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Evaluation of candidate reference genes for qPCR in maize

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ABSTRACT

Quantitative real-time PCR (qPCR) is a powerful tool to measure gene expression levels. Accurate and reproducible results are dependent on the correct choice of the reference genes for data normalization. To date, screenings evaluating candidate reference gene stability for expression studies in maize have not been reported. In the present work, we analyzed the expression patterns of 12 genes in a set of 20 maize samples, obtained from different tissues of plants grown at various experimental conditions. Using genorm^{PLUS}, NormFinder and BestKeeper algorithms, the expression stability of three "classical" reference genes, such as *ACT*, *TUB* and *18S rRNA*, and the newly identified candidates, was assessed. With respect to the algorithms, our results showed similar performance among genorm^{PLUS}, NormFinder and BestKeeper in evaluating the suitability of reference genes. Our data therefore showed that the currently and widely used reference genes for data normalization in maize were not the most stable expressed transcripts. Five of the new putative reference genes (*CUL*, *FPGS*, *LUG*, *MEP* and *UBCP*) exhibited the highest expression stability according to all algorithms. In conclusion, with this study, we provide a list of validated reference genes and their relative primer sequences to conduct reliable qPCR experiments in maize.

Introduction

Quantitative real-time polymerase chain reaction (qPCR) has become the most commonly used method for in-depth analysis of gene expression (Wong and Medrano, 2005; VanGuilder et al., 2008) and a powerful tool for a wide range of applications in biological research (Bustin et al., 2009). Recently, to standardize qPCR experiments, guidelines were published establishing minimum information for publication of quantitative real-time PCR experiments (MIQE guidelines; Bustin et al., 2009). However, qPCR must be performed correctly to prevent pitfalls that can lead to unreliable results (Bustin and Nolan, 2004). For example, the quality and amount of starting material, the variations in RT efficiency and the occurrence of technical errors during PCR setup may dramatically affect the validity of qPCR outcomes (Vandesompele et al., 2002; Udvardi et al., 2008). In addition, the use of a suitable normalization strategy is one of the most critical points for accurate gene expression measurements (Guénin et al., 2009). Even if standardization methods for qPCR have been already developed, the normalizing of the row data to one or more reference genes is the most commonly utilized approach (Hugget et al., 2005). The selection of the optimal reference genes represents, therefore, a crucial step for achieving a reliable normalization, since an incorrect choice would lead to serious misinterpretation of results (Gutierrez et al., 2008).

To date, genes encoding transcripts involved in basic cellular process have been the most frequently used for qPCR normalization, as their expression is assumed to be constant in different organs and tissues and under a wide range of experimental conditions (Czechowski et al., 2005). However, these genes have been selected by examining their expression stability by techniques with limited sensitivity, such as visual examination of RNA gel-blots, densitometry of hybridized blots or semi-quantitative RT-PCR (Brunner et al., 2004; Guénin et al., 2009) and have been often employed for data normalization under experimental conditions different from those in which expression stability was assessed (Guénin et al., 2009). It is therefore not surprising that significant variations in the expression of the commonly used reference genes, such as *actin*, *cyclophilin*, *elongation factor-1 alpha*, *glyceraldehyde-3-phosphate dehydrogenase*, *polyubiquitin*, *tubulin* and ribosomal subunits have been reported by numerous studies (Thellin et al., 1999; Schmittgen and Zakrajsek, 2000; Stürzenbaum and Kille, 2001; Lee et al., 2002; Tricarico et al., 2002; Bas et al., 2004; Dheda

Abbreviations: *ACT*, actin; C_q, quantification cycle; *CUL*, cullin; *FPGS*, folypolyglutamate synthase; *GRP2*, glycine-rich RNA-binding protein 2; *LUG*, leunig; *MEP*, membrane protein PB1A10.07c; NTC, no template control; *PGM*, phosphoglucomutase; qPCR, quantitative real-time polymerase chain reaction; RT, reverse transcription; *TUB*, tubulin; *UBCE*, ubiquitin-conjugating enzyme; *UBCP*, ubiquitin carrier protein; *UCH*, ubiquitin carboxyl-terminal hydrolase; UTR, untranslated region.

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et al., 2004; Radonić et al., 2004; Barber et al., 2005; Czechowski et al., 2005).

It is now well established that the reference genes for data normalization should be validated in each set of experimental conditions (Reid et al., 2006; Expósito-Rodríguez et al., 2008; de Almeida et al., 2010; Migocka and Papierniak, 2010). The adoption of systematic validation of reference genes as a routine practice would allow further improvements in accuracy of qPCR analyses (Guénin et al., 2009). It should also be taken into account that the use of a single internal control is no longer satisfactory and normalization with multiple reference genes is highly desirable and should be considered as necessary (Thellin et al., 1999; Vandesompele et al., 2002; Remans et al., 2008). In this regard, statistical algorithms such as genorm^{PLUS} (Vandesompele et al., 2002), BestKeeper (Pfaffl et al., 2004) and NormFinder (Andersen et al., 2004) have been conceived to aid researchers in validating reference genes. More recently, in *Arabidopsis thaliana*, wide-spectrum transcriptomic approaches have led to the identification of novel reference genes that outperform the "classical" ones in terms of expression stability under a wide range of developmental and environmental conditions (Czechowski et al., 2005). Also, in rice and maize, results obtained from genome-wide atlas have highlighted the inadequacy of the most commonly utilized reference genes to be used as internal controls (Wang et al., 2010; Sekhon et al., 2011). In conclusion, the adoption of statistical tools together with the analysis of whole-genome data sets represents the most appropriate approach to select the best reference genes for transcript normalization (Andersen et al., 2004; Czechowski et al., 2005; Hoogewijs et al., 2008; Sekhon et al., 2011).

