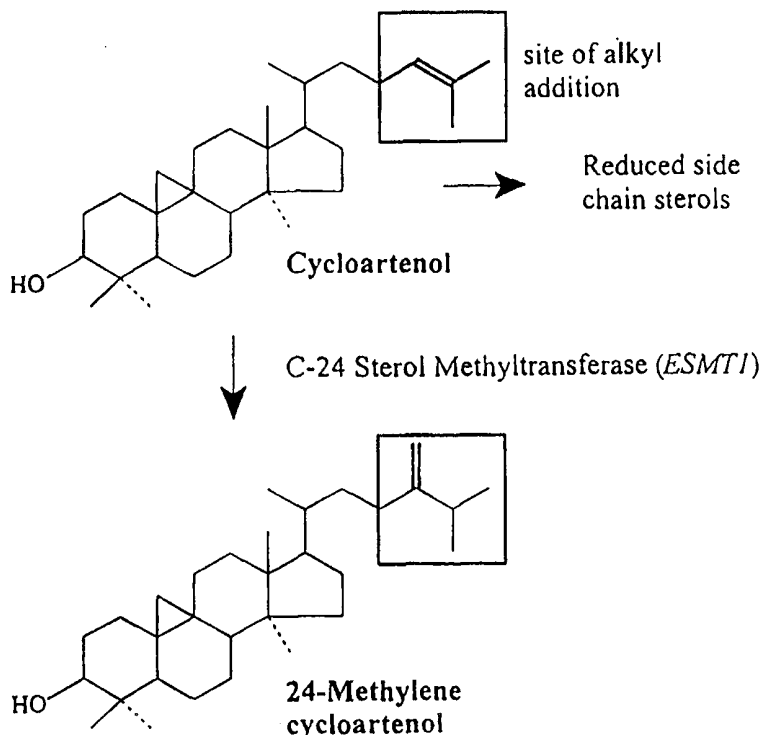




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(21) International Application Number: PCT/US99/17456 (22) International Filing Date: 2 August 1999 (02.08.99) (30) Priority Data: 09/128,339 3 August 1998 (03.08.98) US (71) Applicant: ARIZONA BOARD OF REGENTS ON BEHALF OF THE UNIVERSITY OF ARIZONA [US/US]; 888 North Euclid Avenue, Box 210158, Main Gate 515, Tuscon, AZ 85721-0158 (US). (72) Inventor: GREBENOK, Robert, J.; 9410 Greiner Road, Clarence, NY 14031 (US). (74) Agent: SEAY, Nicholas, J.; Quarles & Brady, P.O. Box 2113, Madison, WI 53701-2113 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: STEROL METHYLTRANSFERASE GENE (57) Abstract Disclosed is an isolated DNA sequence that encodes a <i>Zea mays</i> C-24 sterol methyltransferase. A yeast <i>erg6</i> transformant containing the C-24 sterol methyltransferase gene produces ergosterol, whereas untransformed isolates of yeast <i>erg6</i> do not produce ergosterol. Also disclosed is a method of altering sterol metabolism in a plant by transforming the plant with a heterologous construct comprising a <i>Z. mays</i> C-24 methyltransferase coding sequence operably connected to a plant promoter. Plants having altered sterol metabolism are expected to exhibit unique phenotypes, including reduced ability to support feeding pests that depend on plant sterols for completion of their life cycles.		



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STEROL METHYLTRANSFERASE GENE

CROSS-REFERENCE TO RELATED APPLICATIONS

Not applicable.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

5 Not applicable.

BACKGROUND OF THE INVENTION

Each year, insects, nematodes, and fungi that feed on plants contribute significantly to reduced agricultural productivity and profitability. Traditionally, control of infestations of food crops by plant pests has been achieved through the use of pesticides. It is now recognized that the use of such chemicals can adversely affect the environment, the ecology, and human health.

10 The topical application of pesticides to crops is problematic because it is virtually impossible to effectively target specific fields and specific organisms. Most pesticides exhibit toxicity toward a relatively broad range of organisms, and are effective against species that are not considered to be undesirable, or which are regarded as beneficial.

20 Considerable research has been directed toward developing alternative means of protecting plants against plant pests. Increasingly, plant molecular biologists are seeking to develop genetically engineered plant strains that have desirable characteristics, such as increased resistance to pests. One means by which resistance can be engineered is to alter plant metabolism such that the plant does not provide the feeding pest with nutrients required for completion of its life cycle.

25 Sterols are a class of lipids that have been found to be essential for the synthesis and maintenance of membranes in

30

most eukaryotic organisms studied. Insects, nematodes, and some species of fungi are unable to synthesize sterols and are dependent on plants for the sterols required for the completion of their life cycles. More than 250 different sterols are produced by plants. Certain plant species produce as many as 60 different sterols.

