Physcomitrella PpORS, Basal to Plant Type III Polyketide Synthases in Phylogenetic Trees, Is a Very Long Chain 2′-Oxoalkylresorcinol Synthase*5

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Received for publication, October 24, 2012, and in revised form, December 3, 2012. Published, JBC Papers in Press, December 7, 2012. DOI 10.1074/jbc.M112.430686

Significance: This is the first step toward understanding the co-evolution of the type III PKS family and land plants.

Conclusion: PpORS is a novel very long chain 2′-oxoalkylresorcinol synthase.

Results: PpORS, produced in nonprotonemal moss cells, synthesizes pentaketide 2′-oxoalkylresorcinols using a unique substrate binding site.

Background: Physcomitrella PpORS is an ancient member of the plant type III polyketide synthase (PKS) family.

Type III polyketide synthases (PKSs), which produce diverse secondary metabolites with different biological activities, have successfully co-evolved with land plants. To gain insight into the roles that ancestral type III PKSs played during the early evolution of land plants, we cloned and characterized PpORS from the moss Physcomitrella. PpORS has been proposed to closely resemble the most recent common ancestor of the plant type III PKSs. PpORS condenses a very long chain fatty acyl-CoA with four molecules of malonyl-CoA and catalyzes decarboxylative aldol cyclization to yield the pentaketide 2′-oxoalkylresorcinol. Therefore, PpORS is a 2′-oxoalkylresorcinol synthase. Structure modeling and sequence alignments identified a unique set of amino acid residues (Gln218, Val277, and Ala286) at the putative PpORS active site. Substitution of the Ala286 to Phe instead constricted the active site cavity, and the A286F mutant instead produced triketide alkylpyrones from fatty acyl-CoA substrates with shorter chain lengths. Phylogenetic analysis and comparison of the active sites of PpORS and alkylresorcinol synthases from sorghum and rice suggested that the gramineous enzymes evolved independently from PpORS to have similar functions but with distinct active site architecture. Microarray analysis revealed that PpORS is exclusively expressed in nonprotonemal moss cells. The in planta function of PpORS, therefore, is probably related to a nonprotonemal structure, such as the cuticle.
PKSs are found in bacteria (9) and fungi (5, 10), they are more widely distributed in green land plants (Embryophyta). Each plant has a set of taxon-specific type III PKSs, which produce metabolites that are involved in UV protection (flavonoids), antimicrobial defense (stilbenes, bibenzyls, and alkylresorcinols), flower pigmentation (anthocyanins), spore/pollen protection (hydroxyalkyl pyrones), pollen tube growth (flavonols), and legume nodulation (isoflavonoids). The overall significance and scope of their roles suggest that type III PKSs have successfully co-evolved with land plants. This led us to investigate what major contributions ancestral type III PKSs might have made during the early evolution of land plants, especially during the colonization of land by ancestral plants. Our approach to gain insight into this question was to study the enzymatic properties of a modern-day plant type III PKS thought to closely resemble ancestral plant type III PKSs.

Bryophytes, comprising liverworts, mosses, and hornworts, are the simplest and earliest diverging lineages of land plants (Embryophyta). The model moss *Physcomitrella patens* is currently the only bryophyte whose genome has been sequenced (11), and its genome contains at least 17 putative type III PKS genes (12). Among them, *PpORS* (formerly *PpCHS11*) was shown to be basal to all plant type III PKS genes in phylogenetic trees.
Therefore, it was proposed to encode an extant enzyme that might closely resemble the most recent common ancestor (MRCA) of plant type III PKSs (13). In this study, we cloned PpORS and characterized the enzymatic properties of the recombinant PpORS to demonstrate that PpORS is a 2'-oxoalkylpyrroloresorcinol synthase with substrate preference for very long chain fatty acyl-CoA esters. We then identified putative active site residues by performing structure modeling and mutagenesis studies. We also investigated the expression patterns of PpORS and Phypa126819, a P. patens PKS gene closely related to PpORS, by expressed sequence tag (EST) abundance and microarray analyses, and carried out phytochemical analysis in an attempt to learn about in planta function of PpORS. These studies should help us to understand the roles that type III PKSs may have played during early evolution of land plants.

