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Identification of suitable reference genes in buffalo grass for accurate transcript normalization under various abiotic stress conditions

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ABSTRACT

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) is a sensitive technique for normalization of the gene expression level of target genes. Buffalograss (*Buchloe dactyloides*), a warm-season turfgrass with strong abiotic stress resistance, is widely used in North China. Up to now, no work was performed to evaluate the reference genes in buffalograss. In this study, the expression profiles of ten potential reference genes were examined by qRT-PCR in 24 buffalograss samples, which were subjected to a different treatment (salt, osmotic, cold and heat). Three qRT-PCR analysis methods (GeNorm, NormFinder, and Bestkeeper) were used to evaluate the stability of gene expression. The results indicated that *DNAJ* and *β-ACTIN* were the optimal reference genes for salt-treated leaves, and the combination of *PP2A* and *GAPDH* was better reference genes for PEG-treated leaves. Under cold stress, *DNAJ* and *β-ACTIN* showed less variety of expression level in leaves. *DNAJ* and *GAPDH* exhibited the most stable expression in heat-treated samples. To sum up, glyceral-dehyde-3-phosphate dehydrogenase (*GAPDH*), *β-ACTIN*, DNAJ-like protein (*DNAJ*) and protein phosphatase 2A (*PP2A*) were selected as the most stable reference gene among all tested samples. To further validate the suitability of these reference genes, the expression levels of *DREB2* (homologs of *AtDREB2*) were analyzed in parallel. Our results show that the best reference genes differed across different experimental conditions, and these results should enable better normalization and quantification of transcript levels in buffalograss in the future.

1. Introduction

Gene expression analysis is a basic and important step in the systematic analysis of plant biological processes, such as growth and development as well as biotic and abiotic stress defense pathways. In recent years, quantitative real-time reverse transcriptase PCR (qRT-PCR) has been used as the main analysis technique for quantification and regulating characterization of gene expression, because it provides outputs with high sensitivity, specificity and capacity (Bustin et al., 2005, 2009). A normalization step is an essential pre-requisite needed to avoid experimental errors arising from variation in the quantity and integrity of the RNA template, as well as in the efficiency of the reverse transcription reaction used to synthesize DNA. The most common way to achieve normalization is to use one or several internal control genes, termed reference genes, whose expression is assumed to be constitutive (Huggett et al., 2005; Radonić et al., 2004; Suzuki et al., 2000).

Ideal reference genes should be stably expressed in different plant tissues, at different stages of development or under different experimental environments (Brunner et al., 2004; Jain et al., 2006). In general, housekeeping genes are often selected as reference genes, such as 18S ribosomal RNA (*18S rRNA*), polyubiquitin (*UBQ*), *β-ACTIN*, glyceral-dehyde-3-phosphate dehydrogenase (*GAPDH*), DNAJ-like protein (*DNAJ*), elongation factor 1-α (*EF-1α*), alpha-tubulin (*TUA*), beta-tubulin (*TUB*), and ubiquitin-conjugating enzyme (*UBC*), mostly involving in basic cellular processes, that have been widely used as internal controls for gene expression analyses in many plants (Bustin, 2002; Czechowski et al., 2005; Dheda et al., 2004; Kim et al., 2003; Radonić et al., 2004). However, some studies showed that traditional housekeeping genes did not always keep their stability in any tissues or experimental conditions (Andersen et al., 2004; Guénin et al., 2009). For instance, *ACTIN* was not a suitable reference gene in *Brachypodium distachyon*, at different developmental stages or under various experimental conditions (Hong et al., 2008). Even in *Oryza sativa*, the expression of *ACTIN* was not detected in the apical meristem (Narsai et al., 2010).

