Selection of suitable reference genes for quantitative real-time PCR gene expression analysis in *Salix matsudana* under different abiotic stresses

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*Salix matsudana* is a deciduous, rapidly growing willow species commonly cultivated in China, which can tolerate drought, salt, and heavy metal stress conditions. Selection of suitable reference genes for quantitative real-time PCR is important for normalizing the expression of the key genes associated with various stresses. To validate suitable reference genes, we selected 11 candidate reference genes (five traditional housekeeping genes and six novel genes) and analyzed their expression stability in various samples, including different tissues and under different abiotic stress treatments. The expression of these genes was determined using five programs—geNorm, NormFinder, BestKeeper, ΔCt, and RefFinder. The results showed that α-TUB2 (alpha-tubulin 2) and DnaJ (chaperone protein DnaJ 49) were the most stable reference genes across all the tested samples. We measured the expression profiles of the defense response gene SmCAT (catalase) using the two most stable and one least stable reference genes in all samples of *S. matsudana*. The relative quantification of SmCAT varied greatly according to the different reference genes. We propose that α-TUB2 and DnaJ should be the preferred reference genes for normalization and quantification of transcript levels in future gene expression studies in willow species under various abiotic stress conditions.

Drought, salt, and heavy metal stresses are major abiotic factors that contribute to the risk of environment and affect forestry productivity worldwide1–5; however, plants need to thrive in adverse circumstances6. Plants with short growth cycles, such as *Arabidopsis thaliana*7, soybean8, sorghum9, jute10, *Sedum alfredii*11, rice12, and tobacco13, have been the focus of studies on the effects of various abiotic stresses, and a few studies have been performed on plants with long growth cycles under different stress conditions. Short growth cycle plants are limited by low biomass, while plants (especially woody plants) with high biomass and long growth cycles are more able to deal with severe abiotic stress conditions. Only a small number of reference genes have been reported in trees under drought, salt, and heavy metal stress conditions14–18.

The genus *Salix* (Salicaceae) contains more than 450 willow species worldwide; 275 of these species grow in China19–22. Willow species are used for energy production, afforestation, and greening due to their high biomass, rapid growth, and ability to adapt to different stress conditions23–28. *Salix matsudana* is a deciduous, rapidly growing willow species commonly cultivated in China, which can tolerate drought, salt, and heavy metal stresses29–33. Physiological and biochemical properties have been characterized in *S. matsudana*34,35. Meanwhile, some key genes have been identified to regulate stress response factors in stressed plants at the
molecular level. Understanding the expression patterns of key stress response genes will help elucidate the mechanisms involved in various stresses of *S. matsu-dana*.

Gene expression analysis has been applied to understand different kinds of biological processes. Quantitative real-time polymerase chain reaction (qRT-PCR) is widely used for gene expression analysis due to its high sensitivity, accuracy, specificity, and reproducibility. However, factors such as sample amount, RNA integrity, reverse transcription efficiency, and cDNA quality can significantly influence the reliability of the gene expression results. To reduce the influence of these factors, internal reference genes are used to obtain accurate biologically meaningful expression values; however, unstable reference genes can cause significant biases and misinterpretations of the expression data. Actin (ACT) and β-tubulin (β-TUB) have been used as reference genes for qRT-PCR normalization in gene expression analysis in *S. matsu-dana* under salt and copper stresses, but a systematic study to validate reference genes has not been reported for *S. matsu-dana* under abiotic stresses. To obtain accurate expression data, it is necessary to select suitable reference genes for each plant species and to verify their stability under the specific experimental conditions of interest.

In this study, we determined the expression profiles of 11 candidate reference genes from *S. matsu-dana* in six different tissues and under three kinds of abiotic stresses. The 11 candidate genes were *ACT*, alpha-tubulin 1 (α-TUB1), alpha-tubulin 2 (α-TUB2), chaperone protein DnaJ 49 (DnaJ), E3 ubiquitin-protein ligase AR18 (AR18), F-box family protein (F-box), histone H2A (H2A), heat shock 70 kDa protein (HSP70), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), membrane-anchored ubiquitin-fold protein (MUB), and β-TUB. The transcriptome data of *S. matsu-dana* were used as the source of the potential reference genes (Unpublished data). The stabilities of the 11 reference genes were analyzed using five statistical algorithms—geNorm, NormFinder, BestKeeper, ΔCt method, and RefFinder, a web-based software. The expression stability of the reference genes was evaluated by four algorithms—geNorm, NormFinder, BestKeeper, and the ΔCt method. The results will provide suitable reference genes for qRT-PCR normalization for accurate gene expression analysis in *S. matsu-dana* under different stress conditions.

