Transformation of *Saccharomyces cerevisiae* with a cDNA encoding a sterol *C*-methyltransferase from *Arabidopsis thaliana* results in the synthesis of 24-ethyl sterols

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Abstract Using an EST-cDNA probe, a full-length cDNA (411) sequence of 1411 bp was isolated from *A. thaliana*. This sequence contained features typical of methyltransferases in general and in particular showed 38% identity with *ERG6*, a *S. cerevisiae* gene which encodes the zymosterol-C-24-methyltransferase. A yeast vector containing this ORF (4118-pYeDP60) was used to transform a wild type *S. cerevisiae* which accumulates predominantly ergosterol, a 24-methyl sterol as well as a mutant *erg6* null mutant accumulating principally zymosterol, a sterol non-alkylated at C-24. In both cases, several 24-ethyl- and 24-ethylidene sterols were synthetized indicating that the 4118 cDNA encodes a plant sterol *C*-methyltransferase able to perform two sequential methylations of the sterol side chain.

Key words: *Arabidopsis*; *S*-Adenosylmethionine-sterol-*C*-methyltransferase; *erg6*; Complementation

1. Introduction

Sterols from fungi and higher plants differ from vertebrate sterols by the presence of an extra alkyl group at C-24 [1,2]. Whereas most fungi possess a methyl group at C-24, higher plants contain 24-ethylsterols such as campesterol (I) and sitosterol (II). This alkylation of the side chain is catalyzed by *C*-methyltransferases which have been studied in *Saccharomyces cerevisiae* and in higher plants. In *S. cerevisiae*, the methyltransferase catalyzes the transfer of the methyl group from *S*-adenosyl-L-methionine converting zymosterol (III) to fecosterol (IV) [3]. The stereochemistry of this transfer has been demonstrated [4]. In higher plants, the presence of 24-ethyl sterols results from two distinct methyl transfers from *S*-adenosyl-L-methionine [1,2]. According to the chemical structures of intermediates of sterol biosynthesis and substrate specificity studies, it is generally assumed that cycloartenol (V) is the substrate of the first methylation reaction resulting in 24-methylene cycloartanol (VI) [5,6], whereas 24-methylene lophenol (VII) is the preferred substrate for the second methylation resulting in 24-ethylidene lophenol (VIII) [7] (Fig. 1). Since the chemical structures of V and VII are very different, it has been suggested that the two methylation reactions would be catalyzed by different enzymes [8].

This study reports for the first time the isolation of a cDNA from *Arabidopsis thaliana* encoding a protein possessing 38% identity with the zymosterol C-24 methyltransferase from *S. cerevisiae*. This protein when expressed in a yeast null mutant *erg6* [9] is able to complement the *erg6* deficiency resulting in ergosterol (IX) synthesis but further is able to perform a second methylation reaction in which 24-ethylidene- and 24-ethyl sterols (such as compounds X, XI and XII, Fig. 2) are produced.

2. Materials and methods

2.1. Strains, media and culture conditions

E. coli, XL1 blue recA$^-$ (recA1, lac$^-$, endA1, gyrA96, thi, hsdR17, SupE44, relA1, {F'proAB,lacIq, lacZΔM15, Tn10}).

Saccharomyces cerevisiae, WA6 {(a)ade5, his7-2, leu2-3, 112ura 3-52)}, WAERG6 {(a) ade5, his7-2, leu2-3, 112, ura3-52, erg6Δ::-LEU2}. Strains transformed with pYeDP60 were grown for 48 h on minimum medium (YNB) containing suitable supplements (50 μg/ml each) and where glucose was replaced by galactose (10 g/l).

2.2. Plasmids

A pBluescript SK$^-$ vector (Stratagene) was used for subcloning and sequencing and the plasmid pYeDP60 [10] was used to transform yeast strains. This plasmid contains a *E. coli* replication origin, a yeast 2 μm plasmid replication origin, an *E. coli* ampicillin resistance gene and the yeast gene URA3 encoding an orotidine-5-phosphate decarboxylase for transforming uracil auxotrophic recipient yeast. It utilizes an expression cassette including a hybrid promoter inducible by galactose and a phosphoglycerate kinase (PGK) terminator. The promoter is activated by the upstream activating sequence of the yeast GAL10 and CYC1 genes.