**EXPERIMENTAL PROCEDURES**

**Materials**—Expression plasmids for ArsB and ArsC were provided by Dr. Nobutaka Funada (University of Tokyo). α-Coumaroyl-CoA and cinnamoyl-CoA were purchased from TransMIT (Giessen, Germany). Eicosanoyl-CoA (Ic, C20-CoA), docosanoyl-CoA (Ib, C22-CoA), and tetracosanoyl-CoA (Ia, C24-CoA) were from Avanti Polar Lipids (Alabaster, AL). Other acyl-CoA esters and Fast Blue B salt (ZnCl2) were from Sigma-Aldrich. [2-14C]Malonyl-CoA (55.2 mCi/mmol) was purchased from NEN/PerkinElmer Life Sciences. Wheat bran was obtained from Old Fashion Food (Regina, SK, Canada). Syntheses of 5-pentadecylresorcinol and 4-hydroxy-6-tridecyl-2-pyrrone were reported previously (14, 15).

**Cloning of PpORS—P. patens (Hedw.) Bruch and Schimp**—Subspecies *P. patens*, strain Gransden2004, was cultivated on sterile peat pellets (Jiffy-7; Jiffy Products International AS, Kristiansand, Norway) for 1–1.5 months at 25 °C under continuous light. Upper halves of gametophores without gametangia were collected with scissors. A full-length cDNA library was prepared by the oligo-capping method (16), and the cDNAs were cloned into the DraIII sites of pME18S-FL3 vector (AB009864). The full-length cDNA database in PHYSCObase was searched using the sequence of Contig1663 (17) as a query, and five corresponding clones (ppsp2a16, ppsp2k15, ppsp2n21, and ppsp2n2) were obtained. The coding region of PpORS was amplified by PCR from the ppsp2a16 clone using primer sets shown in supplemental Table S1. The PCR products produced under standard PCR conditions were digested with restriction enzymes and subcloned into pET32a and pET28a expression vectors (Novagen) to give pET32-PpORS and pET28-PpORS, respectively.

**Heterologous Production and Purification of Recombinant Proteins**—Protein production and purification by Ni2+–chelation chromatography were performed as described previously (18) except that purification buffer was 20 mM potassium phosphate (KP, pH 7.6) containing 200 mM NaCl. The enzyme solution was buffer-exchanged to 0.1 M KP buffer, pH 7.6, using a 10DG column (Bio-Rad) for functional assays.

**Enzyme Assay, Kinetic Analysis, and Product Determination**—The standard assay mixture (100 μl) contained purified enzyme (10–20 μg), 0.1 mM starter-CoA (e.g. C24-CoA), and 0.1 mM [2-14C]malonyl-CoA (11 mCi/mmol) in 0.1 M KP, buffer, pH 7.6. Reactions to measure substrate preference and kinetic parameters were performed in 0.1 M KP buffer, pH 7.6, containing 10% glycerol and 0.1% Triton X-100. After incubation at 30 °C for 20–40 min, the reaction was stopped by acidification (7.5 μl of 1 N HCl), and the reaction products were extracted with ethyl acetate (200 μl). The radioactive products were separated and quantified by thin layer chromatography (TLC) and phosphorimaging as described previously (15).

**Expression Analysis**—Two putative genes homologous to PpORS were compared with those produced by ArsC (2) or *P. patens* CHS (PpCHS) (17) by co-spotting on aluminum-backed silica 60 TLC sheets (EMD). Enzyme products were detected by staining with Fast Blue B salt (0.1% in H2O) (19).

**Structure Modeling and Site-directed Mutagenesis**—The structure of PpORS was first modeled with I-TASSER, which utilizes an ab initio multiple-threading approach (20). The quality of the model was further improved by a 10,000-step minimization in NAMD with AMBER ff99SBildn force fields, explicit solvation in TIP3P water, and Particle Mesh Ewald (21, 22).

