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Identification and Validation of Reference Genes for Quantitative Real-Time PCR in
Corydalis saxicola Under Various Abiotic Stresses

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Abstract

Quantitative real-time PCR (qRT-PCR) is highly regarded for its exceptional sensitivity and specificity, making it a popular method for analyzing gene expression. The choice of suitable reference genes (RGs) is crucial for accurately assessing the dynamics of target gene expression through qRT-PCR. This study analyzes a set of eleven candidate RGs and focuses on identifying the most appropriate ones for examining gene expression in *Corydalis saxicola* across various tissues and under different abiotic stress conditions,

including calcium (Ca^{2+}) stress, varying selenium (Se^{2+}) stresses, and distinct drought stresses. *GAPDH2* and *UBCE2-17* emerged as the most appropriate reference genes across various tissues of *C. saxicola*. In response to Ca^{2+} stress, *RPAP3* and *UBCE2-5A* demonstrated the highest expression stability. Under Se^{2+} stress, *GAPDH8* and α -*TUB* were identified as the optimal reference genes, while *ACT4* and *RPAP3* were deemed the best under drought conditions. Furthermore, *GAPDH2*, *TBP*, and *UBCE2-17* were confirmed as the most appropriate comprehensive reference genes applicable to different tissues and abiotic stress scenarios. To substantiate these findings, the expression profiles of *CsBBEL9* and *CsOMT9*, which are pivotal genes in the biosynthesis of benzyloquinoline alkaloids, were analyzed across seven tissues and all stress treatments. The results indicated that employing stable reference genes resulted in consistent expression patterns, whereas the use of unstable reference genes caused artificially inflated expression levels of *CsBBEL9* and *CsOMT9* in stems, old leaves, samples treated with $25 \text{ mmol}\cdot\text{L}^{-1} \text{ CaCl}_2$, and those subjected to 12 days of drought stress, while leading to diminished expression levels under $5 \text{ mmol}\cdot\text{L}^{-1} \text{ CaCl}_2$ and $50 \text{ }\mu\text{mol}\cdot\text{L}^{-1} \text{ Na}_2\text{SeO}_3$ treatments. The varied expression profiles of target genes underscore the essential requirements for meticulous optimization of experimental conditions and careful selection of stable reference genes to guarantee reliable outcomes in gene expression research. This study has effectively identified the most suitable reference genes and their combinations for *C. saxicola* across various tissues and under multiple abiotic stress conditions, establishing a robust basis for further exploration of gene function and regulatory mechanisms in *C. saxicola*.

45 **Keywords** real-time quantitative PCR, reference genes, *Corydalis saxicola*, abiotic stresses, gene
46 expression.

47 *Corydalis saxicola*, a member of the *Corydalis* genus within the Papaveraceae
48 family, is extensively utilized in traditional Chinese medicine for the treatment of
49 conditions such as hepatitis, liver cirrhosis, and liver cancer[1,2]. Currently, proprietary
50 Chinese medicinal formulations derived from *C. saxicola* have been developed
51 specifically for addressing these hepatic disorders[1]. The therapeutic properties of *C.*
52 *saxicola* are attributed to its bioactive compounds, which encompass alkaloids, steroids,
53 triterpenoids, lignans, isoquinolinones, and flavonoids[1,3]. Notably, alkaloids constitute a
54 significant category of pharmacologically active constituents, with 67 unique alkaloids
55 identified thus far. This group includes berberines, protoberberines, benzophenanthridines,
56 benzyltetrahydroisoquinolines, aporphines, protopines, and lignin amides[1,4]. Among
57 these, berberine alkaloids, such as dehydrocavidine, dehydrochelerythrine, and
58 tetrahydroglauconine, are the most commonly studied components found in *C.*
59 *saxicola*[1,2,4].

60 *C. saxicola* is a unique species that thrives exclusively in the crevices of rocks at
61 altitudes ranging from 500 to 1200 meters, as well as in the shaded and humid entrances
62 of caves. This species is endemic to the karst regions of southern China[5]. The karst
63 landscape is characterized by a distinct geomorphological formation resulting from the
64 dissolution, precipitation, erosion, and other processes involving surface and groundwater
65 interacting with soluble rocks, such as limestone and dolomite[5,6]. The soil in these areas
66 is typically shallow, enriched with high levels of calcium, and exhibits poor water
67 retention capabilities, contributing to a sensitive and fragile ecosystem[7]. *C. saxicola*

68 demonstrates remarkable resilience to various environmental stresses, including calcium
69 and drought, due to its adaptation to karst habitats[8]. Notably, selenium (Se) has been
70 shown to alleviate heavy metal stress in crops by regulating antioxidant systems and
71 metabolic genes[9]. In *Plantago ovata*, the application of low-concentration sodium
72 selenate ($<50 \mu\text{mol}\cdot\text{L}^{-1}$) effectively induces the biosynthesis of various polyphenols, and
73 exerts no inhibitory effects on plant growth[10]. Current research on *C. saxicola* primarily
74 emphasizes its physiological traits[11], chemical constituents[12,13], and medicinal
75 properties[14–16]; however, there is a notable lack of studies investigating the correlations
76 between stress levels and the concentrations of bioactive compounds, as well as the
77 molecular mechanisms underlying its stress tolerance. Investigating the relationship
78 between the levels of pharmacologically active substances and the plant stress resistance
79 is crucial for uncovering the genetic traits that contribute to the quality of authentic
80 medicinal herbs, as well as for the conservation and genetic improvement of medicinal
81 plant species.

82 Quantitative real-time PCR (qRT-PCR) is an effective technique for analyzing gene
83 expression profiles and has been extensively utilized in gene expression research[17].
84 This method offers notable advantages, including high sensitivity and excellent
85 reproducibility. Nonetheless, the accuracy of the relative quantification results obtained
86 from qRT-PCR can be influenced by several factors, including RNA integrity, cDNA
87 quality, sample dilution factors, and experimental procedures[18,19]. To minimize
88 potential errors during qRT-PCR analysis, it is essential to incorporate high-quality
89 reference genes that exhibit specificity and stable expression for normalizing target gene

90 expression[20]. Typically, the selection of appropriate reference genes involves
91 identifying candidate genes followed by assessing their expression stability under specific
92 experimental conditions. Housekeeping genes, which are crucial for maintaining
93 fundamental cellular functions, such as *actin*, *18S ribosomal RNA*, *tubulin*, and *ubiquitin*,
94 are commonly considered in this context[21,22]. Ideal reference genes should demonstrate
95 stable expression across various tissues and developmental stages. However, recent
96 research has indicated that no single reference gene consistently maintains high
97 expression stability across all biological studies[19,23]. The transcription levels of
98 housekeeping genes can differ across various species[24], tissues, and organs[25,26].
99 Utilizing unvalidated reference genes may result in discrepancies in the expression
100 outcomes of target genes, thereby raising concerns regarding the reliability of the
101 experimental findings[27]. Consequently, it is essential to identify reference genes with
102 relatively stable expression tailored to specific experimental conditions prior to assessing
103 the relative expression levels of target genes. However, there is currently a lack of studies
104 focused on the screening of reference genes in *C. saxicola*, which hampers the
105 investigation into the mechanisms regulating gene expression in *C. saxicola* under
106 varying conditions.

107 In this research, we selected 11 candidate reference genes—including *actin 1*
108 (*ACT1*), *actin 4* (*ACT4*), *glyceraldehyde-3-phosphate dehydrogenase 2* (*GAPDH2*),
109 *glyceraldehyde-3-phosphate dehydrogenase 8* (*GAPDH8*), *α -tubulin* (*α -TUB*), *RNA*
110 *polymerase II-associated protein 3* (*RPAP3*), *TATA-box-binding protein* (*TBP*),
111 *ubiquitin-conjugating enzyme E2-17 kDa* (*UBCE2-17*), *ubiquitin-conjugating enzyme E2*

112 *5A-like (UBCE2-5A), ubiquitin-conjugating enzyme E2 variant 1C (UEV1C), β -tubulin*
113 *(β -TUB)*—based on the transcriptome data of *C. saxicola*. The expression stability of
114 these candidate reference genes was assessed across various tissues and under abiotic
115 stress conditions (including Ca^{2+} stresses, Se^{2+} stresses, and drought stresses) via
116 real-time fluorescence quantitative polymerase chain reaction (qRT-PCR) and software
117 tools such as geNorm[28], NormFinder[29], and BestKeeper[30]. Furthermore, the online
118 analysis tool RefFinder[31] was employed to provide a comprehensive evaluation of the
119 results obtained from the aforementioned software. To validate the selected reference
120 genes, we utilized the target genes *berberine bridge enzymes like 9*(*CsBBEL9*) and
121 *O-methyltransferase 9*(*CsOMT9*). This study represents the first systematic screening and
122 validation of reference genes for normalizing real-time fluorescence quantitative PCR in
123 different tissues of *C. saxicola* and under abiotic stress conditions, thereby establishing a
124 foundation for gene expression analysis in this species.

