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Identification and Validation of Reference Genes for RT-qPCR Normalization in Shoots and Roots of *Phragmites australis* Exposed to Copper Stress

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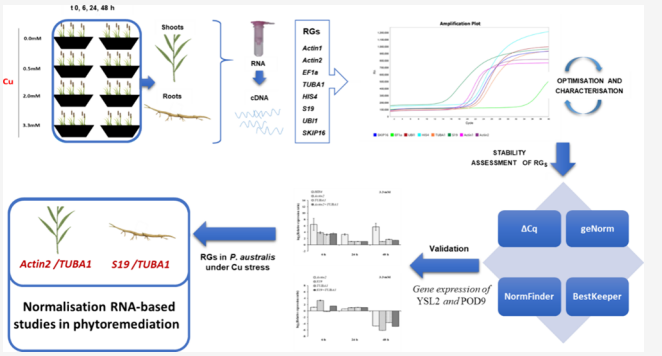
ABSTRACT: The molecular mechanism involved in *Phragmites australis* copper (Cu) phytoremediation remains unclear. This study aimed to identify and validate suitable reference genes (RGs) for RT-qPCR analysis of *P. australis* exposed to Cu stress. The expression stability of eight candidate RGs was evaluated in shoots and roots at different Cu concentrations and exposure times using Δ*Cq*, geNorm, NormFinder, and BestKeeper algorithms. The results revealed that *S19* was the most stable RG in roots but one of the least stable in shoots, while *Actin2* showed the highest stability in shoots, highlighting the necessity of validating tissue-specific RGs. The optimal RG combinations for shoots and roots were *Actin2/TUBA1* and *S19/TUBA1*, respectively. Validation using the target genes *POD9* and *YSL2* confirmed the reliability of the selected RGs. This study is the first to validate RGs in *P. australis* under Cu stress, providing a foundation for further molecular studies in the context of phytoremediation.



KEYWORDS: *copper stress, Phragmites australis, reference gene, RT-qPCR, roots, shoots*

1. INTRODUCTION

The development of agriculture, industry, and urbanization is closely linked to heavy metal pollution, which poses a significant threat to human health and ecosystem stability.[1] Copper (Cu) is a heavy metal commonly found in wastewater that can become potentially toxic at high concentrations, disrupting cellular functions and causing harmful effects in aquatic ecosystems.[2] Therefore, it is important to develop low-cost, feasible, and sustainable wastewater disposal technologies.[3] In this context, in situ remediation technologies like phytoremediation constitute an effective approach for reducing metal toxicity in contaminated environments.[4,5] Among the macrophytes employed in phytoremediation, *Phragmites australis* is particularly notable for its widespread use in treating wastewater contaminated with heavy metals.[6,7]

P. australis, also known as common reed, harbors useful characteristics for phytoremediation, such as its hyperaccumulative character, excessive capacity to grow in various habitats, and extensive root systems, and can be used in the production of bioenergy after the absorption of environmental pollutants.[9] *P. australis* has been shown to take up Cu and store it in the roots at high concentrations.[4,5,10] Nevertheless, although several studies have indicated that the root of *P. australis* is the tissue with the highest Cu accumulation,[10] other studies have detected, to a lesser extent, significant amounts of Cu in stems and leaves.[8,11,12]

Understanding the molecular mechanisms underlying Cu tolerance is crucial for optimizing the efficiency of phytoremediation. Gene expression analyses provide a powerful means of identifying pathways involved in metal uptake, transport, and detoxification. These analyses can be conducted using techniques such as RT-qPCR, RNA sequencing, or microarrays.[13] Since each tissue exhibits a distinct transcriptional profile under environmental conditions, transcript analyses should be performed specifically in tissues with bioaccumulative capacity, such as roots and shoots. Identifying *P. australis* genes associated with tolerance to different Cu concentrations in these tissues can provide valuable insights into metal stress adaptation,

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as has been achieved for other abiotic stresses, such as salinity,[14] CuO nanoparticles, and flood stress.[15] RT-qPCR is one of the most widely used techniques for analyzing gene expression due to its sensitivity and specificity.[16] However, the reliable normalization of RT-qPCR data requires the use of appropriate reference genes (RGs).[17] Notably, the expression of commonly used RGs is not inherently stable and can vary depending on the tissue type, developmental stage, and experimental conditions.[18,19] Therefore, the systematic validation of candidate RGs in different tissues and under specific stress conditions is essential to ensure the accuracy of gene expression analysis.[20] To this end, multiple statistical algorithms, including geNorm,[21] NormFinder,[22] Best-Keeper,[23] and Reffinder,[24] are commonly employed to comprehensively evaluate expression stability.

Conventionally, RGs have been selected from those involved in essential cellular functions since they are assumed to be stably expressed across developmental stages, tissues, biological conditions, and experimental treatments.[25] Accordingly, the RGs, most frequently used in plants, subject to stress by heavy metals include *glyceraldehyde-3-phosphate dehydrogenase* (*GADPH*), *actin* (*ACT*), *tubulin* (*TUB*), *18S ribosomal RNA*, *elongation factor 1-α* (*EF-1α*), and *polyubiquitin* (*UBQ*).[16,26] However, recent studies have indicated that the stability of these RGs is often affected by the type of heavy metal stress, plant species, and tissue analyzed. For instance, although the *RtTUB* gene was identified as the most stable RG in leaves of *Rheum tanguticum* exposed to heavy metal, *RtUBC* (*UBC: ubiquitin-conjugating* enzyme) was the most stable gene in roots and stems.[27] Similarly, Su et al.[28] reported that under cadmium (Cd) stress in seedling of *Iris domestica, UBC9* and *UBC28* were the most stable RGs in leaves and roots, respectively, whereas *GADPH* showed the lowest stability. In *Rumex patientia*, under Cd treatment, *SKD1* (*suppressor of K* $^{+}$*transport growth defect 1*) was the most stable in all tissues.[29] Similar patterns of variability have been reported under Cu stress. For example, *RBD2* (*ribosomal binding protein 2*) was identified as the most stable gene under Cu stress in dayflower, while *ALDH113* (*aldehyde dehydrogenase 113*) and *TUB* showed the greatest stability in leaves and stems, respectively.[30] In the case of *Sedum alfredii*, the most stable RGs under Cu stress were *ACT2* and *APT* (*adenine phosphoribosyltransferase*), while under lead (Pb) stress, they were *UBC9* and *TUB*.[31] For tea plants, the most stable genes under Cu were *EF-1α* and *eIF-4α* (*eukaryotic translation initiation factor 4α–1*), differing from those most stable for manganese (Mn) and aluminum (Al).[32] These findings demonstrate that exposure to heavy metal stress can affect the stability of commonly used RGs. This underlines the importance of investigating the impact of metals on different tissues. However, despite *P. australis* being widely used in phytoremediation, no study has yet examined the stability of RGs under Cu stress conditions in roots and shoots.

