Journal of Applied Genetics (2018) 59:419–430
https://doi.org/10.1007/s13353-018-0466-1

PLANT GENETICS • ORIGINAL PAPER

# Reference gene validation for normalization of RT-qPCR assay associated with germination and survival of rice under hypoxic condition

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Received: 23 March 2018 / Revised: 22 July 2018 / Accepted: 28 August 2018 / Published online: 20 September 2018


## Abstract

Study on expression of genes for the traits associated with hypoxia tolerance during the germination demands robust choice of reference genes for transcript data normalization and gene validation through real-time quantitative polymerase chain reaction (RT-qPCR). However, reliability and stability of reference genes across different rice germplasms under hypoxic condition have not been accessed yet. Stability performance of reference genes such as eukaryotic elongation factor 1 α (*eEF1α*), ubiquitin 10 (*UBQ10*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), 18S ribosomal RNA (*18SrRNA*), 25S ribosomal RNA (*25SrRNA*), β-tublin (*β-TUB*), actin11 (*ACT11*), ubiquitin C (*UBC*), eukaryotic elongation factor 4 α (*eIF4α*), and ubiquitin5 (*UBQ5*) was accessed through statistical algorithms like geNorm, NormFinder, Comparative ΔCt method, BestKeeper, and RefFinder in three rice germplasms (KHO, RKB, and IR-64) with varied level of tolerance to hypoxic condition during germination. Among all genes used, *OsGAPDH* was found to be the most suitable reference gene under hypoxic condition. The performance of the highest-ranking reference gene (Os*GAPDH*) in terms of stability based on statistical algorithms was further validated for its reliability and stability through RT-qPCR with hypoxia-induced target gene *OsTTP7*. The identified stable housekeeping gene could be used as internal control for gene expression analysis in rice under hypoxia.

**Keywords** *Oryzasativa* L. · RT-qPCR · Hypoxia · RefFinder · *OsGAPDH*

## Introduction

Devastation due to flooding during sowing and transplanting of rice leads to severe crop loss (Jackson and Ram 2003; Setter et al. 1997). Several rice genotypes exhibiting a different level of tolerance to hypoxic condition during germination were identified through germplasm screening (Septiningsih et al. 2013). A tremendous advancement in breeding for hypoxic germination-tolerant rice varieties supplemented with high throughput tools for genomic studies for quantitative assessment of genes at transcript level provides a greater opportunity to understand the genetic and molecular basis of hypoxia tolerance. The signal cascade emanating from the perception of stimuli led to activation of hypoxia-responsive genes through various transcription factors, either at the transcriptional or post-transcriptional level results in re-programming of adaptive response at metabolic and physiological level (Sasidharan and Mustroph 2011). Changes in signaling and transcriptional regulation are species-specific (Mustroph et al. 2010). For these instances, quantitative real-time PCR (RT-qPCR), an analytical

Communicated by: Barbara Naganowska

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s13353-018-0466-1) contains supplementary material, which is available to authorized users.

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technique known for high sensitivity and reproducibility, has been used for the gene expression analysis (Chi et al. 2012). The first advantage of RT-qPCR is that it accurately quantifies gene expression without any follow-up intervention, thus reducing the experimental error (Nolan et al. 2006). Reportedly, the RT-qPCR has been used to examine the pattern of gene expression affected by flooding in Arabidopsis(Liu et al. 2005), rice (Kretzschmar et al. 2015; Lasanthi-Kudahettige et al. 2007), maize (Zou et al. 2010), populous (Kreuzwieser et al. 2009), and cotton (Christianson et al. 2010). RNA quality and integrity, variation in the RNA initial amount, and efficiency of complementary DNA (cDNA) synthesis may affect the PCR performance. A typical well-known strategy to correct for analytical variation is to normalize the expression of the target gene against stable endogenous gene(s). In order to qualify for an endogenous control in RT-qPCR, a gene should display stable expression in the plant, irrespective of the developmental and environmental condition (Bo et al. 2008). Yet, to avoid any inconsistency in gene expression of the target genes due to sub-optimal performance of endogenous control, it is necessary to validate the candidate reference genes for a set of treatment and different sample types (Xu et al. 2011). The statistical tools such as geNorm, Norm Finder, and BestKeeper (Setter et al. 1997; Christianson et al. 2010; Xu et al. 2015) have been used for determining the stability analysis of the reference genes. RefFinder is another statistical tool that has been used for grouping all the above-mentioned algorithms (Mallona et al. 2010). Housekeeping genes such as *OsGAPDH* and *ACT-B* have been frequently used in the gene expression studies (Thellin et al. 1999; Bustin et al. 2009). However, reliability and stability of reference genes in rice germplasms under hypoxic condition during germination have not been reported elsewhere. The goal of the present investigation was to identify the best stable candidate reference gene for gene expression studies in rice under hypoxia. We applied open-access software such as RefFinder (Mallona et al. 2010) for the stability analysis and ranking of ten housekeeping genes to narrow-down on the most appropriate gene for normalization under hypoxia. Our findings are likely to reveal that the highest-ranked reference gene obtained using statistical algorithms also performed with greater stability when validated against known hypoxia-induced gene like *OsTTP7* during rice seed germination. The identified reference gene can therefore be used for normalization of candidate gene in rice germination studies under hypoxia.

## Materials and methods

### Plant material selection and hypoxic treatment

Two AG (anaerobic germination)-tolerant rice (*Oryzasativa* L.) germplasms, Khao Hlan On (KHO, obtained from IRRI, Philippines), Rangadhar Kekua Bao (Deepwater rice ecotype from Assam, India), and an AG-susceptible variety IR-64 were used for the gene expression studies under hypoxic condition. Seeds of hypoxia-susceptible and tolerant germplasms were sown in bottles containing sterile water at a depth of 10 cm and maintained at 30 °C in dark in an incubator for up to 96 h ($O_2$ concentration was 2–2.5% as indicated by DO meter at seed depth). These seeds were grown in petri dish containing water-soaked filter to check the viability of the seeds. For stability assessment of housekeeping genes in hypoxic-tolerant and susceptible germplasms, samples for RNA isolation were collected at 24 h, 48 h, and 96 h after sowing and stored immediately in liquid nitrogen until further processing. As *OsTPP7* gene is transcriptionally upregulated under hypoxic condition in tolerant rice, which takes part in the conversion of trehalose-6-phosphate to trehalose (Kretzschmar et al. 2015), this gene was used as a target gene to validate reference genes for the gene expression studies during germination under hypoxia. Each experiment was done taking 3 biological replications. Sampling analysis was done with three technical replicates.

