

Supplementary material

Reference	Species	sequence	bp
XM_017556154.1	Vigna angularis	CCAGCCATCTCCAAGGGGAGTCGGTGCAGGACTGGAGAGAGATTGTGACATACTTTTCCT	412
XM_019604136.1	Lupinus angustifolius	CAAGCCATCTTCAAGGAGAAGCAGTTCAGGATTGGAGAGAGATAGTAACATATTTTCAC	454
XM_019567303.1	Lupinus angustifolius	CTAGCCATCTACAAGGAGAAGCAGTGAGAAATTGGAGAGAGATAATGACATATTCTCAT	460
NM_001249868.3	Glycine max	CCAGCCACCTTCAAGGAGAAGCGGTGCAGGATTGGAGAGAGATAGTGATATATTTTCAC	409
XM_003517127.5	Glycine max	CCAGCCACCTTCAAGGAGAAGCGGTGCAGGATTGGAGAGAGATTGTGATATATTTTCAC	409
XM_019564825.1	Lupinus angustifolius	CCAGCCACCTTCAAGGAGAAACAGTGAAAGATTGGAGAGAGATTGTGACATACTTTTCAT	409
XM_019564826.1	Lupinus angustifolius	CCAGCCACCTTCAAGGAGAAACAGTGAAAGATTGGAGAGAGATTGTGACATACTTTTCAT	409
XM_019564827.1	Lupinus angustifolius	CCAGCCACCTTCAAGGAGAAACAGTGAAAGATTGGAGAGAGATTGTGACATACTTTTCAT	409
CP023129.1	Lupinus angustifolius	CCCTTTGTGTGAAGGGAGAAACAGTGAAAGATTGGAGAGAGATTGTGACATACTTTTCAT	1256
		* * * * *	
Reference	Species	sequence	bp
XM_017556154.1	Vigna angularis	---CTATCTGAGCAATGGAAGTTCAAGAATGCTGATCACCAGCAGTGGTGAACCTCGAA	851
XM_019604136.1	Lupinus angustifolius	---TTATTTGAGTAATGGAAGTTCAAGAATGCTGATCACCAGCAGTGGTGAACCTCAA	893
XM_019567303.1	Lupinus angustifolius	-----TGAGTAATGGGAGGTTCAAGAATGCTGATCATCAAGCAGTGGTGAACCTCAA	899
NM_001249868.3	Glycine max	---TTATCTGAGTAATGGAAGTTCAAGAATGCTGATCACCAGCAGTGGTGAACCTCAAG	848
XM_003517127.5	Glycine max	---TTATCTGAGTAATGGAAGTTCAAGAATGCTGATCACCAGCAGTGGTGAACCTCAAG	848
XM_019564825.1	Lupinus angustifolius	----TATCTGAGCAATGGAAGGTTCAAGAATGCTGATCACCAGCAGTAGTGAACCTCTAA	848
XM_019564826.1	Lupinus angustifolius	----TATCTGAGCAATGGAAGGTTCAAGAATGCTGATCACCAGCAGTAGTGAACCTCTAA	848
XM_019564827.1	Lupinus angustifolius	----TATCTGAGCAATGGAAGGTTCAAGAATGCTGATCACCAGCAGTAGTGAACCTCTAA	848
CP023129.1	Lupinus angustifolius	ACAGTATCTGAGCAATGGAAGGTTCAAGAATGCTGATCACCAGCAGTAGTGAACCTCTAA	1856
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Figure S1. Multiple sequence alignment of partial *F3H* nucleotide sequence from *Glycine max*, *Vigna angularis* and *Lupinus angustifolius* orthologs, illustrating primer location 5'GATTGGAGAGAGATTGTGACATA3' (forward), 5'GTGATCAGCATTCTTGAACC3' (reverse).

LmF3H_a	CRDYSRWPNNPSEWRKVTEQYSDMLMGLGSKLLELLSEAMGSEKDALTAKACIDVDQKIVVNYYPK
	. .
LmF3H_b	ERDYSRWPEKPIGWRKVTEQYSEELMGLSLCKLLQVLSEAMGLDKEAVTKACIEMDQKVNVNFPYK
	. .
LmF3H_a	CQPQDLTLGLKRHTDPGLITLLQLDQVGGGLQATRDNGQTWITVQPIEGAFVVNLGDYG
	. .
LmF3H_b	CQPQDLTLGLKRHTDPGLITLLQLDQVGGGLQATRDNGKWTWITVQPDGAFVVNLGDHG
	. .

