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Evaluation of *Oryza coarctata* candidate reference genes under different abiotic stresses

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Abstract

Oryza coarctata Roxb. is a wild halophytic species found mostly on South Asian seashore. It can tolerate salinity up to 4 S m⁻¹, which is equivalent to 479 mM NaCl. This species has unique morphological, anatomical, as well as physiological adaptations, which helps it to survive under high salinity and submerged conditions. Thus, it is considered to be a reservoir of salinity and submergence tolerance genes. For quantification of gene expressions, it is necessary to identify stable reference genes and validate stress responsive genes by real time quantitative PCR. The present study deals with the expression analyses of eight candidate reference genes, namely, *ubiquitin 2*, *histone 3*, *β-tubulin*, *α-tubulin*, *glyceraldehyde-3-phosphate dehydrogenase*, *eukaryotic initiation factor 4-α (eIF4-α)*, *cyclophilin*, and *actin (ACT)* under different abiotic stresses such as salinity, cold, drought, heat, submergence, submergence with saline water (dual stress), and abscisic acid treatment. The four different statistical algorithms, namely, *Bestkeeper*, *NormFinder*, *geNorm*, and *RefFinder* were used to rank the genes in order of stability. The analysis revealed that *ACT* and *eIF4-α* were the two most stable reference genes, which was further validated by determining the expressions of two well-known stress responsive genes *alcohol dehydrogenase* and *dehydration responsive element binding protein 2A* under submergence and drought stress, respectively. This study is the first report to analyse the reference genes for *O. coarctata*.

Additional key words: abscisic acid, cold, drought, gene expression, halophyte, heat, salinity, submergence.

Introduction

Being sessile, plants encounter many abiotic stresses during their life cycles. Thus, several tolerance mechanisms have been evolved to encounter such stresses. Drought, salinity, submergence, and high as well as low temperatures are important abiotic stresses, which negatively affect the growth of plants. Gene expression studies are important to understand the complex molecular mechanisms involving signal transduction, development, and metabolic pathways in plants. Nowadays, major techniques, such as microarray, northern hybridization, real time quantitative polymerase chain reaction (qPCR) are widely used to study gene expression. Among these, real time qPCR is used extensively for its better reproducibility and high sensitivity (Bustin 2002). Quantitative gene expression can be analysed by two different modules, either by an absolute

quantification method or by a relative quantification method. The absolute quantification is used to quantify the exact transcript expression values based on a standard curve built from known transcript concentrations, whereas the relative quantification calculates fold change values of target genes which in turn utilizes some reference genes for normalization.

However, for any transcript profiling and expression studies through real time qPCR under particular conditions, it is necessary to identify the most stable reference genes. Most of the previously used reference genes were housekeeping genes known to be involved in basic cellular functions, thus assumed to be stable during various developmental stages. But recent studies demonstrated that expressions of these reference genes are not stable under various abiotic stresses (Singh *et al.* 2015, Shivhare and Lata 2016). Thus, it is imperative to

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Abbreviations: ABA - abscisic acid; *ACT* - actin; *ADH* - alcohol dehydrogenase; Cq - quantitative cycle; CV - coefficient of variance; *CYP* - cyclophilin; *DREB2A* - dehydration responsive element binding protein 2A; EC - electrical conductivity; *eIF4-α* - eukaryotic initiation factor 4-α; *GAPDH* - glyceraldehyde-3-phosphate dehydrogenase; *H3* - histone 3; qPCR - quantitative polymerase chain reaction; SD - standard deviation; SV - expression stability value; *α-Tub* - α-tubulin; *β-Tub* - β-tubulin; *UBQ2* - ubiquitin 2.

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have well-studied internal control genes for real time qPCR data normalization to correct variations associated with experimental processes accurately such as sampling, the volume of starting materials taken for RNA extraction, enzymatic efficiencies, and differential expressions of genes across tissues (Expósito-Rodríguez *et al.* 2008, Udvardi *et al.* 2008).

