

Engineered Biosynthesis of Plant Polyketides: Manipulation of Chalcone Synthase

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Materials and Methods

Materials. [2-¹⁴C]Malonyl-CoA (48 mCi/mmol) and [1-¹⁴C]acetyl CoA (47 mCi/mmol) was purchased from Moravek Biochemicals (California). Malonyl-CoA and acetyl-CoA were purchased from Sigma. 4-Coumaroyl-CoA was chemically synthesized as described previously (Abe, I.; Morita, H.; Nomura, A.; Noguchi, H. *J. Am. Chem. Soc.* **2000**, *122*, 11242-11243). Authentic samples of SEK4/SEK4b, 5,7-dihydroxy-2-methylchromone, 2,7-dihydroxy-5-methylchromone, and tetracetic acid lactone were obtained in our previous works (Abe, I.; Oguro, S.; Utsumi, Y.; Sano, Y.; Noguchi, H. *J. Am. Chem. Soc.* **2005**, *127*, 12709-12716). The CHS used in this study was cloned from young leaves of *Scutellaria baicalensis*. The recombinant enzyme with an additional hexahistidine tag at the C-terminal was subcloned into pET-22b(+) (Novagen), expressed in *E. coli*, and purified by Ni-chelate affinity chromatography as described before (Abe, I.; Morita, H.; Nomura, A.; Noguchi, H. *J. Am. Chem. Soc.* **2000**, *122*, 11242-11243).