Figure S2. Alignment of the F3H predicted polypeptide sequence of paralogues LmF3h_a and LmF3h_b of *Lupinus mutabilis*.

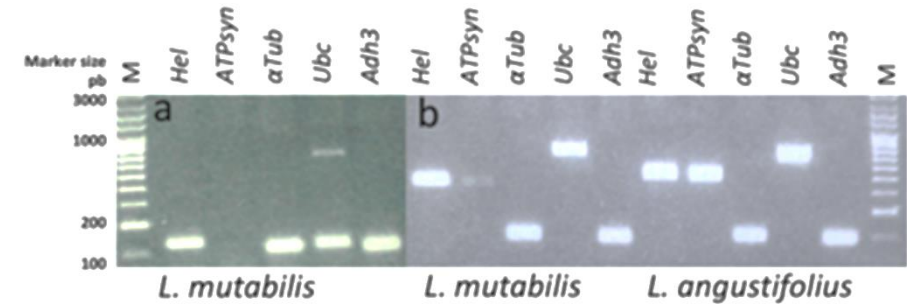
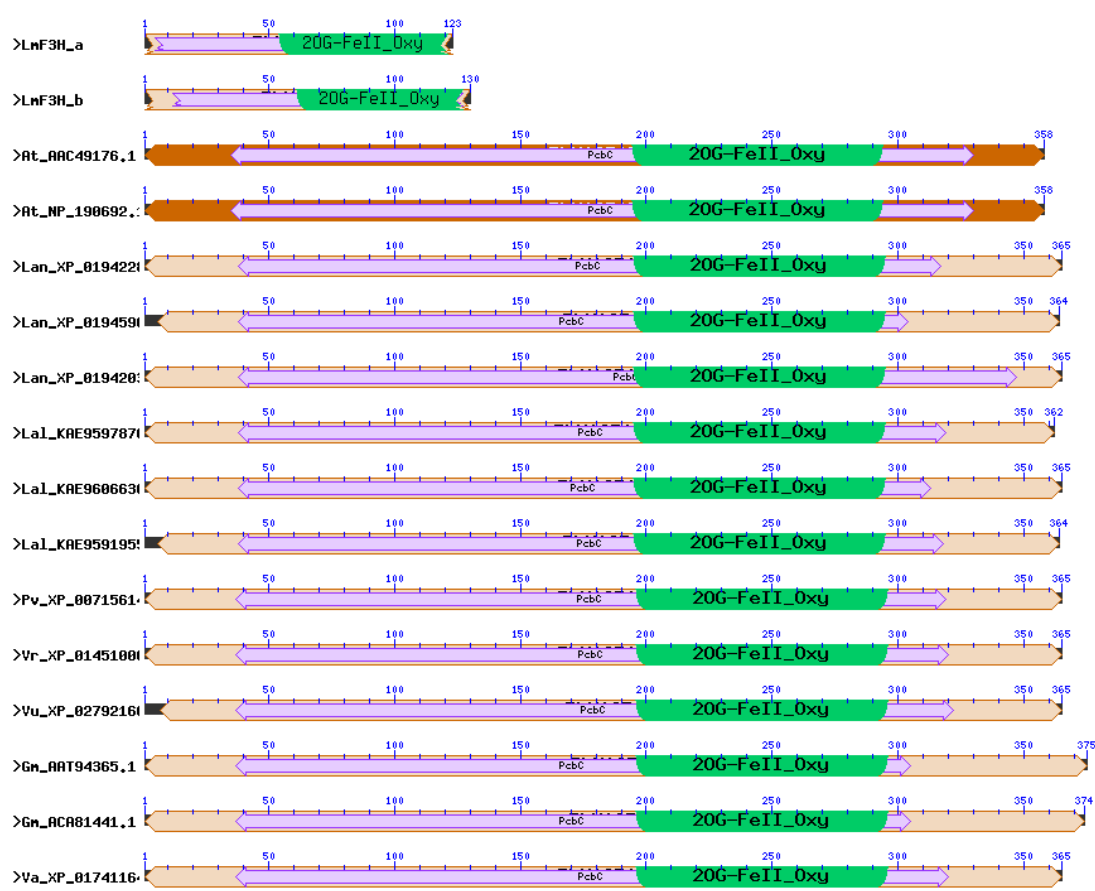


Figure S3. PCR amplification patterns of reference genes using cDNA and genomic DNA as templates. (a) PCR from cDNA and (b) from DNA. M- NZYDNA Ladder VII marker.

