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Building Triketide α-Pyrone-Producing Yeast Platform Using Heterologous Expression of Sporopollenin Biosynthetic Genes

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Sporopollenin is a poorly characterized mixed aliphatic and aromatic polymer with ester and ether linkages. Recent studies have reported that α-pyrone polyketide compounds generated by *Arabidopsis thaliana*, polyketide synthase A (PKSA) and tetraketide α-pyrone reductase 1 (TKPR1), are previously unknown sporopollenin precursors. Here, the yeast *Saccharomyces cerevisiae* was introduced to test potential sporopollenin biosynthetic pathways *in vivo*. A PKSA/TKPR1 dual expressor was generated and various chain-length alkyl α-pyrones were identified by GC-MS. The growth rate of the strain containing PKSA/TKPR1 appeared normal. These results indicate that PKSA/TKPR1-expressing yeast would be a starting platform to investigate *in vivo* sporopollenin metabolism.

Keywords: Sporopollenin monomer, polyketide synthase, triketide α-pyrone, tetraketide α-pyrone reductase 1

Sporopollenin is an extremely robust biopolymer found in exine wall surrounding the spores of bryophytes and the male gametophyte of seed plants. The durable exine wall gives spores and pollen their resistance to high temperatures, prolonged desiccation, and exposure to UV radiation from the harsh terrestrial environment [1–3, 17, 18]. The chemical composition of sporopollenin remains poorly characterized because it is extremely resistant to chemical and biological degradation procedures. Recent studies have shown that hydroxylated α-pyrone polyketide compounds, generated by the sequential action of *Arabidopsis thaliana* acyl-CoA synthetase 5 (ACOS5), polyketide synthase A and B (PKSA/B) and tetraketide α-pyrone reductase 1 (TKPR1), in tapetum cells are key aliphatic constituents of sporopollenin [5, 9–12, 15]. Based on an *in vitro* model for α-pyrone sporopollenin unit synthesis (Fig. 1), after acyl-CoA ester formation by ACOS5, PKSs can catalyze decarboxylative condensation of two or three malonyl-CoAs in the presence of fatty acyl-CoAs to generate tri- and tetraketide α-pyrones. The reduction of the tetraketide α-pyrone at the carbonyl function by TKPRs makes a hydroxylated α-pyrone compound that is the proposed constituent of a sporopollenin polymer. However, it still remains to be determined whether these hydroxylated

polyketides are generated *in vivo* according to models of potential sporopollenin biosynthetic pathways [9], even though these enzymes interact to form a biosynthetic metabolon in *Nicotiana benthamiana* [12].

Saccharomyces cerevisiae offers a useful system for the expression of heterologous genes. Using sets of dual expression vectors with different selectable makers on plasmids, it is possible to express various combinations of genes in a single stain. This system allows for the reconstruction of plant natural product biosynthetic pathways in yeast [14, 16]. Unlike plants, which use the plastid as their fatty acid biosynthetic site, the yeast cell synthesizes fatty acids predominantly in the cytoplasm. The major fatty acids produced in yeast are palmitoleic acid (C16:1), palmitic acid (C16:0), oleic acid (C18:1), and stearic acid (C18:0), which are required to generate phospholipids for biological membranes [6]. Owing to the main location of fatty acid synthesis in yeast, malonyl-CoA (which is a building block to generate fatty acids) is ubiquitously present and easily available in the cytoplasm.

In vitro studies have shown that PKSA produces tri- and tetraketide α-pyrones by condensation of fatty acyl-CoAs with malonyl-CoAs [4, 7, 11, 13] and that TKPR1 reduces the keto function of tetraketide α-pyrone compounds
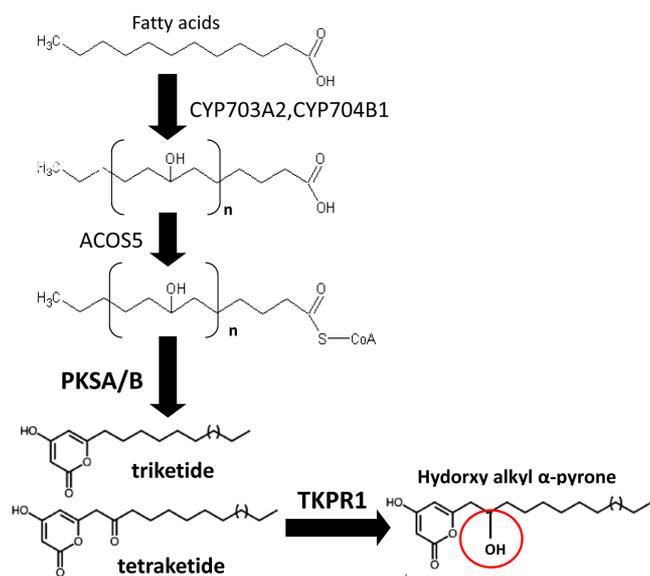
Fig. 1. *In vivo* model of sporopollenin monomer biosynthesis pathway.

