Supporting Information

Engineering a plant polyketide synthase for the biosynthesis of methylated flavonoids

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Supporting Tables

Table S1. Primers used in this experiment

Primers	Templates	Primer sequences (5′ to 3′, mutant site underlined)	Products
Pc4CL del V342 FP Pc4CL RP	C1	AAGCCGGTCCGCTGGCGATGTG GTGGTGCGGGTGCTTTCCGG	C6
Pc4CL Q214A FP Pc4CL RP	C1	CAGCGTTGCACAAGCAGTGGATGGTGAT GTGGTGCGGGTGCTTTCCGG	C8
HvCHS(A228S, D231I, Q232P, L233G, D234V) FP HvCHS (A228S, D231I, Q232P, L233G, D234V) RP	C3	[PHO]GGGTGTTGAGCAGCCGGTATTTCAATTGGTATCGGCGAGTC [PHO]GGGATGGGATCGGATCCGATGATTGCAGCAGC	C7
Os4CL del V340 FP Os4CL del V340 RP	C3	CTGAGGCAGGCCCTCTGGCAATGTGTCT AGACACATTGCCAGAGGGCCTGCCTCAG	C9
Os4CL Q212A FP Os4CL Q212A RP	C3	ATTACATCGGTTGCGCAGGCGGTAGATGGGGAAAACCC GGGTTTTCCCCATCTACCGCCTGCGCAACCGATGTAAT	C10
Os4CL S242A FP Os4CL S242A RP	C3	ATTTATTCGTTAAACGCGGTTCTGCTGGCAGGG CCCTGCCAGCAGAACCGCGTTTAACGAATAAAT	C11
HvCHS A197T FP HvCHS A197T RP	C3	TGCTCGGAGATTACCACCATGGCGTTCCGTGGC GCCACGGAACGCCATGGTGGTAATCTCCGAGCA	C12
HvCHS I265F FP HvCHS I265F RP	C3	GAGGCGGGCTTAACGTTTCACCTGCTGAAAG CTTTCAGCAGGTGAAACGTTAAGCCCGCCTC	C13
PhCHS T197A FP PhCHS T197A RP	C1	GATTACCGCGGTGGCCTTCCGTGGCCC GGGCCACGGAAGGCCACCGCGGTAATC	C14
HvCHS A228S FP HvCHS A228S RP	C3	GCAATCATCGGAtCCGATCCCGACC GAGTTCCAATTGTGAATACCCAAAGGCTCAAACGC	C15
HvCHS D231I FP HvCHS D231I RP	C3	CGGAGCCGATCCCATCCAATTAGACGAG GAGTTCCAATTGTGAATACCCAAAGGCTCAAACGC	C16
HvCHS Q232P FP HvCHS Q232P RP	C3	GCCGATCCCGACCCGTTAGACGAG GAGTTCCAATTGTGAATACCCAAAGGCTCAAACGC	C17
HvCHS L233G FP HvCHS L233G RP	C3	GATCCCGACCAAGGTGACGAGCAGC GAGTTCCAATTGTGAATACCCAAAGGCTCAAACGC	C18
HvCHS D234V FP HvCHS D234V RP	C3	CCCGACCAATTAGTTGAGCAGCCGG GAGTTCCAATTGTGAATACCCAAAGGCTCAAACGC	C19
HvCHS Q232P_D234V_FP HvCHS Q232P_D234V_RP	C15	CCCGACCAATTAGTTGAGCAGCCGG GAGTTCCAATTGTGAATACCCAAAGGCTCAAACGC	C20

*5′ [PHO] means 5' phosphorylation of primers

Table S2. Structure alignment of the new HvCHS structure (8B32) with CHS structures in the PDB with the Dali server.