Recently, some new reference genes that exhibit highly stable expression levels were identified in *Arabidopsis thaliana* and soybean

Abbreviations: *β-ACTIN*, beta-actin; *DNAJ*, DNAJ-like protein; *DREB2*, dehydration responsive element binding protein 2; *EF-1α*, elongation factor 1-α; *GAPDH*, Glyceral-dehyde-3-phosphate dehydrogenase; *PP2A*, protein phosphatase 2A; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; *RAN1*, GTP-binding protein RAN1; *SAMDC*, S-adenosylmethionine decarboxylase gene; *TUA*, alpha-tubulin; *TUB*, beta-tubulin; *UBC*, ubiquitin-conjugating enzyme.

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by microarray (Czechowski et al., 2005; Libault et al., 2008). These reference genes include SAND family protein (*SAND*), protein phosphatase 2A (*PP2A*), TIP41-like family protein (*TIP41*), F-box/kelch-repeat protein (*F-box*), phosphoenolpyruvate carboxylase-related kinase 1 (*PEPKR1*) and others. Many of these reference genes were found to perform better than traditional ones, e.g., *PP2A* in hybrid roses (Klie and Debener, 2011). Therefore, evaluating the expression stability of the reference genes in the target species of interest becomes necessary.

Until now, some statistical algorithms have been developed to validate the expression stabilities of candidate reference genes in order to select the most suitable one for a particular experimental assay. GeNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) are free Visual Basic Applets for Microsoft Excel 2003 that have been commonly used to determine the expression stability of candidate reference genes in plants, such as *A. thaliana* (Czechowski et al., 2005; Hong et al., 2010; Remans et al., 2008), *O. sativa* (Expósito-Rodríguez et al., 2008; Jain et al., 2006; Li et al., 2010), *B. distachyon* (Hong et al., 2008), *Triticum aestivum* (Paolacci et al., 2009), *Hordeum vulgare* (Faccioli et al., 2007), *Brassica napus* (Chen et al., 2010), *Gossypium hirsutum* (Artico et al., 2010), *Glycine max* (Hu et al., 2009), *Pisum sativum* (Die et al., 2010), *Platycladus orientalis* (Chang et al., 2012) and *Caragana intermedia* (Zhu et al., 2013). However, no reliable internal controls for gene expression studies have been defined for buffalo grass (*B. dactyloides*), which limits further studies on this species at the transcriptome level.

Buffalograss, a turfgrass species native to the Great Plains region of the North America, exhibits excellent drought, cold and heat tolerance. Buffalograss is often considered an ideal low input turfgrass, since it requires relatively little irrigation, fertilizer, or pesticide to maintain turfgrass quality compared to more commonly used turfgrass species (Klie and Debener, 2011; Moser et al., 2004). Buffalograss is also dioecious and thus is an obligate outcrossing, highly heterogeneous species which complicates cultivar development and genomic studies (Wachholtz et al., 2013). However, no study has been performed to identify the reference genes under abiotic stress. Systematic exploration and validation of more stable buffalograss reference genes are still requisite.

In this report, the stability of ten candidate reference genes for use in buffalograss gene expression studies was examined, including eight traditional housekeeping genes, *β-ACTIN*, *GAPDH*, *EF-1α*, *TUA*, *TUB*, *DNAJ*, S-adenosylmethionine decarboxylase gene (*SAMDC*) (Hong et al., 2008), and *UBC*, and two novel reference genes *PP2A* and GTP-binding protein RAN1 (*RAN1*) (Mallona et al., 2010). The sequences of these genes were obtained from the transcriptome date of buffalograss. Then, we compared their stabilities across a large set of buffalograss samples representing different abiotic stress treatments using statistical and graphical methods. The results showed that the expressions of the selected genes exhibited different levels of variations across the samples. Furthermore, to validate the selection of candidate reference genes, the expression levels of *DREB2* were assessed using different reference genes. And the aim of this study was to evaluate the usefulness of potential reference genes of buffalograss under abiotic stress for qRT-PCR.

