Stable Internal Reference Genes for the Normalization of Real-Time PCR in Different Sweetpotato Cultivars Subjected to Abiotic Stress Conditions

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Abstract

Reverse transcription quantitative real-time PCR (RT-qPCR) has become one of the most widely used methods for gene expression analysis, but its successful application depends on the stability of suitable reference genes used for data normalization. In plant studies, the choice and optimal number of reference genes must be experimentally determined for the specific conditions, plant species, and cultivars. In this study, ten candidate reference genes of sweetpotato (Ipomoea batatas) were isolated and the stability of their expression was analyzed using two algorithms, geNorm and NormFinder. The samples consisted of tissues from four sweetpotato cultivars subjected to four different environmental stress treatments, i.e., cold, drought, salt and oxidative stress. The results showed that, for sweetpotato, individual reference genes or combinations thereof should be selected for use in data normalization depending on the experimental conditions and the particular cultivar. In general, the genes ARF, UBI, COX, GAP and RPL were validated as the most suitable reference gene set for every cultivar across total tested samples. Interestingly, the genes ACT and TUB, although widely used, were not the most suitable reference genes in different sweetpotato sample sets.Taken together, these results provide guidelines for reference gene(s) selection under different experimental conditions. In addition, they serve as a foundation for the more accurate and widespread use of RT-qPCR in various sweetpotato cultivars.

Introduction

To analyze the expression profile of genes of interest, comparative measurements such as microarray, Northern blot, reverse transcription-PCR (RT-PCR), and real-time RT-PCR (RT-qPCR) are frequently used [1]. Among these, RT-qPCR is the simplest, most sensitive, most precise, and cost-effective quantitative method allowing the detection of both low-abundance mRNAs and slight variations in gene expression. Thus, RT-qPCR has become the preferred approach to the validation of high-throughput or microarray results and the quantitation of gene expression [1,2]. However, there are substantial variations in RNA stability, quantity, and purity that in turn influence the efficiency of RT-qPCRs [3]. In fact, several reports have demonstrated that there is no gene set able to fulfill all the requirements of every experimental condition, and that improper reference-gene selection could yield inaccurate results [1,4,5]. Therefore, the selection of a reliable internal control is essential, as is the use of standardized experimental conditions [6–10]. Current experimental convention includes the use of a single gene for normalization; however, this may lead to relatively large errors in a significant proportion of the samples [9,11]. Alternatively, multiple internal control genes will ensure accurate normalization of the data [9,12,13]. This implies that, prior to their use in RT-qPCR normalization, potential reference genes must be systematically evaluated for their stability under the applied experimental conditions. Several algorithms, such as geNorm [14], are currently available as part of qBasePlus [15], NormFinder [16] and BestKeeper [17]. They have been developed to validate for a given set of experimental conditions the most stable reference gene(s) from a panel of potential genes or candidate genes.

Gene expression analyses under a wide range of experimental conditions have relied on the use of traditional housekeeping genes such as actin (ACT), tubulin (TUB), glyceraldehyde-3-phosphate dehydrogenase (GAP), elongation factor-1 alpha (EF1α), and 18S rRNA, for normalization of the data. However, in many cases, the transcripts expressed from these genes are unstable, such that variations in the respective expression levels can lead to a misinterpretation of the results. Recently, statistical algorithms have been used to identify the best reference genes for RT-qPCR data normalization in a given set of biological samples. These algorithms have been applied to assess the expression stability of
numerous candidate reference genes across a variety of tissues, organs, developmental stages, and stress conditions in various plant species, such as *Arabidopsis* [6,18], tobacco [19], rice [20], tomato [7], potato [21], poplar [13], cotton [12], banana [9], grapevine [22], soybean [23], coffee [24], *Brachiara* grass [25], and petunia [26].

Sweetpotato *Ipomoea batatas* (L.) Lam is, together with cassava, one of the most important commercial and nutritional root crops in Asia. It is used not only as a major food source but also as an important industrial raw material for animal feed, alcohol production and antioxidant pigment syntheses, including anthocyanins and carotenoids [27,28]. Recently, new varieties of colored sweetpotatoes, including yellow-fleshed and purple-fleshed varieties with higher carotenoid and anthocyanin contents and desirable nutritional and physiological properties, have been introduced.