2.3. Isolation of arabidopsis cDNA encoding a sterol C-methyltransferase

400,000 recombinant phages from a cDNA library of developing siliques of *Arabidopsis thaliana* ecotype Columbia in λ-ZAPII [11] were screened with a PCR probe of 782 bp. This probe was synthesized by PCR using a cDNA EST clone as template,

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Abbreviations: I = campesterol = (24ζ)-24-methyl-cholest-5-en-3β-ol; II = sitosterol = (24R)-24-ethyl-cholest-5-en-3β-ol; III = zymosterol = 5α-cholesta-8,24-dien-3β-ol; IV = fecosterol = 5α-ergosta-8,24(24^1)-dien-3β-ol; V = cycloartenol = 4,4,14α-trimethyl-9β,19-cyclo-5α-cholest-24-en-3β-ol; VI = 24-methylene cycloartanol = 4,4,14α-trimethyl-9β,19-cyclo-5α-ergost-24(24^1)-en-3β-ol; VII = 24-methylene lophenol = 4α-methyl-5α-ergosta-7,24(24^1)-dien-3β-ol; VIII = 24-ethylidene lophenol = 4α-methyl-5α-stigmasta-7,Z-24(24^1)-dien-3β-ol; IX = ergosterol = ergosta-5,7,E-22-trien-3β-ol; X = Δ^7-avenasterol = 5α-stigmasta-7,Z-24(24^1)-dien-3β-ol; XI = 5α-stigmasta-8,Z-24(24^1)-dien-3β-ol; XII = stigmasta-5,7,E-22-trien-3β-ol; XIII = episterol = 5α-ergosta-7,24(24^1)-dien-3β-ol; XIV = 5α-cholesta-7,24-dien-3β-ol; XV = cholesta-5,7,9,24-tetraen-3β-ol; XVI = lanosterol = 4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol; XVII = ergosta-5,7,9,E-22-tetraen-3β-ol; XVIII = eburicol = 4,4,14α-trimethyl-5α.-ergosta-8,24(24^1)-dien-3β-ol.

