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Selection of candidate reference genes and validation for real-time PCR studies in rice plants exposed to low temperatures

P.A. Auler[1], L.C. Benitez[1], M.N. do Amaral[1], I.L. Vighi[1], G.S. Rodrigues[1], L.C. da Maia[2] and E.J.B. Braga[1]

[1]Departamento de Botânica, Instituto de Biologia, Universidade Federal de Pelotas, Pelotas, RS, Brasil
[2]Centro de Genômica e Fitomelhoramento, Escola de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, Pelotas, RS, Brasil

Corresponding author: P.A. Auler
E-mail: pri_auler@hotmail.com

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ABSTRACT. Rice is a cereal that presents a great ability to adapt to different soil and climate conditions. However, as it is a tropical crop with C3 metabolism, it performs better in warm temperatures with high solar radiation. Tolerance to stress caused by low temperatures is a highly complex process that involves various metabolic pathways and cellular compartments, resulting in general or specific effects on plant growth and development. In order to observe the true effect of a particular stress on genetic expression, reference genes need to be chosen for real-time PCRs, the expression levels of which should remain stable independent of the situation imposed. In this paper, the expression stability was evaluated of the *actin 11* (*ACT11*), *ubiquitin-conjugating enzyme 2* (*UBC-E2*), *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), *beta tubilin* (β-Tubulin), *eukaryotic initiation factor 4α* (*eIF-4*-α), *eukaryotic initiation factor 1α* (*eIF-1-α*), *ubiquitin 10* (*UBQ10*), *ubiquitin 5* (*UBQ5*),

aquaporin (*TIP41*), and *cyclophilin* genes, in two rice genotypes cultivated in low temperature (13°C) conditions in vegetative stage (V4). The analysis material (leaves) was collected after 0, 6, 24, 48, and 72 h of exposure to the stress. In this study, the geNorm, BestKeeper, ΔCt, NormFinder, and RefFinder methods were used to evaluate the expression stability of the candidate reference genes. The results revealed that the most indicated genes for all the analysis methods were *UBQ10* and *UBQ5* for BRS Bojuru and BRS Pampa, respectively. On the other hand, the *eIF-1-α* gene presents the least expression stability and is not indicated for studies of rice plants subjected to low temperatures. The validation with the antioxidant system genes *SODCc1-Cu/Zn*, *CATC*, *APX2*, and *GR*2 confirmed the importance of using previously tested normalizing genes for adequate real-time PCR results.

Key words: *Oryza sativa* L.; Gene expression; Abiotic stress; Quantitative real-time PCR

INTRODUCTION

Rice (*Oryza sativa* L.) is a crop that evolved in tropical and subtropical areas and is sensitive to the stress of low temperatures, which can affect its geographical distribution and productivity (Kovach et al., 2007; Sang and Ge, 2007). Currently, this cereal is cultivated in a wide range of environments (tropical, subtropical, and temperate). However, because it is a tropical crop with C3 metabolism, it performs better in warm temperatures with high solar radiation (Karki et al., 2013). Different levels of tolerance to low temperatures are observed depending on the subspecies. Typical *japonica* cultivars are grown in regions with lower annual temperatures and generally present greater tolerance to the cold when compared with *indica* cultivars (Ma et al., 2015).

Exposure to low temperatures can cause damage to plants, such as osmotic stress and direct effects on metabolism, via molecular, biochemical, and physiological alterations that can lead to changes in the lipidic composition of membranes and the accumulation of osmolytes (Gilmour et al., 2000; Yamaguchi-Shinozaki and Shinozaki, 2006). Tolerance to stress caused by the cold is a highly complex process that involves various metabolic pathways and cellular compartments (Hannah et al., 2005) and results in general or specific effects on vegetal growth and development (Chinnusamy et al., 2007; Nakashima et al., 2009). Studying the profile of genetic expression in response to stress helps to identify plant signaling and regulation networks for tolerance and provides an effective approach for selecting candidate genes for manipulating and/or crossbreeding superior plants, such as rice (Zhang et al., 2004).

Genes are classified into two types: constitutive and inducible. The first are constantly expressed and codify for essential proteins of the cellular metabolism and undergo little regulation. Inducible genes present a varied expression depending on the cell conditions and determine the production and/or quality of certain momentarily necessary proteins (Munsky et al., 2012). There are numerous studies using strategies that allow for a large number of expressive genes to be identified in response to different stress conditions, with the real-time quantitative polymerase chain reaction (RT-qPCR) technique being widely used, due to its high sensitivity, reproducibility, and specificity (Bustin, 2002; Walia et al., 2005; Derveaux

et al., 2010; Cotsaftis et al., 2011). However, in order to guarantee the efficiency of RT-qPCR and observe the true effect of a particular stress on the vegetal, some genes need to be chosen as normalizers/internal controls, the expression levels of which should remain uniform independent of the situation imposed. Constitutive genes are used for this purpose.

