Functional identification of sterol-4α-methyl oxidase cDNAs from *Arabidopsis thaliana* by complementation of a yeast *erg25* mutant lacking sterol-4α-methyl oxidation[1]

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Abstract Specific primers derived from both genomic sequence data and EST cDNA sequences were used to polymerase chain reaction amplify two full-length cDNA sequences (*At*SMO1 and *At*SMO2), 801 and 783 bp, respectively, from an *Arabidopsis thaliana* cDNA library. The predicted proteins show 32 and 29% identity to the *ERG25* gene from *Saccharomyces cerevisiae* which encodes the sterol-4α-methyl oxidase (SMO), a membrane-bound non-heme di-iron oxygenase involved in lipid metabolism. Heterologous expression of *At*SMO1 and *At*SMO2 in a yeast *erg25* ergosterol auxotroph, lacking SMO activity, restored growth and endogenous ergosterol synthesis. These results represent the first functional identification of SMO genes from plants.

Key words: Hydroxylase; Sterol; Methyl oxidase; ERG25; Non-heme iron oxygenase; *Arabidopsis thaliana*

1. Introduction

Sterols are ubiquitous and essential membrane components found in all eukaryotes. The structure of sterols and their biosynthetic pathway differ significantly between fungi, animals and plants [1–3]. Nevertheless, in these organisms the sterol molecule becomes functional only after removal of the three methyl groups at C4 and C14 [4]. We have shown in higher plants, as well as in animal and fungi, that sterol-4α-methyl oxidation proceeds via three successive monooxygenations of the 4α-methyl groups by sterol-4α-methyl oxidase (SMO) to produce a 4α-carboxylic sterol derivative [5] (Fig. 1). This acid is subsequently oxidatively decarboxylated by the 4α-carboxysterol-C3-dehydrogenase/C4-decarboxylase (4α-CD) [6] with concomitant transposition of the 4β-methyl substituent to the 4α position. The resulting 3-oxosteroid is finally reduced stereospecifically by a NADPH dependent sterone reductase (SR) [7] to yield the corresponding monodemethylated sterol. While in yeast and animals both C4-methyl groups are oxidized by the same SMO [8,9], in higher plants, biochemical data strongly suggest that two distinct SMOs are involved in the removal of the C4-methyl groups of 4,4-dimethyl- and 4α-methyl-sterols, respectively [5]. In addition, membrane-bound cytochrome b_5 was shown to be an obligatory electron carrier from NAD(P)H to the SMO in both animals [10] and plants [11]. In *Saccharomyces cerevisiae*, the SMO gene, *ERG25*, has been isolated and characterized and the disruption of *ERG25* was found to be lethal [9]. In addition, 4α-CD and SR were shown to be encoded by *ERG26* [12] and *ERG27* [13] genes, respectively. Lastly, biochemical data obtained with either the plant or animal microsomal preparations indicated that cytochrome *P450* is not involved in the SMO reaction but suggested the involvement of a non-heme iron oxygenase [5,14].

In contrast to the large amount of knowledge accumulated about cytochrome *P450* systems, very little is known regarding the small but still increasing family of membrane-bound non-heme iron oxygenases which catalyze hydroxylation, desaturation, epoxydation or acetylation of hydrophobic substrates in higher plants [15,16]. Therefore, enzymatic oxidation of the C4-methyl group of plant sterol precursors is of itself interesting in addition to the important role these enzymes have in the sterol biosynthetic pathway. Here we report the cloning and sequencing of two SMO isoenzymes from *Arabidopsis thaliana*. Complementation of a *S. cerevisiae erg25* mutant with the corresponding cDNAs has permitted the first functional identification of SMO genes from higher plants.

2. Materials and methods

2.1. Strains, media and culture conditions

2.1.1. Escherichia coli, XL1Blue. Δ(mcrA) 183 Δ(mcrCB-hsd SMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac. This strain was used to transform and maintain all plasmids used in this study.

