SCIENTIFIC REPORTS

OPEN

Received: 30 August 2016
Accepted: 05 December 2016
Published: 25 January 2017

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

Yunxing Zhang[1,2,3,*], Xiaojiao Han[1,2,*], Shuangshuang Chen[1,2], Liu Zheng[1,2], Xuelian He[1,2], Mingying Liu[1,2], Guirong Qiao[1,2], Yang Wang[4] & Renying Zhuo[1,2]

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 worldwide[1–5]; however, plants need to thrive in adverse circumstances[6]. Plants with short growth cycles, such as *Arabidopsis thaliana*[7], soybean[8], sorghum[9], jute[10], *Sedum alfredii*[11], rice[12], and tobacco[13], 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 conditions[14–18].

The genus *Salix* (Salicaceae) contains more than 450 willow species worldwide; 275 of these species grow in China[19–22]. Willow species are used for energy production, afforestation, and greening due to their high biomass, rapid growth, and ability to adapt to different stress conditions[23–28]. *Salix matsudana* is a deciduous, rapidly growing willow species commonly cultivated in China, which can tolerate drought, salt, and heavy metal stresses[29–33]. 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

[1]State Key Laboratory of Tree Genetics and Breeding, Chinese Academy of Forestry, Beijing 100091, China. [2]Key Laboratory of Tree Breeding of Zhejiang Province, The Research Institute of Subtropical of Forestry, Chinese Academy of Forestry, Hangzhou, Zhejiang 311400, China. [3]School of Architectural and Artistic Design, Henan Polytechnic University, Jiaozuo, Henan 454000, China. [4]College of Plant Protection, Yunnan Agricultural University, Kunming, Yunnan 650201, China. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to Y.W. (email: wangyang626@sina.com) or R.Z. (email: zhuory@gmail.com)

molecular level[36–38]. Understanding the expression patterns of key stress response genes will help elucidate the mechanisms involved in various stresses of *S. matsudana*.

Gene expression analysis has been applied to understand different kinds of biological processes[39]. Quantitative real-time polymerase chain reaction (qRT-PCR) is widely used for gene expression analysis due to its high sensitivity, accuracy, specificity, and reproducibility[40–42]. However, factors such as sample amount, RNA integrity, reverse transcription efficiency, and cDNA quality can significantly influence the reliability of the gene expression results[43–45]. To reduce the influence of these factors, internal reference genes are used to obtain accurate biologically meaningful expression values[46]; however, unstable reference genes can cause significant biases and misinterpretations of the expression data[47,48]. Actin (*ACT*) and β-tubulin (β-*TUB*) have been used as reference genes for qRT-PCR normalization in gene expression analysis in *S. matsudana* under salt and copper stresses[37,49]; however, a systematic study to validate reference genes has not been reported for *S. matsudana* 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. matsudana* 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 ARI8 (*ARI8*), F-box family protein (*F-box*), histone H2A (*H2A*), heat shock 70 kDa protein (*HSP 70*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), membrane-anchored ubiquitin-fold protein (*MUB*), and β-*TUB*. The transcriptome data of *S. matsudana* 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[43], NormFinder[44], BestKeeper[50], ΔCt method[51], and RefFinder, a web-based software[52]. The expression levels of the defense response gene *SmCAT* (catalase) as a target gene were assayed to verify the selected reference genes. The results will provide suitable reference genes for qRT-PCR normalization for accurate gene expression analysis in *S. matsudana* under different stress conditions.

Materials and Methods

Plant materials and stress treatments. Cuttings (approximately 10 cm long) from annual branches of *S. matsudana* were grown in hydroponics. Plants were supplemented with water containing 1/4 strength Hoagland[53] solution on alternate days under normal conditions (25 °C, 16 h light/8 h dark). After 45 days of culture, groups of *S. matsudana* 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 $CdCl_2$). 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 $\geq$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. matsudana* transcriptome data. Primers were designed based on the sequences the 11 genes 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 (Table 1). 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 ddH_2O. 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 $E = [5^{-1/\text{slope}} - 1] \times 100$. Stabilities of the 11 selected reference genes were evaluated by four algorithms—geNorm, NormFinder, BestKeeper, and the ΔCt method. Finally, RefFinder (http://www.

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
			TCCATATCATCCCAGTTGCT			
α-TUB1	alpha-tubulin1	SapurV1A.0005s0080	GAGGATGAAGACGGTGAGGA	197	92.6	0.9995
			GAAGCAAAGGGAGACAGTCG			
α-TUB2	alpha-tubulin2	SapurV1A.0598s0030	ACTACGAGGAAGTCGGAGCA	205	91.0	0.9974
			CAACAAGAACGGAAGCAACA			
DnaJ	chaperone protein DnaJ 49	SapurV1A.0212s0110	GCACCAAATTTGAGCAGGAT	137	101.6	0.9919
			TACAAAACCCCACTGCTTCC			
ARI8	E3 ubiquitin-protein ligase ARI8	SapurV1A.0557s0250	GTAGACGATGCCCCAAGAAA	198	92.9	0.9997
			GGATGCCCTCAAACAAACAT			
F-box	F-box family protein	SapurV1A.1078s0140	CCTGCAACTGCCAGACTACA	121	97.2	0.991
			ACAAGGATTTTCCCCCAAAC			
H2A	histone H2A	SapurV1A.2339s0010	TTGTGCTCCTGTAACGGTGA	165	99.5	0.9979
			AACACCATTGCCCACTTCTC			
HSP 70	heat shock 70 kDa protein	SapurV1A.1370s0010	GTGGAGGTGATGGTGCTTCT	124	95.0	0.9940
			TGAGAGCCGTGTCAAAAATG			
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	SapurV1A.0266s0210	CAGCTGATGAGGAATGCAAA	196	96.2	0.9931
			AGCATTGTTTGGAAGCTTGG			
MUB	membrane-anchored ubiquitin-fold protein	SapurV1A.2454s0040	ATTCAGTCCCAGCTGTCGTT	214	94.5	0.9919
			CGGAATTCCAGAGTGGAAAA			
β-TUB	tubulin beta chain	SapurV1A.1459s0040	CGAGGAAGGCGAGTATGAAG	196	94.1	0.9971
			TGAGCACACCCAGAAACAAG			
Target gene						
SmCAT	catalase	SapurV1A.0016s0660	CACCGAAGCTCAATGTTTCA	190	93.3	0.9978
			GGGCACAGAGCTTGCATTTA			

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

fulxie.0fees.us), a comprehensive evalution 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 (*α-TUB2* and *DnaJ*), as well as one of the least stable reference genes (*β-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.