Therefore, this study focuses on selecting and validating suitable RGs for RT-qPCR analyses in *P. australis* subjected to Cu stress. Specifically, the study aims to evaluate the expression stability of candidate RGs in the roots and shoots exposed to different Cu concentrations while taking into account the tissue-specific transcriptional responses associated with metal uptake and redistribution. To this end, eight candidate RGs (*UBI1-ubiquitin-conjugating enzyme E2, SKIP16-F-box protein, S19-ribosomal protein, TUBA1-tubulin alpha-1 chain, Actin1, Actin2, EF1a-elongation factor 1-alpha, and HIS4-histone H4*) were evaluated using the ΔCq (delta quantification cycle) method, geNorm, NormFinder, and BestKeeper. In addition, the expression of stress-responsive genes, *POD9* (*peroxidase 9*) and *YSL2* (*metal-nicotianamine transporter YSL2*), was analyzed to validate the suitability of the selected RGs.

2. MATERIALS AND METHODS

2.1. Plant Material and Growth Conditions

Seeds of *P. australis* were provided by Viveros la Dehesa (Spain). The full and uniform seeds were surface-disinfected according to Podlipná et al.,[33] with slight modifications. Initially, seeds were submerged in 70% ethanol (v/v) for 1 min. Seeds were then exposed to 1% sodium hypochlorite containing 0.02% Tween-20 for 10 min and rinsed with sterile distilled H_2O until foam disappeared. Following disinfection, five to six seeds of *P. australis* were grown under in vitro conditions in glass jars containing Murashige and Skoog medium with vitamins (Duchefa Biocehmie BV, Haarlem, The Netherlands) at 4.4 and 20 g L^{-1} sucrose and Bacto-agar at 1.5%, adjusting pH to 5.6–5.9. The jars were incubated in a growth chamber (Test Chamber MLR-352H, Panasonic, Osaka, Japan) under controlled conditions for optimal growth (30/18 °C day/night temperature and 16/8 h day/night photoperiod).

2.2. Copper Stress Treatment

One-month-old homogeneous *P. australis* seedlings were transplanted into 2.5 L pots containing autoclaved tap water and acclimatized for 10 days under the same controlled conditions described above. Each pot contained three bucket compartments fixed in a Forexpan support, with two seedlings in each compartment. After acclimatization, the seedlings were subjected to Cu stress treatments by replacing the tap water with $CuSO_4$ solutions at concentrations of 0, 0.5, 2.0, and 3.3 mM. These concentrations were selected to reflect Cu levels encountered in contaminated groundwater systems studied within the European bioremediation project BIOSYSMO (BIOremediation systems exploiting SYnergieS for Improved removal of mixed pollutants, GA 101060211). Plants were exposed to each concentration for 0, 6, 24, and 48 h. At each sampling time, the shoots and roots were harvested separately and immediately frozen in liquid nitrogen and stored at −80 °C. For each experimental condition, plant material from three individual seedlings was pooled and processed as a single sample in a tube.

2.3. RNA Isolation and cDNA Synthesis

Total RNA was extracted from each sample using the RNeasy Plan Mini Kit (QIAGEN NV, Venl, The Netherlands), following the manufacturer's recommendations, using RLT buffer. The final elution of total RNA was performed in 50 μL of RNase-free water. The quantity (RNA yield based on A260) and quality (A260/280 ratio) of extracted RNA were assessed using a NanoDrop 1000 UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA was effectively extracted from the roots and shoots in terms of quantity (60.02 ± 29.67 μg mL^{1} and 334.58 ± 102.79 μg mL^{-1}, respectively) and quality (A260/280: 2.03 ± 0.11 and 2.08 ± 0.07, respectively).

First-strand cDNA was synthesized from total RNA according to the protocol described by the manufacturer's M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using oligo (dT)20 (EURx, Gdansk, Poland) with a few modifications. The volume for each reaction (20 μL) contained 1 μg of total RNA, 4.25 μM oligo (dT)20, 0.5 mM dNTPs, 10 mM DTT, 2 U μL^{-1} RNase OUT recombinant ribonuclease inhibitor (EURx), 1× First-Strand buffer, 10 U μL^{-1} M-MLV reverse transcriptase, and RNase-free water. The cDNA was stored at −20 °C until use.

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2.4. RG Selection and Primer Design

Eight candidate genes from *P. australis* were selected as potential RGs for this investigation: *UBI1*, *SKIP16*, *S19*, *TUBA1*, *Actin1*, *Actin2*, *EF1a*, and *HIS4*. The selection was based on the identity and similarity between the annotated gene sequences and the nucleotide sequences of *P. australis* available at the time the primers were designed (Table S1). The sequences of the RGs were checked after the genome annotation process was completed and published.[34] For the sequence selection, the NCBI database and the Nucleotide BLAST tool were used to identify and align homologous sequences between species. Those with high similarity, greater than 90%, were selected. The primers were designed using the NCBI Primer-BLAST tool. All the primers were synthesized by Metabion International AG (Planegg, Germany). Primer sequences, GenBank accession, gene name and description, and amplicon length are detailed in Table 1.

2.5. qPCR Analysis

Prior to qPCR analysis, PCR conditions were optimized to maximize yield and to ensure assay reliability. Specifically, primer concentrations of 100, 250, and 500 nM were analyzed after determining the optimal hybridization temperature in the range of 55–62 °C.

All assays were performed on the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). All RT-qPCR reactions were performed in triplicate and included no-template controls (NTCs). The final reaction volume was 20 μL, including 4 μL of a 1:5 dilution of the previously synthesized cDNA, 500 nM primer concentration (except for *UBI1* and *TUBA1*, which used 250 nM), and 10 μL of 2× SG qPCR Master Mix (EURx). The reaction conditions were as follows: an initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 58–62 °C (depending on the primer pair, Table 1) for 30 s. The dissociation curve was performed from 60 to 95 °C at 0.5 °C increments.

A standard curve generated using serial fivefold dilutions (100–0.0064 ng) of cDNA was employed to calculate the amplification efficiency ($E = 10^{(-1/\text{slope})} - 1$), the limit of detection (LOD = 3.3 × standard deviation/slope), and the limit of quantification (LOQ = 10 standard deviation/slope). The goodness of fit was determined using the coefficient of determination (R^2).

2.6. Stability Assessment of Candidate RGs

The expression stability and ranking of candidate genes were assessed by four algorithms: the ΔCq method (ΔCq),[35] geNorm (https://genorm.cmgg.be),[21] NormFinder (https://moma.dk/normfinder-software),[22] and BestKeeper (https://www.gene-quantification.de/bestkeeper.html).[23] Raw *Cq* values were employed for the BestKeeper algorithm and the ΔCq method. For geNorm and NormFinder analyses, the *Cq* values were converted to relative quantities (RQ) as follows:

$$\text{RQ} = E^{-\Delta Cq} \tag{1}$$

where *E* represents the PCR efficiency, as described above, and ΔCq for each gene is the difference between the lowest *Cq* and the sample *Cq* value.[21]

The ΔCq method compares the relative expressions of all possible "gene pairs" within each sample. The stability of the RGs was ranked based on the repeatability of gene expression differences between samples.[36] The geNorm algorithm calculates the average expression stability (*M*), which is defined as the average pairwise variation in a given gene compared with all other potential RGs. NormFinder software determines the stability value (SV) for all candidate RGs evaluated. Candidate genes with minimal intra- and inter-group variations exhibit the lowest SV and are ranked first.[22] In this study, group identifiers were incorporated to distinguish between the expression data at different Cu concentrations. BestKeeper, an Excel-based tool, generates a pairwise correlation coefficient (*r*) between each gene and the BestKeeper index (geometric mean of the *Cq* values of all RGs grouped together) and calculates the standard deviation (SD) and coefficient of variance (CV) for the *Cq* values of all individual candidate RGs.[23] Stable RGs had *r* values closer to 1, whereas lower SD and CV values indicated higher stability. In this study, the RGs were ranked according to each of the three criteria (*r*, SD, and CV) and the geometric mean of the individual rankings was calculated to obtain a final ranking of the stability of the candidate genes tested.[37,38] To select the most reliable RGs for RT-qPCR analysis, seven RGs were ranked based on the geometric mean of the stability values obtained using the four software tools.[38]

