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Identification of essential amino acid residues in a sterol 8,7-isomerase from *Zea mays* reveals functional homology and diversity with the isomerases of animal and fungi origin.

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Short Title: Plant sterol 8,7-isomerase essential amino-acids.

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SYNOPSIS

A putative sterol 8,7-isomerase (8,7SI) from *Zea mays* termed *Zm8,7SI* has been isolated from an EST library and subcloned in the yeast *erg2* mutant lacking 8,7SI activity. *Zm8,7SI* restored endogenous ergosterol synthesis. An *in vitro* enzymatic assay in the corresponding yeast microsomal extract indicated that the preferred Δ^8 -sterol substrate possesses a single C4 α methyl group, in contrast to 8,7SIs from animals and fungi, thus reflecting the diversity in the structure of their active site in relation to the distinct sterol biosynthetic pathways. In accordance with the proposed catalytic mechanism, a series of lipophilic ammonium ion containing derivatives possessing a variety of structures and biological properties, potentially inhibited the *Zm8,7SI in vitro*. To evaluate the importance of a series of conserved acidic and tryptophan residues which could be involved in *Zm8,7SI* catalytic mechanism, 20 mutants of *Zm8,7-SI* were constructed as well as a number of corresponding mutants of the *S.cerevisiae* 8,7-SI. The mutated isomerases were assayed *in vivo* by sterol analysis and quantification of $\Delta^{5,7}$ -sterols and directly *in vitro* by examination of the activities of the recombinant *Zm8,7-SI* mutants. These studies have identified His⁷⁴, Glu⁷⁸, Asp¹⁰⁷, Glu¹²¹, Trp⁶⁶ and Trp¹⁹³ that are required for *Zm8,7SI* activity and show that binding of the enzyme-substrate complex is impaired in mutant T124I. They underline the functional homology between the plant and animal 8,7SIs on one hand, in contrast to the yeast 8,7SI on the other hand, in accord with their molecular diversity and distinct mechanisms.

INTRODUCTION

Sterols are essential components of all eukaryotic cell membranes. The structural differences between sterols of animal, plant and fungi are linked to the biosynthetic pathways that differ significantly downstream squalene epoxide [1-3]. Nevertheless, these pathways retain several enzymatic steps in common, as for example those which together are necessary for the migration of the initial unsaturation in the B ring to the Δ^5 position in the functional sterols. This includes isomerization of the C8 double bond to the C7 position catalyzed by the sterol 8,7-isomerase (8,7SI) (Figure 1). The sterol 8,7-isomerization is triggered by an α -protonation of the Δ^8 -double bond from an appropriately positioned general acid residue giving a C8 carbocationic high energy intermediate (HEI). It follows elimination of a proton at C7 accepted by a general base residue (Figure 1) [4]. Loss of the 7β -H occurs in animals [5,6] and plants [7], while in fungi the enzyme proceeds with elimination of the 7α -H [7,8] (Figure 1).

8,7SI is one of the sterol biosynthesis enzymes that deserves special attention. In yeast, sterol 8,7-isomerase is a high regulated step of sterol biogenesis [9]. It is the primary target for a variety of compounds widely used in agriculture and medicine [2, 10-14]. Azadecalines [15], morpholine and piperidine fungicides [12] inhibit the plant and yeast enzymes by mimicking the structure of the carbocationic HEI. The animal 8,7SI is inhibited by a variety of structurally distinct pharmacological compounds including receptor σ -ligand SR31747A [16], trifluoperazine [11,17] and tamoxifen [11]. In addition, the σ -ligands, haloperidol, ifenprodil and verapamil were shown to inhibit the production of ergosterol in wild-type *S. cerevisiae* [11] and in the *erg2* mutant complemented with *A.thaliana* 8,7SI cDNA [18].

cDNAs of sterol 8,7-isomerase genes from fungi, animals and plants have been isolated and in some cases functionally characterized by complementation in the yeast *erg2* mutant deficient in 8,7SI [19, 16, 18]. The mammalian 8,7SI was shown to correspond to the EBP (emopamil binding protein), a high affinity receptor for the phenylalkylamine calcium-antagonist emopamil and other antiischaemic drugs, previously isolated [20]. A number of syndromes caused by EBP gene mutations, such as X-linked dominant Conradi-Hünemann syndrome and CHILD (congenital hemidysplasia with ichthyosiform erythroderma and limb defect), or the "tattered" mouse phenotype, were characterized by abnormally increased levels of cholest-8(9)-en-3 β -ol due to inactivation of 8,7SI. [21-23]. Human 8,7SI (EBP) and the yeast isoenzyme (Erg2p) are structurally unrelated and differ particularly in the substrate

specificity [5,7]. Alanine scanning mutagenesis was used to identify residues of human EBP required for *in vivo* sterol 8,7-isomerization [24]. It is noteworthy that a mammalian protein that is structurally related to the yeast Erg2p has been described as an σ_1 receptor but it exhibits no SI activity upon expression in yeast [25].

In plants, most of reports on enzymes involved in the kinetic control of the post squalene sterol pathway have been described in *Zea mays* [2]. Moreover, *Zea mays* has been widely used as a monocot model to assay both *in vivo* and *in vitro* a variety of fungicides and herbicides inhibiting plant sterol biosynthesis [10]. A plant 8,7SI cDNA has also been isolated by functional complementation of the corresponding yeast sterol mutant (*erg2*) by an *Arabidopsis* cDNA library and characterized by exposure to σ -ligands [18]. The plant 8,7SI shows an homology with the animal enzyme and is not related to the yeast enzyme. The importance of a functional 8,7SI for normal plant growth and development has been shown using the *Arabidopsis hydra* mutants, deficient in 8,7SI [26].

In plants, little attention has been directed towards the *in vitro* enzymological characterization of 8,7SI, including features required for specificity of sterol substrate. This is particularly due to the difficulties in obtaining the substrates and sufficient amounts of microsomal protein required, as the V_{\max} of the enzymes of postsqualene phytosterol synthesis are low [2]. In addition, the integral membrane-bound nature of the 8,7SI renders a structural characterization through crystallization and/or NMR challenging.

Site-directed mutational analysis of plant and yeast 8,7SIs has not been performed so far to establish the importance of select amino-acids in the proteins and to identify amino-acids essential for catalysis.

In this study, we report the cloning and *in vivo* functional expression in yeast, of a 8,7SI cDNA from *Zea mays*, and the first *in vitro* enzymological characterization and inhibition analysis of a plant recombinant 8,7SI. Moreover, to provide a framework for future structural studies, we report here the first site directed mutagenesis analysis of plant and yeast 8,7SIs. We mutated a number of conserved acidic residues, or putative cation stabilizing tryptophane residues, looking for amino acid residues that are essential for catalytic activity. The mutated isomerases were assayed both *in vivo* by sterol analysis and quantification of $\Delta^{5,7}$ -sterols, and directly *in vitro* by examining the activities of the recombinant mutated *Zm*8,7SI in the corresponding yeast microsomes extracts. It allowed particularly the identification of a number of amino acid residues essential for the plant 8,7SI activity. Taken

together, the data revealed functional homology and diversity with the isomerases of animal and fungi.

EXPERIMENTAL.

Materials

Tamoxifen (**16**) (Figure 2) and Trifluoperazine (**15**) were purchased from Sigma. N-benzyl-4 α ,10-dimethyl-8-aza-trans-decal-3 β -ol (**17**), N-[(1,5,9)-trimethyldecyl]-4 α ,10-dimethyl-8-aza-trans-decal-3 β -ol (**18**) and N-benzamido-4 α ,10-dimethyl-8-aza-trans-decal-3 β -ol (**19**) were synthesized as previously described [15,27]. AY9944 (**13**) was a generous gift of Dr. Dvornik (Ayerst Research Lab. Montreal, Canada). We thank the BASF Agrochemical Station (Limburgerhof, Germany) for providing Tridemorph (**11**) and Fenpropimorph (**12**). SR31747 (**14**) was provided by Dr Loison, (Sanofi-Aventis Recherche-Developpement, Labège, France).

Strains and plasmids

The sterol 8,7-isomerase deficient strain, WA10-3-1D (*Mat a*, *erg2-4 ::LEU2*, *ura3-52*, *leu2-3*, *leu2-112*, *his7-2*, *ade5*) used in this study has been previously described [19]. The strain was grown aerobically at 30°C on solid or liquid minimal medium (yeast nitrogen base (DIFCO) 0.67% (w/v), 2% (w/v) glucose) containing suitable supplements (50mg/liter each) and casamino acids (1g/liter), or complete medium (YPD 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose). The yeast strain Δ *erg2*pEMR1235 containing the murine 8,7SI was provided by Dr Loison. The pVT102U [28] *S.cerevisiae* shuttle vector optimized for expressing recombinant proteins in yeast was used for cloning, sequencing and transformation of the *erg2* 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. It contains also an expression cassette including the alcohol dehydrogenase (*ADHI*) promoter and terminator.

