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RESEARCH PAPERS

Selection of Reference Genes for RT-qPCR Studies in Different Organs of Rice Cultivar BRS AG Submitted to Recurrent Saline Stress

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Abstract—Quantitative real-time polymerase chain reactions (RT-qPCR) have become one of the most widely used methods for analyzing gene expression, provided suitable reference genes are available to normalize the data. RNA was isolated from leaves, grain, rachises and sheaths of rice (*Oryza sativa* L. cv. BRS AG) submitted to different saline stress events for seven days, and expression analysis was carried out by RT-qPCR. Expression levels of ten candidate reference genes were assessed, *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-α*), *ubiquitin10* (*UBQ10*), *ubiquitin5* (*UBQ5*), *aquaporin TIP41* (*TIP41-like*). Gene expression stability was calculated using the common statistical algorithms geNorm, BestKeeper and ΔCt method, NormFinder and RefFinder. The most stably expressed genes were *UBC2E* and *GAPDH* for leaves, *UBQ5* and *UBQ10* for sheaths, *TIP41* and *UBQ10* for rachises, and *TIP41* and *cyclophilin* for grain. Gene expression of *triose phosphate translocator* (*TPT1)*, *ADP-glucose transporter* (*BT1-1*), *choline monooxygenase (CMO)* was used to validate the selected reference genes. The results highlighted the importance of using suitable reference gene to normalize gene expression data in rice plants.

Keywords: *Oryza sativa*, rice, abiotic stress, gene expression, plant, real-time quantitative PCR

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INTRODUCTION

Soil and water salinity, pH, flooding, drought and nutrient deficits, can have a devastating impact on plant growth and yield under field conditions. More than 6% of the total world land area, and 19.5% (45 of 230 million hectares) of irrigated land, is affected by excess salts [1]. High salinity is commonly due to high concentrations of Na^+ and Cl^- in the soil solution, resulting in hyperosmotic and hyperionic conditions, which impede plant absorption of water and nutrients from the soil [2].

Rice cv. BRS AG is the result of genetic crossing performed at the Brazilian Agricultural Research Corporation (Embrapa) Temperate Climate, involving genes of the introduced genotype SLG1 (super large grain), whose grain dimensions are larger than those of conventional rice. It has an average thousand-grain weight of 52 g, while the majority of irrigated rice cultivars show lower values, such as 25.6 g for BRS Pampa [3]. This cultivar differs from the traditional cultivars because besides largest grain size, was developed for others purpose other than human consumption, such as ethanol production and animal feed. Although there are already studies with salinity contrasting cultivars, which tested reference genes [4, 5], including by our group, no work has been developed with the cultivar BRS AG. In addition, our previous studies were performed exclusively on leaves and in the present study other organs were analyzed. As it is a promising cultivar and these are initial studies carried out with it is necessary to test reference genes mainly on other organs besides the leaves, because the genes are not expressed in the same way in different organs and the lack of these initial studies can interfere in the results of subsequent analyzes. This can be seen in our results.

In view of this concern, it is necessary to understand whether pre-exposure of rice plants to unfavorable conditions during the vegetative stage can mitigate the effects of a second exposure to the same unfavorable conditions. Imprint or memory of stress, as described by Bruce et al. [6], can be defined as genetic or biochemical modifications which occur as a consequence of a previous exposure to stress and which make plants generally more resistant to future exposure.

Recent understanding of plant responses to salinity has been largely based on genetic and biochemical analysis. Gene expression patterns provide an insight

into gene functioning and gene regulatory networks in plants under salt stress [7]. One wide-spread method used in gene expression analysis is real time quantitative reverse transcription-polymerase chain reactions (RT-qPCR), because it is considered a reliable, sensitive and precise technique [8].

Experimental procedures, amount of RNA, transcriptional efficiency and amplification are among the factors that may affect the accuracy of RT-qPCR. Thus, for greater reliability of RT-qPCR data, it is essential that expression of target genes is compared to stably expressed reference genes. Therefore, selection of a reference gene is unavoidable for the normalization of expression data [9].

In plants, many genes are known to exhibit sufficient stability to make them suitable as reference genes. The genes chosen as references are usually involved in basic cellular processes, such as cell structure maintenance and primary metabolism. Among the most studied in RT-qPCR analysis of plants are *actin* (*ACT*) [10], *tubulin* (*TUB*) [10], *ubiquitin* (*UBQ*) [4], *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *eukaryotic elongation factor4-α* (*elF-4α*) [11].

The choice of reliable reference genes as internal controls to normalize gene expression in RT-qPCR is extremely important to avoid failures in the experimental procedure and to determine the precise expression of target genes [12]. This study evaluated the stability of expression of ten genes under different treatments and in different organs of rice cv. BRS AG, in order to identify suitable reference genes for RT-qPCR analysis under these conditions and for this cultivar.

MATERIALS AND METHODS

Plant material and growth conditions. The experiment was conducted using seeds of rice (*Oryza sativa* L. cv. BRS AG) from the Estação Experimental Terras Baixas (Embrapa, Clima Temperado). The seeds were disinfested with 1% hypochlorite and then germinated on germitest paper in rolls, maintained in a Biological Organism Development (BOD) growth chamber, with a 16 h photoperiod of light and 8 h of dark at 25 ± 2°C for 10 days. The seedlings were then transferred to plastic pots (8 L), perforated at the base and kept on trays. Substrate was sand previously washed with water and 1% hydrochloric acid. Irrigation occurred daily, alternating between water and nutrient solution.

The plants remained under these conditions reaching the V5 stage (with five fully expanded leaves). Half of the plants then received nutrient solution plus 150 mM NaCl for 96 hours. After this period, all plants remained under normal irrigation, with alternating water and nutrient solution, until reaching the reproductive stage R8. During the reproductive stage, the plants belonging to treatment groups T2 and T3 received 150 mM of NaCl for seven days, while the others received only nutrient solution. In this way, there were four treatment groups: T1—control (irrigation only with nutrient solution throughout cycle); T2—irrigation with nutrient solution + 150 mM NaCl in the reproductive stage; T3—irrigation with nutrient solution + 150 mM NaCl in the vegetative and reproductive stages; T4—irrigation with nutrient solution + 150 mM NaCl at the vegetative stage.

At the end of the seven days of stress at the reproductive stage, R8 (grain filling), leaf, sheath, rachis and grain samples were collected from each treatment.

Extraction of RNA and cDNA synthesis. Each macerated plant sample (100 mg) was transferred to a 1.5 mL nuclease-free microtube. Total RNA of all organs (leaf, sheath, rachis and grain) was isolated with TRIzol (Thermo Fisher Scientific, United States) according to the protocol of manufacturer. The quantity and purity of RNA were measured in a ND-1000 NanoDrop spectrophotometer (Thermo Fisher Scientific), while the quality and integrity of the RNA was verified by electrophoresis in 1.5% agarose gels. Total RNA samples were treated with DNase I, and then 1 μg/μL RNA was subjected to reverse transcription for complementary DNA synthesis using the Super Script First Strand System for RT-PCR kit (Invitrogen, United States).

