

## Evaluation of Endogenous Gene Validation: An Important Contrivance for Differential Gene Study in Pearl Millet

Poonam Kanani\* and Y.M. Shukla

Department of Agricultural Biotechnology, Anand Agricultural University,  
Anand-388 110, India

\*Corresponding author

### ABSTRACT

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Nowadays selection and validation of endogenous gene is commonly being studied worldwide for differential gene study. And it is also considered as inevitable for gene validation studies. Endogenous gene expression varies profoundly with every cell and tissue with even mere deviations at nominal levels too, thus a need of normalization. So far, the most refined and steadfast method is preferably Quantitative Real-Time PCR (qRT-PCR) for precise quantification of gene expression to understand explicit gene functions at various development phases and beneath diverse environmental conditions that plant encounters during its life. The data normalization to find most stable gene are obtained through different software programs, such as geNorm, Norm finder and Best keeper. Pearl millet [*Pennisetum glaucum* (L.) R. Br.] a major grain and forage crop, frequently encounter various stresses both biotic and abiotic, and therefore more chances of studying genes responsible for stress adaption. Thus insight into evaluation of endogenous gene (such as *actin*, *tubulin*, *GAPDH*, *ubiquitin* etc.) validation studies can prove to target more exact molecular mechanism for stress tolerance.

### Introduction

Pearl millet (*Pennisetum glaucum* L.) is a monocot, diploid ( $2n=2x=14$ ) cereal crop plant belongs to family *Poaceae*, sub family *Penicedae*, also known as bajra. It is one of most important cereals growing worldwide in tropical semi arid regions mainly as food and forage crop in Africa and Asia, and It is the sixth most important crop after rice, wheat, maize, barley and sorghum, nutritionally rich in zinc, iron, and high antioxidants. It has haploid genome size of 2450 Mbp and has DNA content of  $1C = 2.36$  pg. (Budak *et al.*, 2003, Malik, 2015). Plants being sessile in nature are enforced to face different environmental conditions in time course of

their life span. The stresses such as drought, salinity and temperature: heat or cold are major abiotic and biotic stresses Biotic stress include four major diseases are downy mildew (*Sclerospora graminicola*), ergot (*Claviceps fusiformis*), smut (*Tolysporium penicillariae*) and rust (*Puccinia substriata* var. indica) which develop depending on the weather conditions and genotype are serious restraint for growth and productivity of pearl millet (Neya and Kabore, 1987; Thakur *et al.*, 2008).

All the processes related to physiological, morphological and biochemical changes are

outright affected by changes in abiotic and biotic environmental interactions. Morphological symptoms leads to lower seed germination, decreased seedling growth and reduced dry matter content (Munns *et al.*, 1982; Hasegawa, 2000). Wherein there are series of changes in physiological and biochemical changes occur which comprises elevated production of Osmoprotectant (such as proline, glycine betain and free amino acid), increased activity of antioxidant enzymes (*Peroxidase, Catalase, Superoxide dismutase, Ascorbate Peroxidase, Polyphenol oxidase* and *etc.*) (Alscher, 2002; Hasegawa, 2000).

### **Endogenous genes**

Gene expression profiling represents an elating tool to divulge mechanisms implicated in the response of plants to environmental stress proving as one of the modern molecular biology technique cornerstones. It has provided insight into identification of hundreds of genes induced when plants under stress and helped in understanding of complex biological process, signaling and metabolic pathways (Krebs *et al.*, 2002; Mantri *et al.*, 2007 and VanGuilder *et al.*, 2008). Real-time reverse transcription PCR (real-time RT-PCR) is presently a standard method for precise expression profiling for selected gene study, it is highly specific and sensitive and had broader quantification range than former molecular techniques.

For precise and unwavering gene expression results, real time PCR normalized data is required alongside a control gene, which exhibits a highly uniform expression during various developmental phases and underneath different environmental conditions (VanGuilder *et al.*, 2008). As the expression of endogenous gene is always steady in diverse conditions and they code for essential proteins constantly required by cell, these

genes are referred as constitutively expressed, housekeeping, endogenous, reference or internal control genes (Stephen *et al.*, 2001). Thus, a constant expression of these genes is a prerequisite, used for normalization in real-time PCR analysis, otherwise, erroneous results. Basically housekeeping genes which are involved in primary cellular processes are  $\beta$ -tubulin (*Tub*), Actin (*ACT*), *18S rRNA*, Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and Ubiquitin (*Ubq*) used earlier, with the idea to have uniform expression. But on contrary, reports illustrates that the transcript levels of these genes also vary significantly under different experimental conditions and are therefore not suitable for gene expression studies. So, therefore it is very important to investigate the stability of expression of commonly used endogenous genes in more depth and to identify novel and superior endogenous genes (Czechowski *et al.*, 2005 and Thellin *et al.*, 1999).

