Curcuminoids, major components of the spice turmeric, are used as a traditional Asian medicine and a food additive. Curcumin, a representative curcuminoid, has received a great deal of attention because of its anti-inflammatory, anticarcinogenic, and antitumor activities. Because of these properties, curcuminoid biosynthesis is of great interest.

**In Vitro Synthesis of Curcuminoids by Type III Polyketide Synthase from *Oryza sativa***

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Curcuminoids, major components of the spice turmeric, are used as a traditional Asian medicine and a food additive. Curcumin, a representative curcuminoid, has received a great deal of attention because of its anti-inflammatory, anticarcinogenic, and antitumor activities. Because of these properties, curcuminoid biosynthesis is of great interest. Curcuminoids are synthesized specifically in the rhizomes of *Curcuma longa* L. Curcumin (3e) is the principal component of the turmeric rhizome, which is widely used as a food additive and in traditional Asian medicine (1). In addition to its aromatic, stimulant, and coloring properties in the diet, recent studies suggest that curcumin (3e) possesses anti-inflammatory, anticarcinogenic, and antitumor activities when taken orally or topically (1–3). However, the reaction involving BAS includes deacylation of a β-keto acid and the formation of an α-methylketone (5), which is not a curcuminoid intermediate. According to the well established head-to-tail model of polyketide assembly by type III PKSs, curcuminoid synthesis may begin with the condensation of an acetate unit to yield a phenylpropanoid unit, as is the case with chalcone synthase (Fig. 1C); however, the incorporation of additional phenylpropanoid units appears to violate this head-to-tail rule. The intriguing structure of curcuminoids raises the question of whether a single type III PKS catalyzes all of the steps in curcuminoid synthesis or only some of them, such as β-keto acid synthesis. Although it has been 10 years since Schröder (6) predicted that a type III PKS might be involved in curcuminoid synthesis, no enzymes for the synthesis of the curcuminoid backbone have been identified. Although Ramirez-Ahumada et al. (7) achieved curcuminoid synthesis in vitro using crude extracts of *Rheum palmatum* L., the reaction catalyzed in *Rheum palmatum* by benzalacetone synthase (BAS) (5), a type III polyketide synthase (PKS) that catalyzes the synthesis of benzalacetone from 4-coumaryl-CoA (1a) and malonyl-CoA (Fig. 1B). However, the reaction involving BAS includes deacylation of a β-keto acid and the formation of an α-methylketone (5), which is not a curcuminoid intermediate. Therefore, the well established head-to-tail model of polyketide assembly by type III PKSs, curcuminoid formation from a precursor of the phenylpropanoid backbone have been identified. Although Ramirez-Ahumada et al. (7) achieved curcuminoid synthesis in vitro using crude extracts of *Rheum palmatum* L., they did not characterize the enzyme(s) responsible, *WtPKS1*, a type III PKS from *Wachendorfia thyrsiflora* known to produce curcuminoid-related compounds called phenylphtalenones, is capable of synthesizing benzalacetones from phenylpropanoid-CoAs and malonyl-CoA (8), suggesting that *WtPKS1* may be involved in the synthesis of the β-keto acid during curcuminoid formation. However, *WtPKS1* does not catalyze curcuminoid formation from a precursor of the β-keto acid and phenylpropanoid-CoA, which excludes the possibility that *WtPKS1* alone is capable of synthesizing curcuminoids (8).

Here we report that a single type III PKS, os07g17010, from rice (*Oryza sativa*) (9) is capable of synthesizing bisdemethoxy-

**Bisdemethoxycurcumin (1e), dicinnamoylmethane (2e), and curcumin (3e)** (Fig. 1A) are curcuminoids found exclusively in the rhizomes of *Curcuma longa* L. Curcumin (3e) is the principal component of the turmeric rhizome, which is widely used as a food additive and in traditional Asian medicine (1). In addition to its aromatic, stimulant, and coloring properties in the diet, recent studies suggest that curcumin (3e) possesses anti-inflammatory, anticarcinogenic, and antitumor activities when taken orally or topically (1–3). Because of these properties, curcuminoid biosynthesis is a subject of great interest.