The PpORS mutants (Q218T, V277G, A286F, Q218T/V277G, Q218T/A286F, V277G/A286F, Q218T/V277G/A286F) were generated from the pET28-PpORS plasmid using the QuikChange Site-directed Mutagenesis kit (Stratagene) and mutagenic primers shown in supplemental Table S1. The recombinant mutant proteins were produced in *Escherichia coli* BL21(DE3) or Tuner(DE3) cells. Gene expression in the Tuner(DE3) cells was induced with 0.5 mM isopropyl-β-D-galactopyranoside. Protein purification was carried out in the same manner as the wild-type PpORS except that the KP buffer solutions contained 10% glycerol and 0.1% Triton X-100.

**Characterization of PpORS**—The specific enzyme activity was expressed in pmol of the product produced s−1 mg−1 (picokatal s−1).

Steady-state kinetic parameters of PpORS for C10-CoA and C24-CoA were determined in the presence of 0.1 mM malonyl-CoA and 9 μM PpORS. The concentration of starter substrate varied from 5 to 80 μM, and the reaction time was 10 min. *Km* and *Vmax* were calculated by fitting the data to the Michaelis-Menten equation using a nonlinear curve-fitting program (GraphPad Prism v.5.03).

Large scale reactions for product determination were performed with hexanoyl-CoA (C6-CoA), decanoyl-CoA (C10-CoA), or C24-CoA as the starter substrate under the standard assay conditions except that the concentration of malonyl-CoA was 0.2 mM and the reaction was run for 2 h. After standard work-up procedures, the reaction products were dissolved in methanol for MS analysis. Mass spectra were recorded using a Finnigan-Matt TSQ-700 mass spectrometer equipped with electrospray ionization and a Harvard syringe pump. Solutions were electro sprayed at 4.5 kV, with a capillary temperature of 75 °C. Flow rate was varied between 1 and 10 μl/min, and tube lens voltage was varied between −40 V and −80 V, depending on the compound. 2-Pyrene products of wild-type and mutant PpORS were compared with those produced by ArsC (2) or *P. patens* CHS (PpCHS) (17) by co-spotting on aluminum-backed silica 60 TLC sheets (EMD). Enzyme products were detected by staining with Fast Blue B salt (0.1% in H2O) (19).

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Physcomitrella patens.1.1 database with PpORS as the query sequence. Genomic sequences of these putative genes were manually translated into amino acid sequences based upon exon-intron architecture and homology to other type III PKSs. EST abundance of the two putative PpORS paralogs, Phypa126819 and Phypa72618, was then examined by blastn searches against the NCBI EST database, and the EST profile of PpORS was obtained by examining corresponding ESTs (Ppa.5302) in individual NCBI P. patens UniGene libraries. The expression patterns of PpORS and Phypa126819 were determined with whole genome microarrays (CombiMatrix, Mukileto, WA) based on all gene models v1.2 (11). RNA samples were obtained from protonema from liquid cultures, juvenile gametophores grown on solid medium (23), and freshly isolated protoplasts (24). The microarray experiments were done in biological triplicates. Data analysis with the Expressionist software (Genedata, Basel, Switzerland) was performed as described previously (25).

Phytochemical Analysis—Plants were grown on solid medium with (protonemata) and without (gametophores) ammonium tartrate as described previously (12). Dried and ground tissue (protonema, gametophore, or wheat bran, 0.5 g each) was extracted with 10 ml of acetone for 3 h with a wrist shaker, and the extract was filtered and vacuum-dried. The residue was dissolved in 6 ml of methanol. A portion (0.5 ml) was made basic (pH ~10) by the addition of 0.15 ml of 0.1 M KOH and incubated at 40 °C for 4 h. The resulting hydrolyzed solution was acidified to pH ~2 with 10 μl of 6 N HCl, and partitioned with hexanes (0.4 ml). The organic layer was vacuum-dried and the residue was dissolved in 30 μl of methanol. Extracts were analyzed before and after alkali treatment by silica TLC (toluene/acetone/acetic acid 75/25/1, v/v/v), and stained with Fast Blue B salt (0.1% in H2O).