rank	PDB- chain	Z score	rmsd	lali	nres	%id	enzyme name with ligand	donor species
1	4yjy-A	68.2	0.5	387	393	79	Chalcone Synthase 1	*Oryza sativa*
2	4wum-C	68.2	0.4	386	389	73	Chalcone Synthase	*Freesia hybrida*
7	1cgk-A	67.7	0.5	386	387	72	Chalcone Synthase 2 with naringenin	*Medicago sativa*
8	7bur-A	67.7	0.5	385	388	74	Chalcone Synthase 1	*Gkycine max* (L.)
9	1bi5-A	67.6	0.5	386	389	72	Chalcone Synthase 2	*Medicago sativa*
10	1i86-A	67.6	0.5	386	389	72	Chalcone Synthase 2 G256A	*Medicago sativa*
11	1cgz-A	67.6	0.5	386	387	72	Chalcone Synthase 2 with resveratrol	*Medicago sativa*
12	1d6f-A	67.6	0.5	386	389	72	Chalcone Synthase 2 C164A	*Medicago sativa*
13	1bq6-A	67.6	0.5	386	388	72	Chalcone Synthase 2 with CoA	*Medicago sativa*
14	1cml-A	67.5	0.6	386	389	72	Chalcone Synthase 2 with malonyl-CoA	*Medicago sativa*

Supporting Figures

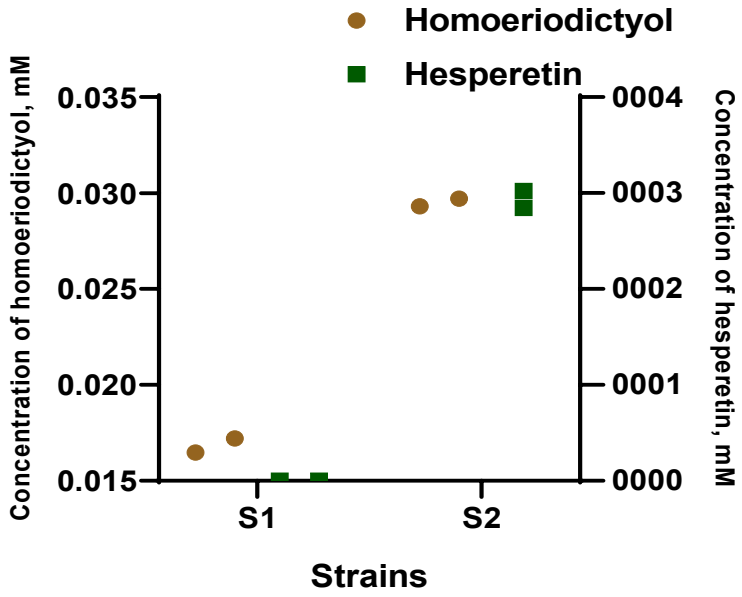


Figure S1. Comparison of different CHS and 4CL variants (s1: PhCHS from *Petunia hybrida* and 4CL from *Petroselinum crispum* and s2: CHS from *Hordeum vulgare* and 4CL from *Oryza sativa*). Small scale fermentation for s1 and s2. 1mM ferulic acid, and isoferulic acid were added as substrates. Samples were taken after 32h fermentation and analyzed by LC-MS. Each experiment was duplicated.

