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Functional identification of AtFao3, a membrane bound long chain alcohol oxidase in *Arabidopsis thaliana*

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Abstract The *Arabidopsis thalina* genome database was searched for homologues of the *Candida cloacae fao1* gene which encodes a membrane bound, flavin-containing, hydrogen peroxide generating, long chain alcohol oxidase. This gene has not been isolated from plants or animals. Four putative candidates were found in the database but their function has not been proven. The cDNAs for two of them were cloned by RT-PCR from *Arabidopis* suspension culture and one of them [AtFAO3] was overexpressed in *Escherichia coli* and shown to functionally express long chain alcohol oxidase activity. The protein has been solubilised and retains biological activity thereby preparing the way for crystallographic studies. This is the first functional proof identifying a long chain alcohol oxidase in higher plants.

Keywords: Alcohol oxidase; Flavin; Haem; RT-PCR; Substrate selectivity; *Arabidopsis*

1. Introduction

The oxidation of alkanes and long chain fatty acids in industrial yeasts occurs via two sequential oxidative pathways: (a) the membrane bound ω-oxidation pathway and (b) the β-oxidation pathway located in the peroxisomes. During ω-oxidation, the methyl end of the molecule is oxidised successively by a cytochrome P450 alkane/fatty acid oxidase, a hydrogen peroxide-generating alcohol oxidase, and an aldehyde dehydrogenase producing ω-alcohols, ω-aldehydes and ω-fatty acids, respectively. The eventual products of ω-oxidation are dicarboxylic acids, which are imported into the peroxisomes following activation to an acylCoA. The ω-oxidation pathway is commercially important in the production of dicarboxylic acids, via yeasts, which have great utility in a number of industrial applications. We have previously purified long chain fatty alcohol oxidases from *Candida tropicalis* and *Candida cloacae* [1]. The protein is strongly induced by growing on alkanes, is membrane bound and is a flavin-dependent enzyme unlike classical alcohol oxidases, which are nicotinamide dependent. The cloned gene from *C. tropicalis* encodes a protein of 70 kDa and has five identifiable functional domains, three of which are shared by the GMC-oxidoreductase family of proteins which includes cholesterol and glucose oxidase, and a unique fourth haem-binding domain present in flavin dependent, fatty alcohol oxidases. Additionally, fatty alcohol oxidases have an extra N-terminal variable region of approximately 200 amino acids. The importance of this region is unknown.

In plants and animals, there is little knowledge on membrane bound alcohol oxidases primarily due to the difficulty in purifying this type of enzyme. *Simmondsia chinensis* [jojoba], which accumulates long chain alcohol-containing waxes as a major storage product, has a lauryl alcohol oxidase in germinating seedlings [2]. This is presumably involved in the mobilisation of seed storage reserves. No genes for long chain membrane bound alcohol oxidases have been cloned from any plant species. With the completion of the *Arabidopsis thaliana* genome sequencing programme, a large number of open reading frames have been identified that have some similarity to known genes from other sources, but their function still remains to be proven, a substantial number of open reading frames correspond to ''unknown proteins'', ''hypothetical proteins'' or ones with ''homology'' to other proteins whose function has not been proven. One of the main challenges following completion of the *Arabidopsis* whole genome sequencing exercise is elucidation of the functional characterisation of all biological activity of these proteins. Four homologues of the *C. cloacae* long chain fatty alcohol oxidase (Fao1) have been identified in the *Arabidopsis* genome [http://Arabidopsis.org/servlets/search] with Accession Nos.: AT1g03990, AT4g19380, AT4g28570, and AT3g23410, these are present on chromosomes 1, 4, 4 and 3, respectively. Hereafter, for convenience we refer to these as AtFAO1, AtFAO4a, AtFAO4b and AtFAO3. In this paper, we report on the cloning of full length cDNA for two of these (AtFAO3: AT3g23410; AtFAO4b: AT4g28570) from suspension cultures of *A. thaliana* and successful overexpression of one of them [AtFAO3] as a his-tagged protein in *Escherichia coli* using the pET101 overexpression system. Purified AtFao3 has higher activity than *Candida* yeast Fao1 when using 1-hexadecanol as substrate. This is the first functional identification of a gene and the corresponding protein of a long chain alcohol oxidase from plants.

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2. Materials and methods

2.1. Arabidopsis *suspension culture*

Suspension cultures of *A. thaliana Landsberg Erecta* were kindly supplied by Dr. Mike May (University of Oxford, UK) [3]. The cultures were maintained by weekly sub-culturing using 10% of a 7-day-o1d culture as inoculum to 100 ml fresh medium. The cultures were grown in 250 ml Erlenmeyer flasks under a 16 h photoperiod at 25 °C on a rotating (120 rpm) platform.