125 Materials and methods

126 Plant materials

127 On March 12, 2024, plants and seeds of *C. saxicola* were collected from Banla
128 Village, Aidong Town, Donglan County, Hechi City, Guangxi Zhuang Autonomous
129 Region, China. The collection process adhered strictly to the guidelines outlined in the
130 *IUCN Policy Statement on Research Involving Species at Risk of Extinction*, the
131 *Convention on the Trade in Endangered Species of Wild Fauna and Flora* (CITES), and
132 the *Regulations on the Protection of Wild Plants of China*. We have obtained the official
133 Plant Collection Certificate for the national second-class protected wild plant *C. saxicola*

134 (Certificate No.: Zhicai Zheng (Gui) 0030784) issued by the Forestry Bureau of Guangxi
135 Zhuang Autonomous Region, which authorizes the collection of this species for scientific
136 research purposes.

137 The collected specimens were identified as *Corydalis saxicola* Bunting (genus
138 *Corydalis*, family *Papaveraceae*) by Senior Engineer Yude Peng from the Guangxi
139 Botanical Garden of Medicinal Plants. The plant specimens were subsequently deposited
140 in the Guangxi Chinese Medicinal Material Herbarium, assigned the specimen number
141 0317019 (Supplementary Figure. S1). The harvested *C. saxicola* plants were then
142 transplanted into the greenhouse, and their seeds were collected later that same year.

143 The seeds of *C. saxicola* were soaked in clean water overnight and then sown in a
144 seedling substrate (sand : vermiculite = 1:1). Subsequently, the seeds were cultured in an
145 artificial climate chamber under the following conditions: a temperature of 25°C, a
146 humidity of 60%, a light intensity of 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and a photoperiod of 12 h light/12
147 h dark. After 30 days of cultivation, healthy plants were transplanted into hydroponic
148 containers (29 cm $\times$ 22 cm $\times$ 9 cm) filled with 1/4-strength Hoagland nutrient solution for
149 7 days. Thereafter, the plants were cultured in 1/2-strength Hoagland nutrient solution for
150 another 30 days. Finally, samples of seven different tissues (including roots, stems, lateral
151 stems, fruits, flowers, old leaves, and young leaves) were collected and stored in a -80°
152 C refrigerator.

153 Ca^{2+} and Se^{2+} stress treatments

154 The seedling raising methods and culture conditions of *C. saxicola* were as
155 previously described. After 30 days of cultivation, healthy plants were transplanted into

156 hydroponic containers filled with 1/4-strength Hoagland nutrient solution and cultured for
157 7 days. Thereafter, based on 1/2-strength Hoagland nutrient solution, different
158 concentrations of CaCl_2 (0, 5, 25, 50 $\text{mmol}\cdot\text{L}^{-1}$) and Na_2SeO_3 (0, 10, 50 $\mu\text{mol}\cdot\text{L}^{-1}$) were
159 added for treatment. After 30 days of treatment, roots and leaves were separately excised
160 as samples for Ca^{2+} stress and Se^{2+} stress, respectively, and stored in a -80°C refrigerator
161 for subsequent use.

162 Drought stress treatment

163 Seedlings of *C. saxicola* were transplanted into pots and maintained under standard
164 water and nutrient conditions within a greenhouse setting. Before initiating the drought
165 treatment, the plants were adequately irrigated, after which watering was discontinued.
166 Sampling was conducted on days 0, 6, 12, and 18 following the cessation of irrigation.
167 Leaves from plants exhibiting uniform growth were selected, quickly frozen in liquid
168 nitrogen, and stored at -80°C for subsequent RNA extraction and additional
169 experimental analyses.

170 RNA extraction and cDNA synthesis

171 RNA was extracted using the MolPure Plant RNA Kit (Yeasen, Shanghai, China) in
172 accordance with the manufacturer's guidelines. In addition, the integrity of RNA was
173 detected by electrophoresis on a 1% agarose gel (Biosharp, Anhui, China). Subsequently,
174 the concentration and purity of RNA were measured using a UV-Vis spectrophotometer
175 (NanoDrop 2000, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

176 To generate the first-strand cDNA, the HiScript III 1st Strand cDNA Synthesis Kit
177 (Novozymes, Nanjing, China) was used in conjunction with 1 μg of total RNA, and the

178 PCR reaction system was as follows: 2 μ L of 5 $\times$ gDNA wiper Mix was mixed with 1 μ g
 179 of RNA template, incubated at 42°C for 2 minutes, and then 2 μ L of HiScript III Enzyme
 180 Mix, 1 μ L of Oligo (dT)₂₀VN, 1 μ L of Random hexamers, and 4 μ L of RNase-free ddH₂O
 181 were added to a total volume of 20 μ L. The samples were reverse-transcribed using a
 182 gradient PCR thermal cycler from Bio-Rad (T100™ Thermal Cycler, Bio-Rad, California,
 183 USA). The PCR reaction program was as follows: first, incubated at 37°C for 120
 184 minutes, and then heated at 85°C for 5 seconds. After the reaction, the reverse-transcribed
 185 cDNA was stored at -20°C for subsequent experiments.

186 Screening of candidate reference genes and designing of primers

187 According to the common reference genes in other plants in the existing literature,
 188 11 candidate reference genes were selected according to the transcriptome database of *C.*
 189 *saxicola*(The list of eleven candidate reference genes and their corresponding FPKM
 190 expression values derived from RNA-seq data from various tissues and CaCl₂-treated
 191 *Corydalis saxicola* experiment is described in supplementary Table S1.), namely: *ACT1*,
 192 *ACT4*, *GAPDH2*, *GAPDH8*, *α -TUB*, *RPAP3*, *TBP*, *UBCE2-17*, *UBCE2-5A*, *UEV1C*,
 193 *β -TUB*. Before conducting qRT-PCR, the best primer was screened from three pairs in
 194 order to confirm the amplification quality and the correctness of the target gene. The
 195 sequences of these primers and alongside the *CsBBEL9* and *CsOMT9* gene as the target
 196 gene, are detailed in Table 1. Primers were synthesized by Tianyi Huiyuan Gene
 197 Technology Co., Ltd., Guangzhou, China.

198 Table 1 Candidate reference genes and primers sequences used for qRT-PCR

Gene	Primers ID	Gene description	Primer sequence (5'-3')	Expected length(bp)
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<i>ACT1</i>	<i>ACT1-F</i>	<i>actin 1</i>	GATGCTCCAAGGGCTGTGTT	187
	<i>ACT1-R</i>		TGCCAGATCTTTTCCATGTCA	
<i>ACT4</i>	<i>ACT4-F</i>	<i>actin 4</i>	ATCTCAAGCCTTAGGATACCGC	211
	<i>ACT4-R</i>		CAACATAAGCTCAGCCGCC	
<i>GAPDH2</i>	<i>GAPDH2-F</i>	<i>glyceraldehyde-3-phosphate dehydrogenase 2</i>	GGTGAGGCTGGTGCTGATTT	164
	<i>GAPDH2-R</i>		TGAGGTGTATTCCTTCTCGTTGAC	
<i>GAPDH8</i>	<i>GAPDH8-F</i>	<i>glyceraldehyde-3-phosphate dehydrogenase 8</i>	GTCCAATCCCATAGTGCCTTC	182
	<i>GAPDH8-R</i>		TTGTCCATAGATACTTTCTTCCTGC	
<i>α-TUB</i>	<i>α-TUB-F</i>	<i>tubulin alpha chain</i>	TGGTGCCATACCCAAGGAT	120
	<i>α-TUB-R</i>		GAGGGCTCAAAGGCACTGTT	
<i>RPAP3</i>	<i>RPAP3-F</i>	<i>RNA polymerase II-associated protein 3</i>	TTTCAAACAAGCACAAACCGTG	224
	<i>RPAP3-R</i>		GCGACCTTCTCGAATACTCAGACT	
<i>TBP</i>	<i>TBP-F</i>	<i>TATA-box-binding protein</i>	GTTTTGCTGCTGTGATTATGAGG	115
	<i>TBP-R</i>		TGCCGCCAACTTTGACTGT	
<i>UBCE2-17</i>	<i>UBCE2-17-F</i>	<i>ubiquitin-conjugating enzyme E2-17 kDa</i>	TGGCAAGCAACAATAATGGG	86
	<i>UBCE2-17-R</i>		TGGATAATCTGGAGGGAAGTGA	
<i>UBCE2-5A</i>	<i>UBCE2-5A-F</i>	<i>ubiquitin-conjugating enzyme E2</i>	TAGTCCTTATTCTGGTGGTGTTC	266
	<i>UBCE2-5A-R</i>		CTTTGATTCTTGTAGATGTGGGC	
<i>UEV1C</i>	<i>UEV1C-F</i>	<i>Ubiquitin-conjugating enzyme E2 variant 1C</i>	CGTGGAGAGAAGGGTATTGGG	191
	<i>UEV1C-R</i>		TGAGTGGAACGAACAGTTGGA	
<i>β-TUB</i>	<i>β-TUB-F</i>	<i>tubulin beta-1 chain</i>	TCAGAGAGGAATACCCAGATAGGA	254
	<i>β-TUB-R</i>		AACAACAGGTGACACCAGACATG	
<i>CsBBEL9</i>	<i>BBEL9-F</i>	<i>berberine bridge enzyme-like 9</i>	GGGCTATAAGAGGAGGAGGTGG	170
	<i>BBEL9-R</i>		TTCAGGAAGTTTACTGGCAACAG	
<i>CsOMT9</i>	<i>OMT9-F</i>	<i>O-methyltransferase 9</i>	AATGCTCATCCTCATCCCAAC	281
	<i>OMT9-R</i>		TACACTCCTTTCTCTTCCACCTG	