M.s CHS	1	-----	MVSVSEIRKA	QRAEGPATIL	AIGTANPANC	VEQSTYPDFY	FKITNSEHKT	ELKKEKFORMC	DKSMIKRRYM	YLTEELKEN	PNVCEYMAPS
S.b CHS	1	-----	MVTVEEYHRA	TRAEGPATVL	AIGTANPPNC	VDQSTYADYV	FRICKSEHMT	ELKKKFQRM	DKSYIKKRYM	HLTEEFLEN	DNMTAEAPS
A.h STS	1	-----	MVSVSGIRKV	QRAEGPATVL	AIGTANPPNC	TDOSTYADYV	FRVTNSEHMT	DLKKKFQRM	ERTAIKKRYL	YLTEELKEN	PNMCAVKAPS
G.h 2PS	1	-----	MGSYS	SDDVEVIREA	GRAQGLATIL	VAQADYADYV	FRVTNSEHMT	DLKKKFQRM	ERTAIKKRYL	ALTEEDYQEN	PIMCEFMAPS
A.a PCS	1	MSSLSNSLPL	MEDVQGIIRKA	QKADGTATVM	AIGTAHPPHI	FPODTYADYV	FRATNSEHKT	ELKKKFQRM	KKTMIGKRYF	NYDEEFLKKY	PNITSYDEPS
A.a OKS	1	MSSLSNASHL	MEDVQGIIRKA	QKADGTATVM	AIGTAHPPHI	FPODTYADYV	FRATNSEHKT	ELKKKFQRM	KKTMIGKRYF	NYDEEFLKKY	PNITSYDEPS
R.p ALS	1	-----	MADVLQELRNS	QKASGPATVL	AIGTAHPPTC	VPQADYPDFY	FRVCKSEHMT	KLKKKMQFIC	DRSGIRQRFM	FHTEENLQGN	PMCTFDGGS
M.s CHS	91		LDARQDMVVV	EVPRLGKEAA	VKAIKEWGQP	KSKITHLIVC	TTSGVDMPGA	DYQLTKLLGL	RFYVKRYMMY	QGGCFAGGTV	LRLAKDLAEN
S.b CHS	91		LDARQDMVVV	ETPKLGKEAA	VKAIKEWGQP	KSKITHLVVFC	TTSGVDMPGA	DYQLTKLLGL	RFSVKRFMMY	QGGCFAGGTV	LRLAKDLAEN
A.h STS	91		LDARQDMVVV	EVPRVGKEAA	VKAIKEWGQP	MSKITHLIFC	TTSGVALPGV	DYELIVLLGL	DPCKRYMMY	QGGCFAGGTV	LRLAKDLAEN
G.h 2PS	96		LNARODLVVT	GVPMLGKEAA	VKAIDEWGLP	KSKITHLIFC	TTAGVDMPGA	DYQLVKLLGL	SPSVKRYMMY	QGGCAAGGTV	LRLAKDLAEN
A.a PCS	101		LNDRODICVP	GVPAALGTEAA	VKAIEEWGRP	KSEITHLVFC	TSCGVDMPSA	DFOCALLLGL	HANVNKYCYV	MOGCAAGGTV	MRVAKDLAEN
A.a OKS	101		LNDRODICVP	GVPAALGTEAA	VKAIEEWGRP	KSEITHLVFC	TSCGVDMPSA	DFOCALLLGL	RTNVNKYCYV	MOGCAAGGTV	MRVAKDLAEN
R.p ALS	92		LNARQDMIM	EVKLGAEAA	EKAIEWGGD	KSRITHLIFC	TTTSNDMPGA	DYQFATLFLG	NGVSRITMVY	QLGCFAGGTV	LRLVKDLAEN
M.s CHS	191		SEITAVTFRG	PSDTHLDSLV	GOALFGDGAA	ALIVGSDPVP	EIEKPIFEMV	WTAOTIAPDS	EGAIDCHLRE	AGLTFHLKUD	VPGLVSKNIT
S.b CHS	191		SEITAVTFRG	PSEAHLDSLV	GOALFGDGAG	ALIVGSDPVP	GVEKPIFELV	SAOTIAPDS	EGAIDCHLRE	TGLTFHLKUD	VPGLVSKNIE
A.h STS	191		SEITAVTFRG	PSETDMDSLV	GOALFADGAA	ALIVGSDPVP	GVEKPIFELV	STQCKLVPGS	HGAIGLLRE	VGLTFYLNKS	VPDIISQNI
G.h 2PS	196		SEITAVTFRG	PNENHLDLSV	GOALFGDGAA	ALIVGSDPVP	AVEKPIFELV	STQCKLVPGS	HGAIGLLRE	GGLTFYLNKS	VPLMVAKNIE
A.a PCS	201		AELTIMLRA	PNETHLDNAI	GISLFGDGAA	ALIVGSDPVP	GVEKPMFEIV	CTKQTVIPNT	EDVTHLHRE	TGMVFYLSKG	SPMTISNNVE
A.a OKS	201		AELTIMLRA	PNETHLDNAI	GISLFGDGAA	ALIVGSDPVP	GVEKPMFEIV	CAKQTVIPNS	EDVTHLHRE	AGLMFYMSKD	SPMTISNNVE
R.p ALS	192		SEITAVTFRG	PHEDHIDSLI	GOALFGDGAA	ALVVGTDIDE	SVERPIFELM	SATQATIPNS	LHTMALHRE	AGLTFHLSKE	VPKVVSDNME
M.s CHS	291		GIS---	DVNS	IFWIAHPGGP	AILDQVEQKL	ALKPEKMNAT	REVLSEYGNM	SSACVLFILD	EMRKKSTQNG	LKTTGEGLEW
S.b CHS	291		GIS---	DVNS	IFWIAHPGGP	AILDQVEEKL	GLKPEIMACT	ROVLSYDGNM	SSACVLFVLD	EMRKSASAKNG	CTTTGEGKDW
A.h STS	291		GIS---	DVNS	IFWIAHPGGP	AILDQVEQKV	NLKPEKMKAT	RDVLSNYGNM	SSACVLFVLD	EMRKSLEEG	LKTTGEGLDW
G.h 2PS	296		GIT---	DVNS	IFWIAHPGGP	AILDQVEQKL	NLKEDKLRA	RHVLSEYGNL	ISACVLFILD	EMRKRSMAG	KSTTGEGLDG
A.a PCS	301		GITPPEDWNS	LFWIPHPGGP	AILDQVEAKL	KURPEKFRAT	RTVLWDGNGM	VSASVGYILD	EMRRKSAAGK	LETYEGLEW	GVLGFGPGI
A.a OKS	301		GMTPPEDWNS	LFWIPHPGGP	AILDQVEAKL	KURPEKFRAT	RTVLWDGNGM	VSACVLFILD	EMRRKSADEG	LETYEGLEW	GVLGFGPGM
R.p ALS	292		GIT---	DVNS	IFWIAHPGGP	AILDKITEKL	ELTKDKMRDS	RYTLSEYGNL	TSACVLFVMD	EMRKRSEFREG	KQTTGEGYEW

Fig. 1 Comparison of primary sequences of *Scutellaria baicalensis* CHS and other CHS-superfamily enzymes. M.s CHS, *Medicago sativa* CHS; S.b CHS, *S. baicalensis* CHS; A.h STS, *Arachis hypogaea* stilbene synthase; G.h 2PS, *Gerbera hybrida* 2-pyrone synthase; A.a PCS, *Aloe arborescens* PCS; A.a OKS, *A. arborescens* OKS; R.p ALS, *Rheum palmatum* ALS. The critical active-site residues 197, 256, and 338 (in pink), the catalytic triad (Cys164, His303, and Asn336) (in red), and the active-site Phe215 and F265 (in blue) were marked with # (numbering in *M. sativa* CHS), and residues for the CoA binding with +.