Labeling studies with *C. longa* showed that curcumin (3e) consists of two phenylpropanoid units connected by an acetate-derived central carbon unit (4). It is hypothesized that the curcuminoid scaffold is synthesized via a three-step reaction from phenylpropanoids (4). These reactions include (i) condensation of malonyl-CoA with feruloyl-CoA (3a) to produce a diketide-CoA, (ii) hydrolysis of the resulting diketide-CoA to its corresponding β-keto acid, and (iii) decarboxylative condensation of the β-keto acid to a second molecule of feruloyl-CoA (3a) (Fig. 1A). The formation of the β-keto acid is analogous to the reaction catalyzed in *Rheum palmatum* by benzalacetone synthase (BAS) (5), a type III polyketide synthase (PKS) that catalyzes the synthesis of benzalacetone from 4-coumaryl-CoA (1a) and malonyl-CoA (Fig. 1B).

This reaction violates the traditional head-to-tail model of polyketide assembly; the growing diketide intermediate is hydrolyzed to a β-keto acid that subsequently serves as the second extender to form curcuminoids. Curcuminoid synthase appears to be capable of the synthesis of not only diarylheptanoids but also gingerol analogues, because it synthesized cinnamoyl(hexanoyl)methane, a putative intermediate of gingerol, from cinnamoyl-CoA and 3-oxo-octanoic acid.

Bisdemethoxycurcumin (1e), dicinnamoylmethane (2e), and curcumin (3e) (Fig. 1A) are curcuminoids found exclusively in the rhizomes of *Curcuma longa* L. Curcumin (3e) is the principal component of the turmeric rhizome, which is widely used as a food additive and in traditional Asian medicine (1). In addition to its aromatic, stimulant, and coloring properties in the diet, recent studies suggest that curcumin (3e) possesses anti-inflammatory, anticarcinogenic, and antitumor activities when taken orally or topically (1–3). Because of these properties, curcuminoid biosynthesis is a subject of great interest.

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**1** The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4.

**2** The abbreviations used are: BAS, benzalacetone synthase; PKS, polyketide synthase; CUS, curcuminoid synthase; LC, liquid chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; APCIMS, atmospheric pressure chemical ionization mass spectrometry; NAC, N-acetylcysteamine.
In Vitro Curcuminoid Synthesis by Type III PKS

FIGURE 1. Proposed mechanism of the CUS reaction (A) compared with that of BAS (B) and chalcone synthase (C). A, CUS produces bisdemethoxycurcumin (1e) from two 4-coumaroyl-CoAs (1a) and one malonyl-CoA. CUS also produces triketide pyrones (1c) as by-products. B, BAS yields benzalacetone from 4-coumaroyl-CoA and malonyl-CoA. C, chalcone synthase, a representative plant type III PKS, synthesizes naringenin chalcone by the sequential condensation of extender substrates onto a starter substrate as follows. 4-Coumaroyl-CoA, acting as a starter substrate, is loaded on the catalytic cysteine via a thioester bond. Repetitive decarboxylative condensation of three malonyl-CoAs, as extender substrates, onto 4-coumarate in a head-to-tail fashion leads to the formation of a tetraketide intermediate. Cyclization and aromatization of the tetraketide intermediate produces naringenin chalcone.

curcumin (1e) from two 4-coumaroyl-CoAs (1a) and one malonyl-CoA. This enzyme can also produce dicinnamoylmethane (2e) and curcumin (3e) from cinnamoyl-CoA (2a) and feruloyl-CoA (3a), respectively; we therefore named it curcuminoid synthase (CUS) (Fig. 1A). Curcuminoids were also synthesized from cinnamoyl-CoA (2a) and 3-oxo-5-phenyl-pent-4-enoic acid (2d) (Fig. 1A), suggesting the involvement of diketide-CoA hydrolysis in the CUS reaction. Therefore, CUS is the first example of a type III PKS that violates the traditional model of head-to-tail polyketide assembly (i.e., a switchover of the role of a diketide intermediate from a growing chain to an extender unit).

