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Supplementary Information for

Supergene evolution via step-wise duplications and neofunctionalization of a floral-organ identity gene

Cuong Nguyen Huu[1], Barbara Keller[2], Elena Conti[2], Christian Kappel[1], Michael Lenhard[1,*]

Michael Lenhard
Email: michael.lenhard@uni-potsdam.de

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Legend for Dataset S1
SI References

Other supplementary materials for this manuscript include the following:

Dataset S1

www.pnas.org/cgi/doi/10.1073/pnas.2006296117

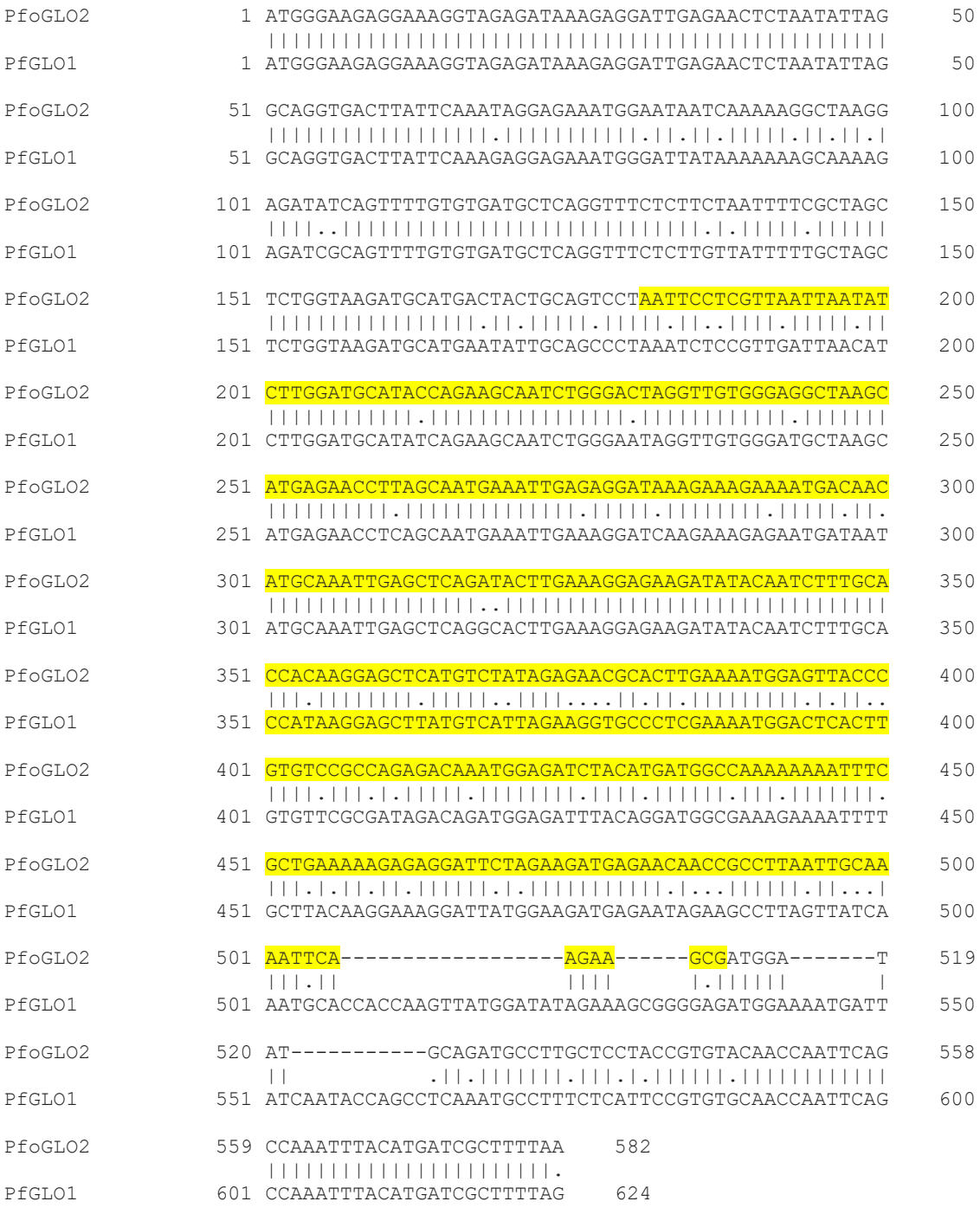


Fig. S1. Nucleotide alignment of *PfoGLO1* and *PfoGLO2*

The alignment was created using Needle (EMBOSS) with default settings. The regions used for VIGS are highlighted in yellow.