Table S1. Similarity matrix between *Lupinus mutabilis* Lm_F3H_a and Lm_F3H_b predicted amino acid sequences, F3H orthologs (with reference to the *L. angustifolius* chromosome location of the respective genes) and FNS, ANS, FLS, H6H and GA20ox proteins

Protein accession numbers	Percent of identity																											
	{A}	{B}	{C}	{D}	{E}	{F}	{G}	{H}	{I}	{J}	{K}	{L}	{M}	{N}	{O}	{P}	{Q}	{R}	{S}	{T}	{U}	{V}	{W}	{X}	{Y}	{Z}	{AA}	{AB}
Dm_AAQ04302.1_H6H {A}	100.00	84.12	27.52	25.68	26.91	26.91	29.94	29.57	30.15	30.15	38.02	28.96	28.96	29.64	29.64	32.24	38.28	31.04	30.65	30.45	31.85	30.06	30.36	30.06	25.23	26.73	30.00	29.06
Bc_ACB40931.1_H6H {B}	84.12	100.00	26.06	26.22	25.76	25.15	30.61	30.30	30.42	31.02	39.67	30.51	30.51	29.09	29.09	32.02	38.28	31.42	30.12	30.42	31.02	29.82	30.42	29.82	25.23	25.76	29.69	29.37
At_NP_175075.1_GA20ox {C}	27.52	26.06	100.00	38.13	44.03	43.24	29.02	28.74	26.07	26.36	39.67	28.74	27.30	27.95	27.67	27.30	33.59	28.03	28.37	27.79	27.51	28.08	27.79	27.79	29.67	27.32	28.22	25.77
Cm_AAB64345.1_GA20ox {D}	25.68	26.22	38.13	100.00	50.80	50.27	26.99	27.33	27.35	27.64	34.71	26.57	26.86	28.37	28.65	26.00	28.91	24.71	26.50	26.57	26.21	26.50	25.93	26.50	25.23	26.11	28.88	27.02
Lan_XP_019449768.1_GA20ox {E}	26.91	25.76	44.03	50.80	100.00	96.34	29.94	30.52	29.86	30.43	37.19	30.81	29.36	30.61	30.61	30.52	35.94	30.23	30.72	30.43	30.14	31.01	30.72	31.01	27.98	27.14	32.21	26.38
Lal_KAE9617323.1_GA20ox {F}	26.91	25.15	43.24	50.27	96.34	100.00	30.23	30.81	30.43	31.01	37.19	31.10	29.94	30.90	30.90	31.40	35.94	31.40	31.01	30.72	31.01	31.30	31.01	31.30	28.57	27.71	32.82	26.38
Cc_ABG78790.1_FNS {G}	29.94	30.61	29.02	26.99	29.94	30.23	100.00	94.68	71.27	71.82	73.17	68.70	68.14	73.18	73.18	72.93	77.69	72.22	74.03	72.85	75.69	74.59	75.69	75.69	30.72	29.89	32.31	31.38
Dc_AAX21536.1_FNS {H}	29.57	30.30	28.74	27.33	30.52	30.81	94.68	100.00	72.19	73.03	73.98	70.14	68.45	74.15	74.43	72.19	76.92	71.47	74.72	73.88	76.12	74.16	75.00	75.28	30.72	30.29	33.23	32.31
Lan_XP_019420370.1_F3H chromosome 17 {I}	30.15	30.42	26.07	27.35	29.86	30.43	71.27	72.19	100.00	91.78	80.49	77.41	76.58	78.77	78.49	78.30	81.54	76.24	80.22	78.79	81.59	79.67	81.59	81.59	29.82	30.45	32.21	31.90
Lal_KAE9606636.1_F3H {J}	30.15	31.02	26.36	27.64	30.43	31.01	71.82	73.03	91.78	100.00	79.67	76.58	75.48	77.09	77.37	79.12	81.54	76.24	80.22	78.79	81.59	79.12	81.04	81.59	30.12	29.89	31.29	30.37
LmF3H_a_F3H {K}	38.02	39.67	39.67	34.71	37.19	37.19	73.17	73.98	80.49	79.67	100.00	91.06	88.62	82.93	82.11	81.30	79.67	82.11	79.67	79.67	82.93	83.74	82.93	82.11	36.36	38.02	38.02	41.32
Lan_XP_019459681.1_F3H chromosome 11 {L}	28.96	30.51	28.74	26.57	30.81	31.10	68.70	70.14	77.41	76.58	91.06	100.00	93.13	74.79	75.07	76.86	78.46	77.56	77.47	77.13	77.75	78.57	78.57	78.57	29.52	30.25	31.60	31.60
Lal_KAE9591955.1_F3H {M}	28.96	30.51	27.30	26.86	29.36	29.94	68.14	68.45	76.58	75.48	88.62	93.13	100.00	75.07	75.07	76.31	79.23	76.73	76.92	76.58	76.92	76.92	77.75	77.47	28.92	29.41	30.98	31.29