Phylogenetic Analysis—Phylogenetic analysis with the Bayesian inference method was performed using the MrBayes program (v. 3.2-cvs) (26), as described previously (13) with some modifications. The search was initialized at a user-defined tree, which was generated from the amino acid sequences by the default slow/accurate option in ClustalW. The Markov Chain Monte Carlo analysis was run for one million generations with four chains, and trees were sampled after every 100 generations. After all trees sampled during the first 250,000 generations were discarded, a consensus tree was constructed based on the remaining trees and displayed using MEGA4 (27).

RESULTS

Cloning and Heterologous Production of PpORS—The full-length coding region of PpORS was obtained from the moss gametophore cDNA library. Attempts to clone the gene from protonema of the moss were unsuccessful, suggesting that PpORS is not expressed during the protonema stage (see below). As discussed earlier (12), the third ATG codon among the four in-frame candidate start codons was assumed to be the translation initiation site of PpORS and used to produce the recombinant PpORS. The enzyme was produced both as a thioredoxin (Trx)-His6-tagged protein (Trx-PpORS, 61 kDa) and as a His6-tagged protein (PpORS, 44 kDa). The Trx tag increased the stability of the recombinant enzyme and had little effect on the product profile. Thus, Trx-PpORS was used for the large scale reactions. The deduced amino acid sequence of PpORS (ABU87504) contains the conserved catalytic residues, Cys185, His323, and Asn356 (28) and the G385FGPG loop (29). The sequence identity of PpORS to other type III PKSs was generally low, and it was 20% to NcORAS, 34% to SbARS1 (4), and 36% to PfCHS and Medicago sativa CHS (MsCHS). Sequence alignments of PpORS with other type III PKSs are shown in supplemental Fig. S1.

In Vitro Analysis of PpORS Activity—We first tested the substrate preference of PpORS. PpORS produced a single major product (3j–3e) when the chain length of the starter fatty acyl-CoA substrate was C6 to C10 (Fig. 2). From C18 to C24-CoA substrates, PpORS produced two additional types of compounds, one with lower RF values (4d–4a) and another with higher RF values (5d–5a). Compounds 5c–5a were the major products from C16 to C24-CoA substrates, as evidenced by the progressively increasing RF value of the product with increasing chain length of the starter substrate (Fig. 2). From C18 to C24-CoA substrates, PpORS produced a molecular ion peak [M–H]− at m/z 237. Minor peaks were also observed at 273 [M+Cl]− and 475 [M+M–H]−, providing further confirmation of the mass. Compound 3h co-migrated on TLC with the reaction product of ArsC from the same starter substrate (C10-CoA), and both products were stained yellow-orange with Fast Blue B salt, similarly to 4-hydroxy-6-methyl-2-pyrones (λmax = 469 nm in
methanol (supplemental Fig. S2, A and B). ArsC was shown to produce triketide alkylpyrones from the starter C6- to C12-CoA substrates and produces both triketide and tetraketide alkylpyrones from C14- to C22-CoAs (2). Therefore, we concluded that 3h is 4-hydroxy-6-nonyl-2-pyrene (Fig. 1B).

Compound 4a as well as 3a showed migration and staining patterns on TLC identical to those of the ArsC products from C24-CoA (supplemental Fig. S2C). Negative mode ESI-MS of the PpORS products from C24-CoA yielded two major ion peaks at m/z 433 and 475. The same molecular ion peaks were also obtained from an ESI-MS analysis of the ArsC products from C24-CoA. Based on these data, we concluded that 3a and 4a are 4-hydroxy-6-tricosyl-2-pyrene ([M−H]− at m/z 433) and 4-hydroxy-6-(2-oxo-pentacosyl)-2-pyrene ([M−H]− at m/z 475), respectively (Fig. 1B).