Oryza coarctata is a wild halophyte, which is found in the South-East coastal region including the Sunderbans delta in India. It can flower and set seeds even under high salinity (electrical conductivity up to 4 S m⁻¹) and under submergence (Bal and Dutt 1986, Chowrasia *et al.* 2018). During its growth in coastal regions, this plant experiences the lunar tide for every 12 h and is almost submerged in salt water. On the other hand, *Oryza sativa* L., the most important cereal crop world-wide, is a glycophytic species. Only few salt tolerant genotypes of rice are available (Thomson *et al.* 2010, Linh *et al.* 2012, Hoque *et al.* 2015). Therefore, *O. coarctata* would serve as an unexplored source of resistant alleles for salinity tolerance. However, to study stress responsive gene expression in this wild species using real time qPCR, there is a need to establish stable reference genes.

Therefore, the present study is focused on identification and validation of stable and well expressed reference genes under different abiotic stresses, such as heat, cold, drought, salinity, submergence and submergence with saline water (dual stress), as well as abscisic acid (ABA) treatments, which will help in normalization of target gene expressions in this plant. Based on published literature, eight candidate genes, namely, *ubiquitin 2 (UBQ2)*, *histone 3 (H3)*, *beta tubulin (β-Tub)*, *alpha tubulin (α-Tub)*, *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, *eukaryotic initiation factor 4-alpha (eIF4-α)*, *cyclophilin (CYP)* and *actin (ACT)* were selected for this purpose.

Materials and methods

Plants and treatments: Young seedlings of *O. coarctata* were grown in soil and kept in a glasshouse under a 16 h photoperiod, an irradiance of 56 μmol m⁻² s⁻¹, day/night temperatures of 25/16 °C, and a relative humidity of 60–70 %. Two-month-old seedlings were exposed to various kinds of stresses. For cold and heat stresses, plants were subjected to temperatures 4 ± 1 °C and 42 ± 1 °C, respectively. Leaf samples were collected after 0, 6, 12, 24, and 48 h. Drought treatment was imposed by withdrawing water for 8 d so that to bring a residual soil moisture content of 13.22 % (DeAngelis 2007), and then leaf samples were collected after 0, 6, 12, 24, and 48 h. For salinity stress, plants were irrigated with 450 mM NaCl [electrical conductivity (EC) of 3.6 S m⁻¹] every alternate day (Mondal *et al.* 2015), and samples were collected after 0, 24, 48, 72, and 96 h. Leaves were also treated with 100 μM ABA (Singh *et al.* 2015), and samples were collected after 0, 1, 2, 6, 12, and 24 h. Submergence stress and dual stress (a salinized water with EC of 3.6 S m⁻¹) were given to plants, and samples were collected after 0, 6, 12, 24, 48, and 72 h. All the treated and control samples

were immediately frozen and stored at -80 °C until RNA extraction. Two biological replicates were taken for each stress treatment.

Extraction of RNA and cDNA preparation: The total RNA from leaf samples (100 mg) was extracted by using a *Trizol* reagent (*Invitrogen*, Carlsbad, USA) as per the manufacturer's protocol, and genomic DNA was removed by *DNase I* treatment (*Thermo Fisher Scientific*, San Jose, USA). Purified RNA samples were then quantified, and RNA integrity was determined by electrophoresis in a 2 % (m/v) formaldehyde-agarose gel. The synthesis of cDNA was performed by using a *Verso* cDNA synthesis kit (*Thermo Fisher Scientific*), where the total RNA of 1 μg was used for a final reaction volume of 0.02 cm³ and stored at -20 °C.

Candidate reference gene identifications and real time qPCR analysis: Eight commonly used reference genes, namely, *UBQ2*, *H3*, *β-Tub*, *α-Tub*, *GAPDH*, *eIF4-α*, *CYP*, and *ACT* were selected from previous studies (Table 1 Suppl.). All gene specific primers were designed manually by employing the *Vector NTI* software (Lu and Moriyama 2004), and details of gene specific primer sequences are provided in Table 1 Suppl. All the cDNA samples were diluted 40 times, amplified with each gene specific primers, and in order to confirm gene identity, the amplified products were sequenced.

