Phytochemistry 71 (2010) 1059–1067

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Alkylresorcylic acid synthesis by type III polyketide synthases from rice *Oryza sativa*

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ARTICLE INFO

Article history:
Received 19 May 2009
Received in revised form 8 January 2010
Accepted 9 February 2010
Available online 5 May 2010

Keywords:
Fatty acid
Oryza sativa
Polyketide synthase
Pyrone
Resorcinol
Resorcylic acid

ABSTRACT

Alkylresorcinols, produced by various plants, bacteria, and fungi, are bioactive compounds possessing beneficial activities for human health, such as anti-cancer activity. In rice, they accumulate in seedlings, contributing to protection against fungi. Alkylresorcylic acids, which are carboxylated forms of alkylresorcinols, are unstable compounds and decarboxylate readily to yield alkylresorcinols. Genome mining of the rice *Oryza sativa* identified two type III polyketide synthases, named ARAS1 (alkylresorcylic acid synthase) and ARAS2, that catalyze the formation of alkylresorcylic acids. Both enzymes condensed fatty acyl-CoAs with three C_2 units from malonyl-CoA and cyclized the resulting tetraketide intermediates via intramolecular C-2 to C-7 aldol condensation. The alkylresorcylic acids thus produced were released from the enzyme and decarboxylated non-enzymatically to yield alkylresorcinols. This is the first report on a plant type III polyketide synthase that produces tetraketide alkylresorcylic acids as major products.

1. Introduction

Alkylresorcinols are amphiphilic compounds distributed widely in plants, bacteria, and fungi (Kozubek and Tyman, 1999). Their major function in plants is thought to be protection against pathogenic fungi (García et al., 1997; Cojocaru et al., 1986). In addition, some health benefits of alkylresorcinols including anti-cancer effects have also been elucidated (Kozubek and Tyman, 1999; Ross et al., 2004). Alkylresorcylic acids, carboxylated forms of alkylresorcinols, rarely occur in the free state except, for example, in *Ginkgo biloba* (Gellerman et al., 1976) and *Ononis speciosa* (Barrero et al., 1989). Suzuki et al. (1996a) identified a mixture of alkylresorcinols in the etiolated seedling of the Indica-type rice (*Oryza sativa*) as antifungal agents. The alkylresorcinol concentrations in the etiolated rice seedlings reached a level comparable to the ED_{50} value, which suggested that alkylresorcinols confer resistance against fungi on its seedlings (Suzuki et al., 1996b). They also found that various types of rice, including the Indica- and Africa-type cultivars, accumulate alkylresorcinols, but the Japonica-type cultivars do not (Suzuki et al., 1996b). They assumed that Japonica-type rice lost the ability for alkylresorcinol accumulation potential during breeding. Although alkylresorcinols were first identified and are widely distributed in plants (Kozubek and Tyman, 1999), alkylresorcinol synthases in plants have not yet been identified. Dayan et al. (2007) reported that a root hair extract of *Sorghum bicolor* exhibited alkylresorcinol synthase activity, but the enzyme system responsible for the synthesis was not characterized. Recently, several type III PKSs from plants which catalyze alkylresorcinol formation, olivetol synthase (OLS) from *Cannabis sativa* (Taura et al., 2009), type III PKS involved in sorgoleone biosynthesis (Cook et al., 2007) and PpCHS1 from *Physcomitrella patens* (Jiang et al., 2008), were described.

Type III PKSs are structurally simple biosynthetic enzymes found in plants, bacteria, and fungi (Austin and Noel, 2003; Funa et al., 2006; Ferrer et al., 1999). They consist of small, homodimeric, ketosynthase that use CoA esters as substrates and synthesize polyketides by using a single catalytic center. Chalcone synthases (CHSs), which is representative of type III PKSs in plants, catalyze synthesis of naringenin chalcone by condensing one *p*-coumaroyl-CoA with three malonyl-CoAs and catalyzing a C-6 to C-1 Claisen condensation (Ferrer et al., 1999). Naringenin chalcone is a common precursor of flavonoids produced by plants. However, there are many type III PKSs whose starter and extender substrate specificity, the number of condensed extender substrate, and the mode of the ring closure reaction, are different. Many of the bioactive polyketides in plants such as stilbenoids and flavonoids are synthesized by type III PKSs (Austin and Noel, 2003).

Abbreviations: APCIMS, atmospheric pressure chemical ionization mass spectrometry; ARAS, alkylresorcylic acid synthase; ArsB, alkylresorcinol synthase B from *Azotobacter vinelandii*; CHES, *N*-cyclohexyl-2-aminoethanesulfonic acid; CUS, curcuminoid synthase from rice; EST, expressed sequence tag; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; OLS, olivetol synthase; ORAS, 2′-oxoalkylresorcylic acid synthase from *Neurospora crassa*; PKS, polyketide synthase; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; SOE, splicing by overlap extension; TLC, thin layer chromatography.

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0031-9422/$ - see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.phytochem.2010.02.012

In bacteria and fungi, some type III PKSs responsible for alkylresorcinol synthesis have been reported. We found that alkylresorcinol synthase B (ArsB) in the gram-negative bacterium *Azotobacter vinelandii* (Funa et al., 2006), which belongs to the type III PKS superfamily, catalyzes synthesis of alkylresorcinols. *In vitro* analysis showed that ArsB releases no alkylresorcylic acids, presumable intermediates to alkylresorcinols. On the other hand, 2′-oxoalkylresorcylic acid synthase (ORAS) in the fungus *Neurospora crassa* synthesized 2′-oxoalkylresorcylic acid as a major product (Funa et al., 2007). 2′-Oxoalkylresorcylic acids are readily decarboxylated non-enzymatically to yield 2′-oxoalkylresorcinol. It is expected that previously reported plant type III PKSs which synthesize alkylresorcinols catalyzes similar reaction to ArsB as they do not synthesize alkylresorcylic acids.