Molecular Cloning

Zea mays 8,7SI

A BLAST search with the *A.thaliana* 8,7SI and the emopamil binding protein from *H.sapiens* revealed significant sequence similarities with the hypothetical protein of a *Zea mays* EST clone (GenBank accession no BG840208) which was obtained from the Schnable laboratory of the Iowa State University of Science and Technology (USA). Sequencing of this EST clone revealed that it contained a complete ORF which was PCR amplified with the primer pair P1 (Table 1) containing an XbaI site, and the reverse primer P2 containing an XhoI site. This 666-bp cDNA termed *Zm8,7SI* (AY533175) was cloned between the XbaI and XhoI sites of the pVT102U shuttle vector and placed under the control of the constitutive alcohol dehydrogenase promoter to generate the plasmid pVT-*Zm8,7SI*. Both strands of the amplified cDNA were sequenced to ensure sequence fidelity.

Zea mays FLAG 8,7SI

In order to check the expression and accumulation of wild-type and mutated *Z.mays* 8,7SIs proteins in yeast microsomes, an N-terminal FLAG epitope (MDYKDDDDK) was fused to the 8,7SI protein. For this purpose a DNA molecule containing at 5'-end the corresponding nucleotide sequence was synthesized by PCR using the following forward primer : P3 containing an XbaI site and the reverse primer P2 using the *Zm8,7SI* cDNA as a template. The *ZmFLAG8,7SI* was further cloned between the XbaI and XhoI sites of the pVT102U vector to generate pVT- *ZmFLAG8,7SI*.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the "Quickchange Site-Directed Mutagenesis Kit" (Stratagene) according to the manufacturer's instructions. pVT- *Zm8,7SI* or pVT- *ZmFLAG8,7SI* were used as a template and the synthetic oligonucleotide primers listed in Table 1. Putative, positive clones were picked, plasmids isolated and sequenced.

Transformations

S.cerevisiae transformations were performed using the lithium acetate procedure as previously described [29]. The transformed *erg2* yeast strain was plated on minimal YNB medium containing adenine and histidine, 50mg mL⁻¹ each. Cells were grown aerobically at 30°C.

Sterol Analysis

Lyophilized yeast cells (10 to 30mg) were sonicated in the presence of methanol/KOH (6%, w :v, 2ml) for 10min and heated in the same medium at 70°C under reflux conditions for 2h. The mixture was diluted with 1 volume of water and total sterols were extracted three times with 1 volume of hexane. The extract was dried on Na₂SO₄ and evaporated to dryness. Sterols were analyzed by gas chromatography. GC analysis was carried out with a Varian GC model 8300 equipped with a flame ionization detector at 300°C, column injector at 250°C and a fused capillary column (WCOT :30m x 0.25mm i.d.) coated with DB1(H₂ flow rate of 2mL/min). The temperature program used included a 30°C/min increase from 60 to 240°C and followed by 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 performed using a GC-MS spectrometer (Agilent 5973N) equipped with an "on column" injector and a capillary column (30m x 0.25 i.d.) coated with DB5. Sterols were unequivocally identified by retention times and an electron impact spectrum identical to that of authentic standards [30].

Substrates

Melting points are uncorrected. Proton magnetic resonance was monitored in a [²H]-chloroform solution with a Bruker 400 or 500Mhz spectrometer. Chemical shifts (δ) (ppm) were determined relative to tetramethylsilane. Coupling constants (J) were in Hertz.

*Δ*⁸-*Sitostenol* (**1**) (24(R)-24-ethyl-5α-stigmasta-8-en-3β-ol(**1**)) was extracted from bramble (*Rubus fruticosus*) suspension cultures treated with AY 9944 as previously described [31]. GC purity > 98% (t_R = 1.233, DB1), MS m/z (rel.int.) M⁺ = 414(100), 399(42), 381(8), 315(13), 273(20), 255(19), 229(20), 213(20). ¹H-NMR (CDCl₃) (acetate derivative): δ : 0 ;605(3H,s,H18), 0,812(3H,d,J=6.6,H26), 0.835(3H,d,J=6.6,H27), 0.843(3H,t,J=6,H29), 0.962(3H,s,H19), 4.698(1H,m,H3α).

Zymosterol (**2**) was isolated from the yeast strain *erg6*, *erg2* and crystallized from methanol : mp 107-109°C. GC purity >98% (t_R= 1.057, DB1) MS m/z (rel.int.) M⁺=384(88), 369(100), 351(25), 271(39), 229(40). ¹H-NMR (CDCl₃) : δ : 0.610(3H,s,H18), 0.944(3H,d,J=5.2,H21), 0.950(3H,s,H19), 1.601(3H,s,H27), 1.681(3H,d,J=0.8,H26), 3.614(1H,m,H3α), 5.093(1H,tt,J=7.1,J=1.4,H24).

4α-Methyl-fecosterol (**3**), *4,4-dimethyl-zymosterol* (**4**) and *4,4-dimethyl-fecosterol* (**5**) were isolated from the wild type yeast strain FL530 grown in the presence of 6-Amino-2-*n*-pentylthiobenzothiazole (APB) or from the mutant *erg-25-25c*, as previously described [32].

4 α -Methyl-fecosterol (3) : GC purity > 98% (t_R = 1.198, DB1 ; t_R = 1.175, DB5), MS, m/z (rel. int.) M^+ = 412(100), 397(68), 379(27), 285(46), 227(31).

4,4-dimethyl-zymosterol (4) : GC purity > 98% (t_R = 1.241, DB1 ; t_R = 1.210, DB5), MS, m/z (rel. int.) M^+ = 412(100), 397(70), 379(39), 299(23), 259(35), 241(46).

4,4-dimethyl-fecosterol (5) : GC purity > 98% (t_R = 1.313, DB1 ; t_R = 1.267, DB5), MS, m/z (rel. int.) M^+ = 426(100), 411(45), 393(26), 342(3), 327(8), 299(26), 259(21), 241(22).

Obtusifoliol (6) was synthesized as previously described [33]. mp 139-141°C from methanol, GC purity > 98% (t_R = 1.165, DB5), MS, m/z (rel. int.) M^+ = 426(30), 411(100), 393(15), 327(13), 259(8), 245(25), 215(10).

Cholest-8(14)-en-3 β -ol (7) was synthesized as previously described [34]. mp = 129-131°C from methanol, GC purity > 98% (t_R = 1.010, DB1), MS, m/z (rel. int.) M^+ = 386(100), 371(33), 353(12), 273(13), 255(10). $^1\text{H-NMR}$ (CDCl_3) : δ : 0.829(3H,s,H18), 0.872(3H,d,J=6.6,H26 or H27), 0.875(3H,d,J=6.6,H26 or H27), 0.906(3H,s,H19), 0.916(3H,d,J=6.4,H21), 3 ;803(1H,tt,J=11, J=4.7,H3 α), 5.159(1H,s, ω =6.5,H15) .

Standard assay for recombinant maize sterol 8,7-isomerase in yeast microsomes.

Yeast microsomes were prepared as previously described [32]. Microsomes (0.4 mL, 1.0 mg of protein) were incubated in the presence of exogenous Δ^8 -sitostenol (**1**) (20-150 μM) emulsified with Tween 80 (final concentration 1.5 g/liter). Incubations were continued aerobically at 30°C with gentle stirring for 90 min. During this period the progression of the reaction was linear. The reaction was stopped by adding 1 ml of 6% (w/v) KOH-ethanol. Sterols were extracted three times with a total volume of 15 ml of *n*-hexane, and after drying with Na_2SO_4 , the extract was concentrated to dryness. The extracts were further analyzed by thin-layer chromatography on silica gel, using dichloromethane as the eluant (developed twice). The 4-desmethylsterols (R_f = 0.30) were separated from 4 α -methylsterols (R_f = 0.40) and from 4,4-dimethylsterols (R_f = 0.45). After elution from the silica gel, an aliquot of the 4-desmethyl fraction was analyzed by gas-liquid chromatography. The residual substrate Δ^8 -sitostenol (**1**) (t_R = 1.233) and the product formed, Δ^7 -sitostenol (**8**) (t_R = 1.265) were readily separated from each other and from the bulk of endogenous 4-desmethylsterols, including ergosterol (t_R = 1.083) (Figure A Sup. Mat.). The Δ^7 metabolite (**8**) produced by the reaction was unequivocally identified by its retention time on GC and by an electron impact mass spectrum identical to that of an authentic standard (Table 2). The conversion ratio was calculated from the areas of the peaks of Δ^7 -sitostenol (**8**) and Δ^8 -sitostenol (**1**) and corrected

with the values obtained in the corresponding assay using boiled microsomes. The rate of substrate isomerization was calculated from the conversion ratio of (1) into (8), and the concentration of substrate used in the reaction. Apparent maximum velocity (V_{max}) and K_m values were determined by fitting the data to the Michaelis-Menten equation using the nonlinear regression program DNRP-EASY derived by Duggleby and Leonard from DNRP53 [35].

In the case of inhibition assays, microsomes were incubated for 90 min at 30°C in the presence of (1) (100 μ M) and a range of concentration of inhibitors (0.01-50 μ M) from which the dose-response curves were obtained allowing the corresponding I_{50} values to be determined. The deviation between two determinations did not exceed 15%.