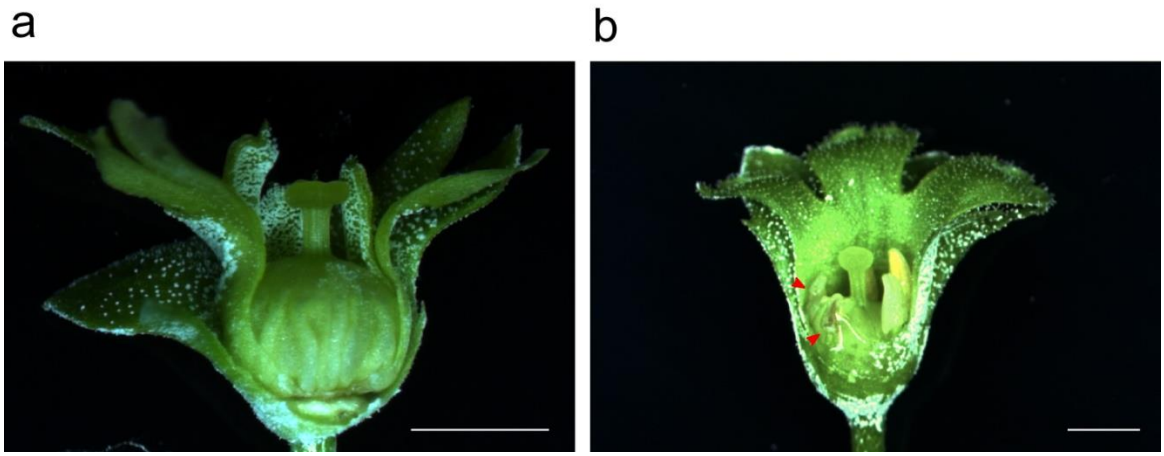


Fig. S2. Silencing of *GLO1* expression

(a,b) In strongly affected flowers of VIGS-*GLO1* treated plants, stamens show signs of homoeotic conversion to carpels.