At_AAC49176.1_F3H {N}	29.64	29.09	27.95	28.37	30.61	30.90	73.18	74.15	78.77	77.09	82.93	74.79	75.07	100.00	99.44	79.33	83.08	76.69	82.40	81.79	84.36	82.68	83.24	83.52	30.51	31.44	30.46	28.92
At_NP_190692.1_F3H TT6 {O}	29.64	29.09	27.67	28.65	30.61	30.90	73.18	74.43	78.49	77.37	82.11	75.07	75.07	99.44	100.00	78.77	83.08	76.69	82.40	81.79	84.64	82.96	83.52	83.80	30.51	31.44	30.46	28.92
Lan_XP_019422848.1_F3H-chromosome 18 {P}	32.24	32.02	27.30	26.00	30.52	31.40	72.93	72.19	78.30	79.12	81.30	76.86	76.31	79.33	78.77	100.00	93.08	91.16	78.36	77.75	81.32	80.49	81.04	81.59	29.52	29.13	30.06	29.75
LmF3H_b_F3H {Q}	38.28	38.28	33.59	28.91	35.94	35.94	77.69	76.92	81.54	81.54	79.67	78.46	79.23	83.08	83.08	93.08	100.00	93.85	80.77	80.00	86.15	83.85	84.62	83.85	36.72	38.28	32.81	35.94
Lal_KAE9597876.1_F3H {R}	31.04	31.42	28.03	24.71	30.23	31.40	72.22	71.47	76.24	76.24	82.11	77.56	76.73	76.69	76.69	91.16	93.85	100.00	78.45	77.84	79.83	79.56	79.56	80.11	29.22	29.01	30.37	30.06
Gm_AAT94365.1_F3H {S}	30.65	30.12	28.37	26.50	30.72	31.01	74.03	74.72	80.22	80.22	79.67	77.47	76.92	82.40	82.40	78.36	80.77	78.45	100.00	98.66	90.14	87.67	88.49	89.86	29.13	30.73	31.19	29.66
Gm_ACA81441.1_F3H {T}	30.45	30.42	27.79	26.57	30.43	30.72	72.85	73.88	78.79	78.79	79.67	77.13	76.58	81.79	81.79	77.75	80.00	77.84	98.66	100.00	89.29	87.64	87.64	89.01	28.83	30.53	30.89	29.36
Pv_XP_007156141.1_F3H {U}	31.85	31.02	27.51	26.21	30.14	31.01	75.69	76.12	81.59	81.59	82.93	77.75	76.92	84.36	84.64	81.32	86.15	79.83	90.14	89.29	100.00	91.51	92.88	93.97	29.43	29.61	30.28	29.05
Vr_XP_014510008.1_F3H {V}	30.06	29.82	28.08	26.50	31.01	31.30	74.59	74.16	79.67	79.12	83.74	78.57	76.92	82.68	82.96	80.49	83.85	79.56	87.67	87.64	91.51	100.00	94.52	95.89	29.43	29.61	31.19	29.66
Vu_XP_027921662.1_F3H {W}	30.36	30.42	27.79	25.93	30.72	31.01	75.69	75.00	81.59	81.04	82.93	78.57	77.75	83.24	83.52	81.04	84.62	79.56	88.49	87.64	92.88	94.52	100.00	96.71	29.13	30.17	31.50	29.66
Va_XP_017411643.1_F3H {X}	30.06	29.82	27.79	26.50	31.01	31.30	75.69	75.28	81.59	81.59	82.11	78.57	77.47	83.52	83.80	81.59	83.85	80.11	89.86	89.01	93.97	95.89	96.71	100.00	29.73	30.17	30.89	29.36
So_BAE54520.1_ANS {Y}	25.23	25.23	29.67	25.23	27.98	28.57	30.72	30.72	29.82	30.12	36.36	29.52	28.92	30.51	30.51	29.52	36.72	29.22	29.13	28.83	29.43	29.43	29.13	29.73	100.00	72.21	42.77	39.94
Fa_AAU12368.1_ANS {Z}	26.73	25.76	27.32	26.11	27.14	27.71	29.89	30.29	30.45	29.89	38.02	30.25	29.41	31.44	31.44	29.13	38.28	29.01	30.73	30.53	29.61	29.61	30.17	30.17	72.21	100.00	42.42	42.90
Cu_BAA36554.1_FLS {AA}	30.00	29.69	28.22	28.88	32.21	32.82	32.31	33.23	32.21	31.29	38.02	31.60	30.98	30.46	30.46	30.06	32.81	30.37	31.19	30.89	30.28	31.19	31.50	30.89	42.77	42.42	100.00	66.15
Rh_BAC66468.1_FLS {AB}	29.06	29.37	25.77	27.02	26.38	26.38	31.38	32.31	31.90	30.37	41.32	31.60	31.29	28.92	28.92	29.75	35.94	30.06	29.66	29.36	29.05	29.66	29.66	29.36	39.94	42.90	66.15	100.00