2.1.2. S. cerevisiae. The methyl oxidase deficient strain, *erg25-25c* (Mat a *ade5, his3, leu2-3, ura3-52, upc2, erg25-25c)* used in this study has been previously described [9]. Sterol auxotrophs were grown an-

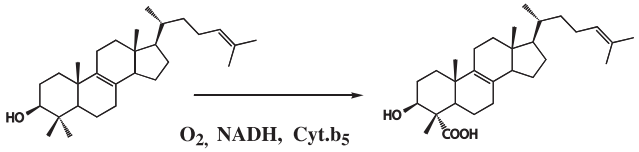

Fig. 1. Reaction catalyzed by the SMO.

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[1] The nucleotide sequences reported in this paper can be accessed through the GenBank/EBI Data Bank with accession numbers AF346734, AF327853 and AF222719.

Abbreviations: SMO, sterol-4α-methyl oxidase; GC, gas chromatography; GC–MS, coupled gas chromatography–mass spectroscopy; ADH, alcohol dehydrogenase; PCR, polymerase chain reaction

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aerobically at 30°C on solid enriched medium (YPG; 1% yeast extract, 2% peptone, 2% glucose) supplemented with 2% ergosterol, or aerobically on liquid minimal medium (YNB; 0.67% yeast nitrogen base, 2% glucose) containing suitable supplements (50 mg/l each), casamino acids (1 g/l) and 0.2% ergosterol or cholesterol. Sterol prototrophic strains were grown aerobically at 30°C for 48 h on solid or liquid minimal medium (YNB) containing suitable supplements (50 mg/l each).

2.2. Plasmid

A pVT102U [17] *S. cerevisiae* shuttle vector was used for cloning, sequencing and transformation of the *erg25* strain. This plasmid contains an *E. coli* origin of replication, a yeast 2 μ origin of replication, an *E. coli* ampicillin resistance gene and the yeast *URA3* gene encoding an orotidine-5-phosphate decarboxylase. This plasmid contains an expression cassette including the alcohol dehydrogenase (ADH) promotor and terminator.

2.3. Polymerase chain reaction (PCR) based cloning and plasmid construction

A BLAST search with the *ERG25* gene from *S. cerevisiae* revealed significant sequence similarity with the hypothetical protein of the EST clone 116C13T7 from the *A. thaliana* EST clone database (accession number T42651). This complete EST clone and the corresponding genomic sequence (accession number At1g07420) were used to construct specific primers SDF17 (ataatactagaatggcttccttcgtggaatctggttg) and SDR8 (ataatactcgagtcacgtttgtttcatgtcaccg), containing *Xho*I and *Xba*I restriction sites for PCR amplification of a 801 bp coding region from *A. thaliana* using a cDNA library of developing siliques [18] as template. This cDNA (accession number AF346734) was cloned into *Xba*I and *Xho*I of pVT102U to generate the plasmid pVT-*At*SMO1. Both strands of the insert were sequenced to ensure sequence fidelity.

A BLAST search also revealed significant sequence similarity to another hypothetical protein of the *Arabidopsis* EST clone 241A21T7 (accession number N65676). After sequencing, this 732 bp incomplete EST clone (AF222719) and the corresponding genomic sequence (accession number At2g29390) were used to construct specific primers SDF7 (ataatatctagaatggattctctcgttgaatccgg) and DL6 (gccgctcgagtcaggtttcttttagggccttaag) containing *Xho*I and *Xba*I restriction sites, respectively for PCR amplification of a 783 bp coding region from *A. thaliana* using the CD4–7 library [19] as a template. This cDNA (accession number AF327853) was cloned into *Xba*I and *Xho*I of pVT102U to generate the plasmid pVT-*At*SMO2. Again both strands of the insert were sequenced to confirm PCR fidelity.

S. cerevisiae ERG25 cDNA was amplified by PCR from a preparation of *S. cerevisiae* genomic DNA using gene specific primers (extended by *Xho*I and *Spe*I restriction sites, respectively). The 900 bp coding region was ligated into the *Xho*I and *Xba*I restriction sites of vector pVT102U yielding pVT-*Sc*ERG25 which showed 100% identity with the known ERG25 gene (accession number U31885).

2.4. Transformations

S. cerevisiae transformations were carried out using the lithium acetate procedure previously described [20]. Transformed *erg25* yeast strains were selected for uracil prototrophy and grown anaerobically at 30°C in the presence of exogenous sterol and subsequently tested for the ability to grow without added sterol.