Compound 5a was stained violet with Fast Blue B salt, similarly to olivetol (λmax = 500 nm in methanol) (supplemental Fig. S2, A and C), suggesting that 5a contains a resorcinol ring. However, compounds 5a–5c exhibited lower Rf values compared with the tetraketide alkylresorcinols produced by ArsB from the same substrates. After reduction with NaBH4, 5a and 4a were converted to polar compounds with lower Rf values, whereas 3a remained intact, suggesting that 5a contains an oxo group as does 4a (data not shown). Positive mode ESI-MS of TLC-purified 5a yielded a molecular ion peak at m/z 950 corresponding to a protonated dimer [M+M+H]+ of 5-(2’-oxo)pentacosylresorcinol (supplemental Fig. S3). Based on these results, we concluded that 5a–5d are pentaketide 2’-oxoalkylresorcinols.

Other CoA esters were also examined as starter substrates for PpORS. PpORS produced a single triketide alkylpyrone product from palmitoleoyl-CoA (C16:1-CoA) or oleoyl-CoA (C18:1-CoA) with specific activity comparable with that for C16-CoA (data not shown). On the other hand, no enzyme activity was observed when the starter substrate was p-menth-8-enoyl-CoA, coumaroyl-, cinnamoyl-, benzoyl-, acetyl-, butyryl-, malonyl-, or arachidonoyl-CoA.

Enzymatic Properties and Kinetics of PpORS—We next tested the effects of different reaction conditions on the PpORS activity and product profile. The optimal activity for production of the pentaketide 2’-oxoalkylresorcinol (5a) from C24-CoA was observed at pH 7.5. The ratio of 5a to 3a decreased progressively as the pH increased, and the ratio was 2.3 at pH 6.5 and 0.67 at pH 8.0 (Fig. 3A). Similarly, the ratio of 5a to 3a varied depending on the concentration of the extender substrate (malonyl-CoA). More 5a was produced at higher concentrations of malonyl-CoA; however, overall activity decreased at 200 μM malonyl-CoA, possibly due to substrate inhibition (Fig. 3B). Incubation time had no effect on the product profile, and the production of 5a, 3a, and 4a from C24-CoA increased steadily as incubation time increased up to 1 h (data not shown).

The steady-state kinetic parameters of PpORS for two representative substrates, C16-CoA and C24-CoA were estimated according to the Michaelis–Menten kinetics model. The km and kcat/Km values for C24-CoA were 26 (±2.1, n = 3) μM and 0.70 M−1 s−1, and those for C10-CoA were 63 (±5.9) μM and 1.1 M−1 s−1, respectively (supplemental Fig. S4).

Structure Modeling and Site-directed Mutagenesis—The modeled PpORS structure closely resembles the x-ray structures of MsCHS and other type III PKSs. Active site residues known to be critical for enzyme function are found at similar positions in the PpORS model compared with known type III PKS structures. Thus, the Cys185-His323-Asn356 catalytic triad, Phe238, Ser358, the G385FGPG loop and others are almost superimposable on the corresponding active site residues of MsCHS (Fig. 4). On the other hand, Thr197, Phe215, and G236 of MsCHS are uniquely substituted by Gin218, Val277, and Ala236, respectively, in PpORS (supplemental Fig. S1). Because these unique substitutions might play roles in the differential enzyme activity of PpORS, these three residues were mutated individually and in combination to the corresponding MsCHS residues by site-directed mutagenesis.

Among the seven single and multiple mutants studied, only the A286F mutant and the V277G/A286F double mutant exhibited activity, and the rest were inactive with all starter substrates examined. Compared with the wild-type enzyme (Fig. 2), it was evident that both mutants lost the ability to produce pentaketide oxoresorcinols and instead produced triketide alkylpyrones (3a–3j) from C6 to C24-CoA starter substrates (Fig. 5). Compound 3j produced by the A286F mutant co-migrated on TLC with 4-hydroxy-6-pentyl-2-pyrylone ([M−H]− at m/z 181) produced by PpORS from C18-CoA. Both mutants exhibited similar substrate preference in that C5- and C16-CoA esters were most preferred. However, the V277G/A286F double mutant was better than the A286F mutant at accepting C22- and C24-CoA esters as the starter substrate. Specific activity for the formation of 3a was 0.019 and 0.072 picomolals mg−1 for the A286F and V277G/A286F mutants, respectively. Tetraketide alkylpyrones (4a–4c) were also produced by both mutants at lower levels. Like the wild-type enzyme, neither mutant accepted p-coumaroyl-, cinnamoyl-, C5-, and C16-CoA esters as starter substrate.

