

[10.1071/FP22051](https://doi.org/10.1071/FP22051)

Functional Plant Biology

Supplementary Material

Combined physiological responses and differential expression of drought-responsive genes preliminarily explain the drought resistance mechanism of *Lotus corniculatus*

Leiting Wang^A, Zhongling Jian^A, Puchang Wang^B, Lili Zhao^{A,C,}, and Keke Chen^A*

^ACollege of Animal Science, Guizhou University, Guiyang 550025, China.

^BGuizhou Institute of Prataculture, Guiyang 550006, China.

^CState Engineering Technology Institute for Karst Rocky Desertification Control, Guiyang 550025, China.

*Correspondence to: Lili Zhao College of Animal Science, Guizhou University, Guiyang 550025, China

Email: zhaolili_0508@163.com

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

To identify the internal control genes of *L. corniculatus* under drought stress, we performed expression level detection and stability analysis on eight candidate genes (Fig. S1). The differences in the expression levels of internal control genes H2A and UBI in *L. corniculatus* were the least obvious under different drought stresses, and the Ct value of UBI was lower (Fig. S1a). Further expression stability analysis of these candidate genes showed that UBI had the best stability (Fig. S1b). In conclusion, UBI is the most reliable reference candidate gene for *L. corniculatus* under drought stress.

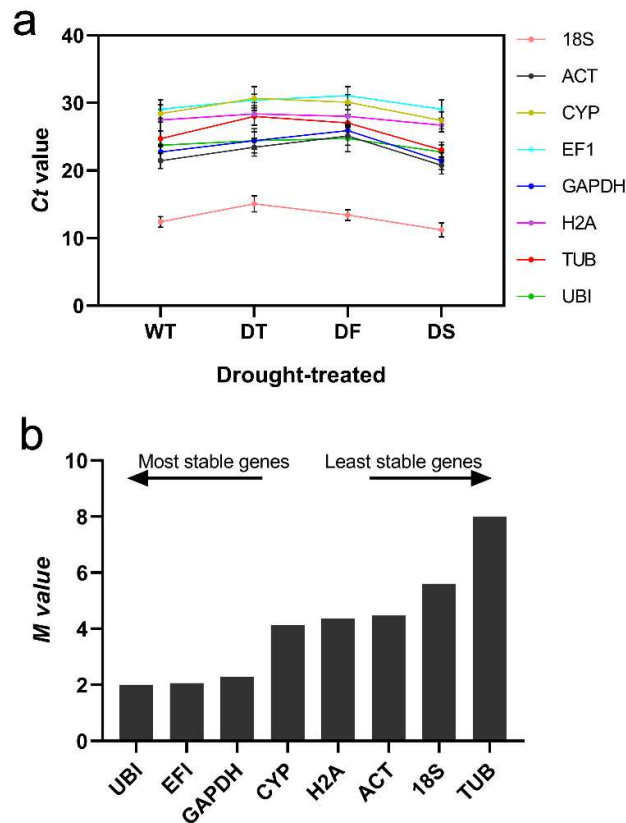


Fig. S1. Analysis of expression stability of reference genes in *L. corniculatus* under drought stress. (a) The expression changes of candidate reference genes under different drought stress. (b) The expression stability of candidate reference genes by an on-line tool in all samples. The value is the comprehensive analysis result of the

comparative ΔCt , Best Keeper, Norm Finde and ge Norm software. *M value*: Average expression stability.

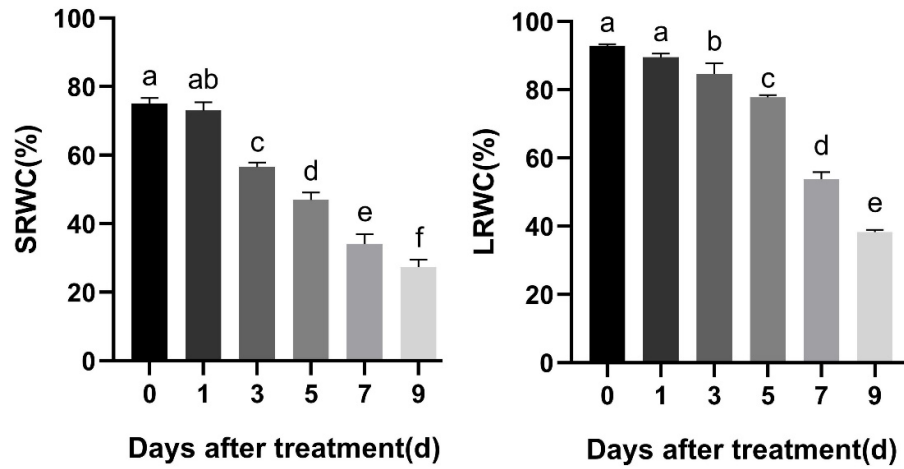


Fig. S2. Changes in the relative water contents of soil and leaves with increasing drought time. Tukey's test was performed to determine the significance of the differences between the drought treatments in the SPSS 19.0 (SPSS Inc. Chicago, IL, USA) to assess the differences between the WC and drought treatments. Values are means \pm SD ($n = 3$). Different lowercase letters indicate $P < 0.05$.