This report deals with two type III PKSs in the rice *O. sativa*, which synthesize alkylresorcylic acids from fatty acyl-CoAs and malonyl-CoA. A BLAST search of the Japonica-type rice genome (Goff et al., 2002) using the CHS sequence in *Medicago sativa* as the query, predicted 31 type III PKS candidates. We have continued to demonstrate the catalytic properties of the enzyme candidates

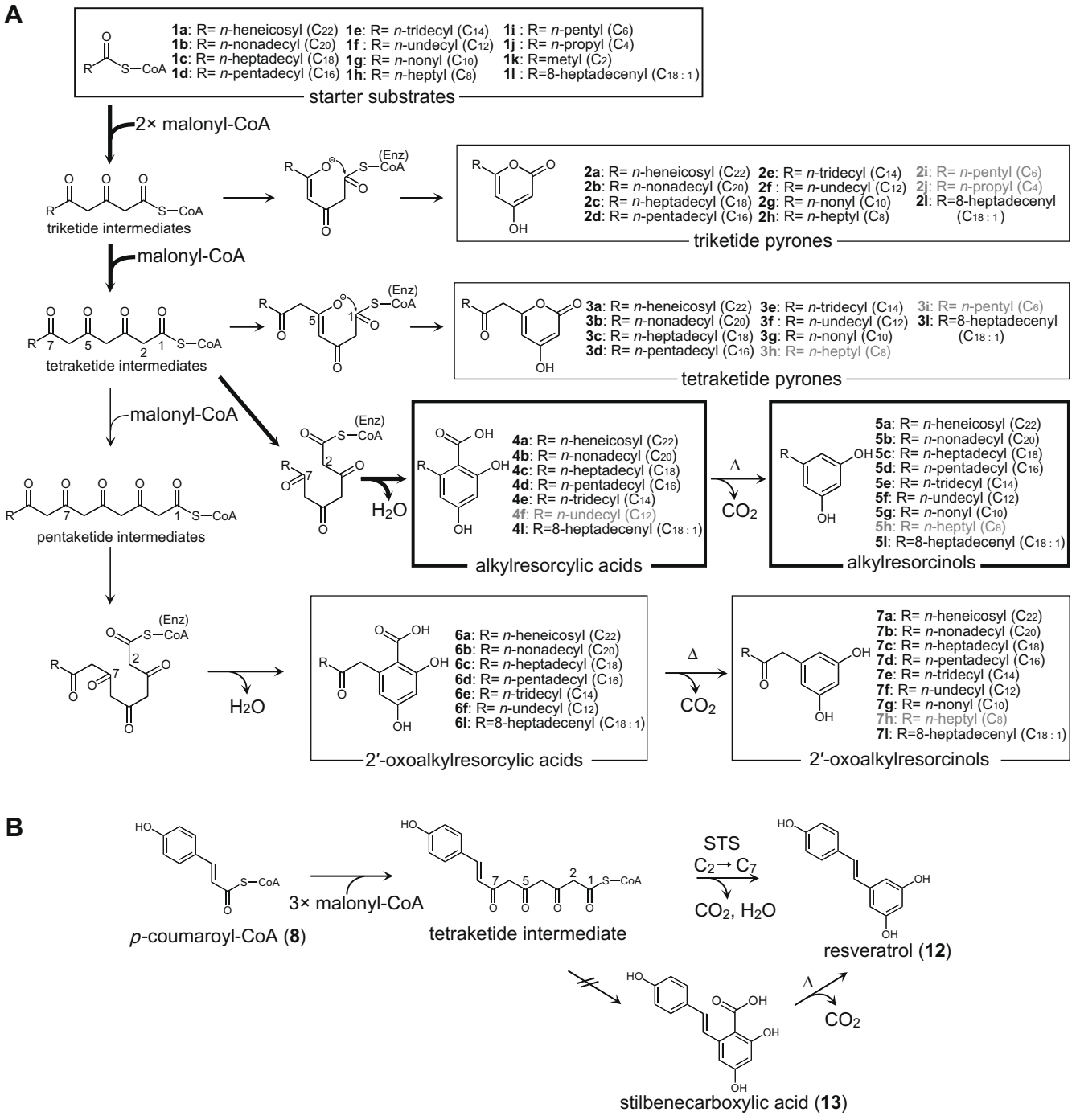

Fig. 1. Summary of the reactions catalyzed by ARAS1 and ARAS2 (A) and STS (B). Non-enzymatic decarboxylation of unstable alkylresorcylic acids (**4a**–**4f**, **4l**), resulting in the formation of the corresponding alkylresorcinols (**5a**–**5h**, **5l**) (A). Products synthesized only by ARAS2 are shown in *gray*. The main pathway is shown by bold arrows. STS synthesizes stilbene (**12**) from one *p*-coumaroyl-CoA (**8**) and three malonyl-CoAs (B). Stilbenecarboxylic acid (**13**) has been shown not to be an intermediate in the STS-catalyzed reaction (Austin et al., 2004a).

one by one and recently characterized one of these enzymes as a curcuminoid synthase (CUS) able to synthesize the curcuminoid scaffold by a mechanism uncommon to the type III PKSs (Katsuyama et al., 2007). Further *in vitro* analysis of the putative type III PKSs identified two gene products, Os10g07040 (alkylresorcylic acid synthase, ARAS1) and Os10g08620 (ARAS2), as enzymes that catalyzed formation of alkylresorcylic acids by condensing three malonyl-CoAs with fatty acyl-CoAs and catalyzing C-2 to C-7 intramolecular aldol condensation. The resultant alkylresorcylic acids were non-enzymatically decarboxylated to yield alkylresorcinols. This is the first report on alkylresorcylic acid synthases from plants. These results suggest that alkylresorcinols found in a wide variety of plants might be synthesized from two pathways; alkylresorcinols might be synthesized by type III PKSs which synthesizes alkylresorcinols, such as olivetol synthase, or via non-enzymatic decarboxylation of the corresponding alkylresorcylic acids synthesized by type III PKSs.

2. Results and discussions

2.1. Characterization of ARAS1 and ARAS2

Our BLAST search against the Japonica-type rice genome databases using the CHS amino acid sequence in *M. sativa* as the query (Ferrer et al., 1999) predicted 31 candidate genes that encode a type III PKS homologue. To characterize the function of these proteins, we carried out *in vitro* experiments by using recombinant proteins produced by *Escherichia coli* and probable substrates, malonyl-CoA as an extender and various CoA esters as a starter. cDNAs of these type III PKS homologues were cloned by using the method of splicing by overlap extension (SOE) PCR (Horton et al., 1989) and overexpressed in *E. coli* BL21 (DE3) to produce recombinant proteins fused with a His-tag at their N-termini by using the pET system. The proteins produced in this way were purified by Ni-NTA affinity column chromatography to give a major protein band on SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Supplementary Fig. 1). The purified enzymes were then incubated with malonyl-CoA and various starter CoA esters that are known to be used by type III PKSs. The starter substrates included *p*-coumaroyl-CoA (**8**), cinnamoyl-CoA (**9**), dihydrocinnamoyl-CoA (**10**), benzoyl-CoA (**11**), and fatty acyl-CoAs (**1a**–**1l**). Products were determined by spectrometric analysis and radio-thin layer chromatography (TLC) analysis. As a result of this screening assay, two type III PKS candidates encoded by *os10g07040* and *os10g08620* were found to catalyze the formation of alkylresorcylic acids from fatty acyl-CoAs and malonyl-CoA (Fig. 1A). Therefore, these type III PKSs were named ARAS1 and ARAS2 (alkylresorcylic acid synthase) from their functions.