Previous studies revealed that inhibition of postsqualene enzymes of sterol biosynthesis by ammonium analogues of carbocationic high energy intermediates follows a non-competitive kinetic inhibition pattern as a consequence of a slow rate of dissociation of the enzyme-inhibitor complex [10, 36]. Assuming that inhibition of 8,7SI by tertiary ammonium derivatives follows such a non-competitive kinetic inhibition pattern, the I_{50} values measured should be independent of the substrate concentration as well as of the Δ^8 -sterol substrate used.

S.cerevisiae 8,7SI and murine 8,7SI were assayed as previously described in respectively [16] and [17].

Membrane proteins were quantified using the Bio-Rad protein assay according to Bradford [37].

Western blots of microsomes (40 μ g protein) or of 100000g supernatants from *ZmFLAG8,7SIs* transformed yeast were achieved after separation of proteins on SDS-14% (w/v) polyacrylamide gel. After electrophoretic transfert to a polyvinylidene difluoride (PVDF) Immobilon P membrane (Millipore), *ZmFLAG8,7SI* was immunoblotted with affinity purified murine monoclonal ANTI-FLAG M2 antibodies from Sigma (1/6000 dilution) according to the manufacturer instructions. Goat anti-mouse IgG-alkaline phosphatase conjugate (Bio-Rad) was used as secondary antibody (1/10000 dilution). The membrane was then treated with the chemiluminescent AP substrate and the blot was further used to expose an instant film for detection.

Incubation of substrate analogs and identification of enzyme-generated products.

The apparent K_m and V_{max} of analogs (1), (2) and (3) were determined by incubating them for 60-90 min at 30°C under standard assay conditions in a yeast microsomal preparation of recombinant *Zm8,7SI*. The concentration of substrate was 40-150 μ M. In the case of (3) and (6), the 4 α -methylsterol fraction was analyzed by GC-MS. In the case of Δ^8 -sterols (4) and (5), the 4,4-dimethylsterols fraction was analyzed by GC-MS. The 8,7SI products were unequivocally identified by their retention time and an electron impact spectrum identical to that of authentic standards (Table 2). The extracts of incubations of Δ^8 -sterols (4), (5), (6), and (7) and of the corresponding controls performed with inactivated microsomes revealed the absence of isomerization product and complete recovery of these compounds which was confirmed by ion monitoring that corresponded to the mass of the substrate and expected Δ^7 -sterol product.

Sequence alignment.

Multiple amino-acid sequences were aligned with Clustal W algorithm.

RESULTS.

Characterization of sterol-8,7-isomerase from *Zea mays*.

Cloning of sterol-8,7-isomerase in Zea mays and sequence analysis.

We identified a maize EST clone encoding a protein presenting 54% identity with the previously identified 8,7SI from *A.thaliana* [18] and 36% identity with the *Homo sapiens* EBP protein [38]. However, the predicted protein showed only 15% identity with the *ERG2* protein from *S. cerevisiae* [19] (Figure 3). Analysis of the *Zm8,7SI* protein sequence indicated the presence of four transmembrane spanning domains similar in order, spacing and length to those found in murine 8,7SI [16] but clearly distinct to the three-hydrophobic domain model observed for the yeast 8,7SI. In addition, *Zm8,7SI* possesses a C-terminal ER retrieval signal KKXX.

Zm8,7SI can complement the erg2 strain deficient in 8,7SI.

To further characterize the function of the cloned full-length *Zm8,7SI*, we performed a yeast complementation assay in the *ERG2*-deficient strain lacking the 8,7-sterol isomerase activity necessary to synthesize ergosterol. The *Zm8,7SI* ORF was cloned into the pVT102U shuttle vector under the control of the constitutive alcohol dehydrogenase promoter. Several pVT transformants were picked from the selection plate and propagated in liquid media. After

sterol extraction, the sterol profiles were analyzed by GC and GC-MS. The strains *erg2*-pVT-*Zm8,7SI* and *erg2*-pVT-*ZmFLAG8,7SI* accumulated ergosterol (44 -57%) as the major sterol, several Δ^7 -sterols (8-10%) and residual amounts (26-37%) of Δ^8 -sterols (Table 3). In comparison, the *erg2*-pVT-*VOID* (without insert) control strain produced exclusively Δ^8 -sterols (Table 3). These results demonstrate that *Zm8,7SI* and *ZmFLAG8,7SI* can efficiently complement the *erg2* strain and restore endogenous ergosterol synthesis.

Zm8,7SI has sterol 8,7-isomerase activity in vitro.

An enzymatic assay was performed to test whether the recombinant putative *Zm8,7SI* protein in the transformed *erg2* strain indeed possesses sterol 8,7-isomerase activity. Purification of plant membrane proteins for functional analysis after expression in yeast is still a relatively unexplored field with little documentation in the literature. Additionally, in the case of an enzyme which is membrane-bound, interactions with other components of the membrane may be necessary for optimum enzymatic activity. Thus, 8,7SI activity was assayed in the microsomes extracts prepared from *erg2*-pVT-*VOID*, *erg2*-pVT-*Zm8,7SI* and *erg2*-pVT-*ZmFLAG8,7SI* by using the standard assay conditions for recombinant 8,7SI described under "Experimental procedure". The results from these studies revealed that microsomes extracts obtained from *erg2*-pVT-*Zm8,7SI* and *erg2*-pVT-*ZmFLAG8,7SI* were able to isomerize the 8,7-SI substrate, Δ^8 -sitostenol (**1**), to produce a single Δ^7 -sterol metabolite, Δ^7 -sitostenol (**8**) which was unequivocally identified by GC-MS analysis (Table 2, Figure A supplementary material). 8,7SI activity was undetectable in reactions with microsomal extract of *erg2*-pVT-*VOID*. The observed catalytical competence of *Zm8,7SI* revealed that it indeed encodes a membrane-bound maize 8,7-SI.

Substrate specificity of *Zm8,7SI*.

A series of Δ^8 -sterols with distinct nucleus or side-chain structures were assayed with the recombinant *Zm8,7SI* (Table 4). The apparent kinetic parameters of *Zm8,7SI* with substrates Δ^8 -sitostenol (**1**), zymosterol (**2**) and 4 α -methylfecosterol (**3**) were determined by varying their concentration under our standard assay conditions. The velocity/substrate concentration curves obey simple Michaelis-Menten kinetics with respect to (**1**), (**2**) and (**3**) (Figure B supplementary material) and the obtained kinetics data are summarized in Table 4. The data indicate that Δ^8 -sterol (**1**), and (**2**) without methyl group at C4 β and C14 α were productive substrates and that (**3**) possessing a single C4 α -methyl substituent had the highest

apparent relative specificity constant (V_{\max}/K_m) in the series and thus appeared to be the preferred substrate of the isomerase. In contrast the presence of an additional C4 β -methyl group in substrates (4) and (5) abolishes activity as did the addition of a C14-methyl group as in substrate (6). While the enzyme can catalyze the isomerization of various Δ^8 -sterols, the $\Delta^{8(14)}$ -monoene analog (7) is not isomerized indicating the strong regioselectivity of the isomerase for the substrate double bond localisation. Finally, the terminal part of the side chain was not essential to activity. As shown in Table 4, absence of substituent at C24 (structure 2) or addition of one or two carbons atom substituent at C24, (3) and (1), were not structural deterrents for the 8,7SI activity.

Inhibition of 8,7SI by lipophilic tertiary ammonium derivatives.

We first synthesized in our laboratory a series of rationally designed carbocationic HEI analogues, including compounds (17) and (18), possessing a nitrogen atom with a steady positive charge in place of carbenium-C, which could simulate the C8-HEI [10,15]. With this mechanism in mind, inhibition of the recombinant *Zm*8,7SI activity by a series of lipophilic tertiary ammonium derivatives susceptible to interact with its active site, and possessing a variety of structures and biological properties, have been examined. This included the agronomical N-substituted morpholine fungicides tridemorph (11) and fenpropimorph (12) [13,14], the fungicide AY-9944 (13) [39], the human receptor σ ligand SR31747A (14) [40], the high affinity human emopamil-binding protein (EBP) ligand trifluoperazine (15) [17], the estrogen receptor modulator tamoxifen (16) [41], and the rationally designed 8-aza-decalins (17), (18) and (19). Table 5 provides a summary of the dose-response curves obtained for *in vitro* inhibition of the recombinant plant 8,7SI by these compounds, allowing the corresponding I_{50} values to be determined. In addition, we similarly determined I_{50} values for a number of these compounds for the yeast and murine 8,7SIs. These data and those found in the literature for *in vitro* inhibition of yeast and animal 8,7SIs are shown in Table 5.

By examining first at pH=7.5 the cationic azadecalin [17] and the corresponding neutral amide analogue [19], yielding I_{50} values of 0.85 μ M and more than 100 μ M, respectively, it was confirmed that the HEI analogues function with a charged ammonium group. Six ammonium-derivatives were potent inhibitors of *Zm*8,7SI with I_{50} values in the range 0.1-2.0 μ M and I_{50}/K_m (1 or 3) = $2 \cdot 10^{-4}$ - $2 \cdot 10^{-2}$. Two ammonium-derivatives (15) and (16) with a higher molecular weight and less structural flexibility were much poorly accommodated,

yielding I_{50} values of 8.5-10 μM . The lower inhibition by these two ammonium-derivatives indicates that the binding site has a limited tolerance in terms of size and flexibility of the lipophilic domain of the HEI analogues.