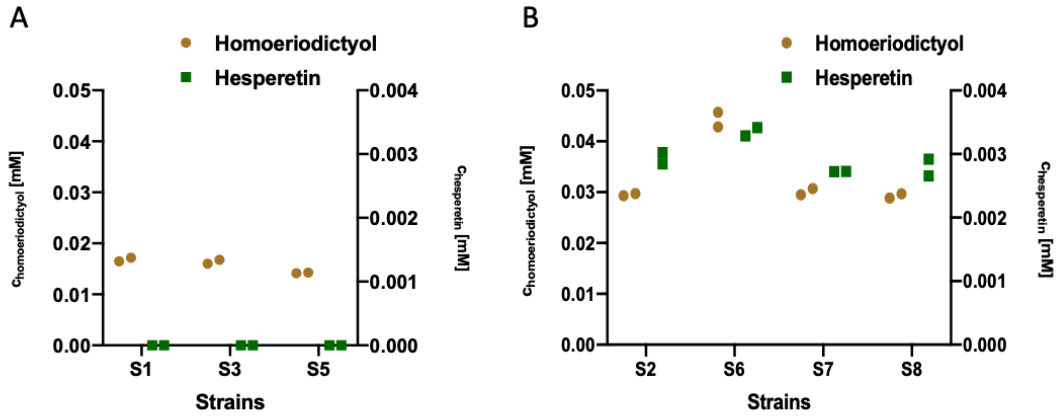


Figure S2. Comparison of different 4CL variants: A) Pc4CL variants (s1 Pc4CL wildtype, s3 Pc4CL del V342 variant, and s5 Pc4CL Q214A variant) and B) Os4CL variants (s2 Os4CL wildtype, s6 Os4CL del V340 variant, s7 Os4CL Q212A variant, and s8 Os4CL S242A variant). Small scale fermentation for those variants. 1mM ferulic acid, and isoferulic acid were added as substrate. Samples were taken after 32h fermentation and analyzed by LC-MS. Each experiment was duplicated.