Although some genes are commonly used and reported as good reference gene in RT-qPCR plant studies, the most traditional ones include *actin* (*ACT*) (Maroufi et al., 2010), *tubilin* (*TUB*) (Wan et al., 2010), *ubiquitin* (*UBQ*) (Chen et al., 2011), codifiers of *rRNA 18S* (*18S*) (Jain et al., 2006) and of *rRNA 40S* (*40S*) (Cruz-Rus et al., 2011), *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), and *elongation factor 1α* (*eIF-1-α*), among others. However, it is known that the stability of some of these genes is relative, since depending on the plant, the stage of development, and/or type and intensity of stress, the expression of even constitutive genes can be significantly altered (Radonić et al., 2004; Czechowski et al., 2005). Therefore, in order to obtain a precise quantification of gene expression, we must analyze the stability of reference gene for the conditions and crop being studied. For this, a different software developed for this purpose is used, which when analyzed together indicate the most suitable gene(s) reference for the condition studied.

In this study, the stability of ten reference genes were analyzed and validated, which are traditionally used as reference genes in genetic expression studies for rice, in order to identify the most adequate for the normalization of transcription in genotypes cultivated at low temperatures in the vegetative stage.

MATERIAL AND METHODS

Vegetal material and stress conditions

The experiment was conducted using two Brazilian rice genotypes that present contrasting responses to stress from low temperatures: BRS Pampa (*indica* subspecies - sensitive) and BRS Bojuru (*japonica* subspecies - tolerant).

The seeds were geminated in *germitest* paper tubes at a temperature of 25° ± 2°C for 10 days in a climatized germination chamber. Then, the plantlets were transferred into plastic trays (3L) containing commercial soil fertilized according to the recommendations for irrigated rice cultivation. Three trays/genotype were used with 50 plantlets, which were kept in a greenhouse at a temperature of 28° ± 2°C and irrigated with Yoshida et al. (1976) nutritive solution until presenting four leaves (vegetative stage V4). In this period, the plants from the control treatment were collected and the remaining trays were transferred to a growth chamber with a temperature of 13°C, where they remained for 72 h for the collections.

The material for the analyses (leaves) was collected in the following way: C1 (collection 1) 0 h = plants not exposed to stress from cold; C2 = 6 h of stress; C3 = 24 h of stress; C4 = 48 h of stress; and C5 = 72 h of stress. The experimental design used was laid in a completely randomized design (CRD), in a 2 x 5 factorial layout (2 genotypes x 5 stress exposure times), with three biological repetitions per treatment. The experimental unit was composed of a bulk of 10 plants.

Total RNA extraction and cDNA synthesis

Total RNA was extracted using 100 mg leaf tissue, in accordance with the method

described for the PureLink Plant RNA Reagent (Invitrogen®) reactant. The quantity and purity of the RNA were measured in NanoDrop (ND-1000), while the quality and integrity were verified in electrophoresis with 1.0% agarose gel. The simple strand cDNAs were synthesized via reverse transcription for 2 µg of total RNA using oligoDT primer and Kit SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen®).

Choosing the reference genes

Ten genes commonly used as internal controls in RT-qPCR analyses were chosen, which supposedly do not present significant variation between treatments. The selected genes were: *actin 11* (*ACT11*), *ubiquitin-conjugating enzyme E2* (*UBC-E2*), *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), *beta tubulin* (β-tubulin), *eukaryotic initiation factor 4α* (*eIF-4- α*), *eukaryotic initiation factor 1α* (*eIF-1-α*), *ubiquitin 10* (*UBQ10*), *ubiquitin 5* (*UBQ5*), *aquaporin* (*TIP41*), and *cyclophilin* (Table 1).

The specificity of each primer set was verified using the melting curve, with only those with specific amplicons being kept in this study; that is, with a single melting peak for the strands of the products of the RT-qPCR. The efficiency of PCR (*E*) was obtained based on four serial dilutions of cDNA (1:1; 1:5, 1:25, and 1:125), with the value of *E* estimated by the equation $E = 10^{(-1/slope)}$ (Rasmussen, 2001). Efficiency values between 1.8 and 2.2 were considered acceptable, which corresponds to efficiency of between 90-110%.

Table 1. Description of the candidate reference genes and target genes for RT-qPCRs in rice plants at a low temperature (13°C).

Gene	Access	F Primer	R Primer	Bibliography
ACT11	AK100267	5'-CAGCCACACTGTCCCCATCTA-3'	5'-AGCAAGGTCGAGACGAAGGA-3'	Zhang and Hu (2007)
β-Tubulin	AK072502	5'-GCTGACCACACCTAGCTTTGG-3'	5'-AGGGAACCTTAGGCAGCATGT-3'	Zhang and Hu (2007)
UBC-E2	AK059694	5'-CCGTTTGTAGAGCCATAATTGCA-3'	5'-AGGTTGCCTGAGTCACAGTTAAGTG-3'	Jain et al. (2006)
eIF-4α	AK073620	5'-TTGTGCTGGATGAAGCTGATG-3'	5'-GGAAGGAGCTGGAAGATATCATAGA-3'	Jain et al. (2006)
Eef-1α	AK061464	5'-TTTCACTCTTGGTGTGAAGCAGAT-3'	5'-GACTTCCTTCACGATTTCATCGTAA-3'	Zhang and Hu (2007)
UBQ10	AK101547	5'-TGGTCAGTAATCAGCCAGTTTGG-3'	5'-GCACCACAAATACTTGACGAACAG-3'	Jain et al. (2006)
UBQ5	AK061988	5'-ACCACTTCGACCGCCACTACT-3'	5'-ACGCCTAAGCCTGCTGGTT-3	Jain et al. (2006)
GAPDH	AK064960	5'-AAGCCAGCATCCTATGATCAGATT-3'	5'CGTAACCCAGAATACCCTTGAGTTT-3'	Jain et al. (2006)
TIP41 - Like	AK103511	5'-GTTTGGATGAACCCCGCAA-3'	5'-GGCAACAAGGTCAATCCGATC-3'	Caldana et al. (2007)
Cyclophilin	AK121304	5'-CCACCATCACAGATCGGATCTT-3'	5'-GCGGTCAGAGCGAAAGTAGCTA-3'	Caldana et al. (2007)
OsAPX2	AK061715	5'-CTCTCCTACGCCGACTTCTAC-3'	5'-AGGTGGTCAGAACCTTGTGT-3'	-
OsGR2	AK100446	5'-CACCTGTTGCACTGATGGAG-3'	5'- GTTCACTCAAGCCCACTACTG-3'	-
OsCATC	AK066378	5'-GTGATTGCCAAGGAGAACAAC-3'	5'- GAGTGCGTCGATCCATCTCT-3'	-
OsSODCc1-Cu/Zn	AK061662	5'-CTGATGATCTTGGAAAGGGTGG-3'	5'- GTGCTGGGAGATGGAAGGT-3'	-