Phylogenetic tree analysis of the 31 type III PKS homologues in the rice showed that ARAS1 and ARAS2 were in the same clade, along with *os10g08670* and *os10g08680* (Supplementary Fig. 2). Our repeated attempts to obtain the Os10g08670 in the soluble fraction of *E. coli* failed, and we were unable to analyze this protein further. The Os10g08680 protein was predicted to be about 15 kDa in size from its nucleotide sequence and was smaller than the half size of other known type III PKSs. Therefore, Os10g08680 would be a pseudogene.

ARAS1 and ARAS2 shared 84% amino acid identity with each other. ARAS1 has 49% amino acid identity with the CHS in *M. sativa* (Ferrer et al., 1999), 46% with the stilbene synthase (STS) in *Pinus sylvestris* (Austin et al., 2004a), 48% with OLS in *C. sativa*, 14% with ArsB in *A. vinelandii* (Funa et al., 2006), and 23% with ORAS in *N. crassa* (Funa et al., 2007). ARAS2 shared 49% amino acid identity with the *M. sativa* CHS, 47% with the *P. sylvestris* STS, 48% with the *C. sativa* OLS, 14% with *A. vinelandii* ArsB, and 24% with *N. crassa* ORAS. A homologue of ARAS1 was found in the Indica-type rice genome whose amino acid identity was 97% (Yu et al., 2002). In addition, both ARAS1 and ARAS2 have a conserved catalytic triad composed of Cys-His-Asn, which is crucial for the decarboxylative condensation activity of all type III PKSs (Austin and Noel, 2003) (Supplementary Fig. 3).

2.2. In vitro analysis of ARAS1 and ARAS2 reactions

We tested whether ARAS1 and ARAS2 could accept C_2 to C_{22} straight-chain fatty acyl-CoAs (**1a**–**1k**) as starter substrates, because already-known alkylresorcinol synthases like ArsB use various long-chain fatty acyl-CoAs as starter substrates (Funa et al., 2006). Products were analyzed by radio-TLC (Fig. 2) and liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC–APCIMS) analysis (Supplementary Table 1). [2-^{14}C]Malonyl-CoA was used for TLC analysis, instead of nonlabeled malonyl-CoA, as an extender substrate to visualize the reaction products. As a result, both ARAS1 and ARAS2 yielded alkylresorcinols (**5a**–**5h**), alkylresorcylic acids (**4a**–**4f**), triketide pyrones (**2a**–**2j**), and trace amounts of 2′-oxoalkylresorcylic acids (**6a**–**6f**), 2′-oxoalkylresorcinols (**7a**–**7h**), and tetraketide pyrones

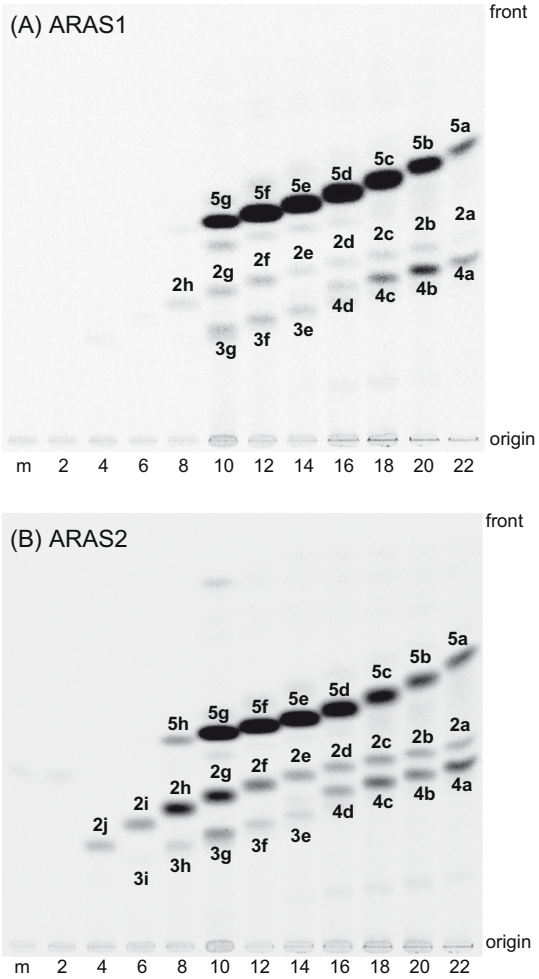

Fig. 2. Radio-TLC analysis of the products synthesized by ARAS1 (A) and ARAS2 (B) from various acyl-CoA starter substrates (**1a**–**1k**) and [2-^{14}C]malonyl-CoA. Starter substrates used were acetyl-CoA (**1k**) (*lane 2*), butyryl-CoA (**1j**) (*lane 4*), hexanoyl-CoA (**1i**) (*lane 6*), octanoyl-CoA (**1h**) (*lane 8*), decanoyl-CoA (**1g**) (*lane 10*), lauroyl-CoA (**1f**) (*lane 12*), myristoyl-CoA (**1e**) (*lane 14*), palmitoyl-CoA (**1d**) (*lane 16*), stearoyl-CoA (**1c**) (*lane 18*), arachidoyl-CoA (**1b**) (*lane 20*), and behenoyl-CoA (**1a**) (*lane 22*). *Lane m* is a control incubation without a starter substrate.

(**3a**–**3i**) from a broad range of CoA esters (**1a**–**1j**) (Fig. 2). The difference between these two enzymes was their preference to the chain length of the acyl-CoAs. ARAS1 preferred relatively longer chain fatty acyl-CoAs (**1c**–**1f**), where ARAS1 displayed a preference to CoAs of C_{12} to C_{18} (**1c**–**1f**), and ARAS2 preferred CoAs of C_{10} to C_{14} (**1g**–**1e**).

