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Supplementary Materials for

A specialized flavone biosynthetic pathway has evolved in the medicinal plant, *Scutellaria baicalensis*

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Supplementary Information

CYP93B6 SbFNSII-1 SbFNSII-2	MALYAALFLLSAAVVRSVLDRK MDLVEVTLYAALFLLSAAFLLLIFAGD MEVTLNVALLLLSAAVCLMVFTGK ::* .**:****	R-GRPPYPPGPFPLPIIGHLHLLGPRLH R-SSPPGPFPLPIIGHLHLLGPKLH RRRRLPNPPGPFPLPLIGNLNLVSPRLH * ******:**:*:*:*:*:*	IQTFHD IQSFHG IHTFHM *::**
CYP93B6 SbFNSII-1 SbFNSII-2	LSQRYGPLMQLRLGSIRCVIAASPELA LSQRHGPLMQIRLGSINCVVASTPELA LAQRYGPIMKFRLGSIPCLVVSTPELA *:**:**:*	KECLKTHELVFSSRKHSTAIDIVTYDSS KEFLKTNELVFSSRKHSTAIDIVTYNSS KDILKTHELIFSSRVKSTAIDIVTYGVS *: ***:**:*** :****	SFAFSP SFAFSP SFAFSP
CYP93B6 SbFNSII-1 SbFNSII-2	YGPYWKFIKKLCTYELLGARNLAHFQP YGPYWKYIKKLCTYELLGARNLHHFQP YGPYWKYIKKLCTYELLGSRMLNHFEP ******:***********	IRTLEVKSFLQILMRKGESGESFNVTEE IRTFEVHTFLRLLMEKSESGESFNVTEE LRALEVREFLKDVMAMGKAGKSFNVTEE :*::**: **: :* .::*:******	ELVKLT ELIKLT ELMKLT
CYP93B6 SbFNSII-1 SbFNSII-2	SNVISHMMLSIRCSETESEAEAARTVI SNVMSNMMLGTRCSATDGEAEAARTVI SNVMSNMMLSIRAAESEEQAEVARTLI ***:*:***. *.: :: :**.**:*	REVTQIFGEFDVSDIIWLCKNFDFQGIF REVTEIFGEFDAADIIWFCKNFDLQGIF REVSQLFGEFDFGDMLWFCKSFDFQGIF ***:::***** .*::*:**	RKRSED RKRSED KRSKD
CYP93B6 SbFNSII-1 SbFNSII-2	IQRRYDALLEKIITDREKQRRTHGGGG IQRRYDALLEKIITDREKLRRSHRGG- IKVRYDALLEKILTDRENVRRQNGVV- *: ********:***: ** :	GGGEAKDFLDMFLDIMESGKAEVKFTRE EAKDFLDIFLDIMDSGNSEVKFSRE EPKDMLDMFLDIMEGGKTDVEFTRE *.**:**:****:.*::*::*	HLKAL HLKAL HLKAV
CYP93B6 SbFNSII-1 SbFNSII-2	ILDFFTAGTDTTAIVCEWAIAEVINNP ILDFFTAGTDTTAISTEWAIAELMNNP ILDFLTAGTDTTAITVEWVLAELMNSP ****:*******	NVLKKAQEEIANIVGFDRILQESDAPNL KVLKKAQEEIQKVVGSCRLMDESDAPNL KAMKKAQDEMDRVVGRERMMAESDAPNL :.:****::::**	_PYLQA _PYLEA _PYFLA ***: *
CYP93B6 SbFNSII-1 SbFNSII-2	LIKETFRLHPPIPMLARKSISDCVIDG IIKETFRLHPPIPMLARKSVSDCVIDG IIKETFRLHPPIPLIIRRSIEDCVIDG :*************	YMIPANTLLFVNLWSMGRNPKIWDYPTA YNIPASTLLFVNIWSIGRNPECWDSPFS YHIPADTLAFINVWSMGRNEKYWDSPLS * ***.** *:*:**	AFQPER SFRPER SFRPER :*:***
CYP93B6 SbFNSII-1 SbFNSII-2	FLEKEKAAIDVKGQHFELLPFGTGRRG FFEKDNASIDIKGQHFQLLPFGTGRRG FLEGDNAAIDIKGMHFELLPFGSGRRG *:* ::*:**	CPGMLLAIQEVVIIIGTMIQCFDWKLPE CPGMLLAIQELLLIIGTMIQCFDWELPE CPGMLSAIQEVLIIAGTVIQCFDWEQAE ***** ****:::* **:******	DGSGHV EGSGPV DGSGRV
CYP93B6 SbFNSII-1 SbFNSII-2	DMAERPGLTAPRETDLFCRVVPRVDPL DMTERAGLTAPRAEDLICRVSCRVDPK DMSERPGLTTPREIDLVCRVVPRVDER **:**.***:** **.*** ***	VVSTQ IVF VISGH ::	

fig. S1. Multiple alignment of CPY93B6, CPY93B24, and CPY93B25.