SSDI S0014-5793(96)00089-0

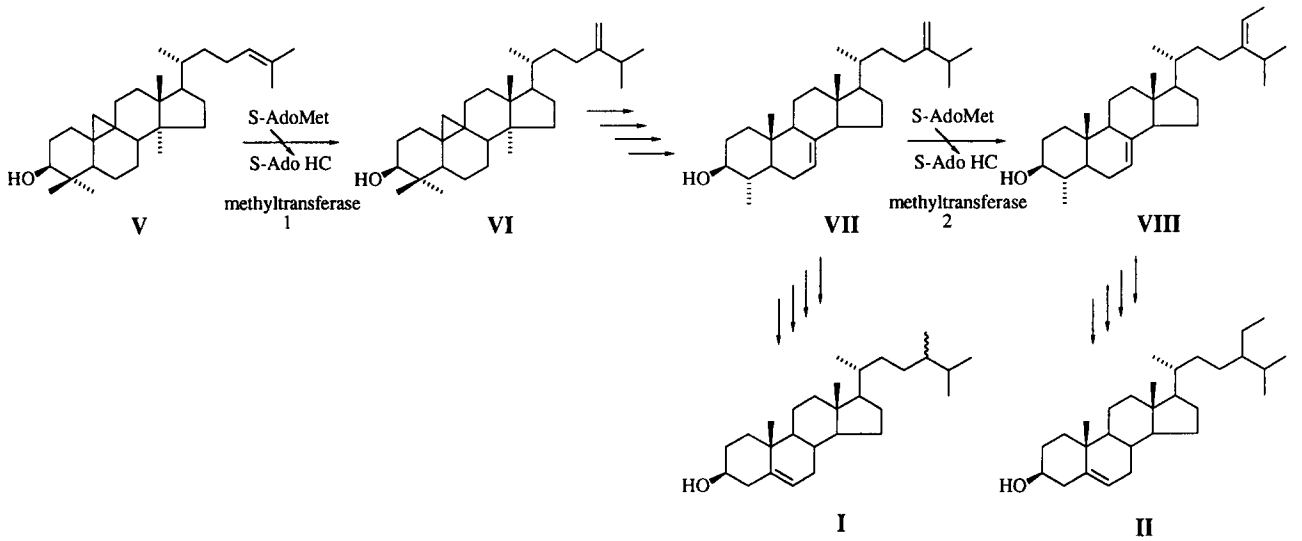

Fig. 1. The two methylation steps involved in sterol biosynthesis in higher plants. S-AdoMet = *S*-adenosyl methionine; S-AdoHC = *S*-adenosyl homocysteine.