**Materials and Methods**

**Plant materials and stress treatments.** Cuttings (approximately 10 cm long) from annual branches of *S. matsu-dana* were grown in hydroponics. Plants were supplemented with water containing 1/4 strength Hoagland solution on alternate days under normal conditions (25 °C, 16 h light/8 h dark). After 45 days of culture, groups of *S. matsu-dana* seedlings were subjected to different abiotic stresses in solutions containing 1/4 strength Hoagland solution at pH 6.0 as follows: drought (15% PEG 6000), salt (100 mM NaCl), and heavy metal (100 μM CdCl₂). Untreated seedlings were used as the control. The roots of the treated plants were sampled at 0 h, 12 h, 24 h, 48 h, and 72 h. Tissues from the root, xylem, bark, stem, leaf, and flower were collected from the untreated plants. All the samples from three biological replicates were carefully harvested, immediately frozen in liquid nitrogen, and stored at −80 °C until total RNA extraction.

**Total RNA isolation and cDNA synthesis.** Total RNA from each sample was isolated from approximately 0.1 g fresh root using a total RNA kit (NORGEN, Thorold, Canada) and treated with DNase I (TaKaRa, Dalian, China) to remove any genomic DNA contamination. The RNA concentration of each sample was determined using a NanoDrop-2000 spectrophotometer (Thermo, Wilmington, USA). Samples with a 260/280 ratio of 1.9–2.1 and a 260/230 ratio ≥ 2.0 were chosen to determine the quality and purity of the RNA preparations. The integrity of the purified RNA was checked by 1.0% (p/v) agarose gel electrophoresis. Subsequently, first-strand cDNA was synthesized in a 20-μL reaction mixture in an Invitrogen SuperScript First Strand Synthesis System (Invitrogen, Carlsbad, USA) following the manufacturer's instructions, and stored at −20 °C until use.

**Screening of candidate reference genes and primer design.** We identified 11 candidate reference genes and one target gene (Table 1) from the *S. matsu-dana* transcriptome data. Primers were designed based on the sequences of each gene using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) with the following criteria: GC content 45–65%, optimal Tm 58–61 °C; primer length 18–22 bp, and amplicon length 120–220 bp. The specificity of each selected primer pair was observed via standard RT-PCR using Premix Ex Taq (TaKaRa, Dalian, China), and each gene was verified by 2% agarose gel electrophoresis and sequenced to ensure its reliability.

**qRT-PCR.** qRT-PCR amplification was performed in 96-well plates with a Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, CA, USA) using SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China). PCR reactions were prepared in 20 μL volumes containing: 2 μL of 50-fold diluted synthesized cDNA, 10 μL 2× SYBR Premix Ex Taq™, 0.8 μL of each primer, 0.4 μL ROX reference dye (50×), and 6.8 μL ddH₂O. The reactions comprised an initial step of 95 °C for 30 s, followed by 40 denaturation cycles at 95 °C for 5 s and primer annealing at 60 °C for 31 s. Next, the melting curves ranging from 60 °C to 95 °C were evaluated in each reaction to check the specificity of the amplicons. Biological triplicates of all the samples were used for the qRT-PCR analysis, and three technical replicates were analyzed for each biological sample. The threshold cycle (Ct) was measured automatically.

**Statistical analysis to determine the expression stability of the candidate reference genes.** Standard curves were generated in Microsoft Excel 2013 to calculate the gene-specific PCR efficiency and the correlation coefficient from 5-fold series dilution of a mixed cDNA (flower, bark, and stem) template for each primer pair. The amplification plots, melting curves and sequencing peaks were shown in Figure S1a,b,c. The PCR amplification efficiency (E) and the correlation coefficient were calculated using the slope of the standard curve according to the equation $E = 5^{(-1/slope)} - 1 \times 100$. Stabilities of the 11 selected reference genes were evaluated by four algorithms—geNorm, NormFinder, BestKeeper, and the ΔCt method. Finally, ReFinder (http://www...
fulxie.0fees.us), a comprehensive evaluation platform integrating the four above algorithms, ranked the overall stabilities of these 11 candidate genes. Pairwise variations based on the geNorm calculation were used to determine the optimal number of candidate reference genes for accurate normalization.