2.7. Validation of Selected RGs

To confirm the expression of the two most stable RGs and the least stable RG in shoots and roots exposed to different concentrations of Cu, genes responsive to heavy metal stress, specifically *POD9* (*peroxidase 9*) and *YSL2* (*metal-nicotianamine transporter YSL2*) (Table 1), were chosen from the study by Shaheen et al.[39] The authors described the antioxidant (*POD*) and transporter (*YSL*) genes as molecular factors contributing to the giant reed's tolerance to copper (Cu) stress. RT-qPCR assays were performed on the RNA obtained after Cu stress treatment, using the same methodology as for the RGs. All samples were analyzed in triplicate for each Cu concentration and exposure time. Shoots and roots were assessed independently. The relative expression ratio of these selected genes was calculated using eq 2[40] or eq 3,[21] depending on whether one or more RGs were considered in the normalization.

$$\text{Relative expression ratio} = \frac{E_{\text{target}}^{\Delta Cq_{\text{target}}(\text{control}-\text{sample})}}{E_{\text{RG}}^{\Delta Cq_{\text{RG}}(\text{control}-\text{sample})}} \tag{2}$$

$$\text{Relative expression ratio} = \frac{E_{\text{target}}^{\Delta Cq_{\text{target}}(\text{control}-\text{sample})}}{\sqrt[f]{\prod_{0}^{f} E_{\text{RG}_0}^{\Delta Cq_{\text{RG}_0}(\text{control}-\text{sample})}}} \tag{3}$$

2.8. Adherence to MIQE Guidelines

To ensure the reliable and unambiguous interpretation of the RT-qPCR data presented in this research, essential experimental information has been reported in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.[35] Compliance with these guidelines is detailed in the Supplemental Table S2.

3. RESULTS

3.1. PCR Amplification Specificity and Efficiency for Candidate RGs

The melting curves of the eight candidate RGs showed unique and characteristic peaks, which demonstrated the specificity of the systems (Figure S1). The *Cq* values ranged between 17.6 and 21.6, with the exception of *EF1a*, which showed higher *Cq* values (33.7). All systems were robust over the range tested (55–62 °C), with variations of less than 1 *Cq* (UBI1) or even less than 0.5 *Cq* between temperatures (TUBA1, HIS4, Actin2). The highest annealing temperature at which the amplification curves showed the highest reproducibility and specificity was selected (Table 1). After establishing the optimal temperature, the effect of primer concentration was analyzed. The optimal concentrations obtained were 500 nM for the S19, SKIP19, HIS4, Actin1, Actin2, and EF1α systems and 250 nM for UBI1 and TUBA1.

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Table 1. Primer Sequences and Amplification Features of the Candidate Reference Genes of *P. australis* Used in This Study

gene name	gen description	GenBank accesion	primer sequence[a](5'–3')	T_a[b](°C)	length[c](bp)	efficiency (%)	R^2
UBI1	*ubiquitin-conjugating enzyme E2 2*	XM_062351582	F: TTCCCTTGAGACCTGACGATG R: GCATCAACCGCTTGAAATCCC	62	70	101.1	0.9962
S19	*psbA-trnH intergenic spacer and ribosomal protein S19*	KP711178	F: TGAAATGCCGAGTAGGCACG R: TTCTACCCGCAATGGTTGGC	58	123	101.4	0.9999
SKIP16	*F-box protein SKIP16*	XM_062342163	F: GCAAAGTTGCTGTGTCTGGG R: TGGCCCATTTACTGGTTGCT	58	119	103.1	0.9998
TUBA1	*tubulin alpha-1 chain*	XM_062358745	F: CGTCTTCGTACTCGCCTCTC R: TCATGGTGTCGTCGAAAGGG	62	98	103.1	0.9993
HIS4	*histone H4*	XM_062363945	F: CGAGCTATGGTTCGACTCCC R: TTGCTAGCCGACCGGAATTA	62	80	100.5	0.9949
Actin1	*actin mRNA*	KP233997	F: GATGATGCTCCAAGGGCTGT R: CTGGGCCTCATCACCAACAT	58	108	101.3	0.9989
Actin2	*actin-7 mRNA*	OY282623	F: AAGTACCCAATCGAGCACGG R: AGGTGCAACACGGAGTTCAT	60	93	100.2	0.9964
EF1a	*elongation factor 1-α*	OQ376571	F: TGCTGCTGCAAYAAGATGGA R: AGGTAYGARGAGACTTCCTTCA	58	86	144.4	0.8970
POD9	*peroxidase 9-like*	XM_062351822	F: ACATGCCCACAAGCTGATGA R: TCACACCCCTGAACAAAGCA	62	122	112.0	0.9745
YSL2	*metal-nicotianamine transporter YSL2-like*	XM_062366618	F: TGCACGACTTCAAGACAGGT R: AAGAACGTGAGTGGTGCGAT	62	109	99.2	0.9999

[a]F, forward; R, reverse. [b]T_a, annealing temperature. [c]Amplicon length was obtained using cDNA as a template.

The *E* and the R^2 values of the eight amplification systems were evaluated (Table 1). The *E* of all the systems remained between 100.2 and 103.1%, with the exception of EF1a, which exceeded 144%. The R^2 values of all systems, except the EF1α, remained between 0.995 and 1.000. As the EF1a system showed non-linear expression (R^2 = 0.897), it could not be used for quantification and was excluded as RG. The seven candidates selected demonstrated a broad dynamic range. Depending on the gene, LOD and LOQ of these systems ranged from 1.08 × 10^5 Eq (genomic equivalent) to 34.4 Eq (SKIP19, TUBA1, and Actin2), and 6.88 Eq (UBI1, HIS4, and Actin1).

3.2. Expression Profiling of Candidate RGs

A boxplot was used to depict the variability in the *Cq* values of the selected candidate RGs with respect to the tissue evaluated (Figure 1). The *Cq* values for the analyzed genes in shoots ranged from 14.0 (*S19*) to 30.8 (*TUBA1*) (Figure 1B), whereas in roots, they varied from 20.3 (*S19*) to 30.7 (*TUBA1*) (Figure 1C). Consequently, the data indicated that *S19* showed the highest expression levels in both tissues, whereas *TUBA1* demonstrated the lowest. Figure 1 also illustrates the variation in gene expression. In both types of tissue, *UBI1* exhibited the least variability, with *Cq* values consistently falling between 24.1 and 29.0 in shoots and between 25.1 and 29.5 in roots. Conversely, *HIS4* in shoots displayed a broader range of *Cq* values, from 20.3 to 28.9, much like *Actin2* in roots, which ranged from 22.3 to 30.4. Consequently, these genes may not be suitable candidates as RGs in each tissue. Furthermore, when data from both tissues were analyzed jointly (Figure 1A), *S19* was the RG with the highest expression levels, and *UBI1* was the RG with the lowest variability. However, the variability of *S19* was notably higher when both tissues were analyzed together.