What is needed in the art is a means of altering sterol metabolism in plants in order to control populations of feeding plant pests.

BRIEF SUMMARY OF THE INVENTION

The present invention is an isolated DNA sequence that is substantially homologous to the *Zea mays* C-24 sterol methyltransferase coding sequence shown in SEQ ID NO:1. The amino acid sequence encoded by the open reading frame (ORF) is shown in SEQ ID NO:2.

The present invention is also a heterologous genetic construct comprising a DNA sequence that is substantially homologous to the *Zea mays* C-24 sterol methyltransferase coding sequence shown in SEQ ID NO:1 operably connected to a promoter that promotes gene expression in plants.

Another aspect of the present invention is a method of altering sterol metabolism comprising the steps of: (a) providing a heterologous genetic construct comprising a DNA sequence that encodes a C-24 sterol methyltransferase having at least 80% amino acid identity with SEQ ID NO:2 operably connected to a promoter that promotes gene expression in plants; and (b) introducing the genetic construct into a plant.

In another embodiment, the present invention is a plant comprising in its genome a genetic construct comprising a DNA sequence encoding a C-24 sterol methyltransferase operably connected to a promoter that promotes gene expression in plants.

It is an object of the present invention to provide a DNA sequence encoding a sterol methyltransferase, which when expressed in plants, alters sterol metabolism in the plant so as to afford protection against infestation by pests to which

the plant is ordinarily susceptible.

It is an object of the present invention to provide a method of protecting plants against pest infestation.

It is an object of the present invention to provide a pest
5 resistant plant.

Other objects, features, and advantages of this invention will become apparent upon review of the specification and claims.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

10 Figure 1 shows the proposed methylation of cycloartenol by a *Zea mays* endosperm C-24 sterol methyltransferase.

Figure 2 shows a comparison between the deduced amino acid sequences of maize ESMT1 (SEQ ID NO:2) and C-24 sterol methyltransferase cDNA sequences from *Arabidopsis*, yeast, and
15 soybean.

DETAILED DESCRIPTION OF THE INVENTION

In one embodiment, the present invention is an isolated DNA fragment comprising a sequence that is substantially homologous to a *Zea mays* endosperm C-24 sterol methyltransferase (ESMT1) coding sequence (SEQ ID NO:1).
20

The methyltransferase of this invention catalyzes the alkylation at carbon 24 of sterol intermediates. In higher plants, the sterols that contain C-24 alkyl additions predominate, whereas unmethylated sterols such as cholesterol
25 are present at low levels. The production of alkylated sterols occurs after cyclization of squalene and involves several stages predicated on the alkylation of the double bond at carbon 24 in the sterol side chain in a reaction catalyzed by an S-adenosyl-L-methionine methyltransferase (SMT) (Benveniste, Annu Rev Plant Physiol 37:275-308, 1986). Alkylation at carbon
30 24 by sterol methyltransferase is believed to be a key step in the regulation of carbon flux through the sterol biosynthetic pathway (Beneviste, *supra*; Chappell et al. Plant Physiol 109:1337-1343, 1995). A proposed reaction catalyzed by ESMT1
35 in maize is shown in Fig. 1.

Sterol methyltransferase cDNA clones have been obtained from *Arabidopsis thaliana* (Husselstein et al. FEBS Lett. 381:87-92, 1996) and soybean (Shi et al., J. Biol. Chem. 271:9384-9399, 1996). The putative proteins encoded by these clones were found to have homology to the yeast ERG6 protein. The expression of the cloned *Arabidopsis* sterol methyltransferase (SMT) gene in *erg6* yeast was found to complement the *erg6* deficiency by allowing low level production of ergosterol, and it was discovered that the enzyme catalyzes a second methyl transfer that results in the formation of large amounts of C-24 ethyl sterols.

We have isolated and characterized a C-24 methyltransferase DNA coding sequence of the present invention from *Z. mays* endosperm as described in the examples below and in Grebenok, et al. (Plant Mol. Biol. 34:891-896, 1997), which is incorporated by reference herein. Briefly, a *Z. mays* endosperm cDNA (53D6) was selected from an EST database based on its sequence identity with the amino terminal portion of the yeast ERG6 protein. A clone containing the cDNA as a *NotI* insert in the plasmid λ Zap was then obtained from Pioneer Hybrid. The DNA sequence of the cDNA is shown in SEQ ID NO:1. It would be well within the ability of one skilled in the art to obtain other maize sterol methyl-transferase DNA coding sequences using known methods. For example, one wishing to obtain a C-24 sterol methyltransferase DNA coding sequence could screen a genomic or cDNA library from any plant with a probe complementary to a portion of the coding region of SEQ ID NO:1.