Expression normalization of SmCAT gene based on different reference genes. The defense response gene SmCAT was selected as the target gene to measure the stabilities of the candidate reference genes by quantifying SmCAT expression levels in all the tested samples. SmCAT gene expression levels were normalized with the two most stable candidate reference genes (\(\alpha\)-TUB2 and DnaJ), as well as one of the least stable reference genes (\(\beta\)-TUB).

Results

qRT-PCR data for the candidate reference genes. The 11 selected candidate reference genes (Table 1) are orthologs of genes in Salix purpurea, for which the whole genome has been sequenced. The specificity and accuracy of the primers designed for the selected genes were determined by 2% agarose gel electrophoresis (Figure S2a), and further confirmed by a single peak in the melting-curve analysis (Figure S2b). The primer sequences, amplicon length, correlation coefficient, and PCR amplification efficiency are shown in Table 1. Furthermore, the qRT-PCR products were sequenced (File S1) to determine the accuracy of the 11 genes.

Table 1. Reference genes and target genes investigated in Salix matsudana by qRT-PCR. \(R^2\), correlation coefficient.

| Gene   | Gene description | S. purpurea ortholog locus | Primer sequence F/R(5’-3’) | Product size (bp) | Efficiency (%) | \(R^2\) |
|--------|------------------|---------------------------|----------------------------|-------------------|----------------|-------|
| ACT    | actin            | SapurV1A.0285s0180        | CAGAAAGACGCCTATGTTGG        | 104               | 98.9           | 0.9941 |
| \(\alpha\)-TUB1 | alpha-tubulin1  | SapurV1A.0005s0080        | GAGGATGAGAAGCGGTAGAGG       | 197               | 92.6           | 0.9995 |
| \(\alpha\)-TUB2 | alpha-tubulin2  | SapurV1A.0598s0030        | ACTAGGAGAAGGGGAGCAGTG      | 205               | 91.0           | 0.9974 |
| DnaJ   | chaperone protein DnaJ 49 | SapurV1A.0212s0110 | GCACCAAATTTTGACAGGAT      | 137               | 101.6          | 0.9919 |
| ARI8   | E3 ubiquitin-protein ligase ARI8 | SapurV1A.0557s0250 | GTAGAGCAGGCCCCCAAGAAAA    | 198               | 92.9           | 0.9997 |
| F-box  | F-box family protein | SapurV1A.1078s0140 | CCTGCAACTGCCAGCAGTACA      | 121               | 97.2           | 0.991  |
| H2A    | histone H2A      | SapurV1A.2339s0010        | TTGTGCTCTTGTAACGGTGA       | 165               | 99.5           | 0.9979 |
| HSP 70 | heat shock 70kDa protein | SapurV1A.1370s0010 | GTGAGGGTGATGGTGCTTCTC      | 124               | 95.0           | 0.9940 |
| GAPDH  | glyceraldehyde-3-phosphate dehydrogenase | SapurV1A.0266s0210 | CAGCTGTAGGAGGAAATGCAA     | 196               | 96.2           | 0.9931 |
| MUB    | membrane-anchored ubiquitin-fold protein | SapurV1A.2454s0040 | ATTCAGTCGCCAGTGGCTTT      | 214               | 94.5           | 0.9919 |
| \(\beta\)-TUB | tubulin beta chain | SapurV1A.1459s0040 | CGAGAGGAGGGATGTGAAG        | 196               | 94.1           | 0.9971 |

Target gene

SmCAT catalase SapurV1A.0016s0660 | CACCGAGTCATAGTTGCA | 190 | 93.3 | 0.9978

Analysis of gene expression stability. Expression stabilities of the 11 candidate reference genes were determined using geNorm, NormFinder, \(\Delta\)Ct, and BestKeeper, and their overall stabilities were ranked by RefFinder across all the stress treatments and tissue samples.

geNorm analysis. The stabilities of the 11 candidate reference genes of S. matsudana calculated using geNorm were ranked in the different tissues and abiotic stress treatments according to their M values, as shown in Figure S1. The lowest M value indicates the most stable reference gene, and the highest M value indicates the least stable one. DnaJ and ARI8 had the highest expression stabilities in the six tissues, and all the genes had M values below the threshold of 1.5 (Figure S2a). The top two most stable genes were DnaJ and \(\alpha\)-TUB2 for drought and heavy metal conditions.
stresses, and $\alpha$-TUB2 and MUB for salt stress (Fig. 2b,c,d). When the stabilities from all the samples were combined, DnaJ and $\alpha$-TUB2 were determined to be the most stable reference genes in all the samples (Fig. 2e), while $\beta$-TUB had the least stability.

