

Transcriptome-Based Selection and Validation of Reference Genes for Gene Expression Analysis in *Roegneria ciliaris* ‘Liao sheng’ across Various Tissues and under Drought Stress

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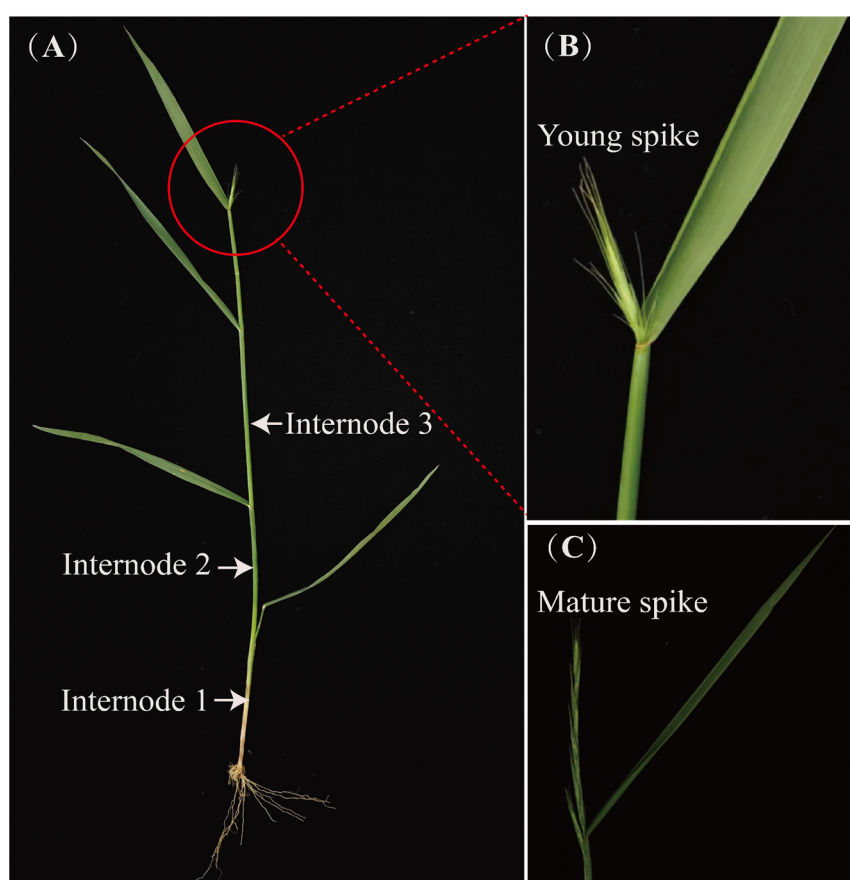


Figure S1. Phenotype diagrams of different tissues of *R. ciliaris* ‘Liao sheng’. (A) A whole plant of *R. ciliaris* at the booting stage, when the young spikes had extended about 2 cm from the flag leaf sheath. Arrows indicate the first three internodes from the base upward. (B) The zoom of young spike of *R. ciliaris*. (C) A mature spike of *R. ciliaris* at the heading stage (spike extended halfway out of the flag leaf sheath).

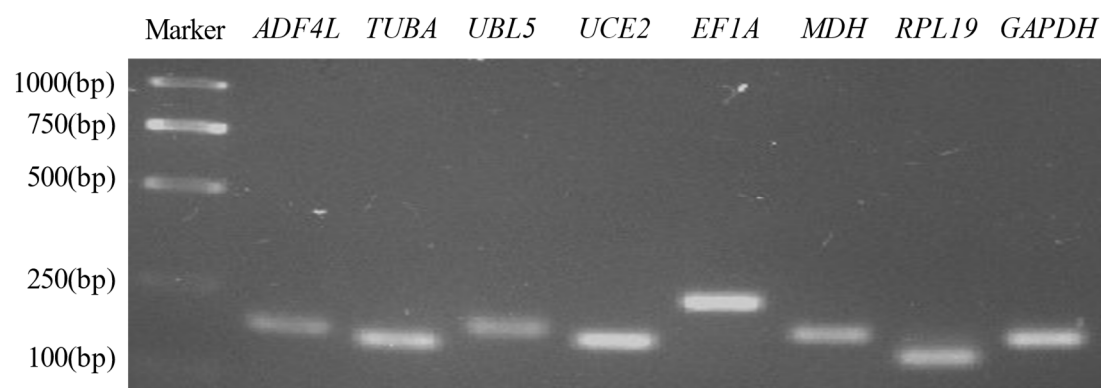


Figure S2. Analysis of PCR amplification specificity for the eight candidate reference genes via agarose gel electrophoresis.

