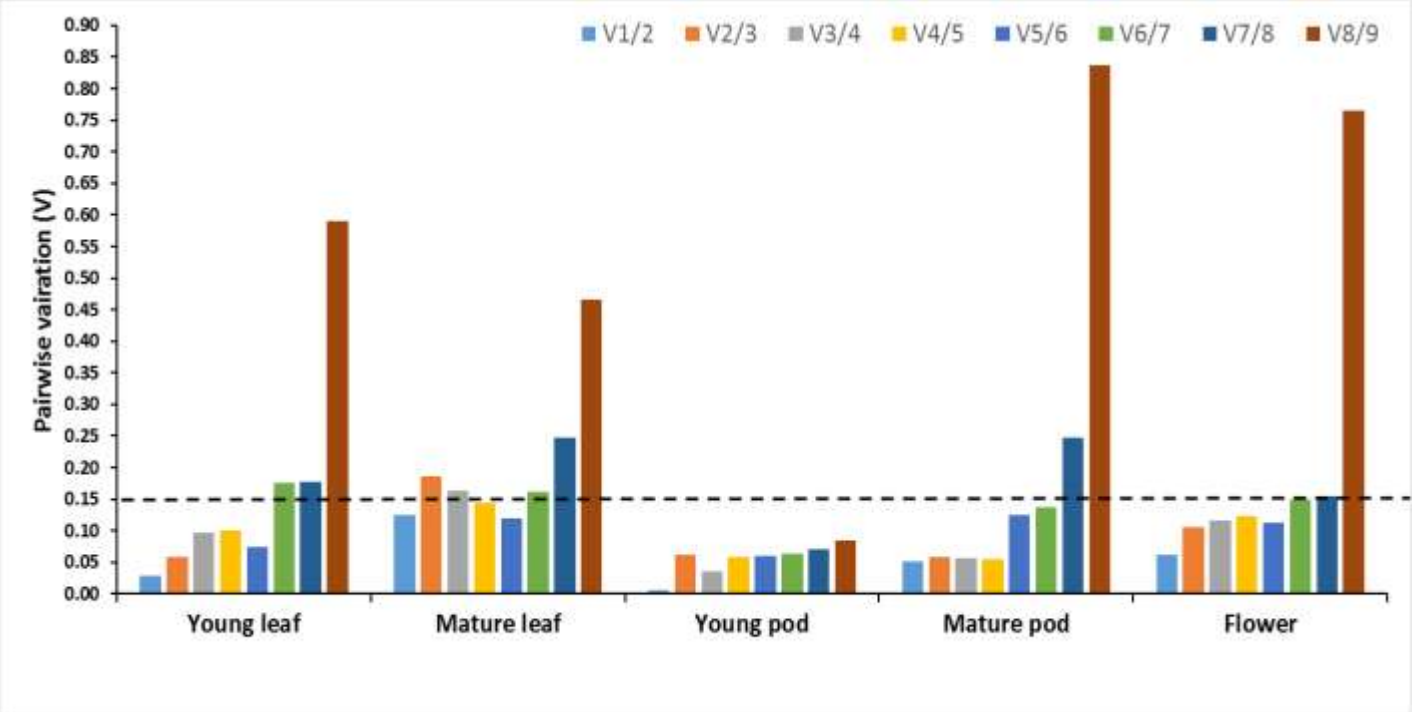

Figure 4: Pairwise variation of the reference genes in tissues of senna (*Cassia angustifolia*).

Czechowski et al., 2005; Maroufi, 2016; Liu et al., 2015). The rankings of the reference genes based on geNorm differed among tissue type used in this study perhaps due to tissue specific regulation of reference genes.

The optimal number of reference genes across different treatments determined by geNorm is presented in Figure 4. It has become clear from our study that a single reference gene is sufficient to normalize gene expression analysis in tissues used in Senna. Kylee et al. (2011) used multiple reference genes for normalizing functional gene expression, while Ling et al. (2011) indicated that an overabundance of reference genes may reduce data-normalization robustness.

Conclusion

Reference genes serve as an important endogenous control for data normalization in gene expression studies using qRT-PCR. We for the first time identified stable reference genes for expression assay using qRT-PCR in leaf (young and mature), pod (young and mature) and flowers in three genotypes of Senna. We provide a list of reference genes as well as specific primers that may be used in the future by researchers working on senna. The study provides the foundation for reference gene(s) selection in senna and will contribute toward more accurate target gene expression analysis studies and facilitate genomic research. Further, our study will also benefit future gene expression studies in other species of the genus Cassia or Senna.

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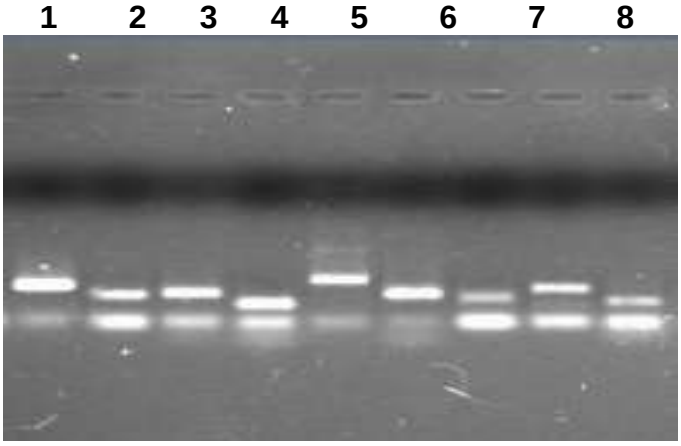

Figure S1: Specificity of qRT-PCR amplicons. (a) Agarose gel electrophoresis showing amplification of a single product of the expected size of reference genes. 1. *ca18srRNA*, 2. *caGAPDH*, 3. *caActin2-7*, 4. *caActin11*, 5.*caADH*, 6. *caATPsyn*, 7. *caTub-β*, 8. *caEEF1-α* and 9. *caEEF1- β*in variety SONA of Senna.

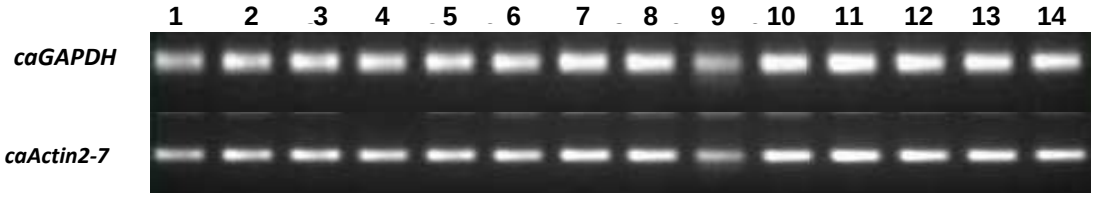

Figure S2: Amplification of *caGAPDH* and *caActin2-7* in leaf, pod and flowers of Senna. 1.Sona (young leaf), 2. Sona (mature leaf), 3. Sona (young pod), 4. Sona (mature pod), 5. Sona (flower buds), 6. DCA-80 (leaf) 7. DCA-80(flower), 8.DCA-80 (pods), 9.DCA-149 (young leaf), 10. DCA-149(mature leaf), 11. DCA-149 (young pod), 12. DCA-149 (mature pod), 13. DCA-149(flower buds) and 14. Sona (leaf)