The pairwise variation ($V_{n/n+1}$) between two sequential normalization factors NF$_n$ and NF$_{n+1}$ was calculated by the geNorm algorithm to determine the optimal number of reference genes for accurate normalization. A cutoff value of 0.15 is the recommended threshold indicating that an additional reference gene will make no remarkable contribution to the normalization. The $V_{2/3}$ values in the tissues, salt, and heavy metal were less than 0.15 (Fig. 3), which suggested that the top two reference genes were sufficient for accurate normalization. For the...

Figure 1. Expression levels of 11 candidate reference genes across all experimental samples.

Figure 2. Expression stability of 11 candidate genes as calculated by geNorm. (a) different tissues, (b) drought treatments, (c) salt treatments, (d) heavy metal treatments, (e) all samples.
drought stress samples V4/5 was 0.123, indicating that the top four reference genes (DnaJ, α-TUB2, MUB, and ACT) were needed for accurate normalization. For the total samples V3/4 was 0.138, showing that three reference genes (DnaJ, α-TUB2, and MUB) were required.

**NormFinder analysis.** As shown in Table 2, DnaJ was the most stable gene (lowest stability value) in the salt and drought subsets calculated using NormFinder. For the heavy metal samples, α-TUB2 was the most stable gene, while ARI8 was the most stable gene in the different tissues. When all samples were taken together to determine the stability of reference genes, the three most stable genes were α-TUB2, ARI8, and DnaJ.

**ΔCt analysis.** The 11 candidate reference genes from the most to least stable expression, as calculated by the ΔCt method, are listed in Table 3. α-TUB2 was the most stable reference gene in the drought, heavy metal, and total samples subsets. MUB and ARI8 were the most stable genes for the salt subset and different tissues, respectively, and were considered the ideal reference genes.

**BestKeeper analysis.** BestKeeper determined the stabilities of the candidate reference genes based on their standard deviation (SD). Genes with SD > 1 was considered unacceptable reference genes. The genes are listed from most to least stable in Table 4. DnaJ and α-TUB2 were the most stable genes in the heavy metal and salt subsets.

**RefFinder analysis.** To acquire reliable results for the expression stabilities of the 11 candidate reference genes of *S. matsudana*, the rankings of the four algorithms were integrated by RefFinder and the results are shown in Table 5. The 11 genes were ranked from the most to least stable expression by RefFinder (Fig. 4). The expression of α-TUB2 was ranked the most stable under the salt and heavy metal stress treatments, and the expression of DnaJ was ranked the most stable under the drought stress treatment. Overall, the best reference gene for accurate transcript normalization in all of the samples was α-TUB2, which had the lowest Geomean (geometric mean) of the ranking values.

**Table 2. Expression stability of candidate reference genes as calculated by Normfinder.**
abiotic stresses; however, the study of reference genes in willows has lagged behind that of other major plant species including drought, salt, and heavy metal. A good understanding of the molecular mechanisms related to these stresses is imperative to improve forestry productivity and decrease the risk of environmental damage caused by ROS

To study gene expression variations and determine gene regulation patterns, suitable reference genes are required. To accurately determine the expression levels of target genes, qRT-PCR is a reliable and accurate technique. It is imperative to understand the regulation and function of the key genes under different abiotic stresses.