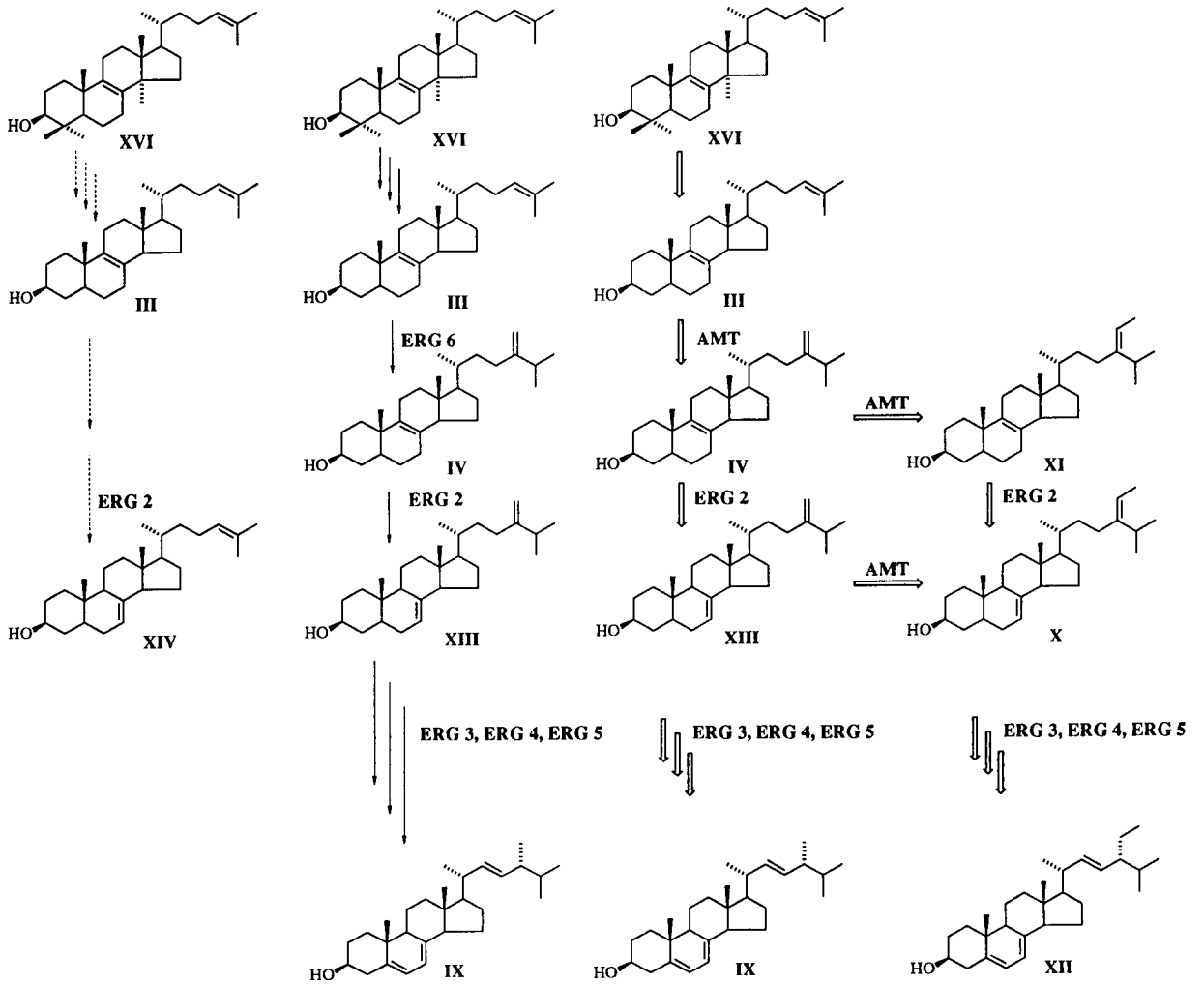

Fig. 2. Comparative sterol biosynthesis in wild type *S. cerevisiae*, in the yeast mutant *erg6* and in the yeast mutant *erg6* transformed with ORF 4118 in pYeDP60. 'Dotted arrow' = sterol pathway in the yeast mutant *erg6*; 'solid arrow' = sterol pathway in WT *S. cerevisiae*; 'hollow arrow' = sterol pathway in *erg6* transformed with ORF 4118. AMT = *Arabidopsis* methyltransferase(s).

EMBL:emb|Z34203|ATTS3237. 48 phages were obtained by hybridation and 10 were shown to contain inserts corresponding to full-length cDNAs. One of them (pSK 411) was sequenced manually in both orientations (Fig. 3).

2.4. Plasmid constructions

The 4117 ORF was synthesized by PCR using the pSK411 vector and the two oligonucleotides, underlined in Fig. 3. *Bam*HI and *Xba*I restriction sites were generated at the ends for subsequent cloning. The PCR product was subsequently cloned into BlueScript to give plasmid pSK4117. pSK4117 was linearized with *Xba*I, blunted using Klenow fragment of DNA polymerase I and subsequently cut with *Bam*HI; the resulting DNA insert (4118) was ligated into pYeDP60 (containing a *Bam*HI site at one end and a blunted *Eco*RI site at the other). The resulting plasmid was called (pYeDP60-4118).

2.5. Transformation of yeast

Preparation of competent cells and the transformation procedure have been described in detail elsewhere [12,13].Transformed yeast strains (either WA6 or WAERG6) were selected for uracil prototrophy. Additionally, the plasmid-mediated ability to synthesize ergosterol was assessed in the case of *erg6* by resistance to cycloheximide (0.04 μg/ml) [22], a concentration lethal to the *erg6* mutant strain.

2.6. Sterol analysis

Sterol isolation from lyophilized yeast cells and purification techniques have been described in detail elsewhere [13].

Identification of purified steryl acetates was finally performed using a computerized gas-chromatograph mass spectrometer (GCMS) (Fison MD800) equipped with an 'on column' injector and a capillary column (30 m×0.25 i.e.) coated with DBS (J&W Scientific). Different