The boxplot, utilizing *Cq* values, not only determined the expression profile of the RGs but also revealed their stability. Nonetheless, given the complexity of their surroundings, it is crucial to perform a comprehensive and systematic evaluation of the stability of RGs under different conditions. Therefore, additional statistical tools and analyses are required.

3.3. Stability Assessment of Candidate RGs

To identify the most suitable RGs in roots and shoots of *P. australis* under Cu stress, gene expression data were collected from samples exposed to different $CuSO_4$ concentrations (0, 0.5, 2.0, and 3.3 mM) and times (0, 6, 24, and 48 h). Expression data were analyzed separately for roots and shoots, as well as jointly using a combined dataset that included both tissues. This approach enabled gene stability to be evaluated across a range of Cu concentrations and exposure times. The gene expression data were analyzed using Δ*Cq*, geNorm, NormFinder, and BestKeeper algorithms.

3.3.1. Δ*Cq*. The Δ*Cq* method assesses the variability in candidate RG expression by comparing the relative expression of gene pairs within each sample, which does not include primer efficiency in the calculation. The average standard deviation (SD) of each gene is inversely proportional to its stability. Using this approach, *Actin1* (1.89) was identified as the most stable gene across all samples, while *TUBA1* (1.41) and *S19* (1.69) showed the highest stability in the shoot and root, respectively. Conversely, the least stable genes were *S19* (2.99) across all samples, *HIS4* (2.14) in the shoots, and *Actin2* (2.24) in the roots (Table S3). Therefore, the Δ*Cq* analysis reveals distinct tissue-specific stability patterns between shoots and roots.

3.3.2. GeNorm Analysis. The geNorm algorithm was used to assess the stability of gene expression for the seven candidate RGs across different tissues of *P. australis* under Cu stress. Genes with the lowest *M* values are identified as the most stable. In general, a candidate gene with an *M* value of less than 1.5 is considered an appropriate RG.[41] Analysis of all samples showed that the *M* values for *TUBA1* and *Actin1* were lower than those for the other genes, indicating that these two are the most stable candidate RGs within this group. However, *S19* had the highest *M* value, suggesting that *S19* was the most unstable gene in this group (Figure 2A). In tissue-specific analysis, *UBI1* and *Actin2* were the most stable genes in shoots

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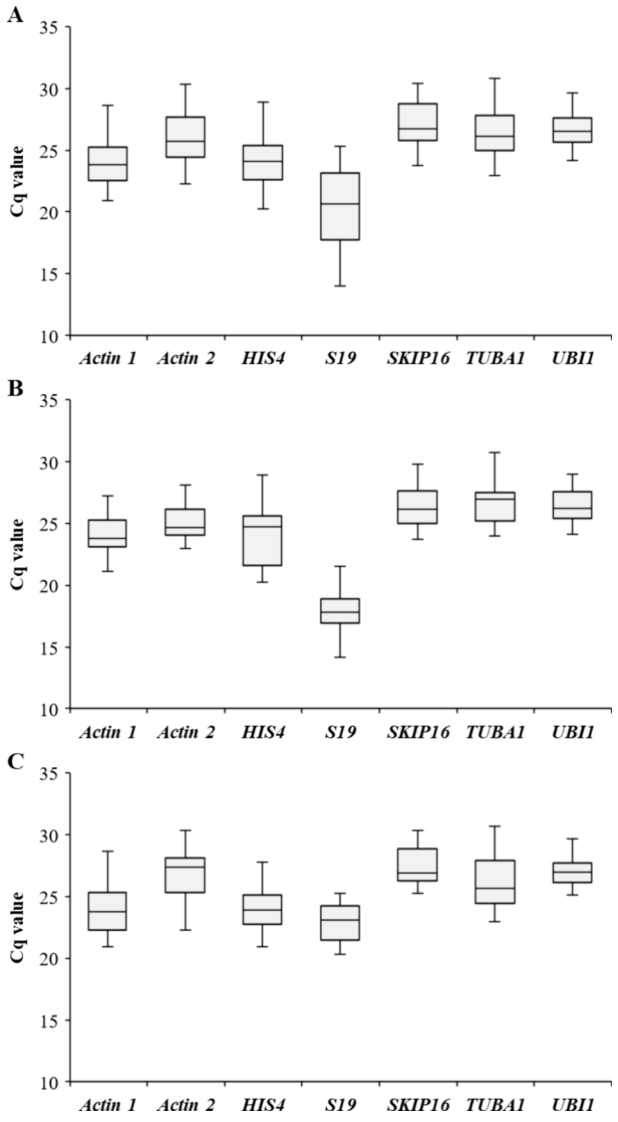

Figure 1. Box plot representing the *Cq* range variation of candidate reference genes in the *P. australis* sample: (A) all samples; (B) shoot samples; (C) root samples. The horizontal line within each box represents the median. Gray boxes and vertical lines indicate the interquartile range and the variance of *Cq* values for each gene, respectively.

(Figure 2B), whereas *TUBA1* and *Actin1* exhibited the greatest stability in roots (Figure 2C). In contrast, *HIS4* and *Actin2* were the least stable genes in shoots and roots, respectively. These results demonstrate distinct tissue-specific stability patterns, with different genes showing optimal performance in shoots and roots when exposed to Cu stress.

When the *M* values calculated for all tissues were analyzed, only one gene was found to have optimal stability ($M < 1.5$). The number of genes increased to three in the roots when tissues were analyzed individually, while the shoots showed the highest number of RGs with optimal stability (4 of 7). Therefore, the gene expression profiles vary depending on the tissue. In roots, for example, finding genes with high stability can be more complex than in shoots.

Although most studies have used a single gene for normalization as an internal control, the use of multiple RGs may provide more reliable results.[21] Pairwise variation values (V_n/V_{n+1}) were calculated using geNorm to determine the minimum RGs required for accurate normalization. When the seven genes were examined in the shoots and roots, the V_n/V_{n+1} values from both tissues under Cu stress conditions exceeded the default geNorm threshold of 0.15 (Figure 3). Nevertheless, the inclusion of additional RGs increases both the instability and complexity of the experimental process.[42]

3.3.3. NormFinder Analysis. The NormFinder Excel add-in evaluates the stability of candidate RGs based on intra- and inter-group variations, ranking them by stability values (SV), where lower values indicate higher stability. Under Cu stress conditions, *S19* (1.46) and *Actin2* (0.28) were the least and most stable, respectively, across all samples (Figure 4A). The algorithm itself suggests the combination of two other genes, *UBI1* and *SKIP16*, to obtain a much more optimal SV value (0.19).

The analysis was performed independently for each tissue type. In the aerial part, the most unstable gene was *HIS4* (0.98), followed by *S19* (0.78), the gene that was shown to be most unstable in the pooled analysis (Figure 4B). The two genes with the highest stability in shoots were *Actin1* (0.48) and *TUBA1* (0.41), but their combination proved to be the most stable (0.18). The graphical representation of the intergroup variation clearly shows, through the width of the confidence intervals, the reduction of this variation in the *TUBA1*, *Actin1*, and even *Actin2* genes, compared to the rest of the genes analysed (Figure S2).