To evaluate the stability of the 11 candidate reference genes at the transcript level under the three abiotic stress conditions, the gene expression levels were determined by the average Ct values, which varied from 17 to 30 (Fig. 1). According to the average Ct values of all the samples, *α-TUB1* was the most abundantly expressed gene, followed by *DnaJ*, *α-TUB2*, and *F-box*, while *H2A* was the least abundantly expressed gene, followed by *β-TUB, ACT* and *MUB*.

Analysis of gene expression stability. Expression stabilities of the 11 candidate reference genes were determined using geNorm, NormFinder, ΔCt, 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 Fig. 2. 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 (Fig. 2a). The top two most stable genes were *DnaJ* and *α-TUB2* for drought and heavy metal

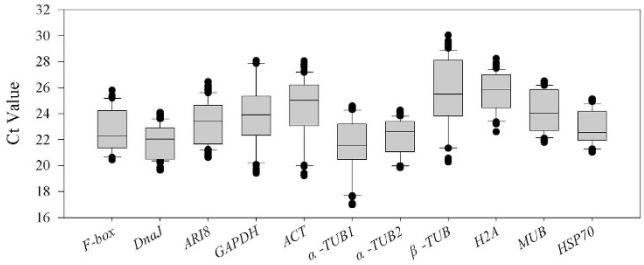


Figure 1. Expression levels of 11 candidate reference genes across all experimental samples.

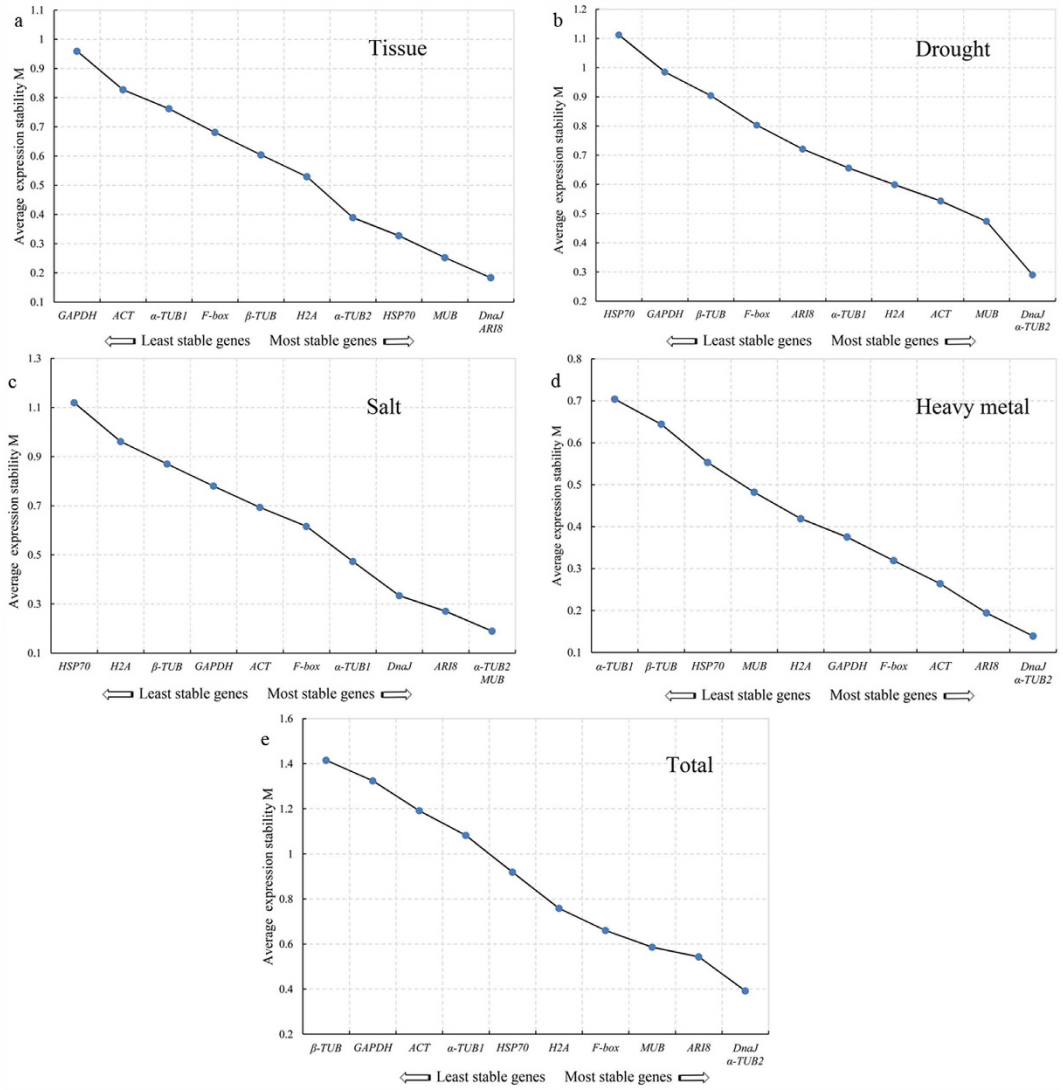
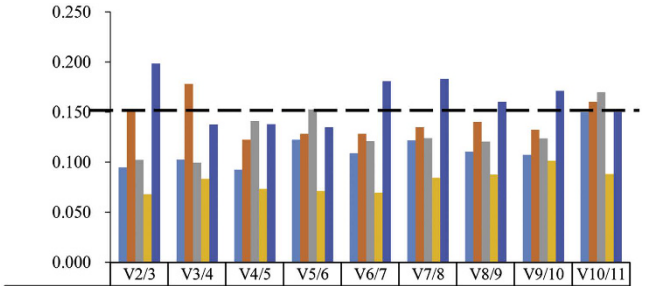


