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Identification of reference genes for real time PCR analysis in Dill seed (*Anethum sowa*)

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Abstract

Dill seed (*Anethum sowa* Roxb. ex Fleming) is an annual herb in the family Apiaceae with chromosome number 2n= 2x= 20. The present work was carried out with an aim to identify housekeeping genes in dill seed (Gujarat dill seed-3) for RT-PCR.Out of 12, total six primers amplified in all samples were subjected to validation through real time PCR.The data showed that *25s rRNA* had the highest expression level with least Ct value while *Tubulin* had lower expression level with higher Ct value that is 37.34. The most stable gene was *25s rRNA*.

The quantification of gene expression is one of the modern molecular biology keystones. It has provided insight into complex biological processes, increasing our understanding of signalling and metabolic pathways that underlie environmental responses and development. Quantitative reverse transcription realtime polymerase chain reaction (qRT-PCR) is a fast and precise method for gene expression study several biological systems (Campos *et al.*, 2015). It is a regular method for accurate expression profiling of a moderate number of selected genes. It is highly sensitive with a broader quantification range (Van Guilder *et al.*, 2008). It allows the detection of amplicon accumulation as qRT-PCR is carried out using fluorophore labelled probes or intercalating dyes like SYBR Green I, rather than by conventional end-point analysis. The basis of amplicon quantification is the expression ratio of a target gene and reference gene (Pfaffl and Hageleit, 2001), the selection of reference gene(s) has a direct and robust impact, given all other variables perfect. Inadequate and non-validated reference genes lead to inaccurate interpretation of expression data (Prunier *et al.*, 2016). Reference genes are relatively expressed constituently across the cell type, growth stage or environment. However, these genes may also participate in other processes and may show substantial variation. Therefore, a given reference gene cannot be universally used during expression study in qRT-PCR in functional studies.

Dill seed (*Anethum sowa* Roxb. ex Fleming) is an annual herb in the family *Apiaceae*. It is an important minor seed spice crop grown under irrigated as well as rain fed condition. It is an annual herb growing in the Mediterranean region, central and southern Asia. The major dill seed producing countries are India, Germany, Hungary, Netherlands, Pakistan and United States. In India, Gujarat, Rajasthan, Madhya Pradesh., Jammu and Kashmir, Orissa and Punjab are the leading states with respect to area and production. Similar to cumin, no molecular tools have been developed for dill seed (Kumar *et al.*, 2014). Therefore, the current study was performed to select robust and reliable reference genes which expressed stably and constitutively for accurate RT-qPCR normalization analysis in dill seed.

For total RNA isolation from root, shoot and developing seeds (25 days after fertilization), seeds of Gujarat Dill Seed-3 genotype were sown in pots during Rabi 2016 (Fig. 1). Total RNA was extracted using trizol method (Sambrook *et al.*, 1989). The quantity and quality (in terms of protein and DNA contamination) of isolated RNA, was estimated through spectrophotometry (Nanodrop, Thermo Scientific, U.S.A.).The first strand cDNA was synthesized from an aliquot of total RNA for each sample using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) and served as template for qRT-PCR. The qRT-PCR was performed using Takara SYBR green mix (Japan) on CFX96™ Real-Time PCR detection system, BioRad, USA following standard qRT-PCR guidelines. A set of 12 candidate genes [GAPDH, TBA, â-TUB, ACT, UBQ, EF-1A, heat shock protein 70 (hsp70), ribosomal protein L2 (rpL2), transcriptional initiation factor

