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Engineered Biosynthesis of Plant Polyketides: Manipulation of Chalcone Synthase

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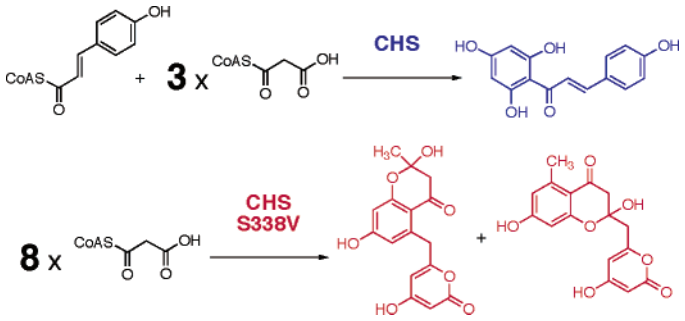
ABSTRACT



Chalcone synthase (CHS) is a plant-specific type III polyketide synthase catalyzing condensation of 4-coumaroyl-CoA with three molecules of malonyl-CoA. Surprisingly, it was demonstrated that S338V mutant of *Scutellaria baicalensis* CHS produced octaketides SEK4/SEK4b from eight molecules of malonyl-CoA. Further, the octaketides-forming activity was dramatically increased in a CHS triple mutant (T197G/G256L/S338T). The functional conversion is based on the simple steric modulation of a chemically inert residue lining the active-site cavity.

The chalcone synthase (CHS) superfamily of type III polyketide synthases (PKSs) share a common three-dimensional overall fold with a conserved Cys-His-Asn catalytic triad to produce structurally diverse polyphenols with remarkable biological activities.[1] CHS is the well-characterized plant-specific type III PKS that produces naringenin chalcone (4,2′,4′,6′-tetrahydroxychalcone), the biosynthetic precursor of flavonoids, through a sequential condensation of 4-coumaroyl-CoA with three molecules of malonyl-CoA (Figure 1A).[2] In an *in vitro* enzyme reaction, CHS also produces bis-noryangonin (BNY) and 4-coumaroyltriacetic acid lactone (CTAL) as early-released derailment byproducts (Figure 1A).[2] On the other hand, recently reported octaketide synthase (OKS) from *Aloe arborescens* catalyzes condensation of eight molecules of malonyl-CoA to produce a 1:4 mixture of octaketide SEK4 and SEK4b (Figure 1B),[3b] the shunt products of the minimal type II PKS from

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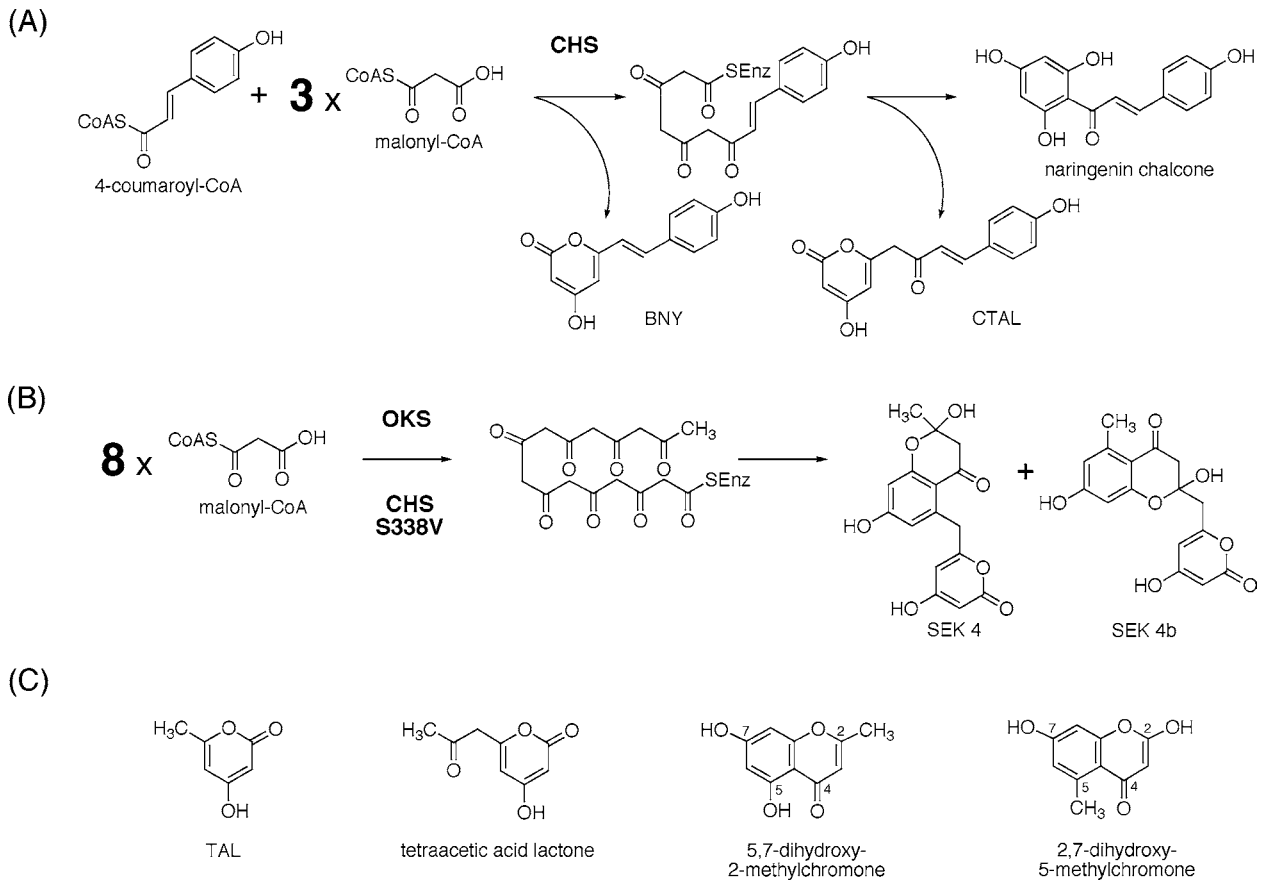