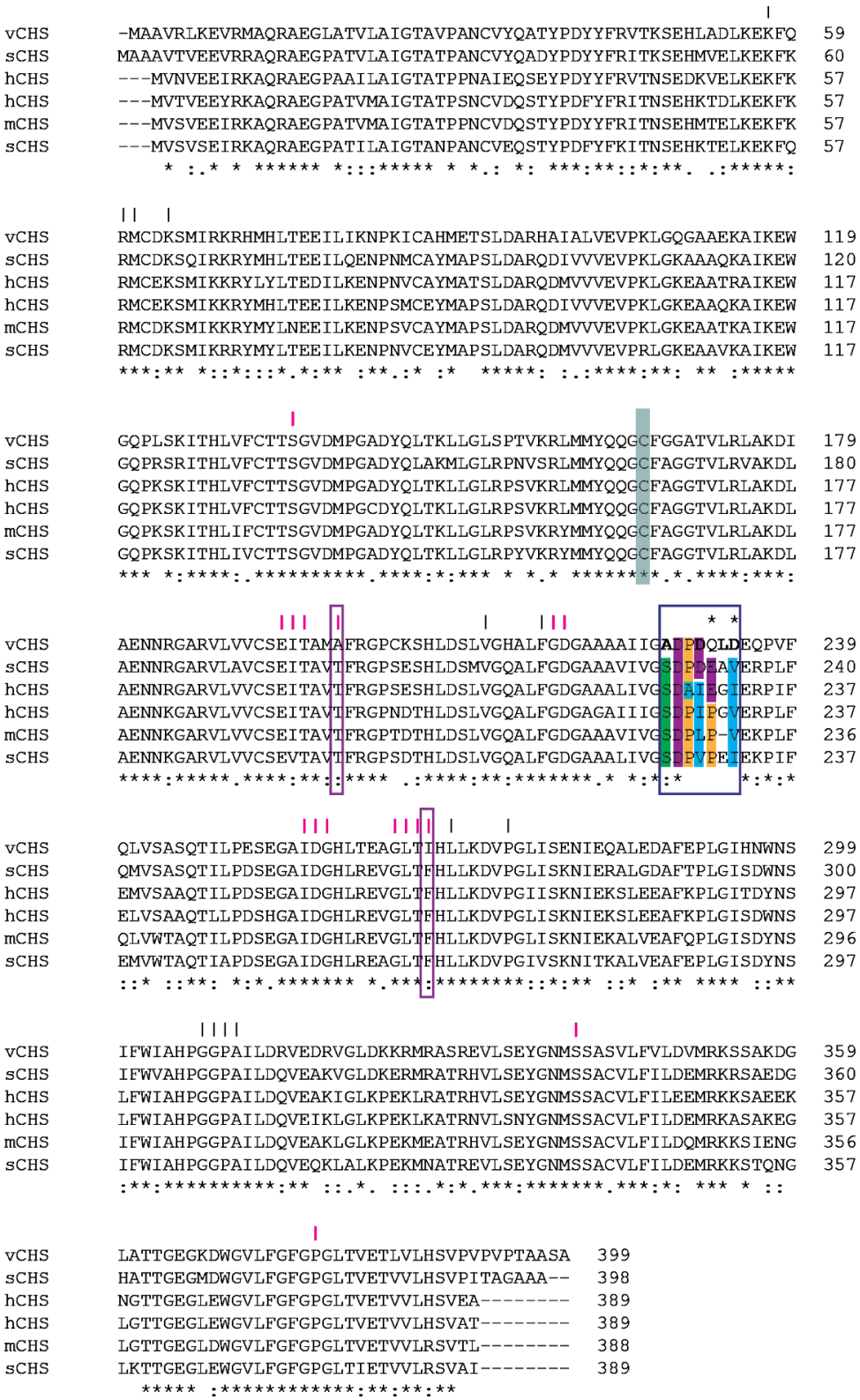


Figure S3: Multiple sequence alignments of those proteins that are structurally most similar to HvCHS as identified by Dali search against the PDB. Gray box: catalytic cysteine; pink bars: flavonoid binding residues, black bars: malonyl-CoA binding residues, purple box: active site residues that were mutated in this study; blue box: surface loop residues that were mutated in this study with stars marking the most beneficial mutations; color coding within black box according to CLUSTAL W color coding.