A reaction mixture for real time qPCR contained 1 mm³ of diluted cDNA, 10 pmol each gene specific forward and reverse primers, and 5 mm³ of 2× *Power SYBR™ Green PCR Master Mix* (*Thermo Fisher Scientific*) in a total reaction volume of 10 mm³. All the reactions were carried out in a *Light Cycler 480 II* (*Roche Molecular Diagnostics*, USA) with cycling conditions as follows: a pre-amplification step for one cycle at 95 °C for 5 min, amplification at 95 °C for 15 s, annealing at 59 - 63 °C for 15 s, and extension at 72 °C for 30 s for 40 cycles. Melting curves were determined by using default parameters to check product specificity through a stepwise increase in a melting temperature from 60 to 95 °C. The PCR amplification efficiency (E) and regression coefficient (R²) were determined for each of the designed primer sets by using serial dilutions (10⁰, 10⁻¹, 10⁻², and 10⁻³) of cDNA and an amplification curve slope was calculated by the Pfaffl efficiency equation, $E = 10^{(-1/\text{slope})}$ (3.6 ≥ slope ≥ 3.1) (Pfaffl 2001).

Statistical analyses of reference gene expressions and validation: Expression stability orders for eight selected reference genes were determined from quantification cycle (Cq) or threshold cycle (Ct) values through commonly used statistical and advanced algorithms such as *BestKeeper* (Pfaffl *et al.* 2004), *NormFinder* (Andersen *et al.* 2004), *geNorm* (Vandesompele *et al.* 2002,) and the *delta Cq* method (Silver *et al.* 2006). In *geNorm* and *NormFinder*, relative quantification values were used. Relative expressions were determined from mean Cq values through the 2^{-ΔΔCq} formula (Schmittgen and Livak 2008). Furthermore, *geNorm* was used for determination of the

Table 1. Means and standard deviations of threshold cycle (Ct) values of studied reference genes, namely *ubiquitin 2 (UBQ2)*, *histone 3 (H3)*, *β -tubulin (β -Tub)*, *α -tubulin (α -Tub)*, *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, *eukaryotic initiation factor 4- α (eIF4- α)*, *cyclophilin (CYP)*, and *actin (ACT)* in *Oryza coarctata* under different abiotic stresses such as cold ($4 \pm 1^\circ\text{C}$), heat ($42 \pm 1^\circ\text{C}$), drought (withdrawing water for 8 d), salinity (450 mM NaCl), abscisic acid (ABA, 100 μM), submergence, and saline water (450 mM NaCl) with submergence (dual stress); (* represents the less variable standard deviations).

Gene	Cold	Heat	Drought	Salinity	Submergence	ABA treatment	Dual stress
UBQ2	31.01 $\pm$ 0.92	30.83 $\pm$ 1.28	31.06 $\pm$ 1.81	31.50 $\pm$ 1.34	29.36 $\pm$ 1.61	29.04 $\pm$ 2.3	29.62 $\pm$ 1.9
H3	25.54 $\pm$ 1.40	27.15 $\pm$ 2.89	25.20 $\pm$ 1.14	25.56 $\pm$ 1.42	26.08 $\pm$ 1.23	25.74 $\pm$ 1.22	24.57 $\pm$ 1.57
α -Tub	28.55 $\pm$ 1.28	31.94 $\pm$ 1.45	29.41 $\pm$ 0.85	29.98 $\pm$ 1.95	31.03 $\pm$ 1.39	34.59 $\pm$ 1.92	29.34 $\pm$ 1.18
β -Tub	31.05 $\pm$ 1.39	33.04 $\pm$ 1.4	30.38 $\pm$ 0.81	31.29 $\pm$ 1.23	31.72 $\pm$ 0.73*	34.35 $\pm$ 1.09	30.53 $\pm$ 0.74
GAPDH	35.58 $\pm$ 1.5	34.58 $\pm$ 1.14	34.17 $\pm$ 1.49	30.89 $\pm$ 1.53	33.82 $\pm$ 1.67	32.57 $\pm$ 2.17	34.43 $\pm$ 1.41
eIF4- α	27.43 $\pm$ 0.52	27.07 $\pm$ 1.26	27.61 $\pm$ 0.53*	27.58 $\pm$ 0.48	27.67 $\pm$ 1.64	27.72 $\pm$ 1.34	27.33 $\pm$ 0.87
CYP	32.59 $\pm$ 1.86	34.41 $\pm$ 1.70	31.29 $\pm$ 0.92	33.01 $\pm$ 2.5	32.55 $\pm$ 1.2	33.04 $\pm$ 1.77	31.43 $\pm$ 1.85
ACT	26.60 $\pm$ 0.49*	25.79 $\pm$ 0.71*	25.42 $\pm$ 0.93	25.80 $\pm$ 0.20*	26.22 $\pm$ 1.18	26.58 $\pm$ 0.56*	26.01 $\pm$ 0.29*