199

200 qRT-PCR reaction conditions

201 qRT-PCR was performed using the SYBR Green fluorescent dye method. The PCR
202 reaction system was prepared according to the instructions of the 2×SYBR Green qPCR
203 Mix Kit (Sikejie Biotechnology Co., Ltd., Shandong, China) as follows: 1 μL of cDNA
204 template (approximately 10 ng), 0.8 μL of forward and reverse primers (10 μmol/L each),

205 0.4 μ L of ROX Reference Dye II, 8.8 μ L of ddH₂O, and 10 μ L of 2 $\times$ SYBR qPCR Mix.
206 All samples were run in triplicate. The samples were amplified using a real-time PCR
207 instrument (Thermo Fisher Scientific Quant StudioTM 3, Waltham, MA, USA). The PCR
208 program consisted of an initial denaturation at 95°C for 30 seconds, followed by 40
209 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 60°C for 30
210 seconds. A melting curve analysis was performed from 65°C to 95°C with a 0.5°C
211 increment per cycle and a 0.05-second hold to determine the melting temperature.
212 Fluorescence signals were collected during the reaction.

213 The stability of candidate reference genes

214 After qRT-PCR, the Ct values of each sample were obtained by analyzing the data
215 using the software with the qRT-PCR instrument(Quant StudioTM Design & Analysis
216 Software). The stability of each candidate reference gene was comprehensively analyzed
217 by geNorm[28], Normfinder[29], BestKeeper[30], and RefFinder[31]. GeNorm analysis
218 ranks the expression stability of genes by calculating the gene expression stability value
219 (M). A lower M value indicates higher gene stability; genes with $M < 1.5$ are considered
220 relatively stable. The optimal number of reference genes was determined by the paired
221 coefficient of variation V_n/V_{n+1} . Generally, when the V_n/V_{n+1} value is less than 0.15,
222 there is no need to add a new reference gene. On the contrary, if this value exceeds the
223 threshold, it is essential to include the $(n + 1)$ th reference gene. NormFinder also
224 evaluates gene expression stability based on the stability value, where a smaller value
225 corresponds to higher stability. BestKeeper assesses reference gene stability by
226 calculating the coefficient of variation (CV) and standard deviation (SD). Lower SD and

CV values indicate greater stability of the candidate reference genes. RefFinder (<http://blooge.cn/RefFinder/?type=reference>) is a comprehensive stability evaluation method that assigns appropriate weights to each gene based on their rankings across multiple algorithms (geNorm, NormFinder, BestKeeper, and comparative ΔCt method), then calculates the geometric mean of these weighted ranks to generate an overall stability ranking.

Evaluation of target gene expression using stable and unstable reference genes

To validate the stability of the screened reference genes, genes responsive to different tissues and abiotic stresses, i.e., *CsBBEL9* and *CsOMT9* were selected as target genes. cDNA templates were prepared from various tissues of *C. saxicola*, the roots under Ca^{2+} stress, the leaves under Se^{2+} stress and the leaves under drought stress. Using qRT-PCR, the optimal candidate reference genes and their combinations were employed as normalization factors, while unstable reference genes were used for comparison. The relative expression levels of *CsBBEL9* and *CsOMT9* in *C. saxicola* were then analyzed using the $2^{-\Delta\Delta\text{CT}}$ method. All experiments were repeated three times, following the reaction system and procedures described in Section “qRT-PCR reaction conditions”.

Results

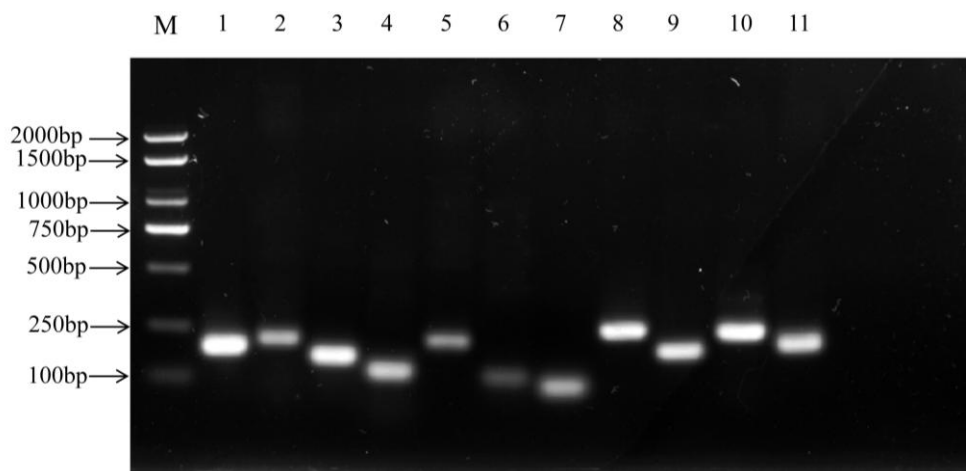
Assessment of total RNA quality

Total RNA was extracted from various tissues and stress-treated samples of *C. saxicola* using the MolPure Plant RNA Kit, followed by analysis via 1% agarose gel electrophoresis. The agarose gel electrophoresis results (Supplementary Fig. S2) revealed

249 distinct and sharp 28S and 18S rRNA bands across all samples, demonstrating that the
250 extracted RNA retained excellent integrity without significant degradation. The nucleic
251 acid purity ratios for all samples were provided in Supplementary Table S2.

252 Determination of primer specificity

253 PCR amplification was conducted using equal quantities of mixed cDNA as the
254 template (Figure 1, Supplementary Fig. S3). All primers successfully targeted fragments,
255 producing distinct and prominent bands without the presence of primer dimers or
256 nonspecific amplification products. The sizes of the bands were in accordance with the
257 anticipated values. The specificity of the primers was confirmed through qRT-PCR
258 technology (Figure 2). The melting curves for each gene displayed a single melting peak,
259 demonstrating that these primers are capable of specific amplification. Consequently,
260 these primers demonstrate high specificity and efficiency in amplifying the candidate
261 genes, indicating their suitability for use in subsequent experiments.