2.5. Sterol analysis

Lyophilized yeast cells (10–30 mg) were sonicated in the presence of methanol/KOH (6%, w/v) (2 ml) for 10 min and heated in the same medium at 70°C under reflux for 2 h. The mixture was diluted with 1 vol of water and total sterols were extracted three times with 3 vol of *n*-hexane. The extract was dried on Na_2SO_4, evaporated to dryness and analyzed by gas chromatography (GC). GC analysis was carried out with a Varain GC model 8300 (Les Ulis, France) equipped with a flame ionization detector at 300°C, column injector at 250°C and a fused capillary column (WCOT: 30 m×0.25 mm i.d.) coated with DB1 (H_2 flow rate of 2 ml/min). The temperature program used included a 30°C/min increase from 60 to 240°C and then a 2°C/min increase from 240 to 280°C. Relative retention times (t_R) are given with respect to cholesterol ($t_R = 1$). Identification of individual sterols was finally performed using a GC–mass spectroscopy (MS) spectrometer (Fison MD800) equipped with an 'on column' injector and a capillary column (30 m×0.25 mm i.d.) coated with DB1. Sterols were unequivocally identified by coincidental retention time and an electron impact spectrum identical to that of authentic standards [21].

2.6. Identity scores and phylogenetic tree

The phylogenetic tree was developed with the aligned sequences from GCG files (version 9.1-UNIX) and the PAUP (Phylogenetic Analysis Using Parsimony, version 4.0.0 d55 for UNIX) program. A bootstrap analysis using a heuristic search was performed in the present case [22]. Distance was chosen as the optimal criterion for tree search. The number of bootstrap replicates was 100. The phylogenetic tree was drawn with TREEVIEW [23].

3. Results and discussion

3.1. PCR based cloning of full-length cDNAs encoding SMO

In order to isolate full-length cDNAs encoding *A. thaliana* enzymes able to oxidize the 4α-methyl group of sterol precursors, we started with an *A. thaliana* EST clone 116C13T7 (accession number T42651) whose deduced protein sequence showed 33% identity with both *Homo sapiens* [8] and *S. cerevisiae* [9] SMO. We used this EST probe and the corresponding genomic sequence (At1g07420) to design specific primers for PCR amplification using an *A. thaliana* cDNA library [18] as template. A DNA fragment corresponding to the expected size was amplified and cloned in the pVT102U shuttle plasmid under the control of the constitutive ADH promotor (see Section 2). Sequencing of this clone indicated an open reading frame (ORF) of 801 bp having 100% identity with both the At1g07420 gene and the EST116C13T7 probe. This fragment designated *At*SMO1 (AF346734) encodes a 267 amino acid polypeptide.

Similarly, the EST clone 241A21T7 (accession number N65676) was shown to contain a 732 bp fragment (AF222719) and the deduced protein sequence had 35 and 40% identity with *H. sapiens* [8] and *S. cerevisiae* [9] SMO, respectively. However, this polypeptide did not contain a complete ORF. A corresponding *A. thaliana* coding sequence deduced from the genomic sequence At2g29390 was totally

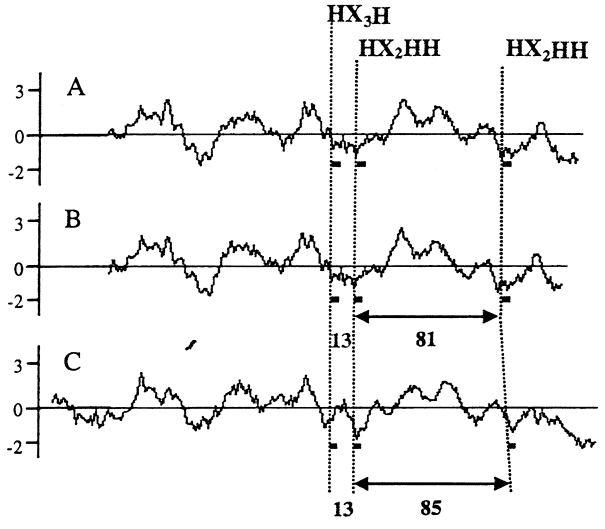

Fig. 2. Comparison of the hydropathy plots of the *A. thaliana* SMO *At*SMO1 sequence (A) and *At*SMO2 sequence (B) with that of the *S. cerevisiae* SMO (ERG25) sequence (C). Vertical lines indicate regions corresponding to the three histidine-rich motifs that show homology to the membrane-bound enzymes of the *ERG3–ERG25* family.