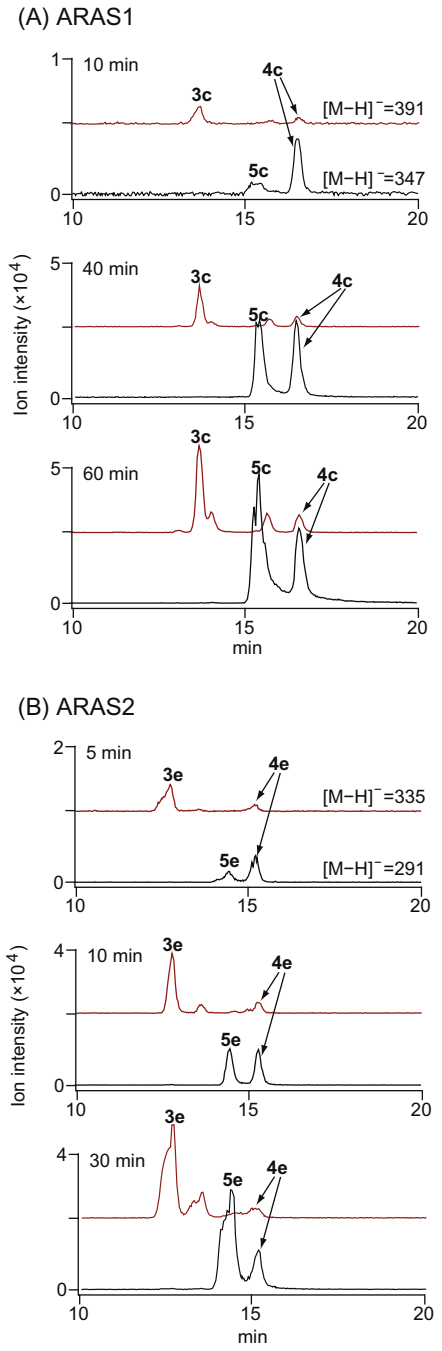

Fig. 3. Time course of alkylresorcylic acids (**4c** and **4e**) formation by ARAS1 (A) and ARAS2 (B). The molecular masses of **3c**, **3e**, **4c**, **4e**, **5c**, and **5e** are 392, 336, 392, 336, 348, and 292, respectively. The *black line*, representing alkylresorcinols (**5c** and **5e**), and the *red line*, representing alkylresorcylic acids (**4c** and **4e**) and tetraketide pyrones (**3c** and **3e**), indicate extracted ion chromatograms of negative LC–APCIMS at m/z = 391 (**3c**), 335 (**3e**), 391 (**4c**), 335 (**4e**), 347 (**5c**), and 291 (**5e**). Because of the instability of **4c** and **4e**, decarboxylation of **4c** and **4e** to give **5c** and **5e** occurred at the ionization step of mass spectrometry after they had been separated by HPLC and therefore **4c** and **4e** are observed in both chromatograms, black and red lines. Thus, the peaks of alkylresorcinols (**5c** and **5e**) were detected with the peaks of alkylresorcylic acids (**4c** and **4e**).

In addition, we checked whether ARAS1 and ARAS2 could produce resveratrol (**12**), which is synthesized from *p*-coumaroyl-CoA (**8**) and malonyl-CoA through the same reaction as in alkylresorcinol synthesis; resveratrol (**12**) is synthesized via tetraketide formation and its C-2 to C-7 aldol condensation (Fig. 1B). Incubation of ARAS1 or ARAS2 in the presence of *p*-coumaroyl-CoA (**8**) and malonyl-CoA yielded no products (data not shown). These results clearly showed that these enzymes are distinct from STS.

There are two types of type III PKSs, represented by ArsB and ORAS, that are responsible for alkylresorcinol synthesis. Fig. 5 shows presumed synthetic pathways of the resorcinol ring by ArsB and ORAS (Funa et al., 2007). After condensation of a fatty acyl-CoA with three malonyl-CoAs, ArsB hydrolyzes the thioester of the tetraketide intermediate before aldol condensation, and the decarboxylation of C-1 carboxyl group occurs along with aldol

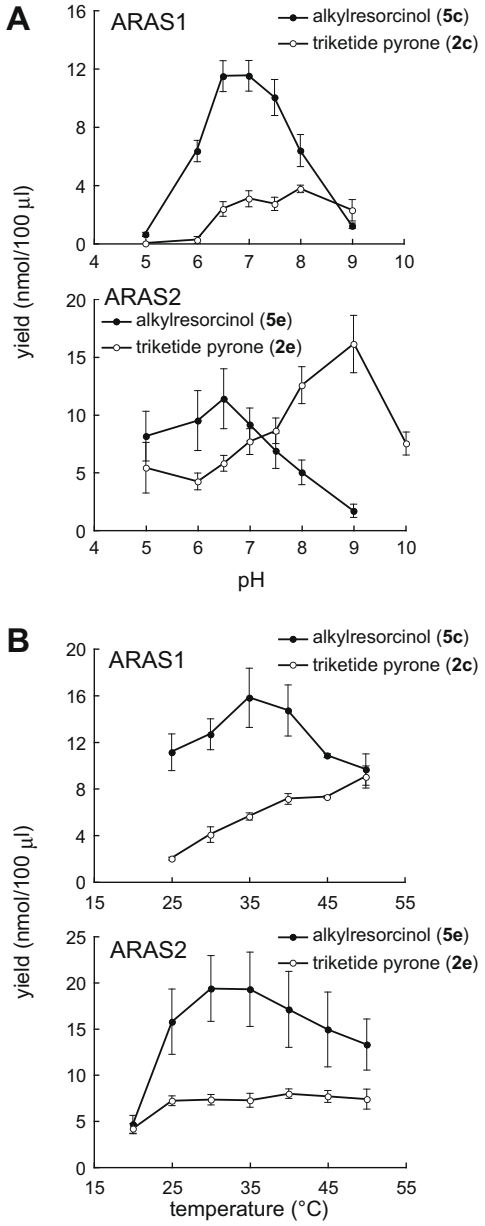

Fig. 4. The pH dependence (A) and temperature dependence (B) of ARAS1 and ARAS2. ARAS1 was incubated with stearoyl-CoA (**1c**) and malonyl-CoA. ARAS2 was incubated with myristoyl-CoA (**1e**) and malonyl-CoA. The yields of alkylresorcinols (**5c** and **5e**) and triketide pyrones (**3c** and **3e**) are plotted in *closed circles* and *open circles*, respectively.