- Pfam03171- 2OG-Fe(II) oxygenase superfamily
- COG3491 - Isopenicillin N synthase and related dioxygenases [Secondary metabolites biosynthesis, transport and catabolism]
- PLN02515 - naringenin,2-oxoglutarate 3-dioxygenase
- PLN02515 - naringenin,2-oxoglutarate 3-dioxygenase

Figure S4. Conserved domains detected in LmF3H_a, LmF3H_b, and other F3H proteins evidencing the putative function of the *Lupinus mutabilis* proteins. LmF3H although incomplete contain the 2-OG-Fe(ii) conserved domain, which is specific of this superfamily.

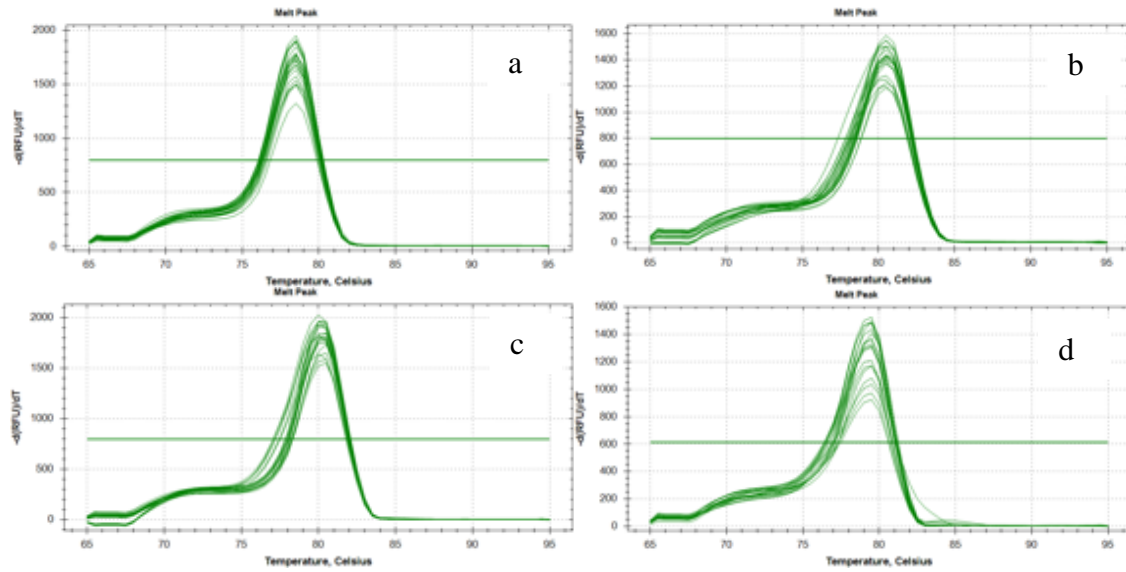


Figure S5. Melting curve analyses of: (a) *Adh3* with detection temperature around 78.5°C; (b) *Hel* (80.5°C); (c) *αTub* (80 °C), and (d) *Ubc* (79.5°C) candidate reference genes for quantitative Reverse-Transcription PCR (RT-qPCR) of *Lupinus mutabilis*. In the four references genes, pure and single amplicons were obtained.