EXPERIMENTAL PROCEDURES

Materials

Escherichia coli JM109, pUC19, restriction enzymes, T4 DNA ligase, and Taq DNA polymerase were purchased from Takara Biochemicals (Shiga, Japan). pET16b (Novagen, Darmstadt, Germany) was used for the expression of His-tagged proteins in E. coli BL21 (DE3). Bisdemethoxycurcumin (1e) was obtained from Chromadex (Santa Ana, CA), and trans-cinnamic acid, hexanoic acid, 4-hydroxycinnamic acid, and trans-4-hydroxy-3-methoxyxycinnamic acid were purchased from Wako (Tokyo, Japan). N-Acetylcycteamine was supplied by Aldrich, and dodecanoyl-CoA (5a), curcumin (3e), hexanoyl-CoA (4a), malonyl-CoA, methylmalonyl-CoA, octadecanoyl-CoA (6a), and succinyl-CoA were purchased from Sigma. 4-Coumaroyl-CoA (1a), cinnamoyl-CoA (2a), and feruloyl-CoA (3a) were synthesized by the procedure of Blecher (10), and cinnamoyl-diketide-N-acetylcycteamine (NAC) (2f) and 3-oxo-octanoyl-NAC (4f) were synthesized according to a published report (11). 3-Oxo-5-phenyl-pent-4-enoic acid (2d) and 3-oxo-octanoic acid (4d) were synthesized according to the methods of Matsumura et al. (12) and Lokot et al. (13), respec-
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tively, with modifications. A triketide pyrone (1c) derived from 4-coumaroyl-CoA (1a) was prepared as described previously (14).

Production and Purification of CUS

os07g17010 (NCBI accession number AK109558) cDNA was purchased from the Rice Genome Resource Center (Tsukuba, Japan). Using the cDNA as a template, a 1.1-kb DNA fragment containing the os07g17010-coding region was amplified by PCR with the primers 5'-CCGAATTCCCATATGGCACGACGACGACCATT-3' (an EcoRI site is shown in boldface, an Ndel site is underlined, and the start codon is shown in italics) and 5'-CGCGGATCCCTTATTCACATGAGAGGTGG-3' (a BamHI site is shown in boldface and the stop codon of os07g17010 is shown in italics). The amplified fragment was cloned between the EcoRI and BamHI sites of pUC19, resulting in pUC19-CUS. The Ndel-BamHI fragment of os07g17010 was excised from pUC19-CUS and cloned between the Ndel and BamHI sites of pET16b, resulting in pET16b-CUS.

To produce His-tagged CUS, E. coli BL21 (DE3) harboring pET16b-CUS was grown at 26 °C overnight in Luria broth containing 100 μg/ml ampicillin. The cells were then harvested by centrifugation and resuspended in a buffer containing 10 mM Tris-HCl (pH 8.0), 10% glycerol, and 20 mM [2-14C]malonate. 1 M sodium phosphate buffer (pH 7.5), 5 mM 4-coumaroyl-CoA, 5 mM malonyl-CoA, and 16.5 μg of CUS in a total volume of 500 μl. After the reaction mixture had been preincubated at 37 °C for 2 min, the reactions were initiated by adding the substrates (4-coumaroyl-CoA and malonyl-CoA) and continued for 20 min. The reactions were stopped with 100 μl of 6 M HCl, and the material in the mixture was extracted with ethyl acetate. The residual material in the organic layer was then dissolved in 20 μl of methanol for thin layer chromatography analysis. Silica gel 60 WF254 thin layer chromatography plates (Merck) were developed in benzene/acetic acid (85:15:1, v/v/v), and the 14C-labeled compounds were detected using a Fuji BAS-MS imaging plate (Fuji Film, Tokyo, Japan).

Radio Thin Layer Chromatography Assay

The standard reaction mixture contained 5 μM [2-14C]malonyl-CoA, 5 μM starter substrate, 100 mM potassium phosphate buffer (pH 7.5), and 6 μg of CUS in a total volume of 100 μl. The reactions were incubated at 37 °C for 1 h before being quenched with 20 μl of 6 M HCl. The products were identified using a Fuji BAS-MS imaging plate detector. Commercially available bisdemethoxycurcumin (1e) and NMR spectroscopically pure triketide pyrone derived from 4-coumaroyl-CoA (1c) were used to prepare calibration curves.