This image has been modified and adapted based on Kim and Douglas [10]. PKSA/B are the first committed enzymes of sporopollenin biosynthesis in *Arabidopsis,* yielding tri- and tetraketide α-pyrones. The tetraketide product is then reduced by TKPR1, which leads to polyhydroxyalkyl α-pyrone.
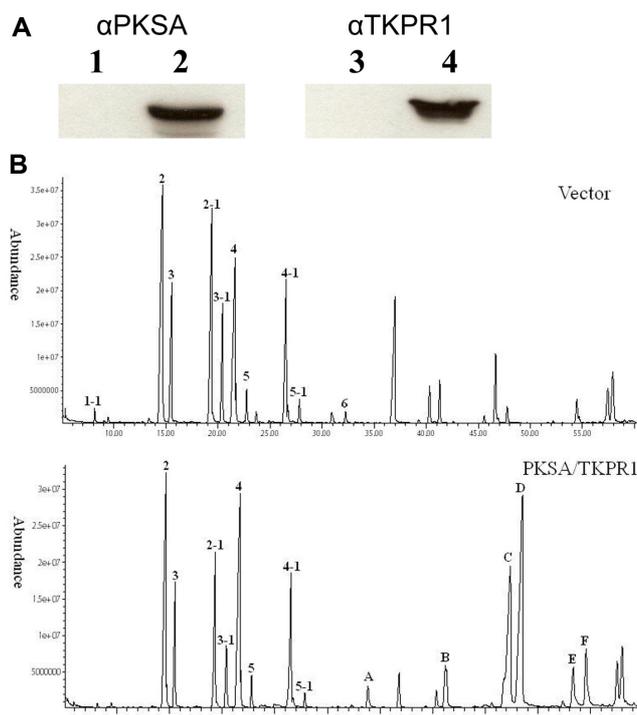

Fig. 2. Immunoblot detection of PKSA and TKPR1 proteins and GC analysis of fatty acid metabolites produced by PKSA/TKPR1-expressing yeast strains.

(**A**) Immunoblot analysis by using polyclonal αPKSA or αTKPR1 antibody. PKSA and TKPR1 antibodies were pre-incubated overnight with an acetonic powder of *S. cerevisiae* to eliminate any nonspecific signal. For immunoblotting experiments, the procedures were as described previously [8] using antibodies at 1/10,000 dilution and a chemiluminescent substrate (Bio-Rad) for phosphatase activity detection. Lanes 1 and 3, vector control; Lanes 2 and 4, PKSA/TKPR1 dual expressor. (**B**) Total fatty acids in yeast cell extracts were transmethylated by adding 1 ml of methanolic-HCl and incubating at 80°C for 1 h. To obtain organic extracts, 1.5 ml of hexane was added followed by vortexing. The organic phases were pooled and evaporated under nitrogen gas. A total of six new compounds (A to F) were detected in the PKSA/TKPR1 expressor. Peaks 1-1, dodecanoic acid trimethylsilyl ether (C12); 2, 9-hexadecenoic acid methyl ether (C16:1); 2-1, 9-hexadecenoic acid trimethylsilyl ether (C16:1); 3, hexadecanoic acid methyl ether (C16); 3-1, hexadecanoic acid trimethylsilyl ether (C16); 4, 9-octadecenoic acid methyl ether (C18:1); 4-1, 9-octadecenoic acid trimethylsilyl ether (C18:1); 5, octadecanoic acid methyl ether (C18); 5-1, octadecanoic acid trimethylsilyl ether (C18); and 6, 9-hexanedioic acid 2,3-*bis* ester.