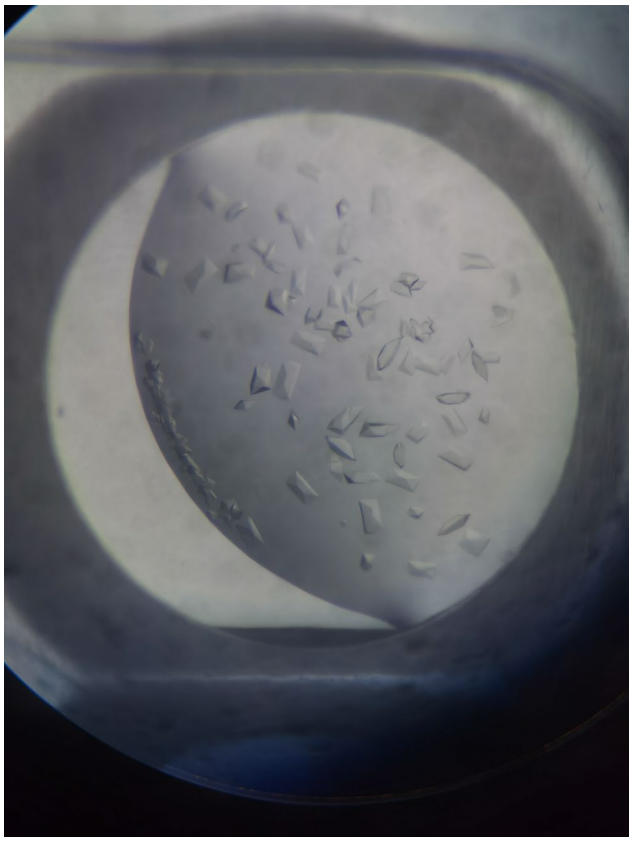
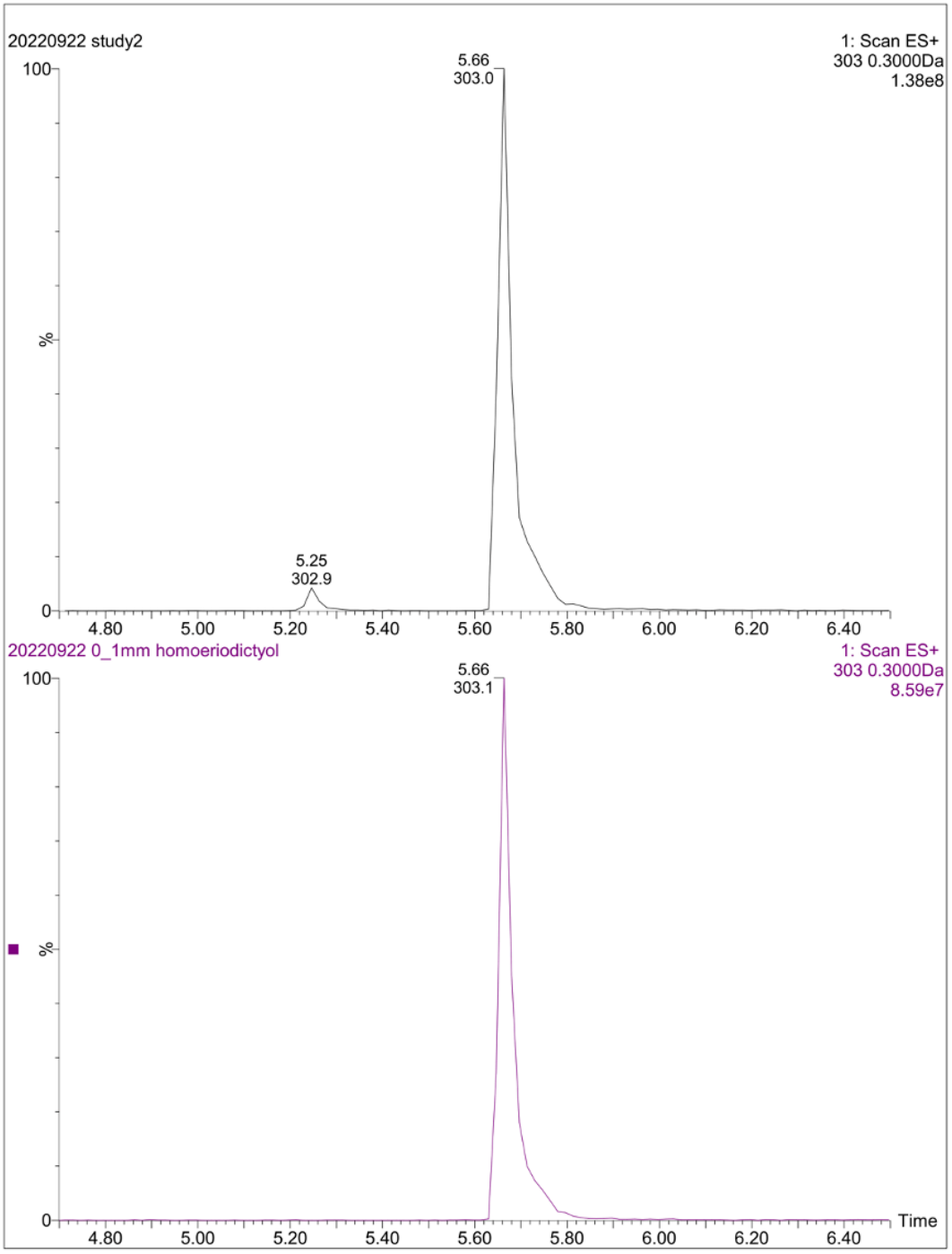


Figure S4. Micrograph of HvCHS crystals in the crystallization experiment. Multiple orthorhombic crystals grew in a clear, sitting drop containing 1µl protein (10mg/ml stock concentration) and 1µl reservoir solution (0.1M MES/Imidazole pH6.5; 0.03M $MgCl_2$, 0.03M $CaCl_2$; 16% (v/v) glycerol, 8% (v/v) PEG4000) in 1-2 days at 4°C.