With respect to the roots, the most unstable genes were *UBI1* (0.66), *Actin2* (0.62), and *TUBA1* (0.62) (Figure 4C). The most stable gene was *S19* (0.37), in contrast with the instability observed in the pooled analysis and for shoots. The SV was reduced to 0.26 when combining the *Actin1* and *SKIP16* genes. It is worth noting that, although the algorithm shows a higher stability value for these genes, the confidence intervals in the root (Figure S2), in general, show more internal variability at each concentration than they did in the aerial part, especially for the control (0.0 mM) and 0.5 mM Cu treatment. This means that the SV was not as low as expected, showing variability due to different exposure times to Cu stress (0, 6, 24, and 48 h).

Overall, the NormFinder analysis revealed pronounced tissue-specific patterns in RG stability, with contrasting behaviour of genes between shoots and roots. Notably, genes that exhibited high stability in shoots (*Actin1* and *TUBA1*) were not necessarily stable in roots, where *S19* exhibited the highest stability, underscoring the strong influence of tissue type on RGs under Cu stress.

3.3.4. BestKeeper Analysis. Based on the rankings obtained using the BestKeeper analysis (Table 2), *UBI1* was the most stable gene in all samples, as well as in both shoots and roots. In contrast, *S19* was the least stable in shoots, while *Actin 2* was the least stable in root samples. As with the other statistical approaches, these results indicate distinct tissue-dependent behavior of RGs, confirming the presence of different stability patterns in shoots and roots under Cu stress.

3.3.5. Overall Ranking Stability Evaluation. The results showed that the stability of RGs depended on the type of tissue. Furthermore, the order in which the candidate RGs were ranked differed depending on the algorithms (Table 3). Although all algorithms ranked *Actin2* as the least stable RG in roots, the most stable gene varied between methods. Only ΔCq and NormFinder approaches consistently ranked *S19* as

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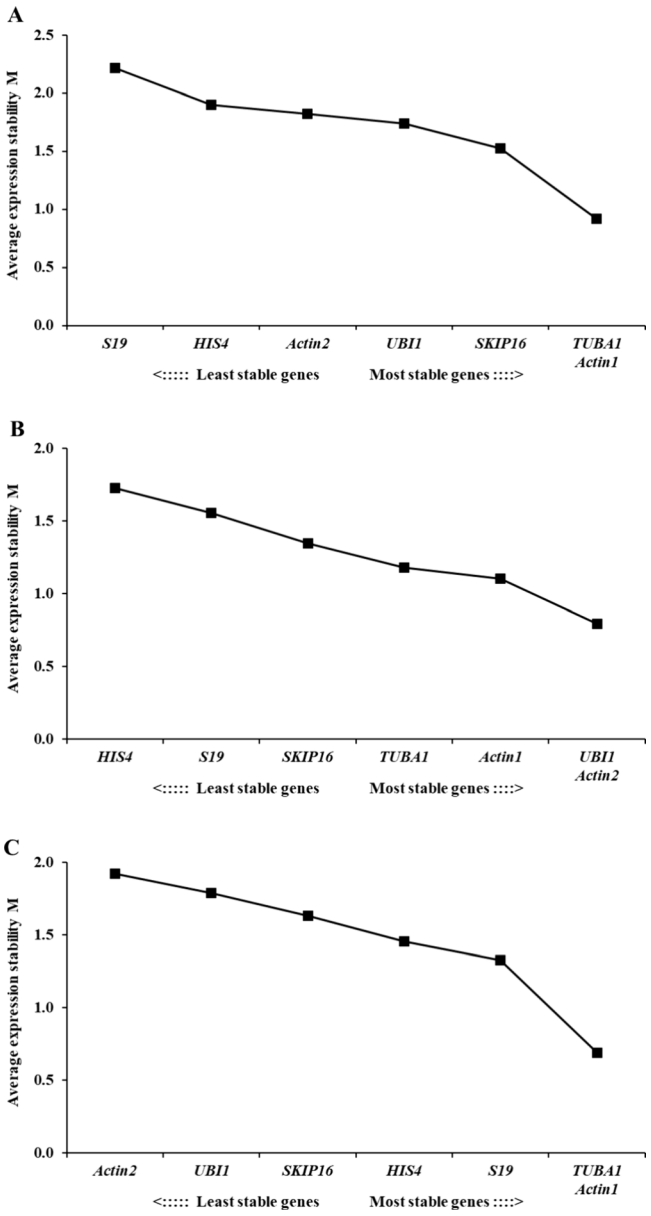
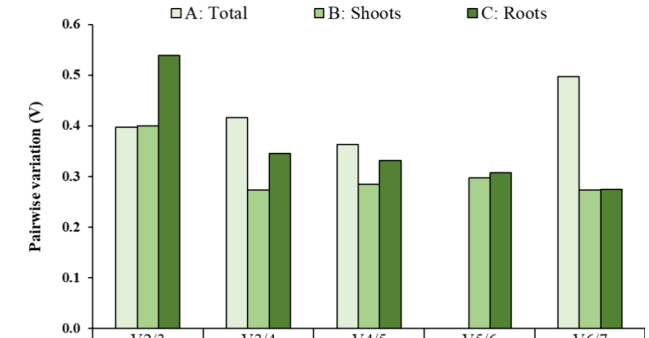


Figure 2. Gene expression stability values (M) of seven candidate reference genes in *P. australis* evaluated using the geNorm program. The least stable genes are positioned on the left with higher M values, while the most stable genes are situated on the right with lower M values. (A) all samples; (B) shoot samples; (C) root samples.



	V2/3	V3/4	V4/5	V5/6	V6/7
A: Total	0.397	0.417	0.363	0.000	0.497
B: Shoots	0.400	0.273	0.285	0.297	0.273
C: Roots	0.539	0.346	0.331	0.307	0.275

Figure 3. Optimal number of reference genes in different experimental groups calculated using geNorm. Pairwise variation (V_n/V_{n+1}) analysis of seven candidate reference genes analyzed in three sample subsets. (A) all samples; (B) shoot samples; (C) root samples.

the least stable gene. Interestingly, these two algorithms also coincided in identifying *TUBA1* as the most stable RG in shoots, whereas the other algorithms selected different candidates. These discrepancies arise from the distinct mathematical approaches and underlying assumptions of each algorithm, highlighting the complexity of RG selection and emphasizing the need for a comprehensive, multi-algorithm evaluation strategy.

A comprehensive joint analysis of the seven candidate RGs was therefore performed to establish an overall stability ranking based on the geometric mean calculated from the four previous analyses: ΔCq, geNorm, NormFinder, and BestKeeper. Overall rankings were established for all samples, as well as for each tissue type separately (Table 3). The two most stable genes across all samples were *Actin1* and *TUBA1*; in the shoots analysis, *Actin2* and *TUBA1;* and in the roots, corresponded to *S19* and *TUBA1*.

3.4. Validation of Selected RGs

The expression profiles of two target genes related to metal stress, *POD9* and *YSL2*, were analyzed using the least stable RG (shoot: *HIS4*; root: *Actin2*) and the two most stable RGs (shoot: *Actin2/TUBA1*; root: *S19/TUBA1*).