263
264 Figure 1. Agarose gel electrophoresis of the conventional PCR products of the candidate reference genes in *C. saxicola*.
265 M: DL2000 DNA Marker, 1 to 11 is *ACT1*, *ACT4*, *GAPDH2*, *α -TUB*, *RPAP3*, *TBP*, *UBCE2-17*, *UBCE2-5A*, *UEVIC*,

266
267

β-TUB and *GAPDH8*, respectively. (The original gel is presented in Supplementary Fig. S2)

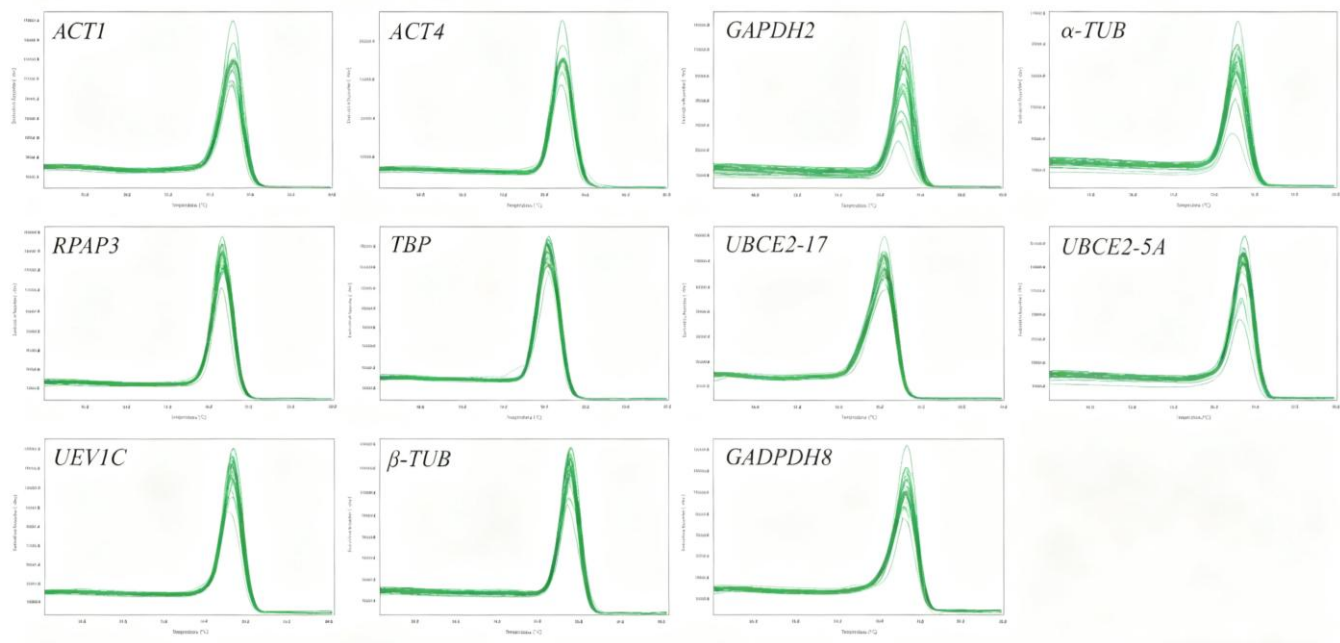


Figure 2. Melting curves of the candidate reference genes in *C. saxicola*.

268
269

270 Expression stability analysis of selected reference genes under
271 different conditions

272 The cycle threshold (Ct) value is inversely proportional to the gene expression level,
273 where a lower Ct value reflects a higher gene expression level. In the box plot analysis
274 (Figure 3), the Ct values of most genes ranged from 18 to 34, indicating moderate
275 expression abundances. As shown in the box plot, the *GAPDH8* gene exhibited a broad
276 Ct value range (22.15–34.27), suggesting low gene expression stability. In contrast, the
277 *ACT4* gene had a narrow Ct value range (26.08–30.32), indicating high expression
278 stability. Therefore, these results demonstrate that different reference genes exhibit
279 distinct expression levels in *C. saxicola* under three types of abiotic stresses (Ca^{2+} stress,
280 Se^{2+} stress and drought stress) and across different tissues. Based on the Ct value range,
281 the *ACT4* gene displayed stable expression, making it the best candidate reference gene.

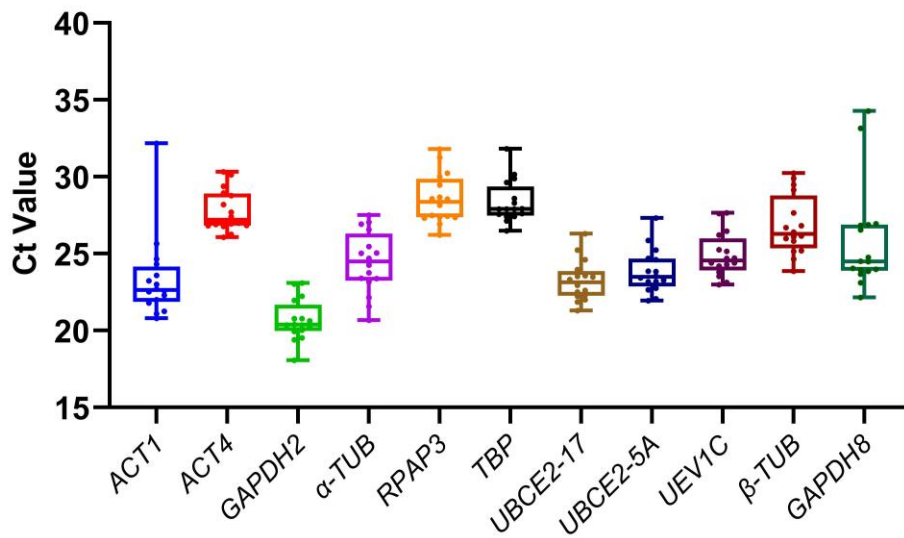


Figure 3. Box plot of the Ct values of the 11 candidate reference genes in *C. saxicola*. The box represents the concentrated range of Ct values. The horizontal line in the middle of the box represents the median value. The upper and lower edges of the box represent the upper and lower quartiles, respectively. The upper and lower ends of the box represent the maximum and minimum values of the gene, respectively.

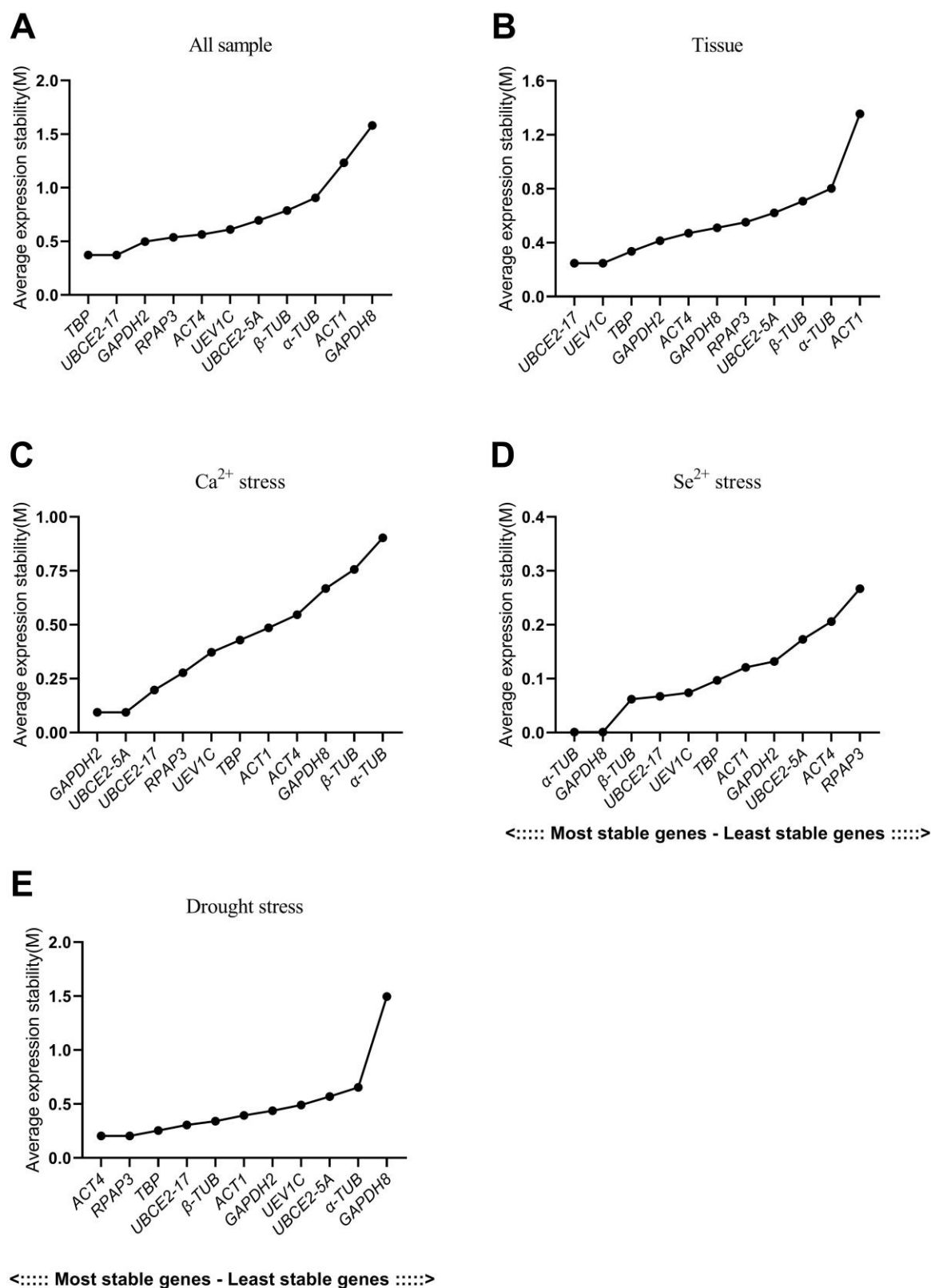
In addition to using Ct values to assess the stability of gene expression, we applied established statistical tools such as BestKeeper, NormFinder, and GeNorm for a thorough data analysis. This approach was employed to validate our results and enhance our understanding of the dataset. By leveraging these statistical methods, we evaluated the stability of reference genes across different experimental conditions, offering crucial guidance for the selection of the most suitable reference genes to ensure precise gene expression analyses.