2.2. RT-PCR quantification of AtFAO3 and AtFAO4b gene expression in Arabidopsis *suspension culture*

RNA samples were prepared from 7-day-old suspension culture. They were treated with RNase-free DNase I (Life Technologies, Inc.) at room temperature for 15 min to remove possible DNA contamination. DNase I was subsequently inactivated at 65 °C for 10 min in the presence of 2.5 mM EDTA. The resulting RNA (2 μg) was used as a template for reverse transcription using the Clontech RT-PCR kit following the manufacturer's instructions. The primer sets used for AtFAO3 were: 5′-gCCATggAgAgCATTgAgCTggTCACg-3′ (start codon ATG included) for the 5′-end sense chain and 5′-ATgAgATAAACCAgTggTCATggAC-3′ (just before stop codon TGA) for the 3′-end anti-sense chain. The other primer sets used for AtFAO4b were: 5′-gCCATggAAgACgTTagAAgAagAAACAgAgg-3′ (start codon ATG included) for the 5′-end sense chain and 5′-TACTTTTgTTTTgTTTTgCAgCgAgTC-3′ (just before stop codon TAG) for the 3′-end anti-sense chain. PCRs were carried out for 35 cycles with denaturing at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 3 min and were performed in a Robocycler (Stratagene).

2.3. Sequencing

All PCR products were completely sequenced by using an Applied Biosystems Model 373 DNA sequencer. Computer analysis of DNA sequences was carried out using DNA Strider [4].

2.4. Sequence alignment

Basic alignment was done by using CLUSTAL W programme [5].

2.5. Expand PCR

To amplify authentic cDNA of AtFAO3 for directional cloning, the Expand PCR system containing a proof reading DNA polymerase (Boehringer–Mannheim Expand Long Template PCR System I) was used. The primers used for the PCR were: 5′-CACC atg cag agc att gag ctg gtc acg-3′ encoding for AtFAO3 N-terminal 8 amino acid residues: M-Q-S-I-E-L-V-T, the 5′-end additional nucleotide CACC is compulsory for directional TOPO-cloning system (Invitrogen) and 5′-ATg AgA TAA ACC AgT ggT CAT ggA C-3′ encoding for AtFAO3 C-terminal 8 amino acids S-M-T-T-G-L-S-H, the stop codon was not included in order to link the his-tag sequence which will then have a stop codon plus T7 polymerase terminator (Invitrogen). PCR was performed under the following conditions: 1 cycle denaturation at 94 °C, 5 min; 10 cycles denaturation at 94 °C, 30 s, annealing at 60 °C, 40 s, elongation at 68 °C, 4 min; 20 cycles denaturation at 94 °C, 30 s, annealing at 60 °C, 40 s, elongation at 68 °C, 4 min, progressively increase 20 s after each cycle; and 1 cycle prolonged elongation at 68 °C for 7 min.

2.6. Overexpression of AtFAO3

Following expand PCR and directional cloning (Invitrogen), the pET101-based expression construct was transformed into *E. coli* BL21(DE3) cells according to the manufacturer's protocol and the cells were grown on an LB/ampicillin plate overnight. One colony was transferred into 5 ml of LB medium containing ampicillin (100 μg/ml), and the culture was grown up to $OD_{600} = 0.4$ at 37 °C after transfer into 500–2000 ml, then grown at 20 °C until $OD_{600} = 0.6$ with shaking at 200 rpm, and grown overnight after 1 mM IPTG was added. Typically, cells from 2 l of culture were harvested by centrifugation ($3000 \times g$, 10 min), resuspended in 1 ml of buffer containing 20 mM Tris–HCl (pH 8.0) and stored at −70 °C till required.

2.7. Membrane isolation

500 ml overnight culture was centrifuged at $5000 \times g$ for 5 min and resuspended in 15 ml in 20 mM Tris–HCl (pH 8.0). Cells were ultrasonicated for 5 min, 10 s intervals while keeping on ice. Membranes were isolated from the supernatant by ultracentrifugation at $140\,000 \times g$ for 2 h and resuspended in 500 μl of 20 mM Tris–HCl (pH 8.0). They were flash frozen in liquid nitrogen and stored at −70 °C until required.