From a scientific standpoint, sweetpotato is a valuable resource for studying the developmental and physiological properties of root crops, such as storage root development, sterility, and cross-incompatibility. In RT-PCR and RT-qPCR studies of sweetpotato cultivars exposed to various stress conditions, *ACT* or *TUB* usually serve as reference genes [29–31], even though their stability has not been verified. Therefore, we aimed to identify stable reference genes for use in RT-qPCR studies in sweetpotato. To this end, four cultivars of pigmented sweetpotato were exposed to four different stress conditions and the validity of the candidate genes as reference genes was subsequently validated. The identification and validation of these reference genes for use in RT-qPCR normalization will significantly improve the accuracy and reliability of gene expression studies in sweetpotato.

### Materials and Methods

#### Plant Materials and Growth Conditions

Four different sweetpotato cultivars, Yulmi (YM, heart-shaped leaves and pale-yellow flesh storage roots), Sinzami (SZM, long spade-like leaves and purple flesh storage roots), Sinhwangmi (SHM, heart-shaped leaves and orange flesh storage roots), and Whitestar (WS, lobed leaves and white flesh storage roots), were placed in a growth chamber at 25±3°C for 3 months (Figure S1). Freshly harvested leaves, petioles, stems, fibrous roots (<5 mm in diameter), and pencil roots (<15 mm in diameter) were sampled 12 weeks after planting. Mature storage roots (>15 mm in diameter) were obtained from the Bioenergy Crop Research Center, National Institute of Crop Science, Rural Development Administration (RDA), Korea. The tissues of all plant materials were immediately frozen in liquid nitrogen and stored at −70°C until further use.

#### Stress Treatment

Fully expanded mature leaves from the third and fourth vine tip of each cultivar were detached approximately 2 months after transplantation of a 10 cm-long segment of the upper part of the vine into a pot (Figure S1). For cold stress treatments, the pots were incubated at 4±1°C for 0, 2, 4, 6, 12, 24, 48 and 72 h in the dark. For drought and salt stress treatments, the petioles of detached leaves were soaked in 30% PEG and 250 mM NaCl, respectively, for 0, 2, 4, 6, 12, 24, 48 and 72 h. For oxidative stress treatments, sweetpotato leaves were treated with 400 mM H2O2 for 0, 2, 4, 6, 12 and 24 h. All treated plant materials were immediately frozen in liquid nitrogen and stored at −70°C until further use.

### Table 1. Primer sequences of selected candidate reference genes, primers, and amplicon characteristics.

| Name | GenBank accession number | Primer sequence (forward/reverse) | Amplicon length | Primer efficiency | R2   |
|------|--------------------------|-----------------------------------|-----------------|------------------|------|
| ACT  | EU250003.1               | GTTATGGTTGGGATGGGACA 199          | 95.8±3.1        | 0.995            |      |
|      |                          | GTGCCTCGGAAGAAAGGACA             |                 |                  |      |
| ARF  | JX177359                 | CTTTGGCAAGAGAGAGATGC 185         | 100±0.5         | 0.999            |      |
|      |                          | TCTTGTACCACCAACCAACA             |                 |                  |      |
| COX  | S73602.1                 | ACTGAAACAGCCAGAGAGA 159          | 99.2±1.7        | 0.998            | 1.7  |
|      |                          | ATGCAACCTTCCATGGGTTC             |                 |                  |      |
| CYC  | EF192427.1               | GGATCGAAGTCAAGGACGA 183          | 93.8±3.1        | 0.999            | 1.3  |
|      |                          | CCTACACCGTCACACACCTT             |                 |                  |      |
| GAP  | JX177362                 | ATACGTGGCGAGAAAATGG 124          | 99.8±0.7        | 0.999            | 0.7  |
|      |                          | TCAACCCCATGAGAACTCTCTC           |                 |                  |      |
| H2B1 | JX177361                 | GTGCCGGAGACAGAAAGAG 110          | 101.9±2.8       | 0.998            | 2.8  |
|      |                          | CTGTCTGGAGATTCTGTCGATG           |                 |                  |      |
| PLD  | JX177360                 | ATGGGAATACGCAGAGATGG 144         | 101.0±3.2       | 0.991            | 3.2  |
|      |                          | ATGTGACGCAAGCAGTGG               |                 |                  |      |
| RPL  | AY596742.1               | TTTGACCGAATGTCCTCTAG             | 150.2±4.7       | 0.997            | 7.4  |
|      |                          | TCTCTGATACCCACCACCACCT           |                 |                  |      |
| TUB  | BM878762.1               | TCCAAACCACCTGGTACC              | 96.8±3.1        | 0.995            | 3.1  |
|      |                          | CTCGTACATCAAGCAGCAA             |                 |                  |      |
| UBI  | JX177358                 | TGCAAAATCTGAAGAAAGAA            | 99.9±3.4        | 0.996            | 3.4  |
|      |                          | TCGATCTCTTCCGGCTTG             |                 |                  |      |

