

Evaluation of reference genes for RT-qPCR studies in the leaves of rice seedlings under salt stress

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ABSTRACT. To obtain accurate and reliable results for the expression of genes of interest using quantitative real-time polymerase chain reaction (RT-qPCR) techniques, it is necessary to normalize the data by comparing them to constitutive genes that exhibit uniform expression levels under experimental conditions. In this study, the stability of expression was evaluated for the following ten candidate reference genes in rice leaves (*Oryza sativa* L.) from the BRS Bojuru and BRS Ligeirinho genotypes that were subjected to salt stress (150 mM): actin 11 (*ACT11*), beta-tubulin (β -TUB), eukaryote elongation factor 1- α (*Eef-1*), eukaryotic initiation factor 4- α (*eIF-4- α*), E2 ubiquitin-conjugating enzyme (*UBC-E2*), ubiquitin 5 (*UBQ5*), ubiquitin 10 (*UBQ10*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *TIP41-like*, and cyclophilin. The stability of expression for the aforementioned genes was then compared to that of three *LTP* genes using *UBQ10*, *Eef-1*, and *eIF-4- α* as references. After analyzing the

expression levels using analysis of variance tests, the results indicated that *UBQ10* was the most stable in all treatments ($M = 0.404$ and $SV = 0.327$). Furthermore, the *eIF-4- α* , *TIP41-like*, and cyclophilin genes exhibited the highest total coefficient of variation ($CV = 269, 169.2, 179.2$, respectively), which signifies that they exhibited the least stable expression. The expression levels of each candidate gene (*LTP7*, *LTP10*, and *LTP13*) were in contrast to the reference genes. Therefore, we concluded that *UBQ10* is the best reference gene for RT-qPCR reactions under the experimental conditions. The expression analysis of *LTP7*, *LTP10*, and *LTP13* confirmed the importance of validating reference genes to achieve accurate RT-qPCR results.

Key words: *Oryza sativa* L.; Salinity; Ubiquitin; Real-time quantitative PCR

INTRODUCTION

The production of rice (*Oryza sativa* L.), like any other crop, can be strongly influenced by several environmental factors. Drought, heat, salinity, UV radiation, and hormones induce numerous physiological stress reactions in plants that alter the chemical composition of crops and the quality of the harvested products (Wang and Frei, 2011). Among the abiotic factors, soil and irrigation water salinity are major problems for irrigated agriculture in many parts of the world. According to data from the Food and Agriculture Organization of the United Nations (FAO, 2008), approximately 20% of cultivated lands have salinization problems. Physiologically, salt stress inhibits the growth and development of plants, which reduces the osmotic potential of the soil solution, restricts water availability, and/or accumulates excess ions in plant tissues. These effects can cause ion toxicity, nutritional imbalance, or both (Tester and Davenport, 2003). Other consequences of salinity involve disturbances to membrane integrity (Eraslan et al., 2007), variations in growth regulator levels (Iqbal et al., 2014), and changes in metabolic activity. Moreover, the changes in metabolic activity may include photosynthesis modifications (Marín-Guirao et al., 2011), increased production of reactive oxygen species, and fluctuations in the concentrations of total soluble carbohydrates, total phenols, glycine betaine, proline, chlorophyll, and proteins (Panda and Khan, 2009).

Rice is typically classified as being sensitive to soil salinity (Munns and Tester, 2008) with decreases in productivity occurring at electrical conductivity values of 3.0 dS/m (deciSiemens per meter), which corresponds to losses of 12% for each dS/m above the tolerable threshold (Maas, 1993). Several rice studies have used strategies that allow the identification of genes expressed in response to different treatment and/or stress conditions (Walia et al., 2005). However, in these experiments, it is necessary to select several candidate genes that must be validated with specific analyses to quantify their expression in response to stress. Quantitative reverse transcription PCR (RT-qPCR) is a widely used molecular biology technique for the validation of gene expression data because of its sensitivity and reproducibility in transcript analyses. The technique is based on the process of reverse transcription (RT), followed by DNA PCR with fluorescent tag molecules covalently bonded to the nucleotides of the synthesized amplicon. The products formed are monitored during each cycle of the reaction, allowing for the rapid and accurate detection of amplified cDNA (Gachon et al., 2004). However, this technique requires standardization to correctly interpret the data obtained. Stan-

standardization is performed by analyzing the expression of target genes simultaneously with one or more constitutive genes that exhibit constant and uniform expression in most cells, during all developmental stages, and under different experimental conditions. Without this standardization, incorrect results may be obtained (Jain et al., 2006). These constitutive genes are termed reference, normalizer, or standard genes in the RT-qPCR technique (de Oliveira et al., 2012).

Sequences from both the constitutive gene and the gene of interest (target) will be present in the tested sample. However, the constitutive gene should not exhibit significant variation in expression because it is a reference gene, but the target may have different expression patterns within the experiment (Expósito-Rodríguez et al., 2008). The genes chosen as references are usually involved in basic cellular processes such as cell structure maintenance and primary metabolism (Czechowski et al., 2005; de Oliveira et al., 2012). Although several genes are routinely used and reported as good reference genes in studies with plants, the genes encoding 18S rRNA (*18S rRNA*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), elongation factor 1 α (*EF-1 α*), actin (*ACT*), and α tubulin (α -TUB) (Benitez et al., 2013) have shown differential expression between tissues, genotypes, and ambient test conditions (Czechowski et al., 2005; Expósito-Rodríguez et al., 2008; Gutierrez et al., 2008). The use of inappropriate reference genes radically changes the interpretation of the expression pattern of a given target gene and introduces flaws into our understanding of the role of the gene. Therefore, the lack of reference gene validation in plants compromises the accuracy of results obtained by RT-qPCR protocols that use non-valid references (Gutierrez et al., 2008). To evaluate the stability of reference gene expression, several algorithms have been developed in recent years, including those used in the geNorm and NormFinder programs (Silver et al., 2006). The algorithm of the geNorm program indicates the two most stable reference genes or multiple combinations of various stable genes by calculating the average expression stability (M) based on the arithmetic mean of the number of candidate reference genes compared pairwise (Vandesompele et al., 2002). This tool uses the principle that the expression ratio of two perfect reference genes should remain constant across different experimental treatments. The algorithm of the NormFinder program identifies the best reference gene in a group of candidate genes based on their expression stability. This algorithm evaluates the total variation in the expression of the candidate genes using the sum of variance (Andersen et al., 2004).