Our *in vitro* assay revealed the potent inhibition of the plant 8,7SI by the σ -ligand SR31747 (**14**) ($I_{50}=0.1\mu\text{M}$) which is comparable to the inhibition of the recombinant yeast ($I_{50}=0.6\mu\text{M}$) [16] and mammalian 8,7SI ($I_{50}=0.40\mu\text{M}$) by this derivative (Table 5). These data indicate that the diverse 8,7SIs contain a high-affinity binding site for the σ -ligand SR31747. In addition, the observed affinity of (**14**) is in the same order as that measured for the *Zm*8,7SI with HEI analogues (**17**) and (**18**) ($I_{50}=0.85$ and $0.10\mu\text{M}$ respectively), and (**18**) is a good inhibitor of the mammalian ($I_{50}=10\mu\text{M}$) [42] and yeast 8,7SI ($I_{50}=0.2\mu\text{M}$). Moreover, we observed a similar affinity of trifluoperazine (**15**) for the plant 8,7SI ($I_{50}=10\mu\text{M}$) and for the human EBP ($I_{50}=7\mu\text{M}$) [17], while it is a poor inhibitor of yeast 8,7SI ($I_{50}>500\mu\text{M}$).

Requirement of acidic amino-acid residues for *Zm*8,7SI activity.

Multiple sequence alignment of 8,7SI across seven species (*Z. mays*, *O. sativa*, *A. thaliana*, *H. sapiens*, *M. musculus*, *R. norvegicus* and *S. cerevisiae*) (Figure 3) revealed that across all seven species, 8,7SIs show identity for a variety of amino acids. In addition, the plant and animal 8,7SIs show additional identities which are not shared with the yeast enzyme.

Based on the catalytic mechanism of 8,7SI, good candidates for both the proton donor and the proton acceptor would be acids as previously suggested [4]. To identify acid residues that might be critically important for the proton delivery and (or) abstraction in the plant sterol isomerase, we mutated a number of acidic residues conserved in the 8,7SIs between plants, animals and yeast, or conserved only between plants and animals, so that they could no longer serve as proton-donating or accepting residues. In addition, we also mutated a conserved histidine residue which could function as a proton-donating or accepting residue during the isomerization reaction. Moreover, previous studies from our laboratory emphasized the importance of electrostatic interactions during the binding of carbocationic HEI analogue inhibitors to the catalytic site [36]. The nature of the amino acid residue of the enzyme interacting with these inhibitors is not known, but it could be a delocalized carboxylate anion such as the two aspartate and glutamate residues which have been shown to be obligatory for ammonium-derivatives binding of the σ_1 receptor [43].

In order to have minimal effects on secondary structure, we chose to mutate the different amino-acid by sterically conservative hydrophobic and electrically neutral residues. Thus we neutralized residues i) H74, E78, and E121 which are conserved in animals, plants and yeast and ii) E102, D107, D114, and D170 which are conserved only in animals and plants. The mutants proteins (H74L, E78V, E102V, D107V, D114V, E121V and D170V) were expressed in *erg2* null mutant and the mutated isomerases were assayed *in vivo* by sterol analysis and quantification of $\Delta^{5,7}$ -sterols. In addition, the activities of the recombinant plant 8,7SIs were examined directly *in vitro* in the corresponding yeast microsomal preparations.

The effects of these mutations on *in vivo* and *in vitro* activities of Zm8,7SI are shown in Table 6. In four out of the seven mutants, H74L, E78V, D107V and E121V, replacement of the proton delivering residue by leucine or valine totally eliminated the activity both *in vivo*, since neither $\Delta^{5,7}$ - nor Δ^7 -sterols were detected, and *in vitro*, since no 8,7SI activity could be detected (Table 6), indicating that these residues were essential for the enzyme activity. The other mutants, D114V and D170V exhibited similar sterol profiles as the wild-type and an *in vitro* activity that was about 60% of the activity of the wild-type enzyme. Therefore, these residues are not essential for the catalysis but contribute to the activity through conformational or other effects.

For the mutants that failed to complement it was conceivable that conservation of functionality and charge at these positions might be sufficient for 8,7SI activity. We explored this possibility in one case, E78, and changed this residue to a number of other residues, such that it could either no longer serve as a general acid or its pK would be significantly perturbed. We constructed 5 additional mutants : mutant E78D, E78H, E78R, E78K and E78Q. None of these mutants complemented *erg2*, suggesting that both functionality and length of the side chain of the residue 78 are critical for 8,7SI activity.

Involvement of critical tryptophan residues for Zm8,7SI activity.

Electron-rich aromatic amino acids have been suggested in general terms as particular stabilizers of intermediate carbocations [44]. The crystal structure of the hopene cyclase indeed revealed that several tryptophanes and phenylalanines residues were well positioned to stabilize different cations of the cyclization cascade [45]. Thus, we mutated i) W183 which is mostly conserved in animals, plants and yeast, ii) W66, W100, W193 which are conserved

only in animals and plants and iii) W67 and W205 which are not conserved, by leucine residues.

In two out of the six mutants, W66L and W193L, replacement of the tryptophane residue by leucine totally eliminated the activity both *in vivo* and *in vitro* (Table 6) indicating that these residues were essential for the enzyme activity. The mutants, W67L, W100L, W183L and W205L exhibited similar sterol profiles as the wild-type and an *in vitro* activity that was 60 to 100% of the activity of the wild-type enzyme. Therefore, these residues are not essential for the catalysis but contribute to the activity through conformational or other effects.

An hydroxyl function at residue 124 is needed for maximal binding of the enzyme-substrate complex.

We looked for conserved residues that could be involved in the binding of the 3 β -OH group of the sterol substrate at the end of the binding pocket. Some of the aforementioned carboxyl residues could form an hydrogen bond with the 3 β -hydroxyl group of the sterol substrate. For exemple, the estrogen receptor donates a hydrogen bond to a glutamate residue [46]. Moreover, in *Mycobacterium tuberculosis* sterol 14 α -demethylase, an aspartate residue is hypothesized to form a hydrogen bond with the 3 β -OH [47]. However, in *Candida albicans* sterol 14 α -demethylase, a threonine residue was predicted to form a hydrogen bond with the 3-OH of the sterol substrate and helped to locate it in the active site [48]. Thus, we mutated the hydroxy-containing residue T124 conserved in all organisms (Figure 3).

While replacement of threonine 124 by a functionally conservative serine residue in mutant T124S led to a sterol profile similar to the wild-type, its replacement by an hydrophobic isoleucine residue in mutant T124I led to a strong decrease in $\Delta^{5,7}$ -sterol content (6%), absence of Δ^7 -sterols and high accumulation of Δ^8 -sterols (Table 6). Because this mutation led *in vivo* to a sterol profile clearly distinct from both those of wild-type-like or inactive mutants, we characterized more precisely its enzymatic kinetics properties *in vitro*. The fact that the K_m for mutant T124I, is increased 25-fold (3.03mM) indicates that Thr124 contributes to the stabilization of the enzyme-substrate complex in the ground state. In contrast, V_{max}/K_m was decreased about only 3-fold, indicating that Thr124 has a minor role in stabilizing the transition state of a rate-controlling step of the 8,7SI reaction, which is much less destabilized in T124I. The result is a substantial decrease of the activation energy to reach

the transition state thus leading to a significant improvement of the isomerase maximum rate [49] ($101\text{nmol}\cdot\text{mg}\cdot\text{h}^{-1}$) in mutant T124I.

Functional diversity between the plant and yeast 8,7SIs.

To know whether sterol 8,7-isomerization reaction in plant and yeast would involve identical essential amino-acid residues, we mutated in *Saccharomyces cerevisiae* 8,7SI conserved residues corresponding to amino-acid residues found to be essential or important for the *Zm8,7SI* activity. Thus yeast mutants H69L, E73V, E116V and T119I, corresponding respectively to H74, E78, E121 and T124 in *Zm8,7SI*, were constructed and analyzed as described above. Mutants H69L, E73V and E116V exhibited similar sterol profiles as the wild-type yeast 8,7SI (Table 6). Therefore, in contrast to *Zm8,7SI* and animal 8,7SI, acidic amino-acid residues at these positions, as well as at position 102 corresponding to D107 in *Zm8,7SI*, are not essential for the yeast 8,7SI catalytic activity (Table 7). In addition, table 7 shows also that two essential *Zm8,7SI* tryptophane residues (W66 and W193) were also not conserved in *Sc8,7SI*.

In contrast, threonine 119 corresponding to T124 in *Zm8,7SI* was found to be essential also for *Sc8,7SI* activity (Tables 6 and 7).

Expression of mutated *Zm8,7SIs*.