Table 1. Primer sequences of selected candidate reference genes, primers, and amplicon characteristics.
RNA Isolation, Quality Control, and cDNA Synthesis

Frozen samples were ground in liquid nitrogen using a mortar and a pestle. Total RNA was extracted with Trizol reagent (Invitrogen, USA) and then treated according to the manufacturer’s instructions with DNase I (Takara, Japan) to remove any traces of genomic DNA. The concentration and purity of the RNA were determined using a Nanodrop 1000 spectrophotometer (Thermo, USA). RNA samples with a 260/280 ratio in the range of 1.8–2.1 and a 260/230 ratio in the range of 2.3–2.6 were used for subsequent analyses. The integrity of the RNA samples was also electrophoretically assessed on a 2.0% agarose/formaldehyde gel. Two micrograms of total RNA were reverse-transcribed using the M-MLV cDNA synthesis kit (Clontech, USA) and oligo-dT primers according to the manufacturer’s instructions.

Primer Design and RT-qPCR

The sequences of ten sweetpotato reference genes were obtained from the GenBank database and from the EST database [32]. The primer pairs were designed from these sequences with the Primer3plus program (Table 1) (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Before RT-qPCR analysis, each primer pair was tested with RT-PCR to determine the size specificity of the amplicon, followed by electrophoresis on a 2% agarose gel and ethidium bromide staining. In addition, target amplicons were sequenced to confirm specificity of the PCR products. The primer specificities were further assessed by melting-curve analysis after amplification in a RT-qPCR study. A standard curve, repeated in three independent plates using a dilution series of the mixed cDNAs obtained from all tested samples as templates. Then we calculated the gene-specific PCR amplification efficiency and correlation coefficient of each gene. The primer sequences and amplicon characteristics, including Tm, length, amplification efficiency with standard deviation, and correlation coefficient, of the ten candidate reference genes are listed in Table 1. RT-qPCR analysis was carried out in 96-well plates with a CFX real-time PCR system and CFX system software (Bio-Rad, USA) using the EvaGreen-based PCR assay. Each reaction (final volume of 20 μl) contained 2 μl diluted cDNAs, 10 μl of EvaGreen PCR Master Mix (Solgent, Korea), and 1 μl of each primer. The PCR conditions were as follows: 95°C for 15 min, followed by 40 cycles of 95°C for 20 s, 60°C for 40 s, and 72°C for 20 s. The melting curves were analyzed at 65–95°C after 40 cycles. Each RT-qPCR was performed in duplicate.

Determination of Reference-gene Expression Stability

The stability of housekeeping-gene expression under different experimental conditions was determined using two statistical algorithms, geNorm and NormFinder, in a stability analysis of 10 candidate reference genes. The geNorm program examines the stability of expression as well as the optimal number of reference genes needed for normalization. It first calculates an expression stability value (M) for each gene and then the pairwise variation (V) of this gene with the others. The lowest stability value represents the gene with the most stable expression within the gene set examined. All tested genes are ranked according to their stability in the tested samples and the number of reference genes required to obtain an optimal normalization is indicated. NormFinder determines the stability of expression as well as the optimal gene or combination of genes for normalization purposes. It ranks the set of candidate normalization genes according to the stability of their expression in a given sample set under a given experimental design.