Therefore, we evaluated the stability of gene expression to identify potential reference genes with stable expression for RT-qPCR studies in the leaves of rice seedlings under salt stress. Furthermore, differences in the expression levels of 10 reference genes were revealed using comparisons of serial analyses with three *LTP* (lipid transfer protein) genes, which were previously identified as being responsive to salt stress.

MATERIAL AND METHODS

Plant material and experimental conditions

Rice seeds (*Oryza sativa* L.) from the BRS Bojuru (salt-tolerant) and BRS Ligeirinho (salt-sensitive) genotypes were disinfected with 1% hypochlorite, germinated on Germitest paper in transparent plastic boxes (Gerbox[®], BRA), and kept in a biological organism development (BOD) growth chamber with a 12 hour photoperiod at 25° \pm 2°C for 10 days. After this period, the seedlings were transferred into plastic pots (500 mL) containing sand as the substrate, which was previously washed with 1% hydrochloric acid. The seedlings were irrigated

every two days with water and Hoagland and Arnon (1938) nutrient solution in a greenhouse. Upon reaching the V₄ (four leaves) growth stage, plants underwent alternating irrigation with nutrient solution and water containing 150 mM NaCl (100 mL/pot). The NaCl concentration was chosen based on data from reported experiments that examined saline stress in rice (Abbas et al., 2013). After the experiment was established, leaves of rice seedlings were collected every 24 hours, and were identified as C1, C2, C3, C4, and C5 based on the following criteria: C1 = plants with no exposure to stress (control); C2 = 24 h of stress; C3 = 48 h of stress; C4 = 72 h of stress; C5 = 96 h of stress in a 150 mM NaCl solution. The experimental design was completely randomized, according to a factorial 2 x 5 (2 genotypes x 5 evaluation times), and used three biological replicates that included five plants each in three replicate pots.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of leaves according to the manufacturer protocol for (Plant RNA Reagent PuriLink[®], USA). RNA quantity and purity were determined using a NanoDrop ND-1000, and RNA quality and integrity were verified via electrophoresis on a 1.0% agarose gel. The single-stranded cDNAs were synthesized by reverse transcription from 2 µg of total RNA using oligo dT primer and the SuperScript First-Strand Synthesis System for RT-PCR Kit (Invitrogen[®]-18080093, USA).

Selection of the reference and candidate genes

Ten genes that were cited in the literature as internal controls for RT-qPCR analyses, which supposedly exhibit no significant differences between treatments, were selected as possible reference genes (Table 1).

Table 1. Description of the candidate reference genes for the qRT-PCR reactions in rice plants under salt stress.

Gene	Accession	F: Primer (5'-3')	R: Primer (5'-3')	Average efficiency	References
<i>ACT11</i>	AK100267	CAGCCACACTGTCCCATCTA	AGCAAGGTCGAGACGAAGGA	2.02	Zhang and Hu (2009)
<i>β-Tubulin</i>	AK072502	GCTGACCACACCTAGCTTTGG	AGGGAACCTTAGCGAGCATGT	1.90	Zhang and Hu (2009)
<i>Eef-1α</i>	AK061464	TTTCACTCTTGGTGTGAAGCAGAT	GACTTCCTTCACGATTTTCATCGTAA	1.96	Zhang and Hu (2009)
<i>UBC -E2</i>	AK059694	CCGTTTGTAGAGCCATAATTGCA	AGGTTGCCTGAGTCACAGTTAAGTG	2.13	Jain et al. (2006)
<i>eIF-4-a</i>	AK073620	TTGTGCTGGATGAAGCTGATG	GGAAGGAGCTGGAAGATATCATAGA	2.05	Jain et al. (2006)
<i>UBQ10</i>	AK101547	TGGTCAGTAATCAGCCAGTTTGG	GCACCACAATACTTGACGAACAG	1.96	Jain et al. (2006)
<i>UBQ5</i>	AK061988	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT	2.00	Jain et al. (2006)
<i>GAPDH</i>	AK064960	AAGCCAGCATCCTATGATCAGATT	CGTAACCCAGAATACCCTTGAGTTT	1.93	Jain et al. (2006)
<i>TIP41 - like</i>	AK103511	GTTTGGATGAACCCCGCAA	GGCAACAAGGTCAATCCGATC	1.95	Caldana et al. (2007)
<i>cyclophilin</i>	AK121304	CCACCATCACAGATCGGATCTT	GCGGTCAGAGCGAAAGTAGCTA	2.00	Caldana et al. (2007)
<i>LTP7</i>	AK058896	GGAGGGTGTGACATCGCAGG	AAGCGTGAGACGACGACAGGT	2.08	-
<i>LTP10</i>	AK061288	ATTAACCAACACCGAGCATCA	CGATCGATCCAGAGGATGCTA	2.09	-
<i>LTP13</i>	CU406598	CTTCTTGTGTGCTGAGGCAATCTG	TTATGGGACGGAGGGAGTAGAG	2.07	-

The genes selected were actin11 (*ACT11*), ubiquitin conjugating enzyme E2 (*UBC-E2*), eukaryotic elongation factor1-α (*Eef-1α*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β-tubulin (β-Tub), eukaryotic initiation factor 4a (*Eif-4-a*), ubiquitin10 (*UBQ10*), ubiquitin5 (*UBQ5*), aquaporin TIP41 (*TIP41-like*), and cyclophilin.