### **Characteristics of endogenous genes as reference gene**

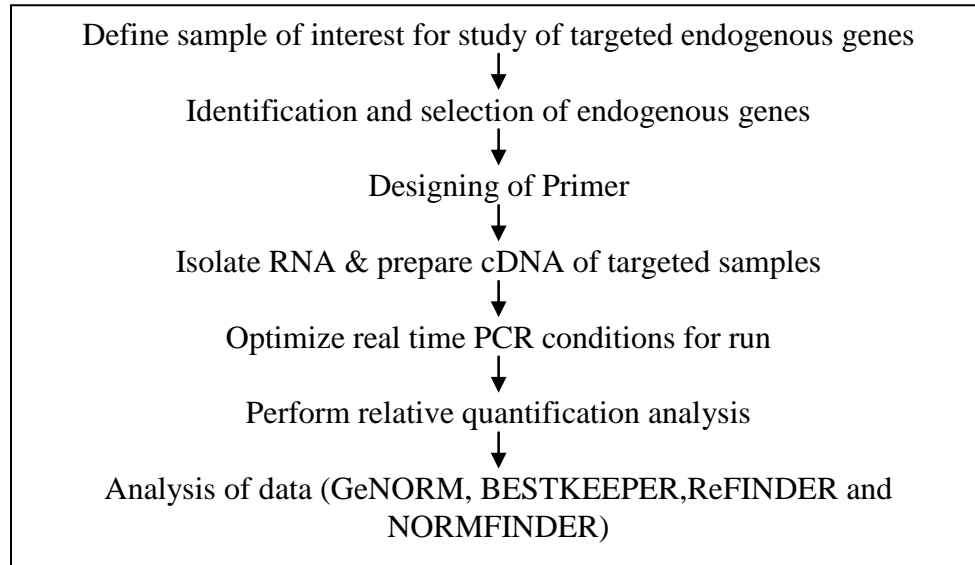
Single (or low) and known copy number  
Low allelic variation in cultivars  
Species specificity  
Ubiquitously expressed in all tissues  
Expression level remains at a steady state as compared to target gene expression.

Gene length of endogenous genes tends to be significantly more compact, shorter and with more exons than tissue specific genes. Scaffold/Matrix Attachment Regions (S/MAR) are less upstream of endogenous gene and poly (dA - dT) and (CCGNN)<sub>n</sub> found to be more abundant upstream of endogenous genes, as compared to tissue specific gene. They destabilize the formation of nucleosomes, leaving the DNA less packaged and maintaining constitutive gene expression (Davis *et al.*, 2002).

Modification of endogenous gene is done by homologous recombination-dependent gene targeting and chimeric RNA/DNA oligonucleotide - directed gene targeting to generate a site-specific base conversion, and homologous recombination-dependent gene targeting to produce either a base change or a gene replacement in a sequence-specific manner (Iida and Terada, 2005).

## Methods

There are different approaches to study endogenous genes are northern blot, southern blot, slot or dot blot, semi and fully quantitative PCRs, microarray and real time PCR. Real time PCR is being in wide use. The procedure follows as:



## Validation of endogenous genes in pearl millet under various biotic and abiotic stresses

Pearl millet is well cultivated in semi-arid climates, so there is more likelihood of having huge number of stress alleviating genes that helps for stress adaptation. As little availability of genome sequence information in pearl millet, has constrained the advancement in gene annotation, discovery, characterization, transcript profiling and in further crop developmental programs. Analysis of genes by qRT-PCR in pearl millet is restricted due to short of sequence information available till now, which can be provided as suitable reference genes. Therefore identification and selection of suitable reference gene remain to be a pre

requisite for future gene expression studies in pearl millet and other allied species of millet (Reddy *et al.*, 2015). With advancement and more focus on studies in pearl millet, few scientists are engrossed for screening endogenous genes in various biotic and abiotic stress condition.