Normalization of swpa2 and IbLEA14

Expression of the stress-inducible sweetpotato marker genes IbLEA14 and swpa2 were quantified during oxidative stress conditions (400 mM H2O2) using one or two of the most stable reference genes and TUB, a gene commonly used as a
**Results**

**Isolation of Sweetpotato References Genes and Verification of their Amplicons, Primer Specificity, and PCR Amplification Efficiency**

We selected ten candidate reference genes for this study based on their use in prior gene expression experiments. These genes include β-actin (ACT), ribosomal protein L (RPL), glyceraldehyde-3-phosphate dehydrogenase (GAP), cyclophilin (CYC), α-tubulin (TUB) [8,21,33], ADP-ribosylation factor (ARF) [34], histone H2B (H2B) and ubiquitin extension protein (UBI) [13], and cytochrome c oxidase subunit Vc (COX) [35]. The expression of phospholipase D1α (PLD) in castor bean [36], Arabidopsis [37] and rice [38] suggests a maintenance role in plant cells and was thus included in the study.

Nucleotide sequences for ACT, CYC, TUB, COX, and RPL were obtained from GenBank database and those for GAP, PLD, ARF, H2B and UBI were obtained from the sweetpotato EST database [32] (Table 1). Specific amplification for each tested primer pair was confirmed by the presence of a unique peak in melting curve analysis proceeding 40 cycles of amplification (Figure S2). Furthermore, each amplicon was cloned and sequenced, matching the predicted target sequence. Sequence analysis of cloned
amplicons revealed that all sequenced amplified fragments were identical or nearly identical to the sequences used for primer design, with 1–5 bp mismatched (but the sequences of amino acids were fully identical). The RT-qPCR amplification efficiency for the ten reference genes varied from 93.8% for CYC to 105.2% for RPL; correlation coefficients ranged between 0.991 (PLD and ARF) and 0.999 (CYC and GAP) (Table 1).

Expression Profiles of the Reference Genes

Analysis of the raw expression levels across all samples identified some degree of variation among the reference genes (Figure 1). The cycle threshold values of the ten reference genes ranged from 20.33 (CYC) to 25.23 (PLD) in all tested samples. Regarding variations in the expression of individual reference genes, the highest values were obtained for RPL (11.97 cycles) and H2B (7.37 cycles), and the lowest values for COX (4.50 cycles) and CIC (4.56 cycles). Thus, none of the selected genes were consistently expressed in all samples, highlighting the importance in sweet-potato studies of identifying a suitable reference gene(s) for use in gene expression normalization under a given set of experimental conditions.

Expression Stability of Candidate Reference Genes

To find the most stably expressed genes for sweetpotato RT-qPCR normalization, the expression stability of ten candidate genes was assessed using statistical methods. Specifically, two widely applied algorithms, geNorm and NormFinder, were used to rank the stabilities of the ten genes and to determine the number of reference genes necessary for accurate gene expression profiling under the experimental conditions selected for this study.

The results obtained with these algorithms are presented in Figure 2 and summarized in Table S1. Among the ten candidate reference genes examined when all samples were considered, geNorm identified ARF (M = 0.669) as the most stable and RPL (M = 1.262) as the least consistently expressed. In contrast, H2B and PLD (0.192) were determined by NormFinder to be the most stable reference genes, whereas RPL (1.152) was again ranked as the most variable (Figure 2F and Table S1). Pairwise variation (\(V_{n/n+1}\)) analysis showed the need for an optimal number of reference genes to obtain a more reliable normalization according to geNorm (\(V_{n/n+1}\#0.15\)), with the genes ARF, UBI, COX, and GAP (\(V_{5/6} = 0.134\)) suitable for proper normalization (Figure 3).

UBI was the most stable gene in geNorm, while in plants under cold stress, H2B was considered by NormFinder as the most stable (Figure 2A and Table S1). A stable combination of two genes according to NormFinder was ACT/ARF. In contrast, COX was identified as the least stable gene both in geNorm and in NormFinder. Analysis of the pairwise variation revealed that the UBI/ARF/H2B/ACT genes (\(V_{4/5} = 0.138\)) are sufficient for normalizing gene expression (Figure 3). These genes were considered the most variable reference genes in most cultivars according to both algorithms, whereas for the WS cultivar,