### Designing of primers

The stability of the qPCR was analyzed using 10 candidate reference genes. All genes used in the present study were present in single copy in rice genome, and the primers were used based on sequence information previously cited in rice reference gene validation studies (Jain et al., 2006). The expected amplicon sizes for reference genes used, namely eukaryotic elongation factor 1 α (*eEF1α*), ubiquitin 10 (*UBQ10*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), 18S ribosomal RNA (*18SrRNA*), 25S ribosomal RNA (*25SrRNA*), β-tubulin (*β-TUB*), actin11 (*ACT11*), ubiquitin C (*UBC*), eukaryotic elongation factor 4 α (*eIF4α*), and ubiquitin5 (*UBQ5*) were obtained from the published report (Jain et al. 2006). Primer pairs were tested for Tm, stability, GC content, and hairpin loop by using oligo-dT analyzer. The primer sequences and amplicon characteristics of the selected reference genes are listed in Table 1. The specificity of the amplicon was verified by dissociation curve analysis (60 to 95 °C) after 40 cycles of PCR and by agarose gel electrophoresis.

### Isolation of total RNA and cDNA synthesis

Total RNA was isolated from seeds collected at different time points using Pure-link reagent (Invitrogen, USA, cat no. 12322-012) following manufacturer's instructions. Purification of RNA was carried out using DNase I (Sigma Aldrich, cat no. AMPD1) and the RNA concentration was determined using Nano-drop 2000 (Thermo Fisher Scientific Wilminton D, USA). The integrity of the RNA samples was analyzed using 1.5% agarose gel

electrophoresis stained with ethidium bromide. One microgram (1 μg) of RNA was used for cDNA synthesis using the Prime Script RT reagent Kit (Takara, Japan cat no. RR047A) following the manufacturer's guidelines.

### Quantitative real-time PCR assay

RT-qPCR reactions were performed on an Applied Biosystem Step One Plus System using SYBR Premix Ex Taq (Takara, Japan). PCR conditions were optimized based on the primer efficiency. Briefly, a 10-μL reaction mixture consisted of 5 μL of SYBR Premix Ex Taq,0.2 μL of ROX dye, 2 μL of cDNA (50 ng/1:20 dilution), and 1.8 μL of Nuclease-Free Water (Ambion, USA cat no AM9930) using three different primer concentrations, along with a negative control (without cDNA). Thermal cycle used for all RT-qPCR reactions was 30 s at 95 °C followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. After 40 cycles the specificity of the amplicon was analyzed through the dissociation curve profile and agarose gel electrophoresis (2%). Each reaction was performed with three biological and three technical replications.

### Selection of candidate reference genes based on ranking

The primer efficiency and specificity of all reference genes were evaluated prior to the expression profile of housekeeping genes during RT-qPCR. Primer specificity was evaluated with the help of dissociation curve. For determination of the best candidate reference gene pair, the following statistical algorithms were used: NormFinder (Andersen et al. 2004), GeNorm (Vandesompele et al. 2002), Best Keeper (Pfaffl et al. 2004), and ΔCt method (Silver et al. 2006), and RefFinder (Mallona et al. 2010), web-based software. A set of 4-fold dilutions (1:20; 1:40; 1:80; 1:160) of cDNA from RKB, KHO, and IR-64were used to make the standard curves; thus, the PCR efficiency ($E$) and correlation coefficient ($R^2$) were determined for each gene using the linear regression model. The PCR efficiency was calculated as E = (10–1/slope) − 1.

### Data analyses

BestKeeper algorithm determined the optimal housekeeping gene employing the pairwise correlation analysis based on the standard deviation (±CP) of all the pairs of tested reference genes (Reena et al. 2009). GeNorm algorithm, which is a stepwise exclusion method based on average expression stability (M) value, provided a pair of two housekeeping genes having equal expression levels (Vandesompele et al. 2002). The NormFinder, an Excel-based algorithm, determined the expression stability value of the candidate gene and identified the stable housekeeping genes based on intra- and inter-group variations among the tested genes (Andersen et al. 2004). For geNorm and NormFinder, the raw Cq values were converted into relative quantity values using the formula $2-\Delta Cq$. Finally, RefFinder was employed for combining all the algorithms used to find the best suitable reference gene (Mallona et al. 2010). All the measurements were carried out in triplicate, and for each primer set, reaction efficiency estimates were derived from standard curves that were generated using serial dilutions of a cDNA pool of all the rice samples. These were then used by qBase to transform the Ct values to relative quantities for analysis with geNorm 3.4 software. After normalization using the geometric mean of the most stable reference genes, the normalized *OsTPP7* expression level ratios were standardized to minimize inter-experimental variation. For the purpose, the normalized expression levels were converted into logarithmic values, divided by their standard deviations and multiplied by the mean standard deviation of all the experiments to calculate the mean standardized expression level per rice sample along with their respective 95% confidence intervals. Finally, all the values were linearized using a power function and plotted in a graph. Differential gene expression was considered to be significant when the 95% confidence interval of the mean expression levels did not overlap (equivalent to $P < 0.05$).