Temporary Change Analysis

The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 5 μM 4-coumaroyl-CoA, 5 μM malonyl-CoA, and 16.5 μg of CUS in a total volume of 500 μl. After the reaction mixture had been preincubated at 37 °C for 2 min, the reactions were initiated by adding the substrates (4-coumaroyl-CoA and malonyl-CoA) and continued for 5, 10, 20, 30, 40, 50, or 60 min. The reactions were stopped with 100 μl of 6 M HCl, and the material in the mixture was extracted with ethyl acetate. The residual material in the organic layer was then dissolved in 20 μl of methanol for HPLC analysis using a Hitachi LaChrom ELITE System. The HPLC was equipped with a Senshu Docosil-B reversed-phase HPLC column (4.6 × 250 mm) eluted with a linear acetonitrile gradient (10–100% over 25 min) in water containing 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. UV spectra were detected on a Hitachi L-2450 diode array detector.

pH Dependence Analysis

The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.0–9.0), 5 μM 4-coumaroyl-CoA, 5 μM malonyl-CoA, and 16.5 μg of CUS in a total volume of 500 μl. After the reaction mixture had been preincubated at 37 °C for 2 min, the reactions were initiated by adding the substrates (4-coumaroyl-CoA and malonyl-CoA) and continued for 10 min. The reactions were stopped with 100 μl of 6 M HCl, and the material in the mixture was extracted with ethyl acetate. The residual material in the organic layer was then dissolved in 20 μl of methanol for HPLC analysis using an Esquire High-Capacity Trap Plus system (Bruker Daltonics, Bremen, Germany), and HPLC analysis was carried out using a LaChrom ELITE system (Hitachi, Tokyo, Japan). The HPLC was equipped with a Pegasil-B C4 reversed-phase HPLC column (4.6 × 250 mm) (Senshu Scientific, Tokyo, Japan) and eluted with a linear acetonitrile gradient (10–100% over 45 min) in water containing 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. UV spectra were detected on a Hitachi L-2450 diode array detector.
RESULTS

Characterization of CUS—To employ a type III PKS as a member of an artificial biosynthesis pathway for fermentative production of plant polyketides by *E. coli* (14), we have screened genomes of various organisms for type III PKSs that catalyze formation of novel skeletons. We conducted a BLAST search using the *O. sativa* genome and found more than 30 proteins that were annotated as type III PKSs (data not shown). For preliminary characterization of the annotated proteins, we expressed them in *E. coli*, purified them, and incubated them with various CoA compounds including malonyl-CoA and 4-coumaroyl-CoA (1a) to determine products by spectrometric analysis. Of these putative type III PKSs, we found that the protein encoded by os07g17010 catalyzed curcuminoid formation from 4-coumaroyl-CoA (1a) and malonyl-CoA (see below). CUS shared 49% amino acid identity with chalcone synthase from *Medicago sativa* (15), 51% identity with BAS from *R. palmatum* (5), and 46% identity with WtPKS1 from *W. thyrsoflora* (8) (supplemental Fig. 1). In addition, CUS had a conserved catalytic triad, composed of Cys-174, His-316, and Asn-349 (CUS numbering), which is crucial for the decarboxylative condensation activity of all type III PKSs (supplemental Fig. 1) (16). The *CtIS* cDNA was expressed in *E. coli* BL21 (DE3), and CUS was purified as a single protein band with a molecular mass of 43 kDa on SDS-polyacrylamide gel electrophoresis (data not shown).

We tested the ability of CUS to synthesize bisdemethoxycurcumin (1e) from 4-coumaryl-CoA (1a) and malonyl-CoA because they are the typical substrates for plant type III PKSs. The reaction was performed with each 5 μM substrate at pH 7.5, which is similar to the cytosolic pH of *O. sativa* (17). Incubation of 4-coumaryl-CoA (1a) with [2-14C]malonyl-CoA yielded two radiolabeled products, as determined by thin layer chromatography (Fig. 2, *lane* 1). The major product was identified as bisdemethoxycurcumin (1e) by comparing its retention time, MS and MS/MS spectra, and UV maximum with those of an authentic sample via LC-APCIMS analysis (supplemental Fig. 2). Similarly, the minor product was identified as a triketide pyrone (1c) (supplemental Fig. 2) formed from one 4-coumaryl-CoA and two malonyl-CoAs. However, neither 5-(4-hydroxyphenyl)-3-oxo-pent-4-enic acid (1d), a possible intermediate, nor 4-hydroxybenzalacetone, a possible by-product, was detected. These findings indicate that CUS is distinct from BAS, which produces 4-hydroxybenzalacetone via decarboxylation of 1d (5).