Expression Profile of PpORS and Phypa126819—Based on homology to PpORS, two gene models, Phypa72618 and
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Phylogenetic Analysis—Expanding previous phylogenetic analyses of type III PKSs (4, 13, 31), a phylogenetic tree was constructed with PpORS, the two *P. patens* paralogs, and other long chain acyl-CoA-utilizing type III PKSs including the gramineous ARSs (ShARS1, ShARS2, OsARAS1, and OsARAS2) (Fig. 7). The tree shows the expected progressive evolution from bacterial to fungal to plant enzymes. PpORS and its two moss paralogs form their own clade at the base of the plant clade. Thus, they are direct descendants of the MRCA of the plant type III enzyme family. The rest of the plant enzymes, in turn, form a sister clade to the PpORS clade. They themselves are divided into two sister clades, one made of anther-specific chalcone synthase-like enzymes (ASCLs) (15) and the other made of non-ASCLs. The gramineous ARSs belong to one of the two sister clades of the non-ASCL clade, reflecting their close evolutionary relationship among themselves. More importantly, PpORS and its moss paralogs are clearly separated from the gramineous ARSs, indicating that the gramineous

Phylogenetic Analysis—To examine whether PpORS products exist in planta either in monomeric or in esterified forms, we attempted to detect putative PpORS products from the moss gametophore before and after alkaline treatment. Wheat bran extracts, a positive control, yielded a major band on TLC, which stained violet with Fast Blue B salt. The extracts were determined to contain 5-nonadecylresorcinol ([M − H]− at m/z 375) and 5-heneicosyresorcinol ([M − H]− at m/z 403), in agreement with the literature (30) (supplemental Fig. S6). However, we failed to detect any resorcinol derivatives from the extracts of the moss gametophores as well as protonemata. No band that responded to the dye in the characteristic manner of resorcinol derivatives was detected. These results led us to conclude that the moss gametophore tissues do not contain (oxo)alkylresorcinols, either as monomeric or esterified forms. Alternatively, the amounts of (oxo)alkyresorcinols present are below the detection limit of this study (~200 ng/g of tissue estimated based on the sensitivity of the dye staining).

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ARSs are not direct descendents of the PpORS lineage and have evolved independently from the PpORS lineage.

DISCUSSION

Pentaketide 2’-oxoalkylresorcinols were previously detected among the reaction products of NcORAS and long chain fatty acyl CoA substrates (5). However, based on a careful time course study, the authors concluded that NcORAS instead produced pentaketide 2’-oxoalkylresorcylic acids and that the detected 2’-oxoalkylresorcinols were indirect products formed by nonenzymatic (or enzymatic (6)) decarboxylation of the resorcylic acids. We also examined the possibility that PpORS may produce 2’-oxoalkylresorcylic acids. We monitored the PpORS reaction with C24-CoA and [2-14C]malonyl-CoA at different time intervals up to 1 h and observed a steady increase of the formation of 5a and no evidence for the formation of a 2’-oxoalkylresorcinol. The same products were produced at different reaction pH values and substrate concentrations. Furthermore, we have demonstrated that 6-tridecyl-β-resorcylic acid, an alkylresorcylic acid, is stable up to several hours in 0.1 M KP buffer (pH 7.8) (14). Based on these results, we conclude that PpORS is a pentaketide 2’-oxoalkylresorcinol synthase. Thus, PpORS condenses a very long chain fatty acyl-CoA with four molecules of malonyl-CoA and cyclizes the pentaketide intermediate to produce 2’-oxoalkylresorcinol, through an aldol reaction accompanied by decarboxylation (Fig. 1B).