optimal number of candidate reference genes required for real time qPCR normalization by calculating the pairwise variation (V_n/V_{n+1} , n - a reference gene number used for normalization). If the average pairwise variation among the sequential normalization factors (NF), NF_n and NF_{n+1} , are less than 0.15, then added reference genes provide significantly improved normalization in comparison to a single reference gene. An average pairwise expression ratio (an M value) was also determined by *geNorm*, and the threshold value of M should be less than 1.5 for determination of stable reference genes (Vandesompele *et al.* 2002). In *BestKeeper* and the *delta Cq* method, the means of Cq values were analyzed directly.

In order to validate the studied reference genes, the relative expression values of two known stress responsive genes, such as *alcohol dehydrogenase (ADH)* (Garg *et al.* 2013) and *dehydration responsive element binding protein 2A (DREB2A)* (Sakuma *et al.* 2006), were determined by normalizing with the eight selected reference genes under submergence and drought stress, respectively.

Two biological replicates each with two independent technical replicates were analysed for seven different stress treatments. Statistical significance was determined using the Student *t*-test (at $\alpha = 0.05$ and 0.01).

Results

The sequences of reference genes *α -Tub*, *β -Tub*, *GAPDH*, *eIF4- α* , *CYP*, and *ACT* of *O. coarctata* were not available in the NCBI GenBank at the time of this study, therefore gene specific primers were manually designed using *O. sativa* as a reference genome. Sequencing PCR amplified products was done and submitted to the NCBI (Table 1 Suppl.). The sequence information is provided in Table 2 Suppl. The percentage of identity with rice homologues was determined (Table 3 Suppl.). The R^2 and E values for each primer pair were determined using serial dilutions of the cDNA. The R^2 was calculated from the standard curve slope, and it ranged from 0.962 for *α -Tub* to 0.998 for *GAPDH* (Table 1 Suppl.). Primer amplification efficiencies,

i.e., E values varied from 79.93 % for *α -Tub* to 106.47 % for *H3*. The dissociation curves were analysed by real time qPCR to ascertain the specificity of the primers for a single PCR product. A single peak represented a single amplicon for each of the primer pairs (Fig. 1 Suppl.).

The expressions of the eight reference genes were determined in terms of Cq values, which were found to be variable under different stresses such as cold, heat, drought, salinity, submergence and the dual stress, and ABA treatment (Fig. 1, Table 1). Higher Cq values represented lesser expressions and *vice-versa*. The mean Cq values of eight reference genes varied from 24.57 to 35.58 across the samples. Standard deviation (SD) was determined from the mean Cq values, and SD less than one was found to be least variable. Among all the candidate reference genes, *H3* was found to have a maximum expression under most stresses, whereas *GAPDH* showed a least expression (Table 1). The analysis of SD values reveals that *ACT* was least variable under all the experimental conditions except in drought

Fig. 1. Quantification cycle (Cq) values represented by boxes; the lines crossing the box represent median values, and the asterisks represent outliers. The top and the bottom are represented by 75 and 25 percentiles, respectively. The whiskers represent 95 percentile confidence intervals. For abbreviations of gene names refer to Table 1.

Table 2. Rank orders of eight candidate reference genes from *Oryza coarctata* under different abiotic stresses in accordance with their standard deviation (SD) and coefficients of variance (CV) values as determined by *BestKeeper*. For abbreviations of gene names and applied stresses refer to Table 1.