To date, studies aimed at evaluating the reliability of reference genes have been reported for several crop plants, such as banana (Chen et al., 2011), coffee (Barsalobres-Cavallari et al., 2009; Cruz et al., 2009), cotton (Tu et al., 2007; Artico et al., 2010), grapevine (Reid et al., 2006), potato (Nicot et al., 2005), rice (Kim et al., 2003; Jain et al., 2006; Narsai et al., 2010), soybean (Jian et al., 2008; Libault et al., 2008; Hu et al., 2009), sugarcane (Iskandar et al., 2004), sunflower (Fernandez et al., 2011), tobacco (Schmidt and Delaney, 2010), tomato (Expósito-Rodríguez et al., 2008; Løvdal and Lillo, 2009), and wheat (Paolacci et al., 2009).

Despite this, to our knowledge no investigations have been yet carried out to validate primer pairs for reference genes in maize, which represents an important crop and a model plant for monocots.

In the present study, we evaluated the performance of twelve candidate reference genes in a set of 20 maize samples obtained from a wide set of experimental conditions, in terms of tissues (root, stem and leaf), developmental stages (3/8/23-day-old plants, for seedlings grown in hydroponics, and 83 days after sowing in the case of field samples) and stress treatments. The reference genes were chosen following the recent literature, including those newly identified by microarray data analyses present in maize atlas (Lin et al., 2009; Zhuang et al., 2010; Sekhon et al., 2011). The expression stability of these genes was subsequently assessed by using genorm^{PLUS} (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) algorithms.

Materials and methods

Plant materials and treatments

Seeds of maize (*Zea mays* L.) hybrid 5783, supplied by DEKALB (Monsanto, Italy), were surface-sterilized in 5% (v/v) sodium hypochlorite for 10 min, washed in distilled water and germinated on wet filter paper at 25 °C in the dark.

After 3 days, roots and leaves were harvested from some seedlings and immediately frozen in liquid nitrogen. The remaining plants were transferred in a controlled environment chamber in 2-L tanks containing a Hoagland-modified nutrient solution, according to the presence or absence of nitrogen source (Quaggiotti et al., 2003). Nutrient solutions were changed every 2 days and the pH of the solutions ranged between 5.6 and 6.0. A day/night cycle of 14 h/10 h at 25 °C/18 °C air temperature, 70/90% relative humidity, and 280 μmol m^{-2} s^{-1} photon flux density were utilized as standard conditions, as illustrated in Table 1. In some cases, different stress treatments were imposed on maize seedlings (Table 1). All collected tissues were immediately frozen in liquid nitrogen and kept at −80 °C for subsequent RNA extraction. Twelve randomly selected plants were used per sample in each experiment. Each experiment was repeated in duplicate.

Regarding the field samples, 40 seeds were sown on 15 April 2010 at the Experimental Farm of the University of Padua (Legnaro, Italy; +45°20′53″, +11°57′10″), in a 1.5 m long and 3 m wide plot (4 rows, 0.75 m apart; 0.18 m between seeds in the row; silty-loam soil). Root samples were collected from four randomly selected plants harvested before flowering on 7 July 2010, and immediately frozen in liquid nitrogen.

RNA isolation, DNAse treatment, quality control and cDNA synthesis

Total RNA was extracted as described by Trevisan et al. (2011) starting from 250 mg of frozen tissue stored at −80 °C and using the TRIzol method as described by the manufacturer (Invitrogen, San Giuliano Milanese, Italy). An aliquot of total RNA was treated with RQ1 RNAse-free DNAse (Promega, Milano, Italy) to avoid genomic contamination as described by Falchi et al. (2010). One μl of total RNA was quantified spectrophotometrically using a Nanodrop 1000 (Thermo Scientific, Nanodrop Products, Wilmington, DE, USA). Samples having A260/A280 < 1.8 were discarded. In order to check RNA integrity and to confirm quantification, 500 ng of total RNA were run into 1% agarose gel. cDNA was synthesized starting from 500 ng of total RNA mixed with 1 μl of Oligo dT 10 μM. Reaction was incubated 5 min at 70 °C. The temperature was lowered to 37 °C and 100 U of M-MLV reverse transcriptase were added after 5 min (Promega, Milano, Italy), along with 25 U RNAse Inhibitor (Affymetrix, High Wycombe, UK) and dNTPs 10 μM each. The mixture was kept at 37 °C for 85 min more, then the temperature was raised to 94 °C for 5 min. cDNA was synthesized twice, and the two reactions were mixed and stored at −20 °C for subsequent analysis.

Choice of candidate reference genes and primer design

Candidate reference genes were chosen mainly on the basis of the maize transcription atlas reported by Sekhon et al. (2011), which identified a number of stably expressed genes (Table 2). In addition, two genes encoding an actin and a tubulin and their respective couples of primer were selected from previously published reports (Lin et al., 2009; Zhuang et al., 2010). An *18S rRNA* primer pair was also synthesized. Primers were designed with the Primer3 web tool (ver. 0.4.0; http://frodo.wi.mit.edu/primer3/; Rozen and Skaletsky, 2000), and the top results were ranked with PRaTo (http://prato.daapv.unipd.it/; Nonis et al., 2011). Where possible, to increase the specificity of amplification primers were designed in the 3′ UTR region of the gene (Table 2). BLASTn against Maize genome (http://www.maizegdb.org/) was performed to avoid multiple amplifications. *GRP2*, *UBCP*, *LUG* and *CUL* amplified two, four, one and two splicing variants, respectively (Table 2). Gene putative function, accession number, primer sequence, their position on transcript and expected size of amplicon are