A


20220922 study2
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5.66
303.0
5.25
302.9
100
%
0
4.80
5.00
5.20
5.40
5.60
5.80
6.00
6.20
6.40
20220922 0_1mm homoeriodictyol
1: Scan ES+
303 0.3000Da
8.59e7
5.66
303.1
100
%
0
Time
4.80
5.00
5.20
5.40
5.60
5.80
6.00
6.20
6.40

B

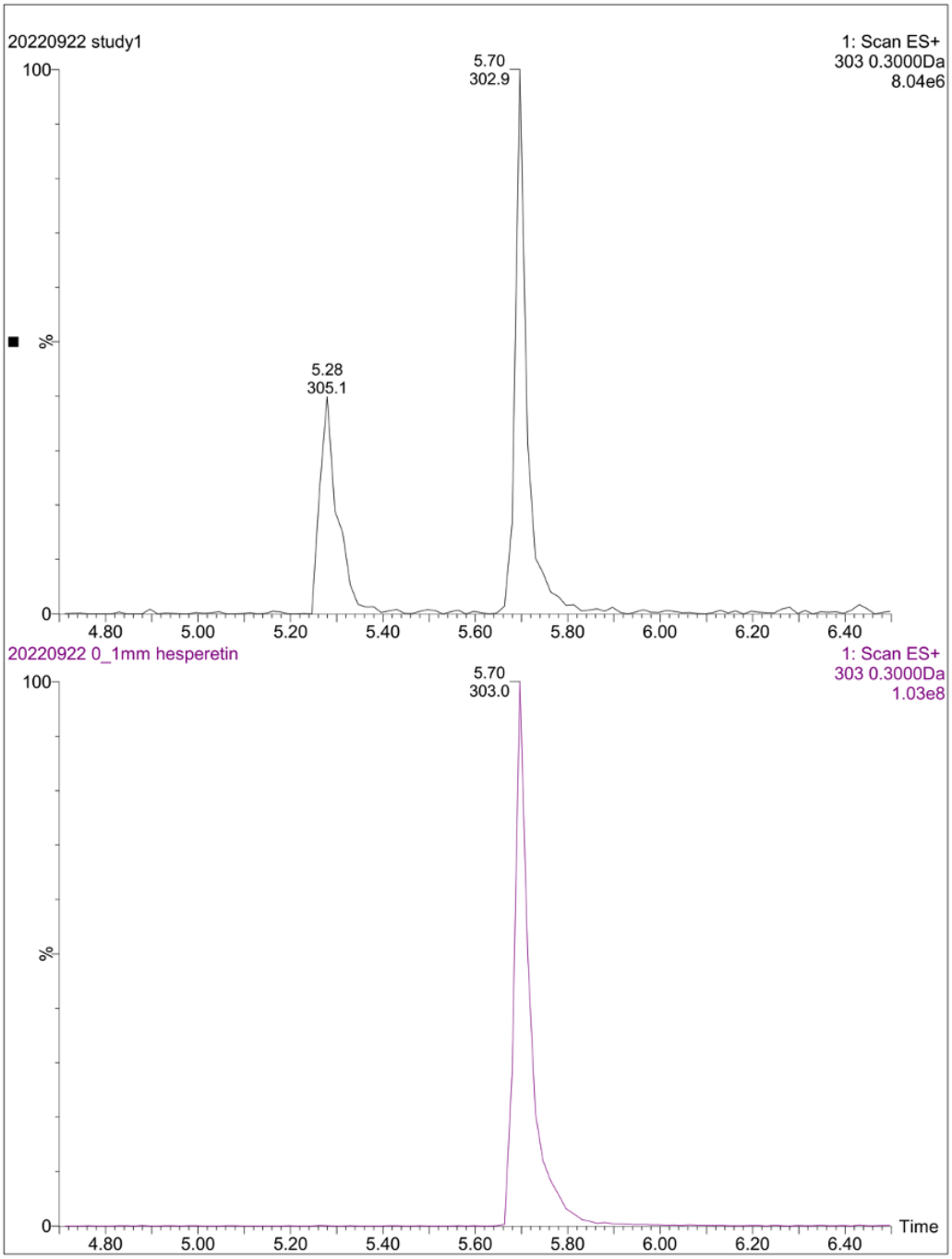

Figure S5. UHPLC- MS extracted ion chromatograms in the positive ion channel (*m/z 303*[H^+]). (A) Chromatogram of homoeriodictyol extracted from s2 (HvCHS wildtype variant, black) and the authentic