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The Induced Evolution of
*Arabidopsis thaliana* Cycloartenol Synthase
and the Cloning of *Oryza Sativa*
Cycloartenol Synthase

by

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A Thesis Submitted
in Partial Fulfillment of the
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Masters of Arts

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ABSTRACT

In the biosynthesis of cholesterol in mammals and ergosterol in fungi, oxidosqualene is cyclized to lanosterol by lanosterol synthase. Similarly, the biosynthesis of plant sterols includes the conversion of the same substrate, oxidosqualene, to cycloartenol by cycloartenol synthase. Cloned lanosterol synthases and cycloartenol synthases are about 30% similar, have over 100 conserved residues and have structurally similar cyclization products. I explored the dissimilarities between these two cyclases by inducing the evolution of a yeast lanosterol synthase mutant expressing cycloartenol synthase. Cycloartenol synthase mutants with lanosterol synthase activity were identified by selecting transformants that were no longer sterol auxotrophs. Once I identified the site of the mutation, I partially characterized the cyclization products of two cycloartenol synthase mutants with lanosterol synthase activity. I also cloned a large fragment of the *Oryza sativa* cycloartenol synthase gene, as concluded by the deduced amino acid sequence.
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INTRODUCTION

Sterol Function and Biosynthesis

Cellular membranes are primarily constructed of lipids and proteins. The lipid composition determines physical characteristics, such as permeability and fluidity. The different types of lipids are common membrane components: phosphoglycerides, sphingolipids and sterols. Characteristically, sterols, which are products of triterpene metabolism, have a 3β-hydroxyl group and a tetracylic backbone, with the rings labeled A, B, C and D (Fig. 1).

Sterols are predominantly found in fungi, plants and animals. One type of fungus, yeast, mainly produces ergosterol to satisfy cellular requirements, whereas plants generate a variety of phytosterols, such as stigmasterol and β-sitosterol. In animals, cholesterol maintains cellular membrane integrity and serves as the precursor of steroid hormones, such as glucocorticoids and sex hormones, and bile acids, like cholic acid. However, cholesterol can be harmful in excess, as in atherosclerosis.
Figure 1  A sterol skeleton with the standard numbering system.
Sterol biosynthesis is subject to feedback inhibition of HMG-CoA reductase (Fig. 2). This rate-limiting enzyme is the target of many antihypercholesterolemic agents like lovastatin and fluvastatin (1). This early inhibition may not be entirely advantageous due to the reduction of downstream intermediates that serve as precursors for other important cellular functions unrelated to sterol production (Fig. 2). Detailed characterization of the structure/function relationship of enzymes downstream in the pathway would provide more specific information for targeting cholesterol biosynthesis. Analysis of late-pathway enzymes in ergosterol biosynthesis has led to the production of an effective antifungal agent, miconazole, a lanosterol demethylase inhibitor that blocks an intermediate step in the conversion of lanosterol to ergosterol (2). As more information is collected about the enzymes in sterol biosynthesis, more effective inhibitors can be designed and applied as antihypercholesterolemic and antifungal agents.
Figure 2  An outline of sterol biosynthesis.
The Conversion of Squalene to Lanosterol

The biosynthesis of squalene, the first step unique to sterol biosynthesis, was described by Woodward and Bloch in 1953 as being made of six isoprene units (3). Ruzicka's isoprene rule is a generalization that all terpene carbon skeletons are constructed of isoprene units (4). From this rule, a rearrangement of the squalene carbon skeleton to form lanosterol was proposed. All tetracyclic or pentacyclic triterpene alcohols are suggested to be derived from one of two carbocation intermediates: protosteryl or dammarenyl (Fig. 3). The protosteryl intermediate has a strained chair-boat-chair configuration of the A, B and C rings, whereas the dammarenyl intermediate has a less strained chair-chair-chair configuration.

Lanosterol is proposed to be derived from the protosteryl intermediate followed by two hydride shifts and two methyl shifts (3,5). The two 1,2 methyl shifts, C-14(CH₃) to C-13 and C-8(CH₃) to C-14 were verified by ¹³C analysis (Fig. 4) (6). The two hydride shifts, C-17(H) to C-20 and C-13(H) to C-17, in addition to the two methyl shifts, were verified using (4R)-[4⁻³H, 2⁻¹⁴C]-mevalonate (Fig. 4) (7). These experiments explain the formation of the A, B, C and D rings, but the initiation of the cyclization was not clear at that time.
Figure 3 The two carbocation intermediates proposed in the cyclization of squalene. The initiation of cyclization by attack on a hydroxyl group is now known not to be true.
Figure 4 The proposed mechanism of lanosterol synthesis from the protosteryl cation.
Squalene cyclization was suspected to be related to the 3\beta-hydroxyl group common to many triterpenes and sterols. One possibility was an attack of OH\(^+\) on one of the termini of squalene (3-5). However, an alternative mechanism involving a new stable intermediate, 2,3-oxidosqualene, was proposed and proven (8). Experiments indicated the requirement of an aerobic environment for the proper conversion of \([^{14}C]\)-squalene to \([^{14}C]\) labeled sterols. In an anaerobic environment, \([^{14}C]\)-2,3-oxidosqualene was sufficient and necessary to produce \([^{14}C]\)-labeled lanosterol. Therefore, in the presence of oxygen, squalene is converted to 2,3-oxidosqualene by squalene epoxidase (ERG1). In a separate step, the oxidosqualene cyclase, lanosterol synthase (ERG7), opens the epoxide ring of 2,3-oxidosqualene and initiates cyclization (Fig. 5). The characteristic 3\beta-hydroxyl substituent is the product of the epoxide ring opening.
Figure 5: The conversion of squalene to lanosterol through 2,3-oxidosqualene.
The Conversion of Squalene to Cycloartenol

Early research of the biosynthesis of sterols and triterpenes in plants showed that \((4R)-[4^{-3}H, 2^{-14}C]\)-mevalonate was the precursor to squalene, which was converted to a variety of 4, 4-dimethyl and 4α-methyl sterols and triterpenes, such as β-sitosterol and α- and β-amyrin (9-12). In animals and yeast, lanosterol was suspected to be a common intermediate in the biosynthesis of sterols. In 1966 Benveniste proposed that cycloartenol was the first cyclic intermediate in phytosterol biosynthesis rather than lanosterol (13). Later, labeled cycloartenol was isolated from incubation of \(1^{-[14}C\)-acetate with a variety of plant tissue cultures (14). After Corey determined that 2,3-oxidosqualene was an intermediate in the conversion of squalene to lanosterol (8), work began to determine if it was also an intermediate in the conversion of squalene to cycloartenol. Corey first proved that 2,3-oxidosqualene was sufficient to enzymatically produce β-amyrin (15). Then, Rees incubated 2,3-oxidosqualene with lysed cells from bean leaves and found cycloartenol to be the only product (16).

Cycloartenol follows the same cyclization mechanism proposed for lanosterol, including the protosteryl carbocation intermediate (17) (Fig. 2), via the oxidosqualene cyclase, cycloartenol synthase (CAS1). Whereas the last step in lanosterol synthesis is proton elimination from C-9 with
formation of Δ8, the last step in cycloartenol synthesis is proton elimination from C-19 with formation of a cyclopropyl ring with C-9. Oxidosqualene is cyclized to cycloartenol, which is further metabolized into numerous phytosterols. Oxidosqualene is also cyclized to other triterpenes, like β-amyrin (15) and lupeol (18).
Cloned Oxidosqualene Cyclase Genes

Many of the early enzymatic studies of cyclization were conducted using liver (hog and rat) microsomal protein extract as a source of oxidosqualene cyclase activity (7,8,19-22). Cyclases are generally associated with the membrane, making isolation of pure protein very difficult (23). However, over the past decade, many oxidosqualene cyclases have been isolated from mammals, fungi and plants (24-27). From a different approach, molecular biology offers a more efficient method of isolating and characterizing proteins.

Cloning oxidosqualene cyclase genes allows us to compare and modify the proteins on a different level. It is possible to infer the evolution of oxidosqualene cyclases based on the genetic information of each cyclase. It is also possible to induce structural and/or functional changes in these cyclases by changing the genetic information. Several oxidosqualene cyclase genes have been cloned recently: lanosterol synthase from Candida albicans (CaERG7) (28,29), Saccharomyces cerevisiae (ScERG7) (30,31), Rattus norvegicus (RnERG7) (32,33), Homo sapiens (HsERG7) (34,35), Schizosaccharomyces pombe (SpERG7) (36); cycloartenol synthase from Arabidopsis thaliana (AtCAS1) (37) and Pisum sativum (PsCAS1) (38).
Figure 6 The amino acid alignment of cloned lanosterol synthases and cycloartenol synthases.