Figure 2. Expression stability of 11 candidate genes as calculated by geNorm. (a) different tissues, (b) drought treatments, (c) salt treatments, (d) heavy metal treatments, (e) all samples..

stresses, and α-*TUB2* and *MUB* for salt stress (Fig. 2b,c,d). When the stabilities from all the samples were combined, *DnaJ* and α-*TUB2* were determined to be the most stable reference genes in all the samples (Fig. 2e), while β-*TUB* had the less stability.

The pairwise variation (V_n/V_{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



	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8	V8/9	V9/10	V10/11
Tissue	0.095	0.103	0.093	0.123	0.109	0.122	0.110	0.107	0.150
Drought	0.151	0.178	0.123	0.128	0.128	0.135	0.140	0.132	0.161
Salt	0.102	0.099	0.141	0.153	0.121	0.124	0.120	0.124	0.170
Heavy metal	0.068	0.083	0.073	0.071	0.069	0.085	0.088	0.101	0.088
Total	0.199	0.138	0.138	0.135	0.181	0.183	0.160	0.171	0.150

Figure 3. Determination of the optimal number of reference genes for normalization by pairwise variation (V) using geNorm. The average pairwise variations (Vn/Vn+1) were analyzed to measure the effect of adding reference gene on the qRT-PCR.

	Tissue		Drought		Salt		Heavy metal		Total	
Rank	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*ARI8*	0.179	*DnaJ*	0.099	*DnaJ*	0.073	*α-TUB2*	0.234	*α-TUB2*	0.388
2	*DnaJ*	0.272	*α-TUB2*	0.145	*MUB*	0.095	*DnaJ*	0.259	*ARI8*	0.392
3	*HSP70*	0.305	*MUB*	0.278	*α-TUB2*	0.255	*ACT*	0.360	*DnaJ*	0.442
4	*MUB*	0.426	*ACT*	0.360	*α-TUB1*	0.362	*ARI8*	0.367	*MUB*	0.578
5	*α-TUB2*	0.486	*H2A*	0.525	*ARI8*	0.383	*H2A*	0.418	*H2A*	0.73
6	*H2A*	0.500	*ARI8*	0.660	*ACT*	0.777	*HSP70*	0.474	*F-box*	0.869
7	*β-TUB*	0.526	*α-TUB1*	0.771	*F-box*	0.899	*GAPDH*	0.482	*α-TUB1*	1.142
8	*α-TUB1*	0.863	*F-box*	1.015	*GAPDH*	1.018	*F-box*	0.594	*ACT*	1.279
9	*ACT*	1.037	*β-TUB*	1.369	*H2A*	1.107	*MUB*	0.669	*HSP70*	1.293
10	*F-box*	1.061	*HSP70*	1.397	*β-TUB*	1.352	*β-TUB*	0.829	*GAPDH*	1.655
11	*GAPDH*	1.514	*GAPDH*	1.487	*HSP70*	1.565	*α-TUB1*	0.861	*β-TUB*	1.755

Table 2. Expression stability of candidate reference genes as calculated by Normfinder.

drought stress samples $V_{4/5}$ 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 $V_{3/4}$ 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* was the most stable gene in the tissue and drought subsets, while *GAPDH* 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.

Rank	Tissue		Drought		Salt		Heavy metal		Total	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*ARI8*	0.69	*α-TUB2*	0.95	*MUB*	0.94	*α-TUB2*	0.58	*α-TUB2*	1.18
2	*DnaJ*	0.71	*DnaJ*	0.99	*α-TUB2*	0.99	*DnaJ*	0.59	*DnaJ*	1.20
3	*HSP70*	0.77	*MUB*	0.99	*DnaJ*	0.99	*ARI8*	0.61	*ARI8*	1.21
4	*MUB*	0.78	*ACT*	1.01	*ARI8*	1.02	*ACT*	0.62	*MUB*	1.27
5	*α-TUB2*	0.82	*ARI8*	1.09	*α-TUB1*	1.08	*H2A*	0.70	*H2A*	1.37
6	*β-TUB*	0.88	*H2A*	1.10	*ACT*	1.23	*GAPDH*	0.71	*F-box*	1.41
7	*H2A*	0.89	*α-TUB1*	1.21	*F-box*	1.28	*HSP70*	0.75	*α-TUB1*	1.60
8	*α-TUB1*	1.07	*F-box*	1.25	*H2A*	1.40	*F-box*	0.75	*ACT*	1.65
9	*ACT*	1.19	*β-TUB*	1.53	*GAPDH*	1.42	*MUB*	0.84	*HSP70*	1.67
10	*F-box*	1.19	*HSP70*	1.60	*β-TUB*	1.63	*β-TUB*	0.97	*GAPDH*	1.94
11	*GAPDH*	1.62	*GAPDH*	1.61	*HSP70*	1.89	*α-TUB1*	0.99	*β-TUB*	1.98