The *POD9* expression profiles, in shoot and root samples, are depicted in Figure 5. In shoots, the patterns of the *POP9* gene over- or under-expression, in relation to Cu concentration and exposure duration, were similar when *TUBA1*, *Actin2,* or their combination (stable genes in this type of tissue) were employed (Figure 5A). In contrast, the expression profile differed markedly in root samples exposed for 6 h to any of the tested Cu concentrations when *Actin2*, an unstable gene in this tissue, was used, compared to results obtained with *S19* or the *S19/TUBA1* combination. Furthermore, a trend toward decreased levels of subexpression was observed, suggesting that the use of *Actin2* for normalization in root tissue would underestimate the reduction in *POD9* expression, particularly in the Cu-affected samples after 24 and 48 h (Figure 5B).

Figure 6 shows the *YLS2* expression profiles. Overexpression of this gene was observed in Cu-treated shoots, especially when *HIS4*, the least stable RG in shoots, was used (Figure 6A). The increased expression was also observed in root samples (Figure 6B), albeit to a lesser extent than in shoots. Moreover, in most cases, *YLS2* expression was higher when *Actin2,* the less reliable RG for roots, was used, compared to the levels obtained with *S19/TUBA1* and *S19/TUBA1*. Furthermore, the least stable RGs exhibited the greatest experimental error, which decreased when expression was normalized using a combination of the two most stable RGs.

4. DISCUSSION

Gene expression is influenced by various factors, including tissue type, environmental conditions, and stress situations. These factors collectively determine which genes are expressed

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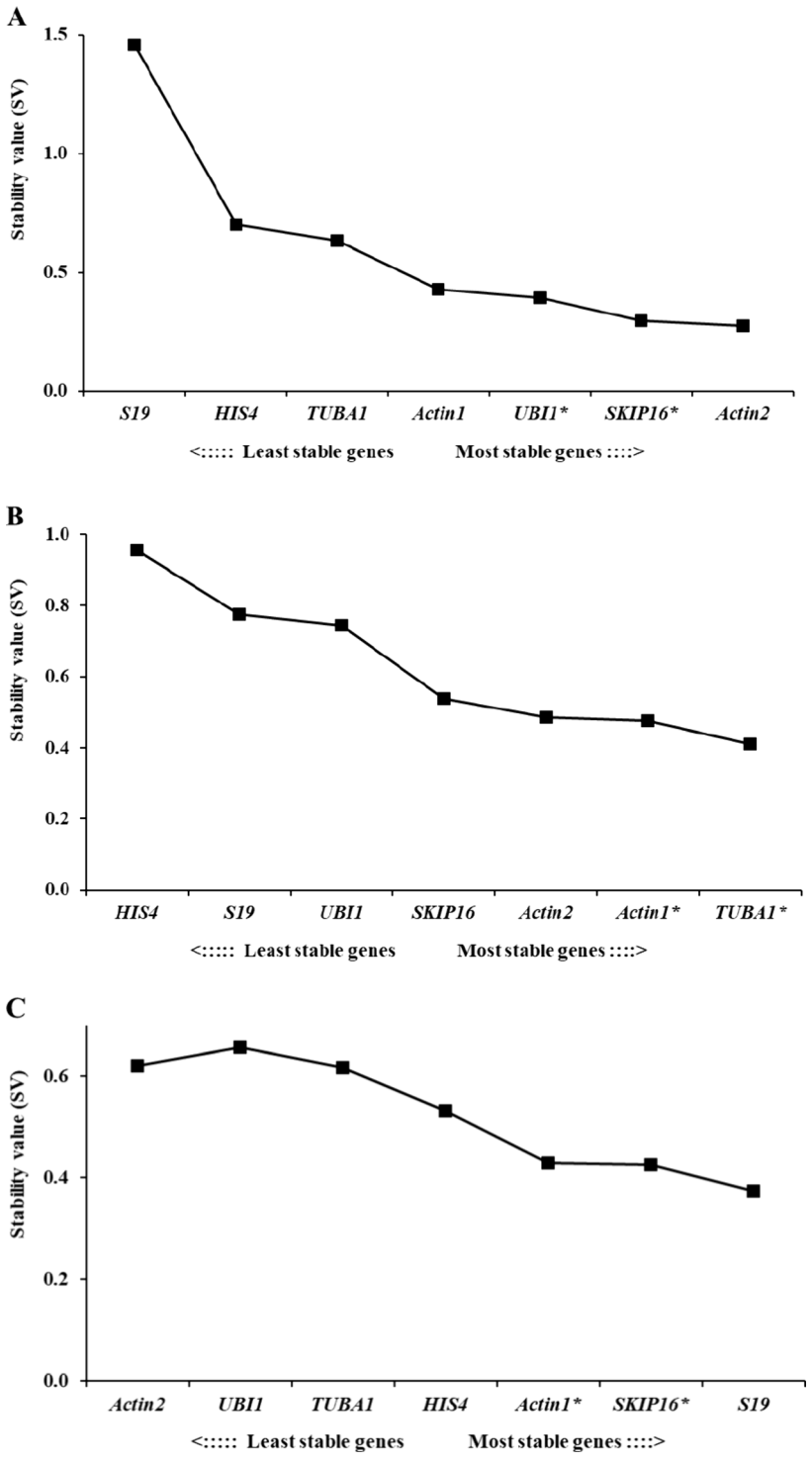

Figure 4. Expression stability values of *P. australis* candidate reference genes as calculated by the NormFinder software. The asterisk represents the best combination for gene expression normalization. (A) all samples; (B) shoot samples; (C) root samples.

and to what extent under a given condition. The accuracy of gene expression analysis forms the basis and key link in research into any plant stress and its potential application in phytoremediation.[43]

RT-qPCR is an accurate and specific method for quantifying gene expression through the analysis of target genes. The reliability of gene expression results is highly dependent on the normalization process, wherein the RGs employed exhibit stable expression across the tissues and conditions under investigation, thereby minimizing variations unrelated to tissue effects or stress.[44]

Based on the reviewed literature, eight amplification systems were designed based on seven genes that have been employed as RG studies in metal stress on different plant species:

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Table 2. Ranking of Candidate RGs in *P. australis*, under Cu Stress, Based on BestKeeper Analysis (SD, CV, and *r*)[a,b]

A: all samples						B: shoot samples						C: root samples					
	ranking based on				final		ranking based on				final		ranking based on				final
gene	SD	CV	*r*	GM	rank	gene	SD	CV	*r*	GM	rank	gene	SD	CV	*r*	GM	rank
UBI1	1	1	7	1.91	**1**	*UBI1*	1	1	7	1.91	**1**	*UBI1*	1	1	7	1.91	**1**
Actin1	3	3	1	2.08	**2**	*Actin2*	2	2	3	2.29	**2**	*TUBA1*	7	2	1	2.41	**2**
SKIP16	2	2	6	2.88	**3**	*Actin1*	3	3	2	2.62	**3**	*HIS4*	2	4	4	3.17	**3**
TUBA1	6	5	2	3.91	**4**	*TUBA1*	6	5	1	4.31	**4**	*SKIP16*	3	3	6	3.78	**4**
Actin2	5	4	3	3.91	**4**	*SKIP16*	4	4	5	4.31	**5**	*S19*	4	5	3	3.91	**5**
HIS4	4	6	5	4.93	**6**	*HIS4*	7	6	4	5.52	**6**	*Actin1*	5	7	2	4.12	**6**
S19	7	7	4	5.81	**7**	*S19*	5	7	6	5.94	**7**	*Actin2*	6	6	5	5.64	**7**

[a]Geometric mean (GM) was calculated for the final ranking. [b]SD, standard deviation; CV, coefficient of variance; *r*, pairwise correlation coefficient between each gene and the BestKeeper index.