A *Zea mays* endosperm sterol methyltransferase coding sequence is any DNA sequence that has substantial homology to SEQ ID NO:1. By "substantial homology" it is meant a DNA sequence that encodes a protein that has at least 80% amino acid identity with SEQ ID NO:2, and which exhibits C-24 sterol methyltransferase activity. Preferably, the DNA sequence encodes a protein that has an amino acid identity with SEQ ID NO:2 of at least 90%. Most preferably, the DNA sequence encodes a polypeptide that has an amino acid identity with SEQ ID NO: 2 of about 95% or higher.

A putative C-24 sterol methyltransferase coding sequence could be confirmed by evaluating the activity of a C-24 sterol methyltransferase gene product by yeast *erg6* complementation as described below in the examples. When expressed in an *erg6* strain of *Saccharomyces cerevisiae*, C-24 sterol methyltransferase allows survival of the yeast on cyclohexamide, presumably by catalyzing a methyl addition to a sterol intermediate to form ergosterol. A putative C-24 sterol methyltransferase coding sequence could be confirmed by expressing the gene in a suitable expression system and evaluating the ability of the gene product to methylate an appropriate substrate, such as cycloartenol, lanosterol, or zymosterol (Nes et al. *supra*).

The C-24 sterol methyltransferase of the present invention is distinguishable from the *Arabidopsis* SMT by its inability to catalyze the transfer of a second methyl group to a 24-methyl sterol to form a 24-ethyl sterol. It is expected that the substrate specificity of the C-24 sterol methyltransferase of the present invention may be altered by introducing regions of the *Arabidopsis* SMT gene or other sterol methyltransferase genes that encode sterol methyltransferases having different substrate specificities into a sequence comprising SEQ ID NO:1.

It is expected that polyploid plants having more than one copy of the C-24 sterol methyltransferase gene may have allelic variations among C-24 sterol methyltransferase gene sequences. It is anticipated that putative C-24 sterol methyltransferase sequences having less than 100% sequence identity to SEQ ID NO:1 encode proteins having sterol methyltransferase activity that are encompassed by the sequence of the present invention.

It is envisioned that minor sequence variations from SEQ ID NO:1 associated with nucleotide additions, deletions, and mutations, whether naturally occurring or introduced in vitro, will not affect C-24 sterol methyltransferase activity. The scope of the present invention is intended to encompass minor variations in C-24 sterol methyltransferase sequences.

It is also envisioned that many DNA sequences can be used to code for the expression of a single protein. For example,

using codon substitution it is known that there are many DNA sequences other than SEQ ID No: 1 which will encode the protein of SEQ ID No: 2. There are also known to be conservative amino acid substitutions that can be made, particularly in portions
5 of the protein not at critical catalytic sites, which are highly unlikely to change protein function. It is intended that the sequence of the present specification be interpreted to encompass such variations.

The expression of C-24 sterol methyltransferase in a yeast
10 *erg6* background allows the formation of ergosterol, which is absent in *erg6* yeast strains. In the examples below, the relative amount of ergosterol produced in *erg6* yeast transformed with the C-24 sterol methyltransferase coding sequence was about 10% of that produced in wild-type yeast. It
15 is expected that a C-24 sterol methyltransferase which when expressed in *erg6* yeast results in ergosterol production at a level that is higher or lower than 10% of wild-type ergosterol production would be suitable in the practice of the present invention.

The C-24 sterol methyltransferase sequence that we
20 identified is a cDNA from *Z. mays* endosperm (ESMT1) encoding a protein with homology to C-24 sterol methyltransferases from soybean (Shi et al., *supra*), *Arabidopsis* (Husselstein et al., *supra*) and yeast (Garber et al. Mol. Cell Biol. 9:3447-3456,
25 1989). Analysis of the deduced amino acid sequence of ESMT1 revealed several conserved motifs (Figure 1), three of which are found in a large number of S-adenosyl-L-methionine-dependent methyltransferases and are thought to contribute to the binding of S-adenosyl-methionine (Kagen and Clarke Arch
30 Biochem Biophys 310:417-427, 1994).

In addition to the conserved SAM motifs in ESMT1, sequence
alignment of corn, *Arabidopsis*, and soybean SMTs and yeast ERG6 protein reveals two other highly conserved regions, designated
SMT I and SMT II, which are unique to the SMTs. We have
35 suggested that the SMTs may define the active site and/or substrate binding sites (Grebenok et al., Plant Mol. Biol. 34:891-896, 1997). Sequence alignments also identified at

least two additional regions, A and B, which share a high level of homology between maize, soybean and yeast but show significant divergence in the *Arabidopsis* SMT. Motifs A and B could define functional sites within the *A. thaliana* SMT that are necessary for multiple methyl additions. We have proposed that two classes of sterol methyltransferase may exist in plants, one defined by ESMT1 and the soybean SMT, and a second defined by the *Arabidopsis* SMT (Grebenok et al., *supra*).