2.8. Protein purification

The cell pellet (from 2 l of AtFao3 overexpression culture) was thawed on ice with 10 ml of sample buffer (1× PBS, pH 7.5, containing 0.3 M NaCl, 10 mM imidazole, pH 7.5), plus protease inhibitors (1 cocktail tablet, Roche), 1 mM DTT and 0.1% Triton X-100 in a 50 ml tube. The Triton X-100 is important for solubilisation of the membranes. The resuspended pellet was sonicated three times at amplitude 15 for 30 s with 1-min intervals on ice, ensuring that the temperature was maintained at 4 °C. It was then centrifuged at $6000 \times g$ for 10 min at 4 °C and the supernatant retained. The pellet was resuspended in 10 ml of sample buffer sonicated and centrifuged as previously described. The supernatants were combined and diluted with sample buffer to 40 ml and centrifugation at $18\,000 \times g$ for 30 min at 4 °C. The supernatant was filtered through 0.2 μm acrodisc filter and loaded onto a Ni–NTA column equilibrated with sample with a flow rate of 1 ml/min. The column was washed with 3-bed volume of washing buffer, 120 ml (PBS containing 0.3 M NaCl, 30 mM imidazole, pH 7.5, and 1 mM PMSF), and eluted with 2-bed volume of elution buffer, 80 ml (1× PBS, pH 7.5, containing 0.3 M NaCl, 250 mM imidazole, pH 7.5, and 1 mM PMSF). The fractions were analysed by SDS–PAGE and those containing target protein pooled. The purified target protein was dialysed (1:50 v/v) against PBS, pH 7.5, containing 0.3 M NaCl, 1 mM DTT and 25 mM Tris–HCl (pH 7.5) for 2 h, the dialysis buffer was changed once. Any precipitate was removed by centrifugation at $14\,000 \times g$ for

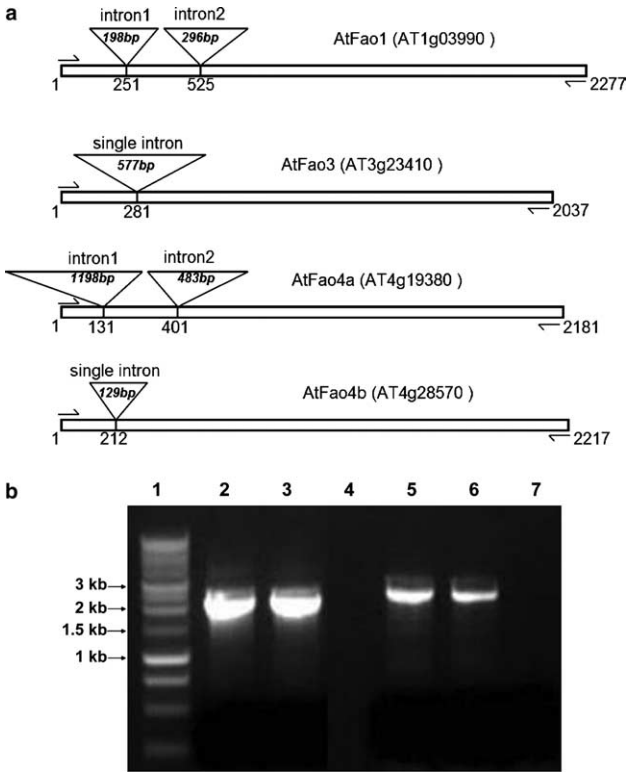


Fig. 1. Schematic diagram of the gene structures of all four *fao*-like genes from *A. thaliana* and RT-PCR quantification of AtFAO3 and AtFAO4b. (a) The length of the predicted coding sequences of AtFao1, AtFao3, AtFao4a and AtFao4b is indicated from start codon to stop codon. The position(s) and the length of intron(s) are also shown. Forward and reverse primers for cloning the full length cDNA are indicated by arrows. Full length AtFao3 and AtFao4b cDNA sequences have Accession Nos. AJ316230 and AJ316231, respectively; (b) lane 1: DNA marker; lanes 4 and 7: negative controls; lanes 2 and 3: AtFAO3 cDNA (2 kb); lanes 5 and 6: AtFAO4b cDNA (2.2 kb).