Table 3. Comprehensive Ranking of the 7 Candidate References Genes Based on Geometric Mean of Δ*Ct*, GeNorm, NormFinder, and BestKeeper Ranking

set	no.	Δ*Cq*	geNorm	NormFinder	BestKeeper	overall ranking
all samples	1	*Actin1*	*Actin1*	*Actin2*	*Actin1*	***Actin1***
	2	*TUBA1*	*TUBA1*	*SKIP16*	*TUBA1*	***TUBA1***
	3	*Actin2*	*SKIP16*	*UBI1*	*Actin2*	***Actin2***
	4	*SKIP16*	*UBI1*	*Actin1*	*S19*	***SKIP16***
	5	*UBI1*	*Actin2*	*TUBA1*	*HIS4*	***HIS4***
	6	*HIS4*	*HIS4*	*HIS4*	*SKIP16*	***UBI1***
	7	*S19*	*S19*	*S19*	*UBI1*	***S19***
shoot samples	1	*TUBA1*	*Actin2*	*TUBA1*	*UBI1*	***Actin2***
	2	*Actin2*	*UBI1*	*Actin1*	*Actin2*	***TUBA1***
	3	*Actin1*	*Actin1*	*Actin2*	*Actin1*	***UBI1***
	4	*UBI1*	*TUBA1*	*SKIP16*	*TUBA1*	***Actin1***
	5	*SKIP16*	*SKIP16*	*UBI1*	*SKIP16*	***SKIP16***
	6	*S19*	*S19*	*S19*	*HIS4*	***S19***
	7	*HIS4*	*HIS4*	*HIS4*	*S19*	***HIS4***
root samples	1	*S19*	*TUBA1*	*S19*	*UBI1*	***S19***
	2	*Actin1*	*Actin1*	*SKIP16*	*TUBA1*	***TUBA1***
	3	*HIS4*	*S19*	*Actin1*	*HIS4*	***Actin1***
	4	*TUBA1*	*HIS4*	*HIS4*	*SKIP16*	***HIS4***
	5	*SKIP16*	*SKIP16*	*TUBA1*	*S19*	***SKIP16***
	6	*UBI1*	*UBI1*	*UBI1*	*Actin1*	***UBI1***
	7	*Actin2*	*Actin2*	*Actin2*	*Actin2*	***Actin2***

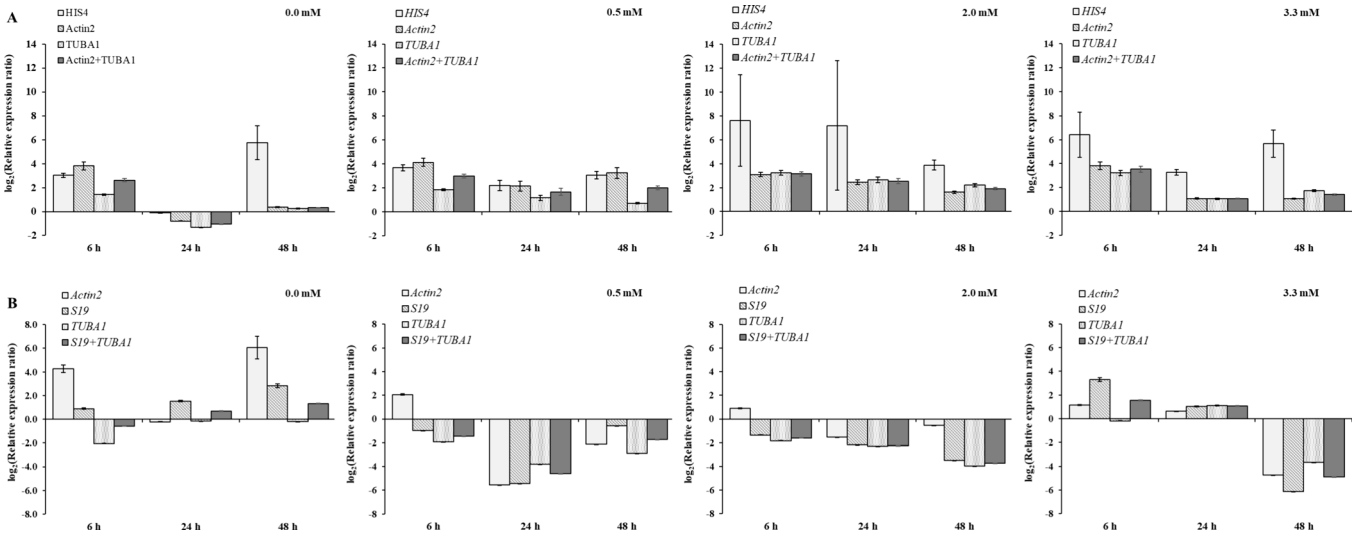

Figure 5. Relative expression levels of *POD9* in the shoots (A) and the roots (B) of *P. australis* under different Cu concentrations (0.0, 0.5, 2.0, and 3.3 mM) at different treatment times (6, 24, and 48 h) normalized to the least stable gene and the top two most stable genes. The error bars represent the standard deviation ($n = 3$).

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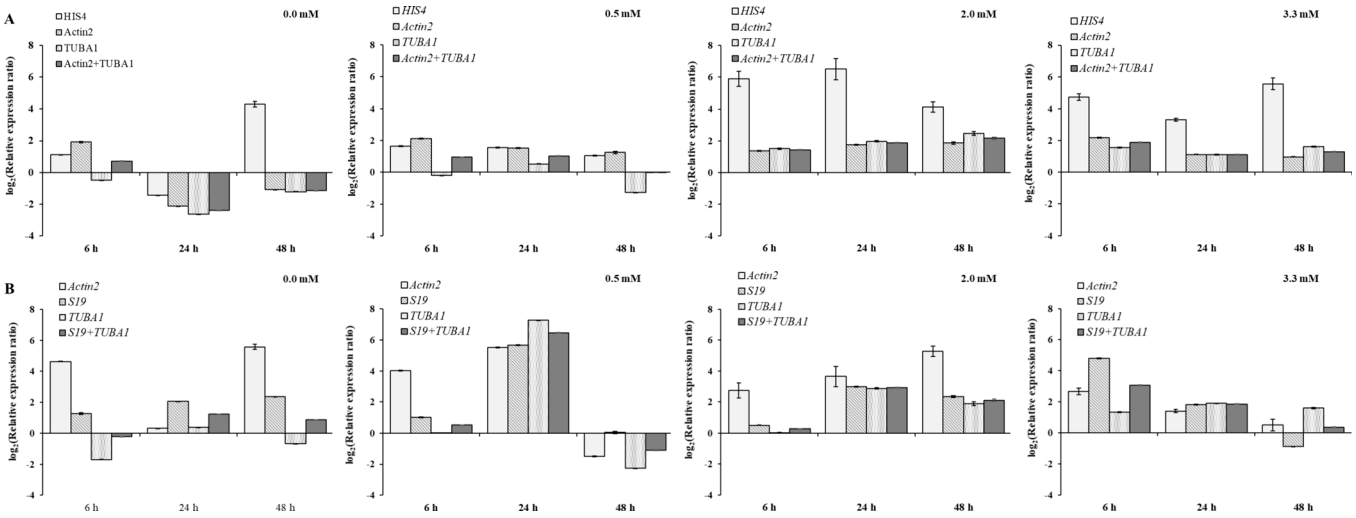

Figure 6. Relative expression levels of *YSL2* in the shoots (A) and the roots (B) of *P. australis* under different Cu concentrations (0.0, 0.5, 2.0, and 3.3 mM) at different treatment times (6, 24, and 48 h) normalized to the least stable gene and the top two most stable genes. The error bars represent the standard deviation ($n = 3$).