PpORS produces different major products from starter substrates of different chain lengths; triketide alkylypyrones from the C6 to C16 substrates and pentaketide oxoresorcinols from the C20 to C24 substrates. The shift is not abrupt, and from C16-CoA, the enzyme produced significant amounts of a triketide alkylypyrone (3d) and a pentaketide oxoresorcinol (5d) along with a tetraketide alkylypyrone (4d). This “substratedirected product specificity” is not uncommon for type III PKS catalyzed reactions. Notably, NcORAS produces triketide alkylypyrones from C4 to C9, starter substrates, tri- and tetraketide alkylypyrones and tetraketide alkylresorcinols from C16 to C14 substrates, and tetra- and pentaketide alkylresorcylic acids from C16 to C20 substrates (5). Also, OsARASs produce alkylresorcylic acids and do not produce tetraketide alkylypyrones when the starter substrate is longer than C14-CoA (7). This raises a question as to the chemical nature of the in planta products of these enzymes. It has been postulated that enzymes that produce more than one product are advantageous in secondary metabolism because they generate chemical diversity at low cost (32). PpORS may well produce in planta alkylypyrones and 2’-oxoalkylresorcinols from fatty acyl-CoA esters of varying chain lengths for similar or different functions. In that case, substrate availability will determine the type of products made by the enzyme in planta. On the other hand, triketide and tetraketide pyrones are produced by most type III PKSs when nonphysiological substrates are given (1). Even with physiological substrates, most type III PKSs produce pyrones as in vitro derailment products. For example, CHS produces bisnoryan-gonin (a triketide pyrone) and coumaroyltriacetic acid lactone (a tetraketide pyrone) in addition to a chalcone (33). Furthermore, type III PKS mutants often produce pyrones when their active sites are compromised. The two PpORS mutants (A286F and V277G/A286F) in which putative active site residues were mutated failed to produce 2’-oxoalkylresorcinols, but still produced alkylypyrones (Fig. 5). These findings suggest that alkylypyrones produced by PpORS in vitro might be derailment products due to nonphysiological substrates or suboptimal reaction conditions. In that case the in planta products might be very long chain 2’-oxoalkylresorcinols.

Long chain alkylresorcinols have been found in higher plants including gramineous cereals. They are particularly abundant...
in the bran layer of cereal grains and are thought to exert antifungal activity (19). Long chain (C19-C25) 2’-oxoalkylresorcinols were found as minor components in wheat and rye grains and etiolated rice seedlings (34, 35). All plant (2’-oxo)alkylresorcinols identified to date are extractable monomers. To the best of our knowledge, no extractable alkylresorcinols have been detected in mosses, and the absence of alkylresorcinols in Sphagnum mosses is well documented (36). In A. vinelandii, monomeric alkylresorcinols and alklypyrones produced by ArsB and ArsC from C20- and C22-CoA esters are the major lipid components of the protective cyst coat (2). PpORS is unique in that it produces exclusively 2’-oxoalkylresorcinols but does not produce alkylresorcinols. In addition to presenting an interesting mechanistic problem for future study, it might also bear significant implications for in planta function of PpORS because it implies important roles for the oxo group in the products. PpORS is expressed in gametophores, and its expression was not largely affected either by light/dark cycle (12) or by UV-B exposure (31). Moreover, PpORS is not expressed in moss protonemata and protoplasts (Fig. 6). Taken together with our failure to detect monomeric or esterified resorcinol derivatives from the moss gametophore extracts, these data suggest that 2’-oxoalkylresorcinols produced by PpORS might be constituents of gametophore-specific materials, such as a cuticle (37) or lignin-like materials (38). The plant cuticle is a waxy covering that protects plant from desiccation. In these materials, chemical components could be bound, at least partly, through alkaline-resistant linkages such as ether bonds. In this context, it is worthwhile to note that the ASCL-produced tetralactone 2’-oxoalkylpyrones have been proposed to be reduced by tetraketide α-pyrone reductases before being incorporated into sporopollenin, a biopolymer found in the pollen and spore.
walls (39). The resultant hydroxyl group of the hydroxyalkylpyr- rones might then form ether or ester linkages in the sporopollenin polymer. The oxo group in 2'-oxoalkylresorcinols might also be reduced in a similar manner in planta.