Rank order	Cold gene	SD	CV	Heat Gene	SD	CV	Drought gene	SD	CV	Salinity Gene	SD	CV
1	<i>ACT</i>	0.37	1.40	<i>ACT</i>	0.51	1.97	<i>eIF4-α</i>	0.33	1.20	<i>ACT</i>	0.12	0.47
2	<i>eIF4-α</i>	0.46	1.69	<i>CYP</i>	0.79	2.34	<i>ACT</i>	0.61	2.42	<i>eIF4-α</i>	0.40	1.43
3	<i>UBQ2</i>	0.71	2.30	<i>UBQ2</i>	0.90	2.90	<i>β-Tub</i>	0.64	2.12	<i>UBQ2</i>	0.96	3.05
4	<i>α-Tub</i>	0.95	3.33	<i>GAPDH</i>	0.95	2.76	<i>CYP</i>	0.76	2.41	<i>β-Tub</i>	1.01	3.22
5	<i>β-Tub</i>	0.99	3.19	<i>eIF4-α</i>	1.05	3.87	<i>α-Tub</i>	0.78	2.65	<i>GAPDH</i>	1.24	4.00
6	<i>H3</i>	1.04	4.06	<i>β-Tub</i>	1.18	3.56	<i>H3</i>	1.01	3.98	<i>α-Tub</i>	1.28	4.25
7	<i>GAPDH</i>	1.24	3.47	<i>α-Tub</i>	1.19	3.73	<i>GAPDH</i>	1.17	3.41	<i>H3</i>	1.32	5.11
8	<i>CYP</i>	1.62	4.94	<i>H3</i>	1.43	5.35	<i>UBQ2</i>	1.44	4.62	<i>CYP</i>	2.10	6.35

Rank order	Submergence gene	SD	CV	ABA treatment gene	SD	CV	Dual stress Gene	SD	CV
1	<i>β-Tub</i>	0.62	1.95	<i>ACT</i>	0.48	1.81	<i>ACT</i>	0.26	0.99
2	<i>ACT</i>	0.68	2.57	<i>eIF4-α</i>	0.98	3.51	<i>β-Tub</i>	0.62	2.03
3	<i>CYP</i>	0.91	2.80	<i>H3</i>	1.03	3.99	<i>eIF4-α</i>	0.69	2.52
4	<i>eIF4-α</i>	0.98	3.55	<i>β-Tub</i>	1.19	3.51	<i>α-Tub</i>	0.86	2.94
5	<i>H3</i>	1.08	4.12	<i>CYP</i>	1.49	4.50	<i>GAPDH</i>	1.22	3.55
6	<i>GAPDH</i>	1.13	3.34	<i>GAPDH</i>	1.86	5.72	<i>CYP</i>	1.32	4.19
7	<i>α-Tub</i>	1.15	3.71	<i>UBQ2</i>	1.94	6.68	<i>H3</i>	1.33	5.41
8	<i>UBQ2</i>	1.20	4.09	<i>α-Tub</i>	2.05	6.11	<i>UBQ2</i>	1.68	5.69

Table 3. Rank orders of eight selected reference genes of *Oryza coarctata* based upon their expression stability value (SV) analysed by *NormFinder*. For abbreviations of gene names and applied stresses refer to Table 1.

Rank order	Cold gene	SV	Heat gene	SV	Drought Gene	SV	Salinity Gene	SV	Submergence gene	SV	ABA treatment gene	SV	Dual stress Gene	SV
1	<i>H3</i>	0.348	<i>ACT</i>	0.511	<i>eIF4-α</i>	0.379	<i>ACT</i>	0.477	<i>β-Tub</i>	0.402	<i>CYP</i>	0.698	<i>β-Tub</i>	0.193
2	<i>α-Tub</i>	0.550	<i>eIF4-α</i>	0.759	<i>β-Tub</i>	0.442	<i>eIF4-α</i>	0.509	<i>ACT</i>	0.530	<i>H3</i>	0.711	<i>eIF4-α</i>	0.408
3	<i>eIF4-α</i>	0.583	<i>CYP</i>	0.767	<i>ACT</i>	0.443	<i>UBQ2</i>	0.656	<i>eIF4-α</i>	0.571	<i>ACT</i>	0.765	<i>α-Tub</i>	0.483
4	<i>β-Tub</i>	0.585	<i>α-Tub</i>	0.789	<i>α-Tub</i>	0.574	<i>β-Tub</i>	0.813	<i>α-Tub</i>	0.613	<i>β-Tub</i>	0.830	<i>GAPDH</i>	0.682
5	<i>ACT</i>	0.657	<i>GAPDH</i>	0.834	<i>CYP</i>	0.576	<i>H3</i>	0.864	<i>CYP</i>	0.641	<i>GAPDH</i>	0.937	<i>H3</i>	0.708
6	<i>GAPDH</i>	0.710	<i>UBQ2</i>	0.859	<i>H3</i>	0.651	<i>α-Tub</i>	0.977	<i>H3</i>	0.708	<i>eIF4-α</i>	1.077	<i>UBQ2</i>	0.730
7	<i>UBQ2</i>	0.859	<i>β-Tub</i>	0.929	<i>GAPDH</i>	0.863	<i>GAPDH</i>	1.067	<i>GAPDH</i>	0.744	<i>UBQ2</i>	1.159	<i>ACT</i>	0.765
8	<i>CYP</i>	0.910	<i>H3</i>	1.008	<i>UBQ2</i>	0.965	<i>CYP</i>	1.104	<i>UBQ2</i>	0.851	<i>α-Tub</i>	1.275	<i>CYP</i>	0.775