Analysis of Cinnamoyl- and Feruloyl-CoA-primed Reactions—We examined whether CUS accepts other phenylpropanoids, such as cinnamoyl-CoA (2a) and feruloyl-CoA (3a), as starter substrates. The reactions, which were performed under conditions as described above, showed that cinnamoyl-CoA (2a) reacted to form a triketide pyrone (2c) with a

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**FIGURE 2. Thin layer chromatography analysis of the products of CUS from various starter substrates and [2-14C]malonyl-CoA.** *Lane* 1, 4-coumaryl-CoA (1a); *lane* 2, cinnamoyl-CoA (2a); *lane* 3, feruloyl-CoA (3a); *lane* 4, hexanoyl-CoA (4a); *lane* 5, dodecanoyl-CoA (5a); *lane* 6, octadecanoyl-CoA (6a); *lane* 7, 4-coumaryl-CoA (1a) and cinnamoyl-CoA (2a); *lane* 8, 4-coumaryl-CoA (1a) and feruloyl-CoA (3a); *lane* 9, 4-coumaryl-CoA (1a) and hexanoyl-CoA (4a); and *lane* 10, 4-coumaryl-CoA (1a) and dodecanoyl-CoA (5a).
In Vitro Curcuminoid Synthesis by Type III PKS

small amount of dicinnamoylmethane (2e) (Fig. 2, lane 2, and supplemental Fig. 2), whereas feruloyl-CoA (3a) yielded no products (Fig. 2, lane 3). The expected products, the triketide pyrone (3c) and curcumin (3e), were detectable only by LC-MS analysis when the reaction volume was scaled up by 5-fold (supplemental Fig. 2). A competition experiment between 4-coumaroyl-CoA and cinnamoyl-CoA yielded both 1e and 2c (Fig. 2, lane 7). Judging from almost the same radioactivity incorporated into 1e and 2c, we supposed that CUS preferred 4-coumaroyl-CoA 2-fold to cinnamoyl-CoA; 2c incorporates two [2-14C]malonyl-CoA molecules and 1e incorporates one molecule. Interestingly, in addition to these products, a very small amount of an asymmetric curcuminoid, cinnamoyl-4-coumaroylmethane, derived from 4-coumaroyl-CoA and cinnamoyl-CoA, was detected by LC-MS analysis (supplemental Fig. 3). A similar competition experiment between 4-coumaroyl-CoA and feruloyl-CoA yielded 1e, indicating that feruloyl-CoA is a poor substrate for CUS (Fig. 2, lane 8). A negligible amount of an asymmetric curcuminoid, 4-coumaroylferuroylmethane, was also detected by LC-MS analysis (supplemental Fig. 3). These results suggested that CUS prefers 4-coumaroyl-CoA (1a) among the typical CoA esters derived from the phenylpropanoid pathway.

Enzymatic Properties of CUS—We determined the temperature and pH dependence of CUS for bisdemethoxycurcumin (1e) synthesis. CUS showed a temperature optimum at 45 °C (Fig. 3A). The velocity of 1e formation was maximal around pH 7.0, whereas that for 1c was maximal around pH 6.5 (Fig. 3B). Furthermore, the ratio of 1c/1e differed depending on the pH; an acidic pH increased the rate of pyrone production, whereas an alkaline pH decreased it. The pH dependence of CUS resembles that of BAS; BAS preferentially produces a triketide pyrone under acidic conditions and benzalacetone under alkaline conditions (18). The production ratio of 1c/1e by CUS was also affected by the concentrations of the substrates. When 50 μM 4-coumaroyl-CoA (1a) and 5 μM malonyl-CoA were incubated with CUS, only 1e was detected by HPLC analysis (data not shown). Conversely, 5 μM 4-coumaroyl-CoA (1a) and 50 μM malonyl-CoA yielded only 1c (data not shown).