Thr197, Gly256, and Phe265 (numbering of MsCHS) that are highly conserved in CHS and many other type III PKSs are uniquely replaced with Gln218, Val277, and Ala286 in PpORS, respectively. All three residues are situated at the opposite side of the active site cavity from the nucleophilic Cys residue to which the growing polyketide chain is attached during catalysis (Fig. 4). Numerous studies have shown that Thr197 and Gly256 play critical roles in determining both substrate preference and the extent of condensation reactions by controlling the size and shape of active site cavity (reviewed in Ref. 1). For example, substitutions of Thr197, Gly256, and Ser238 of MsCHS with the corresponding residues found in 2-pyrene synthase were sufficient to convert the T197L/G256L/S338I triple mutant of MsCHS to a functional 2-pyrene synthase (40). However, only a few mutational studies have been done on Phe265, which sits at the entrance to the active site. Whereas the F233A mutant of a bacterial type III PKS, RppA, was devoid of enzymatic activity (41), the F265V mutant of MsCHS exhibited similar substrate selectivity as the wild-type enzyme (42). We could not properly access the functional role of the Gln218 residue of PpORS because the CHS-like substitution of the Gln218 to a Thr apparently disturbed the folding processes and made the mutant protein insoluble when produced in E. coli BL21(DE3) cells. Replacement of Val277 with a Gly had similar detrimental effects on protein structure, suggesting that Gln218 and Val277 are critical for proper folding and structural integrity of PpORS. Substitution of Ala286 with a bulky Phe had dual effects on enzyme activity. First, the A286F mutant completely lost the ability to form pentaketide 2'-oxoalkylresorcinols. Second, the ability of the mutant to accept the starter substrate gradually decreased as the chain length grew longer than C16. Both effects, although the former was more drastic, could be explained by the constriction of the active site cavity by the bulky Phe residue. The V277G/A286F double mutant exhibited a slightly higher activity than the A286F mutant in accepting the bulky Phe residue. The V277G/A286F double mutant exhibited a slightly higher activity than the A286F mutant in accepting the bulky Phe residue. The V277G/A286F double mutant exhibited a slightly higher activity than the A286F mutant in accepting the bulky Phe residue.

In this study, PpORS was characterized to be a very long chain 2'-oxoalkylresorcinol synthase. A unique set of putative active site residues (Gln218, Val277, and Ala286) were identified through structure modeling and sequence alignments. Replacement of the Ala286 with a bulky Phe affected both the cyclization mode and the substrate preference. Expression profiling and phytochemical studies suggested that in planta products of PpORS might not exist in monomeric form and could be components of a polymeric structure that is specific to nonprotonema tissues, such as the cuticle. More genetic and phytochemical studies are warranted to understand how the type III PKS family, in which ancestral acyl-CoA utilizing type III PKS genes have undergone repeated gene duplication-loss and functional diversification throughout evolution (13).Taken together, PpORS and the gramineous ARSs appear to represent an interesting example of enzyme evolution in which the same functions (the common use of long chain acyl-CoA starter substrates and the same type of cyclization) have evolved more than once within an enzyme family by adopting different structural strategies (different architecture of the binding sites).

Acknowledgments—We thank Matthew Endsin and Fatima Abbas for their contribution in the preparation of pET28-PpORS, and Li Li for the purification of Trx-PpORS. We also thank Dr. Nobutaka Funa (University of Tokyo) for expression plasmids of ArsB and ArsC. CGS-M postgraduate scholarship from NSERC to C. C. Colpitts is also acknowledged.

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