Selection of reference gene. Ten genes that were cited in the literature as internal controls for RT-qPCR analysis, which supposedly exhibited no significant differences between treatments, were selected as possible reference genes. 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-α*), *ubiquitin 10* (*UBQ10*), *ubiquitin 5* (*UBQ5*), *aquaporin TIP41* (*TIP41-like*), and *cyclophilin* (*CYP2*) (Supplementary Table S1).

RT-qPCR analyses were conducted in a Bio-Rad CFX Real-Time thermocycler, using the SYBR Green fluorophore system (Roche, Switzerland). The total reaction volume was 12 μL, which included 6.25 μL fluorophore, 0.25 μL (10 mM) of each primer (sense and antisense), 1 μL cDNA (1 : 5 previously defined dilution), and 4.25 μL ultrapure water. The amplification conditions were 95°C for 10 min, and then 40 cycles at 95°C for 15 s, 60°C for 1 min with the insertion of a melting curve at 65 to 95°C, incrementing 5°C at each fluorescence measure. Three technical repetitions were performed for each biological repetition, including template-free controls.

Data analysis. For the analysis of the stability of expression of candidate reference genes, values obtained from all treatments and organs after seven days of stress in the reproductive period were assessed. The level of expression of the genes in each reaction was determined using the Cq cycle threshold for the different organs of the BRS AG cultivar. To analyze the variation of these reference genes the following

programs were used: geNorm [13], NormFind [14], BestKeeper [15], the ΔCt method [16], and the RefFinder tool (http://fulxie.0fees.us/?type=reference). The RefFinder tool used geNorm, NormFinder, BestKeeper, and the ΔCt method to compare and classify candidate reference genes. The RT-qPCR data were exported to Excel (Microsoft Excel 2010) and graphics were generated using Origin 9.0.

Validation of the reference genes. The expression levels of selected target genes were examined, normalizing the data with the most and least appropriate reference genes in order to illustrate the importance of choosing the correct reference gene. For leaf organ, the target gene chosen was *CMO,* coding for the enzyme choline monooxygenase involved in glycine betaine biosynthesis, expression data of which was normalized with that of the two genes, *UBC-E2* and *GAPDH,* which showed the most stable expression levels, and the two with the least stable expression, *ACT11* and *EeF-1α*. For sheath and rachis the target gene was *TPT1* triose phosphate, involved in carbohydrate transport, expression data of which was normalized with that of two more sets of stably expressed genes, *UBQ5* and *UBQ10* for sheath organ and *TIP41-like* and *UBQ10* for rachises, and with data for the less stably expressed genes *EeF-1α* and β-*tubulin* for sheath organ, and β-*tubulin* and *ACT11* for rachises. For grain, the target gene was the *BT1-1* ADP-glucose transporter, expression data of which was normalized with that of the two most stably expressed genes for this organ, *TIP41-like* and *CYP2,* and two less stably expressed genes, *UBQ5* and *Eef-1α*, as determined by RefFinder when all treatments were analyzed together. Amplification conditions for RT-qPCR were the same as those described above.

Experimental design and statistical analysis. The experimental design was completely randomized with one cultivar (BRS AG), four treatments (T1, T2, T3 and T4) and three replicates. The experimental unit consisted of a pot containing four plants, each pot being a biological replicate.

Expression data were submitted to analysis of variance (ANOVA; $P \leq 0.05$) and the mean values were compared by Tukey's test at 5% probability, using SAS 9.3 statistical software (SAS Institute Inc.). Statistical analysis was performed separately for each organ, leaf, rachis, sheath and grain.

RESULTS

For leaves of rice cv. BRS AG, at the reproductive stage, the most stably expressed genes classified by the comparative ΔCT and NormFinder methods were *UBC-E2* and *eIF-4-α* (Figs. 1a, 1c), whereas the BestKeeper algorithm indicated that β-*tubulin* and *cyclophilin* were more stably expressed (Fig. 1b). The expression stability of the set of reference gene candidates was examined using the geNorm software, which calculated the expression stability (M) for each gene based on the average variation of one gene relative to all others tested, using a threshold of >1.5. Therefore, a lower value of M indicated greater expression stability for the gene. For all samples evaluated, *UBC-2E* and *GAPDH* were the most stably expressed genes (Fig. 1d).

The results obtained from the comparative method ΔCt, BestKeeper, geNorm and NormFinder were confirmed by RefFinder, which integrated the four algorithms and classified the genes tested based on the geometric mean. In leaves of rice cv. BRS AG, at the reproductive stage, RefFinder indicated *UBC-E2* and *GAPDH* were the more stably expressed genes, and *cyclophilin* and *ACT11* were the least stably expressed (Fig. 1e).

For sheath organ (Fig. 2), the *UBQ5* and *UBQ10* genes were the two most stably expressed, as determined by all methods except for BestKeeper, which indicated *cyclophilin* was the second most stably expressed gene for the study conditions. According to the four algorithms used and RefFinder, the β-*tubulin* gene showed the greatest variation, being the least stably expressed gene.

As shown in Figs. 3a, 3c, the genes that showed greater expression stability for rachis organ of rice cv. BRS AG were *TIP41-like* and *UBC-2E* according to the comparative ΔCt and NormFinder methods. Among the candidate genes evaluated by the BestKeeper method, the *UBQ10* and *UBQ5* genes were the most stably expressed (Fig. 3b). The *TIP41-like*, *UBQ10* and *Eif-4-α* genes were identified by geNorm and RefFinder as being the three most stably expressed genes under the treatments tested (Figs. 3d, 3e).

For grains of rice cv. BRS AG, *TIP41-like* and *cyclophilin* genes were the most stably expressed, as determined by all methods with the exception of the NormFinder algorithm, which selected genes *cyclophilin* and β-*tubulin*. In addition, all the software programs identified *UBQ5* and *EeF-1α* as the least stably expressed genes for this organ (Fig. 4).

To determine the expression levels of candidate genes the reference values of the quantification cycle (Cq) were used. For leaf organ, the mean Cq values of the genes ranged from 23.72 to 35.54. The β-*tubulin* gene had the highest mean Cq in leaves of 35.54, while the lowest mean was observed for the *UBQ10* gene, with a value of 23.72 (Fig. 5a). For sheath organ, β-*tubulin* had the highest mean value of Cq at 35.50, while *UBQ10* gene had the lowest value of 23.23 (Fig. 5b). For rachis organ, *UBC-2E* gene had the highest mean Cq of 30.39, while *UBQ10* had the lowest value of 22.41. Finally, for grain, *cyclophilin* had the highest mean value and *UBQ10* the lowest value, with values of 34.23 and 22.41, respectively (Fig. 5d).