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

Rank	Tissue			Drought			Salt			Heavy metal			Total		
	Gene	SD	CV	Gene	SD	CV	Gene	SD	CV	Gene	SD	CV	Gene	SD	CV
1	*DnaJ*	0.44	2.16	*DnaJ*	0.5	2.25	*α-TUB2*	0.91	4.01	*GAPDH*	0.59	2.49	*DnaJ*	1.15	5.26
2	*F-box*	0.5	2.36	*H2A*	0.59	2.22	*DnaJ*	1.02	4.53	*HSP70*	0.67	2.77	*α-TUB2*	1.24	5.58
3	*MUB*	0.53	2.36	*α-TUB2*	0.62	2.72	*ARI8*	1.02	4.26	*DnaJ*	0.77	3.47	*HSP70*	1.26	5.46
4	*ARI8*	0.55	2.56	*α-TUB1*	0.67	3.03	*MUB*	1.05	4.25	*H2A*	0.77	2.93	*H2A*	1.31	5.1
5	*HSP70*	0.72	3.26	*HSP70*	0.78	3.35	*H2A*	1.06	4.06	*α-TUB2*	0.83	3.68	*F-box*	1.41	6.19
6	*α-TUB2*	0.73	3.51	*MUB*	0.94	3.8	*α-TUB1*	1.47	6.46	*ARI8*	0.85	3.58	*ARI8*	1.43	6.13
7	*H2A*	0.97	4.04	*ACT*	1.1	4.38	*F-box*	1.48	6.32	*β-TUB*	0.85	3.43	*MUB*	1.45	5.97
8	*β-TUB*	1.02	4.78	*ARI8*	1.2	4.96	*GAPDH*	1.62	6.5	*ACT*	0.92	3.75	*α-TUB1*	2.08	9.91
9	*α-TUB1*	1.31	7.26	*H2A*	0.59	2.22	*HSP70*	1.64	6.89	*α-TUB1*	0.97	4.44	*GAPDH*	2.31	9.87
10	*ACT*	1.51	7.34	*α-TUB2*	0.62	2.72	*ACT*	1.89	7.33	*F-box*	1.1	4.84	*ACT*	2.35	9.84
11	*GAPDH*	1.57	7.71	*α-TUB1*	0.67	3.03	*β-TUB*	2.21	8.22	*MUB*	1.22	4.83	*β-TUB*	2.62	10.54

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

Reference gene validation. To validate the performance of the best ranked candidate reference genes, the expression patterns of *SmCAT* (catalase) were analyzed (Fig. 5). C*AT* as abiotic stress inducible genes, are up-regulated by drought[54], salt[55], and Cd[56] treatments. The *CAT* with low affinity towards H_2O_2 but with a high processing rate[57], can operate through a complex networking machinery to avoid damage caused by ROS[58]. In this study, we used the most stable reference genes (*α-TUB2* and *DnaJ*) 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 *DnaJ* 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*[59,60] and *DnaJ*[10], 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 (*DnaJ*, *ARI8*, *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 (Better-Good-Average)											
geNorm	*DnaJ* | *ARI8*		*MUB*	*HSP70*	*α-TUB2*	*H2A*	*β-TUB*	*F-box*	*α-TUB1*	*ACT*	*GAPDH*
NormFinder	*ARI8*	*DnaJ*	*HSP70*	*MUB*	*α-TUB2*	*H2A*	*β-TUB*	*α-TUB1*	*ACT*	*F-box*	*GAPDH*
Delta CT	*ARI8*	*DnaJ*	*HSP70*	*MUB*	*α-TUB2*	*β-TUB*	*H2A*	*α-TUB1*	*ACT*	*F-box*	*GAPDH*
BestKeeper	*DnaJ*	*F-box*	*MUB*	*ARI8*	*HSP70*	*α-TUB2*	*H2A*	*β-TUB*	*α-TUB1*	*ACT*	*GAPDH*
Comprehensive ranking	***DnaJ***	***ARI8***	***MUB***	***HSP70***	***α-TUB2***	***F-box***	***H2A***	***β-TUB***	***α-TUB1***	***ACT***	***GAPDH***
Ranking order under drought stress (Better-Good-Average)											
geNorm	*DnaJ* | *α-TUB2*		*MUB*	*ACT*	*H2A*	*α-TUB1*	*ARI8*	*F-box*	*β-TUB*	*GAPDH*	*HSP70*
NormFinder	*DnaJ*	*α-TUB2*	*MUB*	*ACT*	*H2A*	*ARI8*	*α-TUB1*	*F-box*	*β-TUB*	*HSP70*	*GAPDH*
Delta CT	*α-TUB2*	*DnaJ*	*MUB*	*ACT*	*ARI8*	*H2A*	*α-TUB1*	*F-box*	*β-TUB*	*HSP70*	*GAPDH*
BestKeeper	*DnaJ*	*H2A*	*α-TUB2*	*α-TUB1*	*HSP70*	*MUB*	*ACT*	*ARI8*	*F-box*	*β-TUB*	*GAPDH*
Comprehensive ranking	***DnaJ***	***α-TUB2***	***MUB***	***H2A***	***ACT***	***α-TUB1***	***ARI8***	***F-box***	***HSP70***	***β-TUB***	***GAPDH***
Ranking order under salt stress (Better-Good-Average)											
geNorm	*α-TUB2* | *MUB*		*ARI8*	*DnaJ*	*α-TUB1*	*F-box*	*ACT*	*GAPDH*	*β-TUB*	*H2A*	*HSP70*
NormFinder	*DnaJ*	*MUB*	*α-TUB2*	*α-TUB1*	*ARI8*	*ACT*	*F-box*	*GAPDH*	*H2A*	*β-TUB*	*HSP70*
Delta CT	*MUB*	*α-TUB2*	*DnaJ*	*ARI8*	*α-TUB1*	*ACT*	*F-box*	*GAPDH*	*H2A*	*β-TUB*	*HSP70*
BestKeeper	*α-TUB2*	*DnaJ*	*ARI8*	*MUB*	*H2A*	*α-TUB1*	*F-box*	*GAPDH*	*HSP70*	*ACT*	*β-TUB*
Comprehensive ranking	***α-TUB2***	***MUB***	***DnaJ***	***ARI8***	***α-TUB1***	***F-box***	***ACT***	***H2A***	***GAPDH***	***β-TUB***	***HSP70***
Ranking order under heavy metal stress (Better-Good-Average)											
geNorm	*DnaJ* | *α-TUB2*		*ARI8*	*ACT*	*F-box*	*GAPDH*	*H2A*	*MUB*	*HSP70*	*β-TUB*	*α-TUB1*
NormFinder	*α-TUB2*	*DnaJ*	*ACT*	*ARI8*	*H2A*	*HSP70*	*GAPDH*	*F-box*	*MUB*	*β-TUB*	*α-TUB1*
Delta CT	*α-TUB2*	*DnaJ*	*ARI8*	*ACT*	*H2A*	*GAPDH*	*HSP70*	*F-box*	*MUB*	*β-TUB*	*α-TUB1*
BestKeeper	*GAPDH*	*HSP70*	*H2A*	*DnaJ*	*α-TUB2*	*ARI8*	*β-TUB*	*ACT*	*α-TUB1*	*F-box*	*MUB*
Comprehensive ranking	***α-TUB2***	***DnaJ***	***GAPDH***	***ARI8***	***ACT***	***H2A***	***HSP70***	***F-box***	***MUB***	***β-TUB***	***α-TUB1***
Ranking order under total samples (Better-Good-Average)											
geNorm	*DnaJ* | *α-TUB2*		*ARI8*	*MUB*	*F-box*	*H2A*	*HSP70*	*α-TUB1*	*ACT*	*GAPDH*	*β-TUB*
NormFinder	*α-TUB2*	*ARI8*	*DnaJ*	*MUB*	*H2A*	*F-box*	*α-TUB1*	*ACT*	*HSP70*	*GAPDH*	*β-TUB*
Delta CT	*α-TUB2*	*DnaJ*	*ARI8*	*MUB*	*H2A*	*F-box*	*α-TUB1*	*ACT*	*HSP70*	*GAPDH*	*β-TUB*
BestKeeper	*DnaJ*	*α-TUB2*	*HSP70*	*H2A*	*F-box*	*ARI8*	*MUB*	*α-TUB1*	*GAPDH*	*ACT*	*β-TUB*
Comprehensive ranking	***α-TUB2***	***DnaJ***	***ARI8***	***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 others[61]. Therefore, we selected the reference gene(s) determined by geNorm, NormFinder, ΔCt, and RefFinder.