Because it is an integral membrane-bound protein, *Zm8,7SI* has not yet been purified allowing production of antibodies to examine its expression levels in the microsomes extracts used herein. However, two mutational studies of membrane-bound enzymes of the post-squalene sterol synthesis performed in similar yeast expression systems as ours (*erg2* and *erg6* mutants), indicated no major differences in the expression level of the different mutants and wild type enzymes [24,50]. To express our different 8,7SI mutants in the yeast *erg2* strain we used a multicopy plasmid containing a strong promoter (yeast *ADHI* promoter). These conditions should favour the similar expression of these mutants. To check protein accumulation from wild type and mutated *Zm8,7-SI* cDNAs, we constructed for a number of them the corresponding 8,7SI proteins fused to an N-terminal FLAG epitope (*ZmFLAG8,7SI*). Because of the amount of work needed to develop FLAG constructs corresponding to all mutations, we developed FLAG constructs only for a sub-set of essential (D107V) and non-essential (D114V, D170V) acidic residues, as well as for essential

(W193) and non-essential (W205L) tryptophane residues in addition to the WT enzyme. The microsome extracts from the corresponding transformed yeast cells were submitted to SDS/PAGE followed by Western blot analysis using commercial anti-FLAG serum (Figure 4). There is evidence that all mutated and wild-type FLAG8,7SI proteins did accumulate. However there appears to be some differences in the level of accumulation which could be due to the yeast culture conditions which were not optimized for heterologous protein production.

Considering these data together, it seems unlikely that the lack of activity in the mutated 8,7SIs would result from lack of expression or accumulation in the yeast membranes.

DISCUSSION.

The predicted amino-acid sequence of the present *Zm*8,7SI confirms that the plant 8,7SIs, including the *Arabidopsis* isomerase [18], are much more closely structurally related to the animal 8,7SI protein [17] than to the *ERG2* protein from *S.cerevisiae* [19].

In vitro kinetics with a variety of potential substrates revealed that 4 α -methylsterol (**3**) has the highest specificity constant (V_{\max}/K_m) and thus is the preferred substrate of the recombinant maize 8,7SI. The data is in agreement with previous *in vivo* results obtained in plant cells treated with the 8,7SI inhibitor AY9944, indicating an accumulation of 4 α -methyl-5 α -ergosta-8,24(24¹)-dien-3 β -ol (**3**) and 4 α -methyl-5 α -stigmasta-8,24(24¹)-dien-3 β -ol in addition to a variety of C4-demethylated- Δ^8 -sterols [1, 31]. In contrast, substrate specificity studies with membrane-bound rat-liver isomerase indicated that the isomerization occurs primarily after complete nuclear demethylation in the formation of cholesterol from lanosterol [38]. In addition, fecosterol (24-methylene- Δ^8 -cholestenol) was shown to be an isomerase substrate using yeast extracts [8]. Such unique substrate specificity of the plant enzyme is unusual when data for the human and fungal enzymes are compared. The distinct substrate specificities observed for the plant as opposed to human/fungal 8,7SIs presumably reflects the early divergence of the sterol biosynthetic pathway in the plant kingdom and underlines the molecular diversity of 8,7SI substrates in plants, yeast and animals.

The potent inhibition of maize 8,7SI by the σ -ligand SR31747A is in good agreement with the previously reported inhibition by other σ -ligands of the production of ergosterol in *erg2* mutant complemented with an *Arabidopsis* 8,7SI cDNA [18]. Incidentally, drugs such as

the σ -ligand SR31747A which inhibit the activity of the σ receptor and yeast 8,7SI [16], cause defects in a variety of cellular processes including immunosuppressive effects such as inhibition of graft rejection or lymphocyte proliferation [51]. As shown particularly for *Zm8,7SI*, the striking ability of 8,7SIs from different sources to bind a variety of structurally distinct lipophilic tertiary ammonium derivatives with various biological properties is remarkable. It is worth noting that SR31747 (**14**) is structurally not related to the sterol substrate nor to the rationally designed HEI analogs (**17**) and (**18**), although the protonated form of its tertiary amine function presumably interacts with the active site domain that stabilizes the putative HEI. The data underline the primary importance of electrostatic interactions in the binding of such tertiary ammonium derivatives to the isomerase. Accordingly, human 8,7SI (human EBP), has been shown to be able to bind a variety of structurally distinct drugs, suggesting an intimate pharmacological relationship among EBP, Erg2p and σ_1 -receptor [11]. As previously suggested for human 8,7SI [11], the propensity to bind structurally distinct compounds could also be related to the presence of a sterol binding site in the protein. Indeed, plants contain proteins able to bind non-specifically a variety of lipid derivatives including fatty-acids and sterols [52], and the multidrug resistance protein involved in extrusion of xenobiotics takes part in cholesterol biosynthesis [53].

Our *in vivo* and *in vitro* mutational analysis of recombinant 8,7SIs clearly show that acidic residues E78, D107 and E121, and H74 are essential for *Zm8,7SI* enzymatic activity while no essential acidic residue was found in the homologous positions in *Sc8,7SI*. In contrast, the corresponding EBP amino acid residues E81, D108, E122 and H77 have been shown to be also critical for the *in vivo* 8,7SI activity of the human EBP albeit the mutations were not tested directly, *in vitro*, on the enzymatic activity [24]. In the plants and animals 8,7SIs, D107 lies within a sequence box that is completely conserved in a cytoplasmic domain. In contrast H74, E78 and E121 are located into two putative transmembrane segments. H74, E78 and E121 could include the two obligatory distinct residues that deliver and receive a proton during the *trans* addition-elimination of hydrogens atoms during the plant and animal 8,7SIs catalytic process. Incidentally, it has been demonstrated that the initial protonation of the carbon-carbon bond in the squalene hopene cyclase involves a carboxylic acid [45].

For the mutants that failed to complement, we could show in the case of residue E78 that the functionality as well as the length of the side chain residue are critical for 8,7SI activity. The aforementioned detailed catalytical mechanism of the 8,7SI suggests a precise

organization within the Michaelis complex to preferentially add a proton on a C8(9) double bond rather than on a C14 double bond, and to rigidly control the stereochemistry of the proton abstraction at C7. It would be conceivable to disturb the precise molecular interactions of the enzyme-substrate complex by changes in the nature or the distance of the catalytical amino-acid residues to produce an inactive enzyme.

Our data clearly show that tryptophane residues W66 and W193 play crucial roles in the *Zm8,7SI*, either for the correct folding of the protein, or for the catalytical process. In this latter case they could possibly be involved in the stabilization of the C8 carbocationic intermediate. In accord with this hypothesis, W66 and W193 are located into two putative transmembrane segments. Substitution of the homologous residues in the human 8,7SI by alanine was shown to reduce also dramatically the amount of $\Delta^{5,7}$ -sterols in the corresponding yeast transformants compared to the wild-type isomerase [24]. However, no aromatic residue was found in the homologous positions in the yeast isomerase (Table 7), underlying the functional diversity of the different 8,7SIs.

Suppression of the hydroxyl function at residue 124 apparently decreased more substantially the stability of the enzyme-substrate complex in the ground state than in the transition state. The data could reflect the formation of a hydrogen bond between threonine 124 and the 3-OH of the sterol, helping to dock the sterol in the active site in the ground-state as previously proposed in the case of the *Candida albicans* sterol 14 α -demethylase [48]. This residue could have a similar function in the different 8,7SIs since it is important or critical in all of them (Table 7). Incidentally, a more important role for the interaction of the 3 β -hydroxyl group with a specific amino-acid for initial substrate binding than for transition state stabilization would be in accord with previous studies showing that a number of structural features that are critical for binding of the sterol substrates in the ground state (such as the 3 β -hydroxyl group) play a minor role for binding of transition state analogs [36].

The complementing mutations obtained herein did not alter the sensitivity of the transformed yeast strain *erg2* to the inhibitors azadecaline [18] and SR31747 [14], and they showed a similar sensitivity to nystatin as the wild-type, as observed by spotting the corresponding cells on media with or without these inhibitors. In contrast, the non-complementing mutants affected in essential amino-acids were all insensitive to nystatin and, in addition, exhibited a significant increase in sensitivity to [18] and [14] (EC50 decreased 5- to 10-fold, data not shown). It has been previously shown that *ERG2* is not an essential gene in contrast to *ERG24* coding for the sterol C-14 reductase, and that overexpression of *ERG2*

does not lead to fenpropimorph resistance while overexpression of ERG24 does [54]. The increased sensitivity of non-complementing mutants to [18] and [14] could thus reflect a higher concentration of these inhibitors available for blockade of endogenous C14-reductase in the yeast cells because of absence of binding to the mutated *Zm8,7SI*. These data would be consistent with the hypothesis that mutations found to be critical for *Zm8,7SI* activity also lower the binding of these lipophilic tertiary ammonium derivatives to the enzyme. This hypothesis of an intimate structural relationship between the catalytic site and the inhibitors binding domain is in accord with their function as putative HEI analogues and would need a combination of binding assay studies of a radiolabelled inhibitor with the present site-directed mutagenesis study.