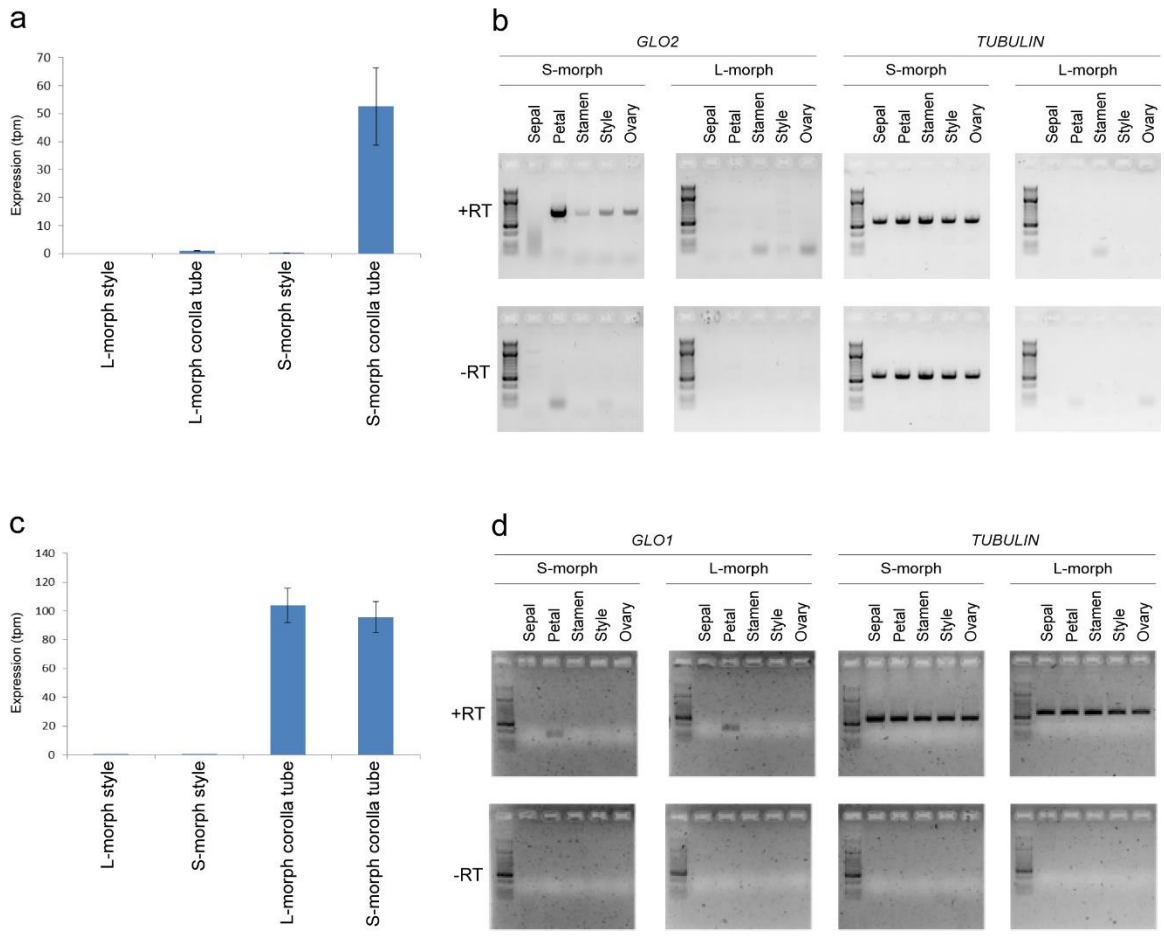


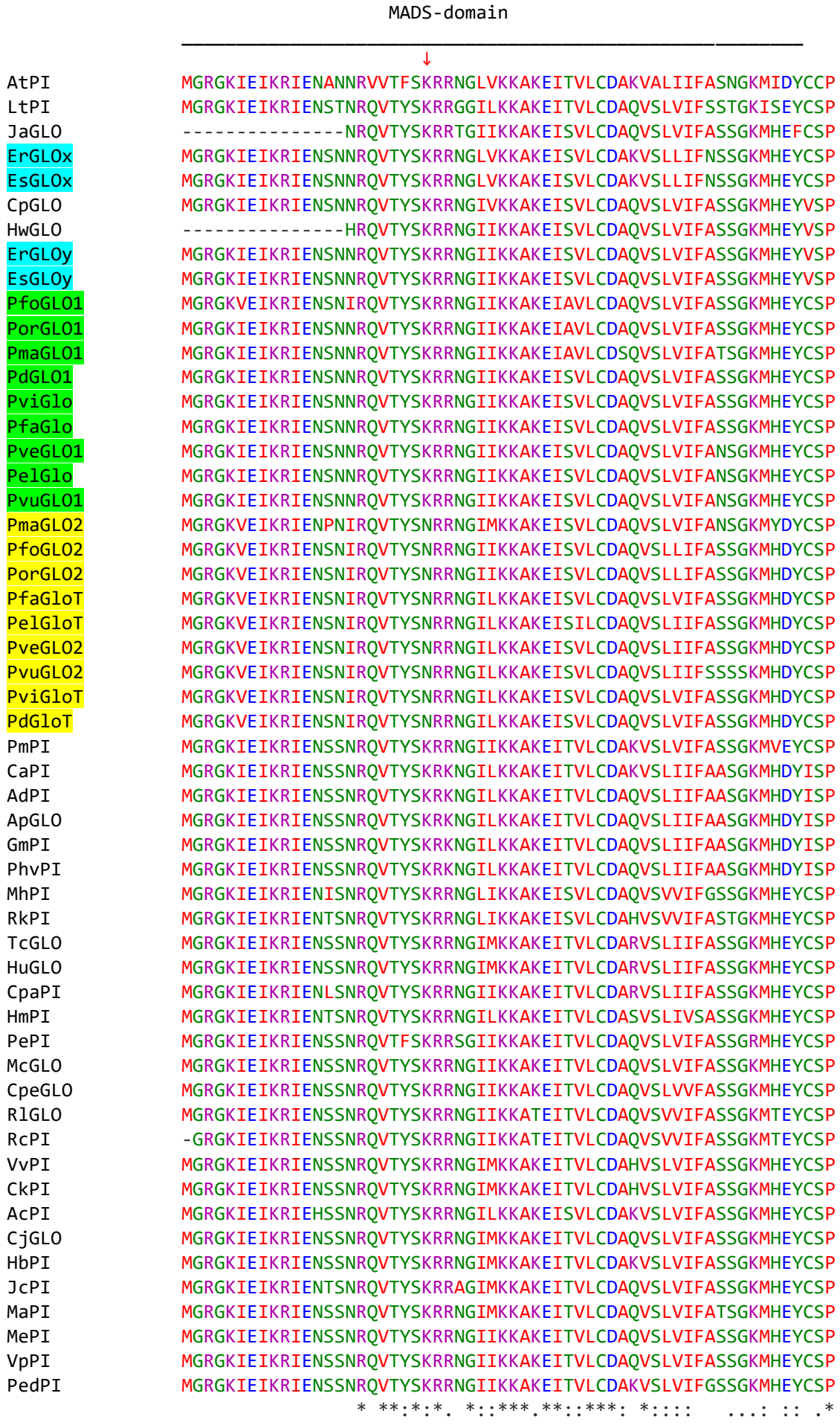
Figure S3: Expression pattern of *GLO2* and *GLO1* in *Primula veris*.

(a) Expression levels of *GLO2* in indicated samples from *P. veris* (n=3) based on RNA-seq.

(b) Expression of *GLO2* and *TUBULIN* in dissected flower organs.

(c) Expression levels of *GLO1* in indicated samples from *P. veris* (n=3) based on RNA-seq.