geNorm analysis

The expression stability values (M values) of candidate reference genes in different tissues and under different stresses were calculated using the geNorm program. This program uses a threshold of $M = 1.5$, where lower M values indicate higher stability of

reference genes. The geNorm analyses are shown in Figure 4. Comprehensive analysis of the expression levels of 11 candidate reference genes across all samples revealed that all genes, except *GAPDH8* ($M > 1.5$), exhibited *M* values below 1.5. The genes were ranked by decreasing stability as follows: *TBP* = *UBCE2-17* > *GAPDH2* > *RPAP3* > *ACT4* > *UEVIC* > *UBCE2-5A* > β -*TUB* > α -*TUB* > *ACT1* > *GAPDH8*. In various tissues, *UBCE2-17* and *UEVIC* demonstrated the most stable expression. Under Ca^{2+} stress, *GAPDH2* and *UBCE2-5A* exhibited the highest stability. In response to Se^{2+} stress, α -*TUB* and *GAPDH8* showed the greatest stability. During drought stress, *ACT4* and *RPAP3* were identified as the most consistently expressed genes.

Determining the optimal number of reference genes can reduce bias and fluctuations caused by a single reference gene. As illustrated in Figure 5, the $V_{2/3}$ for all samples of *C. saxicola* was 0.17, exceeding the critical threshold of 0.15, while the $V_{3/4}$ values were below 0.15. This indicates that three reference genes are required for normalization across all samples to ensure stable and reliable results. Conversely, when examining different tissues, as well as Ca^{2+} , Se^{2+} , and drought stress conditions, the $V_{2/3}$ values for *C. saxicola* were recorded at 0.12, 0.082, 0.031, and 0.087, respectively—all of which were below 0.15. These results imply that the selection of two reference genes is adequate for achieving stable and reliable normalization under these specific conditions.



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Figure 4. Average expression stability of the 11 candidate reference genes in *C. saxicola* analyzed by the geNorm program. (A) all samples, (B) different tissues, (C) Ca^{2+} stress, (D) Se^{2+} stress, (E) Drought stress.

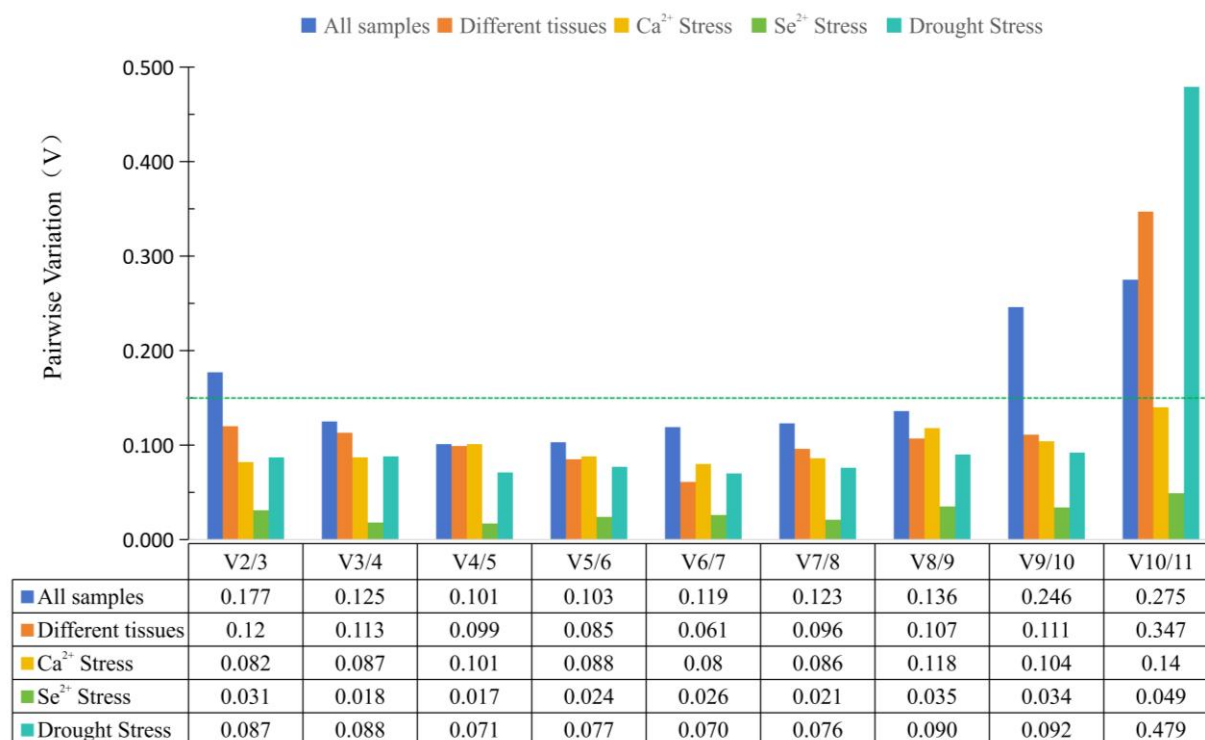


Figure 5. Analysis of the paired variation of the 11 candidate reference genes in *C. saxicola*. by geNorm program. The optimal number of candidate reference genes required for accurate normalization was determined by paired variation $V_n/(n+1)$. The threshold value of $V_n/(n+1)$ was 0.15. When $V_n/(n+1)$ is less than 0.15, n genes can ensure stable and reliable results.

BestKeeper analysis

BestKeeper evaluates gene stability primarily by comparing the standard deviation (SD) values among the Ct values of candidate reference genes. The results of the BestKeeper analysis are shown in Table 2. Across all samples, the ranking of gene expression stability from high to low was: *GAPDH2* (0.979) > *UBCE2-17* (1.016) > *TBP* (1.041) > *UBCE2-5A* (1.067) > *UEVIC* (1.115) > *ACT4* (1.136) > *RPAP3* (1.268) > β -*TUB* (1.536) > α -*TUB* (1.619) > *ACT1* (1.703) > *GAPDH8* (2.466). This indicates that *GAPDH2* (0.979) is the most stable gene across all samples, while *GAPDH8* (2.466) is the least stable. In various tissues, *UBCE2-5A* emerged as the most stable reference gene, while *ACT1* demonstrated the least stability. During Ca²⁺ stress, *ACT4* displayed the greatest expression stability. In the context of Se²⁺ stress, *GAPDH8* was identified as the

336 most stable reference gene. Under drought conditions, *GAPDH2* exhibited the highest
337 level.

338 NormFinder analysis

339 Prior to data analysis, NormFinder transforms the Ct values of genes into relative
340 expression levels and subsequently ranks the stability of potential reference genes
341 through variance analysis. A lower value signifies greater expression stability. The
342 outcomes of the NormFinder analysis are presented in Table 3. The ranking of gene
343 expression stability across all samples from highest to lowest was: *GAPDH2* (0.332) >
344 *UEVIC* (0.423) > *RPAP3* (0.476) > *ACT4* (0.517) > *TBP* (0.542) > *UBCE2-17* (0.636) >
345 *UBCE2-5A* (0.845) > β -*TUB* (0.962) > α -*TUB* (0.969) > *ACT1* (2.478) > *GAPDH8*
346 (3.003). In various tissues of *C. saxicola*, *GAPDH2* emerged as the most stable reference
347 gene, while *ACT1* was identified as the least stable. During Ca²⁺ stress, *RPAP3*
348 demonstrated the highest level of expression stability. In the context of Se²⁺ stress,
349 *GAPDH8* and α -*TUB* were found to be the most stable reference genes. Under drought
350 conditions, *ACT4* exhibited the greatest stability.