Actin,[45,46] *S19*,[39] *TUBA1*,[47] *EF1a*,[48] *HIS4*,[49] *UBI1*,[50] and *SKIP16*,[50] yielding different results depending on the species, tissue, and metal being analyzed. Optimization of the amplification systems and evaluation of the efficiency and effectiveness of quantification within a wide linear range revealed that all systems, except *EF1a*, showed optimal conditions to be considered as RG (Table 1). The remaining seven amplification systems satisfied the criteria required for the stability study and subsequent validation under Cu stress conditions in *P. australis*.

The expression values of RGs were not uniform across the different samples. In particular, exposure to heavy metal such as Cu can induce oxidative stress, metabolic disruption, and transcriptional reprogramming, leading to altered expression of commonly used RGs.[51] As shown in Figure 1, each candidate RG exhibited different *Cq* ranges in all samples, indicating variability in gene stability among samples. The *Cq* values exhibited notable differences in internal variability, with *S19* demonstrating the highest variability among all samples. This was in contrast to the variability observed when root and shoot tissues were evaluated separately. These results suggest differential behavior among tissues and underscore the necessity of validating RGs traditionally employed for each specific tissue type. Previous findings have demonstrated that gene expression varies across different plant tissues. Sun et al.,[52] for example, reported that *GAPDH* expression remained stable in the stems of *Kobresia littledalei* under drought stress, whereas *SOD* exhibited the highest stability in roots. More specifically, various authors have identified differences in gene expression between tissues subjected to Cu stress, as in the case of *Paulownia fortunei*,[53] a species used in phytoremediation of soils contaminated with heavy metals.

To address the potential for bias introduced by a single software package,[54] Δ*Cq*, geNorm, NomFinder, and BestKeeper methods were employed to evaluate the candidate RGs. The results showed discrepancies in gene ranking for each algorithm, as were previously observed in previous studies in plants under heavy metal.[16,26,43] These inconsistencies emphasize the lack of a universally stable RG and highlight the significant impact of tissue-specific expression patterns in response to Cu stress.

In the process of selecting RGs, it is necessary to thoroughly consider the analysis results provided by the comparative Δ*Cq* method, geNorm, NormFinder, and BestKeeper.[55] Although tools such as RefFinder,[56] which is user-friendly, web-based, and comprehensive, have been developed to integrate rankings from multiple algorithms, these assume a PCR efficiency of 100% and do not fully account for inter-group variation in geNorm. To avoid this limitation, the present study identified the most appropriate RGs by calculating an overall ranking based on the geometric mean of the individual rankings obtained from each method. This considers the efficiency of each system (with the exception of the Δ*Cq* method) and inter-group variation in geNorm (Table 3). The comprehensive analysis revealed that *S19* and *Actin2* demonstrated the highest stability in Cu-treated roots and shoots, respectively.

On the other hand, the utilization of multiple RGs can enhance the reliability of gene expression analysis.[21] Therefore, it is essential to determine the optimal number of RGs. In order to determine the most appropriate number of genes, two of the most stable RGs were used both independently and in combination to normalize the expression of two target genes: *Actin2* and *TUBA* in shoots and *S19* and *TUBA1* in roots. Additionally, the results were compared with those obtained using the gene with the lowest stability (*HIS4* in shoots and *Actin2* in roots).

Specifically, to validate the suitability of the RGs, two target genes were examined: one involved in oxidative metabolism and another in metal transport. Antioxidant enzyme systems have been linked to the responses of various plant species to Cu, including tobacco,[57] rice,[58] grape,[59] and *Spirodela polyrhiza*.[60] Among these, the gene *POD9* was selected for its function in mitigating oxidative damage induced by metal-induced stress,[39] and the gene *YSL2* was selected for its role in the translocation of heavy metals.[61] The expression profiles of *POD9* (Figure 5) and *YLS2* (Figure 6), normalized using *Actin2*/

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TUBA1 in shoots and *S19/TUBA1* in roots, align with those reported by other researchers. Species with high phytoremediation potential in Cu-contaminated environments, such as *Paspalum urvillei*, exhibit reduced expression of genes involved in reactive oxygen species scavenging, such as *POD*.[62] This response can reflect alternative defense mechanisms, such as the Cu accumulation in roots, which could explain the tissue-dependent variation in *POD9* expression (Figure 5). In contrast, *YLS2* was overexpressed in Cu-treated *P. australis* (Figure 6), suggesting an enhanced capacity for transporting heavy metals, particularly in aerial tissues, as has been reported for giant reed[39] or rice.[63]

These findings, in line with the literature, are confirmed by a consistent expression pattern when *Actin2/TUBA1* and *S19/TUBA1* were employed as the internal RGs for shoot and roots, respectively. Conversely, irregular expression patterns were observed when *HIS4* (in shoots) and *Actin2* (in roots) were selected as RGs for RT-qPCR analysis, taking into account not only the stress being assessed but also the target tissue. Additionally, it was found that using a combination of two genes to determine the expression of both *POD9* and *YLS2* reduced the errors in the process.

In conclusion, accurate gene expression analysis is essential for optimizing phytoremediation, which relies on the use of stable, tissue-specific RGs. To the best of our knowledge, this study represents the first attempt to validate RGs in shoots and roots of *P. australis* under Cu stress. Based on these findings, we propose using a combination of *Actin2/TUBA1* and *S19/TUBA1* as RGs in the analysis of gene expression in *P. australis* under Cu stress. These results lay the groundwork for further molecular studies in the context of phytoremediation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsagscitech.5c00969.

Information of the genes used in this study (Table S1), ranking of candidate reference genes in *P. australis* under Cu stress, based on Δ*Cq* method (Table S2), MIQE reporting experiment of *P. australis* exposed to Cu (Table S3), *P. australis* melt curves for 8 candidate RGs (Figure S1), and NormFinder analysis of candidate reference genes in *P. australis* at different Cu concentrations (Figure S2) (DOCX)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Δ*Cq*	delta quantification cycle
CV	coefficient of variance
Eq	genomic equivalent
LOD	limit of detection
LOQ	limit of quantification
M	expression stability value
MIQE	minimum Information for publication of quantitative real-time PCR experiments
POD9	peroxidase 9
r	pairwise correlation coefficient
R^2	coefficient of determination
RGs	reference genes
RQ	relative quantities
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SD	standard deviation
SV	stability value

YSL2 metal-nicotianamine transporter YSL2

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