Analysis of subtraction efficiency

When the isolated total RNA was tested for integrity on a 1% denatured agarose gel, the 28S, 18S and 5S bands could be clearly observed, and both the 28S and 18S bands were bright (Fig. S3a). The RNA OD₂₆₀/OD₂₈₀ absorbance ratio ranged from 1.9 to 2.1. The concentrations of total RNA in the WC- and drought-treated plants were 550 ng/ μ L and 520 ng/ μ l (Fig. S2b), respectively. These results indicated that total RNA was successfully isolated.

The cDNA obtained after the successful reverse transcription of mRNA was digested with RsaI. For subtractive hybridization after connecting the adapter, the products were subjected to 2 rounds of inhibitory PCR. It was observed on agarose gels that the bands of the subtracted samples from the second round of PCR (200–650 bp) were more concentrated and smaller than those from the first round of PCR (100–1500 bp) (Fig. S3c, d). We found that the number of genes showing differential expression was sufficiently reduced and that ESTs were effectively enriched.

LB medium was used for the colony culture of *E. coli DH5 α* . The storage capacity of the forward library was 3,000, and the reverse library capacity was 2,800 (Fig. S3h). Twenty-four positive clones were randomly selected from the constructed library for quality identification. PCR fragments ranging from 100 to 500 bp in size were observed on agarose gels (Fig. S3e-g). We considered these genes to represent specifically expressed genes.