When the amino acid sequences deduced from these genes are aligned and compared, regions of conservation between the genes are evident (Fig. 6). By analyzing these regions, I am able to identify residues that may be necessary for catalysis or design primers for polymerase chain reaction (PCR) to detect and isolate additional cyclase genes.
Yeast, The Model System

As previously described, lanosterol is an intermediate in animal cholesterol biosynthesis and fungal ergosterol biosynthesis. The yeast *Saccharomyces cerevisiae* is a useful system to study sterol biosynthesis. It is ideal for complementation analyses due to the homology between the animal, fungal, and even plant sterol biosynthetic pathways. Many of the genes in the ergosterol biosynthetic pathway have been cloned and characterized (39). For complementation studies, a *S. cerevisiae* lanosterol synthase mutant, SMY8, was developed.

Simple lanosterol synthase deletions are not viable. Wild-type yeast obtain sterols exclusively by biosynthesis in aerobic conditions, but import sterols when oxygen is unavailable. Similarly, sterol biosynthetic mutants import sterols only during anaerobiosis and, therefore, cannot import sterols to complement the mutation unless oxygen is excluded. The heme biosynthetic pathway serves as an oxygen sensor for yeast. By deleting a step in this pathway, such as HEM1, the yeast will import sterols due to the pseudo-anaerobic conditions. SMY8 was constructed in a *hem1* background. It lives by importing exogenous sterols, such as ergosterol or cholesterol, unless otherwise transformed with a lanosterol synthase gene and protein production is induced. SMY8 was used to isolate lanosterol
synthase from *S. pombe* by complementation (36).

**Mutagenic Studies of Lanosterol Synthase from *S. cerevisiae***

Putative catalytic residues in ScERG7 were investigated using site-directed mutagenesis. This technique changes the nucleotide sequence that codes for the amino acid of interest into a different defined sequence that codes for a different specified amino acid. Six conserved aspartate residues and nine conserved glutamate residues in ScERG7 were mutated to asparagine and glutamine, respectively (40). An acidic residue was expected to be responsible for opening the epoxide ring of 2,3-oxidosqualene, thereby initiating the cyclization process. Of the fifteen single mutants, only one lost its function, D456N. This residue is included in a stretch of amino acids conserved among the oxidosqualene cyclases (ScERG7: R439-V468) (Fig. 6). Additional site-directed mutants of ScERG7 were made, 76 in all, to identify other catalytic residues. Only thee of the 76 single mutants suggested residues necessary for catalysis: H146, H234, and M532 (41) (Fig. 6). While site-directed mutagenesis is an effective technique for identifying important residues, it is neither practical nor necessary to make the numerous single and combinatorial site-directed mutants of ScERG7 to identify those residues crucial for cyclase activity.
Squalene-Hopene Cyclase

Prokaryotes do not synthesize sterols, but do require lipids for membrane composition. The function and biosynthesis of these prokaryotic lipids is reviewed by Ourisson et al. (42). Interestingly, one of the lipid groups, hopanoids, is derived from hopene, a cyclization product of squalene. Squalene-hopene cyclases appear to be similar in structure and function to oxidosqualene cyclases found in eukaryotes. A few squalene cyclases have been cloned from different organisms, for example, Alicyclobacillus acidocaldarius (AaSHC) (43) and Zymomonas mobilis (ZmSHC) (44). There are several conserved regions between the squalene cyclases and the oxidosqualene cyclases (44). Recently, the crystal structure of AaSHC was determined (45). The active site contains a number of aromatic residues and the region D374-D377 (Fig. 7). D376 is the residue proposed to be the acid responsible for initiating the cyclization of squalene to the pentacyclic triterpene. This residue is equivalent to Asp456 studied in ScERG7. Just as the mutant protein ScERG7D456N lost its function (40), changes to AaSHCD376 resulted in inactive protein (46). The residues around this key aspartate constitute one of the conserved regions between the squalene-hopene cyclases and the oxidosqualene cyclases (Fig. 7).
Figure 7 An alignment of a region conserved in squalene-hopene cyclases, lanosterol synthases, and cycloartenol synthases.
**Objective**

Lanosterol synthase and cycloartenol synthase have noticeable similarities: function, cyclization products, and genetic information. In spite of these similarities, there are differences that have not yet been fully characterized. My objective was to explore the differences between the two oxidosqualene cyclases; more specifically, determine how the difference in protein structure is contributing to the different cyclization products.
MATERIALS AND METHODS

Strains. The *E. coli* strain DH5α (47) served as the host for most of the plasmids constructed. The *E. coli* strain LE392 (48) was the host for bacteriophage λ derivatives. To generate single stranded DNA for site-directed mutagenesis, *E. coli* RZ1032 (49) was used.

Yeast expression constructs were transformed into SMY8 (*MATa erg7::HIS3 hem1::TRP1 ura3-52 trp1-Δ63 leu2-3,112 his3-Δ200 ade2 Gal+*), a *S. cerevisiae* lanosterol synthase (ERG7) mutant (36).

Plasmids. pRS305GAL was a *LEU2*-marked integrative yeast expression vector (30,36,37). pRS426GAL was a *URA3*-marked high copy (2µ) yeast expression vector (18). pSM60.21 was *AtCAS1* inserted into the pRS305GAL vector. pSM60.22 was *AtCAS1* inserted into the pRS426GAL vector. pSM61.21 was *ScERG7* inserted into pRS305GAL.

The plasmid S14946 was identified from a search in the GenBank database (http://www.ncbi.nlm.nih.gov/Recipon/index.html) of sequences similar to *AtCAS1*. S14946, a partial cDNA from an *Oryza sativa* gene in the bacterial vector pBluescript II SK-, was obtained from the Rice Genome Research Program. Sequencing subclones of LD4, a rice cDNA
clone isolated by hybridization with the partial cDNA in S14946, were constructed in pBluescript II KS+ (Stratagene, La Jolla, CA).

*O. sativa* λ libraries. Two rice cDNA libraries, derived from root mRNA and shoot mRNA, were provided by Dr. W. Buchholz (50) from Texas A&M University. The cDNAs were ligated to Not I/Eco R1 polylinkers before they were ligated into EcoR I-digested λ ZAP II. The titers of the root and shoot libraries were 10⁴ and 10⁵ pfu/μl, respectively. The phage were stored at 4°C.

Media. Ampicillin (amp) resistant strains of *E. coli* were maintained in the liquid media LB amp or on the surface of LB amp plates. LB broth was prepared and autoclaved as instructed by the manufacturer (Fisher Scientific, Fair Lawn, NJ). LB agar for plates was prepared by adding 15 g/L agar to the LB solution prior to sterilization. The LB and LB agar solutions were cooled to ~50°C before adding amp (250X stock: 25 mg/mL in dH₂O; filter sterilize; store at -20°C). When ssDNA was prepared, the transformed *E. coli* strain RZ1032 was grown in liquid 2X YT, prepared and autoclaved as described by the manufacturer (BIO 101, Visa, CA). Kanamycin (kan, 500X stock: 25 mg/mL in dH₂O; filter sterilize; store at -20°C) was also used in preparing ssDNA.
The yeast strains were grown on media containing a nitrogen source and a carbon source. Each was prepared, autoclaved and stored as a 2X solution. The nitrogen source used was either the rich medium, 2X YP (2% yeast extract, 4% peptone in dH₂O; autoclave) or the minimal medium, synthetic complete, 2X Sc (1.7 g yeast nitrogen base, 5 g NH₄SO₄, 2 g –leucine amino acid dropout mix or 2 g –uracil amino acid dropout mix (49), 2 pellets NaOH; dilute to 500 mL with dH₂O; autoclave). The 2X Sc medium was either deficient in leucine (2X Sc–leu) or uracil (2X Sc–ura), depending on the plasmid under selection. The carbon source was either 2X dextrose (D, 4% dextrose in dH₂O; autoclave) or 2X galactose (G, 4% galactose in dH₂O; autoclave). For making plates, 30 g/L agar was added to the 2X sugar solutions prior to autoclaving. Before inoculation, equal volumes of a nitrogen source and a carbon source were combined. To support SMY8, both heme (H; 100X stock: 65 mg hemin, 25 mL EtOH, 25 mL dH₂O, 250 μL 1 M NaOH) and ergosterol (E; 100X stock: 100 mg ergosterol, 25 mL EtOH, 25 mL Tween 80) were added to the media prior to inoculation.