(d) Expression of *GLO1* and *TUBULIN* in dissected flower organs.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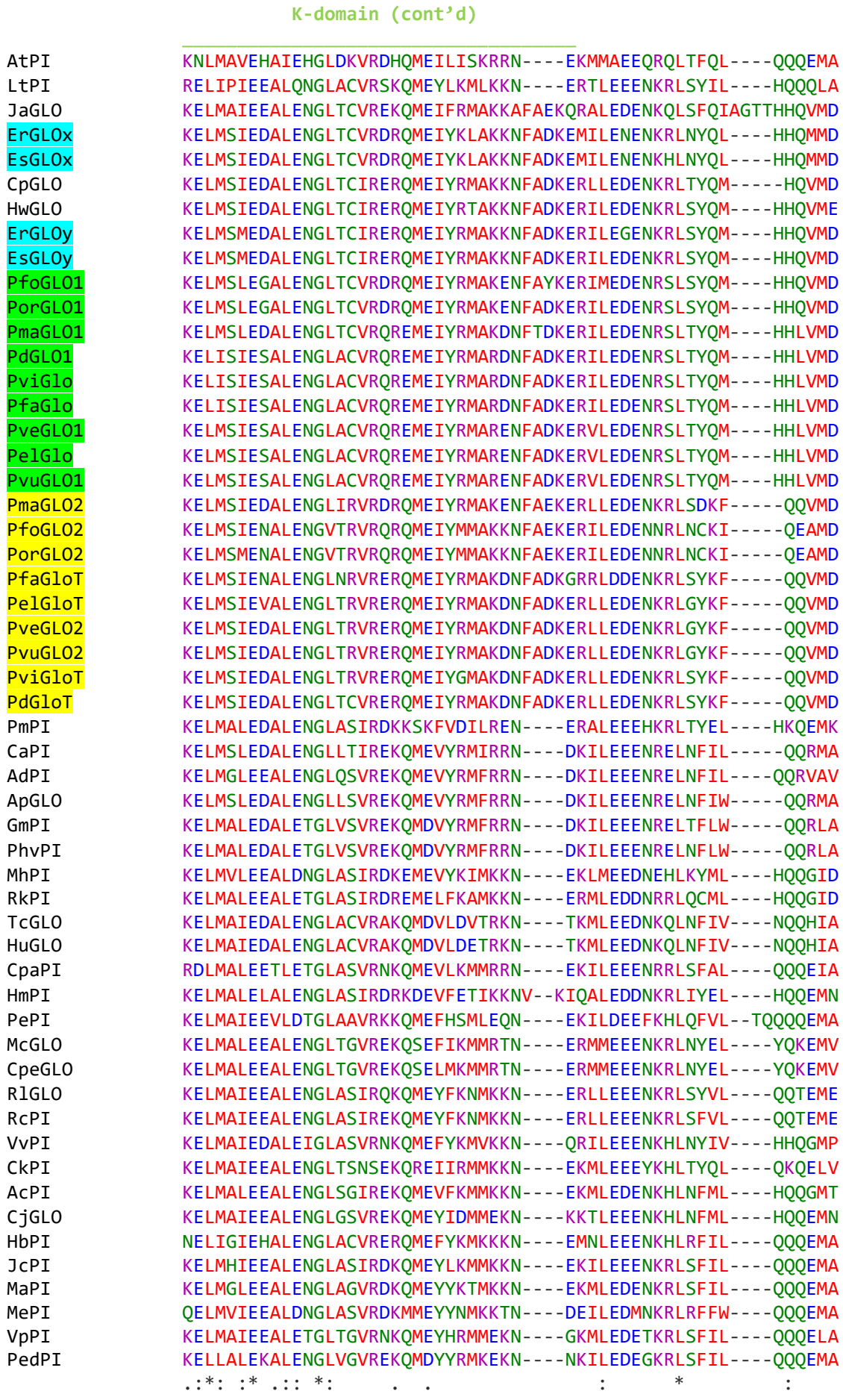
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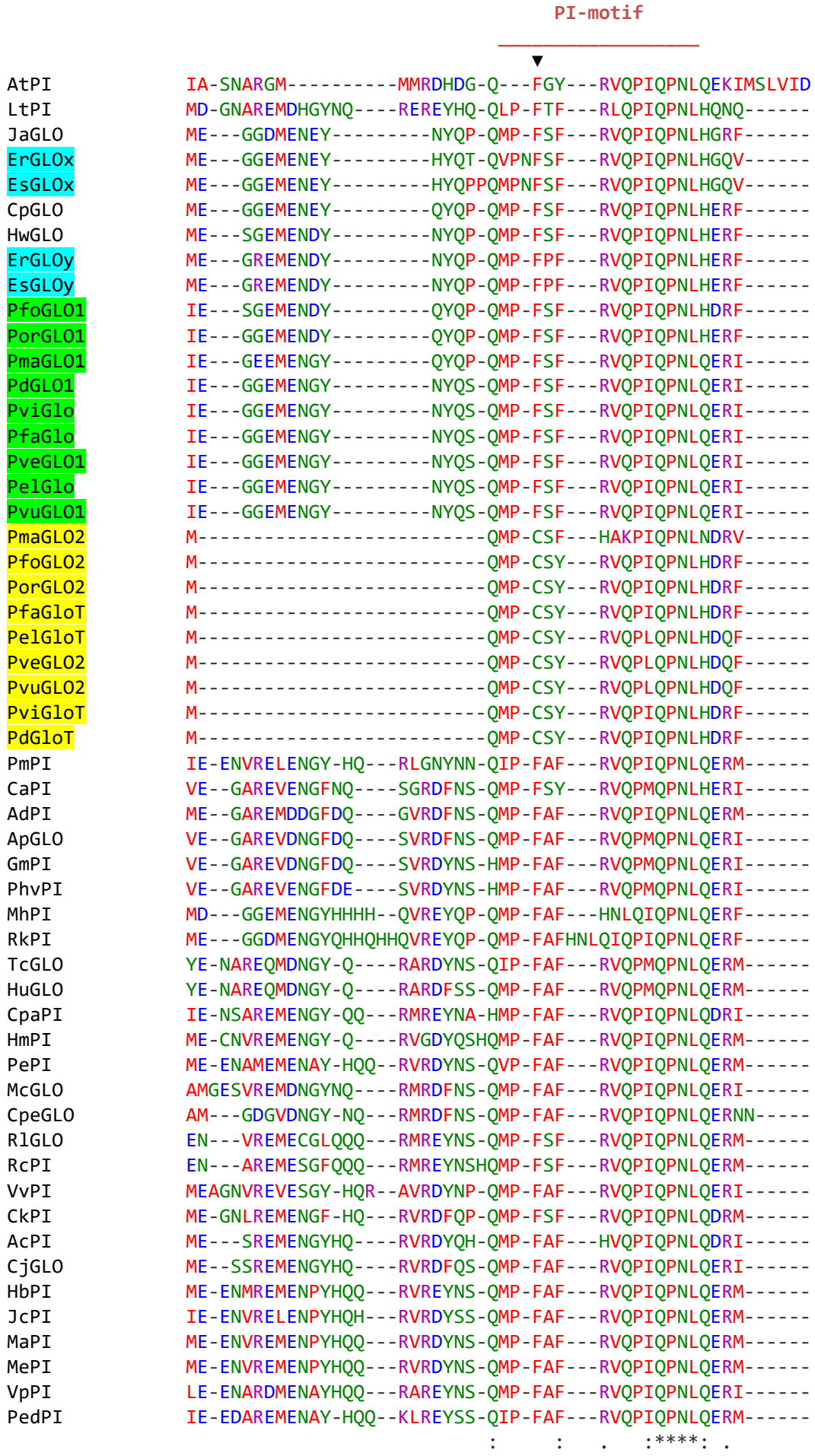
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PfaGlo IE---GGEMENGY---------NYQS-QMP-FSF---RVQPIQPNLQERI------
PveGLO1 IE---GGEMENGY---------NYQS-QMP-FSF---RVQPIQPNLQERI------
PelGlo IE---GGEMENGY---------NYQS-QMP-FSF---RVQPIQPNLQERI------
PvuGLO1 IE---GGEMENGY---------NYQS-QMP-FSF---RVQPIQPNLQERI------
PmaGLO2 M-------------------------QMP-CSF---HAKPIQPNLNDRV------
PfoGLO2 M-------------------------QMP-CSY---RVQPIQPNLHDRF------
PorGLO2 M-------------------------QMP-CSY---RVQPIQPNLHDRF------
PfaGloT M-------------------------QMP-CSY---RVQPIQPNLHDRF------
PelGloT M-------------------------QMP-CSY---RVQPLQPNLHDQF------
PveGLO2 M-------------------------QMP-CSY---RVQPLQPNLHDQF------
PvuGLO2 M-------------------------QMP-CSY---RVQPLQPNLHDQF------
PviGloT M-------------------------QMP-CSY---RVQPIQPNLHDRF------
PdGloT M-------------------------QMP-CSY---RVQPIQPNLHDRF------
PmPI IE-ENVRELENGY-HQ---RLGNYNN-QIP-FAF---RVQPIQPNLQERM------
CaPI VE--GAREVENGFNQ----SGRDFNS-QMP-FSY---RVQPMQPNLHERI------
AdPI ME--GAREMDDGFDQ----GVRDFNS-QMP-FAF---RVQPIQPNLQERM------
ApGLO VE--GAREVDNGFDQ----SVRDFNS-QMP-FAF---RVQPMQPNLQERI------