Site-Directed Mutagenesis. *S. baicalensis* CHS mutants (T197G, G256L, S338V, G256L/S338V, T197G/G256L, T197G/S338V, T197G/G256L/S338V, T197A/G256L/S338V, and T197M/G256L/S338V) were constructed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and a pair of complementary mutagenic primers as follows (mutated codons are underlined); T197G, sense 5'-CCG AAA TCA CCG CCG TCG GAT TCC GGG GAC-3', anti-sense 5'-GTC CCC GGA ATC CGA CGG CGG TGA TTT CGG-3'; G256L, sense 5'-GCG AGG GTG CCA TTG ACC TCC ACC TTC GCG-3', anti-sense 5'-CGC GAA GGT GGA GGT CAA TGG CAC CCT CGC-3'; S338V, sense 5'-CGG GAA CAT GGT CAG CGC CTG CGT GAT CTT CG-3', anti-sense 5'-CGA AGA TCA CGC AGG CGC TGA CCA TGT TCC CG-3';

T197A, sense 5'-GCT CCG AAA TCA CCG CCG TCG CAT TCC GGG GAC-3', anti-sense 5'-GTC CCC GGA ATG CGA CGG CGG TGA TTT CGG AGC-3'; T197M, sense 5'-GCT CCG AAA TCA CCG CCG TCA TGT TCC GGG GAC-3', anti sense 5'-GTC CCC GGA ACA TGA CGG CGG TGA TTT CGG AGC-3'.

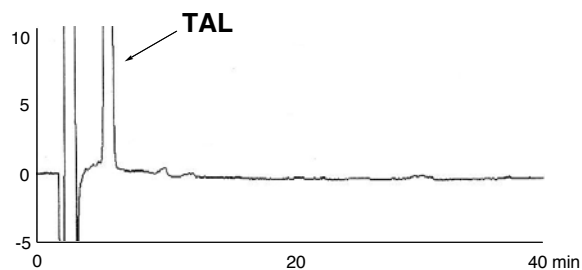
Enzyme Expression and Purification. After confirmation of the sequence, the plasmid was transformed into *E. coli* BL21(DE3)pLysS. The cells harboring the plasmid were cultured to an A_{600} of 0.6 in LB medium containing 100 $\mu\text{g/mL}$ of ampicillin at 30 °C. Then, 0.4 mM isopropyl thio- β -D-galactoside was added to induce protein expression, and the culture was incubated further at 16 °C for 14 h. The *E. coli* cells were harvested by centrifugation and resuspended in 40 mM potassium phosphate buffer, pH 7.9, containing 0.1 M NaCl. Cell lysis was carried out by sonication, and centrifuged at 15,000 g for 40 min. The supernatant was passed through a column of Pro-Bond™ resin (Invitrogen) containing Ni^{2+} as an affinity ligand. After washing with 20 mM potassium phosphate buffer, pH 7.9, containing 0.5 M NaCl and 40 mM imidazole, the recombinant ALS was finally eluted with 15 mM potassium phosphate buffer, pH 7.5, containing 10% glycerol and 500 mM imidazole. Protein concentration was determined by the Bradford method (Protein Assay, Bio-Rad) with bovine serum albumin as standard.

Enzyme Reaction. The standard reaction mixture contained 27 nmol of starter CoA (4-coumaroyl-CoA) and 54 nmol of malonyl-CoA, and 460 pmol of the purified recombinant enzyme in a final volume of 500 μL of 100 mM potassium phosphate buffer, pH 7.0. Incubations were carried out at 30 °C for 12 hours, and stopped by adding 50 μL of 20% HCl. The products were then extracted with 1,000 μL of ethyl acetate, and concentrated by N_2 flow.

