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Validation of housekeeping genes for qPCR in maize during water deficit stress conditions at flowering time

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Abstract

Plant stress studies are increasingly being based on gene expression. The analysis of gene expression requires sensitive, precise, and replicable measurements for specific mRNA sequences. Real-time RT-PCR is nowadays the most sensitive method for the detection of low abundance mRNA. A stable reference gene is mandatory to obtain reliable quantitative real-time PCR (qPCR) analysis results. Real-time RT-PCR is referred with one or several internal control genes, which should not fluctuate during treatments. In this study, we have chosen eight genes as candidates of possible reference genes for maize (*Zea mays* L) during water deficit stress at flowering time: α-tubulin, 3`phosphate glyceraldehyde dehydrogenase (GAPDH), 18S ribosomal subunit, protein 13S ribosomal, actin, zein, invertase, and starch synthase IIB. The eight reference genes candidates were tested on maize plants around flowering time, under three different conditions: before water deficit (BWD), under water deficit (WD) and after water deficit (AWD). Results from the three experimental conditions indicated that protein 13S ribosomal gene was the most stable among all the reference genes tested. This result suggests that protein 13S ribosomal gene can be used as internal control (housekeeping) for qPCR analysis in maize plants under water deficit stress during flowering time.

Keywords: reference genes, water deficit stress, flowering, maize, protein 13S ribosomal

Introduction

Real-time PCR analysis has become the most common method for validating the whole-genome microarray data (Jain et al, 2009). An ideal reference gene must be expressed at constant levels across various conditions, such as developmental stages or tissue types, and its expression is always assumed to be unaffected by experimental parameters. Selection of an appropriate normalization strategy is of crucial importance for the acquisition of biological meaningful data (Tong et al, 2009). Reference genes are the most frequently used to normalize RT-qPCR data and to control the possible experimental errors, since the reference genes are exposed to the same preparation steps as the gene of interest (Kosera and Rapacz, 2013).

Grain yield of most crops is predominantly limited by water availability. The stress caused by water deficit is a major environmental constraint to plant productivity due to its detrimental effects on plant growth and development (Vörösmarty et al, 2010). According to this, drought and flowering periods are excellent as experimental models in many studies due that are critical conditions for yield of most crops (Cicchino et al, 2010).

Maize is the cereal crop with highest worldwide production and the majority of its cultivation is rainfed, with limited possibilities for alleviating water deficit stress. Maize is most susceptible to drought during flowering time, with the most severe reductions in yield occurring in the 3-week period bracketing male (anthesis) and female (silking) flowering events (Cicchino et al, 2010).

It was during the efforts to quantify the expression of the IPT gene in transgenic maize plants subjected to water stress during the flowering period, that the need for a stable housekeeping gene arrived (Décima Oneto et al, 2016).

This research shows how the stability of 8 reference genes in studies of maize gene expression at flowering time and under water deficit stress was evaluated.

Materials and Methods

Plant material and water deficit treatments

Backcrossed 2 (BC2) Hi II x BLS14 maize plants were used for the experiments. BLS14 is an inbreed line obtained in the Genetic Institute of INTA. BC2 seeds were sown and grown in soil (3:1 fertile soil:peat) using 12-liter pots under greenhouse controlled condi-