α-TUB2 and *DnaJ* were the two most stable reference genes in all the sample sets according to the four algorithms. *α-TUB2* encoding a cytoskeleton structure protein[62] and *DnaJ* encoding a cellular chaperone have the ability to repair heat-induced protein machinery damage[63,64]. Our results are in agreement with several previous studies, which showed that *α-TUB2* and *DnaJ* were established as the most stable reference genes in plants under abiotic stresses; for example, in *Syntrichia caninervis* under drought, salt, and heavy metal stresses[65], *Corchorus capsularis* under drought stress[10], *Buchloe dactyloides* under salt stress[66], *and Platycladus orientalis* under salt stress[67]. Normalization with multiple reference genes is an effective way to avoid erroneous data that may result from using a single reference gene[68]. In this study, two top ranked reference genes, *DnaJ* 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, *DnaJ*, *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 sorghum[62], jute[10], and moss[65].

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 *DnaJ*) 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 *DnaJ* 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 *DnaJ* as the most

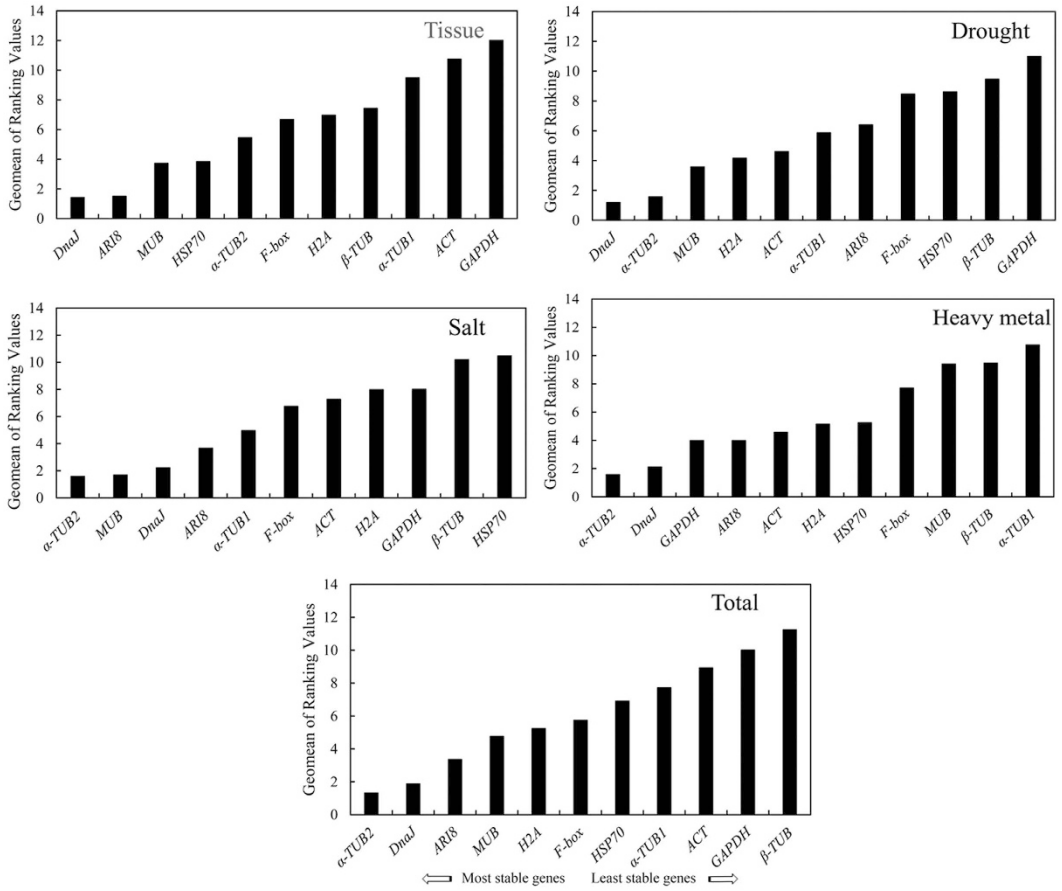


Figure 4. Expression stability of 11 candidate reference genes as calculated by RefFinder. A lower Geomean value indicates more stable expression.