Higher plant sterol methyltransferases have been shown to exhibit substrate specificity with respect to side chain conformation. Nes et al. demonstrated that a side chain stereochemistry identical to that of cycloartenol was necessary for attaining maximal enzyme activity in *in vitro* enzyme assays with purified sunflower SMT (J. Biol. Chem 266:15202-15212, 1991). The precursors zymosterol, which is a precursor for C-24 methylation in yeast, and lanosterol were used less efficiently as substrates by plant enzymes *in vitro*, with reaction velocities of about 30% of maximum SMT activity observed for the plant substrate cycloartenol.

The low level ergosterol production (10% wild-type) found in the complementation of the yeast *erg6* mutant by maize ESMT1 may be due to a lower efficiency in using the zymosterol substrate, as reported for the isolated enzyme (Nes et al., *supra*). Alternatively, in view of the localization of sterol biosynthetic activities to microsomal fractions in yeast and higher plants (Moore and Gaylor, J. Biol. Chem. 244:6334-6340, 1969), the sub-wild-type level of functional complementation with ESMT1 may reflect inappropriate targeting or failure of the enzyme to interact efficiently with the yeast sterol biosynthetic machinery.

Construction of an expression vector comprising a *Zea mays* C-24 sterol methyltransferase coding sequence operably connected to a plant promoter not natively associated with the coding sequence is planned using standard molecular biology techniques known to the art. The plant promoter may be any plant promoter, including a constitutive promoter such as CaMV 35S, which is known to function in a wide variety of plants.

Other promoters that are functional in plants may be used to create the genetic constructs to be used in the practice of this invention. These may include other constitutive promoters, tissue-specific promoters, developmental stage-specific promoters, and inducible promoters. Promoters may also contain certain enhancer sequence elements that improve the efficiency of transcription. Optionally, the construct may contain a termination signal, such as the nopaline synthase terminator (NOS). Preferably, the constructs will include a selectable or screenable marker to facilitate identification of transformants. The constructs may have the coding region in the sense or antisense orientation.

Once a genetic construct comprising a C-24 sterol methyltransferase gene has been obtained, it can readily be introduced into a plant or plant tissue using standard methods known to the art. For example, the *Agrobacterium* transformation system is known to work well with all dicot plants and some monocots. Other methods of transformation equally useful in dicots and monocots may also be used. Transgenic plants may be obtained by particle bombardment, electroporation, or by any other method of transforming plants known to one skilled in the art of plant molecular biology. The experience to date in the technology of plant genetic engineering has taught that the method of gene introduction does not affect the phenotype achieved in the transgenic plants.

A transgenic plant may be obtained directly by transformation of a plant cell in culture, followed by regeneration of a plant. Also, transgenic plants may be obtained from transgenic seeds set by parental transgenic plants. Transgenic plants pass on inserted genes, sometimes referred to as transgenes, to their progeny by normal Mendellian inheritance just as they do their native genes. Methods for breeding and regenerating plants of agronomic interest are known in the art.

It is reasonable to expect that the expression of heterologous C-24 sterol methyltransferase in a transgenic plant will result in alterations in the sterol profile in that

plant. Changes in the sterol profile can be expected to result in unique, advantageous phenotypes, including the reduced ability to support a feeding pest that depends on plant sterols for completion of its life cycle. This invention is intended
5 to encompass other advantageous phenotypes in addition to interfering with the life cycle of feeding pests that may result from alterations in sterol metabolism in plants obtained by the practice of this invention.

The following nonlimiting examples are intended to be
10 purely illustrative.

Examples

Strains

Saccharomyces cerevisiae strain *erg6* (α *leu2 ura3* *erg6::LEU2*) and the corresponding wild type yeast strain were kindly provided by L. Parks (North Carolina State University). The *Z. mays* endosperm cDNA 53D6, contained as a *NotI* insert within plasmid λ ZAP (Stratagene, LaJolla, CA), was provided by T. Helentjaris (Pioneer HiBred).

Growth and transformation conditions

The *erg6* mutant and wild type were grown YPD (1% yeast extract (Difco), 2% bactopectone (Difco), 2% dextrose). Electrocompetent yeast was prepared, electroplated and plated according to the method of Becker and Guarente (Meth. Enzymol 194:182-187, 1991). Yeast strain *erg6* transformants were selected on complete synthetic media without uracil (0.67% yeast nitrogen base without amino acids (Difco) and 2% galactose). *E. coli* strain DH5 α was used for routine cloning according to standard, established procedures.