identical to the above-mentioned EST sequence and encoded a polypeptide containing the missing N-terminal amino acids. We used both sequences to design, as described previously for *At*SMO1, specific primers to PCR amplify a full-length cDNA with the expected size using an *A. thaliana* cDNA library [19] as template. This PCR product was also cloned into the pVT102U plasmid. Sequencing of this clone confirmed an ORF of 783 bp which had 100% identity with both genes At2g29390 and EST241A21T7. It encoded a polypeptide of 261 amino acids designated *At*SMO2 (AF327853). Both *At*SMOs are characterized by the presence of three histidine-rich motifs, HX_3H, HX_2HH, HX_2HH, which exhibit a topology and spacing of amino acids within the histidine motifs typical of the *ERG3–ERG25* family (see Fig. 2) from animal, fungi or plants [24], but distinct from that found in the extended family of membrane fatty acid desaturase/hydroxylase [15]. We have recently demonstrated by mutational analysis of the recombinant *A. thaliana* Δ^7-sterol-C5(6)-desaturase (ERG3), that all of the eight histidine residues were essential for the catalysis by this oxygenase [24]. One possible function for these tripartite motifs would be to provide the ligands for a presumed catalytic Fe center, as has been proposed for a number of integral membrane enzymes catalyzing desaturations and hydroxylations [15].

The dendrogram in Fig. 3 was constructed from the full-length amino acid sequences of SMO and sterol-desaturase-like enzymes. The protein sequences of *At*SMO1 and *At*SMO2 show 29–32 and 33% identity with the yeast and human SMO, respectively. They show 22 and 20% identity with the recently characterized *A. thaliana* SUR2 gene [25], and only 20 and 19% identity compared to the *A. thaliana* Δ^7-sterol-C5(6)-desaturase (*ERG3*) [26]. In addition, the protein sequences of *At*SMO1 and *At*SMO2 are 89% identical to each other. High global and local sequence identity to other SMOs including the presence and spacing of the three histidine-rich motifs and clustering of other amino acids to *ERG25* sequences strongly suggest that *At*SMO1 and *At*SMO2 code for two plant SMO isoenzymes.

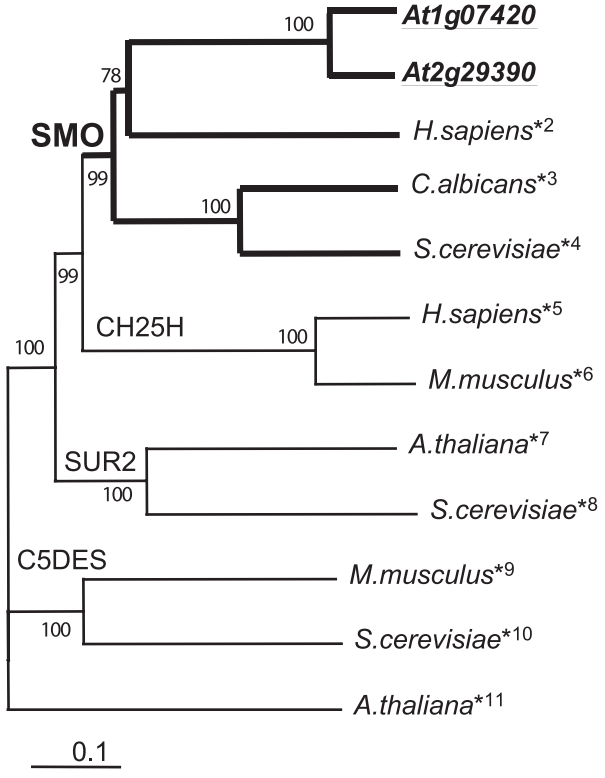

Fig. 3. Dendrogram of SMO and sterol-desaturase-like proteins. Full-length amino acid sequences were aligned and used to generate a phylogenetic tree using the program paupsearch based on method heuristic and distances, coupled with treeview for visualizing. CH25H, cholesterol-25 hydroxylase; SUR2, C4-sphingolipid hydroxylase; C5DES, Δ7-sterol-C5(6)-desaturase. GenBank database accession: At1g07420 (SMO1): AF346734; At2g29390 (SMO2): AF327853; 2: NM 006745; 3: AAC06014; 4: P53045; 5: AF059214; 6: AAC97480; 7: AC013289; 8: AAA16608; 9: BAA33730; 10: NP 013157; 11: AAD38120.