## Results

### Selection of housekeeping genes for the stable expression during hypoxia

The qPCR amplification efficiency ($E$) ranged from 90.30 to 104.10%, with a correlation coefficient ($R2$) ranging from 0.982 to 0.998. The mean Ct values of 10 candidate genes in all samples varied from 10.25468 to 34.37549 (Table 1). For each primer, the dissociation curve displayed a unique peak of fluorescence, indicating that a single fragment was amplified during qPCR amplification for samples of all treatments (Fig. S1). The cycle threshold (Ct) values of 10 reference genes in different germplasms under three different treatments were used to compare the gene expression pattern. Comparative analyses of all the genes tested revealed a wide range of expression variances of the Ct values under all three treatments with the highest variation observed in *Os25SRNA* and *Os18SRNA* and lower variation in *OsGAPDH*, *OseEF1α*, and *OseIF4α*. Even though the variation analysis based on Ct values revealed some of the genes with relatively less variation, a comparison with other statistical algorithms was necessary for determining the most stable gene(s) for normalizing target gene expression.

**Table 1** Housekeeping genes (Jain et al. 2006) and their primer sequences used for the expression study of the target genes through real-time PCR analysis

| Gene name | Gene description | Primer sequence (5′-3′) | Amplicon size (bp) | *Primer efficiency E* (%) | $R^2$ | Gene ID on RapDB/MSU |
|---|---|---|---|---|---|---|
| *ACT11* | Actin 11 | CAGCCACACTGTCCCCATCTA<br>AGCAAGGTCGAGACGAAGGA | 67 | 101.8 | 0.998 | Os03g0718100 |
| *UBC* | Ubiquitin-conjugating enzyme | CCGTTTGTAGAGCCATAATTGCA<br>AGGTTGCCTGAGTCACAGTTAAGTG | 76 | 102.4 | 0.997 | Os02g0634800 |
| *eEF1α* | Eukaryotic elongation factor1-alpha | TTTCACTCTTGGTGTGAAGCAGAT<br>GACTTCCTTCACGATTTCATCGTAA | 103 | 96.9 | 0.990 | Os03g0178000 |
| *GAPDH* | Glyceraldehyde-3-phosphatedehydrogenase | AAGCCAGCATCCTATGATCAGATT<br>CGTAACCCAGAATACCCTTGAGTTT | 79 | 102.7 | 0.992 | Os04g0486600 |
| *β-TUB* | Beta-tubulin | GCTGACCACACCTAGCTTTGG<br>AGGGAACCTTAGGCAGCATGT | 82 | 97.5 | 0.996 | Os01g0805900 |
| *eIF4α* | Eukaryotic initiation factor 4α | TTGTGCTGGATGAAGCTGATG<br>GGAAGGAGCTGGAAGATATCATAGA | 76 | 104.1 | 0.994 | Os02g0221300 |
| *UBQ10* | Ubiquitin 10 | TGGTCAGTAATCAGCCAGTTTGG<br>GCACCACAAATACTTGACGAACAG | 81 | 96.8 | 0.982 | Os02g0161900 |
| *UBQ5* | Ubiquitin 5 | ACCACTTCGACCGCCACTACT<br>ACGCCTAAGCCTGCTGGTT | 69 | 91.9 | 0.991 | Os06g0650100 |
| *18S rRNA* | 18S ribosomal RNA | CTACGTCCCTGCCCTTTGTACA<br>ACACTTCACCGGACCATTCAA | 65 | 90.3 | 0.994 | LOC_Os09g00999 |
| *25S rRNA* | 25S ribosomal RNA | AAGGCCGAAGAGGAGAAAGGT<br>CGTCCCTTAGGATCGGCTTAC | 68 | 97.3 | 0.945 | LOC_Os09g01000 |

## Analysis of the housekeeping genes based on a geNorm algorithm

GeNorm analysis ranked the target reference gene according to their average expression stability (M value) using the Ct value of all samples. Samples with lowest M value were considered as the most stable and vice versa. The M value of housekeeping genes ranged from 0.868 to 3.575 (Min-Max). *OseIF4α*/*Osβ-TUB* showed the highest stability (M value of 0.868) and *Os25SrRNA* (M value of 3.575) was the least stable gene among all reference genes tested (Fig. 1).

## Analysis of the housekeeping genes based on a NormFinder algorithm

The NormFinder analysis, which is based on intra- and intergroup variations, was employed for the estimation of stability values. Following this approach, *OsEF1α* (stability value, 0.569) was identified as the most stable gene followed by *OsGAPDH* (stability value, 0.685), *OsACT 11* (stability value, 4.563), and *Os25SrRNA* (stability value 6.236) (Fig. 2).

## Analysis of the housekeeping genes based on the comparative ΔCт method

The stability of the housekeeping genes was determined by using comparative ΔCт methods based on standard deviation (SD). Higher SD value correlated to the low stability of the housekeeping genes and vice versa. The *OsGAPDH* with SD value of 2.63 was found to be the most stable housekeeping gene while *Os25SrRNA* having SD value of 5.3 was the least stable (Fig. 3).

## Analysis of the housekeeping genes based on the BestKeeper algorithm

The stability of the housekeeping genes was determined by BestKeeper based on the extent of standard deviation (SD [±CP) with higher SD value corresponding to the low stability of the housekeeping genes and vice versa. The descriptive statistics of all the ten housekeeping genes computed by this algorithm (Fig. 4) led to the identification of six housekeeping genes (*OsUBC*,*OsACT11*, *Osβ-TUB*, *OsGAPDH*, *OseIF4α*, and*OsEF1α*) which were consistent and stable. On the other hand, the other 4 genes, namely *Os25SrRNA* (SD, 5.39), *Os18SrRNA* (SD, 4.31), *OsUBQ5* (SD, 3.48), and *OsUBQ10* (SD, 3.39) were found to be inconsistent and less stable. The coefficient of variation (CV %) of all housekeeping genes ranged from 5.37% for *OsUBC* to 27.06% for *Os25SrRNA*, suggesting different levels of variation in candidate housekeeping genes (Table S1).