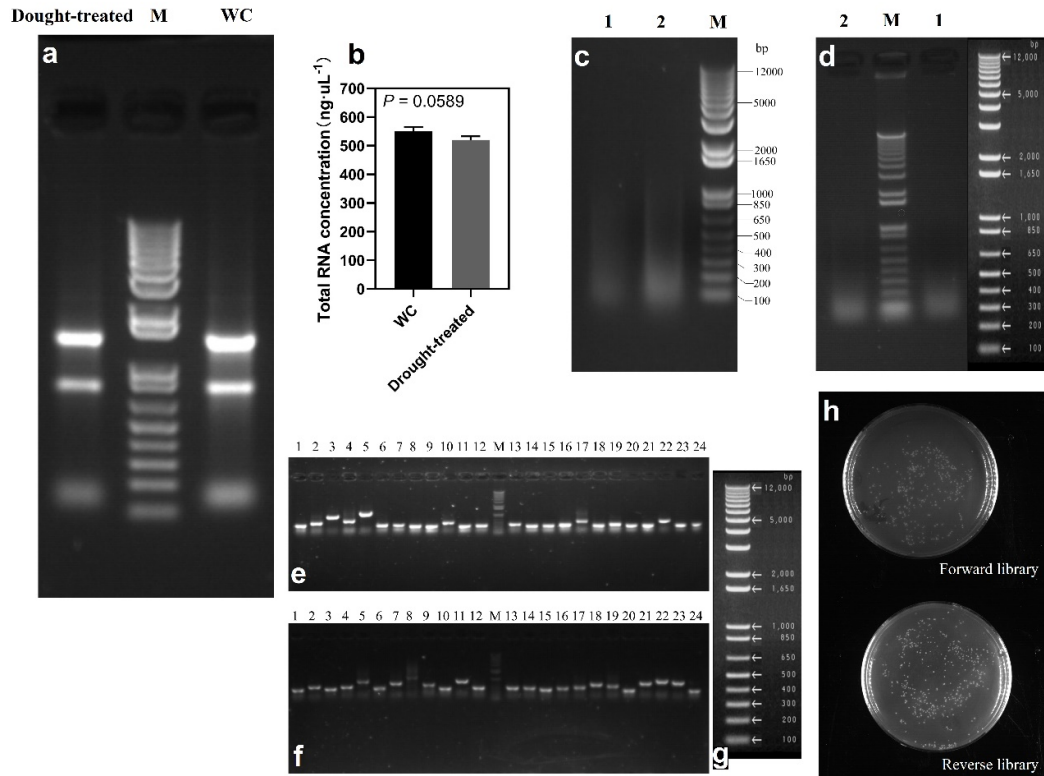


Fig. S3. Generation of the *L. corniculatus* suppression subtraction hybridization (SSH) library. (a) Ethidium bromide-stained denaturing formaldehyde gel electrophoresis of total RNA. Lane M: 1Kb Plus DNA Ladder. (b) The concentration of the isolated total RNA. Values are the means \pm SD of three replicates, with $P = 0.05$ as the significance level. (c) and (d) show the results of the first and second rounds of the PCR-selected cDNA subtraction method. Lane 1: Unsubtracted forward secondary PCR products. Lane 2: Subtracted forward secondary PCR products. Lane M: 1Kb Plus DNA Ladder. (e-g) Lanes 1–24 show the identification of partial positive PCR clones. (e) Forward library, (f) Reverse library. Lane M and (g) 1Kb Plus DNA Ladder. (h) Subtracted library capacity appraisal.

Table S1. Description of primers used for validation of genes by qPCR.

Target gene	Primer (5'→3')	Product size (bp)
<i>BGAL3</i>	F: AGTTGTGCCGCAAATGGT R: ACTGGATTCGGTGCATCACT	83
<i>BGAL4</i>	F: AAGCTGAGGGTTGGCAACAA R: GTGACCGGTCCTAACACTCC	110
<i>BGAL5</i>	F: GGAAGCATGGGAAAGGGTCA R: GGCACCTTTGGCTCTCGGTAT	124
<i>BGAL6</i>	F: GCTTCACAAGGAGGCCCAAT R: TCCTGCTATGCCAAATGCCT	81
<i>BGAL9</i>	F: TGCCTCGATCGAGTATGGTG R: TGAATTCCGGCGGAGATGAG	132
<i>BGAL12</i>	F: GGTCCTTCGTTTGTGCTGA R: CCTTGAACGGCTCATTGTCTG	100
<i>IAA7</i>	F: TGAAGGATGCTTGTGCGGTG R: TTCTCCATGGCTCTTGGTGC	114
<i>IAA29</i>	F: GCAGGTTACACGCTCACCTT R: TCCTCAATATCGCCAAACGC	109
<i>IAA30</i>	F: AGTTGGCAACCAATGCAACC R: TGCCTTCCATGTAGACCTTCAC	103
<i>CML38</i>	F: ACGGGTTCCTGTGTTTGGAG R: CTCTTCAAACCTCCGGCGT	139
<i>CML39</i>	F: TGTAACATGAGGAAGGACGCC R: GAGCTCGGAAGGCGAAATCT	87
<i>CML42</i>	F: CAAAAGCTCACCACCAAGCTC R: GTGACAAGAGCACGGACCAT	102
<i>MPK3</i>	F: CGCACGCTTCGTGAGATTAAG R: GTGTCCATGAGTTCGGTGGT	131
<i>MPK4</i>	F: ATGTCCCTCCTATCCGACCC R: TCGCAACTTCTTCCCGTGT	88
<i>MPK6</i>	F: CATTATCCGAAGAACATTGCCAG R: GTTCAGGAGGAGATTACTTGGTTTC	116
<i>MPK15</i>	F: CAGATGCCACTCGGATCCTT R: AACTCTCTTCGTGAAGGCGG	106
<i>MPK20</i>	F: TGGTGTGTCTGTTTCAGCGA R: TCACGGAGAATACGTGCAGC	108
<i>PP2A</i>	F: GGTCTCTCACCTTCGTTGGA R: ACACATCGGTCCTTCATGCG	84
<i>UBI</i>	F: TGTCCAGTCGTTCTACGGA R: TTGCAGCGTCACTAGCACTT	149

Table S2. BLASTX results of some genes (E -value $\leq 1E-5$) in SSH library from drought stress.