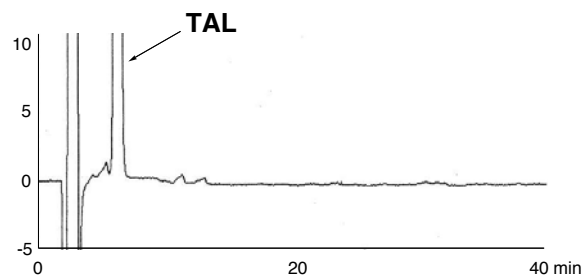
The residue was dissolved in aliquot of MeOH containing 0.1% TFA, and separated by reverse-phase HPLC (JASCO 880) on a TSK-gel ODS-80Ts column (4.6 x 150 mm, TOSOH) with a flow rate of 0.8 ml/min. Gradient elution was performed with H₂O and MeOH, both containing 0.1% TFA: 0-5 min, 30% MeOH; 5-17 min, linear gradient from 30 to 60% MeOH; 17-25 min, 60% MeOH; 25-27 min, linear gradient from 60 to 70% MeOH. Elutions were monitored by a multichannel UV detector (MULTI 340, JASCO) at 290 nm, 330nm and 360 nm; UV spectra (198-400 nm) were recorded every 0.4 s. The retention time (min): SEK4 (19.3), SEK4b (20.6), 6-(2,4-dihydroxy-6-methylphenyl)-4-hydroxy-2-pyrone (16.9), 2,7-dihydroxy-5-methylchromone (22.7), tetracetic acid lactone (4.4), and triacetic acid lactone (6.0).

On-line LC-ESIMS spectra were measured with a Hewlett-Packard HPLC 1100 series (Wilmington, DE) coupled to a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA) fitted with an ESI source. HPLC separations were carried out under the same conditions as described above. The ESI capillary temperature and capillary voltage were 225 °C and 3.0 V, respectively. The tube lens offset was set at 20.0 V. All spectra were obtained in both negative and positive mode; over a mass range of m/z 100-500, at a range of one scan every 2 s. The collision gas was helium, and the relative collision energy scale was set at 30.0% (1.5 eV).

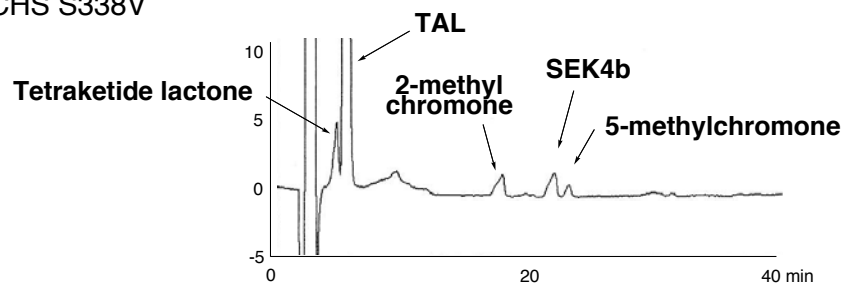
(A) CHS T197G



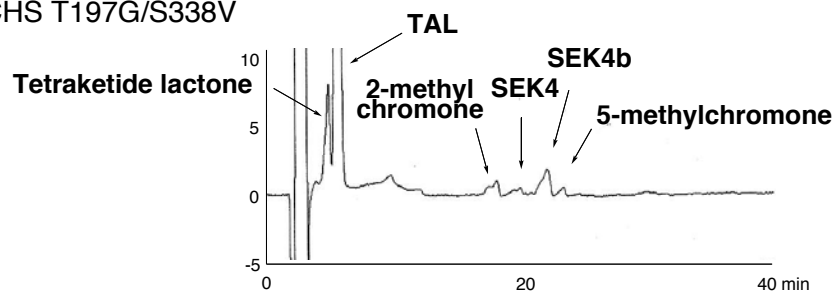
(B) CHS G256L



(C) CHS S338V



(D) CHS T197G/S338V



(E) CHS T197G/G256L/S338V

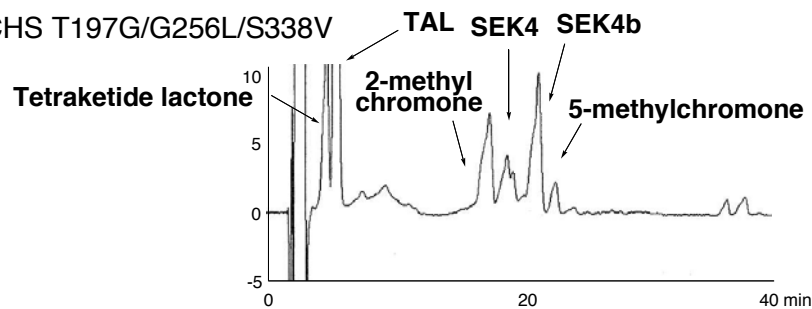


Fig. 2. HPLC elution profiles of enzyme reaction products of *S. baicalensis* CHS mutant; (A) T197G, (B) G256L, (C) S338V, (D) T197G/S338V and (E) T197G/G256L/S338V.