## Comparative expression stability analysis based on RefFinder algorithm

As expression stability analysis of target genes accessed using proven statistical algorithm showed inconsistency in terms of their stability, a comparative gene expression stability and ranking of genes based on their Ct value was performed through RefFinder. Based on the stability value, four housekeeping genes namely *OsGAPDH* (geo mean 2.21), *OseIF4α* (geo mean 2.59), *OsEF1α* (geo mean 2.63), and *Osβ-TUB* (geo mean 2.63) were found to be stable across different treatments, while *UBQ5* and *25SrRNA* with a Geo Mean of 7.74 and 10.0, respectively, were found to be less stable (Fig. 5).Thus, comparative study of all four statistical methods provided a way for ranking the housekeeping genes based on

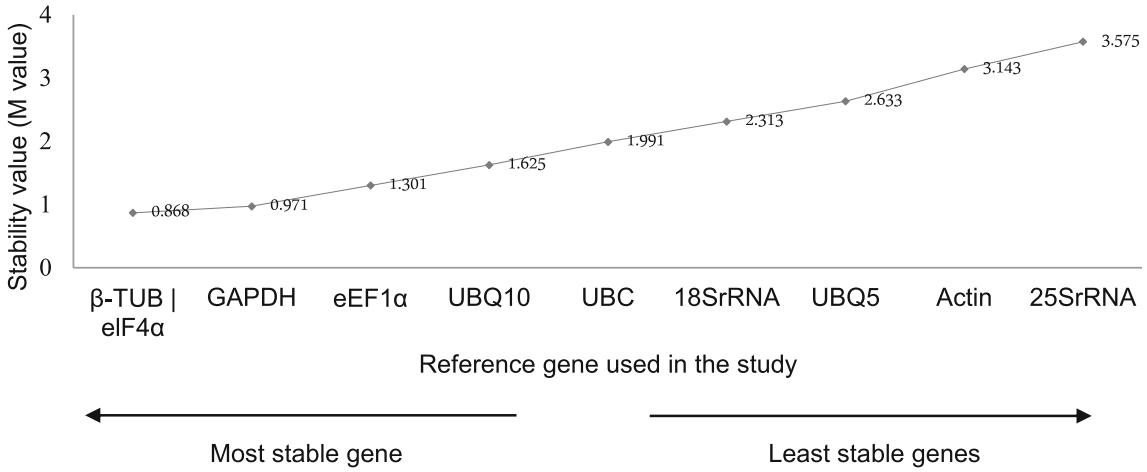


**Fig. 1** Expression stability analysis of reference genes among different anaerobic-treated tissue samples using geNorm algorithm. The lower M value indicates higher stability of the gene and vice versa. The direction of the arrows indicates the most and least stable housekeeping genes

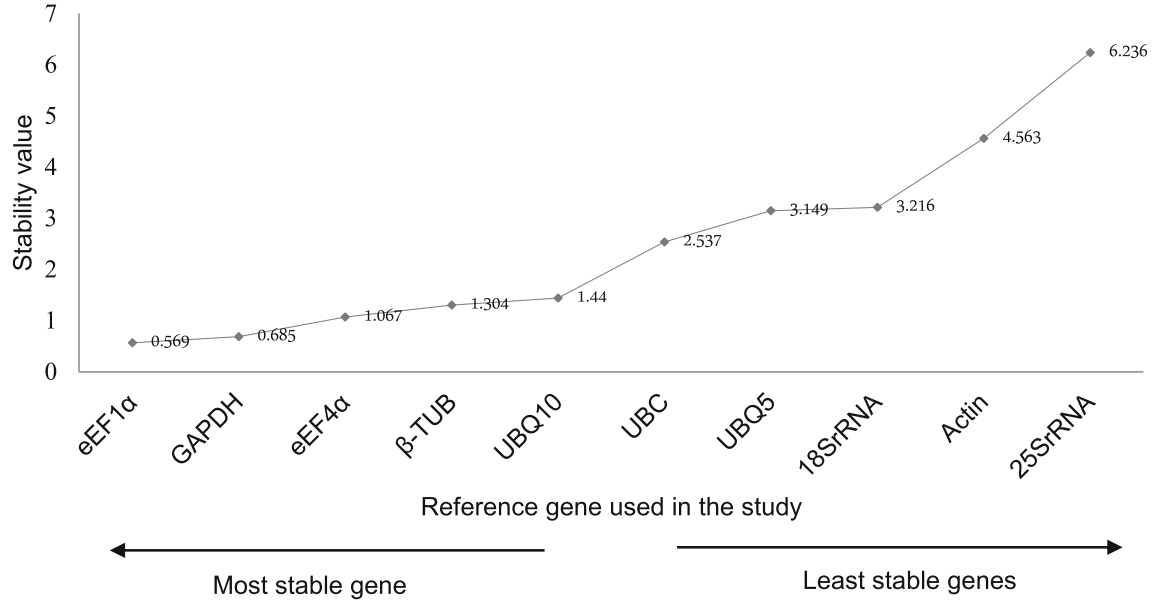


**Fig. 2** Expression stability analysis of each candidate reference gene among different anaerobic treated tissue samples using NormFinder algorithm. Lower stability value indicates higher stability of the housekeeping gene and vice versa. The direction of the arrows indicates the most and least stable housekeeping genes

their performance (Table 2). Among all, *OsGAPDH* was considered to be the most stable housekeeping gene for the gene expression studies under hypoxia in rice during germination and coleoptile elongation.

## Validation of identified stable reference genes

Analyses of data for different hours of treatment (24 h, 48 h, and 96 h) under the hypoxic condition of *OsTPP7* gene revealed differences in the pattern of expression when normalized with the most stable and the least stable housekeeping genes (*25SrRNA*). Relative quantification (RQ) of *OsTPP7* gene varied according to the internal control (stable and least stable) used during normalization of the target gene.