Sample No.	Sequences producing significant alignment	Species	Access number	E -value	Identities (%)
SSH-FH1-contig000 01	Hypothetical protein, partial	<i>Hordeum vulgare</i> <i>subsp</i>	CAG30723. 1	1.60E-11 6	100
SSH-FH1-contig000 02	Predicted: uncharacterized protein LOC106437402	<i>Brassica napus</i>	XP_013733 755.1	3.23E-13	100
SSH-FH1-contig000 04	Predicted: uncharacterized protein LOC103444761	<i>Malus domestica</i>	XP_008381 930.1	7.53E-12	100
SSH-FH1-contig000 05	P-glycoprotein protein	<i>Lactuca sativa</i>	BAF32961. 1	7.27E-07	88.46
SSH-FH1-contig000 16	Hypothetical protein PHAVU_008G179700g	<i>Phaseolus vulgaris</i>	XP_007141 246.1	6.87E-13	93.75
SSH-FH1-233-M13 --A06	Transposon Ty3-I Gag-Pol polyprotein	<i>Cajanus cajan</i>	KYP63270. 1	1.35E-11	96.67
SSH-FH1-346-M13 --B08	Ycf2	<i>Lotus japonicus</i>	NP_084839 .1	2.95E-18	97.37
SSH-FH1-448-M13 --H08	Predicted: uncharacterized ATP-dependent helicase MPN_020-like	<i>Brassica napus</i>	XP_013708 970.1	4.13E-25	94.23
SSH-FH1-A110-M1 3--F02	Hypothetical protein Poptr_cp075	<i>Populus trichocarpa</i>	YP_001109 553.1	3.24E-09	85.71
SSH-FH1-336-M13 --H06	Strain MG-20 mitochondrion	<i>Lotus japonicus</i>	JN872551.2	6.03E-38	88.47
SSH-FH1-352-M13 --H08	Ribosomal RNA gene	<i>Lotus tenuis</i>	KNA06354. 1	3.08E-39	60.38
SSH-FH1-417-M13 --A05	Beta-galactosidase	<i>Lupinus angustifolius</i>	CCH4 7205.1	2.40E-09	67.41
SSH-FH1-A95-M13 --G12	Calmodulin-like protein	<i>Arabidopsis thaliana</i>	AEE35461. 1	3.20E-37	95
SSH-FH2-contig000 01	PREDICTED: uncharacterized ATP-dependent helicase MPN_020-like	<i>Brassica napus</i>	XP_013708 970.1	4.86E-26	98.08
SSH-FH2-contig000 09	hypothetical protein KK1_048196	<i>Cajanus cajan</i>	KYP31477. 1	1.59E-18	91.84
SSH-FH2-contig000 16	PREDICTED: uncharacterized protein K02A2.6-like	<i>Arachis duranensis</i>	XP_015931 971.1	8.70E-11	79.49
SSH-FH2-117-M13 --E03	PREDICTED: uncharacterized protein LOC101495741	<i>Cicer arietinum</i>	XP_012574 812.1	7.35E-13	69.64
SSH-FH2-104-M13 --H01	mitogen-activated protein kinase	<i>Arabidopsis thaliana</i>	BAA09057. 1	2.45E-28	82.30
SSH-FH2-109-M13	PREDICTED: uncharacterized protein	<i>Glycine max</i>	XP_014621	5.97E-22	82.35

--E02	LOC106795569		654.1
SSH-FH2-115-M13	unknown	<i>Lotus japonicus</i>	AFK41271.1 7.17E-09 82.35
--C03			
SSH-FH2-116-M13	Retrovirus-related Pol polyprotein from	<i>Cajanus cajan</i>	KYP76070.1 2.53E-18 78.46
--D03	transposon 17.6		
SSH-FH2-139-M13	envelope-like protein	<i>Glycine max</i>	AAO73524.1 5.65E-11 68.85
--C06			
SSH-FH2-156-M13		<i>Phalaenopsis aphrodite subsp. formosana</i>	YP_358634.1 9.32E-14 78.57
--D08	hypothetical protein PhapfoPp088		
SSH-FH2-158-M13	hypothetical protein AMTR_s03239p00005680, partial	<i>Amborella trichopoda</i>	ERN08425.1 4.01E-16 82.61
--F08			
SSH-FH2-17-M13--A03	maturase (mitochondrion)	<i>Lotus japonicus</i>	YP_005090474.1 2.24E-08 83.87
SSH-FH2-159-M13--G08	hypothetical protein MTR_0333s0010	<i>Medicago truncatula</i>	XP_013442070.1 9.15E-09 92.86
SSH-FH2-267-M13--C10	PREDICTED: uncharacterized protein LOC101503197, partial	<i>Cicer arietinum</i>	XP_004517103.2 2.13E-24 80
SSH-FH2-274-M13--B11	PREDICTED: uncharacterized protein LOC107607039	<i>Arachis ipaensis</i>	XP_016164514.1 1.28E-09 87.88
SSH-FH2-295-M13--G01	Indole-3-acetic acid-induced protein ARG7	<i>Cajanus cajan</i>	KYP71979.1 3.10E-13 96.55
SSH-FH2-311-M13--G03	hypothetical protein MTR_0389s0050, partial	<i>Medicago truncatula</i>	XP_013441978.1 3.69E-06 79.31
SSH-FH2-326-M13--F05	hypothetical protein KK1_039730	<i>Cajanus cajan</i>	KYP38984.1 9.20E-24 72.86
SSH-FH2-18-M13--B03	hypothetical protein VIGAN_10233900, partial	<i>Vigna angularis var. angularis</i>	BAU00723.1 2.39E-11 96.77
SSH-FH2-347-M13--C08	hypothetical protein KK1_047086	<i>Cajanus cajan</i>	KYP32270.1 2.56E-09 100
SSH-FH2-371-M13--C11	ALG2-interacting protein X	<i>Medicago truncatula</i>	XP_003592574.2 4.61E-18 92.86
SSH-FH2-40-M13--H05	PREDICTED: uncharacterized protein LOC100800409	<i>Glycine max</i>	XP_003536822.1 5.38E-10 80
SSH-FH2-456-M13--H09	PREDICTED: uncharacterized protein LOC103420094	<i>Malus domestica</i>	XP_008356376.1 1.27E-08 65.22
SSH-FH2-482-M13--B01	hypothetical protein MTR_3g435560	<i>Medicago truncatula</i>	XP_013459289.1 1.53E-08 90.63
SSH-FH2-484-M13--D01	hypothetical protein VIGAN_01220600, partial	<i>Vigna angularis var. angularis</i>	BAT74520.1 1.70E-12 74.47
SSH-FH2-506-M13--B04	unknown	<i>Lotus japonicus</i>	AFK43749.1 2.49E-09 87.5