Identification and Cloning of *Zea mays* C-24 sterol methyltransferase

A *Z. mays* endosperm cDNA was selected from an EST database based on its sequence identity with the amino terminal portion of the yeast ERG6 protein. The cDNA insert from plasmid 53D6 was excised with *KpnI-HindIII* and ligated into a linearized pBluescript II vector (Stratagene) having compatible ends to form pRJG3. The cDNA insert from plasmid pRJG3 was excised using *EcoRI* and was ligated into the similarly cut yeast expression vector λ YES (Elledge et al. Proc Natl Acad Sci USA 88:1731-1735, 1991). To form pRGJ6. Plasmid DNA was isolated from complemented yeast using previously reported procedures (Hoffman and Winston, Gene 57:267-272, 1987). Automated DNA sequencing was performed at the Arizona Biotechnology Facility after transfer of the *NotI/HindIII* fragment of 53D6 into pBluescript II (Stratagene). The full-length cDNA was sequenced and found to contain an open reading frame of 1.5 kb encoding a 40 kDa protein with 46% identity to the yeast ERG6 protein (Figure 2).

The predicted amino acid sequence of ESMT1 shows 66% similarity and 46% identity to the *S. Cerevisiae* ERG6 protein and 75% and 37% identity to the soybean and *Arabidopsis* SMTs, respectively (Table 1). Alignment of all four methyltransferase cDNAs yields a shared identity of 36%, while alignment of the soybean, maize and yeast methyltransferase cDNA sequences without addition of the *Arabidopsis* cDNA sequence yields 44% identity. Hydropathy analysis indicates the presence of a 25 amino acid leader peptide on the *A. thaliana* protein but no on the *Z. mays*, soybean or yeast SMTs (results not shown). Analysis of the ESMT1 protein sequence indicated the presence of three conserved motifs comparable in sequence, length, order and spacing to those identified in diverse S-adenosyl-L-methionine-dependant methyltransferases described by Kagan and Clarke (Arch Biochem Biohyps 310:417-427, 1994) (Figure 2). The presence of these motifs support identification of *Z. mays* ESMT1 as an S-adenosyl-methionine-dependant methyltransferase. These three SAM motifs are also conserved in sequence, order, length and spacing in the yeast, soybean and *Arabidopsis* SMTs (Figure 2). Alignment of all four SMT sequences identifies two additional highly conserved regions. SMT motif I (SMT I) is 11 amino acids long, begins with the conserved aromatic amino acid phenylalanine found at position 65 of the *Z. mays* sequence and contains 9 amino acids identical in all 4 proteins. SMT motif II (SMT II) begins directly after the SAM II site, Glu-179, and consists of 8 contiguous amino acids which are identical in all 4 proteins. These two SMT motifs, possibly acting in concert may represent an active site (sterol-binding site) for higher plant SMTs. Two additional regions (motifs A and B) were identified based upon sequence identity in the soybean, *Z. mays* and ERG6 SMTs but not in the *Arabidopsis* SMT (Figure 2). Motif A is located directly upstream of the SAM II site beginning with phenylalanine 157 and spans 9 amino acids, 8 of which are identical within maize, soybean, and yeast, while only 3 are conserved in *A. thaliana* Motif B, located very near the carboxy end of the proteins, begins with leucine 304 and spans 13 amino

acids, 10 of which are identical within the maize, soybean and yeast sequences, while only 5 are conserved in *Arabidopsis* (Figure 2).

Complementation of yeast *erg6*

5 Unlike wild-type yeast, the *erg6* strain exhibits sensitivity to cycloheximide, an inhibitor of protein synthesis. Cycloheximide sensitivity is due altered cell membrane permeability that is a consequence of the inability of this strain to produce ergosterol. The *Z. mays* endosperm cDNA
10 was initially characterized based on its ability to relieve cycloheximide sensitivity in *erg6*. The yeast strain *erg6* was transformed with the isolated *Z. mays* endosperm cDNA contained in a yeast expression vector under the transcriptional control of the GAL4 promoter (pRJG6) by electroporation.
15 Transformants were cultured for 4 days at 30°C on complete synthetic minimal media lacking uracil and with 5% galactose as the sole carbon source to induce activity of the GAL4 promoter. Colonies were subsequently replica plated onto fresh complete synthetic media lacking uracil but containing cycloheximide
20 (0.1 µg/ml). After further incubation for 3 days at 30°C, individual colonies demonstrating improved or wild-type growth were selected for sterol analysis.

 A transformation efficiency of 2×10^2 colony-forming units per µg DNA was observed. Complementing the mutant
25 phenotype is a consequence of the production of ergosterol, which restores the wild-type permeability characteristic of the plasma membrane and leads to exclusion of cycloheximide from the cytoplasm (Garber et al. Mol. Cell. Biol. 9:3447-3456, 1989). Depending on the level of ergosterol production,
30 complemented cells exhibit a percentage of wild-type growth in the presence of cycloheximide, whereas non-complemented cells remain sensitive. Without exception, the *erg6* mutant cells containing the 53D6 cDNA in λYES (pRJG6) were able to grow under cycloheximide selection in the presence of galactose, but
35 demonstrated cycloheximide sensitivity in the absence of galactose.