According to Fig. 5, the variation in expression was not constant among the evaluated organs. The β-*tubulin* and *EIF-4-α* genes showed the lowest variation in

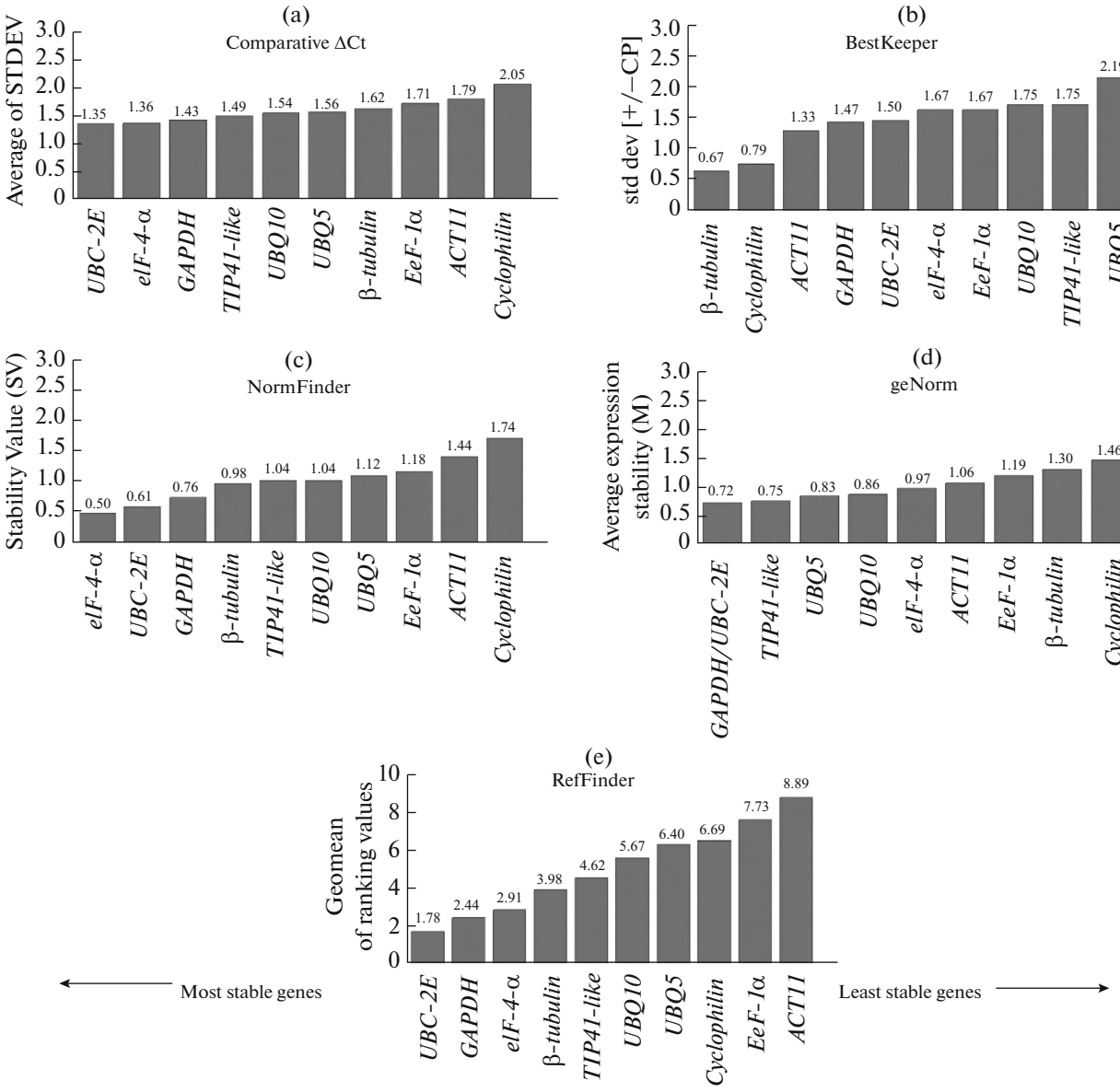

Fig. 1. Stability of expression of ten candidate reference genes in leaves of rice cv. BRS AG, submitted to different treatments, according to comparative ΔCt (a), BestKeeper (b), NormFinder (c), geNorm (d) and RefFinder (e). T1—control (irrigation only with nutrient solution throughout cycle); T2—irrigation with nutrient solution + 150 mM NaCl in the reproductive stage; T3—irrigation with nutrient solution + 150 mM NaCl in the vegetative and reproductive stages; and T4—irrigation with nutrient solution + 150 mM NaCl at the vegetative stage.

expression levels among the genes tested in leaf and grain, respectively. On the other hand, for the sheath and rachis organs, *UBC-2E* had the lowest variation for the study conditions. By contrast, β-*tubulin* showed higher variation of expression for both sheath and rachis organs, while *ATC11* and *Eef-1α* were the least stably expressed genes for leaf and grain organ, respectively.

Calculation of the variation of pairs (Vn/Vn + 1) with the candidate gene combinations was analyzed using the geNorm program to determine the need for adding more reference genes, with a cutoff value of 0.15. According to this criterion, it was found that for all organs evaluated in this study, that the use of only two reference genes was enough to normalize the expression data. It was observed that the value of V2/3 in the leaves of rice cv. BRS AG at the reproductive stage was (Fig. 6a). For the sheath, the value of V2/3 corresponded to 0.0101 (Fig. 6b), for rachis organ it was 0.0094 (Fig. 6c), while in grain the value was 0.0100 (Fig. 6d).

To verify the stability of expression of the reference genes selected above, the relative expression of a gene involved in glycine betaine biosynthesis, CMO, was