GmPI VE--GAREVDNGFDQ----SVRDYNS-HMP-FAF---RVQPMQPNLQERI------
PhvPI VE--GAREVENGFDE----SVRDYNS-HMP-FAF---RVQPMQPNLQERI------
MhPI MD---GGEMENGYHHHH--QVREYQP-QMP-FAF---HNLQIQPNLQERF------
RkPI ME---GGDMENGYQHHQHHQVREYQP-QMP-FAFHNLQIQPIQPNLQERF------
TcGLO YE-NAREQMDNGY-Q----RARDYNS-QIP-FAF---RVQPMQPNLQERM------
HuGLO YE-NAREQMDNGY-Q----RARDFSS-QMP-FAF---RVQPMQPNLQERM------
CpaPI IE-NSAREMENGY-QQ---RMREYNA-HMP-FAF---RVQPIQPNLQDRI------
HmPI ME-CNVREMENGY-Q----RVGDYQSHQMP-FAF---RVQPIQPNLQERM------
PePI ME-ENAMEMENAY-HQQ--RVRDYNS-QVP-FAF---RVQPIQPNLQERM------
McGLO AMGESVREMDNGYNQ----RMRDFNS-QMP-FAF---RVQPIQPNLQERI------
CpeGLO AM---GDGVDNGY-NQ---RMRDFNS-QMP-FAF---RVQPIQPNLQERNN-----
RlGLO EN---VREMECGLQQQ---RMREYNS-QMP-FSF---RVQPIQPNLQERM------
RcPI EN---AREMESGFQQQ---RMREYNSHQMP-FSF---RVQPIQPNLQERM------
VvPI MEAGNVREVESGY-HQR--AVRDYNP-QMP-FAF---RVQPIQPNLQERI------
CkPI ME-GNLREMENGF-HQ---RVRDFQP-QMP-FSF---RVQPIQPNLQDRM------
AcPI ME---SREMENGYHQ----RVRDYQH-QMP-FAF---HVQPIQPNLQDRI------
CjGLO ME--SSREMENGYHQ----RVRDFQS-QMP-FAF---RVQPIQPNLQERI------
HbPI ME-ENMREMENPYHQQ---RVREYNS-QMP-FAF---RVQPIQPNLQERM------
JcPI IE-ENVRELENPYHQH---RVRDYSS-QMP-FAF---RVQPIQPNLQERM------
MaPI ME-ENVREMENPYHQQ---RVRDYNS-QMP-FAF---RVQPIQPNLQERM------
MePI ME-ENVREMENPYHQQ---RVRDYNS-QMP-FAF---RVQPIQPNLQERM------
VpPI LE-ENARDMENAYHQQ---RAREYNS-QMP-FAF---RVQPIQPNLQERI------
PedPI IE-EDAREMENAY-HQQ--KLREYSS-QIP-FAF---RVQPIQPNLQERM------