Figure 1. Formation of (A) chalcone by CHS, (B) SEK4/SEK4b by OKS (and CHS S338V), and (C) other polyketide products.

Streptomyces coelicolor[4] and the longest polyketides generated by the structurally simple type III PKS.[3] In *A. arborescens* OKS, the CHS's active-site residues Thr197, Gly256, and Ser338 (numbering in *Medicago sativa* CHS[2a]) are uniquely replaced with Gly, Leu, and Val, respectively (T197G/G256L/S338V).[3b] Interestingly, the three residues lining the active-site cavity are sterically altered in a number of functionally divergent type III PKSs including *A. arborescens* pentaketide chromone synthase (PCS) (T197M/G256L/S338V)[3a], *Rheum palmatum* aloesone synthase (T197A/G256L/S338T),[5] and *Gerbera hybrida* 2-pyrone synthase (2PS) (T197L/G256L/S338I).[6] These chemically inert residues have been shown to control starter substrate and product specificity by steric modulation of the active-site cavity in *M. sativa* CHS and in *G. hybrida* 2PS.[2d,6b] Further, in previous papers, we reported that the residue 197 determines the polyketide chain length and product specificities in the octaketide-producing *A. arborescens* OKS and the pentaketide-producing *A. arborescens* PCS.[3]

To further study the structure−function relationship between CHS and OKS enzyme, here we constructed a series of *Scutellaria baicalensis* CHS[7] mutants in which the three residues were changed from those in CHS to those in OKS (T197G, G256L, and S338V), and investigated the mechanistic consequences of the mutations using 4-coumaloyl-CoA and/or malonyl-CoA as substrates.

Interestingly, in the absence of the coumaroyl starter, both wild-type and the mutant *S. baicalensis* CHSs initiated decarboxylative condensation of malonyl-CoA, but most of the polyketide chain elongation reactions were terminated at the triketide stage to predominantly produce triacetic acid lactone (TAL) (Figure 2A). This is in good agreement with an earlier report that mutation of *M. sativa* CHS at the residues (T197L, G256L, and S338I) resulted in functional