Prior to bacteriophage infection, the *E. coli* strain LE392 was grown to saturation in LB containing 0.2% maltose (100X stock: 20% maltose in dH₂O; autoclave) and 10 mM MgSO₄ (100X stock: 1 M MgSO₄ in dH₂O;
filter sterilize). The phage were stored in suspension medium (SM, 20 mL 1 M Tris-pH 7.5, 5.87 g NaCl; autoclave, cool; 10 mL 1 M MgSO₄; store at 4°C). SM was also used to make serial dilutions of the phage. Lambda top agar (10 g tryptone, 2.5 g NaCl, 7 g agar diluted into 1L dH₂O; autoclave) was used to plate the phage infected LE392.

**Transformation conditions.** Chemically competent *E. coli* DH5α were prepared by Jennifer Reyna, Ling Hua and Martha Lovato. A single colony of DH5α was used to inoculate 20 mL TYM broth (0.5% yeast extract, 2% tryptone, 0.1 M NaCl, 10 mM MgSO₄ in dH₂O; autoclave) in a 250 mL flask. The culture was incubated at 37°C with shaking until midlog phase (OD₆₀₀ ≈ 0.2 - 0.8), then diluted into 100 mL TYM in a 2 L flask. The culture was incubated with shaking again until OD₆₀₀ ≈ 0.5 - 0.9, then diluted to 500 mL with TYM. Once OD₆₀₀ = 0.6, the flask was put in ice-water and swirled gently to cool the culture quickly. The culture was centrifuged for 15 min at 7000 x g and the supernatant was decanted. The pellet was resuspended in ~100 mL cold TfB I (30 mM KOAc, 50 mM MnCl₂, 100 mM KCl, 10 mM CaCl₂ in dH₂O; autoclave) by gently swirling on ice. The suspension was centrifuged again at 7000 x g for 8 min. The supernatant was decanted and the pellet was resuspended in 20 mL cold TfB II (10 mM Na-MOPS-pH 7.0, 75 mM
CaCl$_2$, 10 mM KCl; autoclave) by gently swirling on ice. Aliquots (100 µL - 300 µL) of the resuspended cells were transferred to prechilled sterile 1.5 mL microcentrifuge tubes, frozen in liquid nitrogen and stored at -80°C. For a transformation, 100 µL of competent cells, thawed on ice, were mixed with 5-10 µg plasmid DNA. The mixture was incubated on ice for 20 min, 37°C for 5 min, plated onto LB amp plates and stored at 37°C 14 to 16 h.

To transform SMY8, a single colony of SMY8 was inoculated into 6 mL YPDHE and incubated at 30°C until the culture was saturated (2 to 3 days). The yeast cells were pelleted by centrifugation for 3 min at 1500 x g. The supernatant was decanted and the pellet was resuspended in 100 µL of sterile dH$_2$O. Then 50 µl of carrier DNA (200 mg high molecular wt. DNA sodium salt type III from salmon testes (#D-1626, Sigma) into 100 mL TE8; stir overnight in cold room; store at -20°C) and 10 µg of plasmid DNA was added to the resuspended yeast and vortexed gently a few seconds. Finally, 2 mL of the transformation buffer (40% PEG-3350, 0.1 M lithium acetate, 10 mM Tris•pH 7.5, 1 mM EDTA, 0.1 M dithiotheitol; filter sterilize, store at -20°C) was added to the yeast/DNA mixture and vortexed. The transformation mixture was incubated 6 to 15 h at room temperature. The yeast cells were then pelleted by centrifugation for 3
min at 1500 x g. The supernatant was decanted and the pellet was resuspended in 5 mL sterile dH₂O. The cells were centrifuged and rinsed once more before the final resuspension in 500 μL sterile dH₂O. Two amounts, 30 and 300 μL, were plated on separate selective plates (Sc-leuDHE or Sc-uraDHE depending on the yeast expression vector used). The plates were incubated at 30°C until colonies appeared (3 to 4 days).

λ hybridization. The titer of both the rice root and shoot libraries was determined (49). To screen the library by hybridization, the dilution of phage that produced 10⁵ pfu/plate was used to infect freshly saturated LE392 cells (49). The infected cells were mixed with 5 mL lambda top agar and spread onto large (150 x 15 mm) petri dishes. After 6 to 8 hours at 37°C, the plates, with visible plaques, were stored at 4°C, 2 to 15 h. Labeled 132 mm filters were placed on the plaques, marked, peeled off and air dried λ side up for 15 min. The filters were placed in 0.2 M NaOH/1.5 M NaCl (a denaturation solution) for 2 min, 1 M Tris pH 7.5 (a neutralization solution) for 2 min, and 2X SSC (20X stock: 3 M NaCl, 0.3 M trisodium citrate•2H₂O in dH₂O; adjust pH to 7.0 with HCl) for 2 min. The filters were then air dried for 30 min. The λ DNA from the plaques was fixed onto the filters by UV-crosslinking with a Stratalinker. The filters were stored dry until used for hybridization.
The filters were soaked in 2X SSC, rolled and inserted into a hybridization tube. Church buffer (35 g SDS, 1 mL 0.5 M EDTA, 8.4 g NaH₂PO₄, 25.6 g Na₂HPO₄, 5 g bovine serum albumin, dilute to 500 mL in dH₂O) was added to the hybridization tube. The buffer had been stored at room temperature and was warmed to 65°C prior to use to redissolve the contents. The tube containing the filters and Church buffer was prehybridized at 65°C in the hybridization incubator for 1 to 4 h.

The labeling of the probe was initiated by purifying the fragment of DNA that complemented the target gene from an agarose gel and then quantifying the fragment. In a 1.5 mL screw cap microcentrifuge tube, 25 ng fragment DNA and 1 μg random 12-mer oligonucleotides were mixed. The volume of the mixture was raised to 16.5 μL with dH₂O. After denaturing the dsDNA for 3 min at 100°C, the tube was placed on ice and 5 μL 5X Klenow buffer with dNTPs (except dCTP) and 1 μL exo- Klenow fragment (New England Biolabs, Beverly, MA). The contents of the tube were mixed, then spun in a microcentrifuge for 2 sec. Once in a shielded rack, 25 μCi α-³²P-dCTP was added and mixed by pipetting up and down gently. The mixture was incubated at room temperature for 1 to 4 h.

The labeling reaction was diluted with TE8 (10 mM Tris-HCl-pH 8.0, 1 mM EDTA in dH₂O) and incubated at 100°C for 3 min. The
prehybridization solution was decanted and fresh Church buffer plus half of the labeled probe was added to the tube. The probe hybridized to the filters at 65°C in the incubator 10 to 15 h. The hybridization solution was decanted. The filters were rinsed 4 - 5 times with 0.2X SSC/0.2% SDS and exposed to film overnight with an intensifying screen. The dark spots on the film indicated which plaques had DNA that hybridized to the probe. These plaques were cored with a sterile glass pipet and stored in 1 mL SM at 4°C. Each plaque was titered and hybridized 1 or 2 more times to collect a pure phage isolate.

**Plasmid isolation.** Plasmids from the *E. coli* strain DH5α were purified using the Qiagen purification protocol (Qiagen, Chatsworth, CA). The concentration of the DNA was determined by UV absorbance at 260 nm.

Isolation of plasmid DNA from yeast was initiated by inoculating 5 mL YPDHE with a single colony of transformed yeast. The culture saturated after 2 - 3 days. The culture was centrifuged for 5 min at 1500 x g to pellet the yeast cells. The supernatant was decanted and the cells were resuspended in the remaining liquid by vortexing. The cells were transferred to a 2.2 mL microcentrifuge tube and pelleted for 5 min at 16,000 x g. The supernatant was decanted and the cells were resuspended
in the remaining liquid. The cells were vortexed for 2 min with 200 µL yeast lysis buffer (1% SDS, 2% Triton X-100, 100 mM NaCl, 10 mM Tris-HCl·pH 8.0, 1 mM EDTA; store at -20°C), 200 µL 1:1 phenol/chloroform and 300 µL volume of glass beads (#G-9268, Sigma,). The lysed cells were centrifuged for 5 min at 16,000 x g and the aqueous layer was transferred to a new microcentrifuge tube. The aqueous layer was extracted twice with 200 µL chloroform. The DNA was precipitated by adding 200 µL isopropanol, mixing and incubating at -20°C for 15 min. The precipitated DNA was pelleted by centrifugation for 15 minutes at 16,000 x g. The supernatant was removed and the pellet was resuspended in 500 µL TE8. The DNA was reprecipitated by adjusting [NaCl] to 100 mM and adding 2 volumes of EtOH, mixing and incubating at -20°C for 15 min. The precipitated DNA was centrifuged for 15 min at 16,000 x g and the supernatant was removed. The pellet was washed once with 70% EtOH and air dried for 15 min. The pellet was dissolved in TE8 and stored at -20°C.