and submergence stresses, followed by *eIF4-α* and *β-Tub*. The variations in the expressions of the studied reference genes under different experimental sets clearly infer that these reference genes were expressed differentially.

In order to screen out the suitable reference genes for normalization, it was necessary to find out their stability values. The expression stability values of the reference genes can be analysed by several statistical algorithms. The Cq values of the eight reference genes from seven experimental conditions were analysed individually by each software.

BestKeeper is an excel based tool, which was used to determine the coefficient of variance (CV) and SD of the

mean Cq values. The genes with a higher SD or CV were the most unstable ones and *vice-versa*. The analyses of the Cq values of the different experimental conditions reveal that *ACT* and *eIF4-α* were most stable under cold, drought, and salt stresses, and under ABA treatment (Table 2). However, *β-Tub* was highly stable under submergence and dual stresses, and *CYP* was most stable under heat and submergence stresses. *UBQ2* was determined to be least stable in most of the treatments, and *α-Tub* was also found to be least stable under heat, submergence, and ABA treatments (Table 2).

NormFinder was employed to calculate the expression stability value (SV) of the eight reference genes depending

Table 4. Rank orders of eight selected reference genes of *Oryza coarctata* depending upon their stability expression values calculated by *geNorm*. M - average pairwise expression ratio. For abbreviations of gene names and applied stresses refer to Table 1.

Rank order	Cold gene	M	Heat gene	M	Drought Gene	M	Salinity gene	M	Submerge Gene	M	ABA treatment gene	M	Dual stress Gene	M
1	β -Tub	0.800	<i>ACT</i>	1.108	β -Tub	0.872	<i>ACT</i>	0.964	<i>CYP</i>	1.173	<i>ACT</i>	1.318	β -Tub	0.803
2	α -Tub	0.831	<i>UBQ2</i>	1.172	<i>eIF4-α</i>	0.885	<i>eIF4-α</i>	0.982	<i>H3</i>	1.203	<i>eIF4-α</i>	1.639	<i>eIF4-α</i>	0.857
3	<i>H3</i>	0.913	<i>eIF4-α</i>	1.412	<i>H3</i>	0.935	<i>UBQ2</i>	1.292	β -Tub	1.335	β -Tub	1.808	<i>ACT</i>	0.958
4	<i>ACT</i>	1.286	<i>CYP</i>	1.545	<i>CYP</i>	1.080	<i>H3</i>	1.764	α -Tub	1.411	<i>H3</i>	1.877	α -Tub	1.119
5	<i>GAPDH</i>	1.413	β -Tub	1.713	<i>ACT</i>	1.367	β -Tub	2.067	<i>ACT</i>	1.776	<i>CYP</i>	2.134	<i>UBQ2</i>	1.366
6	<i>eIF4-α</i>	1.531	<i>GAPDH</i>	1.809	α -Tub	1.515	<i>GAPDH</i>	2.319	<i>eIF4-α</i>	1.827	<i>GAPDH</i>	2.297	<i>GAPDH</i>	1.540
7	<i>UBQ2</i>	1.590	α -Tub	2.044	<i>UBQ2</i>	1.793	<i>CYP</i>	2.686	<i>GAPDH</i>	1.927	<i>UBQ2</i>	2.569	<i>H3</i>	1.683
8	<i>CYP</i>	1.712	<i>H3</i>	2.289	<i>GAPDH</i>	1.885	α -Tub	2.877	<i>UBQ2</i>	2.239	α -Tub	2.847	<i>CYP</i>	2.193