Analysis of dataset for three genotypes (KHO, RKB, and IR-64) at 24 h (hypoxic) time point revealed significant expression difference when normalized against the most stable and the least stable housekeeping genes. RQ of target candidate gene, *OsTPP7*, was higher when normalized against the stable internal control genes, such as *OSGAPDH* (2.70-fold in

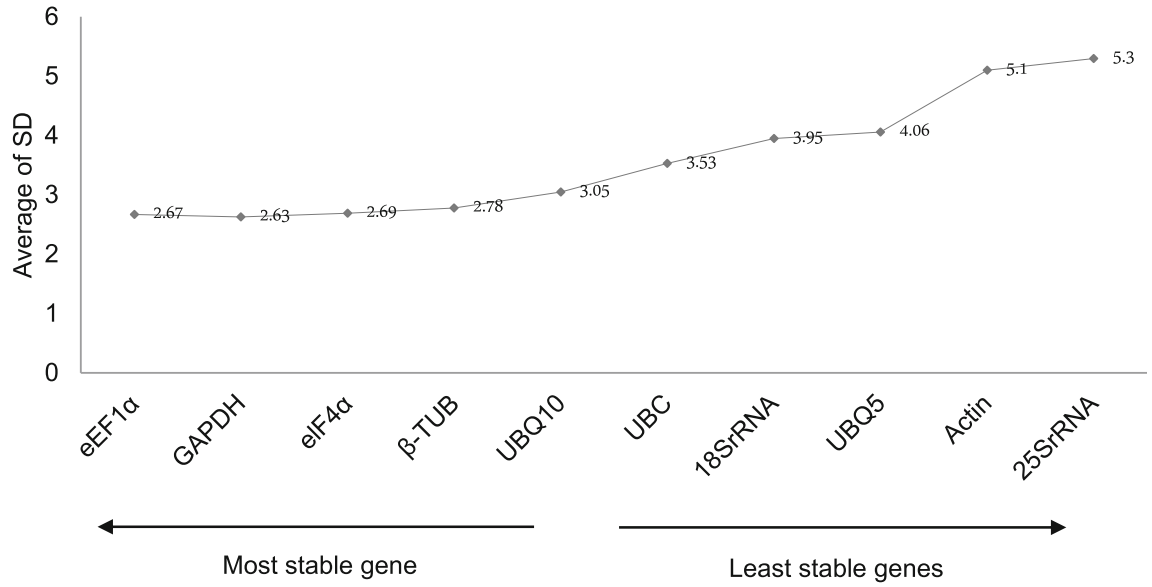


**Fig. 3** Expression stability analysis of each candidate reference gene among different anaerobic-treated tissue samples using comparative ΔCT method. Lower stability value indicates higher stability of the housekeeping gene and vice versa. The direction of the arrows indicates the most and least stable housekeeping genes

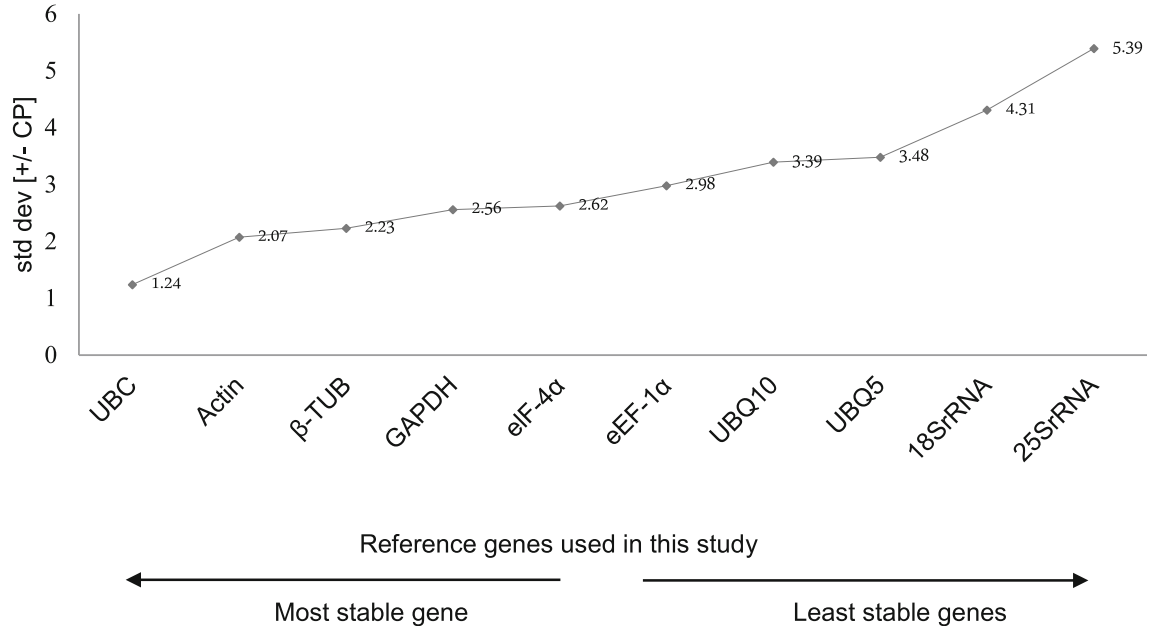


**Fig. 4** Expression stability analysis of each candidate reference gene among different anaerobically treated tissue samples using BestKeeper. The lower standard deviation (SD) [±CP] value indicates the higher the stability of the gene and vice versa. The direction of the arrows indicates the most and least stable reference genes used in the study

RKB and 3.42-fold in KHO), *OsEF1α* (3.53-fold in RKB and 3.21-fold in KHO) and *eIF4α* (4.14-fold in RKB and 3.65-fold in KHO) compared to more commonly used housekeeping gene *OsACT 11* (2.01-fold in RKB and 2.51-fold in KHO).