Sequences of candidate genes (*LTP7*, *LTP10*, and *LTP13*) in rice that code for LTP proteins were obtained from the RAP-DB database (<http://rapdb.dna.affrc.go.jp/>). These three

candidate genes were considered responsive to salt stress according to the Microarrays Genevestigator database (<https://www.genevestigator.com/gv/>) and GEO Profile (<https://www.ncbi.nlm.nih.gov/geo/profiles>).

An alignment analysis of the nucleotide sequences of possible *LTP* isoforms present in the rice genome was performed to guarantee primer specificity. Three pairs of specific oligonucleotide primers were designed based on the deduced nucleotide sequence from putative *LTP* transcripts using the Primer Express v.3.0 and BioEdit v.7.0 software (complementary information about reference genes and candidate gene primers are presented in Table 1).

Specificity and efficiency standard curves

The specificity and efficiency standard curves of the primers were evaluated from a pool of 15 cDNAs (5 times x 3 repetitions) for each genotype. Initially, the specificity of each primer set was found by the dissociation (melting) curve, and only those primers with specific amplicons were retained in this study (i.e., those with a single dissociation peak of the PCR products).

PCR efficiency (E) was obtained from four serial dilutions of cDNA (1:1, 1:5, 1:25, and 1:125) to generate a standard curve for each primer set tested. The value of E was calculated according to the equation of Rasmussen (2001), where the slope corresponds the slope of the regression line between the C_T values (cycle threshold) of the transcripts and the values of the logarithms of different cDNA dilutions (efficiency standard curve). Efficiency values between 1.8 and 2.2 were considered acceptable, which corresponds to a reaction efficiency of 90-110%. The amplification efficiency of each primer pair was calculated individually for the genotypes and used to obtain the average efficiency. All primers functioned at an annealing temperature of 60°C, and the reactions were performed in triplicate for each cDNA dilution.

Stability of the reference genes

To test the stability of the reference genes, changes in the expression levels of samples exposed to 24, 48, 72, and 96 hours of salt stress were compared to control samples (no stress). The formula used to calculate the stability was similar to the one proposed by Chao et al. (2012), where:

$$\Delta C_{T\text{sample}N} = C_{T\text{sample}N} - C_{T\text{control}} \quad \text{(Equation 1)}$$

From the ΔC_T values obtained, the relative quantification (RQ) values were calculated using the modified formula of Pfaffl et al. (2002). The RQ values were subjected to analysis of variance (ANOVA) tests (*proc glm*) to identify possible variation based on the genotype and time of collection. Factors of the F-test with values of $P \leq 0.05$ were considered to be statistically significant. Subsequently, using the *proc univariate* routine, additional information was obtained regarding the overall mean (means), standard deviation (SD), and coefficient of variation (CV%) of different genotypes. Additionally, the total SD and CV% values were calculated for each candidate reference gene. Statistical analyses were performed using SAS v.9.3 (2012, SAS Institute Inc., Cary, NC, USA). Reference genes were considered stable when the ANOVA found no significant difference in the time of exposure to salt stress factors as well as no interaction, and genes with smaller SD and CV% values were also considered stable.

In parallel with the statistical analyses, the stability of the reference genes for both genotypes was assessed using the RefFinder tool available at <http://www.leonxie.com/>, which includes the geNorm and NormFinder computational algorithms to compare and classify the stability of the candidate reference genes. The C_T values for each gene were utilized by these algorithms to determine their relative expression stability.

RT-qPCR reactions

The total volume of the RT-qPCR reactions was 12 μ L, consisting of 6.25 μ L SYBR green fluorophore (Applied Biosystems®, USA), 0.25 μ L 10 mM of each primer (forward and reverse), 1 μ L cDNA (1:25 dilution, previously defined), and 4.25 μ L ultrapure water. The reactions were run on a (Bio-Rad CFX Real Time Thermal Cycler, USA) using the following amplification conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 1 minute with the insertion of the melting curve of 65-95°C, with an increment of 5°C for each fluorescence measurement. For each biological replicate, three technical replicates (triplicates) were performed.

RESULTS

The amplification specificity is determined by the presence of a single peak along the dissociation (melting) curve. At the end of the reaction, the fluorescence of the samples, relative to the continuous increase in temperature, is analyzed along this curve. Furthermore, each amplicon has a specific melting temperature, which makes it possible to differentiate the PCR products. The dissociation curves shown in [Figures S1 and S2](#) indicate that after 40 amplification cycles, all primers used in this study have a well-defined single peak, confirming the specificity of the amplified product.