### Table 3. Expression stability of candidate reference genes as calculated by ΔCt.

| Rank | Gene | Tissue | Drought | Salt | Heavy metal | Total |
|------|------|--------|---------|------|-------------|-------|
| 1    | GAPDH | Drought | 0.91 | 4.01 | 0.59 | 2.49 | 1.15 | 5.26 |
| 2    | DnaL | DnaL | 0.5 | 2.25 | 1.02 | 4.53 | 0.67 | 2.77 | 1.24 | 5.58 |
| 3    | MUB | H2A | 1.06 | 4.06 | 0.83 | 3.68 | 0.84 | 1.67 |
| 4    | SmCAT | H2A | 1.02 | 4.26 | 0.77 | 3.47 | 0.75 | 1.60 |
| 5    | HSP70 | ACT | 0.95 | 2.72 | 0.99 | 3.26 | 0.95 | 1.60 |
| 6    | α-TUB2 | H2A | 1.02 | 4.64 | 0.85 | 3.58 | 0.91 | 2.77 |
| 7    | GAPDH | MUB | 1.05 | 4.25 | 0.77 | 2.93 | 0.77 | 2.93 |
| 8    | α-TUB1 | HSP70 | 1.26 | 5.46 | 1.08 | 4.28 | 1.26 | 5.46 |
| 9    | ACT | F-box | 1.41 | 6.19 | 0.84 | 3.68 | 0.84 | 3.68 |
| 10   | β-TUB | ACT | 0.92 | 3.75 | 0.85 | 3.58 | 0.91 | 2.77 |
| 11   | F-box | F-box | 0.85 | 3.43 | 0.85 | 3.43 | 0.85 | 3.43 |

### Table 4. Expression stability of candidate reference genes as calculated by BestKeeper.

| Rank | Gene | Tissue | Drought | Salt | Heavy metal | Total |
|------|------|--------|---------|------|-------------|-------|
| 1    | GAPDH | Drought | 0.91 | 4.01 | 0.59 | 2.49 | 1.15 | 5.26 |
| 2    | DnaL | DnaL | 0.5 | 2.25 | 1.02 | 4.53 | 0.67 | 2.77 | 1.24 | 5.58 |
| 3    | MUB | H2A | 1.06 | 4.06 | 0.83 | 3.68 | 0.84 | 1.67 |
| 4    | SmCAT | H2A | 1.02 | 4.26 | 0.77 | 3.47 | 0.75 | 1.60 |
| 5    | HSP70 | ACT | 0.95 | 2.72 | 0.99 | 3.26 | 0.95 | 1.60 |
| 6    | α-TUB2 | H2A | 1.02 | 4.64 | 0.85 | 3.58 | 0.91 | 2.77 |
| 7    | GAPDH | MUB | 1.05 | 4.25 | 0.77 | 2.93 | 0.77 | 2.93 |
| 8    | α-TUB1 | HSP70 | 1.26 | 5.46 | 1.08 | 4.28 | 1.26 | 5.46 |
| 9    | ACT | F-box | 1.41 | 6.19 | 0.84 | 3.68 | 0.84 | 3.68 |
| 10   | β-TUB | ACT | 0.92 | 3.75 | 0.85 | 3.58 | 0.91 | 2.77 |
| 11   | F-box | F-box | 0.85 | 3.43 | 0.85 | 3.43 | 0.85 | 3.43 |

### Reference gene validation.

To validate the performance of the best ranked candidate reference genes, the expression patterns of SmCAT (catalase) were analyzed (Fig. 5). CAT as an abiotic stress inducible gene, is up-regulated by drought, salt, and Cd treatments. The CAT with low affinity towards H₂O₂ but with a high processing rate, can operate through a complex networking machinery to avoid damage caused by ROS. In this study, we used the most stable reference genes (α-TUB2 and DnaL) and the least stable gene (β-TUB) as internal controls for normalization of SmCAT according to the RefFinder rankings. The expression profiles of SmCAT were determined in different tissues and under drought, salt, and heavy metal stresses. When the stable reference genes α-TUB2 and DnaL were used for normalization, SmCAT exhibited similar expression trends. However, when the least stable reference gene β-TUB was used for normalization, the expression patterns of SmCAT were different from that obtained using the two stable reference genes.

### Discussion

Abiotic stress conditions including drought, salt, and heavy metals bring great losses to forestry productivity and increase the risk of environment. To guarantee sustainable forestry productivity and decrease the risk of environment, it is imperative to understand the regulation and function of the key genes under different abiotic stresses.

To study gene expression variations and determine gene regulation patterns, suitable reference genes are prerequisite to accurately determine the expression levels of target genes. qRT-PCR is a reliable and accurate technique for measuring gene expression levels. Some suitable reference genes under abiotic stresses, such as GAPDH, and DnaL, have been detected in plants; however, the number of reference genes evaluated is limited, especially for woody plants.