Figure S4: Multiple-sequence alignment of GLO-related proteins

Protein sequence alignment was generated with MUSCLE 3.8. Amino acids are color-coded according to their physicochemical properties. Symbols in the bottom-most line on each page indicate the conservation at each position, with '*' indicating perfect conservation, ':' showing positions with biochemically similar amino acids (score greater than .5 on the PAM 250 matrix), and '.' indicating conservation between amino acids with weakly similar properties (score less than or equal to .5 on the PAM 250 matrix). The protein domains are annotated according to (1).

Primula GLO1 sequences are highlighted in green, GLO2 sequences in yellow. GLO sequences from the most closely related outgroup species *Embelia ribes* and *E. sessiflora* are highlighted in blue (see also Figure 4). Red arrow and black arrowhead indicate amino-acid exchanges of otherwise highly conserved amino acids that are specifically found in *Primula GLO2* orthologues.

The sequences used were chosen from the top 200 hits of a BLAST search with PvuGLO1 to represent a broad sample of angiosperms and are from the following species.

PveGLO1: Primula veris (ANU06251.1); PveGLO2: Primula veris (ANU06256.1); PvuGLO1: Primula vulgaris (AMN15096.1); PvuGLO2: Primula vulgaris (ANU06249.1); PfoGLO1: Primula forbesii (*); PfoGLO2: Primula forbesii (*); PdGLO1: Primula denticulata (ANU06253.1); PdGLOT: Primula denticulata (ANU06257.1); PviGloT: Primula vialii (ANU06258.1); PviGlo: Primula vialii (ANU06254.1); PelGloT: Primula elatior (ANU06259.1); PelGlo: Primula elatior (ANU06252.1; PfaGloT: Primula farinosa (ANU06260.1); PfaGlo: Primula farinosa (ANU06255.1); PorGLO1: Primula oreodoxa (*); PorGLO2: Primula oreodoxa (*); PmaGLO1: Primula maximowiczii (*); PmaGLO2: Primula maximowiczii (*); ErGLOx: Embelia ribes (*); ErGLOy: Embelia ribes (*); EsGLOx: Embelia sessiflora (*); EsGLOy: Embelia sessiflora (*); Vv: Vitis vinifera (NP_001267875.1); Cp: Cyclamen persicum (BAK09618.2); Hw: Hymenandra wallichii (ACY91928.1); Ja: Jacquinia aurantiaca (ACY91926.1); Ac: Actinidia chinensis (ADU15475.1); Cj: Camellia japonica (ADX86812.1); Mc: Momordica charantia (XP_022154226.1); Ma: Mercurialis annua (ALK01328.2); Cpe: Cucurbita pepo (XP_023536339.1); Ped: Passiflora edulis (AER30449.1) ; Hm: Hydrangea macrophylla (BAG68951.1); Vp: Viola philippica (APQ46145.1); Rl: Rhizanthes lowii (AHH28258.1); Ck: Cornus kousa (AGA61757.1); Me: Manihot esculenta (XP_021605746.1); Rk: Rhododendron kaempferi (BBA27231.1)Hb: Hevea brasiliensis (XP_021636651.1); Ca: Cicer arietinum (XP_004498359.2); Rc: Rafflesia cantleyi (AHH28269.1); Jc: Jatropha curcas (XP_012078322.1); Mh: Monotropa hypopitys (AQM52304.1); Ad: Arachis duranensis (XP_015935244.1); Tc: Theobroma cacao (XP_007019220.1); Ap: Abrus precatorius (P_027332702.1); Hu: Herrania umbratica (XP_021284325.1); Cpa: Carica papaya (XP_021902590.1); Pe: Populus euphratica (XP_011027462.1); Pm: Prunus mume (XP_008219575.1); Gm: Glycine max (NP_001235385.1); Lt: Liriodendron tulipifera (AIE44761.1); Phv: Phaseolus vulgaris (XP_007161433.1).

*: The coding sequences of these proteins are given in Supplemental File 1.