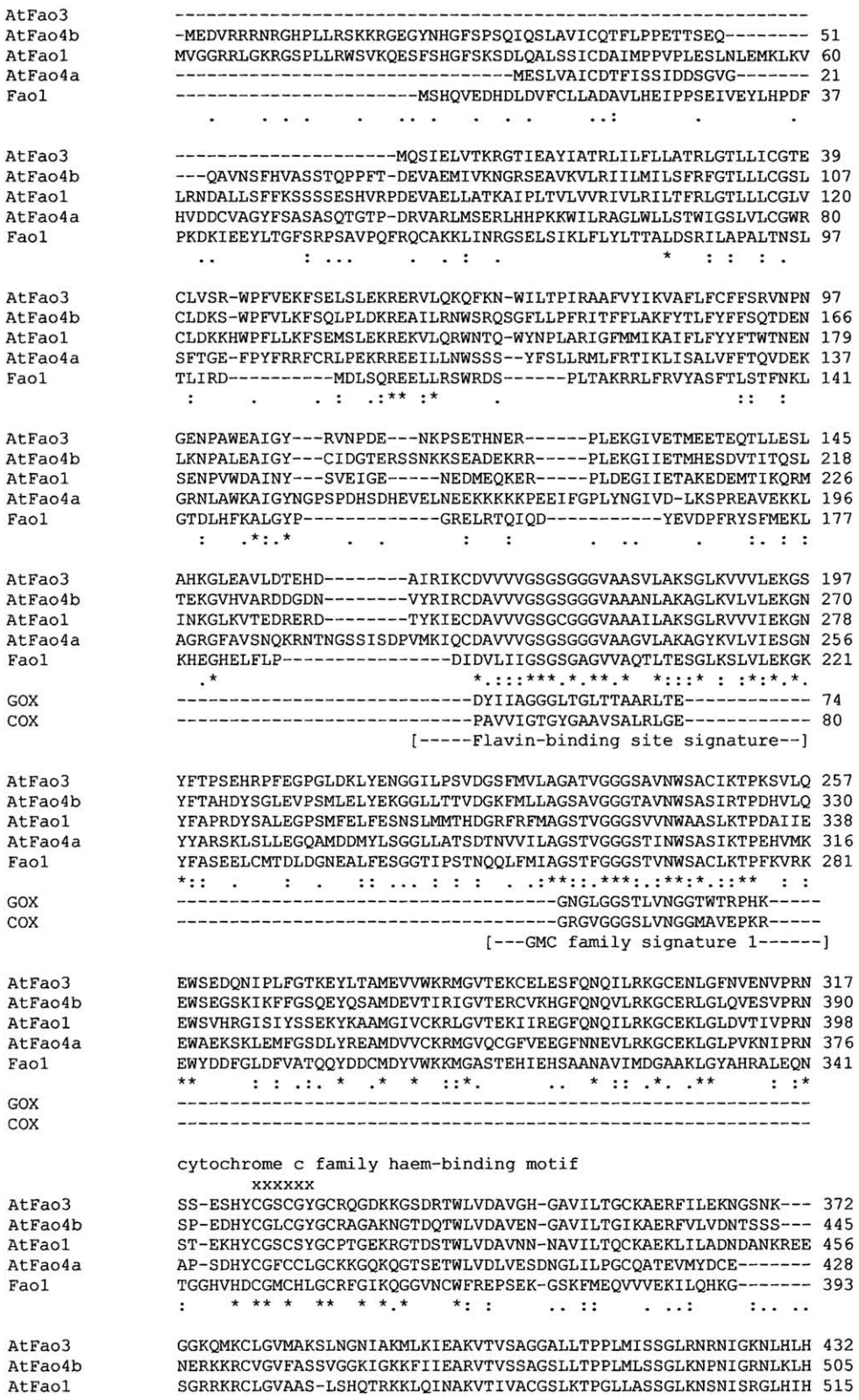


Fig. 2. Sequence alignments of AtFao1, AtFAO3, AtFAO4a and AtFAO4b and comparison with *C. cloacae* long chain alcohol oxidase Fao1 and GMC family proteins (GOX and COX). Four major common domains are indicated by brackets: flavin-binding site [G-x-G-x-G-G/x]; GMC family signature 1: [GA]-[RKN]-x-[LIV]-G(2)-[GST](2)-x-[LIVM]-N-x(3)-[FYWA]-x(2)-[PAG]-x(5)-; GMC family signature 2: [GS]-[PSTA]-x(2)-[ST]-P-x-[LIVM](2)-x(2)-S-G-[LIVM]-G; and substrate-binding site. The unique FAO family haem-binding site is indicated by crosses. Accession Nos. are: AJ242497 for Fao1, P13006 for GOX (glucose oxidase), and P12676 for COX (cholesterol oxidase). The identical amino acids in *A. thaliana* and *Candida* alcohol oxidase sequences are indicated by asterisks.