We next examined the courses of production of bisdemethoxycurcumin (1e) and the triketide pyrone (1c) (Fig. 3C). During the CUS reaction starting with 4-coumaroyl-CoA (1a) and malonyl-CoA (at 5 μM each), the velocity of bisdemethoxycurcumin (1e) synthesis changed and did not become constant; CUS produced bisdemethoxycurcumin (1e) slowly during the early stage of the reaction, for about 10 to 20 min after the reaction initiation, and the production rate became gradually higher thereafter until 40 min. Instead, the velocity of triketide pyrone (1c) synthesis became constant at the early stage of the reaction and the production rate became gradually higher thereafter until 40 min. Instead, the velocity of triketide pyrone (1c) became the major product. The change in the production rate of bisdemethoxycurcumin (1e) during the reaction hampered us in determining the kinetic parameters of CUS. This finding suggested that diketo-CoA (1b) accumulation is important for bisdemethoxycurcumin (1e) synthesis, as discussed later. We also found that the C174S mutant of CUS possessed no PKS activity (data not shown), suggesting that Cys-174 functions as a catalytic center to anchor acyl intermediates, as is the case for other type III PKSs (16).
In Vitro Curcuminoid Synthesis by Type III PKS

Mechanism of the CUS Reaction—According to previous tracer studies (4), CUS would catalyze the condensation of malonyl-CoA with 4-coumaroyl-CoA (1a) to produce a diketide-CoA (1b) (step i in Fig. 1A), hydrolysis of 1b to 5-(4-hydroxyphenyl)-3-oxo-pent-4-enolic acid (1d) (Fig. 1A, step ii), and the decarboxylative condensation of 1d with a second molecule of 4-coumaroyl-CoA (1a) (Fig. 1A, step iii). Consequently, CUS should use two starter substrates; the first condenses with malonyl-CoA to form 1d, whereas the second one condenses with 1d. Similarly, CUS should use two extender substrates; the first, malonyl-CoA, is used to produce 1d, whereas the second is 1d itself (Fig. 1A). To confirm this hypothesis and to obtain additional insight into the CUS reaction, we prepared two intermediates: cinnamoyl diketide-NAC (N-acetylcysteamine, an analogue for CoA) (2f) and 3-oxo-5-phenyl-pent-4-enolic acid (2d) (Fig. 4). We chose cinnamate-derived intermediates because they could be prepared more easily than those derived from 4-coumarate. As expected, CUS produced dicinnamoylmethane (2e) from cinnamoyl-CoA (2a) and cinnamoyl diketide-NAC (2f) (Fig. 4A and supplemental Fig. 4). In addition, CUS efficiently produced dicinnamoylmethane (2e) from 2d and cinnamoyl-CoA (2a) (Fig. 4B and supplemental Fig. 4). In addition, the reaction containing 2d produced more dicinnamoylmethane (2e) than that containing 2f. These results demonstrate that diketide intermediates are accepted as the second extender substrates, which suggests the involvement of enzymatic hydrolysis of a diketide intermediate in the CUS reaction. In contrast, incubation of cinnamic acid with 2d or 2f yielded no products (Fig. 4, C and D). This result excludes the unlikely possibility that cinnamic acid serves as an extender substrate for the diketide intermediate. In addition, thioester formation by the second starter substrate with Cys-174 was found to be essential for elongation of the second extender substrate because use of the C174S mutant in these reactions yielded no products (Fig. 4, A and B).

Substrate Specificity of CUS—As described above, 4-coumaroyl-CoA (1a), cinnamoyl-CoA (2a), and feruloyl-CoA (3a) serve as the first and second starter substrates in curcuminoind production. We next determined whether CUS accepts CoA esters of n-fatty acids as a starter substrate. Incubation of hexanoyl-CoA (4a) and malonyl-CoA yielded only a triketide pyrone (4c) (Fig. 2, lane 4, and supplemental Fig. 2). Dodecanoyl-CoA (5a) also yielded a triketide pyrone (5c) (Fig. 2, lane 5, and supplemental Fig. 2). No curcuminoid-like products derived from hexanoyl-CoA (4a) or dodecanoyl-CoA (5a) were detected even when an excess amount of these starter substrates was added. Therefore, neither hexanoyl-CoA (4a) nor dodecanoyl-CoA (5a) acts as second starter substrate, although they were incorporated as the first starter substrate to produce a diketide-CoA. Incubation of octadecanoyl-CoA (6a) with malonyl-CoA yielded no products (Fig. 2, lane 6). In addition, competition experiments with 4-coumaroyl-CoA (1a) suggested that CUS preferred 4-coumaroyl-CoA (1a) over hexanoyl-CoA (4a) and dodecanoyl-CoA (5a) as its first and second starter substrates (Fig. 2, lanes 9 and 10). Incubation of 4-coumaroyl-CoA (1a) with methylmalonyl-CoA or succinyl-CoA, instead of malonyl-CoA, in the presence of CUS yielded no products (data not shown).