351 Table 2. Analysis of the expression stability by Bestkeeper and the ranking of the reference genes in *C. saxicola*.

Rank	All Samples		Different Tissues		Ca ²⁺ Stress		Se ²⁺ Stress		Drought Stress	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	<i>GAPDH2</i>	0.979	<i>UBCE2-5A</i>	0.782	<i>ACT4</i>	0.993	<i>GAPDH8</i>	2.376	<i>GAPDH2</i>	0.697
2	<i>UBCE2-17</i>	1.016	<i>UEVIC</i>	0.976	<i>GAPDH8</i>	1.206	α - <i>TUB</i>	2.546	β - <i>TUB</i>	0.754
3	<i>TBP</i>	1.041	<i>TBP</i>	1.006	<i>RPAP3</i>	1.277	β - <i>TUB</i>	2.711	<i>ACT1</i>	0.77
4	<i>UBCE2-5A</i>	1.067	<i>UBCE2-17</i>	1.017	<i>GAPDH2</i>	1.304	<i>UBCE2-17</i>	3.557	<i>UBCE2-17</i>	0.779
5	<i>UEVIC</i>	1.115	<i>GAPDH2</i>	1.146	<i>UBCE2-5A</i>	1.357	<i>UEVIC</i>	4.729	<i>TBP</i>	0.793
6	<i>ACT4</i>	1.136	<i>ACT4</i>	1.16	<i>UBCE2-17</i>	1.515	<i>GAPDH2</i>	5.03	<i>ACT4</i>	0.837
7	<i>RPAP3</i>	1.268	<i>GAPDH8</i>	1.376	<i>ACT1</i>	1.574	<i>TBP</i>	5.045	<i>RPAP3</i>	0.946
8	β - <i>TUB</i>	1.536	<i>RPAP3</i>	1.435	α - <i>TUB</i>	1.62	<i>ACT1</i>	5.292	α - <i>TUB</i>	0.957
9	α - <i>TUB</i>	1.619	β - <i>TUB</i>	1.74	β - <i>TUB</i>	1.647	<i>UBCE2-5A</i>	8.452	<i>UEVIC</i>	1.084
10	<i>ACT1</i>	1.703	α - <i>TUB</i>	1.893	<i>UEVIC</i>	1.729	<i>ACT4</i>	9.74	<i>UBCE2-5A</i>	1.292
11	<i>GAPDH8</i>	2.466	<i>ACT1</i>	2.623	<i>TBP</i>	1.767	<i>RPAP3</i>	11	<i>GAPDH8</i>	4.774

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Table 3. Expression stability of the reference genes in *C. saxicola* calculated by NormFinder.

Rank	All Samples		Different Tissues		Ca ²⁺ Stress		Se ²⁺ Stress		Drought Stress	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	<i>GAPDH2</i>	0.332	<i>GAPDH2</i>	0.195	<i>RPAP3</i>	0.141	<i>GAPDH8</i>	0.001	<i>ACT4</i>	0.103
2	<i>UEVIC</i>	0.423	<i>UBCE2-17</i>	0.272	<i>UBCE2-5A</i>	0.264	α - <i>TUB</i>	0.001	<i>UEVIC</i>	0.156
3	<i>RPAP3</i>	0.476	<i>RPAP3</i>	0.408	<i>GAPDH2</i>	0.309	β - <i>TUB</i>	0.029	<i>ACT1</i>	0.156
4	<i>ACT4</i>	0.517	<i>TBP</i>	0.426	<i>ACT1</i>	0.362	<i>UBCE2-17</i>	0.052	<i>RPAP3</i>	0.456
5	<i>TBP</i>	0.542	<i>UEVIC</i>	0.442	<i>ACT4</i>	0.416	<i>UEVIC</i>	0.101	<i>UBCE2-5A</i>	0.494
6	<i>UBCE2-17</i>	0.636	<i>GAPDH8</i>	0.562	<i>UBCE2-17</i>	0.52	<i>TBP</i>	0.196	<i>GAPDH2</i>	0.541
7	<i>UBCE2-5A</i>	0.845	<i>ACT4</i>	0.566	<i>GAPDH8</i>	0.775	<i>UBCE2-5A</i>	0.208	α - <i>TUB</i>	0.545
8	β - <i>TUB</i>	0.962	α - <i>TUB</i>	0.8	<i>UEVIC</i>	0.804	<i>ACT1</i>	0.269	<i>TBP</i>	0.577
9	α - <i>TUB</i>	0.969	β - <i>TUB</i>	0.812	β - <i>TUB</i>	0.852	<i>ACT4</i>	0.272	<i>UBCE2-17</i>	0.828
10	<i>ACT1</i>	2.478	<i>UBCE2-5A</i>	1.088	<i>TBP</i>	0.976	<i>GAPDH2</i>	0.279	β - <i>TUB</i>	0.88
11	<i>GAPDH8</i>	3.003	<i>ACT1</i>	3.811	α - <i>TUB</i>	1.528	<i>RPAP3</i>	0.535	<i>GAPDH8</i>	5.27

354

355 RefFinder analysis

356 To mitigate the potential inaccuracies associated with relying on a single reference
357 gene evaluation program, we utilized the online analysis tool RefFinder to compute the
358 geometric mean of gene expression stability rankings derived from three distinct
359 programs: geNorm, NormFinder, and BestKeeper. A lower geometric mean signifies
360 greater stability in gene expression.

361 As shown in Table 4, the ranking of gene expression stability across all samples was:
362 *GAPDH2* (1.32) > *TBP* (2.59) > *UBCE2-17* (2.78) > *RPAP3* (3.6) > *UEVIC* (4.36) >
363 *ACT4* (4.68) > *UBCE2-5A* (6.09) > β -*TUB* (8) > α -*TUB* (9) > *ACT1* (10) > *GAPDH8* (11).
364 Notably, *GAPDH2* emerged as the most stable reference gene among all samples,
365 whereas *GAPDH8* exhibited the lowest stability. In various tissues, *GAPDH2* emerged as
366 the most stable reference gene, while *ACT1* demonstrated the least stability. During Ca²⁺
367 stress, *RPAP3* exhibited the greatest expression stability. In the context of Se²⁺ stress,
368 *GAPDH8* was identified as the most stable reference gene. Under drought stress
369 conditions, *ACT4* displayed the highest stability, whereas *GAPDH8* was found to be the
370 least stable gene.

371

Table 4. Ranking of the expression stability of the reference genes in *C. saxicola* by RefFinder.

Rank	All Samples		Different Tissues		Ca ²⁺ Stress		Se ²⁺ Stress		Drought Stress	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	<i>GAPDH2</i>	1.32	<i>GAPDH2</i>	2.11	<i>RPAP3</i>	1.86	<i>GAPDH8</i>	2.38	<i>ACT4</i>	1.57
2	<i>TBP</i>	2.59	<i>UBCE2-17</i>	2.21	<i>UBCE2-5A</i>	2.11	<i>α-TUB</i>	2.55	<i>RPAP3</i>	3.03
3	<i>UBCE2-17</i>	2.78	<i>UEV1C</i>	2.51	<i>GAPDH2</i>	2.45	<i>β-TUB</i>	2.71	<i>ACT1</i>	3.22
4	<i>RPAP3</i>	3.6	<i>TBP</i>	2.91	<i>ACT4</i>	3.94	<i>UBCE2-17</i>	3.56	<i>GAPDH2</i>	3.98
5	<i>UEV1C</i>	4.36	<i>UBCE2-5A</i>	5.18	<i>UBCE2-17</i>	4.56	<i>UEV1C</i>	4.73	<i>TBP</i>	4.68
6	<i>ACT4</i>	4.68	<i>ACT4</i>	5.69	<i>ACT1</i>	5.6	<i>GAPDH2</i>	5.03	<i>UEV1C</i>	5.18
7	<i>UBCE2-5A</i>	6.09	<i>RPAP3</i>	5.86	<i>GAPDH8</i>	5.63	<i>TBP</i>	5.05	<i>β-TUB</i>	5.32
8	<i>β-TUB</i>	8	<i>GAPDH8</i>	6.24	<i>UEV1C</i>	7.27	<i>ACT1</i>	5.29	<i>UBCE2-17</i>	5.63
9	<i>α-TUB</i>	9	<i>β-TUB</i>	8.74	<i>TBP</i>	8.78	<i>UBCE2-5A</i>	8.45	<i>UBCE2-5A</i>	7.98
10	<i>ACT1</i>	10	<i>α-TUB</i>	9.46	<i>β-TUB</i>	9.49	<i>ACT4</i>	9.74	<i>α-TUB</i>	8.65
11	<i>GAPDH8</i>	11	<i>ACT1</i>	11	<i>α-TUB</i>	10.16	<i>RPAP3</i>	11	<i>GAPDH8</i>	11