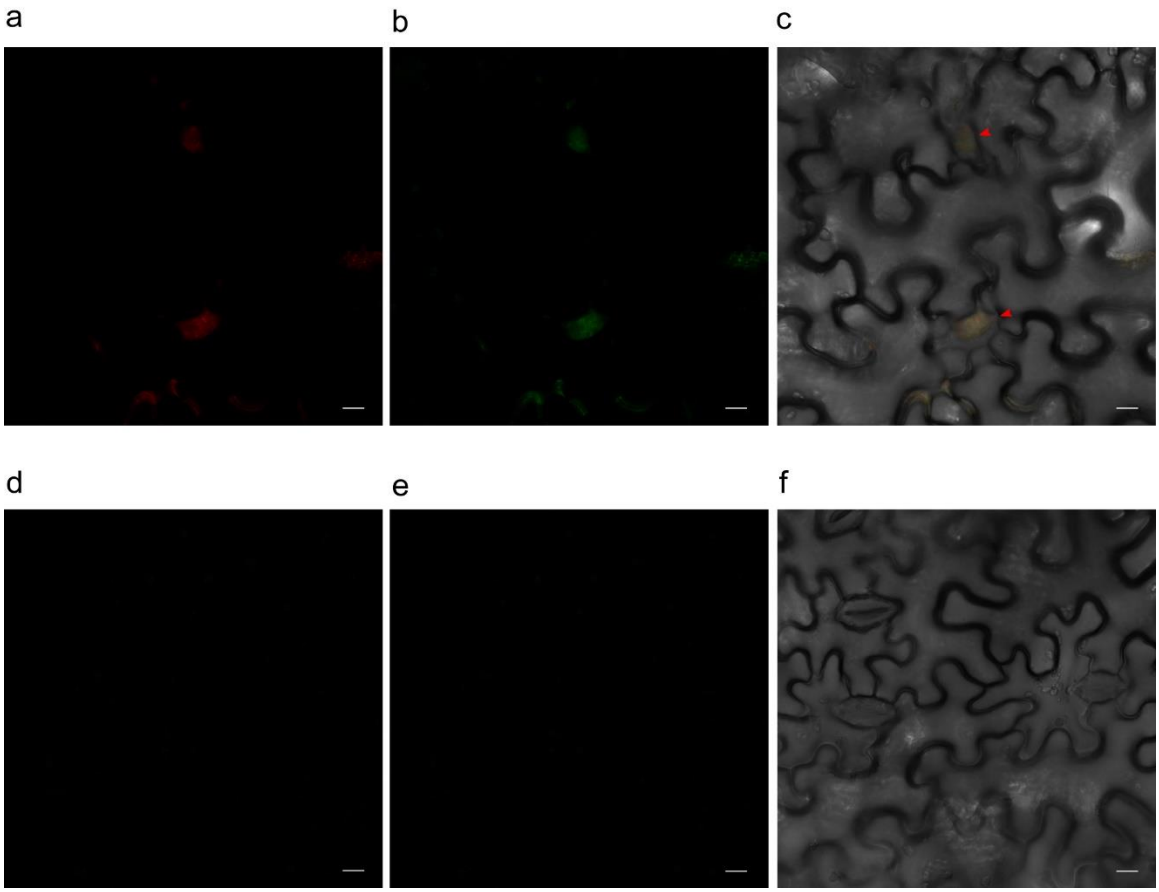

Figure S5: Subcellular localization of GLO1 and GLO2 proteins in *Nicotiana benthamiana*.
(a-c) *N. benthamiana* epidermal cells infiltrated with the construct expressing GLO1-mCherry and GLO2-mGFP fusion proteins. Red arrows indicate the localization of GLO1 and GLO2 proteins.
(d-f) *N. benthamiana* epidermal cells without infiltration as negative control.
(a,d) RFP channel; (b,e) GFP channel; (c,f) overlay with brightfield image.
Scale bar is 5 µm.

Table S1: Genetic mapping of *GLO2* in *Primula veris* and *Primula forbesii*

In *Primula veris*

Morph	Number of morphs tested	*GLO2*	
		Presence	Absence
S	68	68	0
L	72	0	72

In *Primula forbesii*

Morph	Number of morphs tested	*GLO2*	
		Presence	Absence
S	16	16	0
L	16	0	16

Genetic mapping of *GLO2* in both populations of *P. veris* and *P. forbesii* was done using a PCR-based method as described in (Huu et al., 2016). The linkage of GLO2 was detected based on the presence or absence in different morphs. The primers used that bind specifically to the intron region of *GLO2* either in *P. veris* or *P. forbesii* are listed in the Supplemental Table 3.

Table S2: Putative orthologues of *Primula GLO1* and *CYP734A51*

Primula	*Solanum lycopersicum*	*Mimulus guttatus*	*Lactuca sativa*	*Daucus carota*
GLO1	Solyc06g059970.2.1; chromosome 6	Migut.A00326.1; scaffold 1	Lsat_1_v5_gn_1_7541.2; linkage group 1	DCAR_014370; chromosome 4 (22.42 Mb)
	Solyc08g067230.2.1; chromosome 8			
CYP734A51	Solyc03g120060.2.1; chromosome 3	Migut.I00807.1; scaffold 9	Lsat_1_v5_gn_8_57861.1; linkage group 8	DCAR_014861; chromosome 4 (17.72 Mb)
	Solyc12g006860.1.1; chromosome 12	Migut.D01051.1; scaffold 4	Lsat_1_v5_gn_5_97860.1; linkage group 5	DCAR_009214; chromosome 3

Putative orthologues were recovered by BlastN search using *P. veris GLO1* and *CYP734A1* against whole-genome assemblies of the indicated species in Phytozome. The tomato genome contains two duplicates of *GLO*. All four queried genomes contain two very closely related sequences to *CYP734A51*, which are both given, as it is not possible to determine orthology in the absence of a higher-quality *Primula* reference genome. Reverse BlastN searches using the putative *GLO* orthologues from the other species against all *Primula* sequences in NCBI GenBank unambiguously identified *GLO1* or *GLO2* as top matches over all other *Primula* MADS-box genes. Putative *CYP734A51* orthologues from other species also identified *CYP734A51* as best match from all available *Primula* sequences in NCBI GenBank; given the imcomplete coverage, these were also searched against a *P. veris* transcriptome assembly (2), where they identified one of several transcript isoforms representing *CYP734A51* as top hit.

Table S3: Pairwise synonymous-site divergence of *CYP734A* and *GLO* genes

	EriCYP51			*MinCYPb*
EseCYP51	0.053			
MjaCYPb				0.137

	EriGLOx	*EriGLOy*		*MinGLO1*
EseGLOx	0.006			
EseGLOy		0.020		
MjaGLO1				0.065

Ks values of pairwise synonymous-site divergence for the indicated comparisons are shown. Mauve shading indicates comparisons between the two *Embelia* species, and apricot shading shows comparisons between the two *Maesa* species.