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tion (650 µmol of photons m^{-2} sec^{-1},16 h photoperiod, 30ºC-20ºC day/night). BC2 seeds were sown in speedlings and grown in a greenhouse at 16 h photoperiod and 25°C air temperature (Décima Oneto et al, 2010). We evaluated a total of 30 BC2 Hi II x BLS14 maize plants. Plants were watered daily (320 ml $plant^{-1}$ day^{-1}) to keep the soil near 100% field capacity (FC) until 70 days after germination (pre-flowering stage, one week before Vt stage). On that date they were sorted in equal number to two contrasting water regimes, the control group was irrigated daily to keep soil at 100% FC, while the other one was kept under a severe water deficit. Plants in the second group were watered to keep soil at 30% FC for ten days, and subsequently at 10% FC for another 10 days. After 20 days of water deficit (R2) the pots were watered up to FC and plants kept under normal irrigation until final harvest. Soil moisture determinations were made gravimetrically and by means of soil humidity sensors connected to dataloggers (cavadevices.com, Buenos Aires, Argentina). In the latter, hourly records of temperature and humidity inside the greenhouse were obtained in order to calculate the vapor pressure deficit (VPD) as an estimate of atmospheric demand exerted on the plants during the drought period. The VPD was computed according to Abbate et al (2004). Four TC1047 temperature sensors connected to dataloggers (cavadevices.com, Buenos Aires, Argentina) and two humidity sensors (U12-011 Hobo, Onset, Masachusetts, USA) were distributed within the greenhouse.

Total RNA extraction

RNA was extracted from leaf tissue of the BC2 generation plants. Tissue samples were taken during the three periods of water regimes: before water deficit (BWD), under water deficit (WD, R1) and after water deficit (AWD, R2). Extraction of total RNA was done with Trizol (Invitrogen, catalog # 15596-026) following the manufacturer protocol. NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, USA) was used for quantification of the final product of the extraction. Enzymatic amplifications, using EWSF and EWSR oligonucleotides:
EWSF - 5´TACTCTTGGCCTTGATATGTTCC3´; EWSR - 5´AATGCAGGCAAAGCACACAATCTCTA3´ (annealing temperature 58,5°C) were performed in order to verify the absence of DNA in the final product of RNA extractions.

Real time PCR primers design

Eight endogenous genes were selected: 1) α-tubulin; 2) 3` phosphate glyceraldehyde dehydrogenase (GAPDH); 3) 18S ribosomal subunit; 4) protein 13S ribosomal; 5) actin; 6) zein; 7) invertase; and 8) starch synthase IIB. Sequences, for the primer design, were obtained from the GenBank database. Two pairs of primers for α-tubulin, 18S ribosomal subunit, protein 13S ribosomal, actin, zein, and starch synthase IIB were designed. For 3` phosphate glyceraldehyde dehydrogenase gene one pair of primers were designed. The Beacon Designer 7.6 software (PREMIER Biosoft International) was used to design the primer. In all the cases, the primer design conditions were: 100 bp maximum length, optimal T_m at 60ºC, GC% between 20% and 80%.

Verification of amplified products

PCR product sizes were checked on a 4% MetaPhor agarose gel (Lonza, Biocompare). All corresponded to the expected size. Melting curves showed a single amplified product for all genes and the melting temperatures were in accordance to those calculated.

Analysis of transcript levels with real-time quantitative qRT- PCR

The expression pattern of the 8 endogenous genes in maize was measured using qPCR Icycler IQ Real Time Detection System (BioRad, USA). Two µg of total RNA of each sample were reverse transcribed to cDNA with Superscript III reverse transcriptase enzyme (Invitrogen, Catalog No. 18080-044) using oligo dT primers (biodynamics, Catalog No. B071-40). Real-time RT-PCR using SYBR Green technology on Icycler IQ Real Time Detection System (BioRad, USA) was performed. A master mix for each PCR run was prepared with IQ Supermix (BioRad, catalog no 170-8882). Finally, in a total volume of 25 µl, were added: 12,5 µl IQ Supermix, 8 µl of cDNA and 1 µl (5 µM) of each specific sense and anti-sense primers and 8,5 µl H_2O (HPLC quality) were added. The following amplification program was used: 95°C 5 min, 45 cycles at 95ºC for 20s followed by 60ºC for 40s. All samples were amplified in triplicate from the same RNA preparation and the mean value was considered. The real-time PCR efficiency was determined for each gene and each condition with the slope of a linear regression model. For this, each cDNA sample was bulked and then used as the PCR template in a 1, 1/5, 1/25, and 1/125 dilution. Corresponding real-time PCR efficiencies were calculated according to the equation: $E_{eficiency} = 10^{-1/slope}$ (Pfaffl et al, 2001).
for each gene, PCR efficiency was determined by measuring the C_T to a specific threshold for a serial dilution of bulked cDNA.