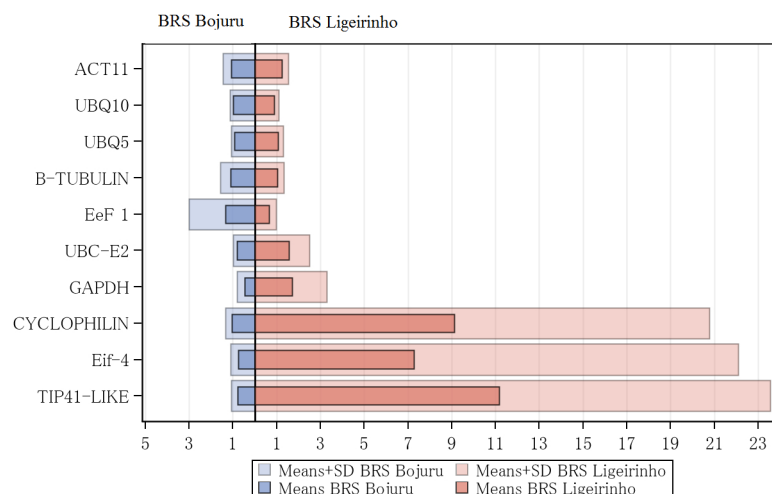


Figure 1. Relative quantification (RQ) and standard deviation (SD) of expression data for ten candidate reference genes that were analyzed using cDNA from the leaves of BRS Bojuru and BRS Ligeirinho rice genotypes after different exposures to 150 mM NaCl.

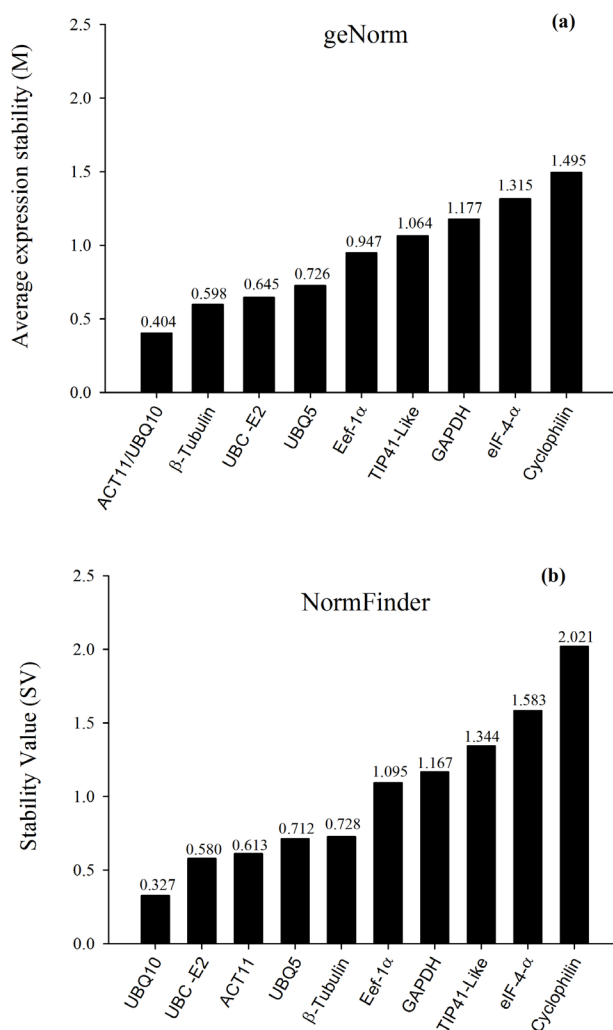


Figure 2. Average expression stability (M) according to the geNorm (a) and the stability value (SV) according to the NormFinder (b) algorithms for ten candidate reference genes that were analyzed using cDNA from the leaves of BRS Bojuru and BRS Ligeirinho rice genotypes after different exposures to 150 mM NaCl.

The efficiency of primer amplification was individually calculated from the logarithm (log) of cDNA dilutions for each genotype, and efficiencies near 100% were observed (ranging between 1.85 and 2.16). This finding indicates that at the end of each cycle, the transcribed template was duplicated with 10% variation, which does not significantly influence the results (Figures S1 and S2).

According to the ANOVA, there was a significant interaction and an isolated effect of the factors (genotype and exposure to salt stress) for *Eef-1 α* , *eIF-4 α* , *GAPDH*, *TIP4-like*, and cyclophilin reference genes, which demonstrates that these genes exhibit variation in their RQ values according to the genotype and stress exposure time. Regarding *UBC-E2*, a significant

effect was only observed for the qualitative factor (genotype); however, no significant effect was found for any of the factors associated with *ACT11*, *β-Tubulin*, *UBQ10*, and *UBQ5*.

The SD and CV% values clearly indicate that *UBQ10* had the lowest total SD (0.18) and CV% (18.52) values among the tested reference genes, and this was followed by *UBQ5* and *ACT11*, which indicates greater expression stability for these genes (Table 2).

Table 2. Overall mean (means), standard deviation (SD), and coefficient of variation (CV%) of the expression data for ten candidate reference genes that were analyzed using cDNA from the leaves of BRS Bojuru and BRS Ligeirinho rice genotypes after different exposures to 150 mM NaCl.