Table S4: Oligonucleotides used in this study

Marker/Name	Primer 1	Primer 2	Used for
PvGlo2	CACAGGAATTTCAAACATCCA	TGCTAGCTCCGGTAAGATGC	Mapping *GLO2* in *Primula veris*
PfGlo2	CAAGAAGCGATGGATATG	GCGATCATGTAAATTTGGC	Mapping *GLO2* in *Primula forbesii*
PfGLO2-VIGS	CGGGAATTCAATTCCTCGTTAATTAATATC	CGGGGATCCCGCTTCTTGAATTTTGCAATTAAG	Design VIGS construct for *GLO2* in *Primula forbesii*
PfGLO1-VIGS	CGGGAATTCATCGCAGTTTTGTGTGATGC	CGGGGATCCAATTTGGCTGAATTGGTTGC	Design VIGS construct for *GLO1* in *Primula forbesii*
Tq_TUB	TTCCACCCTGAACAACTCATT	AAGCACAGGTCCACAATCTC	As control for qRT-PCR *GLO1*/*GLO2* in *Primula forbesii*
Tq_TUB_probe	FAM-ACAACTTCGCCAGAGGCCATTATACC-BHQ1		As control for qRT-PCR GLO1/GLO2 in *Primula forbesii*
Tq_GLO2	CCACAAGGAGCTCATGTCTAT	GGCGGTTGTTCTCATCTTCTA	qRT-PCR *GLO2* in *Primula forbesii*
Tq_GLO2_probe	FAM-TGTCCGCCAGAGACAAATGGAGAT-BHQ1		qRT-PCR *GLO2* in *Primula forbesii*
Tq_GLO1	AGGCTGGTATTGATAATCA	GGTGCCCTCGAAAATGGACTC	qRT-PCR *GLO1* in *Primula forbesii*
Tq_GLO1_probe	FAM-GGTGGTGCATTTGATAACTAAGGCTTC-BHQ1		qRT-PCR *GLO1* in *Primula forbesii*
PvGLO-T-qPCR	CTGCAGTCCAAATTCCTC	AATCCCAGATTGCTTCTG	RT-PCR *GLO2* in *Primula veris*
PvGLO-P-qPCR	TTGCAGCCCTAAAACTCC	CAGATTGCTTCTGGTATG	RT-PCR *GLO1* in *Primula veris*
PfGLO1-mCherry	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGGAAGAGGAAAGGTAGAGATAAA	GGGGACAACTTTGTATAGAAAAGTTGGGTGAAAGCGATCATGTAAATTTGGCTG	Fuse protein GLO1 with mCherry for subcellular localization
PfGLO2-GFP	GGGGACAACTTTGTATAATAAAGTTGTAATGGGAAGAGGAAAGGTAGAGATAAA	GGGGACCACTTTGTACAAGAAAGCTGGGTTAAAGCGATCATGTAAATTTGGCTG	Fuse protein GLO2 with GFP for subcellular localization
PvGLO1-SNP	TGCACATATTGTCATTAGCATTG	GTATTAGGAGAAGTGTAGCCAGACA	Linkage between *GLO1* and *CYP734A51 Primula veris*
PvCYP1-SNP	CCACCTTTATATCCAAAAAACCGCA	AAATTTGGCTGAATCGGTTG	Linkage between *GLO1* and *CYP734A51 Primula veris*
Mi_CYPb_1	GGCTCAAGAAGAAAGCGAGTT	TGCATTAGCTTGATCGGAAA	Amplification of *CYPb* in *Maesa japonica*
Mi_CYPb_2	TTCCCTACATGGTGCAATGA	GGCAAAAGGCAATGTTGATT	Amplification of *CYPb* in *Maesa japonica*
Mi_CYPb_3	TTCTGTCGTCTTCGGCAACA	TAATGTGGGGCCAAGCGAAA	Amplification of *CYPb* in *Maesa japonica*
Mi_CYPb_4	GACCACGTGACATACCCTCC	TTGATTGCTTAGGGTTGGTACA	Amplification of *CYPb* in *Maesa japonica*
Mi_GLO1_1	TTTAAGTAACGGTGGGGGCT	GTATGGGAGGCACACTTCCT	Amplification of *GLO1* in *Maesa japonica*

Mi_GLO1_2	AAGGAGAGATGGGGAGAGGAAAG	CCGCCTAGTCACTCCAATGT	Amplification of *GLO1* in *Maesa japonica*
Mi_GLO1_3	CATTGGAGTGACTAGGCGGG	CTATAGCACGGGTGCGAACA	Amplification of *GLO1* in *Maesa japonica*
Mi_GLO1_4	GCACGCGTGTCAGACATTTA	TCAACCGAGCACAAAGTCCA	Amplification of *GLO1* in *Maesa japonica*
Mi_GLO1_5	CTAAGGAAGTGTGCCTCCCA	AGGGATGGTTCCTGCTACAC	Amplification of *GLO1* in *Maesa japonica*
Mi_GLO1_6	GTGTAGCAGGAACCATCCCT	GAGATATAAAATAAGGGGGAGTCG	Amplification of *GLO1* in *Maesa japonica*

Dataset S1 (separate file): Coding and genomic sequences of genes assembled from whole-genome shotgun sequencing or RNA-seq data and used for phylogenetic reconstruction in Figure 4d,e, along with IGV screenshots of back-mapping reads to gene models.

SI References

1. S. de Bruijn, *et al.*, PISTILLATA paralogs in Tarenaya hassleriana have diverged in interaction specificity. *BMC Plant Biology* **18**, 368 (2018).

2. C. N. Huu, *et al.*, Presence versus absence of CYP734A50 underlies the style-length dimorphism in primroses. *Elife* **5** (2016).