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ctc tct ctc tct ctc tct tgg tct tcc tca ctc tta acg aaa atg gac tct tta aca ctc  60
                                                       M   D   S   L   T   L    6
ttc ttc acc ggt gca ctc gtc gcc gtc ggt atc tac tgg ttc ctc tgc gtt ctc ggt cca  120
F   F   T   G   A   L   V   A   V   G   I   Y   W   F   L   C   V   L   G   P   26
gca gag cgt aaa ggc aaa cga gcc gta gat ctc tct ggt ggc tca atc tcc gcc gag aaa  180
A   E   R   K   G   K   R   A   V   D   L   S   G   G   S   I   S   A   E   K   46
gtc caa gac aac tac aaa cag tac tgg tct ttc ttc cgc cgt cca aaa gaa atc gaa acc  240
V   Q   D   N   Y   K   Q   Y   W   S   F   F   R   R   P   K   E   I   E   T   66
gcc gag aaa gtt cca gac ttc gtc gac aca ttc tac aat ctc gtc acc gac ata tac gag  300
A   E   K   V   P   D   F   V   D   T   F   Y   N   L   V   T   D   I   Y   E   86
tgg gga tgg gga caa tcc ttc cac ttc tca cca tca atc ccc gga aaa tct cac aaa gac  360
W   G   W   G   Q   S   F   H   F   S   P   S   I   P   G   K   S   H   K   D   106
gcc acg cgc ctc cac gaa gag atg gcg gta gat ctg atc caa gtc aaa cct ggt caa aag  420
A   T   R   L   H   E   E   M   A   V   D   L   I   Q   V   K   P   G   Q   K   126
atc cta gac gtc gga tgc ggt gtc ggc ggt ccg atg cga gcg att gca tct cac tcg cga  480
I   L   D   V   G   C   G   V   G   G   P   M   R   A   I   A   S   H   S   R   146
gct aac gta gtc ggg att aca ata aac gag tat cag gtg aac aga gct cgt ctc cac aat  540
A   N   V   V   G   I   T   I   N   E   Y   Q   V   N   R   A   R   L   H   N   166
aag aaa gct ggt ctc gac gcg ctt tgc gag gtc gtg tgt ggt aac ttc ctc cag atg ccg  600
K   K   A   G   L   D   A   L   C   E   V   V   C   G   N   F   L   Q   M   P   186
ttc gat gac aac agt ttc gac gga gct tat tcc atc gaa gcc acg tgt cac gcg ccg aag  660
F   D   D   N   S   F   D   G   A   Y   S   I   E   A   T   C   H   A   P   K   206
ctg gaa gaa gtg tac gca gag atc tac agg gtg ttg aaa ccc gga tct atg tat gtg tcg  720
L   E   E   V   Y   A   E   I   Y   R   V   L   K   P   G   S   M   Y   V   S   226
tac gag tgg gtt acg acg gag aaa ttt aag gcg gag gat gac gaa cac gtg gag gta atc  780
Y   E   W   V   T   T   E   K   F   K   A   E   D   D   E   H   V   E   V   I   246
caa ggg att gag aga ggc gat gcg tta cca ggg ctt agg gct tac gtg gat ata gct gag  840
Q   G   I   E   R   G   D   A   L   P   G   L   R   A   Y   V   D   I   A   E   266
acg gct aaa aag gtt ggg ttt gag ata gtg aag gag aag gat ctg gcg agt cca ccg gct  900
T   A   K   K   V   G   F   E   I   V   K   E   K   D   L   A   S   P   P   A   286
gag ccg tgg tgg act agg ctt aag atg ggt agg ctt gct tat tgg agg aat cac att gtg  960
E   P   W   W   T   R   L   K   M   G   R   L   A   Y   W   R   N   H   I   V   306
gtt cag att ttg tca gcg gtt gga gtt gct cct aaa gga act gtt gat gtt cat gag atg 1020
V   Q   I   L   S   A   V   G   V   A   P   K   G   T   V   D   V   H   E   M   326
ttg ttt aag act gct gat tat ttg acc aga gga ggt gaa acc gga ata ttc tct ccg atg 1080
L   F   K   T   A   D   Y   L   T   R   G   G   E   T   G   I   F   S   P   M   346
cat atg att ctc tgc aga aaa ccg gag tca ccg gag gag agt tct tga gaa agg tag aaa 1140
H   M   I   L   C   R   K   P   E   S   P   E   E   S   S   *                   361
gga aac atc acc gga aaa agt atg gag aat ttt ctc aat ttg ttt tta ttt tta agt taa 1200

atc aac ttg gtt att gta cta ttt ttg tgt ttt aat ttg gtt tgt gtt tca aga att att 1260

agt ttt ttt ttg ttt tgt tgc ata tga gaa tct tac tct tga ttt ctc cgc cgt aga gcc 1320

ggc gag aca tag ggg att att agt att ttt aag tgt gtt taa gat tga tta aca agt tag 1380

taa aat aaa atg tac tta ggt gtc gaa aaa aaa ag                                  1440
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Fig. 3. Nucleotidic sequence of the cDNA contained in the plasmid 411 and deduced amino acid sequence. The putative *S*-adenosyl-methionine binding sequence has been underlined. Sequences underlined with dotted lines have allowed to derive primers to synthesize ORF 4117 by PCR. The accession number of the sequence is X89867.