synthesized by PKSA [9]. To determine the *in vivo* products generated by PKSA or/and TKPR1 using potential substrates present in a wild-type yeast strain (YPH499, genotype *MATa ura3-52 lys2-801 ade2-101 trp-Δ63 his3-Δ200 leu2-Δ1*), *PKSA*, *TKPR1*, and *PKSA/TKPR1* dual-expressing yeasts were generated. Each cDNA was cloned into the pESC-Ura or pESC-Leu vector (Agilent Technologies) under the control of the Gal1 promoter for *PKSA* and the Gal10 promoter for *TKPR1*. To validate the accumulation of PKSA and TKPR1 protein in PKSA/TKPR1 expressor, immunoblotting with PKSA and TKPR1 polyclonal antibodies (kindly provided from Dr. Michel Legrand [9]) was performed and indicated that PKSA/TKPR1 dual-expressing yeast had both abundant PKSA and TKPR1 (Fig. 2A). To monitor metabolic alterations in yeast cells, the cell densities of each strain were measured after 24 h culture in synthetic minimal dropout medium lacking uracil or leucine. In the control strain and *TKPR1* expressor, growth rates reached 5.12 ± 0.13 at OD_{600}, whereas the growth rate of strains containing PKSA appeared abnormal. The PKSA expressor showed that it was close to the initial value, which was 0.40 ± 0.08 (OD_{600}) at 0 h. Moreover, the growth rate of the *PKSA/TKPR1* expressor only reached up to 2.53 ± 0.10. These data suggest that accumulation of

PKSA alone in yeast cells can result in metabolic change and repress growth rate.

To explore the potential products generated by the sporopollenin biosynthetic enzymes in the *PKSA/TKPR1* expressor *in vivo*, total lipophilic compounds ranging from
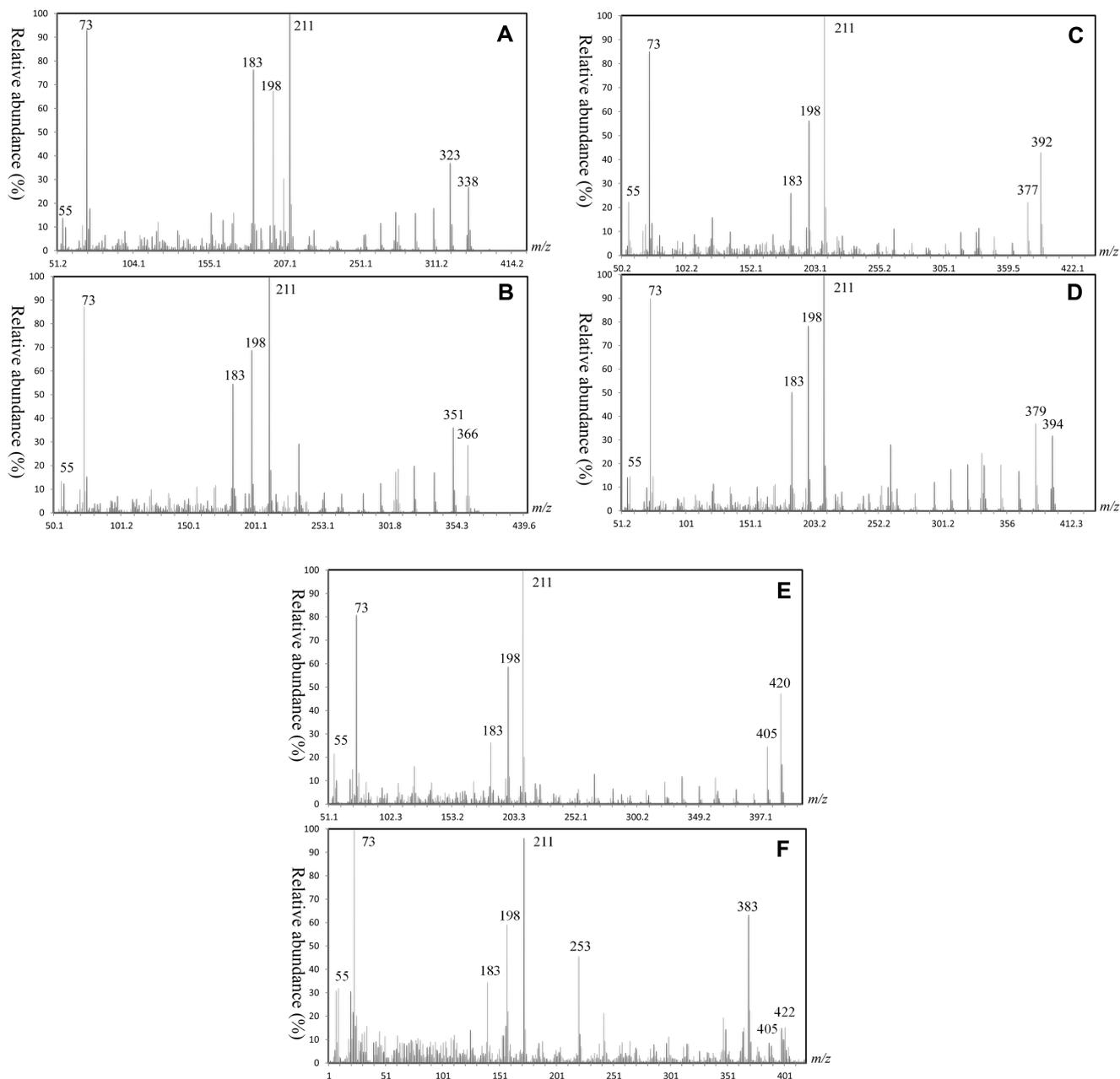
Fig. 3. Mass spectra of novel peaks from the GC chromatogram obtained from the yeast strain expressing PKSA/TKPR1.