condensation and aromatization. As a result, ArsB mainly produces alkylresorcinols and does not synthesize alkylresorcylic acids. On the other hand, ORAS catalyzes aldol condensation and aromatization before hydrolysis of the thioester, and therefore the C-1 carboxyl group remains in the product. Thus, ORAS synthesizes alkylresorcylic acids as the direct product. Then alkylresorcylic acids are readily decarboxylated non-enzymatically and converted to alkylresorcinols. For elucidation of the reaction mechanism of ARAS1 and ARAS2, we examined the product profile dependence on reaction time. In addition, as the long-chain alkylresorcylic acids were readily decarboxylated at room temperature or by heat, we kept the quenched reaction product on ice to avoid non-enzymatic decarboxylation. Stearoyl-CoA (**1c**) (for ARAS1) and myristoyl-CoA (**1e**) (for ARAS2) were used as representative starter CoAs because they seemed to be the most reactive starter substrates as roughly estimated by the radio-TLC analyses (Fig. 2). LC–APCIMS analysis of the reaction products showed that alkylresorcylic acids were the main products of the ARAS1 and ARAS2 reactions during an early period of the reaction (Fig. 3). However, prolonged reactions result in a decrease of alkylresorcylic acids (**4c** and **4e**) and in an increase in the amount of the alkylresorcinols (**5c** and **5e**). Because of the chemical instability of alkylresorcylic acids (**4c** and **4e**), decarboxylation of **4c** and **4e** to give alkylresorcinols (**5c** and **5e**) occurred at the ionization step of mass spectrometry after they had been separated by HPLC. We therefore concluded that the direct products of ARAS1 and ARAS2 were probably alkylresorcylic acids (**4c** and **4e**). Alkylresorcinols (**5c** and **5e**) produced by the reaction of these enzymes result from non-enzymatic decarboxylation of the corresponding alkylresorcylic acids (**4c** and **4e**). However, alkylresorcylic acids (**4h** and **4g**) were not detected when octanoyl-CoAs (**1h**) and decanoyl-CoA (**1g**) were used as starter substrates. These results suggested that ARAS1 and ARAS2 produced alkylresorcinols directly from fatty acyl-CoAs and malonyl-CoA without synthesizing alkylresorcylic acids when the acyl chain of the starter substrate is shorter than lauroyl-CoA. In other words, the reaction mechanism of cyclization might change when a short-chain fatty acyl-CoAs used as a starter substrate.

2.3. Enzymatic properties of ARAS1 and ARAS2

Since alkylresorcylic acids were decarboxylated readily to yield alkylresorcinols during the reaction, extraction and evaporation, it was rather difficult to quantify the alkylresorcylic acids. Therefore, we intentionally decarboxylated the products by exposing the samples to a high temperature, approximately 45 °C, for 30 min and quantified the alkylresorcinols to analyze the enzymatic properties of ARAS1 and ARAS2. It is known that β-resorcylic acids can be decarboxylated non-enzymatically by heat (Clark, 1965). However, alkylresorcylic acids were less stable and decarboxylated at lower temperatures than β-resorcylic acid.

We determined the temperature and pH dependence of ARAS1 and ARAS2 for alkylresorcinol synthesis using stearoyl-CoA (**1c**) and myristoyl-CoA (**1e**), respectively. ARAS1 showed a temperature optimum at 35 °C and pH optimum around 7.0, whereas ARAS2 showed a temperature optimum at 30 °C and pH optimum around 6.5 (Fig. 4). Furthermore, the ratio of alkylresorcinol/triketide pyrone production by ARAS1 and ARAS2 differed depending on pH; the rate of triketide pyrone synthesis increased in alkaline pH. This indicates that the number of condensation reactions increase under acidic conditions. A similar catalytic property was also observed for the benzalacetone synthase (BAS) from *Rheum palmatum* (Abe et al., 2003) and CUS from *O. sativa* (Katsuyama et al., 2007).

We measured the kinetic parameters of ARAS1 and ARAS2 for alkylresorcinols synthesis by varying the concentration of starter CoAs (**1c**, **1d**, **1e** and **1l**). We predicted the *in vivo* substrate utilized by ARASs according to the composition of alkylresorcinols and fatty acids in the etiolated rice seedlings of the Indica-type rice (Suzuki et al., 2003). Extracts from the rice mainly contained alkylresorcinols (**5e**, **5d**, **5d** and **5l**) synthesized from myristoyl, palmitoyl, stearoyl, oleoyl-CoAs (**1e**, **1d**, **1c**, and **1l**, respectively). Among them, the alkylresorcinols (**4l**) from oleoyl-CoA (**1l**) was

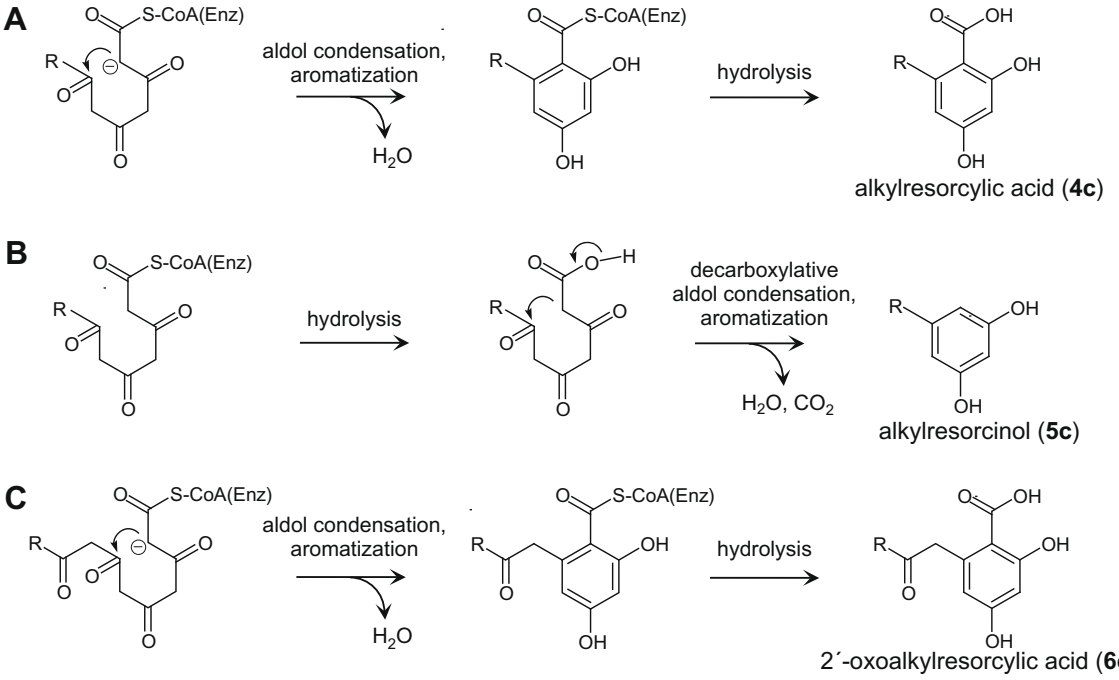

Fig. 5. A proposed mechanism of the reaction catalyzed by ARAS1 and ARAS2 (A), ArsB (B), and ORAS (C). Hydrolysis of the thioester takes place after aldol condensation and aromatization, and therefore the carboxyl group remains on the product (A and C). Hydrolysis of the thioester takes place firstly, and then decarboxylative aldol condensation and aromatization take place to form alkylresorcinols (B).

Table 1
Steady-state kinetic parameters of the ARAS1 and ARAS2 reactions.