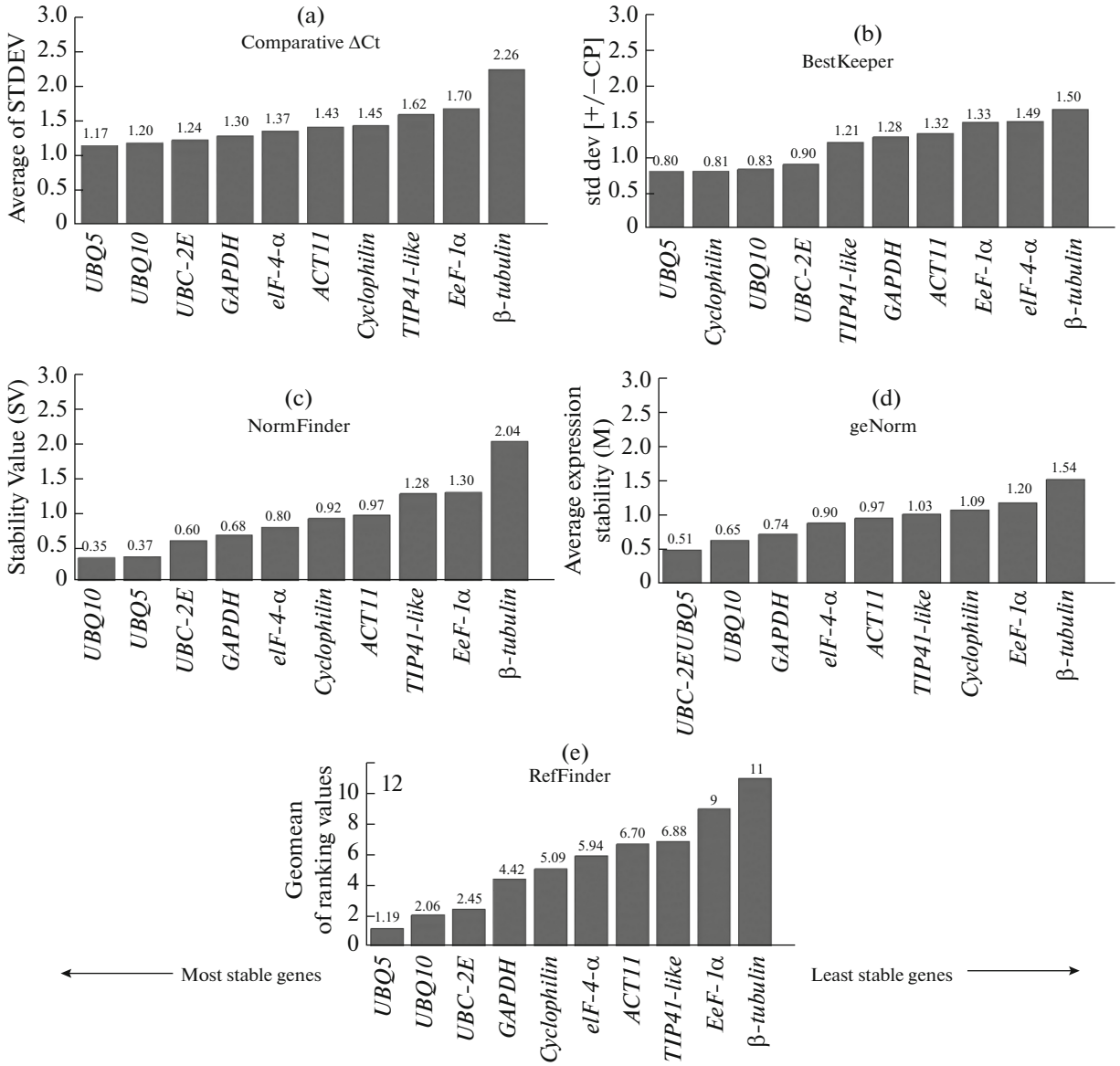

Fig. 2. Stability of expression of ten candidate reference genes in the sheath of rice cv. BRS AG, submitted to different treatments, according to comparative ΔCt (a), BestKeeper (b), NormFinder (c), geNorm (d) and RefFinder (e). T1—control (irrigation only with nutrient solution throughout cycle); T2—irrigation with nutrient solution + 150 mM NaCl in the reproductive stage; T3—irrigation with nutrient solution + 150 mM NaCl in the vegetative and reproductive stages; and T4—irrigation with nutrient solution + 150 mM NaCl at the vegetative stage.

investigated in rice leaves, using the two most and least stably expressed reference genes. It was observed that the expression of the target gene in leaves differed for treatments T2 and T3 statistically when normalized with the most stably expressed reference genes, *UBC-2E* and *GAPDH* (mean values of expression of 0.05 and 0.04, respectively), compared with the least stably expressed reference genes, *EeF-1α* and *ACT11,* (mean values of expression of 0.23 and 0.92, respectively; Fig. 7a).

To validate the results obtained for the reference genes in the sheath and rachis organs, relative expression analysis was conducted using the *TPT1* gene. For the sheath, there was not a significant difference for the evaluated treatments between data normalized with the most stably expressed reference genes and that normalized with the least stably expressed reference genes (Fig. 7b). For rachis organ, *TPT1* was also used to validate the reference genes, with a significant difference in expression values for T2 and T3. Using the most stably expressed genes, the expression values were 1.19 and 1.31, respectively, while using the less stably expressed genes the values were 2.73 and 0.54, respectively (Fig. 7c).

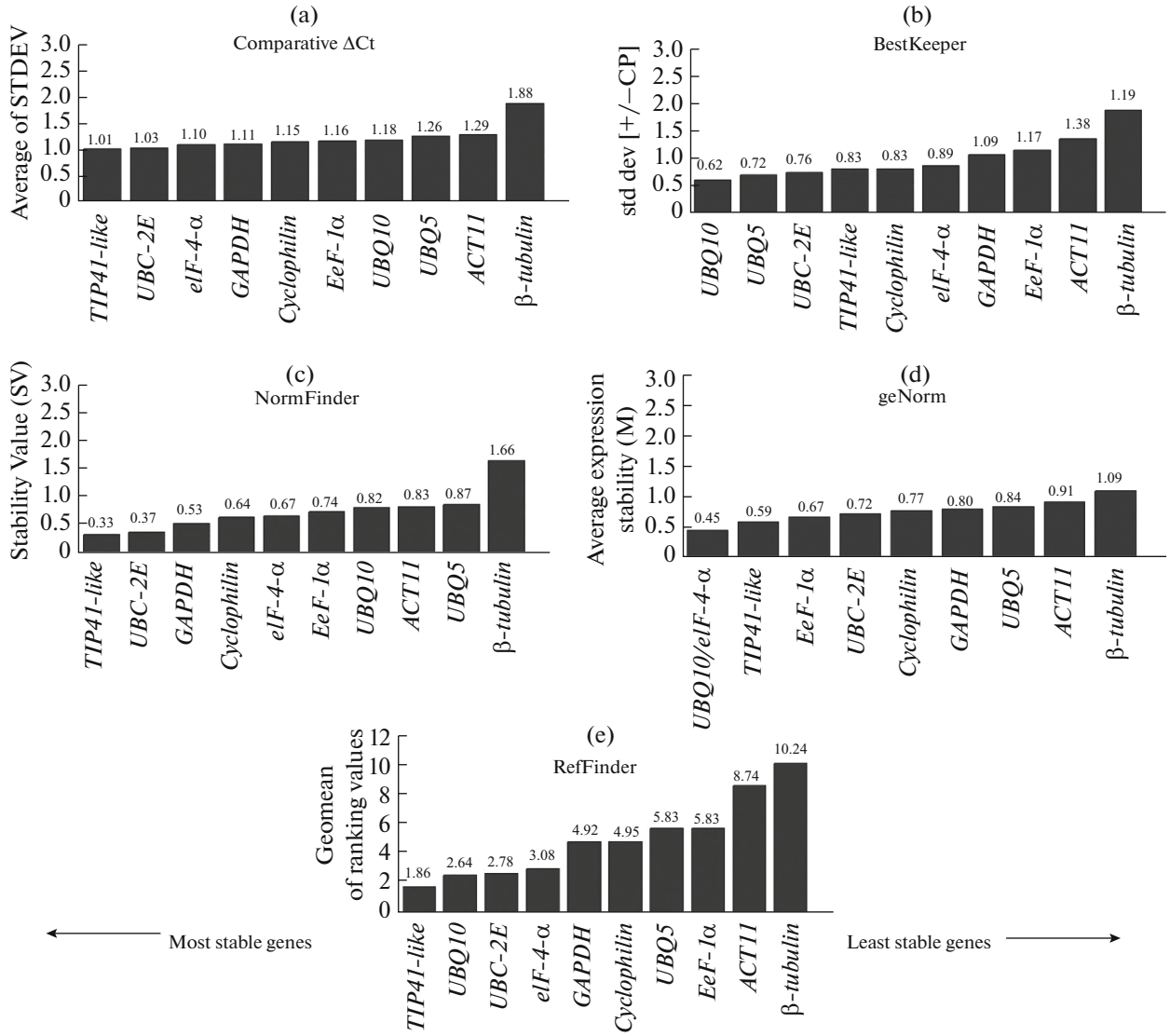

Fig. 3. Stability of expression of ten candidate reference genes in the rachis of rice cv. BRS AG, submitted to different treatments, according to comparative ΔCt (a), BestKeeper (b), NormFinder (c), geNorm (d) and RefFinder (e). T1—control (irrigation only with nutrient solution throughout cycle); T2—irrigation with nutrient solution + 150 mM NaCl in the reproductive stage; T3—irrigation with nutrient solution + 150 mM NaCl in the vegetative and reproductive stages; and T4—irrigation with nutrient solution + 150 mM NaCl at the vegetative stage.