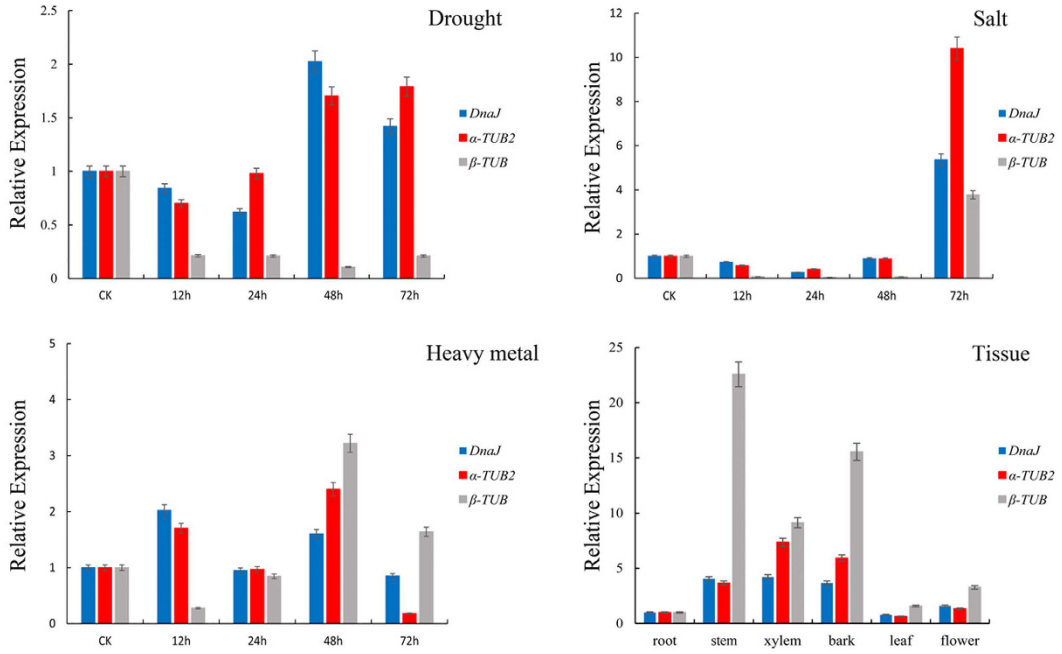


Figure 5. Relative quantification of *SmCAT* expression using validated reference genes.

suitable reference genes for normalization of qRT-PCR expression data in *S. matsudana* 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. matsudana* under abiotic stresses.

Conclusion

To validate suitable reference genes for gene expression normalization in *S. matsudana* 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 *SmCAT* 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.