RT-qPCRs

The total volume of the RT-qPCRs was 12 µL, including 6.25 µL SYBR Green fluorophore (Applied Biosystems®), 0.25 µL 10 mM of each primer (forward and reverse), 1 µL cDNA (1:5 dilution previously defined), and 4.25 µL ultrapure water. The reactions were carried out in a Bio-Rad® CFX Real Time thermocycler, using the following amplification parameters: 95°C for 10 min, 40 95°C cycles for 15 s, 60°C for 1 min with insertion of the melting curve from 65° to 95°C, with a 5°C increase at every fluorescence measure. For each biological repetition, three (triplicate) technical repetitions were carried out, including samples for the control treatment as template-free controls.

Reference gene stability analyses

For the stability analysis of the candidate reference genes, the Cq values of all of the

collections were used for each one of the genotypes. Normal programs for this type of analysis were used, such as geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), the ΔCt comparative method (Silver et al., 2006), and the RefFinder tool. The GeNorm algorithm first calculates an expression stability value (M) for each gene and then compares the variation of pairs (V) of this gene with the others. Genes with highly variable results have a high M value (>0.5), which indicates low expression stability.

NormFinder provides a ranking of the most stable to the least stable gene, based on the stability value of each one, which consists of a direct measure for the estimated variation of expression (Andersen et al., 2004). BestKeeper is a program based on Excel, which uses the gross values (Cq values) and efficiency of PCR to determine the most suitable reference gene, together with a coefficient index and determination of the value of P (Pfaffl et al., 2004). The ΔCt comparative method compares the relative expression of the genes in pairs within the samples in order to indicate the best reference gene (Silver et al., 2006). The RefFinder tool (http://fulxie.0fees.us/?type=reference) was also used, which integrates the geNorm, NormFinder, and BestKeeper programs, and the ΔCt comparative method, to compare and classify the candidate reference genes.

Validation analyses of the reference genes

In order to confirm the choice of potential reference genes as normalizers for subsequent analyses, the transcriptional profiles were observed of codifier genes of the isoforms of enzymes of the antioxidant system: ascorbate peroxidase (*OsAPX2*), glutathione reductase (*OsGR2*), catalase (*OsCATC*), and superoxide dismutase (*OsSODCc1-Cu/Zn*). These genes were quantified and normalized with the most stable gene (*UBQ10*) and less stable gene (*eIF-1α*). The amplification conditions for RT-qPCR were equal to those previously described. The relative expression data were calculated according to the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001).

RESULTS

Reference genes, used for transcription level studies, should have a constant level of expression between the samples studied, and thus the values of the quantification cycle (Cq), also called the threshold cycle (Ct), were used to determine the fluctuation in the expression of the candidate reference genes evaluated in this study.

Observing the gross Cq values, via the BoxPlot graph, it is noted that for BRS Bojuru the genes with the lowest and highest variation around the average Cq were *UBQ10* and *Eef-1α*, respectively, observed by the size of the box (the bigger the box, the greater the variation), and so *UBQ10* presents the best result (Figure 1A). For BRS Pampa, the *UBQ10* gene presents the smallest variation between the maximum and minimum values, while for *cyclophilin*, *Eef-1α*, and *eIF-4α*, the greatest variations were observed (Figure 1B).

Analyzing both genotypes together (BRS Bojuru and BRS Pampa), subjected to a low temperature for different periods of time, the algorithms, with the exception of BestKeeper, indicated *UBQ10* as the most stable reference genes, followed by β-tubulin. However, they were unanimous in establishing *UBQ5* as the gene that presented the greatest variation, with this not being indicated as an ideal normalization gene for both genotypes in the conditions studied (Figure 2).