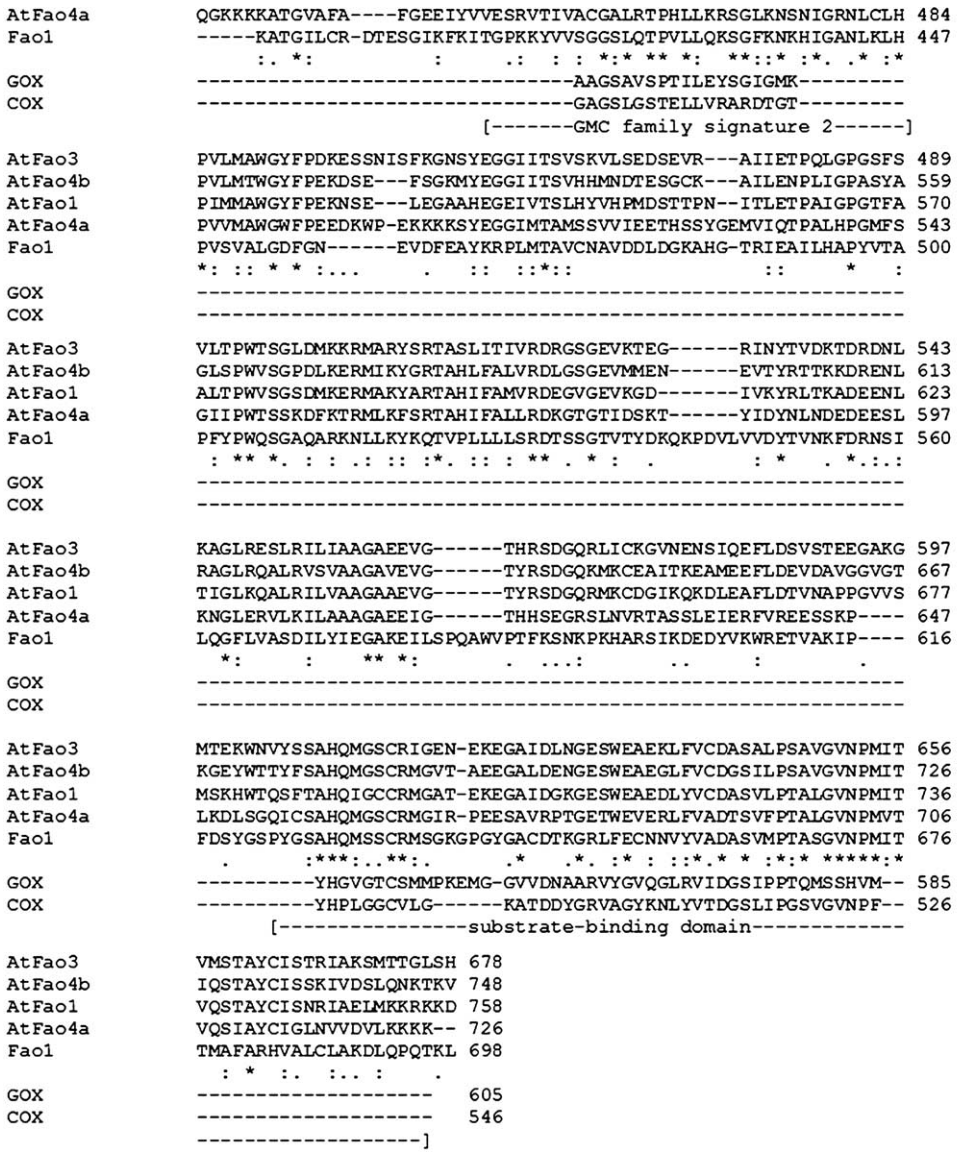


Fig. 2 (*continued*)

10 min. Glycerol was added to a final concentration of 20% and the protein was stored at −70 °C till required.

2.9. Protein analysis

Protein quantification was performed by using a Bio-Rad protein assay kit following the manufacturer's instructions. BSA was used as a standard protein. SDS–polyacrylamide gels consisted of a 5% acrylamide stacking gel with a 10% acrylamide running gel and were run on a Bio-Rad mini-protean gel kit. The buffers used were as described by Laemmli [6].