		<i>ACT11</i>	<i>β-TUB</i>	<i>Eef-1a</i>	<i>UBC-E2</i>	<i>eIF-4-a</i>	<i>UBQ10</i>	<i>UBQ5</i>	<i>GAPDH</i>	<i>TIP41-</i>	<i>Cyclophilin</i>
Means	BRS Bojuru	1.08	1.11	1.32	0.81	0.76	0.97	0.91	0.46	0.77	1.04
	BRS Ligeirinho	1.23	1.03	0.66	1.58	7.28	0.90	1.08	1.71	11.17	9.11
	Overall	1.15	1.07	0.99	0.90	4.02	0.94	1.00	1.09	5.97	5.07
SD	BRS Bojuru	0.38	0.46	1.67	0.17	0.33	0.15	0.17	0.35	0.29	0.29
	BRS Ligeirinho	0.31	0.30	0.33	0.91	14.82	0.19	0.23	1.57	12.39	11.68
	Total SD	0.35	0.39	1.23	0.75	10.00	0.18	0.22	1.29	10.10	9.10
CV%	BRS Bojuru	35.19	41.89	126.00	21.75	43.38	15.35	18.78	76.75	31.37	23.71
	BRS Ligeirinho	25.62	29.83	49.60	57.55	203.00	21.35	21.87	91.91	110.92	128.22
	Total CV	30.41	36.44	123.90	63.04	269.00	18.52	21.91	118.22	169.22	179.22

In contrast, *TIP41-like*, *Eif-4a*, and the cyclophilin gene had SD values between 9.10 and 10.10 and CV% values between 169.22 and 269.00. It was also apparent that there was greater variation in the expression stability of genes from the salt stress-sensitive BRS Ligeirinho genotype, with the exception of the *Eef-1a* gene, which exhibited higher SD values in the BRS Bojuru genotype (Figure 1).

To evaluate the stability of candidate gene expression, in addition to the ANOVA, the average expression stability (M) and the stability value (SV) were calculated and compared using the geNorm and NormFinder algorithms, respectively. The geNorm program is based on the principle that the expression ratio of two reference genes should remain constant across different experimental conditions and/or organs or tissues. The M value is defined as the average variation of a particular gene compared to all others tested. Based on the M values calculated for the ten genes, it was observed that *ACT11/UBQ10* (M = 0.404) were the most stable genes, whereas *β-Tubulin* (M = 0.598) *GAPDH* (M = 1.177), *eIF-4-a* (M = 1.315), and cyclophilin (M = 1.495) were the most variable genes (Figure 2a).

According to the NormFinder results (Figure 2b), the candidate gene with the lowest SV value was *UBQ10* with SV = 0.327, followed by SV = 0.580 and 0.613 for *UBC-E2* and *ACT11*, respectively. The cyclophilin gene (SV = 2.021) had the highest SV value, followed by *eIF-4-a* (SV = 1.583) and *TIP41-like* (SV = 1.344). These data are similar to those found in the ANOVA and geNorm analyses, and this is explained by the fact that these analyses took the relative expression values calculated from the C_T values into account. The determination of a suitable reference gene for gene expression studies is the first step that allows the analysis of relative expression patterns in genes of interest, thus providing more reliable results. In order to validate the stability of reference genes, the relative expression profiles were analyzed for the following genes: *UBQ10* (more stable according to SD intervals), *Eef-1a*, *GAPDH* (intermediate stability), *eIF-4-a* (slightly stable), and the three target genes (*LTP7*, *LTP10* and *LTP13*). When the more stable reference gene (*UBQ10*) was used to validate the stability in the leaves of rice plants exposed to NaCl stress, the expression of *LTP7* increased 13.8-fold in the tolerant genotype and 8.03-fold in the sensitive genotype after 96 h

of exposure to stress compared to control plants (Figure 3a).

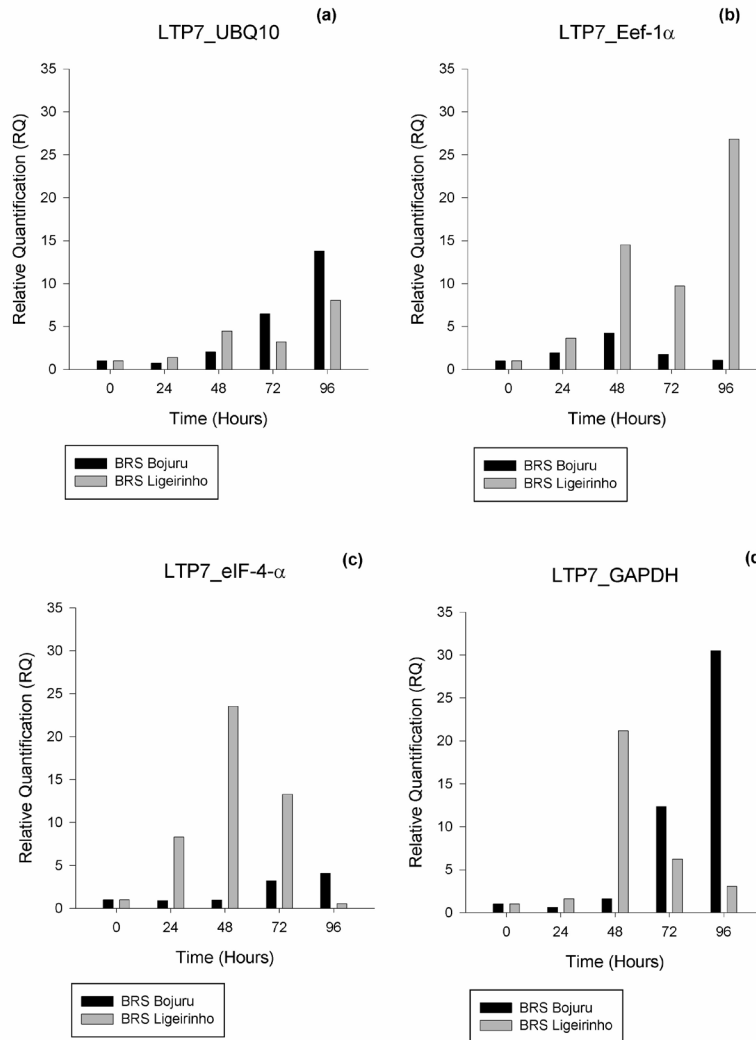


Figure 3. Real-time quantitative PCR analysis of *LTP7* gene expression in the leaves of rice genotypes, BRS Bojuru and BRS Ligeirinho, that were differentially exposed to 150 mM NaCl. *UBQ10*, *Eef-1 α* , *Eif-4- α* , and *GAPDH* were used as reference genes.