*S. matsudana* is an important afforestation and greening material in China that can adapt to harsh environments including drought, salt, and heavy metal. A good understanding of the molecular mechanisms related to abiotic stress responses in woody plants will not only help in improving forestry productivity but also help to decrease the risk of environment. A few studies have explored the ability of *S. matsudana* to withstand different abiotic stresses; however, the study of reference genes in willows has lagged behind that of other major plant species. To address this problem, we analyzed the expression of 11 candidate reference genes, five traditional reference genes (ACT, α-TUB1, α-TUB2, β-TUB, and GAPDH) and six new genes (DnaL, AR18, MUB, HSP70, F-box, and H2A), in various tissues, including the roots of *S. matsudana* under different abiotic stresses using...
| Method                                      | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 |
|--------------------------------------------|----|----|----|----|----|----|----|----|----|----|----|
| Ranking order under different tissues      |    |    |    |    |    |    |    |    |    |    |    |
| geNorm                                     | Dna1 | AR18 | MUB | HSP70 | α-TUB2 | H2A | β-TUB | F-box | α-TUB1 | ACT | GAPDH |
| NormFinder                                 | AR18 | Dna1 | HSP70 | MUB | α-TUB2 | H2A | β-TUB | α-TUB1 | ACT | F-box | GAPDH |
| Delta CT                                   | AR18 | Dna1 | HSP70 | MUB | α-TUB2 | β-TUB | H2A | α-TUB1 | ACT | F-box | GAPDH |
| BestKeeper                                 | Dna1 | F-box | MUB | AR18 | HSP70 | α-TUB2 | H2A | β-TUB | α-TUB1 | ACT | GAPDH |
| Comprehensive ranking                     | Dna1 | AR18 | MUB | HSP70 | α-TUB2 | F-box | H2A | β-TUB | α-TUB1 | ACT | GAPDH |
| Ranking order under drought stress         |    |    |    |    |    |    |    |    |    |    |    |
| geNorm                                     | Dna1 | α-TUB2 | MUB | ACT | H2A | α-TUB1 | AR18 | F-box | β-TUB | GAPDH | HSP70 |
| NormFinder                                 | Dna1 | α-TUB2 | MUB | ACT | H2A | AR18 | α-TUB1 | F-box | H2A | MUB | HSP70 |
| Delta CT                                   | α-TUB2 | Dna1 | MUB | ACT | AR18 | H2A | α-TUB1 | F-box | H2A | MUB | HSP70 |
| BestKeeper                                 | Dna1 | H2A | α-TUB2 | α-TUB1 | HSP70 | MUB | ACT | AR18 | F-box | β-TUB | GAPDH |
| Comprehensive ranking                     | α-TUB2 | MUB | H2A | ACT | α-TUB1 | AR18 | F-box | HSP70 | β-TUB | GAPDH | |
| Ranking order under salt stress            |    |    |    |    |    |    |    |    |    |    |    |
| geNorm                                     | α-TUB2 | MUB | AR18 | Dna1 | α-TUB1 | F-box | ACT | GAPDH | β-TUB | H2A | HSP70 |
| NormFinder                                 | α-TUB2 | MUB | AR18 | Dna1 | α-TUB1 | F-box | ACT | GAPDH | β-TUB | H2A | HSP70 |
| Delta CT                                   | α-TUB2 | Dna1 | MUB | ACT | AR18 | H2A | α-TUB1 | F-box | H2A | MUB | HSP70 |
| BestKeeper                                 | GAPDH | HSP70 | H2A | Dna1 | α-TUB2 | AR18 | β-TUB | ACT | α-TUB1 | F-box | MUB |
| Comprehensive ranking                     | α-TUB2 | MUB | H2A | ACT | α-TUB1 | AR18 | F-box | HSP70 | β-TUB | GAPDH | |
| Ranking order under heavy metal stress     |    |    |    |    |    |    |    |    |    |    |    |
| geNorm                                     | Dna1 | α-TUB2 | AR18 | MUB | F-box | H2A | HSP70 | α-TUB1 | ACT | GAPDH | β-TUB |
| NormFinder                                 | AR18 | Dna1 | MUB | H2A | F-box | α-TUB1 | ACT | HSP70 | GAPDH | β-TUB | |
| Delta CT                                   | α-TUB2 | Dna1 | MUB | H2A | F-box | α-TUB1 | ACT | HSP70 | GAPDH | β-TUB | |
| BestKeeper                                 | Dna1 | α-TUB2 | HSP70 | H2A | F-box | AR18 | MUB | α-TUB1 | GAPDH | ACT | β-TUB |
| Comprehensive ranking                     | α-TUB2 | AR18 | MUB | H2A | F-box | HSP70 | α-TUB1 | ACT | GAPDH | β-TUB | |

Table 5. Expression stability ranking of the 11 candidate reference genes as calculated by RefFinder.

qRT-PCR methods. The best and worst candidate reference genes were further verified by expression profiling of the defense response gene SmCAT.