2.10. Enzymatic assays

Long chain alcohol oxidase activity was assayed by spectrophotometry [7] using Thermo Spectronic UV1. The assay mixture contained 50 mM Tris–HCl (pH 8.5), 0.7 mg/ml ABTS, 7 units of horseradish peroxidase, and 50 mM 1-dodecanol previously dissolved in acetone, in a final volume of 1.0 ml unless otherwise specified. Reactions were initiated by addition of enzyme (10 μg of purified AtFao3 or Fao1 protein was added in each 1 ml reaction mixture) and the increase in absorbance at 405 nm was measured. The value of ε for the radical cation of ABTS is 18.4 $mM^{-1}\,cm^{-1}$, and 1 mol of substrate gives rise to 2 mol of radical cation. One unit of enzyme activity catalyses the conversion of 1 nmol of substrate to product/min. No absorbance change was observed in the absence of either substrate of peroxidase.

3. Results and discussion

The complete genome of *A. thaliana* was searched for homologues of the *C. cloacae fao1* gene, which encodes the membrane bound long chain alcohol oxidase, using the tBlastn programme. Two different homologues AtFAO1 and AtFAO3 were identified on chromosomes 1 and 3, respectively, whilst an additional two different homologues AtFAO4a and AtFAO4b were both found on chromosome 4. Using known intron/exon boundary motif search plus alignment with the amino acid sequence of Fao1 from *C. cloacae*, it seems that two of the candidate *fao1* homologues AtFAO3 and AtFAO4b contain a single intron within the first 100 amino acids of the N-terminal

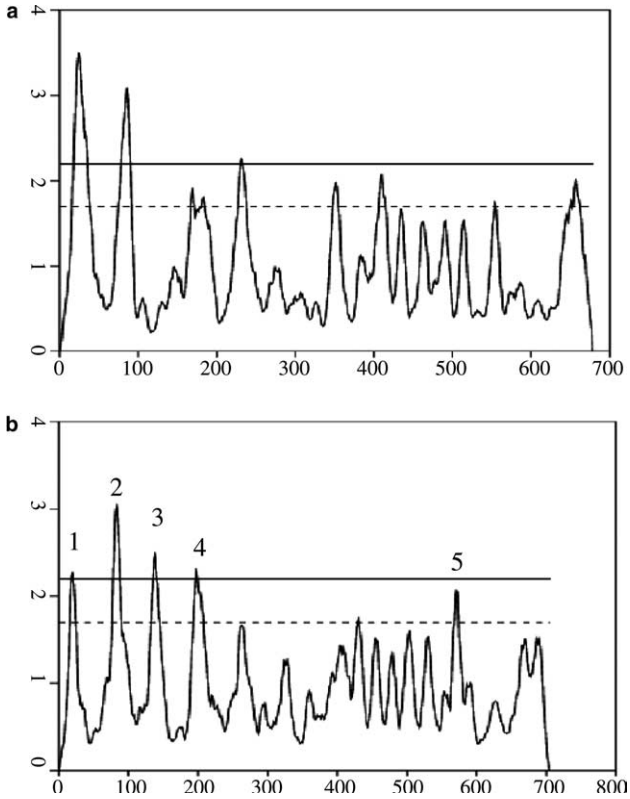


Fig. 3. Transmembrane domain prediction by the DAS method. a and b show the DAS analysis results for AtFAO3 and Faot, respectively. The *x* axis indicates the amino acid locality within the derived sequences and the *y* axis indicates the DAS score. The hit at a DAS score of 2.2 (indicated by the solid lines) is informative in terms of the number of matching segments, whereas the hit at a DAS score of 1.7 (indicated by the dashed lines) gives the actual location of the transmembrane segment.

portion of the protein. The other two candidates AtFAO1 and AtFAO4a appear to contain 2 introns (Fig. 1a).

The full length cDNAs encoding for the putative *fao* genes, from *Arabidopsis*, were amplified from mRNA of suspension cultures of *A. thaliana* by using RT-PCR. It appears that the expression level of AtFao3 gene is much higher than that of AtFao4b gene by approximately 5-fold in suspension culture (Fig. 1b). However, we were unable to obtain the full length cDNA for AtFao1 and AtFao4a. The full length of amplified cDNAs of AtFAO3 and AtFAO4b are 2 and 2.2 kb, respectively. A 577 bp single intron split AtFAO3 gene into two exons of 281 and 1756 bp, while a 129 bp single intron is present in AtFAO4b gene, giving exons of 212 and 2005 bp (Fig. 1a). The translated sequences for the four putative Arabidopsis alcohol oxidases show 45–55% amino acid sequence identity with each other and 25–27% amino acid sequence identical with *C. cloacae* Fao1 protein. The translated sequences contain all five domains present in the *Candida* cDNA, including flavin-binding site [G-x-G-x-G-G/x] [8–10]; GMC family signature 1: [GA]-[RKN]-x-[LIV]-G(2)-[GST](2)-x-[LIVM]-N-x(3)-[FYWA]-x(2)-[PAG]-x(5)-; GMC family signature 2: [GS]-[PSTA]-x(2)-[ST]-P-x-[LIVM](2)-x(2)-S-G-[LIVM]-G; and substrate-binding site [11–13]. The fifth motif, the haem binding domain, which is present in all membrane bound alcohol oxidases but absent from other members of the GMC-oxidoreductase family, such as glucose oxidase and cholesterol oxidase, is also present in the AtFAO1, AtFAO3, AtFao4a and AtFao4b sequences (Fig. 2). The putative proteins do not contain a KDEL or HDEL endoplasmic reticulum retention sequence, a secreatory sequence or known organellar targetting sequences for the peroxisome, chloroplast or mitochondria. Analysis using the DAS programme [14] for transmembrane domains reveals that like the *fao1* gene from *C. tropicalis* there are a number of potential transmembrane regions within the N-terminal 200 amino acids. The second