fig. S2. Flavone accumulation patterns in *S. baicalensis.* (A) Measurements of flavones from different organs of 6 month old *Scutellaria* plants. R, roots; S, stem; L, leaves; F, flowers. (B) Measurements of *Scutellaria* RSFs from hairy root lines subjected to MeJA treatment for 24h and MS profiles of baicalin (C), baicalein (D), wogonoside (E) and wogonin (F). Baicalin (bin), baicalein (bein), wogonoside (wde) and wogonin (win). SEs were calculated from 3 biological replicates. Differences were considered significant (Student's *t*-test) with *P<0.05, **P<0.01, ***P<0.001.



fig. S3. RNAi of *SbFNS II-2* **in hairy root cultures of** *S. baicalensis.* (**A**) HPLC profiles of flavone metabolites in SbFNSII-2-silenced hairy root lines compared with the profiles obtained from hairy roots transformed with an empty vector as control. Bin, baicalin; Wde, wogonoside; Bein, baicalein; Win, wogonin. (B) Relative area of the three new peaks in SbFNSII-2 RNAi lines. SEs were

calculated from 3 biological replicates. Significant differences were considered with *P<0.05, **P<0.01. (C) MS I of peaks I, II and III detected from FNSII-2 RNAi lines (D) MS/MS of m/z 273.26, 257.24 and 287.30 detected from MS I. (E) Structures and fragmentation patterns for MS/MS of m/z 273.26, 257.24 and 287.30 detected from MS I



fig. S4. In vitro assay of SbFNSII-1 and SbFNSII-2 and in vivo assay of SbFNSII-2. (**A**) Structure of flavanones used for in vitro assay. (**B**) Metabolite profiles of assays of extracts of yeast carrying empty vector (EV), SbFNSII-1 or SbFNSII-2 assayed with pinocembrin, naringenin and eriodictyol as substrates, respectively. Pin, pinocembrin; Nar, naringenin; Eri, eriodictyol; Chr, chrysin; Api, apigenin; Lut, luteolin. (**C**) Yeast in vivo assay, both pinocembrin and naringenin were added to yeast media. (**D**) MS I of pinocembrin and chrysin.



fig. S5. HPLC metabolite profiles of *Arabidopsis* plants carrying empty vector or a representative *SbFNSII-2* line, grown on MS with or without supplementation of pinocembrin.

А



В

	At4CL1	At4CL2	At4CL3	At4CLI7	SbCLL-7
At4CL1		92	70	41	42
At4CL2			70	40	43
At4CL3				40	41
At4CLI7					67
SbCLL-7					

С	Dev.4			
At4CL1	210 SSGTTGLPKGVMLTHKGLVTSVAQQVDGENPNLYFHSDDVILCVLPMFHIYALNSIMLCG			
At4CL2	203 SSGTTGLPKGVMLTHKGLVTSVAQQVDGENPNLYFNRDDVILCVLPMFHIYALNSIMLCS			
At4CL3	213 SSGTTGLPKGVVLTHKSLITSVAQQVDGDNPNLYLKSNDVILCVLPLFH <mark>IY</mark> SL <mark>N</mark> SVLLNS			
SbCLL-7	198 <mark>SSGTTGMSKGV</mark> ILTHRNFIASSLMITADQESAGEMHNVFLCVLPMFH <mark>VF</mark> GL <mark>A</mark> VIMLSQ			
At4CL17	198 <mark>SSGTTGTSKGV</mark> ELTHGNFIAASLMVTMDQDLMGEYHGVFLCFLPMFH <mark>VF</mark> GL <mark>A</mark> VITYSQ			
1+1011				
AT4CL1	270LRVGAAILIMPKFEINLLLELIQRCKVIVAPMVPPIVLAIAKSSEIEKYDLSSIRVVKSG			
AT4CL2	263 LRVGATILIMPKFETTLLLEQTQRCKVTVAMVVPPTVLATAKSPETERYDLSSVRMVKSG			
AT4CL3	2/3LRSGATVLLMHKFEIGALLDLIQRHRVTIAALVPPLVIALAKNPTVNSYDLSSVRFVLSG			
SDCLL-/	256 LQRGN I IVSMSKFDFEMILK I VERYGI I HMWVVPPI I LALAKNNVVKK YNLSSLQQIGSG			
AT4CL17	256LQRGNALVSMARFELELVLKNIEKFRVTHLWVVPPVFLALSKQSIVKKFDLSSLKYIGSG			
A+4CL1				
Attcl2	303 AAPLIGKELEDAVNAKFFNAKLEGGTVENTEACDVLANSLGFAKEFFFVKSGACGTVKNAE			
Attcl 3				
shell-7	316 AADLGRELMOECAKNEDOAV/V/OGYGMTETCGTVSVEN/VAGDPHSGSAGTI V/DGVE			
A+4CI 17				
Rov2				
At4CI1	390MKTVDPDTGDSLSRNOPGETCTRG 413			
At4CL2	383MKTLDPDTGDSLPRNKPGETCTRG 406			
At4CL3	393LKVVHLETRLSLGYNOPGEICIRG 416			
SbCLL-7	373COIVSVDKLKPLPPGOLGEICVRG 396			
At4CL17	373A0IVSVETGKS0PPN00GEIWVRG 396			