Biochemical analysis of *A. thaliana hyd1* mutant strongly affected in embryonic development [26, 55], revealed deficiency in 8,7SI activity. The *hyd1-E508* allele was found to encode a D102N substitution in 8,7SI that corresponds to the D107V mutation in *Z.mays*, that we found also essential for the maize isomerase. All the essential amino acids found in the present work are conserved between *Z.mays* and other plant 8,7SIs including *A.thaliana* 8,7SI. Thus, we think that the conclusions obtained with the present maize mutants could be extrapolated with confidence particularly to the *Arabidopsis* isomerase and the data used for appropriate *Arabidopsis* plant mutants for further characterization.

This first series of mutants of plant sterol 8,7SI in this work has allowed the identification of 6 essential amino-acid residues for plant 8,7SI activity by *in vivo* complementation of the yeast *erg2* mutant and corroboration of the results by direct *in vitro* measurement of the 8,7SI activity in the corresponding microsomes extracts. The absence of any structural information for this membrane associated enzyme limits our ability to verify the hypotheses about the specific role played by the essential amino-acid residues identified herein. However, it is likely that they are located at the substrate binding domain of the active-site of the enzyme.

Remarkably, the critical residues identified in the plant 8,7SI are conserved and also crucial in the animal 8,7SI, while they are either not essential or not conserved in the yeast 8,7SI. These data underline the functional homology between the plant and animal 8,7SIs, and their high divergence with the yeast 8,7SI, in accord with their structural diversity and the distinct stereochemistry of their mechanisms. Moreover, enzymes involved in postsqualene sterol biosynthesis in higher plants generally share amino-acid identity ranging from 28% to 38% with their corresponding *S. cerevisiae* counterparts [1]. In contrast, with less than 15% identity, clear distinct substrate specificities and non conserved essential amino-acid residues,

it appears that the 8,7-isomerization step is performed by completely different enzymes in higher plants and animals on one hand, and in *S. cerevisiae* and probably most fungi on the other hand. This finding, however is not unprecedented in the pathway since no orthologs of sterol C24(24¹) reductase isomerase from *A.thaliana* has been reported in the *S. cerevisiae* genome [1, 56]. In addition to the present work, further structural informations about the active site of the fungal, plant, and mammalian 8,7SI could provide a basis for rational design of more efficacious and specific antifungal agents in addition to a better insight into the molecular mechanism of 8,7-sterol isomerase.

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Abbreviations.

t_r : relative retention time to cholesterol in gas chromatography ; GC-MS : coupled gas-chromatography mass-spectroscopy ; I_{50} : Inhibitor concentration required to reduce the reaction velocity one-half ;

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Stage 2(a) POST-PRINT

Table 1. Sequence of the synthetic oligonucleotides used for molecular cloning and site-directed mutagenesis of cDNA of the maize and yeast 8,7SI genes.

Strain	Sense oligonucleotide sequence (5' – 3')
Cloning	
P1	ATAATATCTAGAAATGGCCGACGCGGCGTC
P2	ATAATACTCGAGTTATTTGGTCTTCTTCACCTTCACAGC
P3	ATAATATCTAGAAATGGACTACAAGGACGACGATGACAAGGCCGC AGCGGCGGTCAATG
Site-directed mutagenesis	
<i>Zea mays</i>	
W66L	CCGACCGTCTGCTCATGTGCTTGTGGGCATTACAG
W67L	CTGCTCATGTGCTGGTTGGCATTACAGGGTTG
W66LW67L	CCGACCGTCTGCTCATGTGCTTGTGGCATTACAGGGTTG
H74L	GCATTACAGGGTTGACCCTCATAATGATCGAGGGG
E78V	CCCACATAATGATCGTGGGGCCCTTCGTC
E78D	CCCACATAATGATCGATGGGGCCCTTCGTCTTC
E78H	CCCACATAATGATCCACGGGGCCCTTCGTCTTCACTCC
E78R	CCCACATAATGATCAGGGGGCCCTTCGTCTTCACTCC
E78K	CCCACATAATGATCAAGGGGGCCCTTCGTCTTC
E78Q	CCCACATAATGATCCAGGGGGCCCTTCGTCTTC
W100L	CCCAATTTCTTTGATGAAGTTTGAAAGAGTATAGTAAGGGAGAC
E102V	GATGAAGTTTGCAAAGTGTATAGTAAGGGAGACTCTAGG
D107V	GAGTATAGTAAGGGAGTCTCTAGGTATGTT
D114V	GGTATGTTGCTAGGGTCACTGCAACTGTTACGG
E121V	GCAACTGTTACGGTCGTAGGGATCACCGC
T124S	CGGTTCGAAGGGATCAGCGCTGTATTGGAAGGCC
T124I	CGGTTCGAAGGGATCATCGCTGTATTGGAAGGCC
D170V	CACCGCGTACTTGGTCCGGCTTCAACTTCTGG
W183L	CGGCCCGTTCTACTTCTTGGCGTATTTCAATTGGCGC
W193L	GGCGCAAACAGCTTCTTGATCTGGATAACCGATG
W205L	CGCCATAAGGTCCTTGAAGAAAACCTTGCGCCGC
<i>Saccharomyces cerevisiae</i>	
H69L	GACGCACTTGCCTCTCTATACGGGGACGAATACATC
E73V	GCCTCTCATTACGGGGACGTGTACATCAACAGGTACG
E116V	CCGCTGTTGGTACTGTTGGGCACACAGGTGTTCACTTTGCTGACG
T119I	GGTACTGAAGGGCACATAGGTGTTCACTTTGCTGACG

Table 2. GC-MS analysis of enzymatic products by the recombinant 8,7SI from *Zea mays*.

Substrate	Enzymatic product	GC(DB1) t_R	MS (%relative abundance of molecular and prominent fragment ions)
Δ^8 -Sitostenol (1)	Stigmast-7-en-3 β -ol (8)	1.265	M ⁺ = 414(100), 399(33), 381(5), 273(18), 255(63) 229(15), 213(22)
Zymosterol (2)	Cholesta-7,24-dien-3 β -ol (9)	1.088	M ⁺ = 384(14), 369(30), 300(18), 271(100), 231(10), 213(12).
4 α -Methyl-fecosterol (3)	4 α -Methyl-ergosta-7,24(24 ¹)-dien-3 β -ol (10)	1.227	M ⁺ = 412(8), 397(8), 379(3), 328(42), 313(7), 285(100) 245(5), 227(15).

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Table 3 : Sterol composition of mutant *erg2* transformed with different plasmids.

Plasmid used to transform <i>erg2</i> null mutant	total $\Delta^{5,7}$ -sterols ^a or complementation rate of <i>erg2</i> mutant	total Δ^7 -sterols	total Δ^8 -sterols	others sterols
% total sterol				
pVT-Void ^b	nd	nd	95.6 ± 0.4	4.4 ± 0.5
pVT- <i>Zm8,7SI</i>	56.7 ± 1.3	10.1 ± 0.7	25.4 ± 0.6	7.4 ± 0.02
pVT- <i>ZmFLAG-8,7SI</i>	43.4 ± 1.4	9.2 ± 0.8	37.7 ± 1.0	9.7 ± 0.4

Sterols were analyzed as described in the Materials and Methods. Data shown are means of 4 experiments ± SD.

a) $\Delta^{5,7}$ -sterols : ergosterol ($t_R=1.083$) ; Δ^7 -sterols : 7,22-ergosta-dienol ($t_R=1.136$), 7,24(24¹), ergosta-dienol ($t_R=1.145$) ; Δ^8 -sterols : 5,8,14,22-ergosta-tetraenol ($t_R=1.048$), 5,8,22-ergosta-trienol ($t_R=1.057$), 8,22-ergosta-dienol ($t_R=1.064$), 8,24(24¹)-ergosta-dienol ($t_R=1.115$), ergosta-8-enol ($t_R=1.121$) ; others sterols : lanosterol ($t_R=1.205$), 4,4-dimethylzymosterol ($t_R=1.215$).

b) control plasmid without insert.

nd : not detectable

Table 4. Substrate specificity of recombinant maize sterol 8,7-isomerase and apparent kinetics parameters for productive substrates.

SUBSTRATE	$K_{m,app}$	$V_{max,app}$	relative V_{max}/K_m^a
	mM	nmol.mg ⁻¹ .h ⁻¹	
Stigmasta-8-en-3 β -ol (1)	116 ^b ± 20	28 ± 5	1.00
Cholesta-8,24-dien-3 β -ol (2)	512 ± 41	101 ± 9	0.87
4 α -Methyl-ergosta-8,24(24 ¹)-dien-3 β -ol (3)	522 ± 45	178 ± 20	1.48
4,4-Dimethyl-cholesta-8,24-dien-3 β -ol (4)		not substrate	
4,4-Dimethyl-ergosta-8,24(24 ¹)-dien-3 β -ol (5)		not substrate	
4 α ,14 α -Dimethyl-ergosta-8,24(24 ¹)-dien-3 β -ol (6)		not substrate	
Cholest-8(14)-en-3 β -ol (7)		not substrate	

For each experiment, kinetic parameters of the different substrates were measured in the same microsomal preparation.

a) $V_{max} = k_{cat} \times [\text{Enzyme concentration}]$; thus for two substrates, A and B, using the same enzyme preparation: $(V_{max}/K_m)^A / (V_{max}/K_m)^B = (k_{cat}/K_m)^A / (k_{cat}/K_m)^B$.

b) Values represent the mean ± S.D. of two independent experiments.