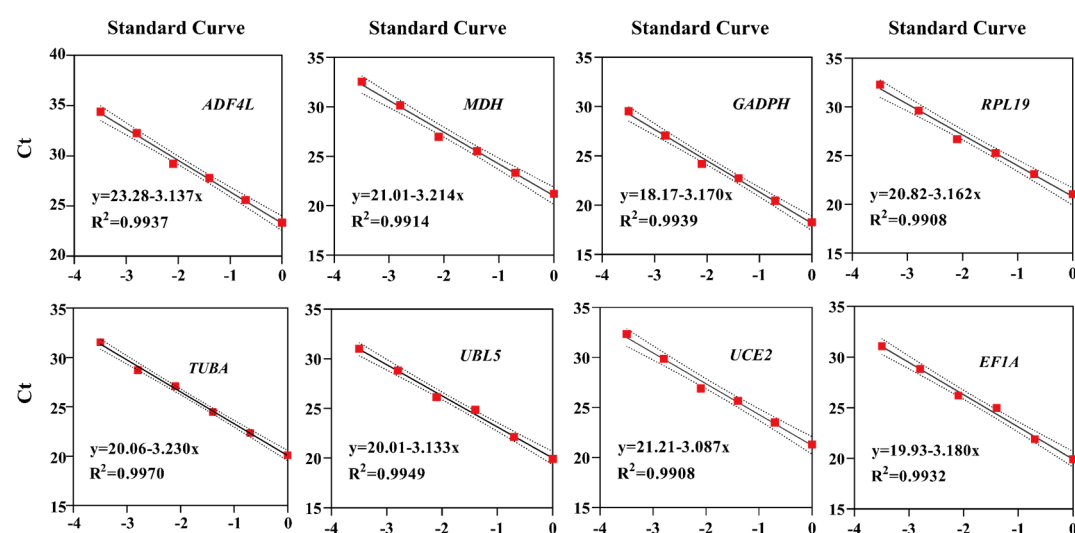


Figure S3. Standard curves of the eight candidate reference genes.