Table 1
Sterol composition of wild type (WA6) and mutant (*erg6*) yeast strains transformed with plasmid pYeDP60 with and without ORF 4118

	WA6 pYeDP60	WA6-4118-pYeDP60	*erg6* pYeDP60	*erg6*-4118-pYeDP60
Lanosterol (XVI)	24[a]	9	10	6
Eburicol (XVIII)	–	3	–	–
Zymosterol (III)	2	3	60	19
5α-cholesta-7,24-dien-3β-ol (XIV)	–	–	25	7
Cholesta-5,7,9,24-tetraen-3β-ol (XV)	–	–	1	1
Total 24-desmethyl sterols	*26*	*12*	*96*	*33*
Fecosterol (IV)	11	10	–	–
Episterol (XIII)	13	5.5	–	–
Ergosterol (IX)	47	7	–	3.5
Ergosta-5,7,9,E-22-tetraen-3β-ol (XVII)	1	2.5	–	4.5
Total methyl sterols	*72*	*28*	*0*	*8*
5α-Stigmasta-8,Z-24(24[1])-dien-3β-ol (XI)	–	27	–	33
Δ^7-Avenasterol (X)	–	15.5	–	18
Stigmasta-5,7,E-22-trien-3β-ol (XII)	–	4.5	–	3
Total ethyl sterol	*0*	*47*	*0*	*54*

[a]Relative percentages.

fragments obtained are designed by the ratio m/e and their relative intensity.

5α-Cholesta-8,24-dien-3β-yl acetate (III): 426 (M^+) (84), 366 (20), 313 (31), 255 (14), 213 (87). 5α-Ergosta-8,24(24[1])-dien-3β-yl acetate (IV): 400 (M^+) (21), 380 (15), 365 (20), 313 (33), 255 (18), 253 (20), 227 (31), 213 (42). 5α-Ergosta-7,24(24[1])-dien-3β-yl acetate (XIII): 440 (M^+) (4), 365 (9), 356 (15), 313 (100), 255 (17), 253 (25), 213 (30). Ergosteryl acetate (IX): 438 (M^+) (4), 378 (100), 363 (19), 253 (27), 237 (7), 211 (10). 5α-Stigmasta-7,Z-24(24[1])-dien-3β-yl acetate (X): 454 (M^+) (2); 356 (36); 313 (100), 296 (4), 255 (6), 253 (8), 213 (9). 5α-Stigmasta-8,Z-24(24[1])-dien-3β-yl acetate (XI): 454 (M^+) (45), 356 (48), 313 (70), 255 (20), 253 (15), 213 (57). Stigmasta-5,7,E-22-trien-3β-yl acetate (XII): 452 (M^+) (2), 392 (78), 376 (13), 377 (20), 378 (8), 253 (34), 251 (15). 5α-Cholesta-7,24-dien-3β-yl acetate (XIV): 426 (M^+) (8), 342 (13), 313 (100), 255 (11), 253 (10), 213 (28). Cholesta-5,7,9,24-tetraen-3β-yl acetate (XV): 422 (M^+) (10), 362 (95), 311 (10), 251 (45), 249 (35). Lanosteryl acetate (XVI): 468 (M^+) (45), 453 (83), 393 (100), 297 (7), 283 (11), 215 (35). Ergosta-5,7,9,E-22-tetraen-3β-yl acetate (XVII): 436 (M^+) (7), 376 (100), 363 (23), 362 (11), 361 (3), 251 (45), 209 (24).