Likewise, analysis of dataset at 48-h hypoxic time point also revealed significant variation in expression level of the target candidate gene (*OsTPP7*). Fold change in expression of *OsTPP7* represented by RQ value was higher when normalized against the stable internal control genes, such as *OsGAPDH* (0.88-fold for IR64, 4.80-fold in RKB, and 4.15-fold-in KHO), *OsEF1α* (0.90-fold in IR-64, 4.70-fold in RKB, and 3.83-fold in KHO) and *OseIF4α* (1.0-fold in IR64, 5.35-fold in RKB, and 3.34-fold in KHO) compared to the commonly used housekeeping gene *OsACT 11* (0.89-fold in *OsACT 11*, 2.76-fold in RKB, and 2.53-fold in KHO). However, the expression of the target gene was more than 3-fold lower in sample normalized with *Os25SrRNA* at 24 h (0.82-fold in IR64, 0.81-fold in RKB, and 0.73-fold in KHO) compared to corresponding stable internal control (*OsGAPDH*, *OsEF1α*, *OseIF4α*) as well as *OsACT 11* normalized samples.

Analysis of dataset at 96-h hypoxia-treated samples showed marked difference in expression levels in susceptible and tolerant genotypes. The fold change of *OsTTP7* gene when most stable housekeeping genes such as *OsGAPDH* were used as internal control (0.58-fold for IR64, 4.77-fold in RKB, and 3.53-fold in KHO), *OsEF1α* (0.97-fold in IR-64, 3.98-fold in RKB, and 3.93-fold in KHO) and *OseIF4α* (0.81fold in IR64, 3.15-fold in RKB, and 3.26-fold in KHO) compared to the commonly used housekeeping gene *OsACT 11* (0.42-fold in IR64, 2.82-fold in RKB, and 2.29-fold in KHO), the performance of least stable gene *Os25SrRNA* in terms of expression of target gene in susceptible and tolerant genotypes were similar (0.38-fold in IR-64, 0.76-fold in RKB, 2.89-fold in KHO) (Fig. 6). This finding shows that the stable genes exhibit similar levels of gene expression highlighting the significance of the choice of internal controls under hypoxia for expression profiling of candidate genes.

## Discussion

Tolerance of rice crop to flooding through enhanced germination and early seedling growth is a prerequisite for successful cultivation of rice in a region where flooding is a recurrent event. Although rice could tolerate flooding, its germination is limited to coleoptile elongation as root and primary leaf fail to develop in susceptible genotypes (Dumbala et al. 2013).

As evident from previous studies, hypoxic germination is a complex process and performance of a tolerant genotype could vary depending on the level of oxygen deprivation (Xie et al. 2011). The process of cell division and elongation is known to occur under hypoxia. However, the ability of cells to divide under the hypoxic condition is determined by the period of submergence, which is around 48 h (Selvey et al. 2005), as beyond which point there would be energy bottlenecks due to lack of oxygen (Ohl et al. 2005). In order to understand the molecular basis of the response of rice to an abiotic stimulus such as hypoxic condition during germination, studies with qPCR have been widely conducted for

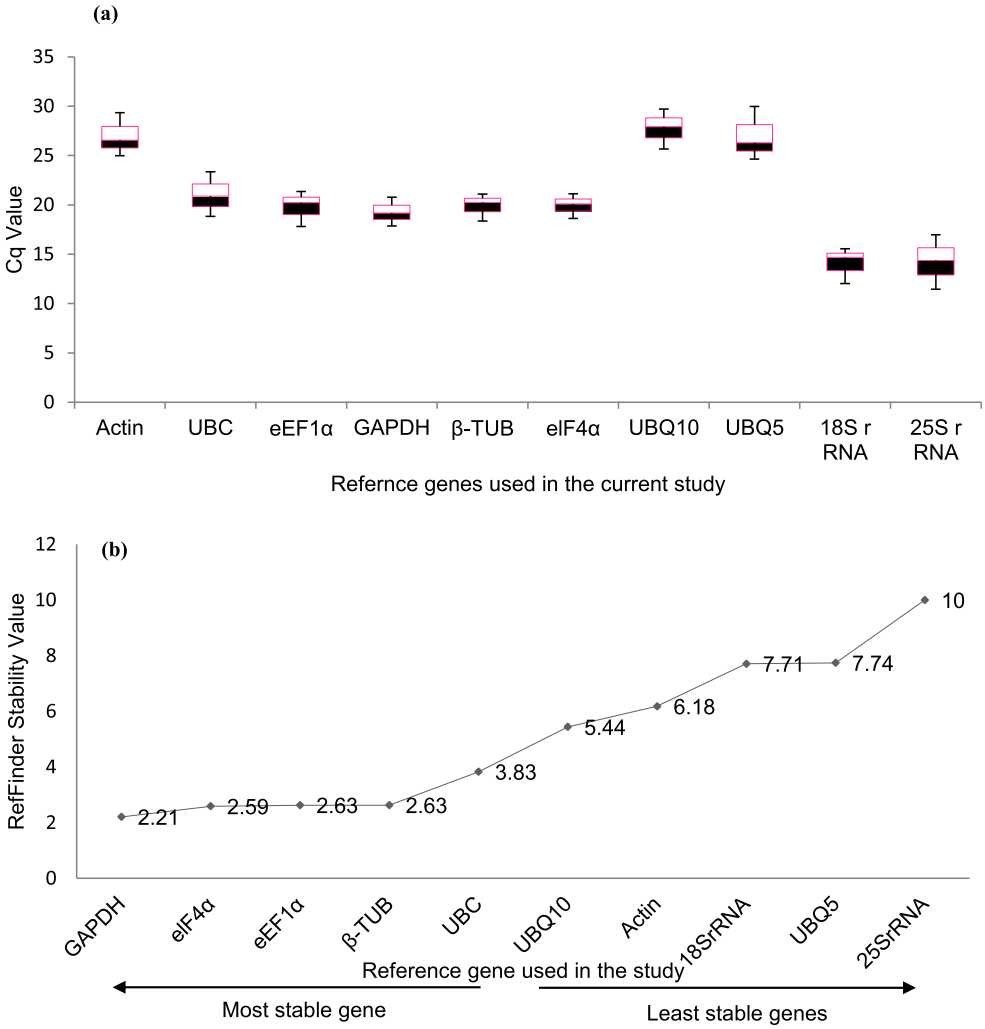


**Fig. 5** Ct variation and expression stability analysis of each candidate reference gene among different tissue samples using RefFinder. **a** Boxplot depicting absolute Ct values, which were calculated using GenEx program. Lower and upper boxes indicate the 25th and 75th percentile, respectively, and median is depicted by the line. **b** Gene expression stability graph using RefFinder algorithm based on stability values of all algorithm. The direction of the arrows indicates the most and least stable housekeeping genes

characterizing gene expression patterns. Normalization of target gene expression via stable endogenous housekeeping genes is a prerequisite to minimize error in relative quantification as the use of inconsistent housekeeping genes may lead to erroneous results leading to misinterpretation of data (Selvey et al. 2005; Ohl et al. 2005). Besides, for accurate quantification of target gene expression, it is essential that expression of reference gene should be independent of