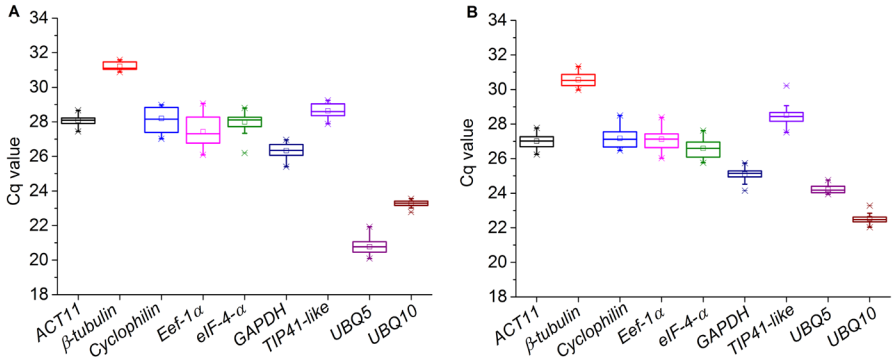

Figure 1. Expression levels of the different candidate reference genes. The expression data are shown by the values of the RT-qPCR quantification cycle (Cq), in the BRS Bojuru (**A**) and BRS Pampa (**B**) genotypes, in the vegetative stage under low temperature (13°C) stress. The line in the middle of the box represents the average expression. The lower quartile is the value at 25% of the ordered sample. The upper quartile is the value at 75% of the ordered sample. The extremities indicate the maximum and minimum values. The bigger the box, the greater the variation.

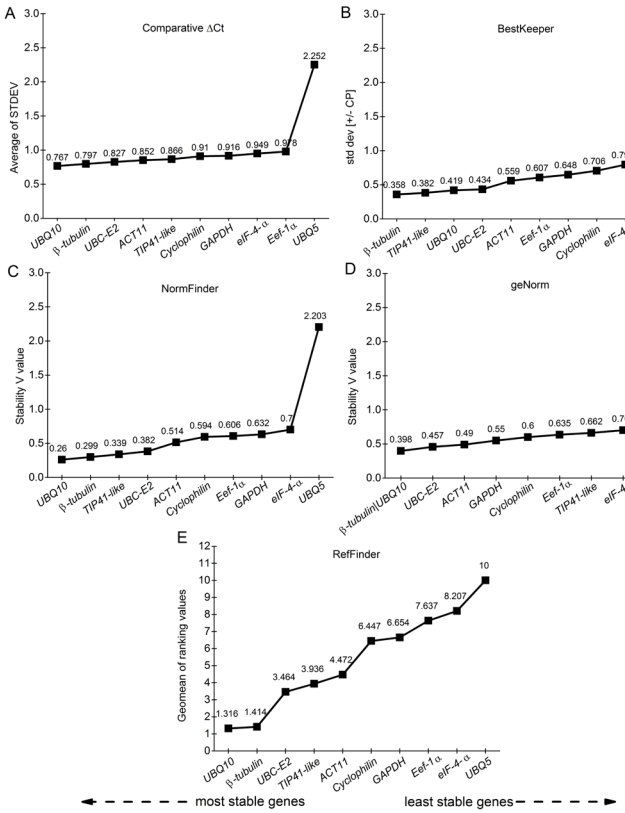

Figure 2. Expression stability of ten candidate reference genes in accordance with the comparative ΔCt (**A**), BestKeeper (**B**), NormFinder (**C**), geNorm (**D**), and RefFinder (**E**) algorithms, in rice leaves from the BRS Bojuru and BRS Pampa genotypes, subjected, in the vegetative stage, to low temperature (13°C) stress during different periods of time.

By analyzing only the BRS Bojuru genotype, it is observed that, according to the software, *UBQ10* and β-tubulin are the most indicated genes as reference genes, due to the smallest variations presented by them. However, in contrast with what was observed by analyzing the genotypes together, *UBQ5* was not the least stable gene, this instead being *Eef-1α* (Figure 3).