Sterol analysis

Those colonies selected for sterol analysis were cultured in complete synthetic liquid media lacking uracil and supplemented with cycloheximide (0.1 μ g/ml). Cells from 50 ml aliquots were collected by centrifugation at 2000 rpm for 10 min. The supernatant was discarded and the pellets were extracted for 12 h in 50 ml methanol. The methanol fraction was removed and evaporated to dryness in a rotary evaporator. Sterols were isolated according to Garber et al. (Mol Cell Biol 9:3447-3456, 1989), and were identified by gas chromatography-mass spectrometry (GC-MS) using a Hewlett-Packard 5890 gas chromatograph and a Hewlett-Packard 5970 mass spectrometer. GC separation was carried out on a HP-1 column (15m x .3mm) with 0.25 μ m film thickness. Column temperature was programmed from 120 to 300°C at 4°C/min and the carrier gas was helium at a velocity of 30 cm/s. The MS was operated at an ionizing potential of 100 eV, and the ion source was maintained at 300°C. Ergosterol produced in the complemented *erg6* yeast cells was identified through co-chromatography with authentic ergosterol on the gas chromatograph, and by a mass spectrum with characteristic ions at m/e: 396 [M⁺], 363, 337, 271, 253, and 211 (Rahier and Benveniste, "Mass spectral identification of phytosterols." In: New WD, Parish EF (eds) Analysis of Sterol and other Biologically Significant Steroids, pp 223-250, Academic Press Publishers, New York, 1989).

Sterols were isolated and characterized from both the *erg6* mutant and the complemented mutant lines. When propagated in complex synthetic liquid medium the *erg6* mutant line produced cholesta-8,24-dien-3 β -ol (M+384) which accounted for 72% and 20% of the isolated sterol, respectively. Two minor sterols, each representing 4% of the isolated sterol, had molecular weights of 382, suggesting cholesta-triene-3 β -ol structure and the final sterol representing 1% of the isolated sterol is presumably the cholesta-tetraene-3 β -ol (M+380). Based upon molecular weights, none of the aforementioned sterols contained methyl additions to their side chains accounting for the cycloheximide sensitivity observed in the *erg6* mutant

(Husselstein et al. FEBS lett 381:87-92, 1996).

When propagated in complete synthetic liquid medium in the presence of galactose and cycloheximide, the transformed colonies produced in addition to the previously mentioned sterols, two new peaks, one of which comigrated with purified ergosterol (results not shown). The molecular weights of the new sterols were 396 and 394, respectively. The sterol with a molecular weight of 394 is presumably a ergostatetraene (Husselstein et al., *supra*). Ergosterol production reached a maximum level of about 5% of the total isolated sterol pool, while in wild-type yeast, ergosterol normally comprises about 45% of the sterol pool (Garber, et al. Mol Cell Biol 9:3447-3456, 1996). The level of ergosterol produced within the transformants, although about 10% of wild type, accounts for the cycloheximide resistance observed (Garber et al. *supra*; Husselstein et al., *supra*).

The present invention is not limited to the exemplified embodiments, but is intended to encompass all such modifications and variations as come within the scope of the following claims.

CLAIM OR CLAIMS

WE CLAIM:

1. An isolated DNA fragment encoding a C-24 sterol methyltransferase from maize comprising a sequence that is substantially homologous to SEQ ID NO:1.

2. The fragment of claim 1, wherein the deduced amino acid sequence of the coding sequence has at least 80% amino acid identity with SEQ ID NO:2.

3. The fragment of claim 2, wherein the amino acid sequence has at least 90% identity with the sequence of SEQ ID NO:2.

4. The fragment of claim 2, wherein the amino acid sequence has at least 95% identity with the sequence of SEQ ID NO:2.

5. The fragment of claim 1, wherein the coding sequence comprises SEQ ID NO:1.

6. A genetic construct comprising the coding sequence of claim 1 operably connected to a plant promoter not natively associated with the coding sequence.

7. The genetic construct of claim 6, wherein the coding sequence comprises SEQ ID NO:1.

8. A method of obtaining a plant comprising in its genome a DNA sequence encoding a heterologous C-24 sterol methyltransferase from maize comprising the steps of:

- 5 a) making a genetic construct comprising a DNA sequence encoding a C-24 sterol methyltransferase having the SEQ ID NO:2 operably connected to a promoter functional in plants; and
b) introducing the genetic construct into a plant cell;
and
c) regenerating a plant from the plant cell of step b.

10 9. The method of claim 8, wherein the genetic construct of step a comprises SEQ ID NO:1.