**Table 2** Ranking orders of all reference genes for anaerobic stress conditions using $\Delta C_T$, BestKeeper, and NormFinder and geNorm algorithms and combined the entire algorithm using RefFinder

| $\Delta C_T$ | BestKeeper | NormFinder | GeNorm | Comparative ranking | Ranking |
|---|---|---|---|---|---|
| *eEF1α* | *ACT11* | *eEF1α* | *β-TUB\|elF4α* | *GAPDH* | 1 |
| *GAPDH* | *UBC* | *GAPDH* | *GAPDH* | *elF4α* | 2 |
| *elF4α* | *eEF1α* | *elF4α* | *eEF1α* | *eEF1α* | 3 |
| *β-TUB* | *GAPDH* | *β-TUB* | *UBQ10* | *β-TUB* | 4 |
| *UBQ10* | *β-TUB* | *UBQ10* | *UBC* | *UBC* | 5 |
| *UBC* | *elF4α* | *UBC* | *18SrRNA* | *UBQ10* | 6 |
| *18SrRNA* | *UBQ10* | *UBQ5* | *UBQ5* | *Actin* | 7 |
| *UBQ5* | *UBQ5* | *18SrRNA* | *Actin* | *18SrRNA* | 8 |
| *Actin* | *18SrRNA* | *Actin* | *25SrRNA* | *UBQ5* | 9 |
| *25SrRNA* | *25SrRNA* | *25SrRNA* | | *25SrRNA* | 10 |

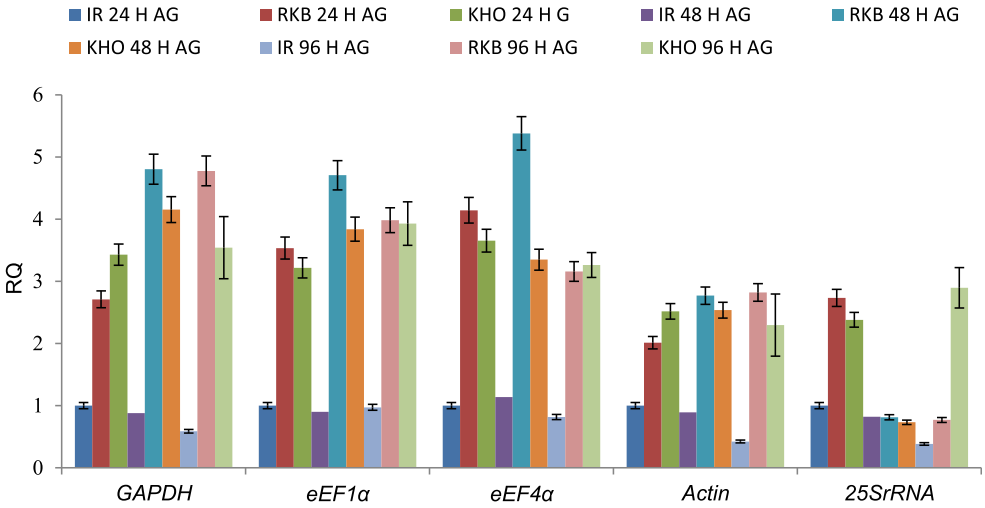


**Fig. 6** Normalization of hypoxia-inducible target gene OsTPP7 in tolerant (KHO, RKB) and susceptible (IR-64) cultivar. The relative quantification (RQ) value of OsTPP7 was obtained by normalization with housekeeping genes such as (i) GAPDH, (ii) eEF4α, (iii) eEF1α, (iv) actin, and (v) 25SrRNA gene using susceptible IR-64 at 24 HAG as treatment control

genotypes, conditions, and treatments (Isaiah et al. 2016; Maksup et al. 2013; Manoli et al. 2012).Therefore, to perform the accurate candidate gene expression analysis, number of housekeeping genes have been tested and identified in many crops such as rice (Xu et al. 2015; Isaiah et al. 2016; Jain et al. 2006; Maksup et al. 2013), maize (Manoli et al. 2012; Yueai et al. 2014), wheat (Long et al. 2010; Paolacci et al. 2009), soybean (Hu et al. 2009; Le et al. 2012; Ma et al. 2013; Nakayama et al. 2014), peanut (Chi et al. 2012; Reddy et al. 2013), and tea (Ming-Le et al. 2017).