Plasmid excision protocol for λ ZAP. Plasmids were excised from λ ZAP as described by Short et al (51). The plasmid DNA was isolated by Qiagen Miniprep (see Plasmid isolation).
**DNA analysis.** Double-stranded DNA was fragmented with restriction enzymes provided by New England Biolabs. Generally, the conditions for the digests included the appropriate restriction enzyme's buffer, 1 to 3 units of enzyme per μg DNA, and 100 ng of DNA per μL reaction volume. The majority of the digests were incubated at 37°C for 1 h; a few restriction enzymes required the reaction to be incubated at room temperature for 2 h. Fragments of DNA were separated by gel electrophoresis and visualized under UV light in the presence of ethidium bromide (0.5 μg/mL). DNA fragments were purified from an agarose gel using Qiaex II (Qiagen, Chatsworth, CA), according to the manufacturer's instructions.

**DNA ligation.** Fragments of DNA were joined together or subcloned into vector plasmids by the enzyme ligase. The reaction mixtures contained 50 ng of vector DNA, 5 molar excess of insert DNA, ligase buffer and 5 units of ligase. The reaction was incubated at room temperature for 5 to 15 h. The success of the ligation reaction was determined by gel electrophoresis. An aliquot of the ligation mixture was transformed into DH5α.

**DNA sequencing.** Sequencing of the genes and gene fragments was completed by the Applied Biosystems International Sequencer and the
dye primer method at the UT-Houston Medical School Molecular Genetics Core Facility. The primers used for sequencing were EXT7, T3 or gene specific primers.

Site-directed mutagenesis. The plasmid to be mutagenized was transformed into RZ1032, a dut ung strain that permits uracil to be incorporated into the DNA and not repaired. A single colony was used to inoculate 1 mL 2X YT amp. The culture was incubated, while shaking, at 37°C for thee hours. After 5 μL helper phage M13K07 was added, the culture was incubated at 37°C and shaken for 1.5 h. The cloudy culture was diluted into 20 mL 2X YT amp kan. The culture was shaken for 12 to 15 h at 37°C. The cells were centrifuged for 10 min at 4°C, 7000 x g. The supernatant was transferred to a new tube and mixed well with 5 mL 20% PEG-8000/2.5 M NaCl. The mixture was incubated on ice for 1 h and spun again at 7000 x g, 4°C for 10 min to pellet the phage. The supernatant was decanted and the pellet was dried for 2 min. The pellet was resuspended in 1.2 mL TE8, transferred to a microcentrifuge tube and incubated on ice for 15 min. The tube was spun in a microcentrifuge for 10 min to remove any remaining cellular debris. The supernatant was transferred to another microcentrifuge tube and mixed well with 300 μL 20% PEG-8000/2.5 M NaCl. The mixture was incubated on ice for 15 min
and centrifuged for 10 min, 16,000 x g to pellet the phage. The supernatant was removed and the pellet was resuspended in 200 µL TE8. The ssDNA was purified by adding 4 µL 5 M NaCl and extracting with 200 µL phenol. The mixture was vortexed and spun in the microcentrifuge for 5 min. The aqueous layer was transferred to a new microcentrifuge tube and extracted once with 200 µL chloroform. The aqueous layer was transferred to a new tube and mixed with 2 volumes of EtOH. The mixture was incubated at -20°C for 15 min and the precipitated DNA was pelleted by centrifugation at 16,000 x g for 15 min. The supernatant was removed and the ssDNA pellet was air dried for 15 min. The pellet was dissolved in 50 µL TE8 and quantified by UV absorbance at 260 nm. The concentration of the uracil-containing ssDNA was then adjusted to 0.5 µg/µL with TE8 and stored at -20°C.

An oligonucleotide complementary to the region to be mutated, with the exception of the few base changes for the mutation, was designed. The mutated region included the change of a base or two to code for a different amino acid and a silent mutation to introduce or remove a restriction site. There were 15 to 18 unchanged bases before the first mutation site and after the last mutation site. The oligo was designed either in the coding or the noncoding direction depending on the orientation of the f1 E. coli
origin of replication. For example, if the ssDNA were the noncoding strand, the oligo was designed as if part of the coding strand. The oligos were synthesized by the Great American Gene Company. The oligo was phosphorylated by mixing 360 pmoles of the oligo, 1 μl 10X kinase buffer, 1 μL 10 mM ATP and 5 U T4 DNA kinase. The reaction was incubated at 37°C for 1 h before 0.5 μL of 0.5 M EDTA was added. The mixture was incubated at 70°C for 10 min. The phosphorylated oligo was diluted to 10 pmoles/μL with dH₂O and stored at -20°C.

The oligo was annealed to the template by mixing 1 μg ssDNA, 25 pmoles phosphorylated oligo, 1 μL 20X SSC and dH₂O to 20 μL total reaction volume. The mixture was placed in a heat block at 70°C. The heat block was removed from the heat source and allowed to cool to below 35°C. The primed DNA was placed on ice and the second strand was synthesized by adding ligase buffer, 500 μM dNTPs, 1 μL single-stranded binding protein, 3 U T4 DNA polymerase, 200 U T4 DNA ligase and dH₂O to 100 μL. The reaction was incubated on ice for 5 min, room temperature for 5 min and 37°C for 2 h. The DNA was precipitated by adjusting [NaCl] to 100 mM, adding 2 volumes of EtOH and incubating at -20°C for 15 min. The DNA was pelleted by centrifugation at 16,000 x g for 15 min. The pellet was air dried for 15 min. After the pellet was
resuspended in 10 μL sterile dH₂O, 4 μL of the mutagenized DNA was use to transform DH5α and 2 μL was used to verify synthesis of dsDNA by gel electrophoresis.

**Synthesis of oxidosqualene.** Racemic 2,3-oxidosqualene was prepared as described by Nadeau and Hanzlik (52). The final product, a pale yellow oil, was stored at -20°C. The oxidosqualene was purified by gel chromatography and was determined to be > 95% pure by TLC.

**Evolution of AtCAS1.** The evolution of AtCAS1 was induced by *in vivo* mutagenesis.* SMY8[pSM60.22] is a yeast heme mutant in which lanosterol synthase is replaced with cycloartenol synthase on a high-copy plasmid. A single colony of this strain was inoculated into 5 mL YPGH supplemented with 20 mg/L ergosterol and incubated at 30°C with shaking. The saturated culture was diluted into 500 mL YPGH supplemented with limiting amounts of ergosterol (0.5 mg/L) and incubated for 24 h, at which point the culture was slightly turbid (OD₆₀₀ = ~0.5). Most of the medium was decanted and discarded (~5 mL was left in the flask to serve as inoculum for the next generation) and another 500 mL YPGH supplemented with limiting amounts of ergosterol (0.5 mg/L) was added. This process was repeated daily for 17 days, at which point the culture

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*The induced evolution of SMY8[pSM60.22] was performed by Prof. Seiichi Matsuda.*
reached a dramatically higher density ($\text{OD}_{600} = \sim 10$), indicating that mutants had evolved that no longer required exogenous ergosterol for growth. A 100 $\mu$L aliquot of the culture was spread on a YPGH plate, and several colonies displayed sterol-independent growth. One colony was inoculated into 5 L YPGH and allowed to grow to saturation for large-scale analysis of the triterpene products.