372

373 Evaluation of target gene expression using stable and unstable
374 reference genes

375

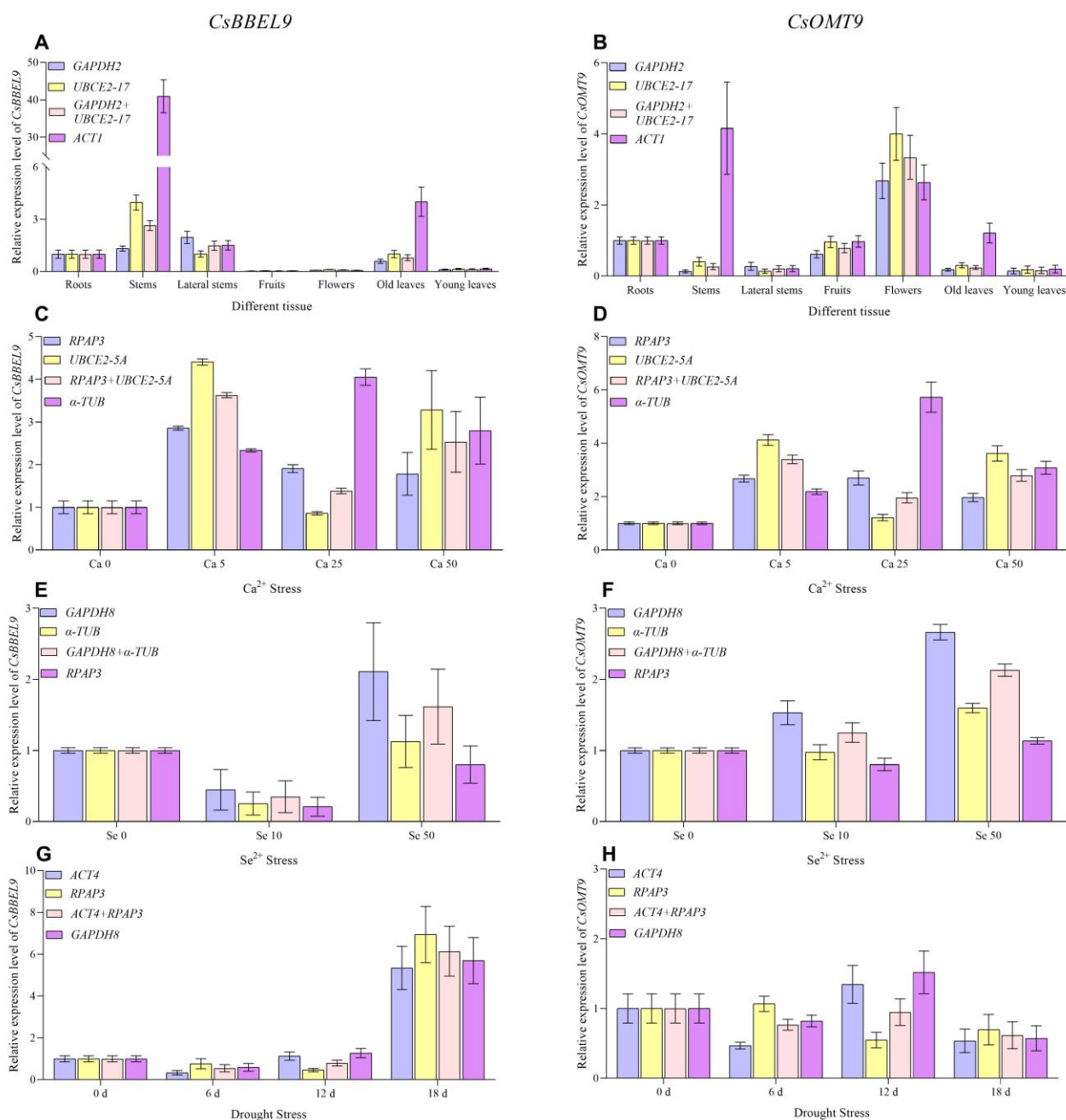


Figure 6. Using *CsBBEL9* and *CsOMT9* to verify the expression stability of reference genes screened under different tissues and abiotic stresses in *C. saxicola*. (A,B): Different tissues; (C,D): Ca^{2+} stress (roots), Ca 0, Ca 5, Ca 25, and Ca 50 represent treatments with 0, 5, 25, and 50 $\text{mmol}\cdot\text{L}^{-1}$ CaCl_2 , respectively; (E,F): Se^{2+} stress (leaves), Se 0, Se 10, and Se 50 represent treatments with 0, 10, and 50 $\mu\text{mol}\cdot\text{L}^{-1}$ Na_2SeO_3 , respectively; (G,H): Drought stress (leaves). The error bars indicate the standard deviations (SDs)

To assess the reliability of the chosen reference genes, the most stable and least stable reference genes identified by the RefFinder program were utilized as normalization factors. Notably, dehydrocavidine was the primary bioactive component of *C. saxicola*,

385 while *CsBBELs* and *CsOMTs* were well-recognized as key enzyme families mediating
386 dehydrocavidine biosynthesis in this plant. Therefore, *CsBBEL9* and
387 *CsOMT9*—representative members of the *CsBBELs* and *CsOMTs* families,
388 respectively—were chosen as target genes to independently validate their expression
389 profiles. As illustrated in Figure 6, the expression levels of *CsBBEL9* and *CsOMT9*, when
390 measured using different reference genes, exhibited significant variation. When only the
391 selected stable genes were employed as normalization references, the relative expression
392 patterns of *CsBBEL9* and *CsOMT9* displayed consistent trends. In contrast, the use of
393 relatively unstable genes for relative quantification resulted in considerable fluctuations
394 in the relative expression levels of *CsBBEL9* and *CsOMT9*.

395 In various tissues, the most stable reference genes, *GAPDH2* and *UBCE2-17*,
396 indicated that the *CsBBEL9* and *CsOMT9* genes had the highest expression levels in
397 stems and flowers, respectively. Conversely, when the least stable gene, *ACT1*, was used
398 for calculations, both *CsBBEL9* and *CsOMT9* displayed the highest expression in stems,
399 with expression levels differing by 10.5 to 31.5-fold between the two genes(Figure 6A,
400 B). Under Ca^{2+} stress, the expression levels of *CsBBEL9* and *CsOMT9* exhibited an initial
401 increase followed by a decrease as Ca^{2+} concentrations rose, reaching a maximum at Ca5
402 when assessed using the most stable reference genes *RPAP3* and *UBCE2-5A*. Conversely,
403 when the least stable gene α -*TUB* was utilized for calculations, the expression levels of
404 *CsBBEL9* and *CsOMT9* peaked at Ca25(Figure 6 C, D). Under Se^{2+} stress, the relative
405 expression of *CsBBEL9* exhibited an initial decrease followed by an increase as Se^{2+}
406 concentrations rose, peaking at Se50 when assessed using the stable reference genes

407 *GAPDH8* and α -*TUB*. In contrast, the relative expression of *CsOMT9* showed a steady
408 increase with rising Se^{2+} concentrations, also peaking at $\text{Se}50$. However, when the
409 unstable gene *RPAP3* was used for calculations, both *CsBBEL9* and *CsOMT9* displayed a
410 similar pattern of first decreasing and then increasing, but their expression levels
411 remained consistently low throughout(Figure 6E, F). Under drought stress conditions, the
412 expression levels of *CsBBEL9* exhibited an initial decrease followed by an increase,
413 reaching a peak after 18 days of drought when analyzed with the stable reference genes
414 *ACT4* and *RPAP3*. In contrast, when the unstable gene *GAPDH8* was employed, a similar
415 trend was observed, although there were no significant differences in expression levels.
416 For *CsOMT9*, the expression levels initially declined, subsequently increased, and then
417 decreased again over the duration of drought when using the stable reference genes *ACT4*
418 and *RPAP3*. The analysis with the unstable gene *GAPDH8* revealed a comparable trend to
419 that observed with the stable reference genes(Figure 6G, H).

420 Therefore, the selection of reference genes significantly influences the expression
421 levels of target genes. Selecting suitable reference genes is vital for achieving precise
422 qRT-PCR results, while the use of unstable reference genes can result in inconsistent
423 findings.