Hypoxic condition prevailing during the time of germination affects global transcript change (Reena et al. 2009). Choice of internal control is of utmost importance when expressions of genes are studied in metabolically active tissues, particularly during early seed germination and coleoptile elongation when there is a rapid change in transcript dynamics (Reena et al. 2017). *OsACT11* and *OsUBQ* have been previously used as reference genes for assessment of transcript perturbation in hypoxic responsive genes in hypoxic-sensitive and tolerant rice (Kretzschmar et al. 2015). We did not come across any published report that systematically analyzes the expression stability of housekeeping genes in rice under hypoxic condition. In order to assess any shortcomings with regard to the stability of housekeeping gene that could affect the performance of target genes for hypoxic germination studies, 10 well-known housekeeping genes were studied on a set of AG-tolerant viz KHO and RKB and susceptible IR-64 rice germplasm through different statistical approaches. Use of different statistical algorithms for validation, normalization, and statistical modeling of selected reference gene has been reported (Ward et al. 2015; Aniko et al. 2004). The BestKeeper was previously used for analyzing descriptive statistics of different reference genes, while geNorm, comparative ΔCT, and NormFinder were used to determine the ranking of genes according to their stability values (Manoli et al. 2012; Ming-Le et al. 2017; Vandesompele et al. 2002). *OsGADPH* was identified as most stable gene when assessed through NormFinder and $\Delta C_T$, while geNorm identified *Osβ-TUB* and *OseIF4α* as the most stable. Variation in the ranking of the gene, as evident from our study, could be attributed to the use of different algorithm programs such as geNorm and NormFinder (Vandesompele et al. 2002; Andersen et al. 2004). Such discrepancies were also reported while identifying *OseIF4α* as the most stable housekeeping gene in pigeon pea under drought stress using similar statistical method (Sinha et al. 2015). NormFinder and GeNorm algorithms have been previously reported in soybean for identification of Elongation factor 1-beta and *ACT11* as stable internal reference gene during anoxic stress (Nakayama et al. 2014).

Variation in stability of known housekeeping genes under the hypoxic condition is not unexpected as similar findings have been reported for genes used as internal reference control, like *GAPDH*, *β-Actin 11*, *β-TUBLINE*, *PGK*,*UBQ*, *RPL-19*, and *18S rRNA* in a wide variety of tissues and cells (Rebouças et al. 2013). Importantly, our analysis suggests that housekeeping genes like *Os25SrRNA* should be avoided as an internal control for studying the expression profile of candidate genes under hypoxic conditions in rice. This is because the expression of endogenous target gene *OsTPP7* normalized with *Os25SrRNA* was lower in rice genotypes tested under different hypoxic treatments. Suboptimal performance of *Os18SrRNA* and *Os25SrRNA* gene had been reported previously while using them as internal controls for normalizing the drought-induced gene *uspA* (Sinha et al. 2015). Finally, comparative study on expression dataset obtained from four different statistical methods using RefFinder (Mallona et al. 2010) and ranking the genes based on geo mean also identified *OsGADPH* as the most stable internal control gene under hypoxia.

It has been previously reported that the performance and gene stability of certain housekeeping genes may get altered depending on the type of treatment. The stable housekeeping gene (*OsGAPDH*) identified through normalization of candidate genes in the present study is known to be cytosolic glyceraldehyde 3-phosphate dehydrogenase (*OsGAPDH*, E.C. 1.2.1.12), present in all organisms and catalyzes the oxidation of trios phosphate during glycolysis (Giulietti et al. 2001). Some commonly used reference genes like *ACT 11* and *OsGAPDH* performed inconsistently in different plants, tissues, and experimental conditions, and that warrants for caution in its use as an internal control, primarily because transcript levels of reference genes could vary considerably in response to experimental conditions, cellular process, and tissue types. It is also true that *OsGAPDH* acts as a component of the glycolytic pathway, and gluconeogenesis, processes that are integral to the survival of the organism. Yet, studies in other crop species have shown that *OsGAPDH* has constant expression in total developmental stages in crops like pearl millet (Reddy et al. 2015) and sugarcane (Larissa Mara de Andrade et al. 2016) while being the least stable transcript in wheat developmental series. Also *OsGAPDH* was reported as an appropriate candidate for reliable normalization of target gene expressions levels during biotic and abiotic stresses in rice (Bevitori et al. 2014). Although it is reported that expression level of 37 kDa *OsGAPDH* subunit changes during early stages of germination during anoxia (Ricard et al. 1989), in our study, we focused on hypoxic condition (2–3% oxygen level) for reference genes whose transcript does not vary in tolerant and susceptible rice cultivars under hypoxia germination stage oxygen deficiency (GSOD). Although *Os*eIF-4a and *OseIF-1a* were found to be stable besides *OsGAPDH* in GSOD-tolerant and susceptible cultivar in hypoxia, cytosolic form *OsGAPDH*2 was ranked as most stable when all the datasets from different algorithms were analyzed together. The transcript level of target gene *OsTPP7* was also expectedly found higher in GSOD-tolerant rice germplasm while using *OsGAPDH* as an internal control. For our study, we used *OsGAPDH*2 cytosolic form located on the chromosome 4, (Locus: chr04:24305454.24309055) as there is precedence for its use in reference gene evaluation in rice (Jain et al. 2006). The *OsTPP7* has been used as a hypoxic germination biomarker gene in the present study. High fold change in expression of *OsTPP7* gene, normalized against stable endogenous candidates compared to less stable ones, across different time intervals in tolerant cultivar was also in tune with the functional aspect of the gene. It would be of interest to investigate in future if the selected endogenous genes could maintain similar stability and performance levels when validated with other biomarkers of hypoxic germination like *Ramy3D*. Notwithstanding the effect of stable candidate endogenous control on other hypoxic biomarker genes, it would be safe to assume that the most stable housekeeping genes identified from the combination of the algorithms can be used as an internal control for global transcript study during hypoxic germination of rice.

## Conclusion

We have compared and ranked the performance of reference genes in RT-qPCR using three different germplasms (2 hypoxic tolerant and 1 susceptible) under hypoxic condition, analyzed using RefFinder. Reliability of two genes, namely *OsGAPDH* and *OsEF1α*, for its use as endogenous control was further validated through expression of known hypoxia-induced gene *OsTPP7* during germination in tolerant germplasms. Our findings suggest that the identified reference genes *OsGAPDH* and *OsEF1α* could be considered as the most stable housekeeping genes for expression studies in rice germination studies under hypoxia.

**Author contributions** Conceived and designed the experiments: DK, PKD, and BKS. Performed the experiments: DK. Analyzed the data: DK, PKD, and BKS. Wrote the manuscript: DK, PKD, and BKS. Corrected the manuscript: PKD and BKS.

**Funding information** This work was financially supported by the Department of Biotechnology, Government of India.

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.



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