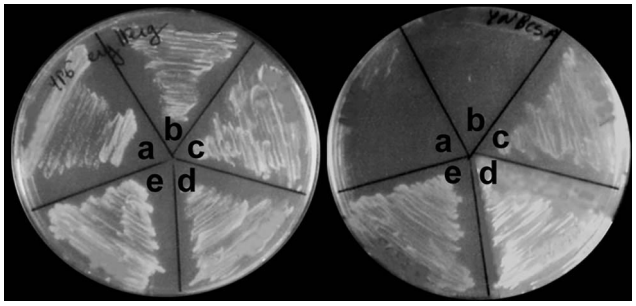

Fig. 4. Growth responses of untransformed *erg25* (a), or *erg25* transformed with pVT-VOID (b), pVT-*At*SMO1 (c), pVT-*At*SMO2 (d) and pVT-*Sc*ERG25 (e) grown anaerobically on rich medium, YPG supplemented with ergosterol (2%) (left panel), or aerobically on minimal medium (right panel). All plates were grown for 48 h.

3.2. *Restoration of ergosterol prototrophy and reconstitution of ergosterol biosynthesis in a yeast erg25 mutant by expression of A. thaliana AtSMOs*

For a functional identification, the cloned SMOs pVT-*At*SMO1, pVT-*At*SMO2, pVT-*Sc*ERG25 and the empty control vector, pVT102U (pVT-VOID) were transformed into the *erg25* mutant strain of *S. cerevisiae* lacking the SMO. In *Saccharomyces*, the *ERG25* product, the SMO, is an essential enzyme since disruption of *ERG25* is lethal as the *erg25* strain requires ergosterol (or cholesterol) supplementation for viability [9]. Thus the pVT-VOID transformants were first plated onto an ergosterol containing medium then replicated onto a medium devoid of sterol. As shown in Fig. 4, the *erg25* strain transformed with pVT-*Sc*ERG25, pVT-*At*SMO1 and pVT-*At*SMO2 was capable of growing aerobically without ergosterol supplementation while *erg25*-VOID transformants and untransformed *erg25* strains could grow only on an ergosterol supplemented medium. These data indicate that *At*SMO1 and *At*SMO2 as well as *ScERG25* inserts could restore growth in the absence of ergosterol.

Sterol auxotrophic strains, *erg25* and *erg25* transformed with the empty vector, pVT102U (*erg25*-VOID), were grown in cholesterol containing media for sterol analysis by GC and GC–MS and sterol profiles are listed in Table 1. In addition, sterol profiles of prototrophic strains *erg25*-*Sc*ERG25, *erg25*-*At*SMO1 and *erg25*-*At*SMO2 grown in a non-sterol medium were also determined. Under these conditions, the *erg25* and *erg25*-VOID strains accumulated lanosterol (peak 1, Fig. 5) and 4,4-dimethyl-zymosterol (peak 2, Fig. 5) but no ergosterol as previously shown [9] (Fig. 5, Table 1). In contrast, strains *erg25*-*Sc*ERG25, *erg25*-*At*SMO1 and *erg25*-*At*SMO2 accumulated C4-demethylated sterols including ergosterol (peak 4,