References

1. Farouk, S., Mosa, A. A., Taha, A. A. & Ibrahim, H. M. Protective effect of humic acid and chitosan on radish (*Raphanus sativus*, L. var. sativus) plants subjected to cadmium stress. *Journal of Stress Physiology & Biochemistry* **7** (2011).
2. Agarwal, P. K., Agarwal, P., Reddy, M. K. & Sopory, S. K. Role of *DREB* transcription factors in abiotic and biotic stress tolerance in plants. *Plant Cell Reports* **25,** 1263–1274 (2006).
3. Burke, E. J., Brown, S. J. & Christidis, N. Modeling the recent evolution of global drought and projections for the twenty-first century with the Hadley Centre Climate Model. *Journal of Hydrometeorology* **7,** 1113 (2006).
4. Shao, H.-B., Chu, L.-Y., Jaleel, C. A. & Zhao, C.-X. Water-deficit stress-induced anatomical changes in higher plants. *Comptes Rendus Biologies* **331,** 215–225 (2008).
5. Zhang, J. *et al.* The heat shock factor gene family in *Salix suchowensis*: a genome-wide survey and expression profiling during development and abiotic stresses. *Frontiers in Plant Science* **6,** 748 (2014).
6. Shivhare, R. & Lata, C. Selection of suitable reference genes for assessing gene expression in pearl millet under different abiotic stresses and their combinations. *Scientific Reports* **6** (2016).
7. Remans, T. *et al.* Normalisation of real-time RT-PCR gene expression measurements in *Arabidopsis thaliana* exposed to increased metal concentrations. *Planta* **227,** 1343–9 (2008).
8. Hu, R., Fan, C., Li, H., Zhang, Q. & Fu, Y. F. Evaluation of putative reference genes for gene expression normalization in soybean by quantitative real-time RT-PCR. *Bmc Molecular Biology* **10,** 93–93 (2009).
9. Reddy, P. S. *et al.* Evaluation of sorghum [*Sorghum bicolor*(L.)] reference genes in various tissues and under abiotic stress conditions for quantitative real-time PCR data normalization. *Frontiers in Plant Science* **7** (2016).
10. Niu, X. *et al.* Selection of reliable reference genes for quantitative real-time PCR gene expression analysis in Jute (*Corchorus capsularis*) under stress treatments. *Frontiers in Plant Science* **6,** 848 (2015).
11. Sang, J. *et al.* Selection and validation of reference genes for real-time quantitative PCR in hyperaccumulating ecotype of *Sedum alfredii* under different heavy metals stresses. *Plos One* **8,** e82927–e82927 (2013).
12. Jain, M., Nijhawan, A., Tyagi, A. K. & Khurana, J. P. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. *Biochemical & Biophysical Research Communications* **345,** 646–651 (2006).
13. Schmidt, G. W. & Delaney, S. K. Stable internal reference genes for normalization of real-time RT-PCR in tobacco (*Nicotiana tabacum*) during development and abiotic stress. *Molecular Genetics & Genomics* **283,** 233–241 (2010).
14. Basa, B. Housekeeping gene selection in poplar plants under Cd-stress: comparative study for real-time PCR normalization. *Functional Plant Biology* **36,** 1079–1087 (2009).
15. Fries, J., Pfeiffer, S., Kuffner, M. & Sessitsch, A. Spirosoma endophyticum sp. nov., isolated from Zn- and Cd-accumulating *Salix caprea*. *International Journal of Systematic & Evolutionary Microbiology* **63,** 4586–90 (2013).
16. He, J. *et al.* A transcriptomic network underlies microstructural and physiological responses to cadmium in *Populus x canescens*. *Plant Physiology* **162,** 424–39 (2013).
17. Atkinson, N. J. & Urwin, P. E. The interaction of plant biotic and abiotic stresses: from genes to the field. *Journal of Experimental Botany* **63,** 3523–43 (2012).
18. Seki, M., Kamei, A., Yamaguchi-Shinozaki, K. & Shinozaki, K. Molecular responses to drought, salinity and frost: common and different paths for plant protection. *Current Opinion in Biotechnology* **14,** 194–199 (2003).
19. Newsholme, C. Willows: the genus *Salix* (B.T. Batsford, 1992).
20. Argus, G. W. Infrageneric classification of *Salix* (Salicaceae) in the new world. *Systematic Botany Monographs* **52,** 1–121 (1997).
21. USA, F.o.N.A.E.C. Flora of North America, Volume 7. Magnoliophyta: Salicaceae to Brassicaceae (2010).
22. Zhang, J. *et al.* A high-density genetic map of tetraploid *Salix matsudana* using specific length amplified fragment sequencing (SLAF-seq). *Plos One* **11** (2016).
23. Gullberg, U. Towards making willows pilot species for coppicing production. *Forestry Chronicle* **69,** 721–726 (1993).
24. Åhman, I., Larsson, S. & Dietrichson, J. Genetic improvement of willow (*Salix*) as a source of bioenergy. *Norwegian Journal of Agricultural Sciences* (1994).
25. Lindegaard, K. N. & Barker, J. H. A. Breeding willows for biomass. *Aspects of Applied Biology* 155–162 (1997).
26. Greger, M. & Landberg, T. Use of willow in phytoextraction. *International Journal of Phytoremediation* **1,** 115–123 (2006).
27. Aronsson, P., Dahlin, T. & Dimitriou, I. Treatment of landfill leachate by irrigation of willow coppice – Plant response and treatment efficiency. *Environmental Pollution* **158,** 795–804 (2010).
28. Mirck, J. & Volk, T. A. Mass balances and allocation of salt ions from Solvay storm water for shrub willow (*Salix spp.*). *Biomass & Bioenergy* **39,** 427–438 (2012).
29. Yang, J., Yi, J., Yang, C. & Li, C. Agrobacterium tumefaciens-mediated genetic transformation of *Salix matsudana* Koidz. using mature seeds. *Tree Physiology* **33,** 628–639 (2013).
30. Hangs, R. D., Schoenau, J. J., Rees, K. C. J. V. & Steppuhn, H. Examining the salt tolerance of willow (*Salix spp.*) bioenergy species for use on salt-affected agricultural lands. *Canadian Journal of Plant Science* **91,** 509–517 (2011).
31. Pulford, I. D. & Watson, C. Phytoremediation of heavy metal-contaminated land by trees—a review. *Environment International* **29,** 529–40 (2003).
32. Peng, X., Fan, J., Wang, Q. & Warrington, D. Discrepancy of sap flow in *Salix matsudana* grown under different soil textures in the water-wind erosion crisscross region on the Loess Plateau. *Plant & Soil* **390,** 383–399 (2015).
33. Yang, W. D., Chen, Y. T. & Ming-Hua, Q. U. Subcellular distribution and chemical forms of cadmium in *Salix matsudana*. *Acta Botanica Boreali-Occidentalia Sinica* **29,** 1394–1399 (2009).
34. Hai-Yan, W. U. *et al.* Cadmium tolerance of and cadmium transportation and accumulation in *Salix matsudana*. *Chinese Journal of Ecology* **30,** 1222–1228 (2011).