For grain, for the *BT1-1* target gene, there was variation in the expression response between the less and more stably expressed reference genes. When the more stably expressed reference genes were used for normalization, the expression values of the target gene were 0.34 and 1.1 for treatments T2 and T3, respectively, differing statistically from the expression values when normalized with the least stably expressed genes, which produced values of 9.22 and 9.72, respectively (Fig. 7d).

DISCUSSION

When carrying out gene expression analysis, particularly by RT-qPCR, it is essential that there is correct standardization, in order to guarantee the control of non-specific variation between samples. The most commonly used method for normalizing data with this technique is based on the use of one or more reference genes [17]. According to previous studies on the selection of reference genes in plants for RT-qPCR, the expression level of a reference gene may not be constant between species. In addition, variation may occur within the same species in response to various treatments or different plant organs. Thus, genes involved in metabolism may exhibit significant variation in expression in different situations [9]. Therefore, choosing appropriate reference genes is a crucial step in experimental design, in generating data that is reliable and less likely to be misinterpreted.

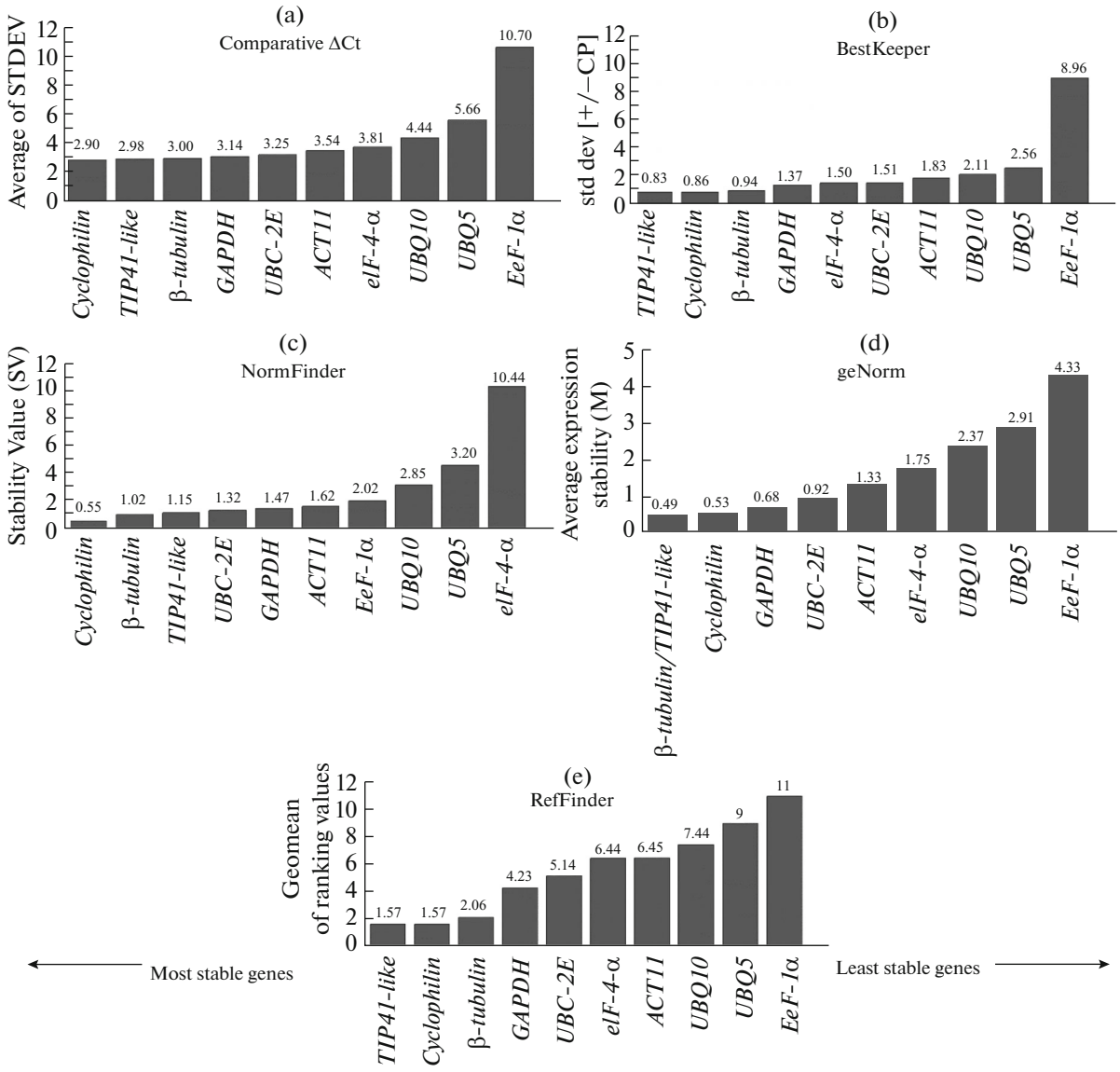

Fig. 4. Stability of expression of ten candidate reference genes in the grain of rice cv. BRS AG, submitted to different treatments, according to comparative ΔCt (a), BestKeeper (b), NormFinder (c), geNorm (d) and RefFinder (e). T1—control (irrigation only with nutrient solution throughout cycle); T2—irrigation with nutrient solution + 150 mM NaCl in the reproductive stage; T3—irrigation with nutrient solution + 150 mM NaCl in the vegetative and reproductive stages; and T4—irrigation with nutrient solution + 150 mM NaCl at the vegetative stage.

In this current study, it was possible to observe that expression of potential reference genes changed in different organs in the same species. For leaves of rice cv. BRS AG, the most appropriate reference genes were *UBC-E2* and *GAPDH*. A study with cotton plants (*Gossypium hirsutum* L.) showed that, depending on the conditions, the reference genes behaved differently, with *UBQ7*, *GAPDH*, *EFIA8* being the most appropriate genes when studying saline stress in leaves of this species [18]. Evaluation of expression levels of candidate reference genes in Amaranthus (*Amaranthus hypocondriacus*) indicated *AhyMDH*, *AhyGAPDH*, *AhyEF-1a* and *AhyACT* were ideal for normalization in this species for the study conditions [19]. Auler et al. [20] obtained similar results studying leaves of rice plants subjected to water deficit in the vegetative and reproductive stages, showing that *UBC-E2* and *UBQ5* could be used as reference genes in all treatments tested. On the other hand, β-*tubulin*, *eIF-4α* and *GAPDH* showed high instability of expression, as was also observed in the present study, where β-*tubulin* was the least appropriate gene, except in the case of sheath, rachis and grain organs.