	1e: myristoyl-CoA (C14)			**1d**: palmitoyl-CoA (C16)			**1c**: stearoyl-CoA (C18)			**1l**: oleoyl-CoA (C18: 1)		
	K_m (μM)	k_{cat} ($\times 10^{-4}$ s^{-1})	k_{cat}/K_m ($\times s^{-1}$ M^{-1})	K_m (μM)	k_{cat} ($\times 10^{-4}$ s^{-1})	k_{cat}/K_m ($\times s^{-1}$ M^{-1})	K_m (μM)	k_{cat} ($\times 10^{-4}$ s^{-1})	K_{cat}/K_m ($\times s^{-1}$ M^{-1})	K_m (μM)	k_{cat} ($\times 10^{-4}$ sec^{-1})	k_{cat}/K_m ($\times s^{-1}$ M^{-1})
ARAS1	10.7 ± 2.41	7.18 ± 0.77	67.4 ± 8.64	18.3 ± 4.23	10.3 ± 2.21	56.3 ± 8.70	9.53 ± 4.01	8.37 ± 1.66	87.9 ± 32.3	17.0 ± 2.21	9.07 ± 0.90	53.4 ± 8.48
ARAS2	106 ± 59.0	240 ± 111	226 ± 15.7	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.

Results are mean ± S.E. (n = 3). n.d., not determined.

the most abundant component, reaching 50% of the total. However, the most abundant fatty acid in the etiolated rice seedlings was palmitic acid (**0d**). Thus the alkylresorcinol synthase in the rice could prefer oleoyl-CoA (**1l**). Since ARAS1 preferred lauroyl-(C_{12}) to stearoyl-CoAs (C_{18}) and did not accept short-chain fatty acyl-CoAs, we assumed that the substrate preference of ARAS1 reflected the alkylresorcinols composition *in vivo*.

To clarify whether or not the substrate preference of ARAS1 reflects the *in vivo* alkylresorcinol composition, we measured kinetic parameters of ARAS1 for alkylresorcinols synthesis from myristoyl, palmitoyl, stearoyl, oleoyl-CoAs (**1e**, **1d**, **1c**, **1l**). In the case when palmitoyl-CoA (**1d**) as a starter substrate, substrate inhibition was observed and the activity decreased when substrate concentration was higher than 8 μM (data not shown). Contrary to our expectations, however, the k_{cat}/K_m value for alkylresorcinol synthesis was highest (88 s^{-1} M^{-1}) when C_{18}-CoA (**1c**) was used, and the k_{cat}/K_m values were not significantly different among myristoyl, palmitoyl, stearoyl, and oleoyl-CoAs (**1e**, **1d**, **1c**, and **1l**, respectively) (Table. 1). Kinetic parameters of ARAS2 from myristoyl-CoA (**1e**) were estimated in the same way. The k_{cat}/K_m value of ARAS2 was 226 s^{-1} M^{-1}, about 2.5-fold higher than that of ARAS1.

Determination of kinetic parameters for triketide pyrone synthesis was hampered because the yields of triketide pyrones were not detectable under these reaction conditions, in which an excess amount of malonyl-CoA was contained. This could be interpreted to mean that the elongation reaction would be promoted when the concentration of the extender substrate (malonyl-CoA) was higher than that of starter substrates. Therefore, the ratio of triketide pyrones became much lower than that of the alkylresorcinols (tetraketides). A similar property was also observed for CUS (Katsuyama et al., 2007). CUS mainly produces a triketide pyrone, not a curcuminoid, when the concentration of malonyl-CoA is higher than that of the starter substrate.

2.4. Insight into the activity of ARAS1 and ARAS2

Two type III PKSs found on the Japonica-type rice genome sequence, ARAS1 and ARAS2, were established to catalyze the synthesis of alkylresorcylic acids (**4a**–**4f**) from long-chain fatty acyl-CoAs (**1a**–**1f**) as starter substrates and malonyl-CoA as an extender substrate. The mechanism of the resorcinol ring formation by ARAS1 and ARAS2 seems to resemble that catalyzed by ORAS (Funa et al., 2007). Thus, the reactions catalyzed by ARAS1 and ARAS2 can be summarized as shown in Fig. 5. After condensation of one fatty acyl-CoA and three malonyl-CoAs, the C-2 to C-7 aldol condensation and aromatization occurs before the thioester bond cleavage between C-1 and the active site cysteine (or CoA), therefore, the carboxyl group remains on the product (Fig. 5). Then the alkylresorcylic acids produced were released from the enzyme and decarboxylated non-enzymatically to yield alkylresorcinols. This synthetic pathway is crucially different from that catalyzed by ArsB, STS, OLS, type III PKS involved in sorgoleone biosynthesis and PpCHS1. The C-2 to C-7 aldol condensation catalyzed by these enzymes precedes thioester bond cleavage, and the aldol condensation and aromatization that occur accompanied by decarboxylation because alkylresorcylic acids and stilbenecarboxylic acid (**13**) were not detected in the reaction products (Funa et al., 2006; Austin et al., 2004a; Cook et al., 2007; Jiang et al., 2008; Taura et al., 2009).

ARAS1 and ARAS2 catalyze a similar cyclization including aldol condensation with ORAS. However, the former two gave alkylresorcylic acids (**4a**–**4f** and **4l**) as major products, being distinct from the latter that gave 2′-oxoalkylresorcylic (**6c**) acids as major products. In other words, the difference between ARASs and ORAS is the number of malonyl-CoA condensation that occur. It is known that the size of active-site cavity of type III PKSs is important for the control of the number of malonyl-CoA condensation (Morita et al., 2007). That is because a larger active-site can keep a longer polyketide intermediate. Therefore, we assume that ARASs have a smaller active-site than ORAS.

A hydrophobic tunnel, which accounts for binding of long-chain aliphatic substrates, is present in the structure of PKS18 (Sankaranarayanan et al., 2004), ORAS (Goyal et al., 2008), and other type III PKSs (Austin et al., 2004b), all of which accommodate long-chain fatty acyl-CoAs as starter substrates. A similar tunnel may also exist in ARAS1 and ARAS2. ARAS1 formed a resorcinol ring only from decanoyl to behenoyl-CoAs (**1a**–**1g**), and ARAS2 formed it from octanoyl to behenoyl-CoAs (**1a**–**1h**). These results imply that ARASs catalyze condensation of three malonyl-CoAs and C-2 to C-7 intramolecular aldol condensation only when an appropriate fatty acyl-moiety of the starter substrate is accommodated in the hydrophobic tunnel. In addition, alkylresorcylic acids (**4a**–**4f**) were detected only when lauroyl to behenoyl-CoAs (**1a**–**1f**) were used as starter substrates. Similar results were also observed in analysis of ORAS (Funa et al., 2007); 2′-oxoalkylresorcylic acids (**6a**, **6b**, **6c**) were detected only when acyl-CoAs whose alkyl chains are longer than stearoyl-CoA (**1c**) were incorporated. This may be due to a relatively weak interaction of a short acyl-CoA with the putative hydrophobic tunnel. This weak interaction might change the order of the reaction. The cleavage of the thioester bond might occur prior to the C-2 to C-7 aldol condensation in this case. As *os10g08670* belongs to the same clade with in ARAS1 and ARAS2, the protein encoded by this gene or its ancestor might also catalyze formation of alkylresorcylic acids.