Table 1
Sterol composition of mutant *erg25* transformed with different plasmids

Sterol	GC t_R (DB1)	Strain							
		Erg25	*erg25*+VOID plasmid	*erg25*+*Sc*ERG25		*erg25*+*At*SMO1		*erg25*+*At*SMO2	
		(% Σ)							
Cholesterol added to the medium		yes	yes	yes	no	yes	no	yes	no
Lanosterol	1.225	24.6 ± 5.6	18.2 ± 7.0	3.8 ± 0.4	3.7 ± 0.2	8.4 ± 2.2	20.7 ± 2.0	7.7 ± 2.2	23.2 ± 2.0
4,4-diMe-zymosterol	1.236	46.8 ± 8.4	44.3 ± 5.8	7.2 ± 0.2	6.8 ± 1.7	57.9 ± 0.4	45.2 ± 4.2	53.5 ± 4.5	36.6 ± 6.2
Eburicol	1.267	4.6 ± 4.6	5.9 ± 3.9	0.5 ± 0.5	–	1.5 ± 1.5	3.2 ± 0.3	2.4 ± 0.2	3.8 ± 0.6
4,4-diMe-fecosterol	1.306	22.9 ± 8.3	30.1 ± 5.0	0.8 ± 0.8	0.7 ± 0.5	20.9 ± 6.4	17.7 ± 2.0	21.7 ± 3.0	17.2 ± 0.8
Total 4,4-dimethyl-sterols		**100.0**	**100.0**	**12.3 ± 2.0**	**11.3 ± 2.4**	**89.1 ± 2.6**	**86.8 ± 2.8**	**85.1 ± 3.6**	**80.7 ± 8.4**
Ergosta-tetraenol	1.040	–	–	3.4 ± 0.7	4.5 ± 0.5	2.4 ± 2.4	5.3 ± 0.6	2.7 ± 0.3	5.4 ± 0.9
Zymosterol	1.052	–	–	6.3 ± 2.0	6.3 ± 1.9	–	–	–	–
Ergosterol	1.085	–	–	75.1 ± 7.6	75.2 ± 6.5	8.4 ± 0.2	7.9 ± 0.6	12.1 ± 3.4	13.8 ± 7.4
Fecosterol	1.126	–	–	0.7 ± 0.7	0.7 ± 0.7	–	–	–	–
2,2-Dihydroergosterol	1.147	–	–	1.2 ± 1.2	1.1 ± 1.1	–	–	–	–
Total 4-desmethyl-sterols		**0**	**0**	**87.7 ± 2.0**	**88.7 ± 2.4**	**10.9 ± 2.6**	**13.3 ± 2.8**	**14.8 ± 3.6**	**19.2 ± 8.4**

Values represent the mean ± S.E.M. of two or three independent transformations.

Fig. 5) in addition to 4,4-dimethyl-sterols. These sterols accumulated both in presence and absence of added cholesterol in the medium. However, while *erg25-Sc*ERG25 accumulated large amounts of ergosterol and minor amounts of residual 4,4-dimethyl-sterols, the complemented strains *erg25-At*SMO1 and *erg25-At*SMO2 accumulated both ergosterol and 4,4-dimethyl-zymosterol, indicating only partial complementation by the *A. thaliana* genes. Taken together, these results can be ascribed to the activity of SMOs coded by *At*SMO1 and *At*SMO2.

The complementation levels obtained with *At*SMO2 appeared to be slightly higher than with *At*SMO1. Although these two isoenzymes share 89% identity, their substrate selectivity and particularly their catalytic efficiency towards 4,4-dimethyl-zymosterol (the physiological substrate of *Sc*SMO), could be different. Coincidentally, C4-methylated-Δ^8-sterols such as 4,4-dimethyl-zymosterol were shown to be poor substrates of SMOs from *Zea mays* [5]. Although the substrate selectivity of the *At*SMOs could be different from that of the *Z. mays* SMOs, 4,4-dimethyl-zymosterol is probably metabolized with a poorer efficiency by the *At*MSOs than by the *Sc*SMO. In addition, the absence of accumulation of 4α-monomethylated sterols following transformation with both *At*SMO isoenzymes suggests that they catalyze oxidation of both 4,4-dimethyl- and 4α-methyl-sterol intermediates when expressed in a yeast *erg25* mutant background. These data would be consistent with a reduced substrate specificity for the *At*SMOs compared to SMOs from *Z. mays* which appear to be specific for the oxidation of 4,4-dimethyl- or 4α-methyl-sterols, respectively [5], possibly reflecting a significant divergence between SMOs from different plants. Similarly, a reduced substrate specificity was also observed in vivo for an *Arabidopsis* sterol-methyl transferase (SMT) [27] when compared to that of *Z. mays* SMT [28], when the two plant genes were expressed in the same yeast *erg6* mutant background. The apparent lower demethylation efficiency obtained with the demethylation complex comprising the heterologous *At*SMO isoenzymes would suggest that in vivo, the initial oxidation steps catalyzed by the SMO are partially rate-limiting relative to the overall demethylation process in the *erg25* mutant background.