The *GAPDH* gene, according to Jonge et al. [21], is one of the most frequently used reference genes, to the extent it is considered "classical". For leaves of rice cv.

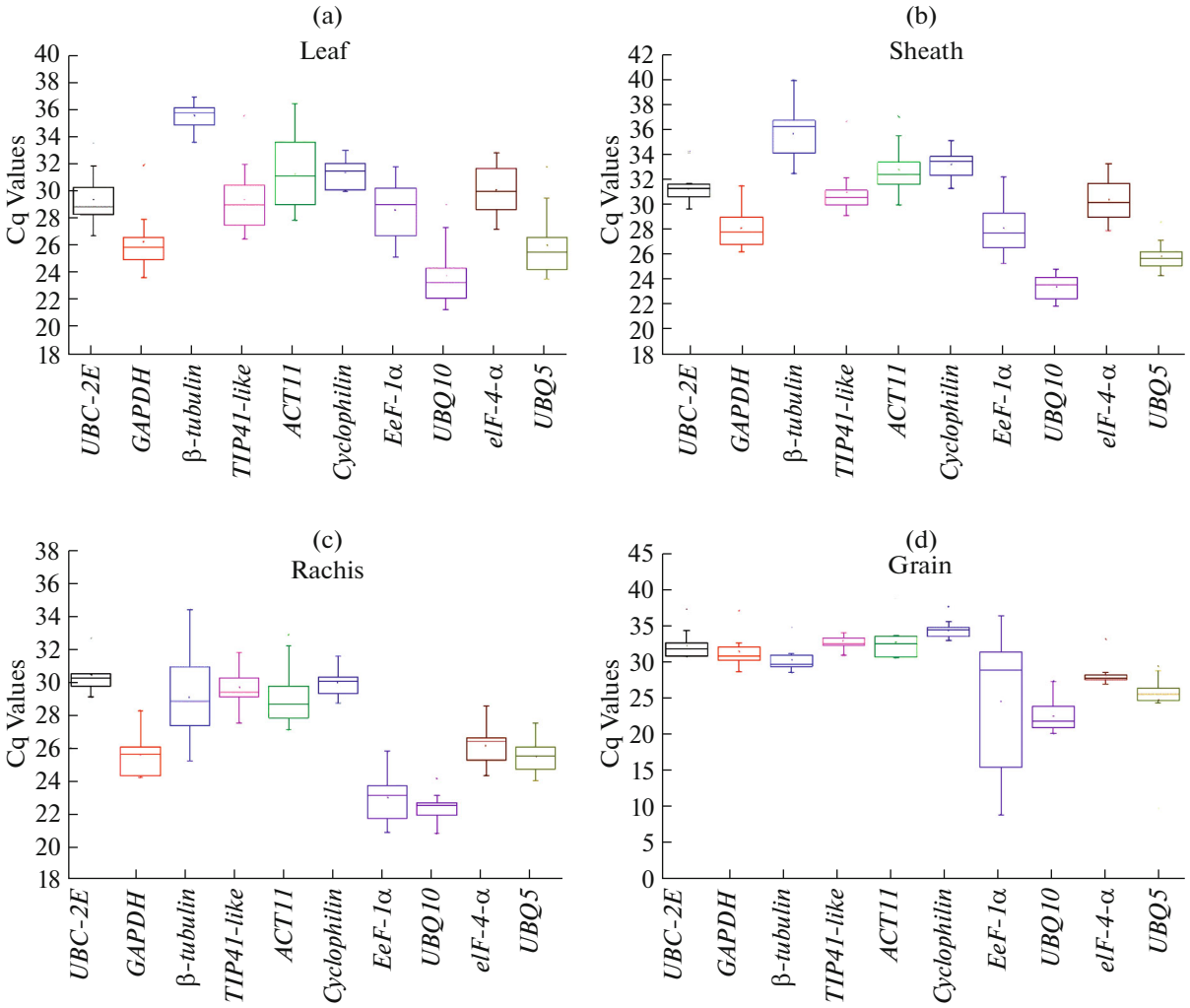

Fig. 5. RT-qPCR quantification cycle values of ten candidate reference genes in leaves (a), sheaths (b), rachises (c) and grains (d) of rice cv. BRS AG, under the different treatments. T1—control (irrigation only with nutrient solution throughout cycle); T2—irrigation with nutrient solution + 150 mM NaCl in the reproductive stage; T3— irrigation with nutrient solution + 150 mM NaCl in the vegetative and reproductive stages; and T4—irrigation with nutrient solution + 150 mM NaCl at the vegetative stage. The lower quartile is the value for 25% of the sample. The upper quartile is the value for 75% of the sample. The edges indicate the maximum and minimum values. The bigger the box, the greater the variation.

BRS AG, *GAPDH* showed high expression stability. The enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) participates in one step of glycolysis, converting glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate, with concomitant reduction of NAD^+ to NADH [21].

In the present study, the *UBQ5* and *UBQ10* showed a relatively stable pattern of expression, and were considered the most appropriate reference genes in sheath organ. Jain et al. [11] evaluated 25 samples, 7-day-old light-grown seedlings (7dL), 7-day-old dark-grown seedlings (7dD), 7dL shoots, 7dL roots, mature leaf organ, rachises, young inflorescences (5–10 mm), pre-pollinated (PP) flowers, post-fertilized (PF) flowers and mature seed, and verified that among the most stably expressed genes was *UBQ5* gene, consistent with our results. By contrast, the same authors indicated that *UBQ10* was less stably expressed, whereas in our study we found that it was the most appropriate reference gene for sheath and rachis organs.

For the rachis and grain, the most stably expressed genes were *TIP41-like* and *UBQ10*, and *TIP41-like* and *cyclophilin*, respectively. Tian et al. [22], when evaluating carrot plants under saline stress using NormFinder, indicated that *TIP41* was the most stably expressed gene. In our study, both NormFinder and BestKeeper identified the same gene as more stably expressed for both rachis and grain, while the same authors recommended *UBQ* under cold conditions. Moraes et al. [4] also suggested *UBQ10* was more suitable for studies on rice leaves subjected to saline stress because it exhibited a continuous expression pattern.