For GC-MS analyses, the extraction from the reaction mixture, derivatization of organic compounds, and running condition of samples in GC-MS were previously described [19], except for the following: the oven temperature was programmed for 2 min at 50°C, followed by a 40°C/min ramp to 120°C, held at 120°C for 2 min, increased by 2°C/min to 225°C, and held at 320°C for 10 min. Each mass spectrum has three common characteristic peaks at *m/z* 183, 198, and 211, together with different possible total molecular weights of 338, 366, 392, 394, 420, and 422, respectively. Spectra were obtained at 33.89 min (**A**), 41.21 min (**B**), 47.30 min (**C**), 48.35 min (**D**), 53.34 min (**E**) and 54.33 min (**F**), of retention time.

medium fatty acid (FA) derivatives to very long-chain FA derivatives were profiled by gas chromatography-mass spectrometry (GC-MS). To assay the FA metabolic profile in each strain, lipophilic compounds were extracted with

hexane and then derivatized [19]. The FA derivatives were identified by comparing their GC-MS characteristics with literature data. In comparison with the *TKPR* expressor (data not shown) and the empty vector strain, *PKSA/*

TKPR1 expressors exclusively generated a total of six unidentified compounds in the GC chromatograms (A to F in Fig. 2B). Each peak had three fragments of the same characteristic masses (183, 198, and 211 *m/z*), while each peak had different total masses (338, 366, 392, 394, 420, and 422 *m/z*, respectively) (Fig. 3). Since these novel peaks were present only in PKSA/TKPR1-expressing strains, they are likely to be polyketide products, supporting the presence of active PKSA enzymes in the strains even though they could not be identified in mass spectrum library and by the literature.

To identify novel products found in PKSA/TKPR1-expressing strains, the molecular weight for possible triketide and tetraketide α-pyrones was calculated and compared with the mass spectra. This result indicated that the chemical structures likely are triketide α-pyrone in these strains (Fig. 4). This is consistent with previous *in vitro* studies suggesting that PKSA catalyzes the condensation of malonyl-CoA units with fatty acyl-CoAs of various chain lengths [4, 7, 11, 13]. Based on the total masses of the putative α-pyrone polyketides that accumulated, it appears that PKSA accepts C12:0, C14:0, C16:0, C16:1, C18:0, and C18:1 fatty acids *in vivo* to generate triketide α-pyrones.