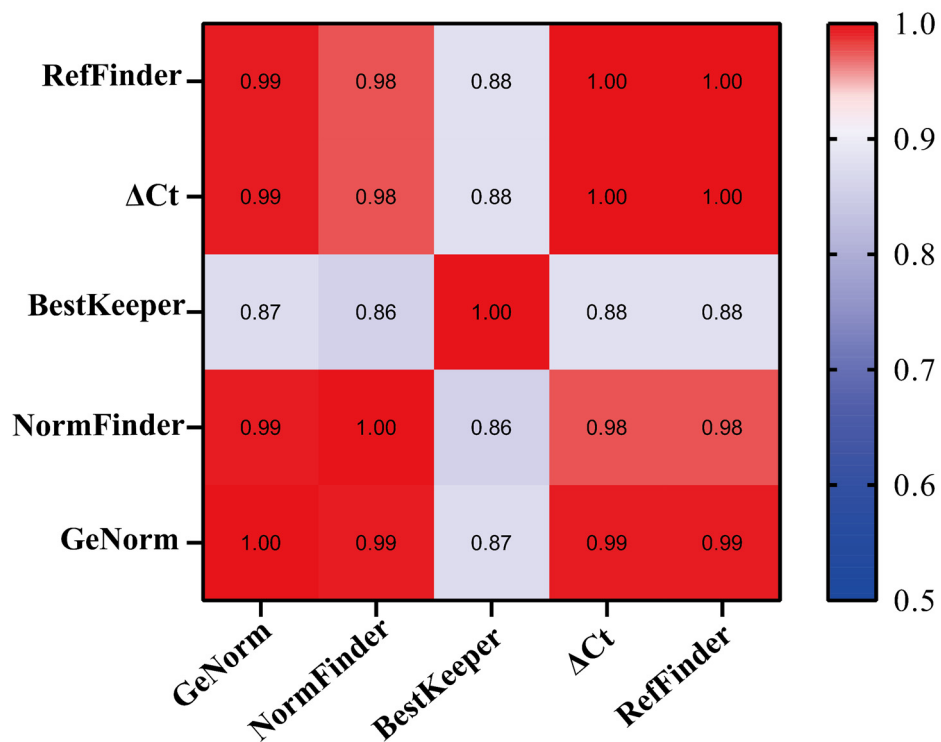


Figure S4. Spearman's rank correlation heatmap of candidate reference genes ranking. The heatmap illustrates the pairwise correlation coefficients (r) between the five algorithms used in this study (geNorm, NormFinder, BestKeeper, Δ Ct, and RefFinder). The color scale ranges from blue (lower correlation) to red (higher correlation). Numerical values within each cell indicate the specific Spearman's r . All correlations are statistically significant ($p < 0.01$).

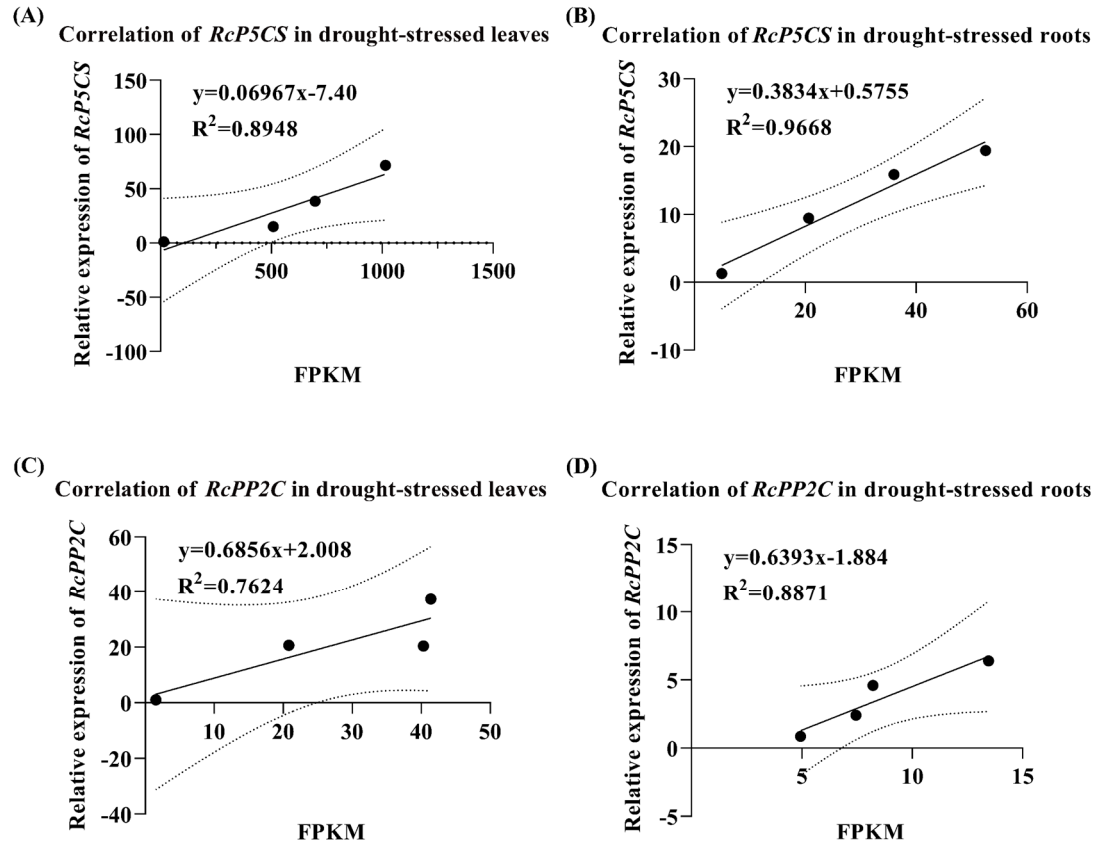


Figure S5. Correlation analysis validating the consistency between RNA-seq (FPKM) and qRT-PCR expression profiles for *RcP5CS* and *RcPP2C* under drought stress. The scatter plots illustrate the linear regression of transcript abundance (X-axis, FPKM) versus relative expression levels determined by qRT-PCR (Y-axis). Panels (A) and (B) show the correlation for *RcP5CS* in drought-stressed leaves and roots, respectively. Panels (C) and (D) show the correlation for *RcPP2C* in drought-stressed leaves and roots, respectively. The qRT-PCR data were normalized using the geometric mean of the optimal reference gene combination (*MDH* and *RPL19*). The solid lines represent the linear regression fit, while the curved dotted lines indicate the 95% confidence intervals. The linear equation and coefficient of determination are displayed within each graph.