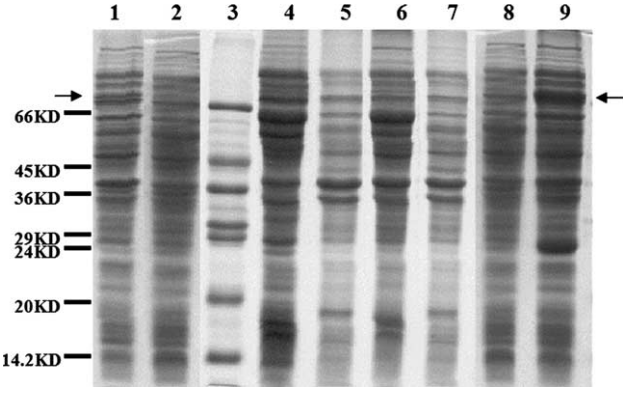


Fig. 4. SDS–PAGE profile of *E. coli* BL21(DE3) transformed with pET101 vectors. Lanes 1, 4, 5, 9: *E. coli* cells transformed with pET101-AtFAO3; lanes 2, 6, 7, 8: *E. coli* cells transformed with pET101 empty vector. Lanes 1 and 2: microsomal fractions; lanes 8 and 9: total cellular fractions; lanes 4 and 6: cell debris and inclusion body fractions; lanes 5 and 7: soluble fractions; lane 3: molecular mass markers. The position of the protein corresponding to AtFAO3 is indicated by the arrows.

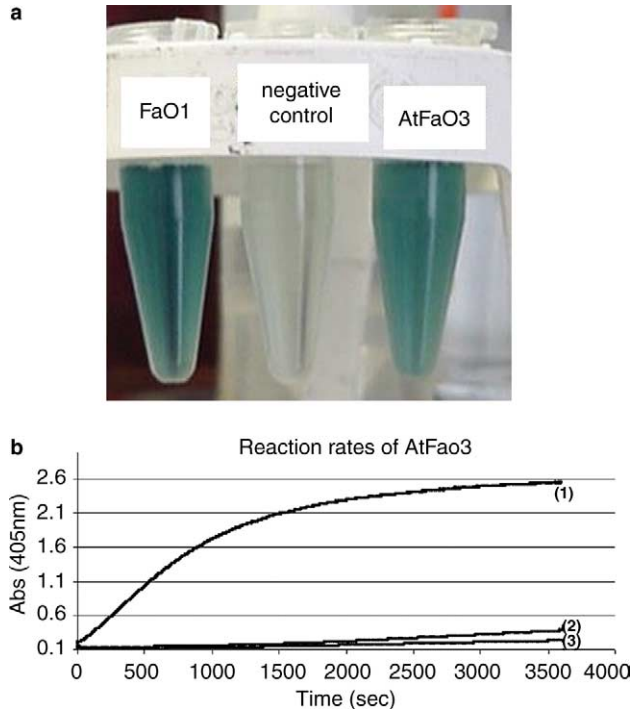
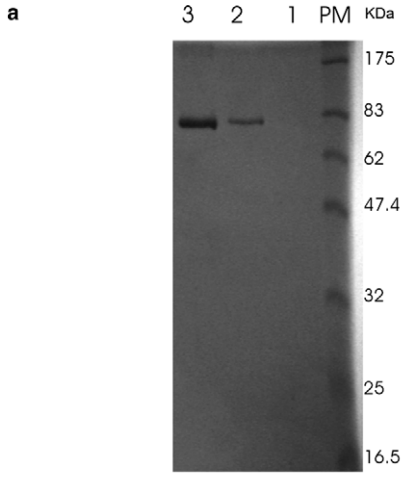