Table 5 : *In vitro* inhibition by lipophilic tertiary ammonium derivatives of recombinant Zm8,7SI.
Comparison with data obtained with the animal and yeast 8,7SIs.

Enzyme source	maize 8,7SI	yeast 8,7SI	mammalian 8,7SI
Inhibitor		I₅₀ (10⁻⁶ M)	
N-Geranyl-8-azadecalin (18)	0.10 ^a	0.2	10 [42] ^b
AY9944 (13)	0.10	ND	16 [11]
SR31747 (14)	0.10	0.60 [16]	0.4
Fenpropimorph (12)	0.21	0.03	ND
N-Benzyl-8-azadecalin (17)	0.85	ND	20 [42]
Tridemorph (11)	1.7	1.0	3 [11]
Tamoxifen (16)	8.5	ND	1.8 [11]
Trifluoperazine (15)	10	>500 [16]	7 [16], 8 [11]
N-benzamido-decalin (19)	>100 : not inhibitory	ND	ND

a) 8,7SI activity and I₅₀ values were determined as described in Experimental using a 100μM concentration of substrate Δ⁸-sitostenol (**1**). Values are the mean of two distinct determinations ; the standard deviation was less than 15%.

b) references refer to values taken from the literature.

ND : not determined or data not found in the literature.

Table 6 : *In vivo* sterol composition and *in vitro* 8,7SI activity of *erg2* null mutant and transformants carrying the wild-type or mutagenized *Z.mays* and *S.cerevisiae* 8,7-sterol isomerases.

Enzyme used to transform <i>erg2</i> null mutant	total $\Delta^{5,7}$ -sterols ^a or complementation rate of <i>erg2</i> mutant	total Δ^7 -sterols	total Δ^8 -sterols	others sterols	Relative <i>in vitro</i> <i>Zm8,7SI</i> activity ^b
<i>erg2</i> null mutant	ND	ND	97.7	2.3	0
<i>Zea mays</i>					
wild-type	56.6 ^c	10.1	25.4	7.4	100 ^d
H74L	ND	ND	92.3	7.7	0
E78V,D,H,R,K,Q	ND	ND	96.5	3.5	0
E102V	50.4	8.4	34.8	6.0	90
D107V	ND	ND	96.8	3.2	0
D114V	37.2	6.0	50.0	9.8	57
E121V	ND	ND	96.9	3.1	0
D170V	42.4	9.7	42.3	9.3	60
W66L/W67L	ND	ND	95.8	4.2	0
W66L	ND	ND	96.0	4.0	0
W67L	57.3	10.2	28.8	5.7	na
W100L	30.8	7.3	55.5	6.5	68
W183L	51.0	11.2	28.4	9.4	88
W193L	ND	ND	96.9	3.1	0
W205L	39.9	6.1	48.8	10.4	110
T124I	5.8	ND	89.8	4.4	34
T124S	46.7	10.2	28.6	14.5	na
<i>S.cerevisiae</i>					
wild-type	56.5	3.8	36.7	3.0	na
H69L	40.1	2.0	51.6	6.3	na
E73V	60.0	ND	31.7	8.3	na
E116V	40.6	2.3	54.0	3.1	na
T119I	ND	ND	97.9	2.1	na

a) Sterols were those described in Table 3. b) The relative *Zm8,7SI* activity was measured in the corresponding microsomal extracts at a 100 μM concentration of (**1**) under the standard assay conditions. c) Percentage of total sterols ; sterols were analyzed as described in the Experimental. Data shown are means of 2 to 4 experiments ; the standard deviation was less than 5%. d) Data shown are means of 2 experiments ; the standard deviation was less than 10% ; 100% activity corresponded to 17 nmole.h⁻¹.mg prot⁻¹ product formed .

ND: not detectable ; na : not analyzed

Table 7. Comparative mutational analysis of plant, animal and yeast sterol 8,7-isomerases.

	Corresponding mutation and (% complementation ^a of the corresponding wild type 8,7SI)									
<i>Zea mays</i> (this work)	WT (100)	H74L (ND)	E78V (ND)	D107V (ND)	E121V (ND)	W66L (ND)	W193L (ND)	T124I (10)		
<i>Homo sapiens</i> (Ref.24)	WT (100)	H77A (9)	E81A (5)	D108A (2)	E122A (10)	W69A (5)	W196A (5)	T125A (4)		
<i>S.cerevisiae</i> (this work)	WT (100)	H69L (71)	E73V (100)	NC	E116V (72)	NC	NC	T119I (ND)		

ND : not detectable

NC : not conserved

a) calculated as the sum of $\Delta^{5,7}$ -sterols, assuming 100% complementation for the wild type enzyme.

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LEGENDS OF FIGURES.

Figure 1 : Reaction catalyzed by the sterol 8,7-isomerase. Proposed mechanism for isomerization of 4 α -methyl-ergosta-8,24(24¹)-dien-3 β -ol (**3**) to produce 4 α -methyl-ergosta-7,24(24¹)-dien-3 β -ol (**10**) by *Zm8,7SI* involving C-8 α protonation and C-7 β H elimination. The putative carbocationic high energy intermediate is in brackets.

Figure 2 : Chemical structure of compounds considered in this study. (**1**) Δ^8 -Sitostenol ; (**2**) Zymosterol ; (**3**) 4 α -Methyl-fecosterol ; (**4**) 4,4-Dimethyl-zymosterol ; (**5**) 4,4-Dimethyl-fecosterol ; (**6**) Obtusifoliol ; (**7**) Cholest-8(14)-en-3 β -ol ; (**8**) Δ^7 -Sitostenol ; (**9**) Cholesta-7,24-dien-3 β -ol ; (**10**) 4 α -Methyl-ergosta-7,24(24¹)-dien-3 β -ol ; (**11**) Tridemorph ; (**12**) Fenpropimorph ; (**13**) AY9944 ; (**14**) SR31747A ; (**15**) Trifluoperazine ; (**16**) Tamoxifen ; (**17**) N-benzyl-4 α ,10-dimethyl-8-aza-trans-decal-3 β -ol ; (**18**) N-[(1,5,9)-trimethyldecyl]-4 α ,10-dimethyl-8-aza-trans-decal-3 β -ol ; (**19**) N-benzamido-4 α ,10-dimethyl-8-aza-trans-decal-3 β -ol.

Figure 3 : Sequence alignment of the sterol 8,7-isomerases. *Z.mays* 8,7SI : AY533175 ; *O.sativa* 8,7SI : AK059848 ; *A.thaliana* 8,7SI : AF030357 ; *H.sapiens* 8,7SI : Z37986 ; *M.musculus* 8,7SI : X97755 ; *R.norvegicus* 8,7SI : AF071501 ; *S.cerevisiae* 8,7SI : M74037. Conserved residues throughout at least four sequences are shaded in gray. *Z.mays* 8,7SI mutated amino acids are boxed. Essential amino acid residue (\star). Important amino acid residue (\bullet). Four putative membrane-spanning domain sequences (1-4) of *Zea mays* 8,7SI are indicated.

Figure 4 : Expression of the FLAG-tagged 8,7SIs. Western blot analysis of 40 μ g of microsomal proteins from *erg2* yeast strain overexpressing the FLAG-tagged wild type *Zm8,7SI* cDNA (WT) and cDNAs with the shown mutations, and of yeast transformed with vector without cDNA (Void). Proteins were resolved by SDS/PAGE and were submitted to immunoblotting with an anti-FLAG serum as described in the Experimental section.