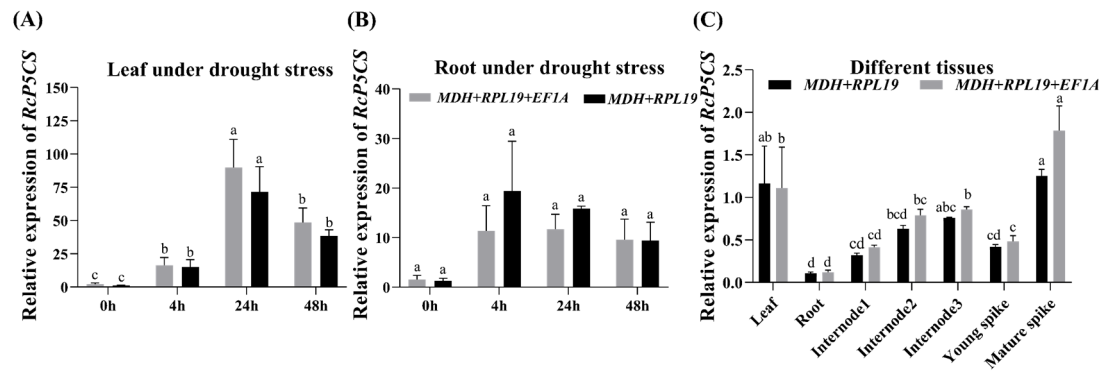


Figure S6. Comparison of *RcP5CS* relative expression normalized by the top two and top three reference genes. (A-C) qRT-PCR analysis of *RcP5CS* expression in leaves (A) and roots (B) under drought stress, and across different tissues (C). Relative expression levels of *RcP5CS* were calculated using the dual-gene combination (*MDH* and *RPL19*) and the three-gene combination (*MDH*, *RPL19*, and *EF1A*) for normalization.

Table S1. FPKM values of the eight reference genes in leaf and root samples under drought stress.

Gene name	Gene ID	FPKM								average	SD	CV	τ
		L_0h	L_4h	L_24h	L_48h	R_0h	R_4h	R_24h	R_48h				
<i>ADF4L</i>	isoform_66084	214.33	241.80	218.14	191.74	242.69	229.68	187.69	202.69	216.10	19.81	0.09	0.11
<i>TUBA</i>	isoform_17485	58.77	69.05	51.60	65.29	506.73	491.80	526.78	398.38	271.05	26.40	0.10	0.08
<i>UBL5</i>	isoform_95957	212.78	222.82	291.70	292.67	140.17	150.91	425.48	381.38	264.74	96.40	0.36	0.35
<i>UCE2</i>	isoform_77241	154.88	166.18	144.10	163.31	193.06	163.01	176.67	165.88	165.88	5.74	0.03	0.06
<i>EF1A</i>	isoform_20927	88.45	76.23	55.79	71.27	157.36	174.15	136.18	128.03	110.93	15.24	0.14	0.15
<i>MDH</i>	isoform_64311	108.97	79.31	80.66	90.52	179.75	110.24	157.59	152.90	119.99	20.27	0.17	0.26
<i>RPL19</i>	isoform_57359	117.55	131.22	115.21	140.37	243.83	237.18	212.74	176.38	171.81	12.57	0.07	0.09
<i>GAPDH</i>	isoform_36556	724.12	942.20	948.87	849.17	2789.04	2319.44	3321.59	4340.95	2029.42	433.77	0.21	0.29

Note: “L” and “R” represent leaf and root, respectively.

Table S2. Log₂ fold-change values of the eight reference genes under drought stress.