Data acquisition

Expression levels were determined as the number of cycles needed for the amplification to reach a threshold fixed in the exponential phase of PCR reaction (C_T) (Walker, 2002). iCycler™ iQ Optical System (BIO-RAD) was used to perform qPCR. The Cts obtained was transformed into quantities using PCR efficiencies according to Pfaffl et al (2002) in order to use REST software (Relative Expression Software Tool) (Pfaffl et al, 2002). The geNORM software was used to analyze the expression stability of tested genes in the three treatments (BWD, WD, and AWD), and ranked them accordingly. The geNORM software is a statistical algorithm to determine the most stable reference (housekeeping) genes from a set of tested candidate reference genes in a given sample panel.

From this, a gene expression normalization factor can be calculated for each sample based on the geometric mean of a user-defined number of reference genes (Vandesompele et al, 2002).

Statistical analysis

Ct values from the Icycler IQ Real Time Detection System (BioRad, USA) were analyzed. A two sample F-test was performed in order to compare two population variances.

A p-value superior to 0.05 indicated that no difference of variation of expression could be deduced.

Results

Identification of reference genes candidates

Eight reference genes were selected from the bibliographic search conducted, to be validated as reference genes under water stress conditions during flowering period assays performed on maize plants. qPCR primers were designed according the published sequences of each gene at the National Center for Biotechnology Information (NCBI). The primer sequences were selected according to the program ranking. The gene name, accession number, gene description, primer sequences, melting temperatures and amplicon length are shown in Supplementary Table 1. The expression stabilities of these eight housekeeping genes were assessed by real-time PCR in a set of maize plants. A total of 30 BC2 maize plants and their biological replicates were analyzed for the three treatments: BWD, WD, and AWD (Figure 1).

Expression stability of the reference genes candidates

geNORM software was used to analyze the expression stability of the eight tested genes at the three stages (BWD, WD, and AWD). For this assay, the primer pairs which had lower standard deviation in Ct average in the efficiency test were selected. These primers were: TUB2, STS2, ACT1, 18S2, INV2, 13S2, Z1, and GAPDH (Supplementary Table 1). The parameter considered to evaluate the stability of these genes was the average expression stability (M). This parameter indicates the average expression stability value of the reference genes at each step during stepwise exclusion of the least stable reference gene. In Figure 2 it can be observed that during the BWD period the three least stable genes ($1 > M > 0.7$) were TUB, ACT, and STS. At the WD period the three least stable genes were ACT, TUB, and STS, and finally during the AWD period ACT, TUB, and 18S were the least stable genes. However, during all the evaluated periods, the most stable genes were also three ($M < 0.5$): GAPDH, INV, and 13S (Figure 2).

These results showed that the expression of 13S gene was the most stable in maize plants during the evaluated period (one week before Vt, R1, and R2). It was for this reason that the 13S gene was selected as the amplification target for the qPCR, and the 13S2 primers were particularly selected due to their lower standard deviation in Ct average in the efficiency calculation. Taking these results into account, primer ef-