| Table 2. Optimal number of stability gene(s) estimated by geNorm and Normfinder under abiotic stresses and in the tissue of each cultivar and all cultivars. |
|---|
| **Cultivar** | **Samples** | **geNorm** | **Normfinder** | **Cultivar** | **Samples** | **geNorm** | **Normfinder** |
| YM | Tissue | UBI, ACT | ARF | SHM | Tissue | COX, TUB | ARF |
| Cold | UBI, ARF | UBI | Cold | H2B, UBI | Cold | H2B, UBI | ARF |
| Oxidative | PLD, RPL | ARF | Oxidative | COX, GAP | ARF |
| Salt | UBI, ARF | ARF | Salt | H2B, ARF | ARF |
| Drought | GAP, ARF | GAP | Drought | ARF, GAP | UBI |
| All stresses | ARF, UBI, COX, CYC | UBI | All stress | ARF, UBI | UBI |
| SZM | Tissue | PLD, RPL | GAP | WS | Tissue | COX, UBI | ARF |
| Cold | UBI, ARF | H2B | Cold | PLD, RPL | H2B |
| Oxidative | PLD, RPL, ARF | ARF | Oxidative | ARF, COX | ARF |
| Salt | COX, PLD | COX | Salt | ARF COX, UBI | ARF |
| Drought | TUB, UBI | GAP | Drought | COX, H2B | PLD |
| All stresses | ARF, UBI | GAP | All stress | COX, UBI, H2B, ARF | PLD |

| Cultivar | **Samples** | **geNorm** | **NormFinder** | **NormFinder best pair** |
|---|
| All cultivars | Tissue | PLD, RPL, ARF, COX | ARF | ARF, COX |
| Cold | UBI, ARF, H2B, ACT | H2B | ACT, ARF |
| Oxidative | RPL, PLD, H2B, ARF | ARF | CYC, GAP |
| Salt | ARF, COX, UBI | ARF | ARF, UBI |
| Drought | COX, UBI, PLD | PLD | CYC, PLD |
| All stresses | UBI, COX, ARF, H2B | H2B | ARF, COX |
| Total | ARF, UBI, COX, GAP, PLD | H2B | PLD, UBI |

doi:10.1371/journal.pone.0051502.t002
geNorm identified only PLD and RPL as reference genes (Table 1, 2 and Figure 2).

During oxidative stress conditions, elicited by 400 mM H2O2, RPL was selected by geNorm as the most stable gene among all reference genes, whereas ARF, followed by RPL, was the most stable in NormFinder (Figure 3B and Table S1). TUB was identified by geNorm as the least stable gene, whereas GAB was the most stable in NormFinder. A combination of four reference genes, RPL/PLD/H2B/ARF, comprised the optimum and most stable gene set under oxidative stress, according to geNorm (V4/5 = 0.131). While ARF was the most stable gene in every cultivar according to NormFinder (Table S1), geNorm identified different gene sets in cultivars SHM and WS (COX/GAP and ARF/COX, respectively) (Table 2 and Figure S3).

ARF was calculated by both geNorm and NormFinder to be the most stable gene under salt stress, followed by COX or UBI (Figure 2C). The two-gene combination according to NormFinder comprised ARF and UBI. Analysis of the pairwise variation revealed ARF/COX/UBI (V3/4 = 0.125) as the suitable reference genes in geNorm (Figure 3). ACT was the least stable gene in geNorm and GAB in NormFinder. Two cultivars, SHM and SZM, differed in their reference gene sets (H2B/ARF and COX/PLD, respectively) in geNorm, whereas in other cultivars, the gene set ARF/COX/UBI was identified as the most suitable (Table 2 and Figure S3).

Under conditions of drought stress, COX was calculated to be the most stable gene in geNorm and PLD in NormFinder (Figure 2D). The least stable gene under drought stress was RPL in geNorm, while in NormFinder it was ACT. For all PEG-treated cultivars, even though PLD/UBI/COX constituted the most appropriate reference gene set (V3/4 = 0.122), the values for each cultivar differed (Figure 3 and Table 2). According to geNorm, in cultivars YM and SHM the optimal combination was GAP/ARF, while in cultivars SZM and WS it was TUB/UBI and COX/H2B, respectively. In NormFinder, the best gene was GAP in cultivars YM and SZM, while UBI/PLD were the most stable genes in SHM and WS (Table 2, S1).