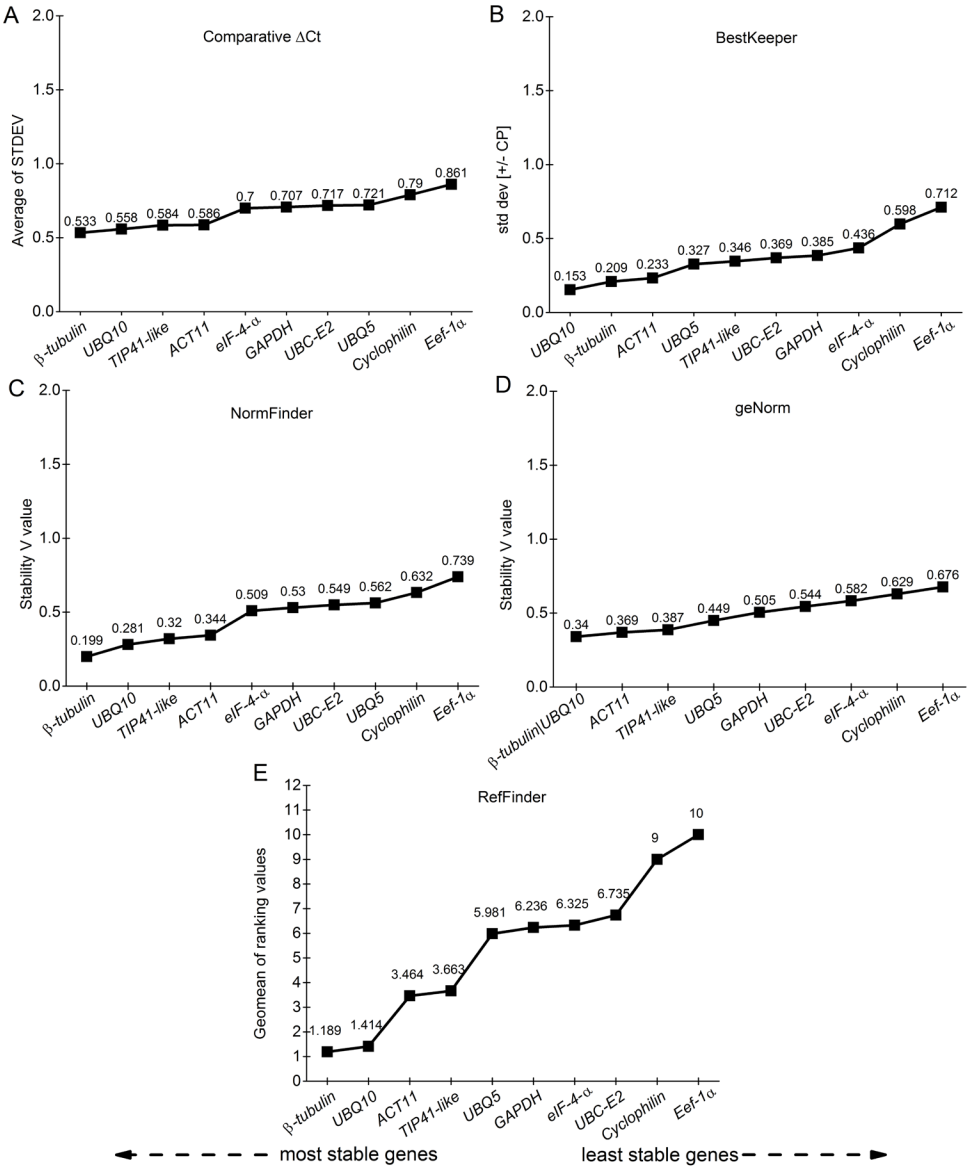

Figure 3. Stability of expression of ten candidate reference genes, in accordance with the comparative ΔCt (**A**), BestKeeper (**B**), NormFinder (**C**), geNorm (**D**), and RefFinder (**E**) algorithms, in rice leaves from the BRS Bojuru genotype, subjected, in the vegetative stage, to low temperature (13°C) stress during different periods of time.

In the BRS Pampa genotype the results were the opposite of those that were observed by analyzing the genotypes together, given that for this genotype *UBQ5* was indicated by all of the softwares as the gene with the smallest variation. In relation to the least indicated, the results were similar, revealing the *Eef-1α* gene to be the most variable (Figure 4).