These expression patterns were modified when the RQ values were measured using the other reference genes. For instance, RQ = 30.51 for the tolerant genotype at 96 h of exposure using *GAPDH* as a reference (Figure 3b-d). Similar results were observed when *LTP10* expression was evaluated, and the largest variation was observed in the sensitive genotype at 96 h of exposure to salt stress. In comparison to the expression of *LTP10*, the expression value was 71.10-fold for *UBQ10*, whereas a RQ = 517.56 was registered for *Eef-1 α* (Figure 4a and b).

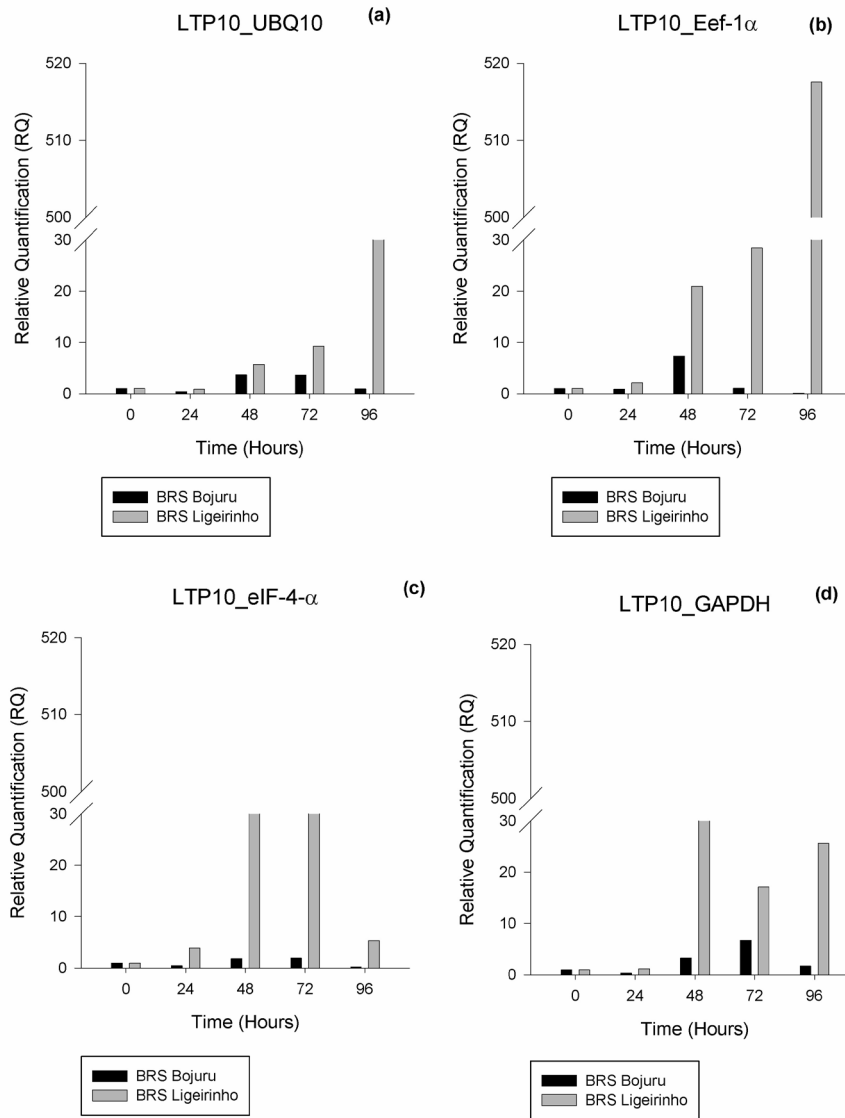


Figure 4. Real-time quantitative PCR analysis of *LTP10* gene expression in the leaves of rice genotypes, BRS Bojuru and BRS Ligeirinho, that were differentially exposed to 150 mM NaCl. *UBQ10*, *Eef-1α*, *Eif-4-α*, and *GAPDH* were used as reference genes.

Thus, inappropriate use of the reference genes gives rise to the misinterpretation of the relative expression profile (Figure 4c and d). Evaluating the expression of the target gene *LTP13* relative to reference genes, we observed variations when the more stable gene (*UBQ10*) was used. Variations were also detected when the least stable reference genes for both genotypes (Figure 5a-d) were used, which further corroborates the importance of validating reference genes before experimental applications.