Analysis of the best reference genes in each experimental subset showed several differences (Table 2). Six different tissues, leaf, petiole, stem, fibrous root, pencil root and storage root, were analyzed. In geNorm, PLD was ranked as the most stable reference gene followed by RPL and ARF, whereas ARF was the most stable gene according to NormFinder (Figure 2E). In contrast, H2B was identified as the least stable gene by both geNorm and NormFinder. The two-gene combination in NormFinder was ARF/COX. Analysis of the pairwise variation revealed that the PLD/RPL/ARF/COX genes (V4/5 = 0.127) are sufficient for normalizing gene expression (Figure 3). These genes were considered by both algorithms as the most variable reference genes. ACT/TUB (YM), COX/TUB (SHM), COX/UBI (WS) and PLD/RPL (SZM) were identified by geNorm as the most stable gene set, while according to NormFinder, ARF ranked highest in terms of stability in every cultivar except SZM (GAP) (Figure 2 and Table2, S1). The stable gene sets specific to each cultivar under different stress conditions are presented in Table 2. A comparison of the results obtained with geNorm and NormFinder identified ARF/UBI/COX/PLD/GAP as the most stable reference gene combination for all samples and subsets tested in this study, thus supporting its use as reference gene sets for accurate transcript normalization in sweetpotato.

Reference Gene Validation
To demonstrate the usefulness of the validated candidate reference genes in RT-qPCR, the relative expression levels of
two sweetpotato marker genes, \textit{IbLEA14} and \textit{swpa2}, already reported to be stress-inducible [31,39] were investigated under oxidative stress conditions (400 mM H\textsubscript{2}O\textsubscript{2}), together with one or two of the most stable reference genes, validated by geNorm and NormFinder (Figure 2 and Table S1), as well as \textit{TUB}, a gene commonly used to normalize expression (Figure 4). The results showed that, for normalization, the expression levels of two of the chosen stable reference genes, \textit{ARF}/\textit{COX}, underwent similar changes, while slight differences were observed when \textit{ARF} was used alone. In response to H\textsubscript{2}O\textsubscript{2} treatment, \textit{IbLEA14} expression increased progressively for 6 h and then decreased between 12 and 24 h, whereas \textit{swpa2} expression increased gradually until 24 h. Although these changing expression patterns were confirmed when normalized using \textit{TUB}, the expression levels of the examined reference genes suggested a completely different result, in that the two genes were initially expressed at extremely high levels followed, after 6 h of stress treatment, by a sharp decrease. This variation illustrates the adverse consequences of using an unsuitable reference gene for normalization.

**Discussion**

In this study, the results identified \textit{ARF}, \textit{UBI}, \textit{COX}, \textit{GAP} and \textit{PLD} as the most stably expressed reference genes in all samples and subsets studied in sweetpotato (Figure 2 and Table 2, S1). This
observation reinforces the necessity to assay the stability of expression of candidate genes to select suitable reference genes, allowing a reliable normalization in a specific biological assay. In addition, in sweetpotato cultivars subjected to various abiotic stress conditions, ARF and UBI were shown to be good candidate reference genes (Table 2). Consistent with our results, Carvalho et al. [34] reported that ARF and EF-1a yielded the most stable transcript accumulation in *Svingle citrumelo* subjected to drought stress. In the adventitious or lateral root development of poplar, the most stable reference gene set consisted of *UBI* and *RPL* [13]. Therefore, present study is the first to identify stable internal reference genes in different sweetpotato cultivars.

There is increasing evidence that a single reference gene cannot be used to accurately normalize RT-qPCR data; rather, a combination of multiple reference genes is needed. However, although increasing the number of reference genes for normalization will improve the accuracy of the analysis, this is expensive as well as time-consuming. It also has been suggested that the number of reference genes that must be analyzed is dependent on the purpose of the study [9,40]. The use of two stable reference genes provides a valid normalization for most experimental conditions [9]. This was confirmed in the present study, in which for most sample sets, two genes were shown to be sufficient to obtain a more accurate and more reliable normalization than achieved with the use of a single reference gene (Figure 3 and Figure S3). In addition, our results showed that the choice of the best combination of reference genes depends on the experimental conditions. Interestingly, the best reference genes differed for the various samples (Figure 3 and Table 2, S1). For example, in samples obtained from six different tissues, *ACT* and *TUB* ranked as a stable gene set in cultivar YM, whereas in the cultivars SHM, SZM, and WS, the gene sets *COX/TUB*, *PLD/RPL*, and *COX/UBI*, respectively, were better. Under drought stress, *GAP* and *ARF* were the best reference genes in YM and SHM, but when these two cultivars were placed under oxidative stress, *PLD/RPL* and *COX/GAP* were the most stable genes. Finally, *UBI* was the highest ranked reference gene for all stresses in all cultivars, whereas *UBI* and *H2B* appeared to be the least stable reference genes in all tissues, except those of cultivar WS (*H2B/GAP*). These results provide further support for both the need to use a specific set of reference genes and the importance of validating those genes for each experimental condition tested, especially in greatly differing samples. Findings similar to ours have been reported for banana [9], citrus [40], and tomato [7].