Although ARAS1 and ARAS2 were very similar in the primary sequence and catalytic function, their substrate specificities were slightly different; ARAS2 accepted shorter chain fatty acyl-CoAs and mainly produced triketide pyrones from them. As in the case of PKS18 and its mutants (Sankaranarayanan et al., 2004), a slight difference in the size of the hydrophobic tunnel may result in the difference in the preferred chain length of the starter CoA.

ARAS1 did not particularly prefer oleoyl-CoA (**1l**), which is thought to be used most *in vivo*. However, the substrate specificity of ARAS1 regarding the chain lengths of starter-CoAs virtually reflects the side-chain length of alkylresorcinols abundant in the etiolated seedling of the Indica-type rice. The composition of alkylresorcinols might be regulated by the substrate specificity of fatty acid CoA ligase, which catalyzes fatty acyl-CoA formation.

The genome sequence of the Indica-type rice genome shows that the genome of this type of rice encode 23 type III PKS homo-

logues (Supplementary Fig. 5). ARAS1 belongs to the same clade with EAY77763, EAY94420 and EAY94419. EAY77763, EAY94420 and EAY94419 shared 97%, 96% and 94% amino acid identities with ARAS1 and 85%, 84% and 85% amino acid identities with ARAS2, respectively. ARAS2 belongs to the same clade with EAY77809 and EAY77810. EAY77809 and EAY77810 shared 75% and 84% identities with ARAS1 and shared 88% and 85% identities with ARAS2, respectively. It is highly probable that these homologues are responsible for the biosynthesis of alkylresorcinols (**5c**, **5d**, **5e**, **5l**) in the Indica-type rice. Because alkylresorcinols (**5c**, **5d**, **5e**, **5l**) were not produced at any detectable level by the Japonica-type rice, *ARAS1* and *ARAS2* in this rice have probably become inactive during breeding. The expression level of ARAS1 and ARAS2 also suggested this hypothesis. The expression level of ARAS1 and ARAS2 was estimated by using expressed sequence tag (EST) database (http://rice.plantbiology.msu.edu/). The EST sequence derived from ARAS1 and ARAS2 had not been obtained from any tissue of *O. sativa*, including seedling, flower, sheath, root, immature seed, stem pistil, endosperm, panicle, root tip, shoot, seed, leaf and callus. This phenomenon suggested that ARAS1 and ARAS2 are not expressed in the Japonica-type rice.

At any rate, this is the first demonstration of the plant type III PKSs that can produce alkylresorcylic acids. Alkylresorcinols, synthesized by decarboxylation of alkylresorcylic acids, are in-born fungicides of the rice, wheat, maize, barley and other plants. Therefore, analyzing ARAS homologues in other plants will uncover antifungal defense mechanisms of these plants.

3. Concluding remarks

Two type III PKS, ARAS1 and ARAS2 were cloned from *O. sativa* and characterized *in vitro*. The *in vitro* analysis of ARAS1 and ARAS2 demonstrated that these enzymes synthesize alkylresorcylic acids (**4a**–**4f**, **4l**) from acyl-CoAs (**1a**–**1f**, **1l**) and malonyl-CoA. This is the first report describes plant type III PKSs which synthesize alkylresorcylic acids as the major products.

4. Experimental

4.1. Materials

E. coli strains JM109 and BL21 (DE3), plasmid pUC19, restriction enzymes, T4 DNA ligase, and PrimeSTAR DNA polymerase were obtained from Takara Biochemicals (Shiga, Japan), whereas pET16b was purchased from Novagen (Darmstadt, Germany). [2-^{14}C]Malony-CoA was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, USA). *p*-Coumaroyl-CoA, cinnamoyl-CoA and dihydrocinnamoyl-CoA were synthesized by the procedure of Blecher (1981). Un-labeled malony-CoA, benzoyl-CoA and fatty acyl-CoAs were purchased from Sigma. The genomic DNA of *O. sativa* (Nipponbare) was a gift from Y. Tozawa (Ehime University).

4.2. Production and purification of ARAS1 and ARAS2

Splicing by overlap extension (SOE)-PCR (Horton et al., 1989) was used to obtain *ARAS1* and *ARAS2* cDNAs. Using the rice genome DNA as a template, the 0.2-kb exon 1 and 1-kb exon 2 DNA fragments containing the *ARAS1* or *ARAS2*-coding regions were amplified and connected by PCR with the primers *ARAS1*_1F 5′-CCG **GAATTC**CAT*ATG*CCTGGAGCAACTACCGC-3′ (an EcoRI site is shown in boldface, an NdeI site is underlined, and the start codon is shown in italics), *ARAS1*_1R 5′-GATTTATGACATATCCTCTTCATCTTTTCC-3′, *ARAS1*_2F 5′-AAGAGGATATGTCATAAATCTGGTATAGAA-3′, *ARAS1*_2R 5′-CGC**GGATCC***TTA*ATTTTCCTTCAAACCAC-3′ (a BamHI site is shown in boldface and the stop codon is shown in italics), *ARAS2*_1F 5′-CCG**GAATTC**CAT*ATG*CCTGGAGCAGCTACCAC-3′ (an EcoRI site is shown in boldface, an NdeI site is underlined, and the start codon is shown in italics), *ARAS2*_1R 5′-GATTTTTTACATATCCTCTTCATCTTGTCC-3′, *ARAS2*_2F 5′-AAGAGGATATGTAAAAAATCAGGCATAGAG-3′, and *ARAS2*_2R 5′-CGC**GGATCC***CTA*ATTTTGCTTAAGACCAC-3′ (a BamHI site is shown in boldface and the stop codon is shown in italics). The amplified fragments were cloned between the EcoRI and BamHI sites of pUC19, resulting in pUC19-ARAS1 and pUC19-ARAS2. The NdeI-BamHI fragments containing *ARAS1* and *ARAS2* were excised from pUC19-ARAS1 and pUC19-ARAS2, respectively, and cloned between the NdeI and BamHI sites of pET16b, resulting in pET16b-ARAS1 and pET16b-ARAS2. For production of His-tagged ARAS1 and ARAS2, *E. coli* BL21 (DE3) harboring pET16b-ARAS1 or pET16b-ARAS2 was grown at 26 °C overnight in Luria broth containing 100 μg/ml ampicillin. The cells were then harvested by centrifugation and resuspended in 100 mM potassium phosphate buffer (pH 7.5), 10 mM imidazole and 10% glycerol. After sonication, cellular debris was removed by centrifugation and the cleared lysate was applied to a column with a His-bind metal chelation resin (Qiagen, Hilden). The purified His-tagged protein was dialyzed against 10 mM Tris–HCl (pH 8.0) and 10% glycerol.