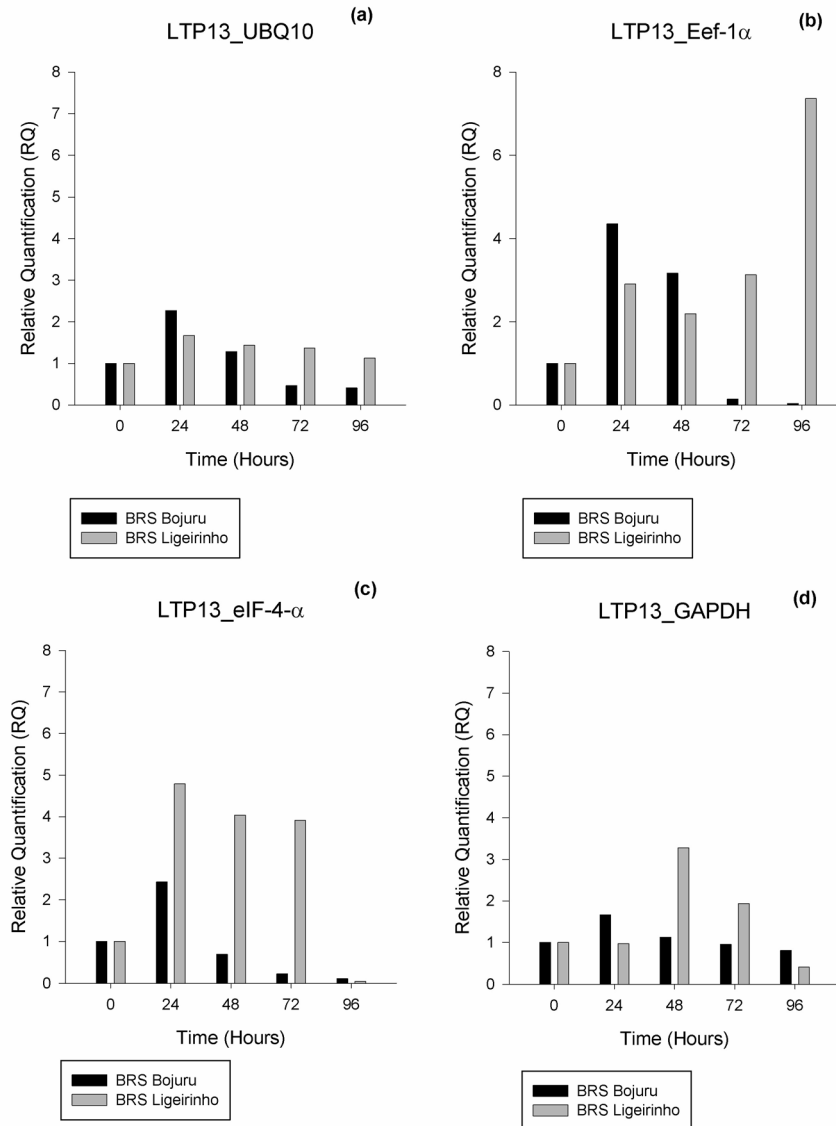


Figure 5. Real-time quantitative PCR analysis of *LTP13* gene expression in the leaves of rice genotypes, BRS Bojuru and BRS Ligeirinho, that were differentially exposed to 150 mM NaCl. *UBQ10*, *Eef-1 α* , *Eif-4- α* , and *GAPDH* were used as reference genes.

DISCUSSION

In the context of gene expression analysis, the RT-qPCR technique has become one of the most important methods for research on gene expression profiles (Gachon et al., 2004). However, for correct interpretation of the data analyzed, efficient standardization becomes a fundamental condition for reliable results, particularly for verifying the

biological relevance of minor differences or in the study of samples from different organs or tissues (Expósito-Rodríguez et al., 2008; Gutierrez et al., 2008; Guénin et al., 2009). Several genes are commonly known to be constitutive, and are regularly used as reference genes in RT-qPCR analyses (Wang et al., 2012). However, even genes involved in basic cellular metabolism processes can exhibit significant variations in expression that make them unsuitable as internal controls in certain situations (Czechowski et al., 2005; Gutierrez et al., 2008; Guénin et al., 2009; de Oliveira et al., 2012). The ideal reference gene should have the same expression, regardless of the experimental conditions. Given the concerns about the choice of stable reference genes, this study examined the expression patterns of ten candidate reference genes in the leaves of rice plants after various lengths of salt exposure (150 mM NaCl). Actin is the most abundant intracellular protein in eukaryotic cells, and it is present in higher concentrations in the cell cortex. Genes encoding actin are commonly used as references in gene expression analyses (Jain et al., 2006; Caldana et al., 2007). In the present study, the *ACT11* gene showed a relatively stable pattern of expression, and was considered, along with *UBQ10*, to be one of the most appropriate reference genes under the experimental conditions. These results are in agreement with those found by Caldana et al. (2007) who identified *ACT1* (AK071586) as one of the most stable genes for salt stress experiments that analyzed the expression profiles of rice transcription factors. In contrast, Nicot et al. (2005), suggested that the use of *ACT* (X55749) as an internal control is inappropriate when studying biotic and abiotic stress in *Solanum tuberosum* ssp *tuberosum* L. The results reported above imply that the stability of orthologous actin genes varies in different plant species and under different conditions. Thus, the adequacy of a reference gene must be tested (Zhang and Hu, 2009).

Tubulin is a heterodimeric protein consisting of two highly conserved subunits, α and β (Whittaker and Triplett, 1999), and it is the main structural component of microtubules in eukaryotic cells. These microtubules play a critical role in numerous basic processes, such as cell division, intracellular transport, cell movement, morphogenesis, and the maintenance of cell shape (Whittaker and Triplett, 1999). Although it is involved in essential cellular processes, tubulin may not exhibit a constituent pattern in all species. According to the results obtained in the present study, the expression pattern observed for the β -*Tubulin* gene was among the five most stable, with total SD = 0.39 and total CV% = 36.44%. Similarly, Brunner et al. (2004) obtained good results using tubulins as reference genes in studies of expression in plants of the genus *Populus*. In contrast, Expósito-Rodríguez et al. (2008) found that tubulin genes were the least stable genes in studies of different organs and developmental stages in *S. lycopersicum*. Together, these results suggest that constitutive genes may be regulated differently in different species, organs, and developmental stages of plants. Thus, a reference gene with stable expression in one particular organism may not be suitable as a normalizing gene in another organism under a given set of conditions, requiring its validation before use (Jain et al., 2006).