We used five different statistical algorithms to determine the stabilities of candidate reference gene(s) under various abiotic stress conditions in *S. matsudana*. The results listed in Table 5 showed that, for the most parts, geNorm, NormFinder, ΔCT, and RefFinder consistently ranked the same genes as the most stable candidate reference genes. The BestKeeper algorithm is different from the other algorithms, which explains why the BestKeeper results showed the least correlation with the others61. Therefore, we selected the reference gene(s) determined by geNorm, NormFinder, ΔCT, and RefFinder.

α-TUB2 and Dna1 were the two most stable reference genes in all the sample sets according to the four algorithms. α-TUB2 encoding a cytoskeleton structure protein62 and Dna1 encoding a cellular chaperone have the ability to repair heat-induced protein machinery damage63,64. Our results are in agreement with several previous studies, which showed that α-TUB2 and Dna1 were established as the most stable reference genes in plants under abiotic stresses; for example, in *Syntrichia caninervis* under drought stress, salt, and heavy metal stresses65, *Corchorus capsularis* under drought stress66, *Buchloe dactyloides* under salt stress66, and *Platycladus orientalis* under salt stress67. Normalization with multiple reference genes is an effective way to avoid erroneous data that may result from using a single reference gene68. In this study, two top ranked reference genes, Dna1 and α-TUB2 under heavy metal stress and α-TUB2 and MUB under salt stress, were appropriate for gene expression normalization. Meanwhile, four reference genes, Dna1, α-TUB2, MUB, and ACT under drought stress, were needed for accurate normalization. Two reference genes were found to be sufficient to analyze the expression of target genes in sorghum62, jute10, and moss65.

Significant differences were revealed in the expression patterns of the target gene SmCAT when was normalized with the two most stable genes (α-TUB2 and Dna1) compared with one of the least stable genes (β-TUB) (Fig. 5). The results emphasize the importance of using stable reference genes for normalization. Our findings indicated that α-TUB2 and Dna1 either singly or in combination are suitable for normalization of gene expression in *S. matsudana* under different abiotic stresses. Consequently, we recommend α-TUB2 and Dna1 as the most...
Figure 4. Expression stability of 11 candidate reference genes as calculated by RefFinder. A lower Geomean value indicates more stable expression.

Figure 5. Relative quantification of SmCAT expression using validated reference genes.
suitable reference genes for normalization of qRT-PCR expression data in *S. matusdana* under diverse abiotic stress conditions.

To the best of our knowledge, this is the first report on the identification and validation of suitable reference genes for qRT-PCR analysis in *S. matusdana* under abiotic stresses.

**Conclusion**

To validate suitable reference genes for gene expression normalization in *S. matusdana* under drought, salt, and heavy metal stresses, we selected 11 candidate reference genes using four systematic statistical algorithms (geNorm, NormFinder, ΔCt, and BestKeeper). The obtained results were compared and ranked using RefFinder. Based on the gene stability analysis, we identified α-TUB2 and DnaJ as the most stable reference genes for normalization of gene expression under drought, salt, and heavy metal stress conditions. Furthermore, the expression profiles of SmtCAT validated α-TUB2 and DnaJ could be used as suitable reference genes. The reference genes identified in this study will facilitate accurate and consistent expression analysis of stress tolerance genes in willows and woody plants under various abiotic stress conditions for functional genomic studies.

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**Author Contributions**

Y.Z., X.H. conceived and designed the experiments. Y.Z., X.H., S.C., and L.Z. performed the experiments. Y.Z., X.H., M.L. and G.Q. analyzed the data, and Y.Z., X.H. wrote the manuscript and coordinated its revision. Y.W. and R.Z. contributed reagents/materials/funds support. All authors read and provided helpful discussions, and approved the final version.
Additional Information

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