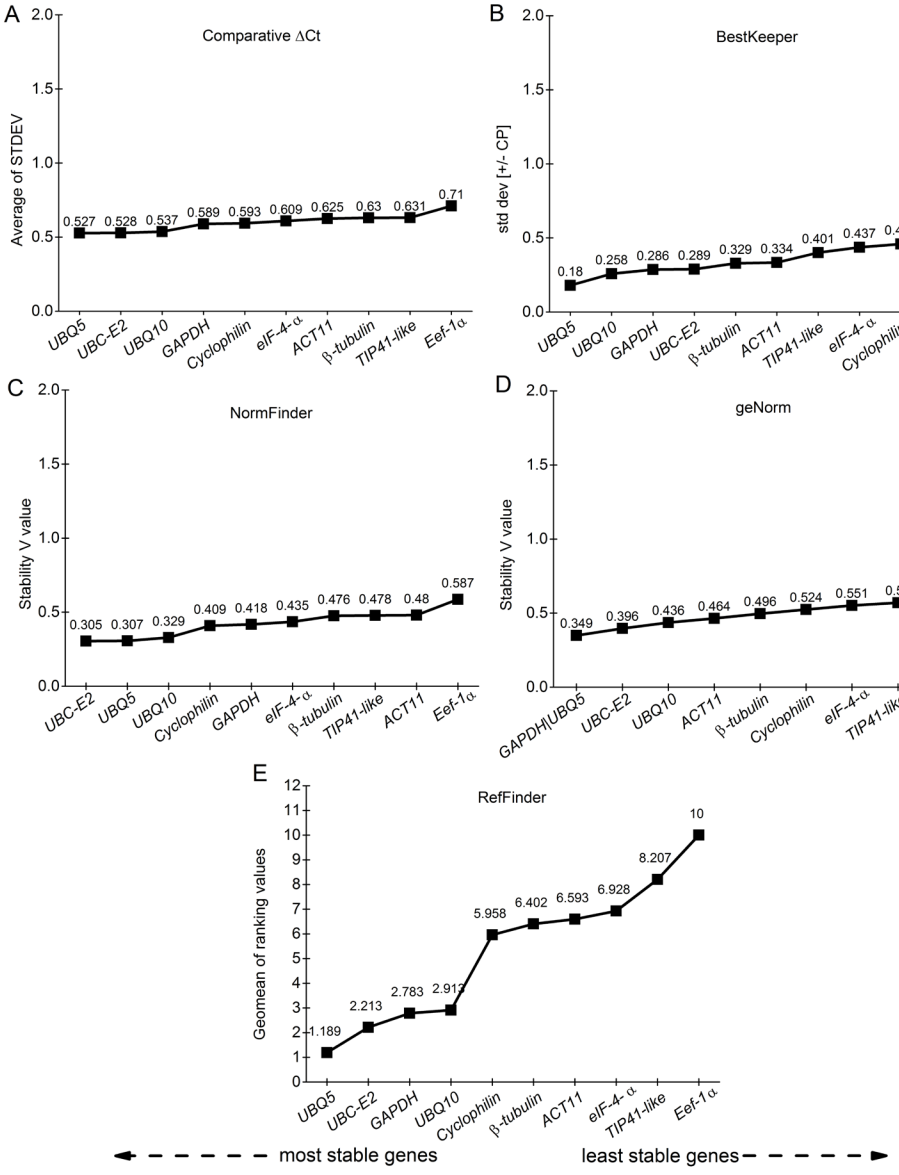

Figure 4. Expression stability of ten candidate reference genes, in accordance with the comparative ΔCt (**A**), BestKeeper (**B**), NormFinder (**C**), geNorm (**D**), and RefFinder (**E**) algorithms, in rice leaves from the BRS Pampa genotype, subjected, in the vegetative stage, to low temperature (13°C) stress during different periods of time.

The geNorm software also indicated the minimum number of genes for the ideal reference genes, via the Pairwise calculation between two sequential normalization factors (Vn/Vn +1), and thus determined the need to add more normalization genes. Knowing that the minimum value to determine the need to add more normalization genes is 0.15, by observing this calculation in both genotypes analyzed together, it is believed that the use of two reference genes is sufficient, since the V2/3 value was 0.0078. If only one of the genotypes, BRS Bojuru or BRS Pampa, was used in the studies, two reference genes would also be sufficient given that V2/3 was lower than the cut-off point in both genotypes (Figure 5).

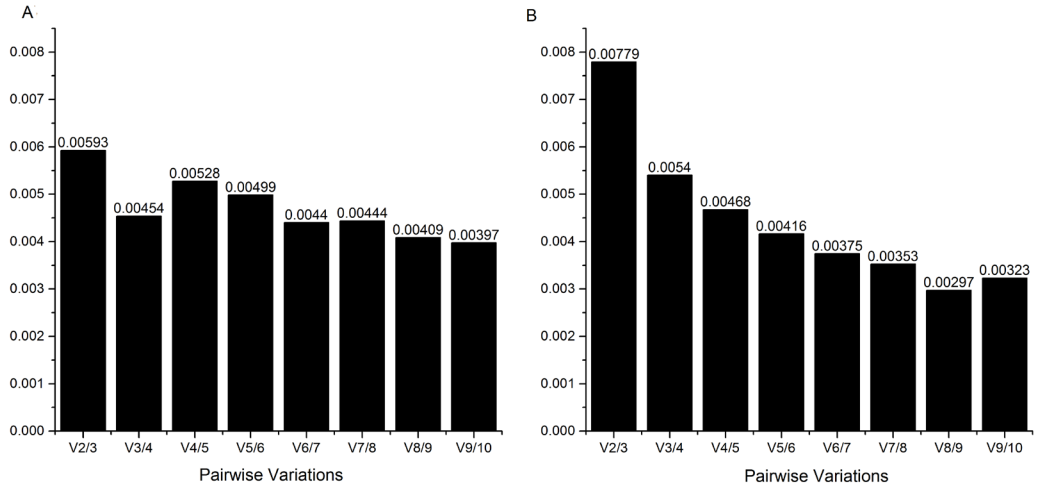

Figure 5. Pairwise variation (V) calculated by the geNorm algorithm to determine the minimum number of reference genes for adequate normalization in leaves of rice genotypes BRS Bojuru (**A**) and BRS Pampa (**B**), in the vegetative stage, subjected to low temperature (13 C) stress during different periods of time.

In order to validate and demonstrate the importance of adequate use of reference genes, the relative quantification (RQ) of expression value was calculated for some codifier genes of the isoforms of the enzymes ascorbate peroxidase (APX), glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT), using the most and least indicated genes for normalization in each genotype.

For the gene of the APX2 isoform, both for Bojuru and BRS Pampa, the greatest difference in relative expression values was observed at 72 h. For the most indicated reference genes, *UBQ10*, the values were 13.21 and 10.88, and for the least indicated reference genes (*Eef-1α*), they were 3.07 and 3.70, respectively (Figure 6A and 6B). As for the gene that codifies the GR2 isoform in 72 h, for BRS Bojuru with *UBQ10* the expression value was 3.25 and for BRS Pampa it was 5.63, while with the *Eef-1α* gene the RQ values were 0.73 and 2.92, respectively (Figure 6C and 6D).

For the gene of the *CATC* isoform, in BRS Bojuru and BRS Pampa the greatest difference in relative expression values was observed at 72 h. For the most indicated reference gene, *UBQ10*, the values were 5.56 and 4.15 and for the least reference gene, *Eef-1α*, they were 1.49 and 1.78, respectively (Figure 7A and 7B). As for the gene that codifies the *SODCc1*-Cu/Zn isoform in BRS Bojuru, at 72h with *UBQ10*, the expression value was 14.28 and in BRS Pampa it was 10.87, while for the *Eef-1α* gene the RQ values were 3.13 and 4.35, respectively (Figure 7C and 7D).