Gene name	Gene ID	log ₂ (Leaf_4h/Leaf_0h)	<i>P</i> value	log ₂ (Leaf_24h/Leaf_0h)	<i>P</i> value	log ₂ (Leaf_48h/Leaf_0h)	<i>P</i> value
<i>ADF4L</i>	isoform_66084	0.05	0.96	0.01	1.00	-0.24	0.97
<i>TUBA</i>	isoform_17485	0.14	0.96	-0.19	0.93	0.10	0.97
<i>UBL5</i>	isoform_95957	-0.10	0.96	0.48	0.82	0.37	0.97
<i>UCE2</i>	isoform_77241	-0.02	0.98	-0.12	0.96	-0.01	1.00
<i>EF1A</i>	isoform_20927	-0.31	0.67	-0.67	0.01	-0.36	0.97
<i>MDH</i>	isoform_64311	-0.58	0.10	-0.49	0.59	-0.36	0.93
<i>RPL19</i>	isoform_57359	0.05	0.96	-0.05	0.96	0.18	0.97
<i>GAPDH</i>	isoform_36556	0.29	0.85	0.39	0.60	0.17	0.97

Gene name	Gene ID	log ₂ (Root_4h/Root_0h)	<i>P</i> value	log ₂ (Root_24h/Root_0h)	<i>P</i> value	log ₂ (Root_48h/Root_0h)	<i>P</i> value
<i>ADF4L</i>	isoform_66084	0.15	0.96	-0.34	0.77	-0.13	0.96
<i>TUBA</i>	isoform_17485	0.16	0.96	0.03	1.00	-0.24	0.96
<i>UBL5</i>	isoform_95957	0.41	0.46	1.75	0.00	1.63	0.00
<i>UCE2</i>	isoform_77241	-0.01	0.99	-0.08	0.95	-0.08	0.96
<i>EF1A</i>	isoform_20927	0.35	0.66	-0.24	0.92	-0.19	0.96
<i>MDH</i>	isoform_64311	-0.49	0.54	-0.18	0.95	-0.11	0.96
<i>RPL19</i>	isoform_57359	0.18	0.96	-0.20	0.95	-0.35	0.87
<i>GAPDH</i>	isoform_36556	-0.06	0.96	0.23	0.94	0.75	0.13

Table S3. The Ct values of the eight reference genes in *R. ciliaris* across various tissues and under drought stress.

Gene Name	Leaf	Root	Internode 1	Internode 2	Internode 3	Young spike	Mature spike	Tissues	Drought stress	All samples
<i>MDH</i>	20.00±0.33	18.94±0.14	20.20±0.09	19.15±0.06	19.52±0.12	18.79±0.36	19.53±0.40	19.45±0.13	19.89±0.14	19.68±0.10
<i>TUBA</i>	22.00±0.19	18.82±0.38	17.37±0.08	17.08±0.34	18.30±0.14	17.67±0.29	19.63±0.51	20.91±0.33	18.80±0.34	19.93±0.28
<i>UBL5</i>	20.13±0.30	20.84±0.31	21.39±0.09	22.70±0.02	23.26±0.12	21.29±0.07	22.14±0.35	21.68±0.23	20.27±0.15	20.93±0.17
<i>UCE2</i>	21.39±1.07	19.21±0.12	20.63±0.05	21.28±0.88	20.94±0.19	20.02±0.19	21.03±0.34	20.64±0.23	20.16±0.20	20.39±0.15
<i>EF1A</i>	20.30±0.31	17.84±0.45	19.35±0.16	17.89±0.14	17.86±0.14	17.18±0.18	18.61±0.44	19.14±0.23	18.43±0.24	18.81±0.17
<i>RPL19</i>	20.67±0.29	19.13±0.16	21.40±0.07	20.12±0.17	19.75±0.19	19.31±0.08	20.24±0.37	20.09±0.17	20.36±0.18	20.23±0.12
<i>ADF4L</i>	24.89±0.67	23.92±0.41	23.36±0.47	21.80±0.03	22.34±0.19	21.29±0.38	22.46±0.07	22.87±0.28	22.74±0.27	22.80±0.19
<i>GAPDH</i>	-	-	-	-	-	-	-	-	17.91±0.37	-

Note: The Ct values for *GAPDH* are only presented for the drought stress dataset, as its expression was undetectable in tissues other than leaves and roots. “-” indicates that data are not available. Data are presented as means $\pm$ SE (n=3).