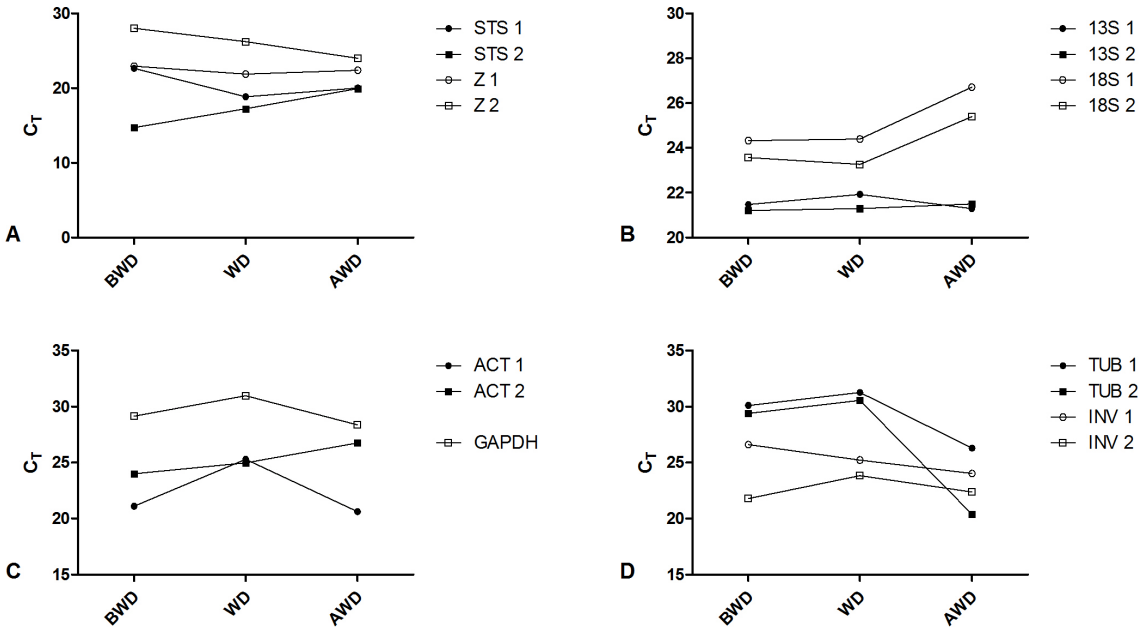

Figure 1 - RNA transcription levels of housekeeping genes tested, presented as C_T mean value in the different water conditions. BWD - before water deficit, WD - water deficit, AWD - after water deficit. **A**) STS and Z gene pair of primers 1 and 2, **B**) 13S and 18S gene pair of primers 1 and 2, **C**) ACT gene pair of primers 1 and 2; GAPDH gene pair of primers 1, **D**) TUB and INV gene pair of primers 1 and 2. n= 30 maize plants, C_T values are mean of three replicates.

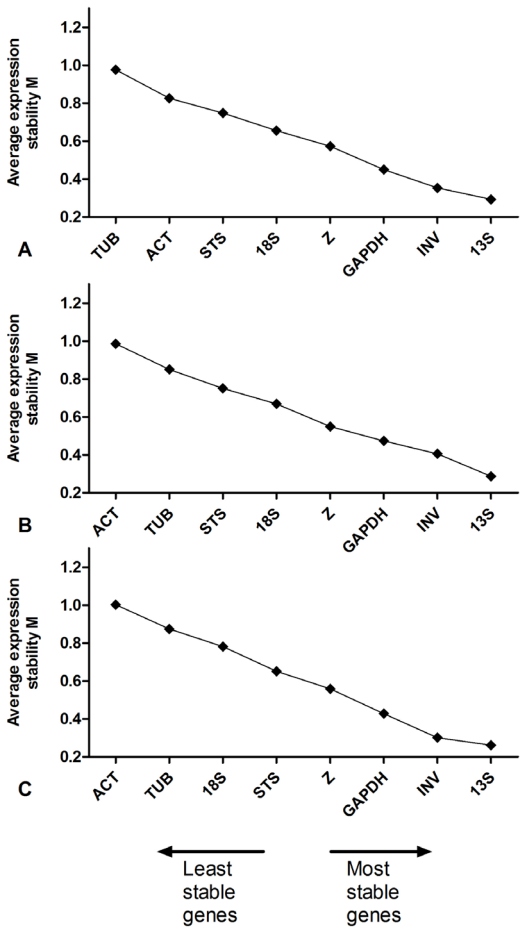

Figure 2 - Average expression stability values of candidates genes via geNorm analysis. **A**) Before water deficit (BWD) (n= 30), **B**) Water deficit stress (WD) (n= 30), **C**) After water deficit (AWD) (n= 30).

ficiency was evaluated for the set 13S2. The slope value was assessed on serials dilutions of a cDNA pool (Figure 3A). Amplification efficiency for the pair of primers 13S2 was 2.08 (108.8%) (slope -3,128) (Figure 3B).