424 Discussion

425 Research into the biosynthesis and regulation of secondary metabolites in medicinal
426 plants offers significant potential for generating genetic resources that can enhance the
427 breeding of high-quality germplasm and support effective quality control measures for
428 herbal medicines. It is commonly observed that the expression levels of key enzyme

genes involved in secondary metabolic pathways positively correlate with the accumulation of secondary metabolites across various plant tissues[32 – 35]. Consequently, accurately quantifying target gene expression using techniques such as qRT-PCR is a crucial step in the identification and characterization of these genes. While the expression of reference genes typically remains stable under normal physiological conditions and is vital for essential cellular processes, their transcriptional levels can vary significantly under specific circumstances, such as environmental stress responses[21,36,37] or different developmental stages[21,38]. Therefore, selecting reference genes with stable expression is critical for ensuring the reliability of target gene expression analyses. This study identified a set of putatively stable candidate reference genes through transcriptomic database mining. These genes, including *GAPDH2*, *GAPDH8*, *TBP*, *UBCE2-17*, *UEVIC*, *UBCE2-5A*, *ACT1*, *ACT4*, β -*TUB*, α -*TUB*, and *RPAP3*, have been validated as stable reference genes in plants[39 – 42]. Their expression stability was systematically assessed across seven distinct tissue types and under specific abiotic stress conditions, including Ca^{2+} stress, Se^{2+} stress, and drought stress.

This study began by assessing the stability of housekeeping genes through descriptive statistical methods, including the calculation of mean Ct values and standard deviation analyses. The findings indicated that *GAPDH2* demonstrated the highest expression stability across all samples, with a mean Ct value of 20.68 ± 1.33 , while *RPAP3* exhibited the lowest expression level (mean Ct = 28.56 ± 1.61). Although evaluating gene stability using mean Ct values and standard deviations is a straightforward approach, its utility is somewhat restricted due to inherent limitations in accounting for biological and

451 technical variations[43]. Wang et al.[42] identified *GAPDH* and *TBP* as the most reliable
452 reference genes for qRT-PCR normalization in *Nelumbo nucifera*, showing consistency
453 across various tissues and developmental stages. Similarly, in the traditional Chinese
454 medicinal plant *Dracocephalum moldavica*, the *GAPDH* gene was recognized as the
455 stable reference gene under PEG6000 treatment[44]. A prior meta-analysis indicated that
456 *GAPDH* is the preferred reference gene in over 90% of high-impact journal
457 publications[45], a finding that aligns well with the results of the current study.

458 The ideal reference gene should exhibit minimal transcriptional variation across
459 various experimental conditions. However, empirical investigations across different crop
460 species have identified significant inconsistencies in reference gene expression profiles
461 among distinct tissues, developmental stages, and stress conditions[41]. For instance,
462 variations in the expression of standard reference genes have been observed in *Solanum*
463 *tuberosum* under drought, cold, and salinity stress[46]; in *Solanum lycopersicum*
464 experiencing nitrogen deficiency, cold, and light treatments[47]; and in *Helianthus annuus*
465 throughout six leaf developmental stages[48]. These observations highlight the urgent
466 need to validate reference gene stability for specific experimental contexts to ensure the
467 reliability of plant gene expression analyses. The results of this study indicate that no
468 universally stable reference genes exist across various tissues and abiotic stress
469 conditions, emphasizing the lack of a one-size-fits-all reference gene panel for
470 environmental stress research. This finding is crucial as it reinforces the importance of
471 context-specific validation of reference genes. In light of the limited availability of
472 validated reference genes in *C. saxicola* research, this study serves as a valuable resource

473 for the scientific community. Our research provides essential insights into the selection of
474 robust reference genes for *C. saxicola* under varying stress conditions, which is vital for
475 accurate gene expression analysis and the development of high-yielding medicinal
476 varieties.

477 Conventional normalization methods that depend on a single reference gene may
478 undermine the precision of standardization outcomes[28,49]. This highlights the essential
479 need for thorough validation of reference genes and the adoption of advanced statistical
480 techniques to determine the optimal number of reference genes necessary for accurate
481 gene expression normalization. In this investigation, three bioinformatics tools—geNorm,
482 NormFinder, and BestKeeper—were utilized to evaluate the stability of candidate genes,
483 revealing minor variations in stability rankings due to the differing computational
484 algorithms and statistical methodologies employed by each program[50,51]. Specifically,
485 geNorm identified *TBP* and *UBCE2-17* as the most stable reference genes across all
486 sample sets, while NormFinder ranked *GAPDH2* and *UEVIC* as the leading candidates.
487 BestKeeper confirmed *GAPDH2* and *UBCE2-17* as the most consistently expressed
488 genes. Notably, *GAPDH2* consistently ranked among the top three in stability across all
489 analytical software, positioning it as a relatively optimal reference gene for qRT-PCR
490 analysis in *C. saxicola*. The results of this study are consistent with previous research that
491 emphasizes the importance of selecting reference genes based on specific contexts. The
492 variability inherent in different algorithmic methods can lead to inconsistent results when
493 analyzing the same datasets, as demonstrated by conflicting findings across various
494 species such as *Papaver somniferum*[52], *Angelica dahurica*[53], *Panax ginseng*[54,55],

495 *Glycyrrhiza*[56], and *Ludisia discolor*[51], which were assessed using different analytical
496 frameworks. To address the potential bias associated with using a single tool, we
497 employed RefFinder, a well-established integrative platform for evaluating reference
498 genes, to consolidate the findings from three distinct programs. Our thorough analysis
499 identified *GAPDH2*, *TBP*, and *UBCE2-17* as the most stable reference genes, while
500 α -*TUB*, *ACT1*, and *GAPDH8* showed the least expression consistency. Importantly,
501 normalization using a single gene was found to be inadequate for precise qRT-PCR
502 analysis; therefore, geNorm was utilized to ascertain the optimal number of reference
503 genes required for various tissues and abiotic stress conditions, facilitating the
504 development of context-specific reference gene panels.

505 Previous research has indicated that the concentration of dehydrocavidine in the
506 roots of *C. saxicola* is greater than that in the stems[8], with this variation linked to the
507 stages of plant growth and development[57]. In the current study, the expression level of
508 *CsBBEL9* in the stems was found to be 1.32 times higher than in the roots, while the
509 expression of *CsOMT9* in the stems was reduced by 86.57% compared to the roots.
510 However, when normalization was performed using unstable reference genes, the
511 expression levels of *CsBBEL9* and *CsOMT9* appeared to be abnormally high.
512 Additionally, it has been shown that appropriate levels of calcium and selenium ion stress
513 can enhance dehydrocavidine accumulation in *C. saxicola*[8]. In line with these findings,
514 treatments with Ca5 and Se50 were observed to upregulate the expression of *CsBBEL9*
515 and *CsOMT9*. Nevertheless, normalization with unstable genes resulted in lower
516 expression levels of *CsBBEL9* and *CsOMT9*, highlighting the significant influence of

517 using unsuitable reference genes.

518 In conclusion, the reference genes identified in this study are suitable for gene
519 expression analysis in *C. saxicola* across multiple organs and various abiotic stress
520 conditions. We advise utilizing these reference genes in qRT-PCR experiments involving
521 *C. saxicola* to prevent any potential inaccuracies.

522 Conclusion

523 Understanding the molecular mechanisms that regulate the biosynthesis of
524 secondary metabolism in *C. saxicola* has increasingly relied on functional genomics,
525 particularly transcriptomics. To date, although extensive transcriptomic data for *C.*
526 *saxicola* exist, a thorough analysis of large-scale gene expression datasets to identify
527 stable reference genes has not been performed. This study aims to identify potential
528 housekeeping gene candidates that can act as reliable reference genes for quantitative
529 gene expression analysis. To evaluate the validity and stability of these reference genes
530 comprehensively, we employed four different tools to assess their expression stability
531 across various tissues and under diverse abiotic stress conditions. We ranked the known
532 reference genes based on their stability, revealing that the combination of *GAPDH2*, *TBP*,
533 and *UBCE2-17* is appropriate for normalizing gene expression data across all *C. saxicola*
534 samples. Additionally, we found *ACT1* and *GAPDH8* to be the least stable among the
535 candidate genes. Importantly, results from the same tool showed inconsistencies in
536 identifying stable reference genes across samples subjected to different treatments.
537 Therefore, we recommend selecting stable reference genes tailored to the specific
538 treatment conditions of the samples for normalizing gene expression in *C. saxicola*, with

the use of at least two highly stable reference genes. The reference genes identified in this study not only enhance the reliability of gene expression analyses but also support functional genomics and metabolomics research related to abiotic stress tolerance in *C. saxicola*.

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Author contributions

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Declarations

Ethics approval and consent to participate

This article does not involve any studies conducted on human participants or animals by any of the authors.

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Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

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