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(TIF1) and the ribosomal18S, 25S and 5.8S RNAs reported by Campos *et al.* (2015) in carrot were deployed in present study for identification of reference gene for qRT-PCR. For each of the reference genes, the cycle threshold (Ct) was determined. Subsequently each data point was transformed according to the formula 2(-delta Ct) resulting in raw reference gene quantities which are the required input data for "geNorm" (Vandesompele *et al.*, 2002). For analysis of gene expression stability and rank, geNorm plus software was used. Out of 12, six endogenous genes namely actin (90 bp), rpl2 (85 bp), tubulin (108 bp), 25s rRNA (78 bp), 18S rRNA (133 bp) and 5.8S rRNA (130 bp) gave single amplicon in PCR (Fig. 2). The specificity of amplification reaction was verified before actual proceeding for quantitative Real-Time PCR. Melt curve analysis was performed in real time thermal cycler as a quality control step. The melting temperature of primers ranged between 74.50-84.50°C. 18s rRNA had highest melting temperature that is 84.50°C while Tubulin had lowest with Tm value 74.50°C. To evaluate the stability of expression of endogenous genes the Cq value of each gene of interest was recorded. Cq value ranged from 16.11 (low Cq value) to 37.34 (high Cq value) as shown in table 1, suggested that 25s rRNA had the highest expression level with least Cq value showed strong positive reactions. While Tubulin (Fig. 3) had lower expression level with higher Cq value that is 37.34.

Data on variation of internal control genes in various samples are presented in Fig.4. In Shoot tissue 5.8s rRNA gene was found to be the most stable followed by Tub,

Table 1. Cq values of endogenous primers obtained through qRT- PCR in shoot, root and seed tissue of Dill seed

Sample/Reference gene	Threshold cycle (Cq) value					
	Actin	*Tubulin*	*rpl2*	*18S rRNA*	*5.8s rRNA*	*25s rRNA*
Shoot	32.53	34.01	25.58	14.53	14.64	13.18
Root	25.26	39.30	30.57	29.92	27.94	25.58
Seed	35.90	38.72	24.50	10.13	9.72	9.57

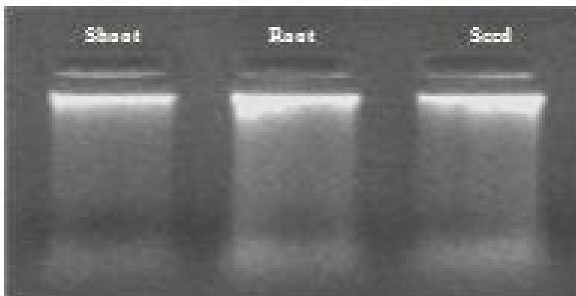

Fig 1. Agarose gel electrophoresis(2%) image of cDNA from shoot, root and seed

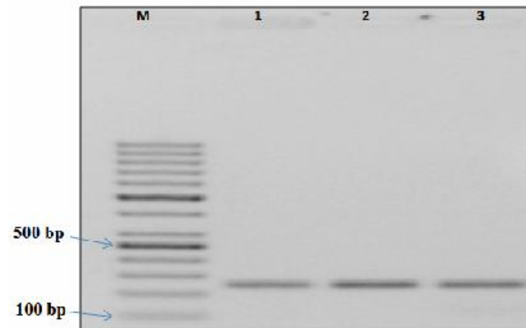

Fig 2. PCR amplification of reference gene using Tubulin primer. M: marker, 1- Shoot, 2- Root, 3- Seed