Ubiquitins are highly conserved proteins in eukaryotes, and are involved in the signaling complexes of other proteins (Sun et al., 1997). These proteins also participate in the structure and transcription of chromatin, DNA repair, the regulation of endocytosis, and protein trafficking (Hernandez-Garcia et al., 2009). Among the genes analyzed in the present study on salt stress, the gene encoding ubiquitin 10 (*UBQ10*) was among the three most stable genes in the analyses using the geNorm and NormFinder. According to Gutierrez et al. (2008), the values of average expression stability (M) obtained from geNorm algorithms, are considered stable when $M < 0.5$. This claim suggests that the *UBQ10* gene is the most suitable for use as a reference gene for RT-qPCR analysis in rice plant leaves subjected to 150 mM NaCl, because

according to geNorm and NormFinder analyses, $M = 0.404$ and $SV = 0.327$, respectively. Furthermore, this gene presented the lowest SD and CV% values for both BRS Bojuru and BRS Ligeirinho rice genotypes. Corroborating the data obtained, Tong et al. (2009) investigated the expression of 11 reference genes in different samples of *Prunus persica*, and found that the genes encoding translation elongation factor 2 (*TEF2*) and ubiquitin 10 (*UBQ10*) showed stable expression and can be used as reference genes for this species.

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is a key enzyme in glycolysis, converting glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate with concomitant reduction of NAD^+ to $NADH_2$. In plants, glycolysis occurs in both the cytosol and plastids, and is a fundamental process in the generation of ATP (Muñoz-Bertomeu et al., 2009). The SD and CV% values obtained in this study for *GAPDH* were 1.29 and 118.22, respectively. These are relatively high values, which indicate the instability of this gene for these specific experimental conditions in rice.

The respiratory pathway plays a crucial role in supplying energy for several metabolic activities in cells. It has been demonstrated that the expression of transcripts corresponding to genes that encode glycolytic enzymes are activated to maintain homeostasis in rice cells under stress conditions, including the lack of O_2 (Pareek et al., 1998), which increases the expression and instability of these genes as was observed in this study. Like *GAPDH*, *Eef1- α* had a variable expression pattern, with SD and CV% values of 1.23 and 123.90, respectively. Moreover, geNorm and NormFinder algorithms calculated $M = 0.947$ and $SV = 1.095$, respectively, which differed from the results of Jain et al. (2006) that identified the *Eef-1 α* gene as one of the most stable in rice tissue samples under different experimental conditions.

Cyclophilins are proteins linked to the function of modulating mitochondrial membrane permeability. Tip41 aquaporins are important transport proteins related to maintaining cellular metabolism. Eukaryotic initiation factors 4 α are proteins that are continuously produced in cells and are highly conserved in the eukaryotic genome. These three families of proteins correspond to the cyclophilin, *TIP41-like*, and *eIF-4 α* genes that were tested in this study. For these genes, higher SD, CV%, and average expression stability (M) values were observed, and they were not indicated as reference genes for the experimental conditions tested. Conversely, Caldana et al. (2007) obtained stable expression patterns for these three genes, and they were recommended by the authors as good references for rice plants subjected to 100 mM NaCl. The contradiction of these and other results confirm the importance of correctly choosing the constitutive gene to be used when analyzing the expression of transcripts by RT-qPCR.

To compare the expression pattern of the *UBQ10* reference gene, which was identified as the best reference due to its high stability under our experimental conditions, the relative expression of target genes *LTP7*, *LTP10*, and *LTP13* were analyzed together with *Eef-1 α* , *GAPDH*, and *eIF-4 α* . Comparing the expression level of *LTP7* as a target gene relative to *UBQ10*, the RQ values increased for both genotypes. However, the expression was higher in the salt tolerant genotype compared to the sensitive genotype. Nevertheless, the same expression trend was not observed even when the target gene was normalized with *Eef-1 α* , *eIF-4 α* , and *GAPDH*. Contrasting expression levels were also verified for *LTP10* and *LTP13* target genes based on the reference gene used. The relative expression of *LTP13* using *UBQ10* as a reference, at all sampling times, did not increase RQ values above 2.3 for either cultivar. However, the RQ values were higher when the reactions were conducted with *Eef-1 α* , followed by *GAPDH* and *eIF-4 α* as reference genes.

The necessity for accurate choice of reference genes in studies of expression has been

confirmed in other studies. For example, when evaluating the expression of *PAL* and *HPPD* genes in lettuce plants (*Lactuca sativa* L.), Borowski et al. (2014) observed significant differences in the expression of these genes depending on both the reference gene and stress condition used. The results of this study confirm the importance of a specific choice of reference genes for transcript analyses by RT-qPCR. As discussed above, erroneous and inconsistent interpretations can occur if prior validation of reference genes is not performed.

CONCLUSIONS

The analysis of the expression stability of ten candidate reference genes for RT-qPCR studies suggests that the constitutive gene *UBQ10* is a suitable gene for studies of rice leaves subjected to salt stress because it exhibited a consistent and continuous expression pattern. In contrast, *eIF-4 α* , cyclophilin, and *TIP41-like* genes are the least suitable for these experimental conditions. The expression analysis of *LTP7*, *LTP10*, and *LTP13* confirmed the importance of validating reference genes to achieve accurate RT-qPCR results.

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[Supplementary material](#)

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