35. Surhone, L. M., Tennoe, M. T. & Henssonow, S. F. *Salix Matsudana* (Betascript Publishing, 2013).
36. Rao, G. *et al.* De novo transcriptome and small RNA analysis of two Chinese willow cultivars reveals stress response genes in *Salix matsudana*. *Plos One* **9,** 134–134 (2014).
37. Yang, J. *et al.* Overexpression of the *Tamarix hispida ThMT 3* gene increases copper tolerance and adventitious root induction in *Salix matsudana* Koidz. *Plant Cell Tissue & Organ Culture* **121,** 469–479 (2015).
38. Konlechner, C. *et al.* Expression of zinc and cadmium responsive genes in leaves of willow (*Salix caprea* L.) genotypes with different accumulation characteristics. *Environmental Pollution* **178,** 121–127 (2013).
39. Bustin, S. A., Benes, V., Nolan, T. & Pfaffl, M. W. Quantitative real-time RT-PCR–a perspective. *Journal of Molecular Endocrinology* **34,** 597–601 (2005).
40. Gachon, C., Mingam, A. & Charrier, B. Real-time PCR: what relevance to plant studies? *Journal of Experimental Botany* **55,** 1445–54 (2004).
41. Kumar, K., Muthamilarasan, M. & Prasad, M. Reference genes for quantitative real-time PCR analysis in the model plant foxtail millet (*Setaria italica* L.) subjected to abiotic stress conditions. *Plant Cell Tissue & Organ Culture* **115,** 13–22 (2013).
42. Bustin, S. A. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology* **25,** 169–93 (2000).
43. Vandesompele, J. *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3,** research0034.1-research0034.11 (2002).
44. Andersen, C. L., Jensen, J. L. & Ø, T. F. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research* **64,** 5245–50 (2004).
45. Janská, A. *et al.* The choice of reference gene set for assessing gene expression in barley (*Hordeum vulgare* L.) under low temperature and drought stress. *Mgg Molecular & General Genetics* **288,** 639–49 (2013).
46. Gutierrez, L. *et al.* The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. *Plant Biotechnology Journal* **6,** 609–18 (2008).
47. Ferguson, B. S., Nam, H., Hopkins, R. G. & Morrison, R. F. Impact of reference gene selection for target gene normalization on experimental outcome using real-time qRT-PCR in Adipocytes. *Plos One* **5,** 5525–5556 (2010).
48. Mafra, V. *et al.* Reference genes for accurate transcript normalization in citrus genotypes under different experimental conditions. *Plos One* **7,** e31263–e31263 (2012).
49. Qiao, G. *et al.* Comparative proteomic analysis of responses to salt stress in Chinese willow (*Salix matsudana* Koidz). *Plant Molecular Biology Reporter* **32,** 814–827 (2014).
50. Pfaffl, M. W., Tichopad, A., Prgomet, C. & Neuvians, T. P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. *Biotechnology Letters* **26,** 509–515 (2004).
51. Silver, N., Best, S., Jiang, J. & Thein, S. L. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *Bmc Molecular Biology* **7,** 1–9 (2006).
52. Xie, F., Peng, X., Chen, D., Xu, L. & Zhang, B. miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Molecular Biology* **80,** 75–84 (2012).
53. Hoagland, D. R. Minerals, Plants, and Men. (Book Reviews: Lectures on the inorganic nutrition of plants). *Scientific Monthly* **59** (1944).
54. Yang, G., Zhang, X. Q. & Xie, W. G. Study on the drought tolerance of dactylis glomerata Lines. *Hubei Agricultural Sciences* (2007).
55. Fadzillah, N. A. M., Gill, V., Finch, R. P. & Burdon, R. H. Chilling, oxidative stress and antioxidant responses in shoot cultures of rice. *Journal of Experimental Botany* **199,** 552–556 (1996).
56. Singh, H. P., Batish, D. R., Kaur, G., Arora, K. & Kohli, R. K. Nitric oxide (as sodium nitroprusside) supplementation ameliorates Cd toxicity in hydroponically grown wheat roots. *Environmental & Experimental Botany* **63,** 158–167 (2008).
57. Scandalios, J. G. Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. *Brazilian Journal of Medical & Biological Research* **38,** 995–1014 (2005).
58. Arora, A., Sairam, R. K. & Srivastava, G. C. Oxidative stress and antioxidative system in plants. *Soviet Physics Doklady* **16,** 1227–1238 (2002).
59. Sinha, P., Saxena, R. K., Singh, V. K., Krishnamurthy, L. & Varshney, R. K. Selection and validation of housekeeping genes as reference for gene expression studies in pigeonpea (*Cajanus cajan*) under heat and salt stress conditions. *Frontiers in Plant Science* **6** (2015).
60. Galli, V. *et al.* Validation of reference genes for accurate normalization of gene expression for real time-quantitative PCR in strawberry fruits using different cultivars and osmotic stresses. *Gene* **554,** 205–14 (2015).
61. Guo, J., Hui, L., Wu, Q., Xu, L. & Que, Y. The choice of reference genes for assessing gene expression in sugarcane under salinity and drought stresses. *Scientific Reports* **4,** 7042–7042 (2014).
62. Palakolanu, S. R. *et al.* Evaluation of sorghum [*Sorghum bicolor*(L.)] reference genes in various tissues and under abiotic stress conditions for quantitative real-time PCR data normalization. *Frontiers in Plant Science* **7** (2016).
63. Cheetham, M. E. & Caplan, A. J. Structure, function and evolution of *DnaJ*: conservation and adaptation of chaperone function. *Cell Stress & Chaperones* **3,** 28–36 (1998).
64. Schröder, H., Langer, T., Hartl, F. U. & Bukau, B. *DnaK, DnaJ* and *GrpE* form a cellular chaperone machinery capable of repairing heat-induced protein damage. *Embo Journal* **12,** 4137–44 (1993).
65. Li, X. *et al.* Characterization of reference genes for RT-qPCR in the desert moss *Syntrichia caninervis* in response to abiotic stress and desiccation/rehydration. *Frontiers in Plant Science* **6,** 38 (2015).
66. Li, W., Qian, Y. Q., Han, L., Liu, J. X. & Sun, Z. Y. Identification of suitable reference genes in buffalo grass for accurate transcript normalization under various abiotic stress conditions. *Gene* **547,** 55–62 (2014).
67. Chang, E. *et al.* Selection of reference genes for quantitative gene expression studies in *Platycladus orientalis* (Cupressaceae) using real-time PCR. *Plos One* **7,** 65–65 (2012).
68. Le, D. T. *et al.* Evaluation of candidate reference genes for normalization of quantitative RT-PCR in soybean tissues under various abiotic stress conditions. *Plos One* **7,** 1602–1603 (2012).

Acknowledgements

This work was supported by the National Nonprofit Institute Research Grant of Chinese Academy of Forestry (CAFYBB2017ZY007, No. TGB2013008, No. RISF2014010), the National High Technology Research and Development Program of China (No. 2013AA102701-3), the Science and Technology Department of Zhejiang Province (No. 2016C32027), and a Nonprofit Research Grant of Zhejiang Province (No. 2016C32G3030016).

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., 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

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zhang, Y. *et al.* Selection of suitable reference genes for quantitative real-time PCR gene expression analysis in *Salix matsudana* under different abiotic stresses. *Sci. Rep.* **7**, 40290; doi: 10.1038/srep40290 (2017).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.