upon intra- and inter-group variations among different experimental sets. This programme ranks reference genes depending upon their SV; a lower SV of a gene indicates its greater expression stability and *vice-versa* (Table 3). Across all the samples, *eIF4- α* had a low SV under most stresses (cold, heat, drought, salinity, submergence, and dual stress). *ACT* was also found to be stable under heat, salinity, drought, and submergence. However, in the case of ABA treatment, *CYP* and *H3* were the most stable reference genes.

The average pairwise expression ratio, the M value, of the reference genes was analysed by *geNorm* (Table 4). The threshold value of M should be less than 1.5 for stable reference genes. Reference genes with higher M values were less stable. In the present study, *ACT* was found to be one of the most stable reference gene across all the treatments except submergence (Fig. 2). Additionally, β -Tub was also shown to be stable under cold, drought, submergence, and dual stresses with M value < 1.5. However, *GAPDH* was found to be consistently unstable under all the stresses except cold and dual stresses with a rather high M value ($1.809 < M < 2.319$). Similarly, *UBQ2* was one of the least stable genes under cold, drought, submergence, and ABA treatments (Table 4).

For all the seven experimental sets except cold and heat stresses, the pairwise variation value of $V_{2/3}$ was less than 0.15, which indicates that for all the sets, the use of two reference genes is sufficient for accurate normalization of real time qPCR data (Fig. 3).

The average SD values were analysed by the *delta Cq* method among different samples and reference genes. From the results, it was observed that *ACT* followed by *eIF4- α* and β -Tub were most stable in a maximum of experimental sets (Table 4 Suppl.) and *UBQ2* was found to be least stable under most stress conditions.

It was clearly observed that the best reference gene for one stress condition may not be suitable for other stress conditions. Therefore, integrating the results from the above described programmes, the best reference genes for individual stress conditions were summarized as: cold (*H3*, α -Tub), heat (*eIF4- α* , *CYP*, *ACT*), drought (*eIF4- α* , *ACT*, β -Tub), salinity (*UBQ2*, *eIF4- α* , *ACT*), ABA treatment (*ACT*, *H3*), submergence (*ACT*, β -Tub), and

dual stress (β -Tub, *eIF4- α*). Thereby, we can generalize that *ACT* and *eIF4- α* were the most stable reference genes under most of the treatments, whereas *GAPDH* and *UBQ2* were the least stable reference genes under cold, drought, submergence, and dual stresses.

Further, for validation of the studied reference genes, the two already reported stress inducible genes, *viz.*, *ADH* under submergence stress and *DREB2A* under drought stress were normalized with eight selected reference genes, where it was found that results were good when normalized with *ACT* and *eIF4- α* under submergence and drought stresses.

Additionally, the expressions of *ADH* and *DREB2A* were normalized with a combination of two genes, *i.e.*, *ACT* along with *eIF4- α* , which provided a better normalization of the real time qPCR data in terms of a more statistically significant result as compared to individual reference genes. Conversely, when the expression data of the same stress responsive genes were normalized with least stable reference genes, *i.e.*, *GAPDH* and *UBQ2* individually and in combination, it resulted in statically insignificant normalization (Fig. 4).

Discussion

Oryza coarctata is a wild rice species, which is able to withstand high salinity and submergence for a certain period of time. Thus, it serves as a great resource for candidate genes for salinity as well as submergence stress tolerances, and it is being considered as a good plant model to study salt stress tolerance (Garg *et al.* 2013, Mondal *et al.* 2015). Apart from transcriptomic (Garg *et al.* 2013) and proteomics studies (Sengupta and Majumder 2009), its genome has been decoded recently (Mondal *et al.* 2018). Thus, the data can be utilized for identification of stress responsive genes, and real time qPCR analysis is an excellent technique for validation of such stress responsive genes. For an accurate interpretation of real time qPCR data in any particular species, it is necessary to find out stable reference genes in that species. Although several reference genes have been established for various abiotic stress conditions in different plant species (Manoli *et al.*

Fig. 2. Expression stability values and rank order of eight candidate reference genes as analysed by *geNorm* under different abiotic stresses: A - cold, B - heat, C - drought, D - salinity, E - submergence, F - ABA treatment, and G - dual stress. The x-axis represents average expression stability (M-value). For abbreviations of gene names and applied stresses refer to Table 1.