Fig. 5. Biological assay of lauryl alcohol oxidase activity for AtFAO3. (a) Membrane proteins (100 μg each) from samples transformed with pET17b-Fao1, pET101-AtFAO3 and pET101 empty vector were assayed. The colour of the reaction mixture changes from colourless to green due to the amount of excited radicals released from ABTS catalysed by horseradish peroxidase linked to H_2O_2-generating AtFAO3 oxidising lauryl alcohol. Fao1 was used as positive control and empty vector was negative control. Photo was taken at 1 h after reaction was initiated. (b) Reaction rates of AtFao3 against three substrates: (1) 1-dodecanol, (2) 1,16-hexadecandiol and (3) 1-hexadecanol.



b

Specific activities (nmoles/mgprotein/min) of AtFao3 and Fao1

	1-dodecanol	1-hexadecanol	1,16-hexadecandiol
AtFao3	215 ± 5	6 ± 1	14 ± 2
Fao1	1075 ± 21	0.9 ± 0.2	311 ± 10

Fig. 6. Protein purification of AtFao3 and FAO activity comparison between AtFao3 and Fao1. (a) Fractions of purified AtFao3: lanes 2 and 3, the protein size is indicated on the right, lane 1 is empty. (b) Specific activities (nmol/mgprotein/min) of AtFao3 and Fao1 against three substrates: 1-dodecanol, 1-hexadecanol and 1,16-hexadecandiol. Assays were repeated three times and the S.E. is shown.

transmembrane domain, in the region of amino acid 90 of the AtFAo3 sequence, seems absolutely conserved with the corresponding region in *C. tropicalis* and is a region with a very high DAS score (Fig. 3). In order to try and prove the function of the isolated gene, we subcloned it into the expression vector pET101, transformed *E. coli* BL21(DE3) and looked for production of the protein. Whilst the cells transformed with the AtFAO3 cDNA construct produced a detectable additional protein product, none were detected in cells transformed with the AtFAO4b cDNA. This is a similar situation to that observed when we previously tried to overproduce protein from cDNAs for the two isoforms of *C. cloacae* fao1 in *E. coli* and only one gave detectable product, the reason for this is unknown [1]. From Fig. 4 it can be seen that an additional band at M_r of ~73 000 Da is present in membrane fraction [lane 1] but is absent from the soluble fraction [lane 5]. The microsomal fraction from *E. coli* containing the pET101-AtFAO3, and a negative control (empty vector pET101) and the positive control (pET17b-Fao1) [1] were assayed for lauryl alcohol oxidase activity. The colour of the reaction mixture with AtFao3 and Fao1 changes from colourless to a dark green due to the generation of radical cation, this is not seen in the empty vector control (Fig. 5a).

Despite the membrane protein nature of AtFao3, we eventually managed to solubilise and purify it for crystallisation trials (Fig. 6a). Three substrates (1-dodecanol, 1-hexadecanol, and 1,16-hexadecandiol) were used for assaying purified AtFao3 protein activities by measuring the absorbance change at 405 nm (Fig. 5b). The specific activities of AtFao3 with 1-dodecanol, 1-hexadecanol, and 1,16-hexadecandiol as substrates were 215, 6, and 14 nmol/mgprotein/min, respectively. Each assay was done at least three times and extensive kinetic analysis of AtFao3 is currently being carried out.

The activities of the *A. thaliana* AtFao3 were compared with the purified *C. cloacae* Fao1 in Fig. 6b. Intriguingly, AtFao3 has higher activity than Fao1 when using 1-hexadecanol as substrate whilst Fao1 appears more active when 1-dodecanol was used as substrate. The difference of substrate selectivity between 1-hexadecanol and 1,16-hexadecandiol is less significant in AtFao3 than in Fao1.

In conclusion, homology based database searches have allowed us to identify four homologues of the *Candida*, peroxide generating, long chain alcohol oxidase in the *Arabidopsis* genome. Functional proof has been obtained by overexpressions and biological assays. Despite the protein being membrane bound, we have solubilised it by using Triton X-100. The recombinant protein has been purified to homogeneity and retains biological activity enabling us to initiate crystallisation trials. This is the first report of a gene with this function in plants or animals. The gene could be involved in a ω-oxidation pathway of lipid degradation in plants as has been reported for cytochrome P450 proteins [15,16]. Further investigations into the importance of FAO in *Arabidopsis* can be elucidated by identification of gene knockouts, which are currently underway.

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