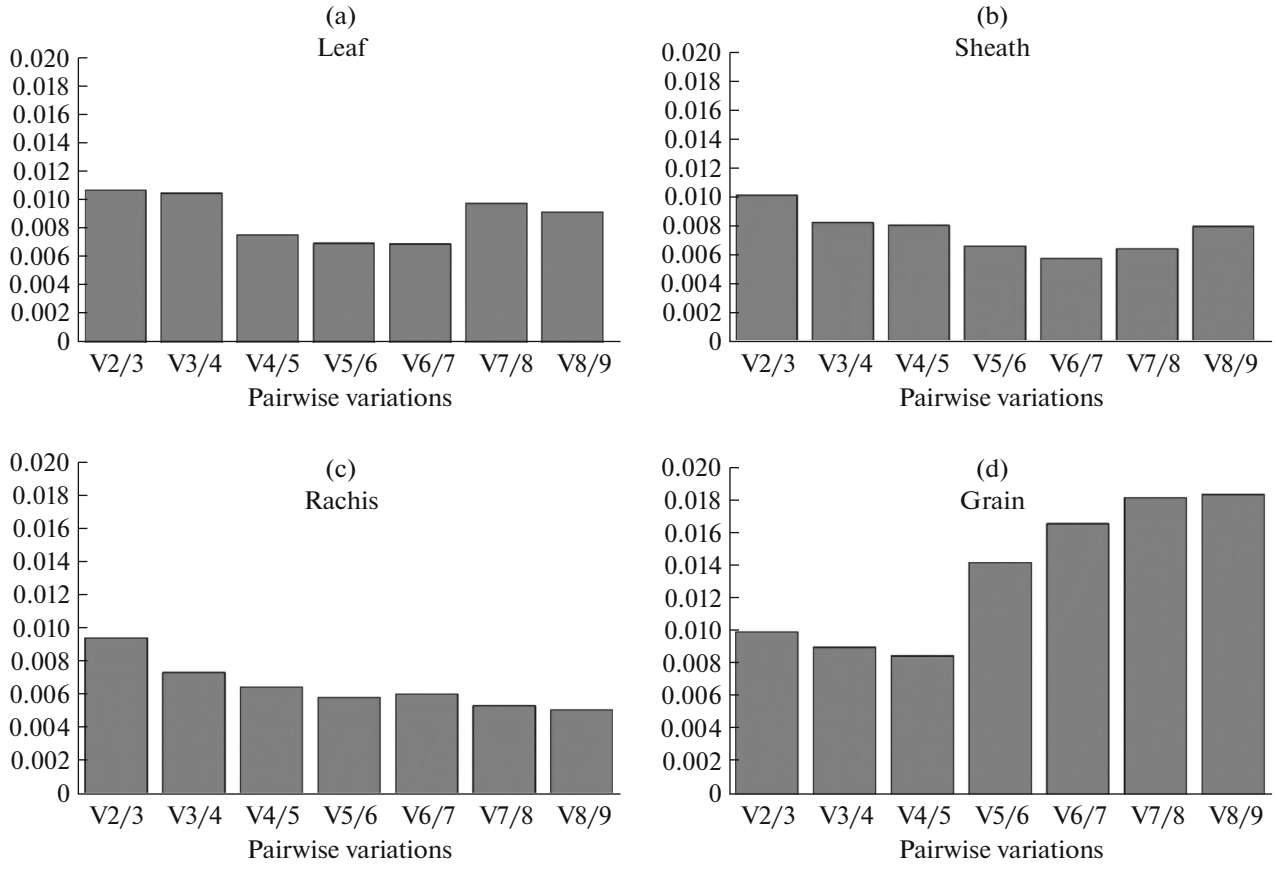

Fig. 6. Pairwise variation (V) calculated by the geNorm algorithm to determine the minimum number of reference genes for proper normalization in leaves (a), sheaths (b), rachises (c) and grains (d) of rice cv. BRS AG, under the different treatments. T1—control (irrigation only with nutrient solution throughout cycle); T2—irrigation with nutrient solution + 150 mM NaCl in the reproductive stage; T3—irrigation with nutrient solution + 150 mM NaCl in the vegetative and reproductive stages; and T4— irrigation with nutrient solution + 150 mM NaCl at the vegetative stage.

This same study indicated *eIF-4α, cyclophilin* and *TIP41* as less suitable reference genes. In contrast with the current study, Li et al. [23] found that the most stable genes in rice grains collected at 3, 6, 10, 15 and 20 days after flowering, and in samples from different organs, were *eIF-4a*, *ACT1* and *UBC*.

According to Stone [24], a well-studied function of ubiquitin is its role in selective proteolysis by the ubiquitin-proteasome system. Ubiquitination is a process involving the action of three enzymes, with ubiquitin conjugation enzyme (UBC-E2) binding ubiquitin to the substrate. Among several processes, ubiquitination is involved in plant responses to environmental stresses, such as drought, salinity, and low temperatures. Of the genes analyzed in the current study with rice cv. BRS AG, under saline stress, the genes encoding *ubiquitin 2E* (*UBC2E*), *ubiquitin 10* (*UBQ10*) and *ubiquitin 5* (*UBQ5*) were among the most stably expressed genes in the organs investigated.

Aquaporins are membrane proteins that function as water-conducting pores in plant and animal cells. They occur on the plasma membrane (PIPs) and the tonoplast membrane (TIPs). They participate in the transport of water in the whole plant, as well as performing important functions at the cellular level, acting as buffers to osmotic fluctuations that may occur in the cytosol, especially in situations of saline and/or water stress [25]. The *TIP41* gene, coding for an aquaporin, was shown to be stably expressed under the conditions of the current study for rachis and grain organ.

For the boxplot graph, where no selection program was used and only the Cq analysis was taken into account, differences in the expression stability of genes could be identified. This demonstrated the importance of using these software programs, which allowed the identification of stably expressed genes in a set of samples. These tools included geNorm, NormFinder, and Bestkeeper [13–15], which have been widely used by researchers to find suitable reference genes. These programs allow the calculation of a normalization factor based on multiple reference genes, improving its robustness. BestKeeper employs quantification cycle values directly for expression stability calculations, while geNorm and NormFinder