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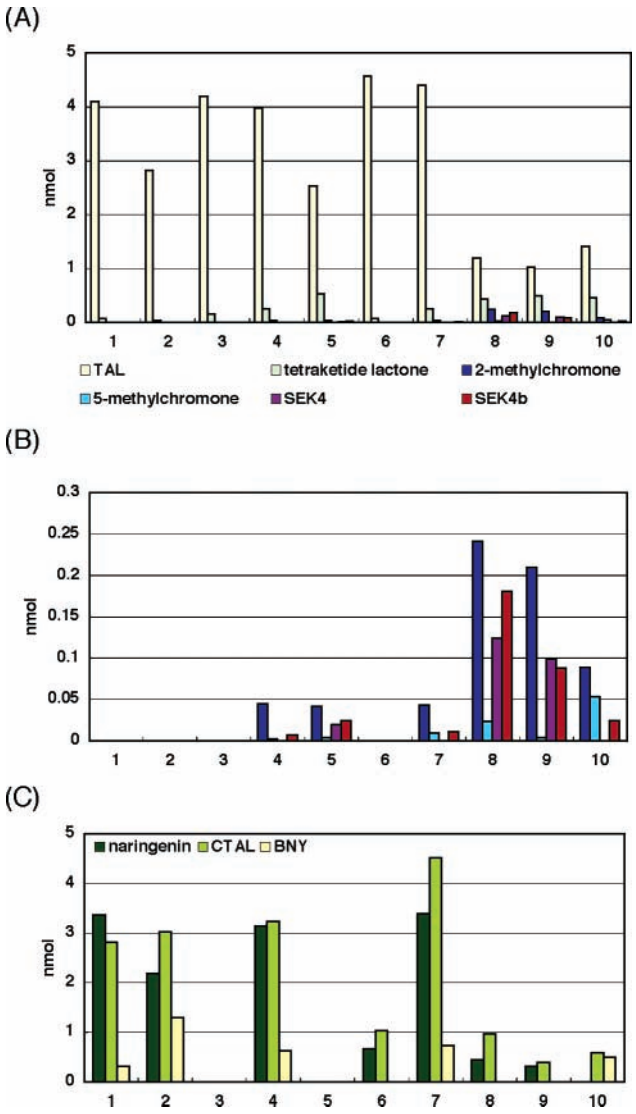

Figure 2. Distribution pattern of polyketides produced by CHS mutants: (A) from malonyl-CoA (all products), (B) from malonyl-CoA (pentaketides and octaketides), (C) from 4-coumaroyl-CoA/malonyl-CoA. (1) wild-type, (2) T197G, (3) G256L, (4) **S338V**, (5) G256L/**S338V**, (6) T197G/G256L, (7) T197G/**S338V**,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

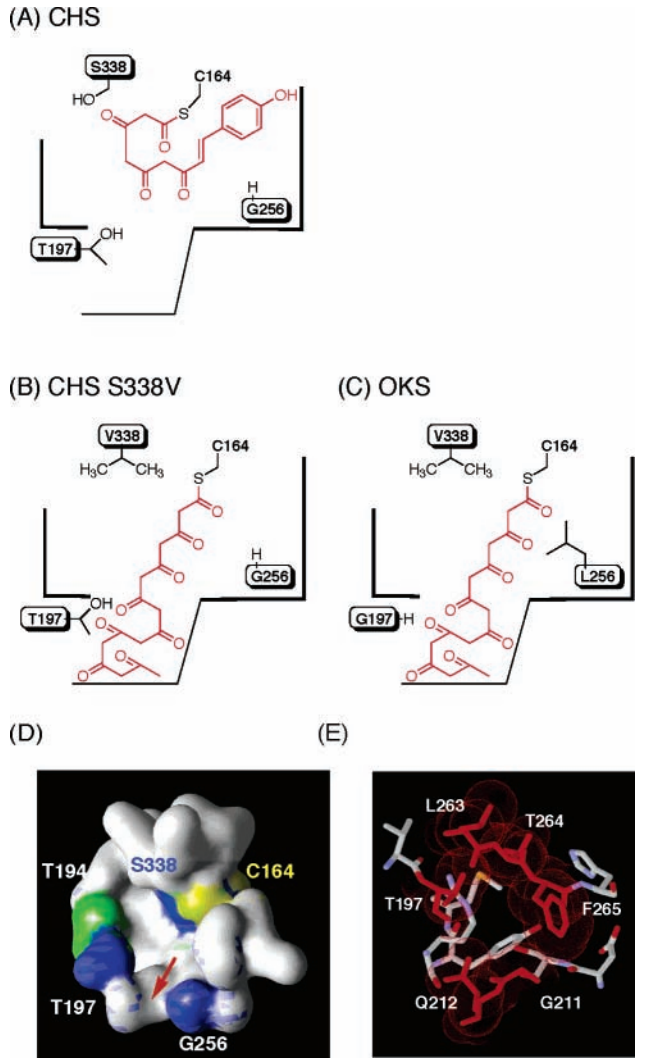

Figure 3. Schematic representation of the active-site architecture of (A) CHS, (B) CHS S338V mutant, and (C) *A. arborescens* OKS. (D) CHS's active-site cavity and an entrance to a novel buried pocket, and (E) the entrance of the buried pocket (a putative polyketide elongation tunnel) that extends into the "floor" of the active-site cavity.