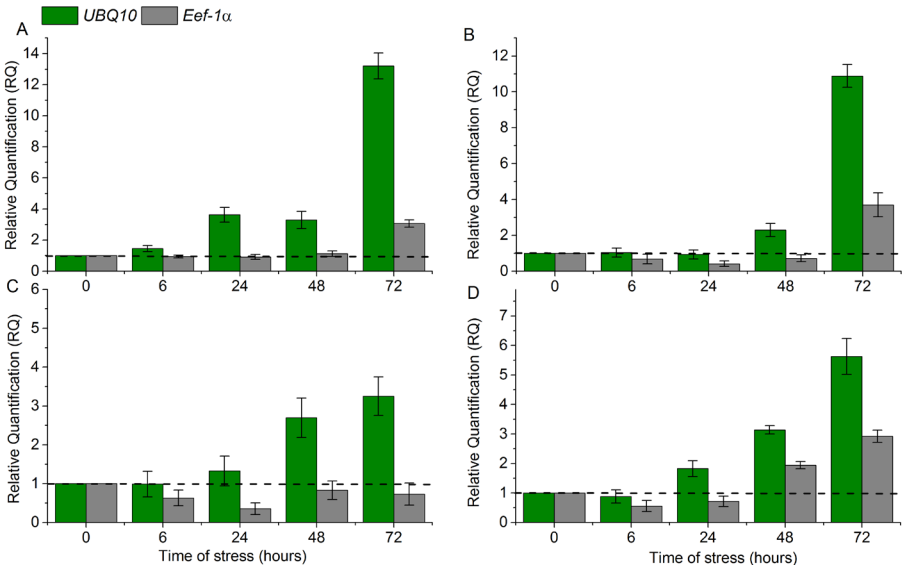

Figure 6. Relative quantification (RQ) of expression of the genes that codify the APX2 (**A** and **B**) and GR2 (**C** and **D**) isoforms in leaves of the rice genotypes BRS Bojuru (A and C) and BRS Pampa (B and D) subjected to a low temperature (13°C) in the vegetative stage. The most adequate reference genes (*UBQ10*) is represented in green, while the least indicated (*eIF-1α*) is represented in gray. The control samples were used as reference samples. The data show the average ± expression and standard deviation calculated based on three biological replicates.

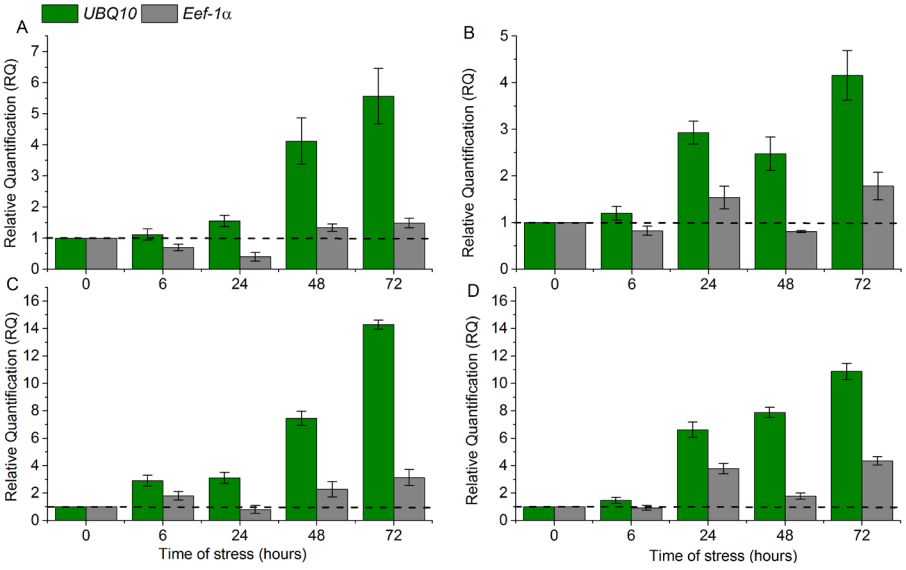

Figure 7. Relative quantification (RQ) of expression of the genes that codify the *CATC* (**A** and **B**) and *SODCc1-Cu/Zn* (**C** and **D**) isoforms in leaves of the rice genotypes BRS Bojuru (A and C) and BRS Pampa (B and D) subjected to low temperature (13°C) in the vegetative stage. The most adequate reference gene (*UBQ10*) is represented in green, while the least indicated (*eIF-1α*) is represented in gray. The control samples were used as reference samples. The data show the average ± expression and standard deviation calculated based on three biological replicates.

DISCUSSION

Protein synthesis in eucaryotes basically occurs in three stages: initiation, elongation, and termination. For these three stages to be able to occur, not only ribosome, mRNA, and aminoacyl RNA transferase (aa-tRNA) are needed, but also many other soluble proteins that facilitate these processes. Once the translation is initiated, the first codon is linked to subunit 80C of the ribosome, followed by the elongation phase in which the peptide chain increases its length, cyclically adding one amino acid at a time. The aa-tRNA addition step is catalyzed by the *1α* (*eIF-1α*) elongation complex. Besides this primordial function, studies have shown that this complex is involved in a variety of cellular processes (Sasikumar et al., 2012).

Relationships have been established between the organization of the cytoskeleton and the protein translation process. Actin is the principal component involved, playing a significant role in the regulation and efficiency of translation. It has been observed that small perturbations in the cells produce a negative effect on translation, although the levels of global components remained similar to those of the intact cells (Stapulionis et al., 1997). Since the discovery of the interaction between *eIF-1α* and actin (Yang et al., 1990), various studies have characterized and shown that cellular alterations, such as change of pH, decrease the affinity of actin with *eIF-1α* (Murray et al., 1996).