SSH-FH2-530-M13 --B07	hypothetical protein GLYMA_13G015600	<i>Glycine max</i>	KRH17782. 1	7.39E-08 100
SSH-FH2-534-M13 --F07	Transposon Ty3-I Gag-Pol polyprotein, partial	<i>Cajanus cajan</i>	KYP47135. 1	1.82E-31 96.55
SSH-FH2-540-M13 --D08	gag-pol polymerase	<i>Arabidopsis lyrata</i> <i>subsp. lyrata</i>	ABW81017 .1	8.72E-10 83.33
SSH-FH2-552-M13 --H09	PREDICTED: uncharacterized protein LOC106796443	<i>Glycine max</i>	XP_014624 207.1	1.36E-09 92
SSH-FH2-561-M13 --A11	GDP-D-mannose-4, 6-dehydratase (MUR1)	<i>Arabidopsis thaliana</i>	CAB62638. 1	6.80E-11 92.86
SSH-FH2-58-M13-- B08	Transposon Ty3-I Gag-Pol polyprotein	<i>Cajanus cajan</i>	KYP37665. 1	1.76E-07 95.83
SSH-FH2-93-M13-- E12	Retrovirus-related Pol polyprotein from transposon 412 family	<i>Cajanus cajan</i>	KYP56985. 1	5.38E-18 72.73
SSH-FH2-A101-M1 3--E01	Retrovirus-related Pol polyprotein from transposon 17.6	<i>Cajanus cajan</i>	KYP50545. 1	6.42E-24 73.75
SSH-FH2-A113-M1 3--A03	hypothetical protein MTR_0008s0310	<i>Medicago truncatula</i>	XP_013443 534.1	1.16E-15 97.3
SSH-FH2-A149-M1 3--E07	hypothetical protein OsJ_00596	<i>Oryza sativa Japonica</i> <i>Group</i>	EEE53976. 1	3.81E-07 64.44
SSH-FH2-A165-M1 3--E09	putative gag protein	<i>Coffea canephora</i>	AJE29373.1	2.13E-06 60.34
SSH-FH2-A169-M1 3--A10	Retrovirus-related Pol polyprotein from transposon TNT 1-94	<i>Cajanus cajan</i>	KYP40337. 1	2.03E-09 100
SSH-FH2-A44-M13 --D06	orf116d (mitochondrion)	<i>Batis maritima</i>	YP_009045 821.1	7.55E-06 95.65
SSH-FH2-A51-M13 --C07	Retrotransposable element Tf2	<i>Cajanus cajan</i>	KYP71657. 1	2.25E-23 82.46
SSH-FH2-A59-M13 --C08	Serine/threonine-protein phosphatase	<i>Cicer arietinum</i>	XP_012572 055.1	5.14E-10 90
SSH-FH2-A60-M13 --D08	Pol polyprotein, partial	<i>Cajanus cajan</i>	KYP36967. 1	2.03E-22 77.05
SSH-FH2-A146-M1 3--B07	Putative ribonuclease H protein, partial	<i>Glycine soja</i>	KHN14248. 1	2.62E-09 75.51