Recent studies performed with a yeast two-hybrid strain

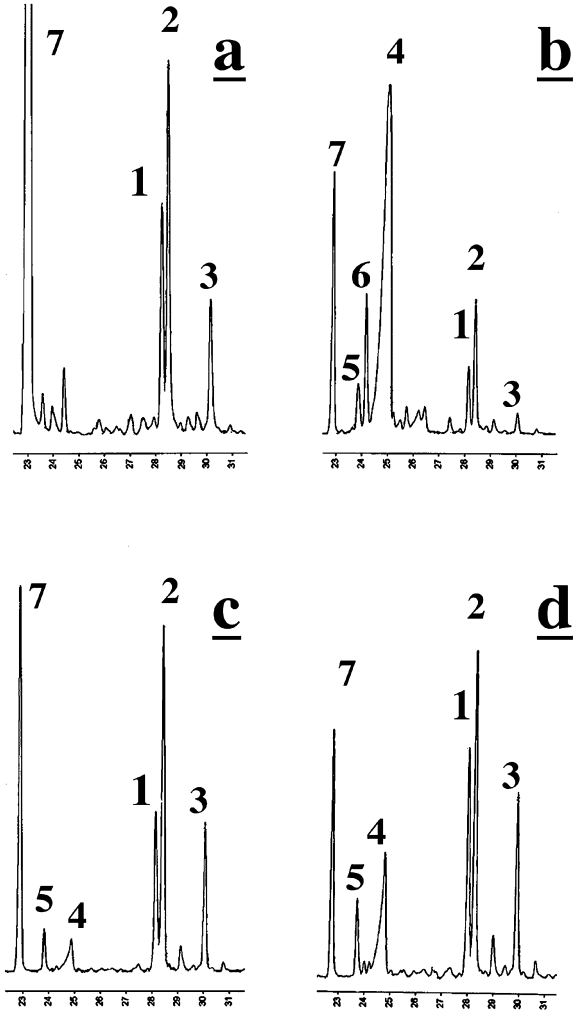

Fig. 5. GC profiles of *erg25* strain complemented with different plasmids: with the empty vector pVT102U (a), the major sterols are lanosterol (peak 1), 4,4-dimethyl-zymosterol (peak 2), and 4,4-dimethyl-fecosterol (peak 3). Formation of ergosterol (peak 4) is observed in *erg25* cells expressing the *S. cerevisiae* Erg25 protein (b) and the *A. thaliana* SMO1 (c) and SMO2 (d) proteins. Peak 7 is cholesterol from the medium (a), or added as internal standard (b,c,d). Peak 5 is dehydroergosterol; peak 6 is zymosterol.

indicated that a *Sc*SMO protein, Erg25p, physically associates with Erg26p and Erg27p within the cell and suggested that they form a heterocomplex within the yeast ER [29]. In addition, a novel but functionally uncharacterized gene, *ERG28*, has been identified in yeast which could be involved in tethering Erg26p and Erg27p to the ER or facilitating interaction between these proteins [30]. Our data suggest that in vivo integration of the recombinant *At*SMOs into the multienzymatic C4-demethylation system appears to be functional. Particularly, interaction and subsequent electron transfer between endogenous yeast cytochrome b_5 and the recombinant plant SMO proteins appear to be efficient.

Our data do not yet allow the determination as to which of the two putative plant SMO isoenzymes catalyzes the various enzymological reactions and the answer will require a thorough enzymological study performed with the microsomal preparation from the corresponding yeast strains and putative substrates of plant SMO.

Finally, the physiological consequences of the presence of C4-methylated sterols remain to be explored in plants. In this regard, no mutants affecting the plant C4-demethylation system have been described, so it is unclear whether such mutants may or may not result in lethality. Thus, a functional biochemical and genetic analysis of plant C4-demethylation enzymes and genes may suggest possible new targets for obtaining inhibitors that might have antifungal or herbicidal properties.

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