Discussion

Quantitative RT-PCR has become a powerful tool for analysis of gene expression because of its high throughput, sensitivity, and accuracy (Lee et al, 2010). However, to get reliable results from real-time PCR analysis, an accurate validation of gene expression against a control gene is required. A reliable internal control should show minimal changes, whereas a gene of interest may change greatly over the course of an experiment (Dean et al, 2002). Thus, choosing an internal control is crucial to quantify gene expression (Nicot et al, 2005). However, no one gene has a stable expression under every experimental condition. Numerous studies reported that expression of housekeeping genes can also vary considerably with experimental conditions (Vandesompele et al, 2002). The use of one or more stably expressed reference genes to validate the variation introduced by RNA sample quality, RNA input quantity, and RT enzymatic efficiency is essential to achieving reliable results (Lee et al, 2010). To obtain a solid basis for normalization of gene expression data, it is advisable to validate the expression stability of candidate reference genes under the conditions studied.

According to bibliography, ACT, TUB, and GAPDH are the most used reference genes to test the genetic expression in maize under abiotic stress (Manoli et al, 2012). However, in this particular study, the expression of these genes was tested during flowering time together with water deficit stress conditions and too many variations were detected for it to be properly used as reference genes. Other authors, such as Migocka (2011), Manoli (2012), and Lin (2014), have observed variations in the expression of the genes GAPDH, TUB, ACT, and 18S, and suggest that they should be carefully handled as reference genes, because their transcript levels are variable under particular experimental conditions.

The lack of stability of expression of the commonly utilized reference genes was what prompted the search and validation of other reference genes for the evaluated conditions.

In this study the expression levels of eight housekeeping genes were analyzed, which were tested on maize plants during flowering time under water deficit. According to our results, 13S was the most suitable gene under all conditions (flowering and water deficit stress). The ACT, TUB, STS, and 18S genes did not satisfy the selection criteria and had an unacceptably high maximal variability.

A gene that could successfully be used as the reference gene for maze plants under both these conditions (during flowering time and under water stress) had not been reported until now.

The ACT gene was often used to normalize the quantification of expression (Thomas et al, 2003; Manoli et al, 2012). In fact, Galli et al (2013) determined that ACT was the most suitable reference gene in maize grains and GAPDH should be avoided because it performed poorly in ge-NORM program. However, the fact that these results were obtained in maize grains must be taken into account. The results obtained here, indicated that both ACT and GAPDH were some of the least stable genes under flowering and water deficit stress. Other earlier reports showed that ACT expression could be regulated in response to various factors (Tricarico et al, 2002; Dheda et al, 2004). Consequently, as it was also suggested by Gutierrez et al (2008) and Liu et al (2015), ACT may not fully satisfy basic requirements for a suitable reference gene. Indeed, many studies on different plant species (for example Arabidopsis, potato, rice, tomato, wheat, peach, cucumber, and chrysanthemum)