Fig. 3. The average pairwise variations (V) of candidate reference genes determined by *geNorm*. The $V \leq 0.15$ is represented by a line, $V_{2/3} \leq 0.15$ indicates that two genes could be used in combination for an effective normalization of target genes. For abbreviations of applied stresses refer to Table 1.

2012, Wang *et al.* 2016.), yet it has not been studied in depth for *O. coarctata*.

For this purpose, in the current study, we have examined the expressions of eight potential reference genes by real time qPCR. To analyse the expression data, we have used

four statistical algorithms such as *BestKeeper*, *NormFinder*, *geNorm*, and *RefFinder*. *BestKeeper* revealed that *ACT* and *eIF4-α* had the lowest CV and SD values, thus the highest expression stability among all the reference genes in most of the treatments. From *NormFinder* analysis, it

Fig. 4. The validation of the most stable (*ACT* and *eIF4-α*) and the least stable (*GAPDH* and *UBQ2*) reference genes with stress induced genes *ADH* (A) and *DREB2A* (B) under submergence and drought, respectively. The values represent means $\pm$ SEs; and the means were compared with *t*-test (* - $P < 0.05$, ** - $P < 0.01$). For abbreviations of gene name refer to Table 1.

was found that *eIF4-α* and *ACT* had the least SVs, which suggests their most stable expression in all the stress conditions. The M-value of *ACT* from *geNorm* analysis was found to be less than 1.5 in six stress treatments out of seven and for *eIF4-α*, the M-value was less than 1.5 in five out of seven experimental sets, which indicates their stability in expression. The comprehensive results from these algorithms show consensus in identifying the top ranked reference genes depending upon their stability values. For example, *ACT* and *eIF4-α* were ranked among the best reference genes across all the software in most of the treatments. Contrastingly, *GAPDH* and *UBQ2* were identified by most softwares as the least stable genes in a maximum number of the experimental sets. These differences in rank order given by different software tools may be due to the individual software sensitivity towards the expressions of co-regulated internal control genes as described in previous reports (Shivhare and Lata 2016, Wu *et al.* 2016). In several cases, the use of a single reference gene is not sufficient for normalization of real time qPCR data to significant levels, thus the use of more than one reference gene is preferred for data normalization. In the present study, the combination of the two genes in the average pairwise variation is $V_{2/3} < 0.15$, which indicates that the two genes can be used in combination for an effective normalization of the target genes.

In addition, the validation of the two most stable reference genes, *i.e.*, *eIF4-α* and *ACT*, and the two least stable reference genes, *i.e.*, *GAPDH* and *UBQ2*, as determined in our study, was also undertaken. For this, the real time qPCR data of the known stress inducible genes, such as *DREB2A* under dehydration and *ADH* under submergence, were normalized with *ACT* and *eIF4-α*, and in combination of both (*ACT + eIF4-α*). Results showed a significant upregulation of the positive stress marker genes during each stress (Sakuma *et al.* 2006, Garg

et al. 2013). In contrast, the normalization of these stress responsive genes with the most variable and least stable reference genes, such as *GAPDH* and *UBQ2* along with their combination (*GAPDH + UBQ2*), provided results with a significant disparity, which highlights the need to use stable and well correlated internal control genes for statistically significant real time qPCR data analyses.

Conclusions

In conclusion, the present study focussed on the identification as well as validation of reference genes under different abiotic stresses in a halophyte species *O. coarctata*. Our findings demonstrate that out of eight studied reference genes, four genes, *H3*, *eIF4-α*, *ACT* and *β-Tub*, were the most stable ones for individual stress conditions. However, *eIF4-α* and *ACT* showed themselves as the highest ranked reference genes under most of the studied stress conditions. Additionally, the statistical analysis revealed that the use of two reference genes for normalization of real time qPCR data was more reliable than the use of a single reference gene. In conclusion, the present work provides valuable information to facilitate gene expression studies and validation of candidate stress responsive genes in *O. coarctata*.

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