2. Materials and methods

2.1. Plant materials and treatment

Buffalograss seeds were washed three times with tap water, and then sown in plastic pots filled with peat soil in a growth chamber with a 16 h light/8 h dark photoperiod at 25 °C/22 °C day/night temperatures and ~70% relative humidity. For salt and osmotic stress treatments, three-week-old seedlings were carefully removed from the soil while avoiding injury, their roots were washed cleanly with tap water, and groups of seedlings were placed in either NaCl (200 mM) or PEG6000 (20%) solutions for 0, 1, 3, 6, 12, or 24 h in the growth chamber. For the cold and heat stress treatments, the seedlings in pots were grown at either 4 °C or 42 °C, for 0, 1, 3, 6, 12, or 24 h. Leaves were collected from the three-week-old seedlings subjected to all four treatments. These were immediately frozen in liquid nitrogen and stored at −80 °C. Samples above were collected from three seedlings to provide three replicates.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Only RNA preparations having an A_{260}/A_{280} ratio of 1.8–2.0 and an A_{260}/A_{230} ratio of >2.0 were used for subsequent analysis. RNA integrity was verified by 1.5% agarose gel electrophoresis followed by Goldview staining. Before cDNA synthesis, the RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA), according to the manufacturer's instructions, and first-strand cDNA synthesis was carried out using 1 μg RNA for each sample. The cDNAs were diluted 1:10 with nuclease-free water prior to qRT-PCR analyses.

2.3. qRT-PCR analysis and test of amplification efficiency

Primers for the ten reference genes of buffalograss were designed using the Primer Premier 5 software with melting temperatures of 58–62 °C, primer lengths of 20–24 bp, GC content of 45–55% and amplicon lengths of 50–230 bp (Table 1). qRT-PCR reactions were carried out in 96-well blocks with an Applied Biosystems 7500 Real-Time PCR system using UltraSYBR Mixture (CWBio. Co., Ltd., China) in a 20 μl reaction volume (containing 2 μl cDNA reaction mixture, 10 μl 2× UltraSYBR Mixture (with ROX Reference Dye I), 0.4 μl each primer, and 7.2 μl sterile distilled water). The reaction conditions were those recommended by the manufacturer (10 min at 95 °C, 45 cycles of 95 °C for 15 s, and 60 °C for 60 s). The dissociation curve was obtained by heating the amplicon from 60 °C to 95 °C (Fig. S1). All qRT-PCR reactions were carried out in technical and biological triplicate.

A standard curve for each gene was generated using serial dilutions of pooled cDNAs (1, 1/5, 1/25, 1/125, 1/625, 1/3125; each gene in triplicate) (Fig. S2). To determine their amplicon specificity, electrophoresis analysis of the PCR products was also carried out (Fig. S3). Expression levels of the ten genes in all samples were determined by their cycle threshold values (Ct). Only Ct values of less than 40 were used to calculate correlation coefficients (R^2 values) and an estimate of PCR efficiency (E) was derived from the expression $[10^{(1/-S)} - 1] \times 100\%$, where S represents the slope of the linear regression (Ginzinger, 2002).

2.4. Reference gene expression stability determination

The expression stability of the ten candidate genes was evaluated using three commonly used algorithms, GeNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper (Pfaffl et al., 2004). Following qRT-PCR data collection, Ct values were converted to relative quantities using the formula: $2^{-\Delta Ct}$ (ΔCt = each corresponding Ct value − minimum Ct value). The sample with the maximum expression level (the minimum Ct value) was used as a calibrator and was set to a value of 1. Relative quantities were used for GeNorm and NormFinder, while BestKeeper analyses were based on raw Ct values. All three software packages were used according to the manufacturer's instructions.

3. Results

3.1. Expression profiling of candidate reference genes

Ten candidate reference genes were assessed using qRT-PCR to quantify their mRNA levels. The expression levels of the candidate reference genes were determined as cycle threshold (Ct) values, and the transcripts of these genes showed different levels of abundance (Fig. 1). The mean Ct values of the genes ranged from 21 to 32 across