3. Results

3.1. Isolation of a cDNA encoding a putative higher plant sterol C-methyltransferase

The systematic screening of an *Arabidopsis thaliana* cDNA library (expressed sequence tag project) resulted in the identification of a cDNA (VEVECO7) EMBL: emb|Z34203|ATTS 3237, having significant identity (~30%) with *ERG6*, a yeast gene encoding a methyltransferase capable of converting zymosterol (III) to fecosterol (IV) [9,14,15] (Fig. 2). Complete sequencing of this cDNA indicated 38% identity with yeast *ERG6* but also indicated that the cDNA was truncated at the 5′ end. A probe was synthesized from this cDNA by PCR using two oligonucleotide primers deduced from the sequence. This PCR product was then used to screen a cDNA library of *Arabidopsis thaliana* siliques resulting in the isolation of a full-length cDNA of 1421 bp (pSK411). This sequence (Fig. 3) possesses an ORF of 1086 bp encoding a protein of 361 amino acids, 38% identical with *ERG6* (Fig. 4) and 82% identical with VEVECO7. This 411 ORF contains a 10 amino acids sequence, ILDVGCGVGG, which is a conserved motif found in all methyltransferases and which is considered to be the *S*-adenosyl methionine binding site [16–18]. The presence of a hydrophobic peptide of 25 amino acids at the N-terminal position may correspond to a transmembrane region required for the association of plant sterol methyltransferases with the endoplasmic reticulum [19,20].

3.2. Expression of the methyltransferase cDNA in S. cerevisiae

Since the biosynthesis of plant 24-ethyl sterols involves two distinct methylation steps (and possibly two different enzymes) we have used two yeast strains as recipient cells. The first is the yeast null mutant (*erg6*) [9] in which *ERG 6* is disrupted with *LEU2*. This mutant is unable to synthetize 24-methyl sterols and accumulates only C_{27} sterols, non alky-

Fig. 4. Alignment of protein sequence of the sterol-*C*-methyltransferase from *A. thaliana* (AMT) with yeast ERG6 product.

lated at C-24. Zymosterol (III) is the major accumulating sterol [9]. If 411 encoded a methyltransferase (MT) capable of methylating the sterol side chain at C-24, it might complement *erg6* leading to ergosterol production. The second yeast strain is the wild type strain WA6 which contains ergosterol (IX), a 24-methyl sterol, as a major component but does not contain any 24-ethyl sterol [21]. If 411 encoded the second methyltransferase that is to say a C-24[1]-methyltransferase, the wild type WA6 transformed with 411 should produce 24-ethyl sterols.

The cDNA clone pSK411 was used as a template in a PCR experiment in order to delete 5′ and 3′ non-coding sequences (ss in Fig. 3). This sequence cloned into the yeast expression vector, pYeDP60 that contains a galactose inducible promoter [9], yielded 4118-pYeDP60. Sequence analysis confirmed that the PCR product was identical to the 411 ORF. These constructions are detailed in the experimental section.

Transformation of the yeast strains was performed by the lithium acetate method [12] modified according to Gachotte et al. [13]. Transformants were selected for uracil prototrophy. Transformation of the wild type WA6 strain yielded WA6-4118-pYeDP60 and transformation of *erg6* mutant yielded *erg6*-4118-pYeDP60. Controls consisted of WA6 and *erg6* transformed with pYeDP60. The sterol composition of strains transformed with the different plasmids is presented in Table 1. The WA6 and *erg6* strains when transformed with the control plasmid pYeDP60 (minus ORF 4118) showed upon galactose induction, a sterol composition identical to untransformed WA6 and *erg6* strains with ergosterol (IX) and zymosterol (III) as the major sterols, respectively [9]. However, WA6 and *erg6* strains transformed with the plasmid 4118-pYeDP60 both contained a novel and unexpected sterol profile. The most remarkable result was the presence in both transformants of large amounts of sterols possessing two additional carbons at C-24 among which 5α-stigmasta-7,Z-24(28)-dien-3β-ol (X), 5α-stigmasta-8,Z-24(24[1])-dien-3β-ol (XI), stigmasta-5,7,E-22-trien-3β-ol (XII) were the major components. These compounds were identified by their mass spectra and represented about 50% of total sterols. In the case of the strain WA6-4118-pYeDP60, 24-ethyl sterols accumulated at the expense of ergosterol which accounts for only 7% of total sterols in WA6-4118-pYeDP60. In the case of the strain *erg6*-4118-pYeDP60, significant amounts of ergosterol (IX) were formed but the majority of the new sterols that accumulated were three ethyl sterols (X, XI and XII) totaling more than 54% of all sterols.