conversion into a TAL-producing enzyme.[6b] However, very surprisingly, careful examination of the enzyme reaction products revealed that CHS **S338V** mutant yielded a trace amount of SEK4/SEK4b in addition to 5,7-dihydroxy-2-methylchromone,[3a] 2,7-dihydroxy-5-methylchromone,[3b] and tetracetic acid lactone[3b] (Figures 1C and 2B). It was thus for the first time demonstrated that CHS could be engineered to produce longer octaketides by the single amino acid replacement. Furthermore, the SEK4/SEK4b-forming activity was dramatically increased in an OKS-like triple mutant (T197G/G256L/**S338V**) (Figure 2B). On the other hand, in the presence of 4-coumaroyl-CoA in the assay mixture, most of the *S. baicalensis* CHS mutants still accepted the coumaroyl starter to produce chalcone, whereas interestingly G256L and G256L/S338V mutant did not afford any products from 4-coumaroyl-CoA (Figure 2C).

Out of the three point mutations tested (T197G, G256L, and S338V), the hydrophobic replacement **S338V** is critical for production of the longer chain polyketides including SEK4/SEK4b.[8] In contrast, T197G and G256L mutant did not produce any malonate-derived polyketides except TAL and tetraacetic acid lactone. On the basis of the published X-ray crystal structure of *M. sativa* CHS at 1.56 Å resolution,[2a] the residue 338 is located in proximity of the catalytic Cys164 at the "ceiling" of the active-site cavity and plays a crucial role in the polyketide chain elongation

(8) *S. baicalensis* CHS S338I (2PS-like) mutant also produced a trace amount of SEK4/SEK4b, whereas S338A, S338F, and S338T (ALS-like) mutant only produced the pentaketide chromones. Interestingly, S338C mutant did not produce any malonate-derived polyketides except TAL.

reactions. Further interestingly, the crystal structure also revealed presence of an additional buried pocket that extends into the "floor" of the traditional CHS active site (Figure 3).[9] Presumably, the **S338V** mutaion provided steric guidance so that the linear polyketide intermediate extends into the buried pocket, thereby leading to formation of the longer polyketides (Figure 3). Most of the polyketide elongation reactions were, however, terminated at the triketide stage to yield TAL as a predominant product. A similar active-site architecture with a downward expanding polyketide tunnel has been recently reported for a bacterial pentaketide-producing type III PKS, 1,3,6,8-tetrahydroxynaphthalene synthase, from *S. coelicolor* that shares only ca. 20% amino acid sequence identity with CHS.[9]

On the other hand, Thr197 in *S. baicalensis* CHS functions as a gate keeper at the entrance of the buried pocket along with Gly211, Gln212, Leu263, Thr264, and Phe265 (Figure 3E). The replacement of Thr197 with less bulky Gly in **T197G**/S338V and **T197G**/G256L/S338V mutant widely opens the gate, thereby expanding the putative polyketide chain elongation tunnel, which led to significantly increased production of the longer chain polyketides including the octaketide SEK4/SEK4b (Figure 2A and 2B). Whereas, small-to-large substitutions in place of the residue 197 (**T197A**/G256L/S338V and **T197M**/G256L/S338V) resulted in decrease of the octaketide-forming activity and the concomitant formation of shorter chain polyketides (Figure 2A and 2B), which is well consistent with our previous reports that the residue 197 determines the polyketide chain length and product specificities in *A. arborescens* OKS[3b] and *A. arborescens* PCS.[3a]

Finally, the OKS- and 2PS-like bulky **G256L** substitution[6b,9] contributes to a steric constriction of the coumaroyl binding pocket,[2a] thus controlling the starter substrate selectivity in *S. baicalensis* CHS (Figure 3). In fact, **G256L** and **G256L**/S338V mutant did not yield any polyketide products from the bulky coumaroyl starter (Figure 2C), but instead efficiently produced TAL from the malonyl starter (Figure 2A). It was interesting that the additional, the downward expanding, T197G replacement recovered chalcone-forming activity of the CHS mutants (T197G/**G256L** and T197G/**G256L**/S338V) (Figure 2C).

In summary, it was for the first time demonstrated that the CHS active-site can be extended to allow for the synthesis of larger polyketides including SEK4/SEK4b by a single amino acid substitution **S338V**. Further, the octaketide-forming activity was dramatically increased in the OKS-like *S. baicalensis* CHS triple mutant (T197G/G256L/S338T). The functional conversion is based on the simple steric modulation of a chemically inert residue lining the active-site cavity. These results provided structural basis for understanding the functional diversity of type III PKS enzymes and suggest strategies for engineered biosynthesis of plant polyketides.

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Supporting Information Available: Experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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