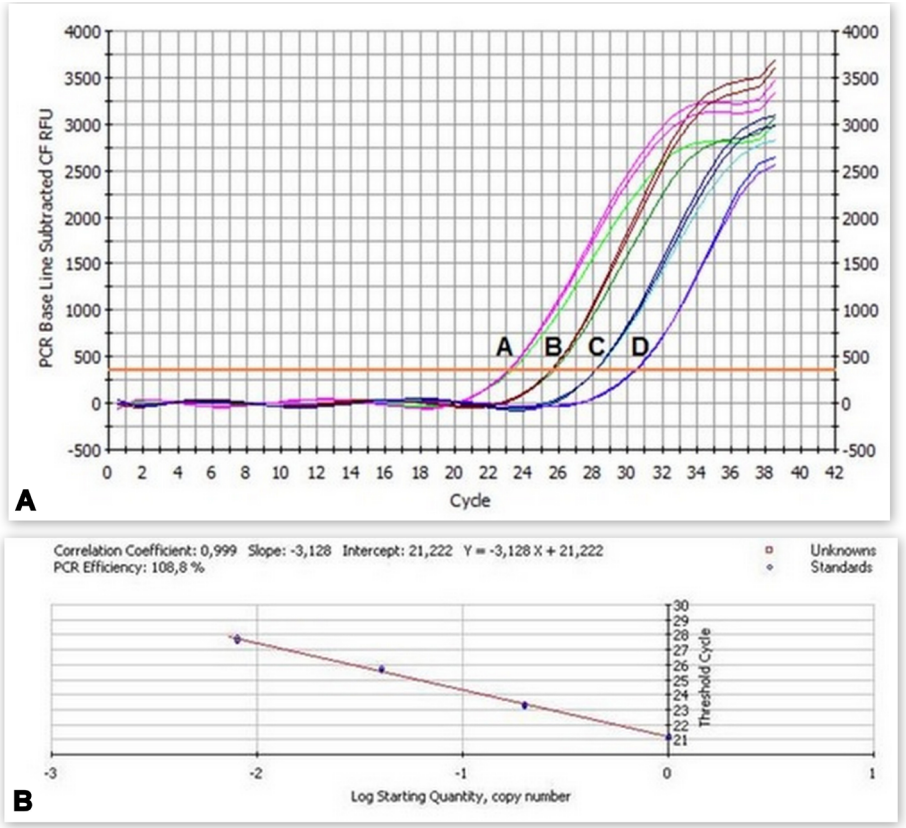

Figure 3 - **A**) Amplification of the 13S ribosomal protein gene using the primer pair 13SII. A set of cDNA maize plants at BWD, WD and AWD (n= 30) was used: A - undiluted and serial dilutions, B - 1/5, C - 1/25, and D - 1/125. The iCycler (BioRad) software were used. **B**) Primer amplification efficiency of the 13S ribosomal protein gene using the primer pair 13SII. A set of cDNA maize plants at BWD, WD and AWD (n= 30) was used. The iCycler (BioRad) software was used.

have now confirmed the unsuitability of the ACT gene for qPCR normalization (Migocka et al, 2011; Gu et al, 2011).

Particulary in maize, Manoli et al (2012) have demonstrated that the gene expression of the most frequently used reference genes, such as ACT, TUB, and 18S rRNA, was significantly less constant under different stress treatments when compared with the expression stability of some genes extrapolated from a maize transcription atlas (Sekhon et al, 2011).

The 18S ribosomal subunit is another example of commonly used internal controls that is subject to controversy (Klok et al, 2002). Thellin et al (1999) recommended the use of 18S rRNA as reference gene for mRNA quantification studies because mRNA variations were weak and could not highly modify the total RNA level. Nevertheless, there are several arguments against the use of 18S rRNA as reference gene (Nicot et al, 2005) because ribosomal subunit transcription is affected by biological factors and drugs (Vandesompele et al, 2002). Moreover, our results indicate that 18S is not the more suitable housekeeping gene for the tested conditions. Further drawbacks of the use of 18S rRNA molecule as standards are its absence in purified mRNA samples, and their high abundance compared with target mRNA transcripts. This characteristic makes the 18S gene suitable as a reference gene only in cases where the gene to be quantified is one of high expression levels. The latter makes it difficult to subtract the baseline value in real-time RT-PCR data analysis accurately (Vandesompele et al, 2002; Nicot et al, 2005).

Finally, this report shows that housekeeping genes are highly specific for a particular experimental model, and validation for each situation is a crucial requirement.

Conclusion

This is the first study on the selection and validation of reference genes for maize under water stress deficit during flowering period. According to these results, the 13S gene is recommended for validation as reference gene in studies regarding the expression of genes under the conditions described in this work. This can enable further research about the variation of the gene expression, and validate the results of large-scale gene expression analysis methods during flowering time and under water stress conditions.

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