4. Discussion

A cDNA of 1411 pb encoding a protein of 361 amino acids has been cloned from a cDNA library of *Arabidopsis thaliana* siliques. The amino acid sequence of this protein shows 38% identity with a zymosterol-C-24-methyltransferase from *S. cerevisiae* and contains consensus motifs of methyltransferases in general [16–18].

The methyltransferase ORF inserted into a yeast expression vector results in a plasmid which complements a yeast null mutant *erg6*. Indeed ergosterol, the major sterol of WT *S. cerevisiae* WA6 is synthetized. Moreover sterol biosynthesis continues alkylating unknown intermediates to 24-ethylidene sterols such as Δ^7-avenasterol (X), a typical plant sterol [1] and 5α-stigmasta-8,Z-24(24[1])-dien-3β-ol (XI), found in *Rubus fructicosus* suspension cultures treated with AY 9744, an inhibitor of the Δ^8-Δ^7-sterol isomerase [23] (Fig. 2). Therefore the *Arabidopsis* methyltransferase expressed in yeast, is not only able to methylate the 24-desmethyl sterols of *erg6* (zymosterol presumably) at C-24, but is capable of performing a second methylation reaction at C-24[1] leading to 24-ethylidene sterols. The nature of the endogenous sterol acceptors of this second methylation step is unknown but fecosterol (IV) and episterol (XIII) are likely. According to enzymological studies, these sterols have previously been shown to be substrates of the second methylation reaction [8]. Both sterols normally accumulate in the WA6 strain and this may explain the occurrence of large amounts of 24-ethylidene sterols (X and XI) in WA6 transformed with 4118-pYeDP60.

Stigmasta-5,7,E-22-trien-3β-ol (XII) is found in addition to X and XI in strains transformed with ORF 4118 (Fig. 2). XII has been reported previously in Terbinafin-treated celery (*Apium graveoleus*) suspension cultures [24]. This compound differs from ergosterol by the presence at C-24 of an ethyl group in place of a methyl group. The low amounts of XII is interesting. 24-ethylidene sterols (X and XI), products of the *Arabidopsis* sterol-*C*-methyltransferase activity must be metabolized by enzymes present in *erg6* or WA6 strains. These enzymes are indicated in Fig. 2 and are: (i) from XI to X, a Δ^8-Δ^7-sterol isomerase (product of ERG2); and (ii) from X to XII a Δ^7-sterol-5-desaturase (product of *ERG3*), a Δ^{22}-desaturase (product of *ERG5*) and a Δ^{24}-reductase (product of *ERG4*) [15,25]. Low amounts of the end pathway sterol (XII) may be explained by high substrate specificity of one or all these enzymes which would not work optimally on substrates possessing an additional methyl group at C24[1].

Taking these results into account we are faced with the question of the exact nature of the enzyme encoded by ORF 4118. Three hypotheses are suggested: (i) a cycloartenol-C-24-methyltransferase (methyltransferase 1 in Fig. 2) of low substrate specificity; (ii) a 24-methylene-lophenol-C-24[1]-methyltransferase (methyltransferase 2 in Fig. 2) of low substrate specificity; and (iii) a single methyltransferase capable of performing both methylation reactions. The two first hypotheses imply the existence in higher plants of two discrete enzymic entities whereas the last one implies one enzyme capable of performing both methylation reactions. Presently, it is not possible to determine the exact specificity of the methyltransferase encoded by ORF 4118. The answer requires a thorough enzymological study performed on the null mutant *erg6* expressing the product of ORF 4118. Two groups of substrates should be used: zymosterol and cycloartenol for methylation at C-24; fecosterol, episterol and 24-methylene lophenol for methylation at C-24[1].

Finally, physiological consequences of the presence of 24-ethyl- and 24-ethylidene sterols on growth and metabolism of *S. cerevisiae* remain as yet unexplored. Possible growth, metabolic, and other physiological responses to ethyl-sterols may be probed using various inhibitors of sterol biosynthesis, polyene antibiotics, and other indicators of membrane function.

In recent months a paper dealing with the isolation of a cDNA clone from soybean presenting 57% identity upon 47 amino acids with ERG6 has been published [26].

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