Production of Cinnamoyl(hexanoyl)methane by CUS—Cinnamoyl(hexanoyl)methane (4e) (Fig. 5), an analogue of gingerol found in ginger (Zingiber officinale) (19), is composed of hexanoyl- and cinnamoyl-CoA-derived moieties and a single acetate. The structural similarity between 4e and the asymmetric curcuminoids described above prompted us to examine the ability of CUS to synthesize 4e. We first incubated hexanoyl-CoA (4a) and malonyl-CoA along with cinnamoyl-CoA (2a), but the reaction produced dicinnamoylmethane (2e) along with a triketide pyrone derived from cinnamoyl-CoA (2c) (data not shown). As described above, incubation of hexanoyl-CoA (4a) with malonyl-CoA yielded no symmetric curcuminoid-like compounds, although it yielded a triketide pyrone (4c) (Fig. 2, lane 4). These observations suggested that CUS relatively preferred cinnamoyl-CoA (2a) to hexanoyl-CoA (4a) as the first and second starter substrates. Cinnamoyl(hexanoyl)methane (4e) synthesis was achieved by incubation of cinnamoyl-CoA (2a) with either 3-oxo-octanoyl-NAC (4f) or 3-oxo-octanoic acid (4d) (Fig. 5 and supplemental Fig. 4), suggesting a relaxed specificity for the second extender substrate. Having a β-keto acid moiety in the second extender substrate may be sufficient to be recognized as a substrate by CUS. In contrast, hexanoyl-CoA (4a), when incubated with 4d or 2d, did not yield any products (data not shown), suggesting a strict preference for the second starter substrate. Thus, other enzyme system(s) may be
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FIGURE 5. Synthesis of asymmetric curcuminoids by CUS from diketide intermediates. The front chromatograms indicate the products of CUS, whereas the rear chromatograms indicate the products of the negative control (boiled enzyme). Cinnamoyl(hexanoyl)methane (4e) was produced by incubation of CUS with cinnamoyl-CoA (2a) and 3-oxo-octanoyl-NAC (4f) (A) and with cinnamoyl-CoA (2a) and 3-oxo-octanoic acid (4d) (B).

involved in preparing 3-oxo-octanoic acid (4d) or its acyl carrier protein/CoA esters in gingerol-producing *Z. officinale*.

**DISCUSSION**

The field of plant natural products has dramatically changed in recent years because of the advent of genomic sequencing. Instead of classical homology-based reverse transcription-PCR strategies using RNA pools, a BLAST search of a single data base allows one to identify a target gene. This strategy, sometimes called genome mining, (20), can uncover cryptic or untranslated genes that are missed by classical RNA-based methodologies. Using genome mining instead of a classical reverse genetic approach, we identified a novel type III PKS that catalyzes the synthesis of curcuminoids, polyphenols specific to Zingiberaceae. The *in vivo* function of CUS in rice is still unclear. However, we believe that the curcuminoids are biosynthesized in Zingiberaceae in a manner similar to the unique biosynthesis of the first extender substrate, malonyl-CoA, with 4-coumaroyl-CoA bound to Cys-174 (i.e. 4-coumaroyl-CoA bound to Cys-174) to produce bisdemethoxycurcumin (1e) (Fig. 1A). When another molecule of malonyl-CoA is added to the diketide intermediate in place of curcuminoid synthesis, CUS produces a triketide pyrone (1c) as a by-product.