For the purpose of experimental data analysis, this study evaluated candidate genes in terms of their expression stability, using statistical methods to rank gene stabilities and to determine the number of reference genes necessary for accurate gene expression profiling under specific experimental conditions. The most widely used algorithms, geNorm, NormFinder or Bestkeeper were selected for the analysis. Some studies in which both geNorm and NormFinder were applied reported minor differences in the gene stability rankings [4,9,12], while in studies of citrus and wheat, the differences were substantial [40,41]. In the present work, the two methods yielded slightly different rankings (Table S1). Because the Bestkeeper algorithm can process 100 samples, whereas our study was based on 144 samples, ruling out the use of this software herein, NormFinder employs a model-based variance estimation approach to identify genes suitable for normalization. In practice, it estimates both the intra- and intergroup variation and combines them into a stability value. This model-based approach ranks the top genes with minimal estimated inter- and intra-group variation. By contrast, the pairwise approach of geNorm selects two genes with the highest degree of expression profile similarity and the lowest intra-group variation. It is therefore not surprising that the two algorithms differed in their rankings of the best candidate genes. Nonetheless, regardless of such differences in the ranking order, the most unstable gene was almost always the same in all sample sets, as also observed in other studies [4,9,12].

In summary, this study is the first attempt to validate a set of candidate reference genes in different cultivars of sweetpotato for the normalization of gene expression analysis using RT-qPCR. Our results suggest that, for normalization, different suitable reference genes or combinations thereof should be selected based on the given experimental conditions and the particular cultivars. The results of the analysis of *IbLEA1* and *sap1* expression emphasized the importance of validating reference genes to achieve accurate RT-qPCR results. These findings provide a foundation for the more accurate and widespread use of RT-qPCR in the analysis of gene expression in sweetpotato.

**Supporting Information**

**Figure S1** Leaves, storage roots, and effects of stress conditions in four different sweetpotato cultivars. (A) Leaves and storage roots of different colored sweetpotato cultivars. (B) Visible damage under cold, salt, drought and oxidative stress conditions. YM (Yulmi), SZM (Sinzami), Sinhwangmi (SHM), and WS (Whitestar).

**Figure S2** Melting curves of tested reference genes: For each reference gene melting curves were analyzed to verify the presence of a single product. (A) Actin, (B) Cyclophilin, (C) Glyceraldehyde-3-phosphate dehydrogenase, (D) phospholipid-acyltransferase D1a, (E) a-tubulin, (F) ADP-ribosylation factor, (G) cytochrome c oxidase subunit Vc, (H) histone H2B, (I) ribosomal protein L, (J) ubiquitin extension protein.

**Figure S3** Pairwise variation (V) analysis of the candidate reference genes. The pairwise variation (Vn/Vn+1) was analyzed between the normalization factors NFn and NFn+1 by the geNorm software to determine the optimal number of reference genes required for RT-qPCR data normalization. *Arrow* indicates the optimal number of genes for normalization in each sample sets A) YM, B) SHM, C) SZM D) WS

**Table S1** The ten candidate genes for normalization and their expression stability values in various sample pools as calculated by geNorm or Normfinder.

**Author Contributions**

Conceived and designed the experiments: SSK SCP HSL. Performed the experiments: SCP YHK CYJ. Analyzed the data: SCP JCJ HSL. Contributed reagents/materials/analysis tools: SSK SCP HSL. Wrote the paper: SSK SCP YHK HSL.

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Sweetpotato Reference Genes