4.3. PKS assay

The standard reaction mixture for radio-thin layer chromatography (TLC) assay contained [2-^{14}C]malonyl-CoA (5 μM), starter substrate (5 μM), 100 mM potassium phosphate buffer (pH7), and enzyme (50 μg) in a total volume of 500 μl. The standard reaction mixture for liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC–APCIMS) analysis contained malonyl-CoA (100 μM), starter substrate (100 μM), 100 mM potassium phosphate buffer (pH 7), and 50 μg of enzyme in a total volume of 500 μl. After the reaction mixture had been preincubated at 30 °C for 2 min, the reactions were initiated by adding the substrates. The reactions were stopped with addition of 6 M HCl (100 μl) after incubation for 20 min, and the products in the mixture were extracted with EtOAc. The organic layer was sampled for TLC and LC–APCIMS analyses. Silica gel 60 WF_{254} thin layer chromatography plates (Merck) were developed in benzene/acetone/AcOH (85:15:1, v/v/v), and the ^{14}C-labeled compounds were detected using a Fuji BAS-MS imaging plate (Fuji Film, Tokyo). LC–APCIMS analysis was carried out by using the Esquire High-Capacity Trap (HCT) plus system (Bruker Daltonics, Bremen) and Agilent1100 series (Santa Clara, CA) equipped with a Pegasil-B C4 reversed-phase HPLC column (4.6 × 250 mm; Senshu Scientific, Tokyo). The compounds were eluted with a linear gradient [70–90% CH_3CN (solvent A), 60–90% CH_3CN (solvent B), 50–90% CH_3CN (solvent C) in H_2O (each containing 0.1% AcOH)] over 30 min at a flow rate of 0.8 ml/min. The triketide pyrone (**2a**) and alkylresorcinol (**5a**) were identified by comparing their LC–MS and MS/MS spectra with authentic standards prepared by chemical synthesis and isolation from an *A. vinelandii* cell culture (Funa et al., 2006). Triketide pyrones (**2c**–**2j**), tetraketide pyrones (**3d**–**3g**), alkylresorcylic acids (**4c**), alkylresorcinols (**5b**–**5g**), 2′-oxoalkylresorcylic acids (**6b**–**6c**), and 2′-oxoalkylresorcinols (**7b**–**7c**) were identified by comparing their LC–MS and MS/MS spectra with those from authentic standards that had been prepared with ORAS from *N. crassa*, as previously described (Funa et al., 2007). Other pyrones and resorcinols were characterized by comparing the LC–MS and MS/MS spectra with those of authentic standards prepared with previously reported ArsB and ArsC (Funa et al., 2006).

4.4. pH and temperature dependence analysis

The standard reaction mixture for pH dependence analysis contained [2-^{14}C]malonyl-CoA (5 μM), starter substrate [stearoyl-CoA

(**1c**) for ARAS1, myristoyl-CoA (**1e**) for ARAS2] (5 μM), 100 mM potassium phosphate buffer (pH 5.0–9.0) or *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer (pH 10), and enzyme (10 μg) in a total volume of 100 μl. The standard reaction mixture for temperature dependence analysis contained [2-^{14}C]malonyl-CoA (5 μM), starter substrate [stearoyl-CoA (**1c**) for ARAS1, myristoyl-CoA (**1e**) for ARAS2] (5 μM), 100 mM potassium phosphate buffer (pH 7 for ARAS1, pH 6.5 for ARAS2), and enzyme (10 μg) in a total volume of 100 μl. After the reaction mixture had been preincubated for 2 min at 30 °C (pH dependence analysis) or 25–50 °C (temperature dependence analysis), the reactions were initiated by adding the substrates. The reactions were continued for 20 min. The reactions were stopped with 6 M HCl (20 μl), and the products in the mixture were extracted with EtOAc. The organic layer was evaporated, and kept at 45 °C for 30 min to decarboxylate the alkylresorcylic acids and the residual material was then dissolved in methanol (10 μl) for TLC analysis. TLC conditions were as described in Section 4.3. After separation by TLC, the amounts of polyketides were quantified by means of [2-^{14}C]malonyl-CoA incorporation.

4.5. Determination of kinetic parameters

The standard reaction mixture contained starter substrate, [2-^{14}C]malonyl-CoA (61,700 dpm) (100 μM), 100 mM potassium phosphate buffer (pH 7 for ARAS1, pH 6.5 for ARAS2), and enzyme (4 μg) in a total volume of 100 μl. The concentrations of starter substrates were varied between 1.0 and 7.0 μM for palmitoyl-CoA (**1d**), stearoyl-CoA (**1c**), and oleoyl-CoA (**1l**), and 1.5–7.0 μM for myristoyl-CoA (**1e**) when incubated with ARAS1 and 15–50 μM for myristoyl-CoA (**1e**) when incubated with ARAS2, respectively. The reaction mixtures containing ARAS1 were incubated at 35 °C and containing ARAS2 were incubated at 30 °C. After 2 min incubation, reaction was initiated by adding substrates, starter substrates and malonyl-CoA. After 20 min incubation, reaction was quenched by adding 6 M HCl (20 μl). Reaction products were extracted by EtOAc and evaporated to dryness. The reaction products were kept at 45 °C for 30 min to enhance decarboxylation of the alkylresorcylic acids. The reaction and TLC conditions were as described in Section 4.3. After separation by TLC, the amounts of polyketides were quantified by means of [2-^{14}C]malonyl-CoA incorporation. Kinetic parameters were deduced by nonlinear least-squares fitting to the Michaelis–Menten equation. Lineweaver–Burk plot was exceptionally used for determination of kinetic parameters for palmitoyl-CoA (**1d**) because fitting to the Michaelis–Menten equation was unsuitable due to substrate inhibition occurring in a linear region.

Acknowledgments

We thank Y. Tozawa (Ehime University) for providing us with the *O. sativa* genomic DNA. This work was supported by a research grant from the New Energy and Industrial Technology Development Organization of Japan and a Grant-in-Aid for Scientific Research on Priority Area "Applied Genomics" from Monkasho. Y. katsuyama was supported by the Japan Society for Promotion of Science.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.02.012.

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