**Large-scale preparation of the mutant SMY8[pSM60.22].** Once the 5 L culture reached saturation ($\text{OD}_{600} = \sim 10$), the cells were pelleted by centrifugation for 10 minutes at 4°C, 3000 x g. The supernatants were removed and the pellets were combined. The final pellet ($\sim 80$ g) was resuspended in 100 mM $\text{NaH}_2\text{PO}_4$, pH 7.0 to make a $\sim 40\%$ w/v slurry of the yeast cells and was stored in ice-water. The cellular suspension was passed though a French Press twice at 20,000 psi to lyse the cells. The lysate was collected in a flask stored in ice-water. After the lysate was spun in the centrifuge for 20 min at 4°C, 3000 x g, the supernatant was decanted into a 1 L flask. Oxidosqualene solution (20X stock: 20% Triton X-100 w/v, 20 mg/mL racemic oxidosqualene in 100 mM $\text{NaH}_2\text{PO}_4$, pH 7.0) was added to the supernatant, gently swirled and incubated at room temperature for 24 h. The production of a triterpene sterol was visualized by thin layer chromatography, as described below.
**Thin layer chromatography (TLC).** TLC was used as a quick assay to determine the production of triterpene alcohols. Next to a lanosterol standard, 5 μL of the incubation reaction mixture, was spotted in a line, about 1 cm long, lengthwise on a TLC plate. Lanosterol was used as a standard because all known β-hydroxy triterpenes have similar Rfs. After the TLC plate air-dried for 20 - 30 min, it was developed with ether three times to allow the solvent front to travel just above the top of the spotted line to carry all ether-mobile compounds to a common baseline. The solvent system used to resolve the compounds was 1:1 ether/hexane. The plate was viewed under a UV light (λ = 254 nm) which indicated a UV-active spot from ergosterol (Rf = 0.37). Then the TLC plate was dipped in an anisaldehyde stain (15 mL p-anisaldehyde, 10 mL conc. H₂SO₄, 10 mL glacial acetic acid, 350 ml EtOH) and heated to visualize the separated compounds as colored spots. The resolved spots corresponded with spots generated by oxidosqualene (yellow-green, Rf = 0.93), lanosterol/cycloartenol (blue-purple, Rf = 0.56), and ergosterol (brown-green, Rf = 0.37).

**Large-scale analysis of the mutant SMY8[pSM60.22].** For detailed analysis of the products, the compounds were purified from the large-scale incubation. After the reaction was quenched with 2 volumes EtOH, the
mixture was filtered though a cellulose extraction thimble (#2800432, Whatman) in a Soxhlet extractor connected to a 1L collection flask; the flask contained ~200 mL EtOH and a stir bar. A condensor was attached to the extractor and the flask was heated to maintain a steady boil. As hot EtOH fluxed though the apparatus, it extracted various compounds, including sterols and triterpenes, from the solids in the extraction thimble. After 24 h, the apparatus was allowed to cool to room temperature. The filtrate in the collection flask was evaporated to dryness by a rotary evaporator and a warm-water bath. The red oily residue was dissolved in ~100 mL of 1:1 EtOAc/water. The organic layer was washed twice with water (~60 mL) and the aqueous layer was extracted twice with EtOAc (~60 mL). After determining by TLC that the products of interest were only in the organic layers, those layers were combined, dried over MgSO₄, filtered, and evaporated to dryness by a rotary evaporator and a warm-water bath. The oily orange residue was dissolved in ~20 mL of ether and passed though a silica column to remove additional polar impurities. The column, which was packed with 70 - 230 mesh silica gel (15 X 20 mm), was loaded with the material and eluted with ~60 mL ether. TLC indicated that all the nonpolar compounds had eluted. The filtrate was evaporated to dryness in a warm water bath.
The oily yellow residue was dissolved in ~5 mL hexane and loaded onto a 25 X 150 mm column. The column had been packed with 230 - 400 mesh silica gel and equilibrated with hexane. The compounds were eluted from the column with a stepwise gradient of ether/hexane (1% to 20%); 8-mL fractions were collected. The fractions were assayed by TLC and those with material that co-migrated with the lanosterol standard (fractions 19 - 22) were pooled together. The combined triterpene alcohol fraction was evaporated to dryness by a rotary evaporator and a warm-water bath. The pale yellow oily material was then analyzed by NMR. The shifts from the NMR spectrum clearly showed multiple products, including cycloartenol, parkeol, and possibly lanosterol (see Results section). The ability of the mutant SMY8[pSM60.22] to live in the absence of exogenous sterol by producing lanosterol was suspected to be linked to one or more mutations in the CAS1 gene in the transformed plasmid.

Isolation and characterization of the plasmid from the mutant SMY8[pSM60.22]. The plasmid was isolated from the mutant SMY8[pSM60.22] (see Plasmid isolation) and labeled pLD2.0. The plasmid was transformed into DH5α (see Transformation conditions). Individual DH5α transformants were prepared as described in Plasmid isolation. The DNA collected were digested with several restriction enzymes (see DNA
analysis) which verified that pLD2.0 was derived from pSM60.22. pLD2.0 was transformed into SMY8 (see Transformation conditions) and plated onto Sc-uraDHE plates. Single colonies of SMY8[pLD2.0] were streaked onto YPGA, Sc-uraGH and Sc-uraDHE plates alongside the mutant SMY8[pSM60.22] and the original SMY8[pSM60.22] and incubated at 30°C. SMY8[pLD2.0] and the original mutant SMY8[pSM60.22] lived on all three plates, whereas SMY8[pSM60.22] only lived on the Sc-uraDHE plate.

The mutant plasmid was further characterized by subcloning the gene into a smaller vector for sequencing. A Xho I/Not I fragment of pLD2.0, which contained the entire LD2 mutant gene, was ligated into Xho I/Not I digested pBluescript II KS+ to make LD2.1 (see DNA ligation); both fragments had been gel-purified (see DNA analysis). The ligation was transformed into DH5α and the transformants were analyzed by restriction enzymes following plasmid purification (see Plasmid isolation). Constructs were made from an Apa I digest of pLD2.1 and sequenced. Sequencing indicated two base changes, resulting in two residue changes: Q158H and I481V. Subclones with single mutations were made by digesting pLD2.0 and pSM60.22 with BspE I/Sac II; two fragments were purified from both reactions (see DNA analysis). The small fragment containing the 5’-end of
pLD2.0, was ligated to the large fragment of pSM60.22 to make pSM60.22Q154H, and vice versa to make pSM60.22I481V (see DNA ligation). pSM60.22Q154H and pSM60.22I481V were transformed into DH5α (see Transformation conditions) for plasmid purification (see Plasmid isolation).

Characterization of pSM60.22Q154H and pSM60.22I481V. The plasmids pSM60.22Q154H and pSM60.22I481V were transformed into SMY8 (see Transformation conditions) and plated onto Sc-uraDHE plates. Single transformant colonies were streaked onto YPGH and Sc-uraDHE plates beside the controls, SMY8[pSM61.22] and SMY8[pSM60.22], and incubated at 30°C. SMY8[pSM61.22] and SMY8[pSM60.22I481V] lived on the YPGH plate. A single colony of SMY8[pSM60.22I481V] from the Sc-uraDHE plate was inoculated into 6 mL Sc-uraDHE which, upon saturation, was diluted into 20 mL of YPGH. The 20 mL saturated culture served as inoculum for 4 L of YPGH. The saturated culture was harvested for large-scale analysis of the cyclization products.

Large-scale preparation of the mutant SMY8[pSM60.22I481V]. After the 4 L culture of SMY8[pSM60.22I481V] reached saturation (OD<sub>600</sub> = ~10), the cells were pelleted by centrifugation for 10 minutes at 4°C, 3000 x g. The supernatants were removed and the pellets were combined.
The final pellet (~60 g) was resuspended in 100 mM NaH$_2$PO$_4$, pH 6.2 to make a 30 - 40% w/v slurry of the yeast cells and was stored in ice-water. The cellular suspension was passed though a French Press twice at 20,000 psi to lyse the cells. After the French Press, more buffer was added to dilute the slurry to 10 - 20% w/v. Oxidosqualene solution was added to the lysed cells, gently swirled and incubated at room temperature for 15 h. After visualizing the production of a triterpene sterol by TLC, the reaction was quenched with 2 volumes of EtOH to precipitate the proteins and DNA.

Large-scale analysis of the mutant SMY8[pSM60.22I481V]. For detailed analysis of the products, the compounds were purified from the large-scale incubation. After the reaction was quenched with 2 volumes EtOH, the mixture was filtered though a cellulose extraction thimble (#2800432, Whatman) in a Soxhlet extractor connected to a 1 L collection flask. The solids collected in the thimble were not extracted with hot EtOH as was performed in the previous experiment. In a separate extraction experiment, a decrease in sterol yield was detected by TLC following the use of the Soxhlet extractor, therefore a less harsh extraction method was developed for future extractions. The solids filtered from the quenched reaction were extracted once with ~50mL EtOH and once with ~50 mL
EtOAc. The filtrate was evaporated to dryness by a rotary evaporator and a warm water bath. The red oily residue was dissolved in ~50 mL of EtOAc and water. The organic layer was washed twice with ~30 mL water and the aqueous layer was extracted twice with ~30 mL EtOAc. The organic layers were combined, dried over MgSO₄, filtered and evaporated to dryness as done previously. The oily orange residue was dissolved in ~5 mL of EtOAc and passed through a silica plug to remove additional nonpolar impurities. The silica plug was a 15 x 25 mm column packed with 70 - 230 mesh silica gel and, once loaded with the material, was eluted with ~50 mL EtOAc. TLC indicated that all the polar compounds had eluted. The filtrate was evaporated to dryness as before.