10. A plant comprising in its genome the genetic construct of claim 6.

11. A seed of the plant of claim 10.

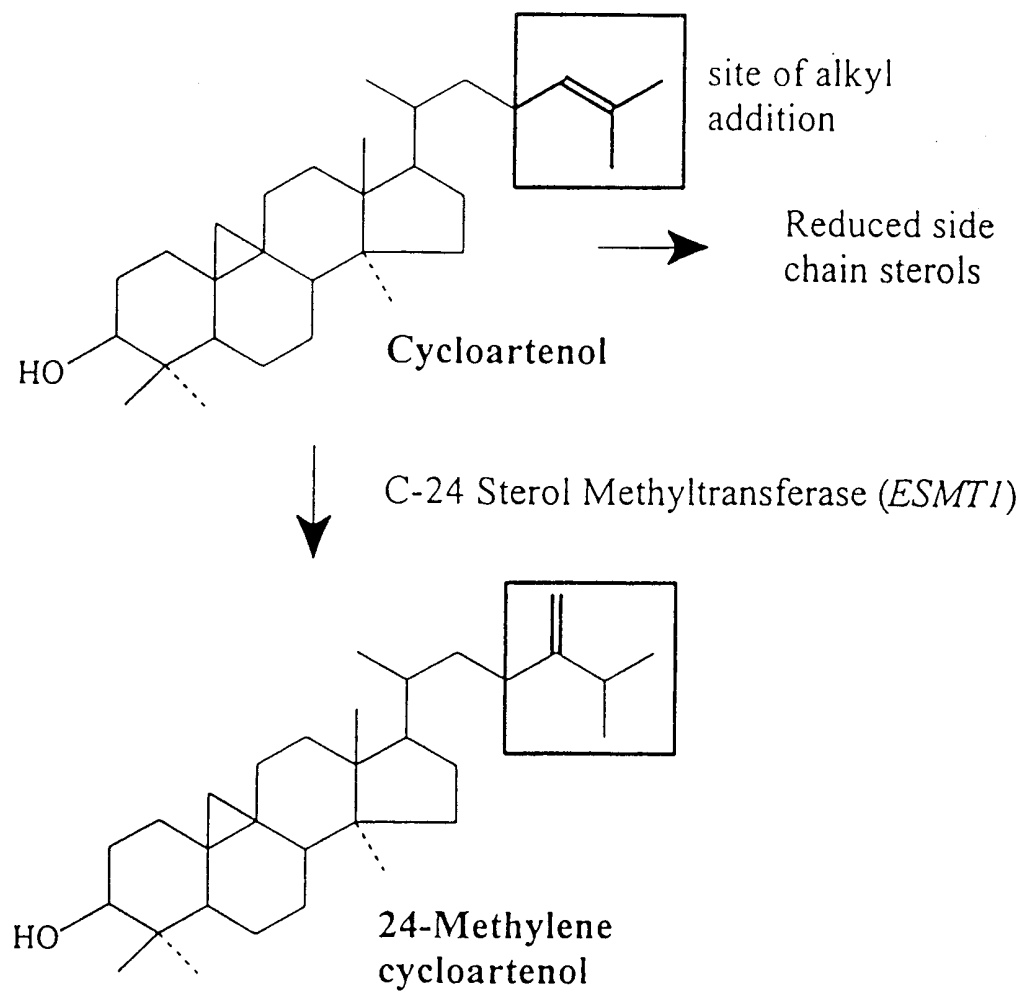


FIG 1

Arabidopsis -MDSLTLFFTGALVAVGIYWFLCVLGAERKKGKRAVDLSGGSSISAEKVQDNYKQYWSFFR
 yeast ERG6 -MSETELRKRQAQFTRELHG--DDIGKKTGLSALMSKNN--SAQKEAVQKYLNRNWDGRTD
 soybean MQKKKKNRNEVVLCSAEGTGGCSRLAAMD LASNLGGKID--KAEVLSAVQKYEKYHV CYG
 maize -----MSKSGALDLASGLGGKIN--KVEVKSAVDEYEKYHGYG 37

Arabidopsis RPKEIETAEKVPDFVDTFYNLVTDIYEWGWGQSFHFSPIPGKSHKDATRLHEEMAVDLI
 yeast ERG6 KDAEERRLEDYNEATHSYYNVVTDFYEGWGSSFHFSRFYKGESFAASIRHEHYLAYKA
 soybean GQEEER-KANYTDMVNKYD LVT SFYEGWGGSFHFAPRWKGESLRESIKRHEHFLPLQL
 maize GKEEAR-KSNYTDVNKYD LATS FYEGWGGSFHF AHRWNGESLRESIKRHEHFLALQL 96