## Primer selection

Due to inadequate accessibility of sequence information of pearl millet in the NCBI GenBank and EBI, therefore different approaches were carried out for sequence information retrieval. EST database was used for retrieving of the sequences and degenerate primers were designed based on the homologous genes of other closely related plant species (Reddy *et al.*, 2015). Like

GenBank accession numbers of selected candidate reference genes from Arabidopsis and rice prior were used for study, also by searching locus IDs and/or GenBank accession numbers of foxtail millet (a close relative of pearl millet) orthologous locus IDs from Phytozome v.9.0., Sequences (Saha and Blumwald, 2014; Shivhare and Lata, 2016).

Few studies on Pearl millet endogenous gene validation selection integrated both traditional housekeeping as well as several new reference genes. Saha and Blumwald, 2014 assessed about 18 candidate reference genes namely Actin (*ACT*), Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*), Cyclophilin (*CYC*), Phosphoenol Pyruvate Carboxylase-related Kinase (*PEPCK*), F-Box domain containing protein (*FBX*),  $\alpha$  Tubulin (*TUA*), Eukaryotic Elongation Factor 1 alpha (*eEF1a*), Eukaryotic Initiation Factor 4a2 (*eIF4a2*), Protein Phosphatase 2A (*PP2A*), Rubisco Activase (*RCA*), S-Adenosyl Methionine Decarboxylase (*SAMDC*), Tonoplast Intrinsic Protein (*TIP41*), Ubiquitin-Conjugating Enzyme 2 (*UBC2*), Ubiquitin-Conjugating Enzyme 18 (*UBC18*), Transmembrane Protein 56 (*UNK*), Ubiquitin 5 (*UBQ5*), 18S Ribosomal RNA (*18S rRNA*) and 25S Ribosomal RNA (*25S rRNA*) in 234 samples (three important pearl millet genotype). Analyses of Ct values via BestKeeper, Stability Index, Norm finder, DCt, ge Norm and Ref Finder programs ranked *PP2A*, *TIP41*, *UBC2*, *UBQ5* and *ACT* as the most reliable endogenous genes for normalization study under different experimental condition. Reddy *et al.*, 2015 reported 11 endogenous genes Protein Phosphatase 2C (*PP2C*), Ubiquitin-Conjugating Enzyme (*UBC*), Malate Dehydrogenase (*MDH*),  $\beta$ -tubulin ( *$\beta$ -TUB*), Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*), Elongation Factor 1-alpha (*EF-1a*), Eukaryotic Initiation Factor 4A (*EIF4A*),  $\beta$ -Actin ( *$\beta$  ACT*), Acyl Carrier Protein (*ACP*),

CYP Cyclophilin (*CYP*) and Ribosomal Protein (*S2440S*). Result showed that *EF-1a* and *EIF4A* expression was most stable indifferent plant tissues, *MDH* and *EIF4A* were found to be stable under different abiotic stress conditions. In the midst of the different genotypes of pearl millet evaluated, while *UBC* and *MDH* genes showed most stable expression, *MDH* and *ACP* showed superior stability in all samples set. Shivhare and Lata, 2016 illustrated 10 potential internal control genes *viz.* Actin (*ACT*), Elongation Factor 1 $\alpha$  (*EF-1a*), Malate Dehydrogenase (*MDH*), Eukaryotic Initiation Factor 4A (*eIF4A*), Heat shock protein 90 (*HSP90*), Ribosomal Protein L20 (*RPL20*), Glutaredoxin (*GlutR*), Protein Phosphatase 2A (*PP2A*), Ubiquitin-Conjugating Enzyme E2 (*UBC-E2*) and Tonoplast intrinsic protein 41 (*TIP41*) in various developmental tissues and different individual abiotic stresses and also their combinations at 1 h (early) and 24 h (late) of stress using Norm Finder, geNorm and RefFinder algorithms. Data analysis revealed that *EF-1a* and *UBC-E2* was best internal control genes across all samples.

In conclusion, in this present paper, it is tried to show the importance of validating endogenous genes in pearl millet for further differential gene expression studies is must for precise and accurate data obtaining. Profoundly different case studies reviewed so far showed the variability in expression levels at minor levels among endogenous genes. Thus it is demand of gene expression studies based on real time PCR to start with appropriate selection and validation of endogenous genes at even single experimental condition.

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