Figure 1

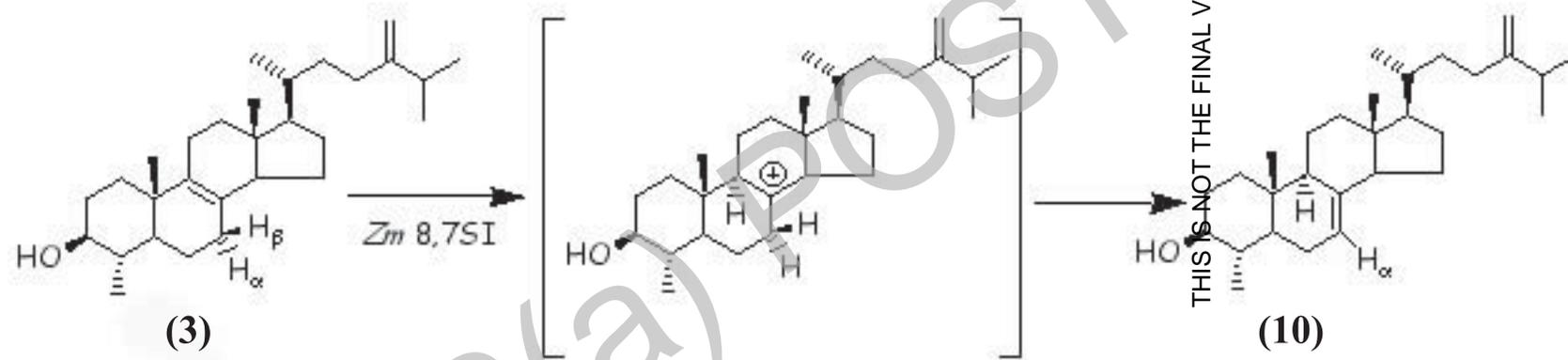
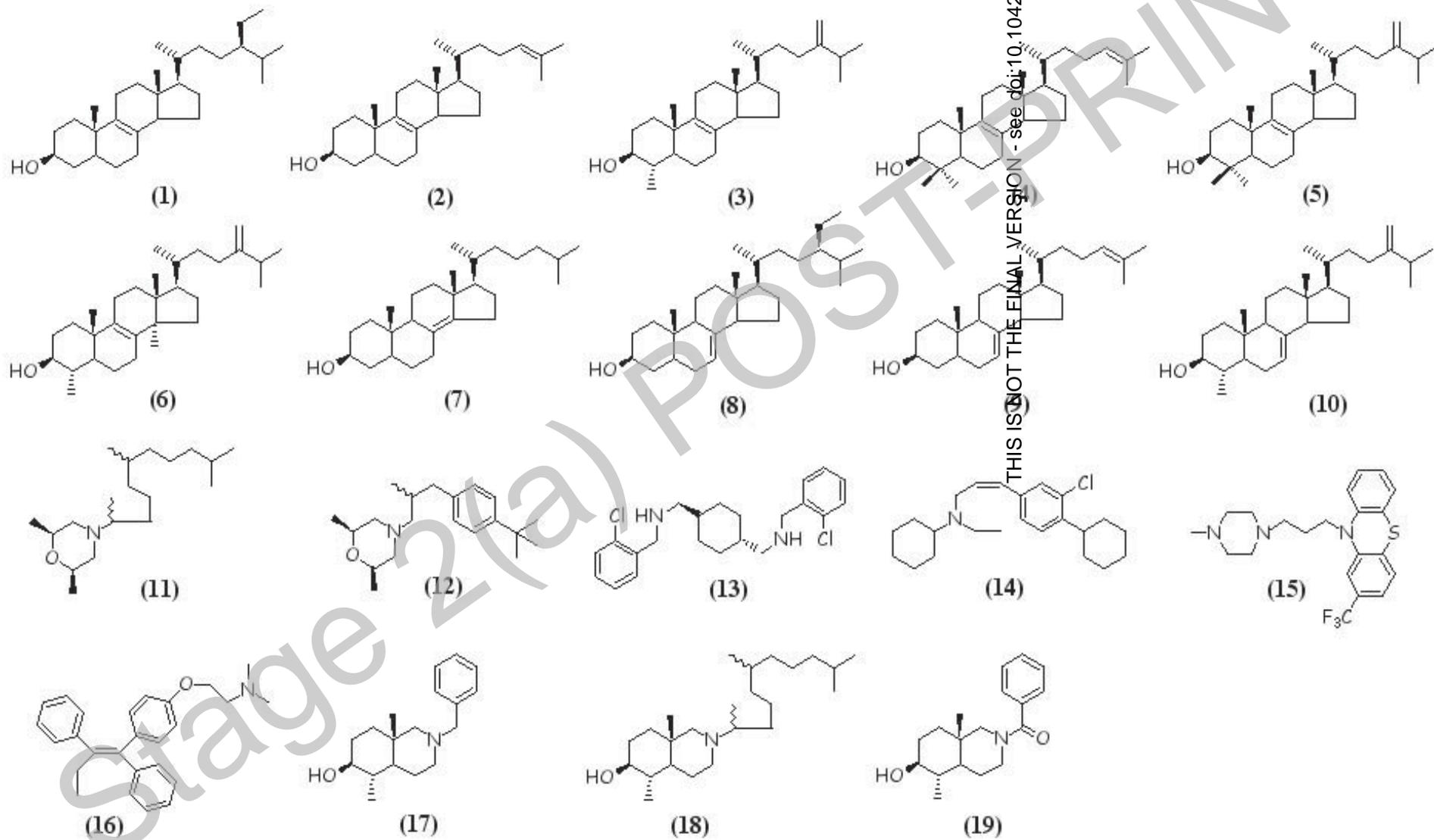


Figure 2



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Figure 3

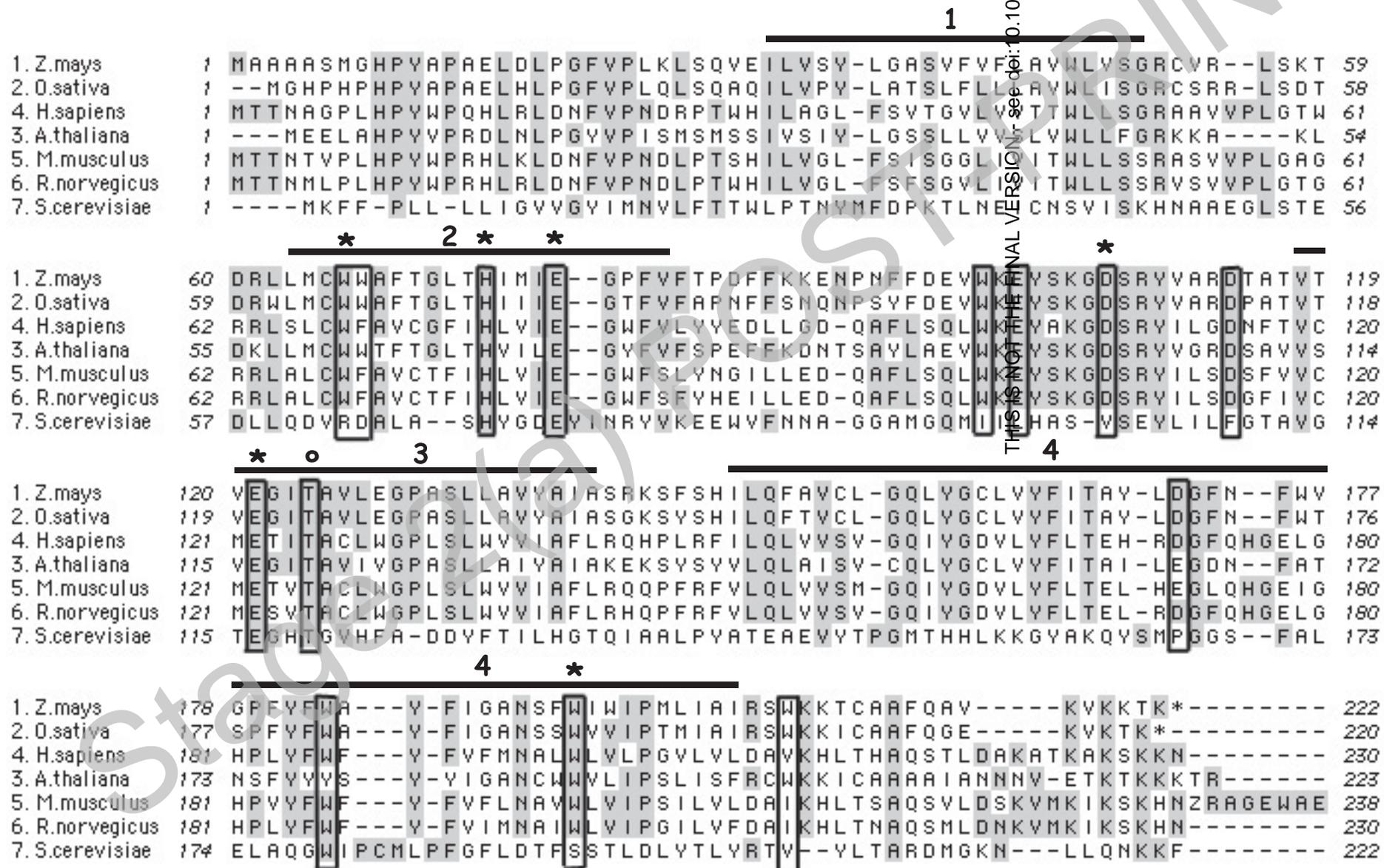
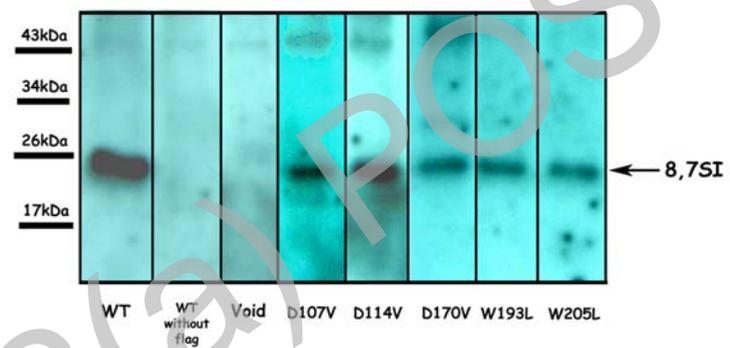


Figure 4



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