The oily yellow residue was dissolved in ~5 mL of 5% EtOAc/hexane and loaded onto a 25 x 200 mm column. The column had been packed with 230 - 400 mesh silica gel and equilibrated with hexane. After the sample was loaded, the compounds were eluted from the column with 5% EtOAc/hexane and 5-mL fractions were collected. The fractions were assayed by TLC and those with common material were pooled together. The potential triterpene alcohols, those fractions with the same R_f as the lanosterol standard were collected and derivatized for further
characterization. The products were later shown by Elizabeth Hart to be lanosterol, parkeol, and cyloartenol, roughly 1:1:2, respectively (53).

**Mutagenesis of AtCAS1.** A new mutant of AtCAS1 was made to characterize other possible cyclization products derived from the mutation of the amino acid at position 481 to a residue smaller than valine. pSM60.21 was mutagenized as described in *Site-directed mutagenesis*. The oligo, SM60I481G, was designed to replace the isoleucine at position 481 with a glycine and to introduce a BsrG I restriction site by a silent mutation: 5'-TTT CAG TCC TTC TGC TGT ACA GTC AGA GGC GGG CCA ACC GTG ATC. The plasmid, pSM60.21I481G, was purified and the mutations were verified by digestion with BsrG I and sequencing with a gene specific primer. After pSM60.21I481G was digested with BstE II, the linear DNA was transformed into SMY8 and plated onto Sc-leuDHE plates. Single colonies were streaked onto YPGH and Sc-leuDHE plates beside the controls, SMY8[pSM61.21] and SMY8[pSM60.21], and incubated at 30°C. SMY8[pSM61.21] and SMY8[pSM60.21I481G] lived on the YPGH plate. A single colony of SMY8[pSM60.21I481G] from the Sc-leuDHE plate was used to inoculate 6 mL Sc-leuGHE for small-scale analysis.

**Small-scale analysis of SMY8[pSM60.21I481G].** Two 2 mL aliquots of saturated SMY8[pSM60.21I481G] culture (OD$_{600}$ = ~10) were
transferred to separate 2.2 mL microcentrifuge tubes for small-scale analysis. The remainder of the saturated culture was diluted into 20 mL of the appropriate medium, which upon saturation was used to inoculate 1 L, for large-scale analysis. Each 2.2 mL tube containing the saturated culture was centrifuged for 5 min at 16,000 x g and the supernatants were removed. A 300 µL volume of glass beads (#G-9268, Sigma) and 200 µL of 100 mM NaH₂PO₄, pH 6.2 was added to each yeast pellet. To one tube, oxidosqualene solution (20X stock: 20% Triton X-100 w/v, 20 mg/mL racemic oxidosqualene in 100 mM NaH₂PO₄, pH 6.2) was also added. The other tube served as a control. The mixtures were vortexed for 2 min, incubated on ice for 2 min and vortexed again for 2 min before incubation at room temperature for 5 to 15 h. The products of the reactions were visualized by TLC. The TLC did not indicate the presence of ergosterol when viewed under UV light. The stained TLC, however, did indicate a significant amount of a new compound (more than the amount of compound visible for cycloartenol in the pSM60.21 control) that was less polar (Rᵢ = 0.7, 1:1 ether/hexane) than the lanosterol standard. The saturated 1 L culture was prepared for large-scale analysis of the triterpene products.
Large-scale preparation of the mutant SMY8[pSM60.21I481G]. Once the 1 L culture of SMY8[pSM60.21I481G] reached saturation (OD$_{600}$ = ~10), the cells were pelleted by centrifugation for 10 minutes at 4°C, 3000 x g. The supernatants were removed and the pellets were combined. The final pellet (~8 g) was resuspended in 100 mM NaH$_2$PO$_4$, pH 6.2 to make a 30 - 40% w/v slurry of the yeast cells and was stored in ice-water. The cellular suspension was passed though a French Press twice at 20,000 psi to lyse the cells. After the French Press, more buffer was added to dilute the slurry to 10 - 20% w/v. Oxidosqualene solution was added to the lysed cells, gently swirled and incubated at room temperature for 15 h. After visualizing the production of the new compound by TLC, the reaction was quenched with 2 volumes of EtOH to precipitate the proteins and DNA.

Large-scale analysis of the mutant SMY8[pSM60.21I481G]. After the reaction was quenched with 2 volumes EtOH, the mixture was filtered though a cellulose extraction thimble (#2800432, Whatman) in a Soxhlet extractor connected to a 500 mL collection flask. The solids filtered from the quenched reaction were extracted once with ~20 mL EtOH and once with ~20 mL EtOAc. The filtrate was evaporated to dryness by a rotary evaporator and a warm-water bath. The red oily residue was dissolved in ~20 mL of EtOAc and water. The organic layer was washed twice with
~20 mL brine and the aqueous layer was extracted twice with ~20 mL EtOAc. The organic layers were combined, dried over MgSO₄, filtered and evaporated to dryness as done previously. The oily orange residue (430 mg) was dissolved in ~5 mL of 1:1 EtOAc/hexane and passed though a silica plug to remove additional nonpolar impurities. The silica plug was a 15 x 25 mm column packed with 70 - 230 mesh silica gel and, once loaded with the material, was eluted with ~30 mL 1:1 EtOAc/hexane. TLC indicated that all the polar compounds had eluted. The filtrate was evaporated to dryness as before.

The oily yellow residue (270 mg) was dissolved in ~5 mL of 5% EtOAc/hexane and loaded onto a 25 x 200 mm column. The column had been packed with 230 - 400 mesh silica gel and equilibrated with hexane. After the sample was loaded, the compounds were eluted from the column with 5% EtOAc/hexane and 5-mL fractions were collected. The fractions were assayed by TLC and those with pure new compound (less polar than the lanosterol standard) were combined (fractions 9 - 11). The material was evaporated to dryness by a rotary evaporator and a warm-water bath. The oily residue (13 mg) was further purified by HPLC, as follows.
**HPLC purification of the 1481G triterpene product.** A 250 mm x 10 mm YMC-Pack-SIL (S-10P mm, 120 Å) column was equilibrated with 85% hexane, 15% t-butyl methyl ether on a Hewlett-Packard 1100 HPLC. The new compound (13 mg) isolated by column chromatography of the cyclization products of SMY8[pSM60.21I481G] was dissolved in 1 mL of the equilibration solvent. Aliquots (100 μL) were chromatographed at 5 mL/min using an isocratic system of the same solvent. The major peak visible at 204 nm eluted at 11.8 min, and fractions containing this peak from ten runs were pooled and concentrated to yield 7.8 mg of a colorless oil. An NMR spectrum was collected from the HPLC purified triterpene. The NMR shifts corresponded with the shifts reported for the monocyclic triterpene, achilleol (see Results section).

**Mutagenesis of ScERG7.** The reverse mutation in ScERG7 was made to identify changes in the cyclization products by replacing valine at position 454 with a larger residue, isoleucine. pSM61.21 was mutagenized as described in *Site-directed mutagenesis*. The oligo, SM61V454I, was designed to replace the valine at position 454 with an isoleucine and to introduce a BsrG I restriction site by a silent mutation: 5’-TTT AAT TGC TTC TGC TGT ACA ATC TGC AAT TGT ATA GCC CTG TGT. The

* HPLC purification of SMY8[pSM60.21I481G] triterpene product was performed by Prof. Seiichi Matsuda.
plasmid, pSM61.21V454I, was purified, sequenced, digested, transformed into SMY8 and plated onto Sc-leuDHE plates as described in *Mutagenesis of AtCAS1*. Single colonies were streaked onto YPGH and Sc-leuDHE plates beside the controls SMY8[pSM61.21] and SMY8[p60.21] and incubated at 30 °C. SMY8[pSM61.21] and SMY8[pSM61.21V454I] lived on the YPGH plate, suggesting that the mutant gene was still able to generate ergosterol by cyclizing oxidosqualene to lanosterol. A single colony was used to inoculate 6 mL YPGH for small-scale analysis of the triterpene products. Under UV light, the TLC indicated the presence of ergosterol. The stained TLC indicated the presence of a compound that co-migrated with the lanosterol standard.