Table S2. Stability values, classification by algorithm and general for the four reference genes under validation for subsequent analyses of *Lupinus mutabilis* seed coat development

Gene	BestKeeper	Genorm	Normfinder	ΔCt	Ranking
<i>Adh3</i>	2.204	1.273	1.085	1.38	4
<i>Hel</i>	1.257	0.532	0.997	1.25	2
<i>αTub</i>	2.382	1.164	1.048	1.37	3
<i>Ubc</i>	1.406	0.532	0.494	1.09	1

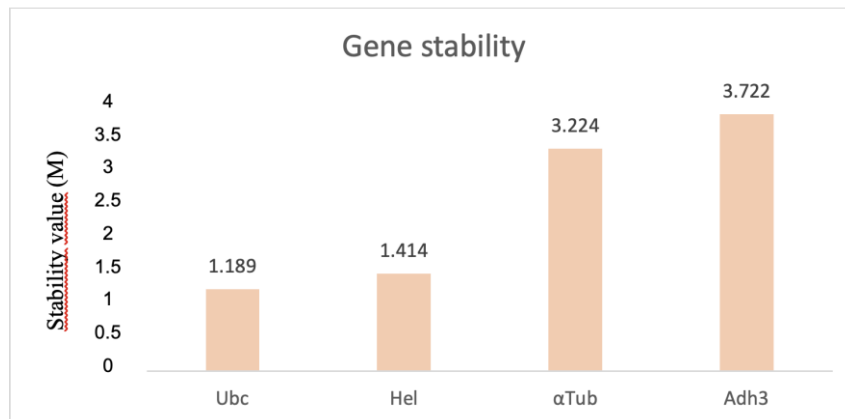


Figure S6. Analysis of the stability of the reference genes (under validation for subsequent analyses of *Lupinus mutabilis* seed coat development) based on the comprehensive classification of RefFinder. The most stables are *Ubc* and *Hel* and the least stables are *αTub* and *Adh3*.

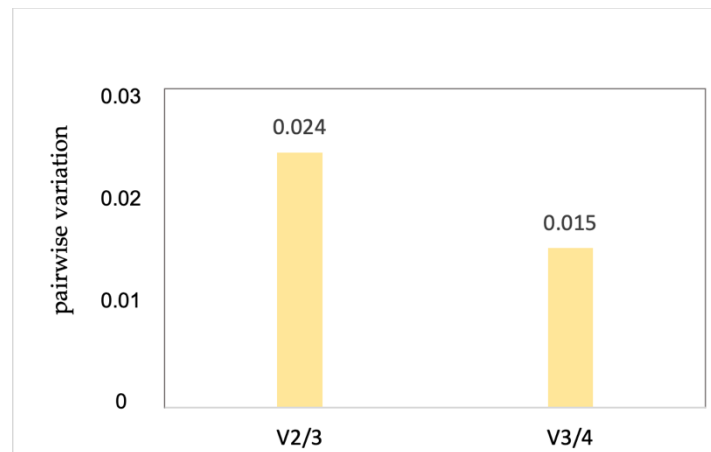


Figure S7. Variation in pairs to identify the optimal number of genes for normalisation.