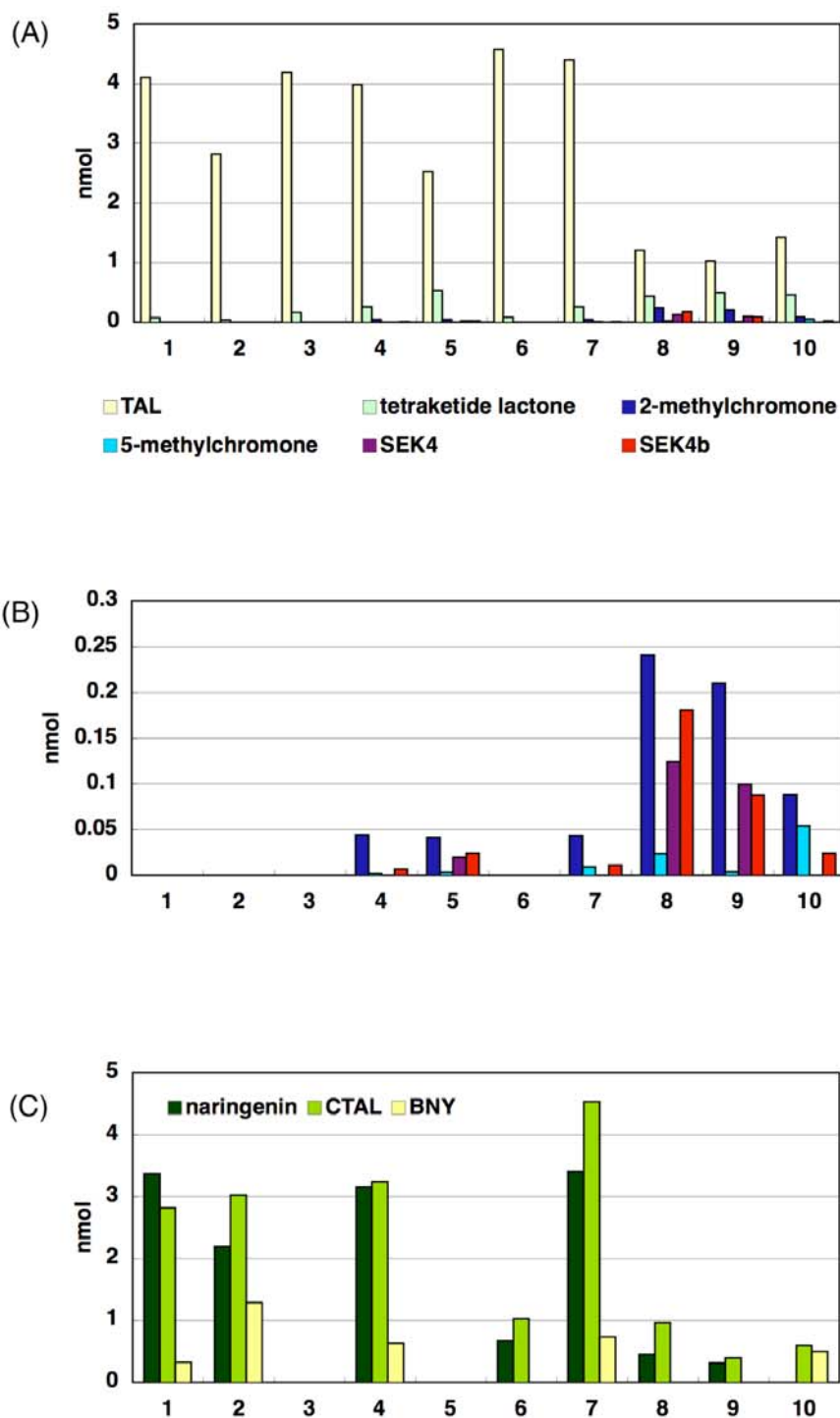


Fig. 3. Distribution pattern of polyketides produced by CHS mutants; (A) from malonyl-CoA (all products), (B) from malonyl-CoA (pentaketides and octaketides), (C) from 4-coumaroyl-CoA/malonyl-CoA. (1) wild-type, (2) T197G, (3) G256L, (4) S338V, (5) G256L/S338V, (6) T197G/G256L, (7) T197G/S338V, (8) T197G/G256L/S338V, (9) T197A/G256L/S338V, (10) T197M/G256L/S338V.