The *eIF-1α* gene has been identified as a stable reference gene in some species, including members of the Poaceae family, such as *Brachiaria* (Silveira et al., 2009), *Lolium* (Dombrowski and Martin, 2009), perennial ryegrass (Lee et al., 2010), and rice (Li et al., 2010). However, in our study, it is presented as the most unstable gene in both genotypes, which may be the result of the low temperatures, which physically (Almeida et al., 2016) and biochemically alter the cells. Similarly to this study, by evaluating rice plants subjected to saline stress, Moraes et al. (2015) showed that the *eIF-1α* gene presented considerable instability when used as a reference gene.

However, another structural gene evaluated, β-tubulin, was shown to have considerable stability in the BRS Bojuru genotype, and can thus serve as a reference gene for studies of the *japonica* subspecies involving low temperatures. Brunner et al. (2004), in a study on plants of the *Populus* genus, obtained significant results using tubulin genes as housekeeping. However, Expósito-Rodríguez et al. (2008) identified tubulin genes as the most unstable in studies of *Solanum lycopersicum* involving different organs and stages of development. Thus, we can perceive the plasticity of the reference gene function of these genes, which varies according to the species and situation studied, thus showing the importance of prior studies involving housekeeping in order to analyze transcripts.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a key enzyme involved in glycolysis, and is also a gene that is commonly used as reference gene in RT-qPCR analyses (Goidin et al., 2001). In this study, it was verified that the level of stability in the expression of *GAPDH* was different between the *O. sativa* genotypes analyzed. For BRS Bojuru, the values obtained showed that this gene is among the most stable in low temperature conditions. On the other hand, for BRS Pampa, the stability values of *GAPDH* were better, with this being among the most indicated by the geNorm software. Thus, care should be taken when using the *GAPDH* gene as housekeeping, as it has already been shown that the transcript expression corresponding to genes that codify glycolytic enzymes is activated to maintain homeostasis in rice cells under stress conditions (Pareek et al., 1998).

In plants, cyclophilic proteins are present in different organelles and tissues, and one

of their main functions is to accelerate the isomerization reaction of the *cis* link to the *trans* link and facilitate the binding process (Romano et al., 2004a). However, due to their ubiquitous nature, it becomes difficult to limit the attribution of functions to cyclophilins. Nevertheless, it has been shown that the expression of cyclophilins in plants is induced in various stress situations, such as exposure to $HgCl_2$, viral infections, and high and low temperatures, among others, with its role in the binding of proteins and its redox modulation appearing to be essential for inhibiting the detrimental effects resulting from these stress situations (Romano et al., 2004b). This claim supports the results obtained in our paper, which showed that the *cyclophilin* gene presented significant variations of expression, and consequently high instability values, especially in the BRS Bojuru genotype.

Ubiquitinization frequently occurs via the formation of an isopeptide link between C of the ubiquitin and the amino group of lysine residues of a substrate, marking the protein to be degraded via proteasome (Heride et al., 2014). Ubiquitin genes have been associated with the DNA repair mechanism, regulation of the cellular cycle, modification of kinase, endocytosis, and regulation of other cellular signaling pathways, in order to protect and control vegetal metabolism (Walton et al., 2016).

In our study, the genes related to ubiquitinization, *UBQ10*, *UBQ5*, and *UBC-E2*, were shown to be stable under abiotic stress conditions involving low temperatures, for both rice genotypes, possibly indicating that this environmental condition does not have a significant influence on the expression of these constitutive genes. A similar result was found in a study involving rice plants subjected to hydric deficit, in which *UBQ5* and *UBC-E2* were the most indicated reference gene (Auler et al., 2017).

The production of reactive oxygen species (ROS) occurs naturally and is associated with respiratory and photosynthetic metabolism, representing a normal metabolic condition of vegetal cells. In stress conditions, an alteration occurs in the cellular balance, causing an accumulation of ROS in the cell, and can thus result in oxidative damage (Ahmad et al., 2010a). It is known that in rice plants, genes exist that codify different isoforms of the enzymes involved in the antioxidant defense system, described as genetic families (Yamane et al., 2010).

In this paper, variations were observed in the relative expression values of the *SODCc1-Cu/Zn*, *CATC*, *APX2*, and *GR*2 genes when normalized with the most stable reference gene in comparison with the least stable. This observation shows that the use of inadequate genes as housekeeping leads to erroneous results and thus masks any real understanding of genetic behavior in the face of a particular condition.

CONCLUSION

By observing the genotypes separately, the analysis of the expression stability of ten candidate reference genes for RT-qPCR studies in leaves of rice subjected to a low temperature (13°C) in the vegetative development stage, shows that the *UBQ10* and β-tubulin genes are the most stable for the genotype of the *japonica* subspecies (BRS Bojuru), while *UBQ5* is the most stable for the *indica* subspecies (BRS Pampa). However, the *UBQ10* gene can be used as reference gene option for the *indica* subspecies, as it presents acceptable stability values. On the other hand, the *eIF-1α* gene does not present expression stability in both genotypes, and is not indicated for studies involving rice plants subjected to low temperatures. The expression analysis of *SODCc1-Cu/Zn*, *CATC*, *APX2*, and *GR*2 confirm the importance of validating reference genes for adequate results in RT-qPCR. Because of this, this study is of considerable

relevance for subsequent analyses of genetic expression in rice leaves in low temperature conditions.

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