**Cloning of the putative OsCAS1 gene.** S14946, a partial rice cDNA in the bacterial vector pBluescript II SK-, was transformed (see *Transformation conditions*) into DH5α and purified by Qiagen Miniprep (see *Plasmid isolation*). A Sal I/BspD I fragment was purified (see *DNA analysis*) and radiolabeled (see *λ hybridization*). The rice root library was probed with this labeled fragment. The plaques with DNA that hybridized to the probe were isolated and the plasmids were excised from the bacteriophage and purified (see *Plasmid isolation protocol for λ ZAP*). The inserts of the clones isolated were one of two sizes: 2.3 kb and 0.8 kb. A
series of single and double restriction digests determined that the 0.8 kb fragment was from the 3'-end of the 2.3 kb insert. Many sequencing subclones were made of the long insert. Sequence data indicated that the 2.3 kb insert was not full-length; it did not contain a start site. A Sal I/Sma I fragment containing the 2.3 kb insert was ligated into pRS426GAL digested with Sal I/Sma I (see DNA analysis and DNA ligation). An oligo, LD4-MW, was designed to insert the codons for a methionine and a tryptophan at the beginning of the insert while removing a Not I restriction site: 5'-AAG CTT GAT ATC GAA TTC GCG ATG TGG CGG CTG AGG GTG GCG GAG GGC. Methionine and tryptophan are the first two residues in both AtCAS1 and PsCAS1.
RESULTS

The Mutagenesis of A. thaliana Cycloartenol Synthase

Evolution of AtCAS1. The strain SMY8[pSM60.22] was inoculated into liquid YPGH medium containing 0.5 mg/L ergosterol.* The stress of the low ergosterol environment selected for mutants able to synthesize ergosterol over many replications. The new strain was able to live in the absence of ergosterol, as was determined by its ability to grow on a YPGH plate. The plasmid was recovered from the mutant and transformed into original unstressed SMY8. The transformants were also able to live on a YPGH plate, therefore, the new phenotype was linked to the plasmid. The mutant pSM60.22 was sequenced and two base mutations were identified in the gene, resulting in two amino acid changes: AtCAS1Q158H and AtCAS1I481V. Individual mutations were introduced into pSM60.22 to construct pSM60.22Q158H and pSM60.22I481V. Both plasmids were independently transformed into original SMY8; only SMY8[pSM60.22I481V] transformants were able to live on a YPGH plate. The ability of SMY8[pSM60.22I481V] to live in the absence of exogenous

* The induced evolution of SMY8[pSM60.22] was performed by Prof. Seiichi Matsuda.
ergosterol implied that the mutant protein was able to cyclize oxidolsqualene to lanosterol, which was metabolized to ergosterol.

**Analysis of SMY8[pSM60.22I481V].** SMY8[pSM60.22I481V] was inoculated into YPGH for analysis of the cyclization products as described in Material and Methods. The TLC plate of the small-scale preparation indicated production of ergosterol (a UV-active spot; \( R_f = 0.37 \), 1:1 ether/hexane). After staining the TLC plate, there was a purple-blue spot that co-migrated with the purple-blue spot for the lanosterol standard. The NMR of the products purified from the large-scale preparation of SMY8[pSM60.22I481V] clearly showed multiple products, including cycloartenol and another triterpene alcohol, probably parkeol. Parkeol is derived from the protosteryl intermediate, just as cycloartenol and lanosterol, but it is deprotonated at C-11 (17) (Fig.8). Itoh et al. (53) collected the proton magnetic resonance spectra for a series of tetracyclic triterpene alcohols with CDCl\(_3\) on a 60 MHz machine at 28°C. The chemical shifts reported were only the measurements of the methyl signals and a few select protons. The triterpene alcohols Itoh et al. studied included cycloartenol: \( \delta 0.33 \) (d, 1H), 0.57 (d, 1H), 0.81 (s, 3H), 0.90 (s, 3H), 0.97 (s, 6H), 1.62 (s, 3H), 1.69 (s, 3H), 3.27 (m, 1H), 5.11 (m, 1H); lanosterol: \( \delta 0.70 \) (s, 3H), 0.82 (s, 3H), 0.88 (s, 1H), 1.01 (s, 6H), 1.62 (s,
3H), 1.69 (s, 3H), 3.26 (m, 1H), 5.11 (m, 1H); and parkeol: δ 0.66 (s, 3H), 0.75 (s, 3H), 0.82 (s, 3H), 0.99 (s, 3H), 1.06 (s, 3H), 1.63 (s, 3H), 1.70 (s, 3H), 3.25 (m, 1H), 5.10 (m, 1H), 5.25 (m, 1H). The following shifts were identified in the NMR spectrum of the acetylated SMY8[pSM60.22I481V] triterpene products (250 MHz, CDCl₃, 25°C) δ 0.34 (d, cyc), 0.58 (d, cyc), 0.63 (s, par), 0.68 (s, lan), 0.73 (s, par), 1.60 (s, cyc, lan), 1.64 (s, par), 1.67 (s, cyc, lan), 5.10 (m, cyc, lan, par), 5.25 (m, par). The relative concentrations of the triterpenes within the mixture were calculated to be 55% cycloartenyl acetate, 22% lanosteryl acetate and 23% parkeyl acetate.∗

∗ This ratio was determined by the GC analysis of the acetylated cyclization products. This analysis was conducted by Elizabeth Hart.
Figure 8. The structures of cycloartenol, lanosterol and parkeol.
Construction of SMY8[pSM60.22I481G]. Site-directed mutagenesis was used to make pSM60.22I481G as described in Materials and Methods. The mutated plasmid was transformed into SMY8; the transformants were able to live on a YPGH plate. The ability of SMY8[pSM60.22I481G] to live in the absence of exogenous ergosterol suggests the mutant protein was able to cyclize oxidosqualene to lanosterol, which was metabolized to ergosterol.

Analysis of SMY8[pSM60.22I481G]. SMY8[pSM60.22I481G] was inoculated into Sc-leuGHE for analysis of the cyclization products as described in Materials and Methods. The TLC plate of the small-scale preparation did not indicate the presence of ergosterol under UV light. A new compound (light blue spot) that was less polar than a triterpene alcohol ($R_f = 0.7$, 1:1 ether/hexane) was visualized after staining and heating; a triterpene alcohol co-migrating with the lanosterol standard was not obvious. The new compound was purified by column chromatography as described in Materials and Methods. The compound was purified by HPLC*; a pure fraction was analyzed by NMR. The NMR spectrum identified the new compound as achilleol (Fig. 9), a monocyclic triterpene alcohol. The spectrum was compared to the few shifts noted by Barrero et

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* HPLC purification of SMY8[pSM60.21I481G] triterpene product was performed by Prof. Seiichi Matsuda.
al. (55) as characteristic of achilleol: $^1$H NMR (80 MHz, CDCl$_3$) δ 0.72 (s, 3H), 1.02 (s, 3H), 1.60 (s, 12H), 1.69 (s, 3H), 2.02 (bs), 3.42 (dd, J=10.0, 4.5 Hz, 1H), 4.62 (m, 2H), 4.88 (m, 2H), 5.12 (m, 4H).
Figure 9 The structure of achilleol.
Construction of SMY8[pSM61.21V454I]. Site-directed mutagenesis was used to introduce the reverse mutation, V454I, into ScERG7 (see Materials and Methods). The plasmid, pSM61.21V454I, was transformed into SMY8; the transformants were able to live on a YPGH plate. The ability of SMY8[pSM61.21V454I] to live in the absence of exogenous ergosterol implied the mutant protein was still able to convert oxidosqualene to lanosterol, which was metabolized to ergosterol.