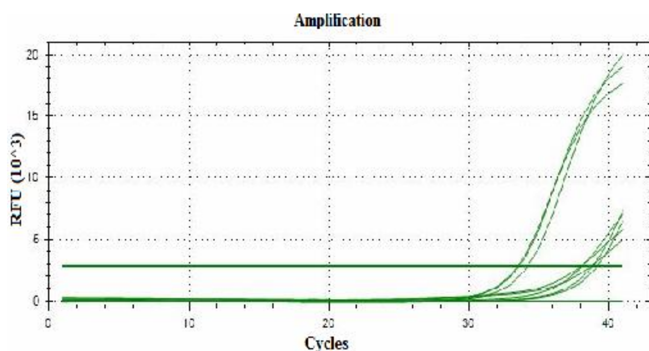

Fig 3. Amplification plot of Tubulin

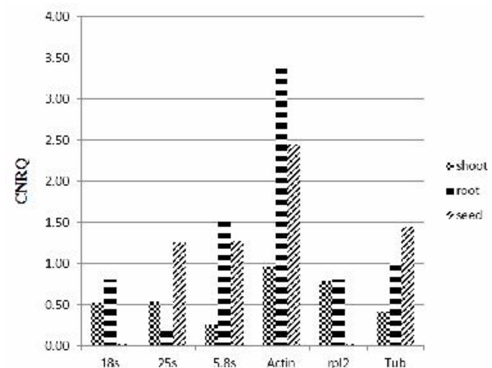

Fig 4. Variation of internal control genes in different tissues

18s rRNA, 25s rRNA and rpl2 (Fig. 4). In Root tissue sample 25s rRNA found to be most stable followed by rpl2, 18s rRNA, Tub and 5.8s rRNA. In Seed sample rpl2 and 18s rRNA found to be most stable gene followed by 25s rRNA, 5.8s rRNA and Tub, whereas Actin showed the least stable gene in all tissue samples. On the basis of M value, across the samples, the most stable gene was 25s rRNA followed by 18s rRNA, 5.8s rRNA, rpl 2, Tub and Actin (Fig 5). The most two stable genes were 25s rRNA and 18s rRNA. The M value ranged from 6.49 (highly stable) to 12.282 (least stable).The expression analysis of all six primers taken for the study revealed that the maximum expression level was observed in seed was for Actin and rpl 2. While in root tissues Actin and rpl 2 expression levels was decreased. The expression level was in normal range in shoot tissue while 25s rRNA showed maximum expression. 18s and Tubulin showed very low expression in seed (Table 2).

The analysis of stability index of studied reference genes will facilitate the selection of one or more than one housekeeping gene as internal control for gene expression studies in dill seed and will provide insight into the knowledge of gene expression of target genes to understand several metabolic and developmental processes during transcriptome analysis.

Table 2. Fold change (RQ) of gene expression in dill seed

Tissue	Fold change (RQ)				
	18s rRNA	*rpl 2*	*5.8s rRNA*	*Actin*	*Tubulin*
Shoot	1.04	0.36	2.52	3704.55	2.91
Root	0.47	1.43	1.23	4155.96	2.43
Seed	0.47	0.74	5.10	8945.73	2.79

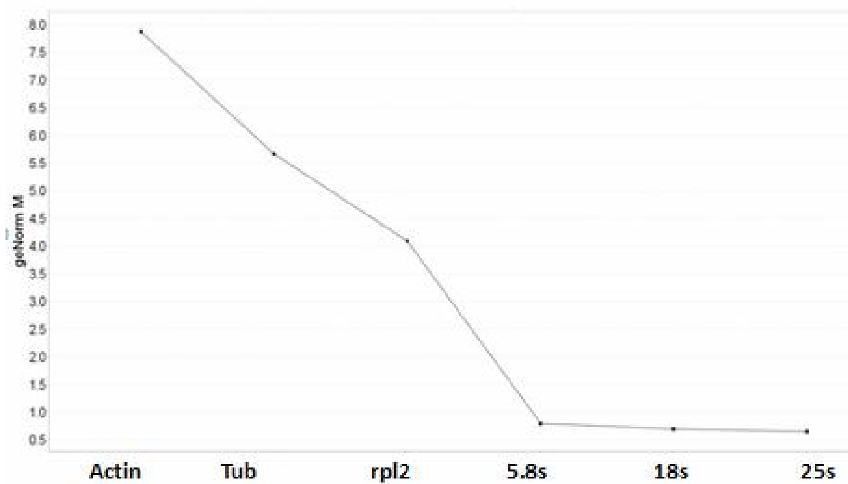

Fig 5. Average expression stability values of genes by geNorm analysis

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