standard of homoeriodictyol (pink). (B) Chromatogram of hesperetin extracted from s2 (HvCHS wildtype variant, black) and of the authentic standard of hesperetin (pink).

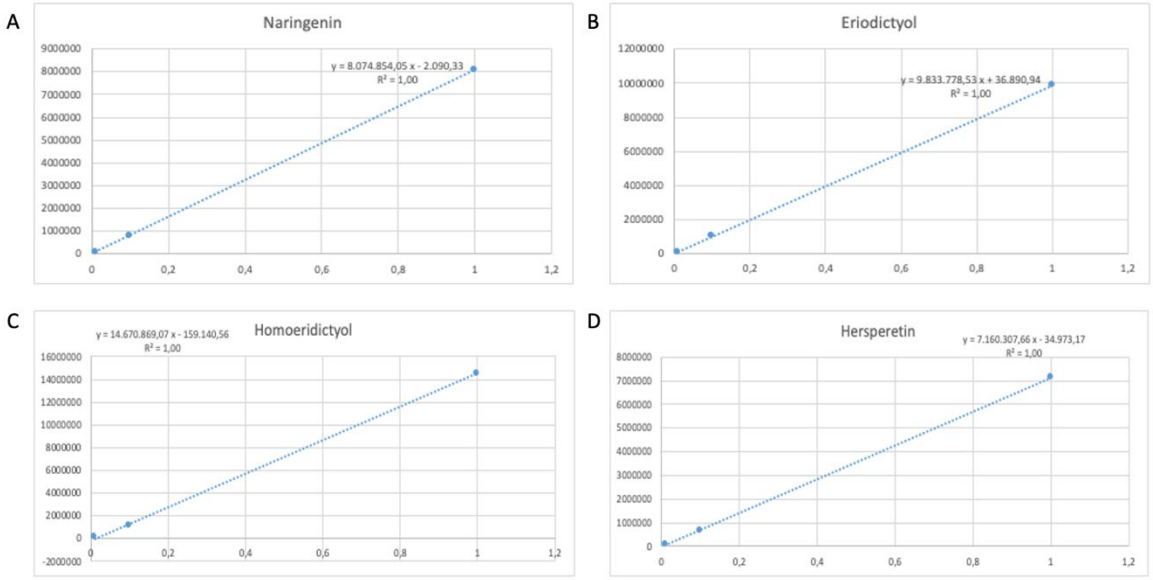

Figure S7. Calibration plot of flavonoids (A: naringenin, B: eriodictyol, C: homoeriodictyol, and D: hesperetin) dissolved in DMSO and analyzed by HPLC. The compounds were detected at 288 nm and the analysis was performed as described in the method section. The range of calibration curve was 0.01mM to 1mM.