Analysis of SMY8[pSM61.21V454I]. SMY8[pSM61.21V454I] was inoculated into YPGH for small-scale analysis as described in Materials and Methods. The TLC plate indicated the production of ergosterol (UV-active spot; Rf = 0.37, 1:1 ether/hexane), and, after staining, one or more triterpene alcohols (purple-blue spot co-migrating with lanosterol standard).
The Cloning of the Putative *O. sativa* Cycloartenol Synthase

A rice gene was hybridized from an *Oryza sativa* root cDNA library with a Sal I/BspD I fragment of S14946 (see Materials and Methods). The gene, LD4, was not expressed in yeast because the clone did not contain a start site, as was determined by sequence data. It is probable that LD4, considering its similarity to AtCAS1 and PsCAS1 (Fig. 10), is only missing the first six base pairs that code for methionine and tryptophan. A Sal I/Sma I fragment containing LD4 was ligated into pRS426GAL. An oligo for site-directed mutagenesis, LD4-MW, was designed to insert six base pairs that code for methionine and tryptophan. Noting the similarity of LD4 to AtCAS1 and PsCAS1 and the presence of isoleucine in the position equivalent to AtCAS1I481, it is probable that LD4 is the cycloartenol synthase gene of *O. sativa* (OsCAS1).
Figure 10 An alignment of AtCAS1, PsCAS1 and LD4.
DISCUSSION

*S. cerevisiae* lanosterol synthase is a remarkable enzyme able to transform 2,3-oxidosqualene, essentially an unsaturated hydrocarbon, into lanosterol, a tetracyclic compound with seven stereocenters. *A. thaliana* cycloartenol synthase is able to convert the same substrate to an extremely similar compound, cycloartenol. From the homology of these sterol compounds, it is evident that ScERG7 and AtCAS1 are homologous enzymes. The residues necessary for the proper cyclization of the A, B, C, and D rings should be conserved between the two enzymes. Among the residues not conserved should be those responsible solely for lanosterol or cycloartenol formation. I am interested in identifying the residues that contribute to the cyclization of oxidosqualene to one compound versus the other. The techniques I used for this investigation include induced evolution, site-directed mutagenesis, and cloning.

The cyclization of oxidosqualene to lanosterol is very similar to the cyclization of oxidosqualene to cycloartenol. The proposed mechanisms have the protosteryl carbocation as a common cyclization intermediate (Fig. 3). The formation of lanosterol differs from the formation of cycloartenol by the final site of deprotonation. The common cyclization intermediate is deprotonated at C9 to form the C8 double bond in lanosterol, versus deprotonation at C10 to form the cyclopropyl ring in
cycloartenol. The formation of the cyclopropyl ring is catalytically unfavorable.

Several lanosterol synthases (Candida albicans, Saccharomyces cerevisiae, Rattus norvegicus, Homo sapiens, Schizosaccharomyces pombe) and two cycloartenol synthases (Arabidopsis thaliana, Pisum sativum) have been cloned and published. As determined by the alignment of the amino acids translated from the cloned DNA (Fig. 6), there are 180 conserved residues among the five cloned lanosterol synthases. Out of those 180, 142 residues are also conserved in the two cloned cycloartenol synthases. Of course, it is not possible for all 142 residues to be part of the active site, but probably 10 to 15 are responsible for the common cyclization of oxidosqualene. Among the 38 residues that are conserved in the lanosterol synthases but not with the cycloartenol synthases, 31 are conserved between the two cycloartenol synthases. Again, not all 31 residues are responsible for the different deprotonation of the common cyclization intermediate to form cycloartenol instead of lanosterol, but a few of them most certainly are.

One method of identifying which of the 142 residues are necessary for catalysis is site-directed mutagenesis. As previously discussed, the Corey group made 76 different site-directed mutants of the lanosterol synthase gene from S. cerevisiae (41). Only four of the 76 single mutants suggested residues essential for catalytic activity: D456, H146, H234, and
M532. Thee of the residues are conserved in all seven cloned oxidosqualene cyclases. M532 is one of the 31 residues that is differentially conserved; a methionine is conserved among the five lanosterol synthases and a valine appears to be conserved between the two cycloartenol synthases. Using site-directed mutagenesis to identify catalytic residues one at a time is slow and inefficient.

A more effective method of identifying residues in the active site would be to analyze the crystal structure of an oxidosqualene cyclase. Unfortunately, no oxidosqualene cyclases have been successfully crystalized. However, the crystal structure of a squalene-hopene cyclase has been published recently. Such cyclases have been cloned from prokaryotes and are similar in function to oxidosqualene cyclases; they cyclize squalene, the parent molecule of oxidosqualene, to hopene, a pentacyclic triterpene.

My first approach to identifying residues that contribute to the formation of lanosterol versus the formation of cycloartenol began with the induced evolution of AtCAS1. My goal was to determine whether or not ergosterol selective conditions would force AtCAS1 to evolve to allow the formation of lanosterol along with or instead of cycloartenol. To accomplish this task, it was necessary to use a yeast lanosterol synthase mutant. In wild type yeast, oxidosqualene is cyclized to lanosterol. Lanosterol is further metabolized to form ergosterol, the sterol preferred
to satisfy cellular requirements (Fig 2). In a yeast lanosterol synthase mutant, oxidosqualene is not cyclized to lanosterol and is therefore unable to form ergosterol (Fig. 11). This mutant is a sterol auxotroph, requiring exogenous ergosterol or cholesterol for survival. A yeast lanosterol synthase mutant expressing cycloartenol synthase is able to cyclize oxidosqualene to cycloartenol (Fig 12). However, this mutant is still a sterol auxotroph because there is no ergosterol production. These mutant yeast were used for the induced evolution experiment.

The lanosterol synthase mutant expressing cycloartenol synthase was forced to replicate under the stress of a low ergosterol environment. Over a number of days, mutants developed that were no longer sterol auxotrophs. I suspected that one of these mutant strains was now able to make lanosterol. A TLC of the lysed yeast cells indicated the presence of ergosterol which would be possible if cycloartenol synthase evolved to allow the formation of lanosterol (Fig 13).
Figure 11 Sterol biosynthesis in a yeast lanosterol synthase mutant.
Figure 12: Sterol biosynthesis in a lanosterol synthase mutant expressing cycloartenol synthase.
Figure 13 Sterol biosynthesis after evolution of cycloartenol synthase to lanosterol synthase.
Upon sequencing of the cycloartenol synthase gene from the mutant SMY8[AtCAS1], I found two mutations: H137Q and I481V. I used site-directed mutagenesis to create single mutants. Only SMY8[AtCAS1I481V] was able to complement the sterol auxotrophy. The region surrounding this residue is well conserved among the seven cloned oxidosqualene cyclases. Upon comparing the amino acid sequence of the cloned cycloartenol synthases, isoleucine appears to be conserved. More obvious is the conservation of valine at the homologous position in the lanosterol synthases. The NMR of the SMY8[AtCAS1I481V] cyclization products indicated the presence of multiple triterpene alcohols. Per personal communication with E. Hart, there were thee triterpene alcohols: cycloartenol, lanosterol, and parkeol. The difference in formation among these thee compounds is the final site of deprotonation. I believe with the loss of a methyl group in AtCAS1I481V, a hole is created in the protein which allows deprotonation at all thee positions, thereby allowing thee cyclization products (Fig 14).
Figure 14 Oxidosqualene cyclization in the CAS1481V mutant.
I decided to create another mutant, AtCAS1II481G, to study the effect on the cyclization products by generating an even larger hole in the protein. AtCAS1II481G is also able to complement the mutant yeast sterol auxotrophy. The NMR of the HPLC purified SMY8[AtCAS1II481G] was consistent with the monocyclic triterpene alcohol, achilleol. The large hole generated by the absence of any side chain at position 481 appears to interrupt the cyclization process, thereby only allowing the formation of the A-ring.

The crystal structure of squalene-hopene cyclase suggests residues that are most likely to be present in the active site. This information may be applied to oxidosqualene cyclases to propose which homologous residues may be in the active site. Two residues away from isoleucine-481 in AtCAS1 is an aspartate that is conserved in both the oxidosqualene cyclases and the squalene-hopene cyclases (Fig 7). This is the same aspartate that was identified by Corey as necessary for catalysis in yeast lanosterol synthase. The homologous aspartate in squalene-hopene cyclase was identified by Feil as necessary for catalysis (46). As additional support for both experiments, the crystal structure of squalene-hopene cyclase indicates the aspartate is a likely member of the squalene cyclase active site, therefore, potentially a member of the oxidosqualene cyclase active sites. This lends support to my theory of generating a hole in the protein,
considering the proximity of isoleucine-481 to this highly conserved aspartate.

The experiments described above provided additional characterization of the differences between cycloartenol synthase and lanosterol synthase. I determined that the mutation of a single residue in cycloartenol synthase will allow the formation of other cyclic compounds. The mutation of isoleucine-481 to a smaller residue creates a hole in the protein that allows lanosterol formation. By cloning the *O. sativa* cycloartenol synthases gene, I determined that isoleucine-481 is conserved among thee cycloartenol synthases. A homologous residue, valine-454, is conserved among five lanosterol synthases. Future experiments directed towards such differentially conserved residues will provide even more information regarding the differences in formation of lanosterol and cycloartenol.
REFERENCES