Pentadecyl triketide α-pyrones, generated from condensation of C16:0 or C16:1 with malonyl-CoA, were the major products in these yeast strains, suggesting that C16 and C16:1 are preferable substrates for PKSA *in vivo*. Interestingly, although C12 and C14 fatty acid derivatives were not detected by GC in the *PKSA/TKPR1* expressor, undecyl and tridecyl triketides α-pyrones generated by condensation reaction with C12 and C14, respectively, were present in this co-expressor (Fig. 2B). This suggests that PKSA might have a higher affinity for medium chain fatty acyl-CoAs than acyl-CoA thioesterases catalyzing the hydrolysis of acyl-CoAs to the free acids and CoAs for further reaction.

The estimated molecular weights of triketides and tetraketides as well as the congruence of predicted and observed fragmentation patterns of triketide α-pyrones suggest that PKSA generated triketide α-pyrones rather than tetraketide α-pyrones (the TKPR1 substrate *in vitro*; [9]). Moreover, no significant changes in the metabolite profiles were detected in strains expressing TKPR1, even though TKPR1 was clearly present in both soluble and in cell debris fractions of these strains (data not shown). The likely reason for the lack of TKPR1-specific products in these strains is that TKPR1 substrates were absent. We previously suggested that triketide α-pyrone corresponds to a derailment reaction product due to incomplete catalysis
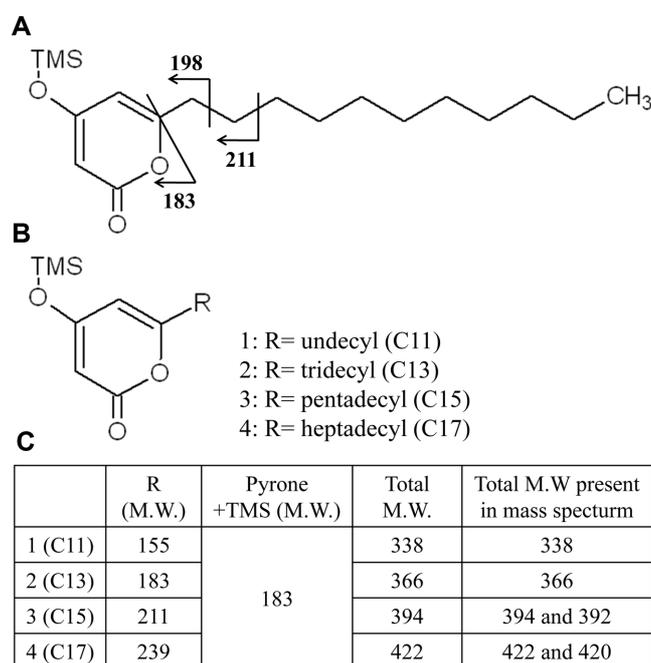

Fig. 4. Predicted GC-MS fragmentation patterns and estimated molecular weights of α-pyrones containing various alkyl chain lengths.

(**A**) Based on characteristic *m/z* values from the mass spectra in Fig. 3, predicted fragments are drawn for the triketide compound (**A**) and possible alkyl chains on the R position (**B**). (**C**) Calculated molecular weights (M.W.) of alkyl α-pyrones are 338, 366, 394, and 422 from R-group alkyl chain lengths of C11, C13, C15, and C17, respectively. These values correspond to the total molecular weight (*m/z*) present in Fig. 3. Both 392 and 420 (*m/z*) would be unsaturated alkyl chains containing one double bond, of which start molecules are likely 9-hexadecenoic acid (C16:1) and 9-octadecenoic acid methyl ether (C18:1).

[11]. Thus, heterologously expressed *Arabidopsis* PKSA appears to perform only two rounds of condensation with malonyl-CoA in yeast cells, generating triketide α-pyrones that do not provide substrates for TKPR1. Interestingly, the total cell density at OD_{600} was much lower in the *PKSA* expressor than in the *PKSA/TKPR1* expressor. Under microscopy, *PKSA* expressors appear abnormal in morphology and devoid of budding daughter cells, showing that *in vivo* novel compounds generated by PKSA could inhibit cell growth or proliferation. These data indicate that TKPR1 could metabolize endogenous PKSA substrates into unknown products, making them less available for PKSA activity and resulting in restored yeast cell proliferation. Alternatively, the TKPR1 enzyme could act on the product generated by PKSA, leading to new compounds not detected in this metabolic profile. Altogether, our data suggest that yeast co-expressing PKSA and TKPR1 could be a platform

strain to test the potential sporopollenin biosynthesis pathway *in vivo*.

Acknowledgments

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