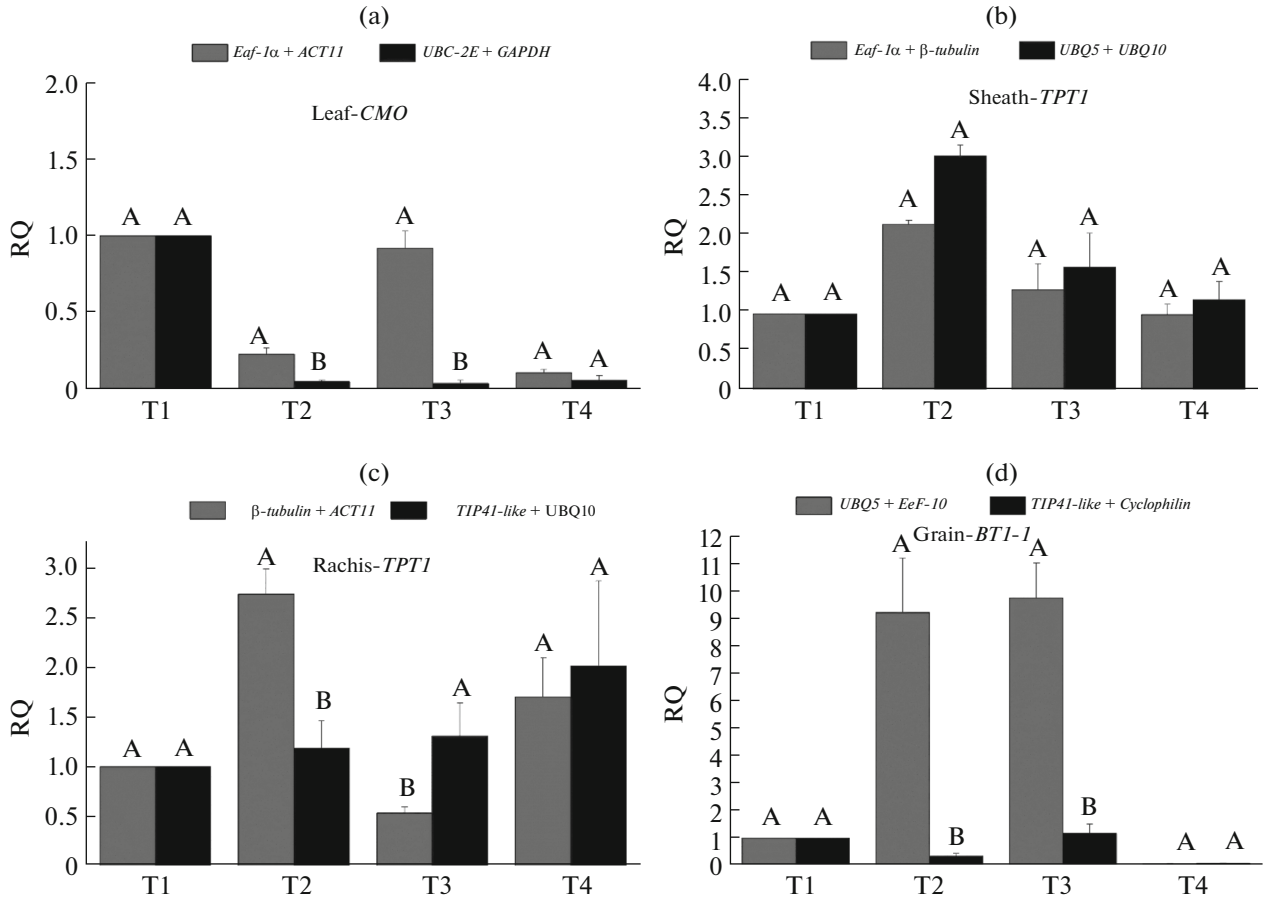

Fig. 7. Expression profile of the *CMO* (a) *TPT1* (b) *TPT1* (c) and *BT1-1* (d) genes in leaves, sheaths, rachises and grains, respectively, of rice cv. BRS AG, submitted to the different treatments. T1—control (irrigation only with nutrient solution throughout cycle); T2—irrigation with nutrient solution + 150 mM NaCl in the reproductive stage; T3—irrigation with nutrient solution + 150 mM NaCl in the vegetative and reproductive stages; and T4— irrigation with nutrient solution + 150 mM NaCl at the vegetative stage. The combinations of the two best reference genes for leaves (*UBC-E2* + *GAPDH*), sheaths (*UBQ5* + *UBQ10*), rachises (*TIP41* + *UBQ10*), and grain (*TIP41* + *cyclophilin*) are shown in black, while those of the reference genes with the least stable expression for leaves (*EeF-1α* + *ACTIN 11*), sheaths (*EeF-1α* + β-*tubulin*), rachises, (β-*tubulin* + *ACT11*) and grain (*UBQ5* + *EeF1α*) are represented in gray. Data are presented as means ± standard deviation, calculated from three biological replicates. The upper-case letters indicate a significant difference ($P \leq 0.05$) between the most and least appropriate reference genes in each treatment.

have these values changed for relative quantities using the normalization factor [13, 14]. The RefFinder tool has also been used, which integrates geNorm, NormFinder, BestKeeper and the comparative ΔCt method.

The *cyclophilin* for leaves, β-*tubulin* for sheaths and rachises, and *EeF-1α* for grains were shown to be less stably expressed in the current study. Cyclophilin proteins are involved in increased mitochondrial membrane permeability [26]. Tubulin protein has two α and β subunits, with structural functions in eukaryotic cell microtubules. In addition to the role of *elF-1α* in protein synthesis, it also seems to act in the organization of the cytoskeleton [27]. Expósito-Rodríguez et al. [28] found that *tubulin* genes were the least stable genes in their study of different organs and development stages of *Solanum lycopersicum*, consistent in part with the current study, since β-*tubulin* and *eIF-1-α* were judged to be the least appropriate reference genes for sheath, rachis and grain organ of rice cv. BRS AG.

Plant productivity and yield are governed by the ability to synthesis, transport and use photo-assimilates by sink organs, especially during the reproductive phase [29]. Plants of rice cultivar BRS AG have this characteristic, and thus the presence of highly effective transporters and/or enzymes involved in the metabolism and translocation of carbohydrates may be responsible for the accumulation of starch in the grain. Studies have shown that the triose phosphate translocator (TPT) family, located on the inner membrane of the chloroplast, carries out the exportation of triose phosphate into the cytosol in exchange with inorganic phosphate (Pi), and play an important role during the filling of rice grains [29]. Likewise, some previous studies have shown that the ADP glucose

transporter (BT1-1) is essential during the synthesis of starch in cereals, such as rice.

Throughout their life cycle, plants are exposed to unfavorable environmental conditions, such as saline stress. Glycine betaine is an osmoprotector, which can act in reducing oxidative stress by stabilizing cell macromolecules under adverse conditions [30]. It is synthesis from choline by two stages of oxidoreduction, with choline monoxigenase (CMO) participating in the first stage of synthesis. Using *TPT1, BT1-1* and *CMO* to validate the candidate reference genes, we could observe changes in expression values. The results of the current study showed the importance of carefully choosing reference genes in experiments comparing the expression of target genes, due to their potential variations in expression. In fact, it was possible to observe that within the same species, the most appropriate reference genes were different depending on the organ being analyzed.

The results presented in this study will aid in future studies involving gene expression. In addition, our work differs from others because it involves more than one stress event, as it can be observed in the experimental design, i.e., how a previous stress event contributes to a better adaptive response to plants to a new unfavorable event. In the present study, it was tested in the vegetative and/or reproductive stages.

To conclude, from the evaluation of ten potential reference genes in rice cv. BRS AG, under salt stress (150 mM NaCl) at different times of its cycle, we identified two appropriate reference genes for each organ analyzed. The most appropriate genes to normalize RT-qPCR data in this species were *UBC2E* and *GAPDH* for leaves, *UBQ5* and *UBQ10* for sheaths, *TIP41* and *UBQ10* for rachises, and *TIP41* and *cyclophilin* for grain. In contrast, in these organs, the genes that presented the greatest variation in gene expression were *cyclophilin*, β-*tubulin*, *EeF1α* and *ACT11,* making them inappropriate reference genes for these organs, under the conditions of this study.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflicts of interest.

Statement on the welfare of humans or animals. This article does not contain any studies involving animals performed by any of the authors.

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: TR, PAA, MNA, CM and EJBB. Performed the experiments: TR, PAA, MNA, AMMJ. Analyzed the data: TR, PAA, MNA and EJBB. Wrote the paper: TR. Corrected the manuscript: EJBB.

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