Supporting information

Sequences of synthetic genes

Pc4CL *(Petroselinum crispum*):

ATGGGTGACTGCGTTGCCCCGAAAGAGGATCTGATCTTCCGCAGCAAACTGCCGGACATTTACATTCCAAAGCATCTGCCGCT
GCATACCTATTGTTTTGAGAACATCAGCAAGGTTGGCGACAAGAGCTGTCTGATCAACGGCGCAACCGGCGAAACCTTTACCT
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CTGCTGCCGAACTCCCCGGAGTACTTTTTCGCTTTCCTGGGTGCGAGCTATCGCGGTGCAATCAGCACTATGGCGAACCCATT
CTTTACCAGCGCAGAAGTGATCAAGCAACTGAAAGCGAGCCAAGCGAAGCTGATTATCACCCAGGCATGCTATGTTGACAAGG
TTAAGGACTACGCAGCGGAGAAAAACATCCAGATCATTTGTATTGACGATGCACCGCAGGATTGCCTGCACTTTAGCAAGCTG
ATGGAAGCGGATGAGAGCGAAATGCCGGAAGTGGTTATTAACAGCGATGATGTGGTGGCACTGCCGTACAGCTCTGGCACCAC
CGGCCTGCCGAAAGGCGTTATGCTGACCCACAAGGGTCTGGTTACCAGCGTTGCACAACAGGTGGATGGTGATAACCCGAACC
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CAATTTGTGAGCAAACAGGTTGTGTTCTACAAACGTATCTTCCGCGTTTTCTTCGTTGACGCTATTCCGAAATCCCCGAGCGG
CAAGATTCTGCGTAAGGATCTGCGCGCTCGTATTGCGAGCGGCGACCTGCCGAAGTAA

Os4CL (*Oryza sativa*):

ATGGGGTCAGTTGCAGCCGAGGAAGTAGTCGTCTTCCGCTCTAAGCTGCCGGATATCGAAATCGATAACTCTATGACCCTGCA
AGAATACTGCTTTGCACGTATGGCAGAGGTGGGAGCCCGCCCATGTCTTATCGACGGGCAAACTGGAGAGTCATACACTTATG
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GTTGCCAAAGAGGTGGTGTTCTACAAGCGTATTAACAAAGTGTTCTTTACAGATTCCATCCCTAAAAACCCTTCCGGTAAGAT
CTTGCGCAAGGATCTTCGTGCTCGTCTGGCGGCGGGTATTCCGGACGCTGTCGCTGCGGCAGCTGCAGATGCTCCTAAAAGTA
GCTAA

HvCHS (*Hordeum vulgare*):

ATGGCAGCGGTGCGTTTGAAGGAGGTGCGCATGGCGCAGCGCGCCGAGGGTTTAGCTACAGTGCTGGCGATCGGAACGGCTGT
ACCTGCAAATTGTGTTTACCAAGCGACATATCCTGACTACTACTTTCGTGTTACTAAGTCAGAGCATTTAGCCGATCTTAAAG
AAAAGTTTCAGCGCATGTGTGACAAGAGTATGATCCGCAAACGCCACATGCACCTTACGGAAGAGATTTTAATTAAGAACCCC
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CTCTGAAAACATCGAGCAGGCTCTTGAGGATGCGTTTGAGCCTTTGGGTATTCACAATTGGAACTCTATTTTTTGGATCGCTC
ATCCGGGCGGGCCTGCAATTTTAGACCGCGTCGAAGATCGCGTTGGATTAGATAAAAAACGTATGCGTGCTTCACGCGAGGTC
CTTAGCGAATACGGCAACATGTCTTCAGCCTCTGTCTTATTCGTGCTTGACGTTATGCGCAAGTCGAGTGCAAAGGATGGGTT
GGCGACGACGGGCGAGGGGAAAGATTGGGGGGTGCTGTTCGGATTTGGCCCAGGACTGACCGTCGAAACCCTGGTATTACACT
CTGTTCCTGTTCCCGTTCCCACTGCGGCTTCTGCTTAA

PhCHS (*Petunia hybrida*):

ATGGTGACCGTGGAAGAATACCGTAAGGCGCAACGTGCGGAAGGCCCGGCGACCGTGATGGCGATTGGCACCGCGACCCCGAG
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CGACCTAA

MsCHI (*Medicago sativa*):

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ACTAA