fig. S6. Western blot analysis of the recombinant SbCLLs and SPB domain analysis of At4CLs and SbCLL-7. (**A**) Five SbCLLs were expressed as fusion proteins with an N-terminal His₆-tag in E. coli. The total proteins (10 μg each lane), were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with His-tag antibody. Protein extracts from bacteria containing the empty expression vector served as a control. Protein sizes are: SbCLL-1, 59.96 kD; SbCLL-5, 61.40 kD; SbCLL-6, 59.39 kD; SbCLL-7, 59.33 kD ; SbCLL-8, 56.12 kD. (**B**) The amino acid identity within the SPB domain of SbCLL-7 to 4CL-like proteins from *Arabidopsis thaliana*. (**C**) Multiple alignment of At4CL 1,2, 3, At4CL17 and SbCLL-7. * Indicates Asn-256 of At4CL2 and Ala-249 of SbCLL-7 respectively.



fig. S7. Metabolite profiles by HPLC from empty vector line and a representative *SbCLL-7* RNAi line.



fig. S8. Metabolite profiles of HPLC analysis of infiltrated *N. benthamiana* **leaves.** (A) Metabolite profiles of infiltrated tobacco leaf without supplementation of cinnamate. Pin and Chr means pinocembrin and chrysin standards, respectively; GFP, *N. benthamiana* leaf infiltrated with GFP as a control; 3X, leaf infiltrated with *SbCLL-7*, *SbCHS-2* and *SbCHI*. 4X, leaf infiltrated with *SbCLL-7*, *SbCHS-2*, *SbCHI* and *SbFNSII-2*. (B) MS/MS of peak I, which was identical to the pinocembrin standard. (C) MS/MS of peak II, which was identical to the chrysin standard.



fig. S9. Transcript levels of *SbC4H* relative to actin. SEs were calculated from 3 independent biological replicates. R = roots, S = stems, L = leaves, F = flowers.

table S1. Primers used in this study. Underlined sequences mean recombination cites for Gateway cloning.

Names	Used for	Sequences
AP	3'RACE	GGCACGCGTCGACTAGTACT[T] ₁₆
3'RACE AUAP	3'RACE	GGCCACGCGTCGACTAGTAC
SbFNSII1 GSP 1	3'RACE	GCTGGAAAAGATTATCACCGACAG
SbFNSII1 GSP 2	3'RACE	CCAGAAAGTGGTGGGATCTTGTAG
SbFNSII1 SP1	5'RACE	GCAAATCGAAGTTCTTACAGAACCAG
SbFNSII1 SP2	5'RACE	GCAAATCGAAGTTCTTACAGAACCAG
SbFNSII1 SP3	5'RACE	GATTCGCTCTTCTCCATGAGAAGC
FNSII1 CDS F	Cloning	GGGGACAAGTTTGTACAAAAAGCAGGCTTC
		ATGGACTTAGTAGAAGTCACAC
FNSII1 CDS R	Cloning	<u>GGGGACCACTTTGTACAAGAAAGCTGGGTT</u> CTAGAAAAC
		AATTTTCGGGTCAACC
FNSII1 QPCR F	QPCR	AAACCAACGAGCTGGTGTTC
FNSII1 QPCR	QPCR	CGGAGAAAAGTGTGGACCTC
SbFNSII1 RNAi F	RNAi	<u>GGGGACAAGTTTGTACAAAAAGCAGGCTTA</u> CTTCTCAT
		GGAGAAGAGCGAATC
SbFNSII1 RNAi R	RNAi	<u>GGGGACCACTTTGTACAAGAAAGCTGGGTA</u> ACAAGATCC
		CACCACTTTCTGG
SbFNSII 2 CDS F	Cloning	<u>GGGGACAAGTTTGTACAAAAAAGCAGGCTTC</u> CCGATGGA
		AGTCACACTGAATGTGG
SbFNSII 2 CDS R	Cloning	<u>GGGGACCACTTTGTACAAGAAAGCTGGGTA</u> CTCTCCATA
		AAAGCAGCAGCAACAG
Sb FNSII2 RNAi F	RNAi	<u>GGGGACAAGTTTGTACAAAAAGCAGGCTTA</u> GATGTGAT
		GGCTATGGGGAAGG
Sb FNSII2 RNAi R	RNAi	<u>GGGGACCACTTTGTACAAGAAAGCTGGGTA</u> CTCTCTTCC
		AACCACTCTGTCC
SbFNSII 2 QPCR F	QPCR	TCACCTATGGCGTCTCCTTC
SbFNSII 2 QPCR R	QPCR	CAGCTCCTCAGTGACGTTGA
SbCLL1 F	Cloning	<u>GGGGACAAGTTTGTACAAAAAGCAGGCTTC</u> ATGGAGA
		CTGTAGAAAACCACG
SbCLL1 R	Cloning	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAATTTGA

		AAGAGTAGCTGCTTTTG
SbCLL5 F	Cloning	<u>GGGGACAAGTTTGTACAAAAAGCAGGCTTC</u> ATGTTGTC
		GGTGGCCTCCGTG
SbCLL5 R	Cloning	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAAGATGA
		GGATGGTGGAGTAGCAG
SbCLL6 F	Cloning	GGGGACAAGTTTGTACAAAAAGCAGGCTTCATGGCGG
		AATTGATAGATCCACACAG
SbCLL6 R	Cloning	<u>GGGGACCACTTTGTACAAGAAAGCTGGGTT</u> CTTCACATC
		TTGGAGAGAGCAAGC
SbCCL7 F	Cloning	GGGGACAAGTTTGTACAAAAAGCAGGCTTCATGGAGA
		AATCGGGCTATGG
SbCCL7 R	Cloning	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTATAACTT
		CGATCGGACCTTTTC
SbCCL8 F	Cloning	<u>GGGGACAAGTTTGTACAAAAAAGCAGGCTTC</u> ATGTCTAT
		TTCAACCCTAACCGGTTTG
SbCCL8 R	Cloning	<u>GGGGACCACTTTGTACAAGAAAGCTGGGTT</u> TTAAGCTCC
		AAACTTAGGGACCTTAGC
SbCCL1 F	QPCR	ATAATCAAGTACAAAGGGTTCCA
SbCCL1 R	QPCR	ACCTGTTTGGATATAAATTGCTT
SbCCL5 F	QPCR	TGGAAACCCAGAATCCAGAG
SbCCL5 R	QPCR	GTTTCCGACAAAGAGGCAAG
SbCCL7 F	QPCR	ATTGAGGCCACCGTTGTATC
SbCCL7 R	QPCR	CGACTTTTGAAACCGTGGAT
SbCCL7 RNAi F	RNAi	GGGGACAAGTTTGTACAAAAAGCAGGCTTCCCATGGTT
		TACTTCAACTGG
SbCCL7 RNAi R	RNAi	GGGGACCACTTTGTACAAGAAAGCTGGGTTGCAGCTGTA
		TCATCTTGCTTG
SbCHS1 CDS F	Cloning	<u>GGGGACAAGTTTGTACAAAAAGCAGGCTTC</u> ATGGTGAG
		CGTCGAAGAGTTC
SbCHS1 CDS R	Cloning	<u>GGGGACCACTTTGTACAAGAAAGCTGGGTT</u> TTAGTTGAG
		AGGCACACTACGAAG
SbCHS1 QPCR F	QPCR	AACTGCCTCCACCAGTCCACC
SbCHS1 QPCR R	QPCR	GTCAACATCTCCTGCCTGACG
SbCHS2 CDS F	Cloning	<u>GGGGACAAGTTTGTACAAAAAGCAGGCTTC</u> ATGGTGAC
		AGTTGAAGAATTCCAC

SbCHS2 CDS R	Cloning	<u>GGGGACCACTTTGTACAAGAAAGCTGGGTT</u> TCAATTGAG
		AGGCACACTATGC
SbCHS2 QPCR F	QPCR	GCAGTCCACTTATGCTGATTAC
SbCHS2 QPCR R	QPCR	GTGAAGTTGTCGTTCTCCTTC
SbCHI CDS F	Cloning	<u>GGGGACAAGTTTGTACAAAAAGCAGGCTTC</u> ATGTCTGC
		TTCGCCATCCGT
SbCHI CDS R	Cloning	<u>GGGGACCACTTTGTACAAGAAAGCTGGGTT</u> TTAAAACAA
		CTCCGATAGTCTTGC
SbCHI QPCR F	QPCR	AAGGCAGTAATAGAGAACAAACAG
SbCHI QPCR R	QPCR	TTAAAACAACTCCGATAGTCTTG
SbC4H F	QPCR	GCCGATTCTCTGTATCACTATC
SbC4H R	QPCR	ATGATTAAAATGATCTTGGCTTT