Type III PKSs catalyze the sequential condensation of extender substrates onto a starter substrate in a head-to-tail fashion. CUS follows this head-to-tail rule to produce a diketide-CoA, which in turn is hydrolyzed to a β-keto acid as described above. However, CUS catalyzes the decarboxylative Claisen condensation of the resultant β-keto acid with the second starter substrate. Intramolecular condensation of a CoA-free intermediate is not uncommon in type III PKS reactions. For example, stilbene synthase cyclizes a tetraketide intermediate via an intramolecular aldol reaction (21). A unique feature of CUS is that the decarboxylative Claisen condensation of the β-keto acid occurs intermolecularly. It is interesting that CUS accepts two structurally distinct extender substrates, a β-keto acid and malonyl-CoA.

Interestingly, 1d is a common intermediate in both the CUS and BAS reactions. BAS synthesizes 4-hydroxybenzalacetone via decarboxylation of 1d from 4-coumaroyl-CoA (1a) and malonyl-CoA (Fig. 1B) (5). The difference between CUS and BAS is that the former decarboxylatively condenses a β-keto acid with a second starter substrate to yield a curcuminoid, whereas the latter simply decarboxylizes it to yield benzalacetone. On the basis of this proposed reaction mechanism, the CUS reaction should include a thioesterase activity to produce β-keto acids. Nevertheless, we could not detect β-keto acids (i.e. benzalacetone analogues) even when CUS was incubated with the diketide-NAC (2f) (data not shown). These observations raise a question as to how the cryptic thioesterase activity of CUS is controlled. It may be that the thioesterase activity of CUS toward its diketide intermediate emerges only in the presence of the second starter substrate. Furthermore, the hydrolysis of the diketide intermediate to yield the β-keto acid (1d) and condensation to yield bisdemethoxycurcumin (1e) may occur in concert without releasing the β-keto acid. Although the expected intermediates (i.e. the diketide intermediate and 1d) were not detected experimentally, we assumed that the diketide-CoA (1b) is once released from a CUS molecule and bound by another molecule of CUS, in which Cys-174 anchors a 4-coumarate moiety. Then, hydrolysis of the diketide-CoA and decarboxylative condensation of the β-keto acid produced results in the formation of bisdemethoxycurcumin (1e). This hypothesis could explain the course of production of bisdemethoxycurcumin (1e) and the triketide pyrone (1c), shown in Fig. 3B. According to this hypothesis, the velocity of bisdemethoxycurcumin (1e) production depends on the concentration of the diketide-CoA (1b). The early stage of the reaction is for accumulation of the diketide-CoA (1b). As the diketide-CoA accumulates in the solution, the velocity of bisdemethoxycurcumin (1e) synthesis becomes larger. On the other hand, the velocity of triketide pyrone synthesis was constant during the early stage of the reaction, which suggests that the triketide pyrone synthesis does not involve diketide-CoA release.
Recently, Austin et al. (21) reported a novel mechanism leading to the hydrolysis of a thioester, which in turn results in an aldol-type folded ring, on the basis of the crystal structure of stilbene synthase, a type III PKS responsible for stilbene synthesis. The thioesterase activity of stilbene synthase is also cryptic, and the hydrolyzed tetraketide intermediate is cyclized into stilbene without releasing the derailment product from the enzyme (21).

Curcuminoids and phenylphenalenones are characteristic compounds in Hemodorales (22, 23) and Zingiberales (24). The Zingiberales, which include turmeric, ginger, and banana, are closely related to Hemodorales (25). CUS is derived from rice O. sativa, which is phylogenetically distinct from Hemodorales and Zingiberales. To date, curcuminoids and phenylphenalenones have not been reported in O. sativa. CUS synthesizes curcuminoids in vitro under near-physiological conditions from 4-coumaroyl-CoA (1a) and malonyl-CoA, both of which are common in plants and the typical substrates for plant type III PKSs. Speculatively, the rice CUS produces curcuminoids but in undetectable amounts. Additional studies are apparently necessary to determine the exact in vivo function of CUS. It is notable that os07g17010 was identified from a panicle cDNA library constructed two weeks after flowering. An interesting question is whether CUS is found in Hemodorales and Zingiberales. Homology-based cloning using the CUS sequence will answer this question.

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