SMTI

Arabidopsis QVKPGQKILDVGCVG GPMRAIASHSRANVVGITINEYQVNRARLHNKKAGLDALCEVVC
 yeast ERG6 GIQRGDLVLDVGCVG GPPAREIARFTGCNVIGLNNNDYQIAKAKYYAKKYNLSDQMD FVK
 soybean GLKPGQKVL DVGCGIGG PLREISRFSSSTSITGLNNNEYQITRGKELNRIAGVDKTCNFVK
 maize GLKPGMKVLDVGCVGIGG PLREIARFSSTSVTGLNNNEYQITRGKELNRLAGISGTCDFVK 156

SAMI

Arabidopsis GNFLQMPFDDNSFDGAYSIEATCHAPKLEEVYAEIYRVLKPGSMYVS YEWVTTEKFAED
 yeast ERG6 GDFMKMDFEENTFDKVYAIEATCHAPKLEGVYSEIYKVLKPGGTFAYYEWVMTDKYDENN
 soybean ADFMKMPFDDNSFDVYAIEATCHAPDAYGCYKEIFRVLKPGQYFAAYEWCMTDSFDPQN
 maize ADFMKMPFDDNTFDVYAIEATCHAPDPVGCYKEIYRVLKPGQCFAYYEWCI TDHYDPNN 216

A SAMII / SMTII SAMIII

Arabidopsis DEHVEVIQGIERG DALPGLRAYVDIAETAKKVGFEIVKEKDLAS-PPAEPWWTRLK----
 yeast ERG6 PEHRKIAYEIELGDGIPKMFHVDVARKALKNCGFEVLVSED LADNDDEIPWYYPLTGEWK
 soybean PEHQKIKAEIEIGDGLPDIRLTAKCLEALKQAGFEVIWEKDLAV-DSPLPWYLPD----
 maize ATHKRIKDEIELGNGLPDIRSTRQCLQAVKDAGFEVVWDKDLAE-DSPLPWYLPD---- 272

Arabidopsis -----MGRLAYWRNHIVVQILSAVG VAPKGTVDVHEMLFKTADCLTRGGETG
 yeast ERG6 YVQNLANLATFFRTSYLGRQFTTAMVTVM EKLGLAPEGSKEVTAALENA AVGLVAGGKSK
 soybean ---KSHFSLSSFRLTAVGRLFTKNMVK VLEYVGLAPKGLRVQDFLEKAAEGLVEGGKRE
 maize ---PSRFSLSFRLTSVGRMITRTMVKALEYVGLAPQGSERVS NFLEKAAEGLVEGGKKE 329

B

Arabidopsis IFSPMHMILCRKPESPEESS-----
 yeast ERG6 LFTPMMLFVARKPENAE TP SQT SQEATQ
 soybean IFTPMYFFLARKPD LDRN-----
 maize IFTPMYFFLV RKPLLE----- 345

FIG 2

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Grebenok, Robert J.
Galbraith, David W.
DellaPenna, Dean
- (ii) TITLE OF INVENTION: STEROL METHYLTRANSFERASE GENE
- (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:
- 10 (A) ADDRESSEE: Quarles & Brady
(B) STREET: 1 South Pinckney Street
(C) CITY: Madison
(D) STATE: WI
(E) COUNTRY: US
(F) ZIP: 53701-2113
- 15 (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- 20 (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
- 25 (A) NAME: Seay, Nicholas J.
(B) REGISTRATION NUMBER: 27,386
(C) REFERENCE/DOCKET NUMBER: 920214.90166
- (ix) TELECOMMUNICATION INFORMATION:
- 30 (A) TELEPHONE: (608)251-5000
(B) TELEFAX: (608)251-9166

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
- 35 (A) LENGTH: 1383 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (ix) FEATURE:
- 40 (A) NAME/KEY: CDS
(B) LOCATION: 78..1112

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

	CCCACGCGTC CGAGACTCTG GTTCTGACAT GCAGCAATTA TTGCAGGTGC ATTTGATCCG	60
	TCCCGGCCGC CTACACG ATG TCC AAG TCG GGA GCG CTG GAT CTT GCT TCT	110
	Met Ser Lys Ser Gly Ala Leu Asp Leu Ala Ser	
5	1 5 10	
	GGC CTC GGA GGG AAG ATC AAC AAG GTG GAA GTC AAG TCG GCC GTC GAT	158
	Gly Leu Gly Gly Lys Ile Asn Lys Val Glu Val Lys Ser Ala Val Asp	
	15 20 25	
	GAG TAT GAG AAA TAT CAT GGA TAC TAT GGA GGG AAG GAG GAA GCA AGG	206
10	Glu Tyr Glu Lys Tyr His Gly Tyr Tyr Gly Gly Lys Glu Glu Ala Arg	
	30 35 40	
	AAG TCC AAC TAT ACT GAT ATG GTT AAT AAA TAC TAT GAT CTT GCC ACT	254
	Lys Ser Asn Tyr Thr Asp Met Val Asn Lys Tyr Tyr Asp Leu Ala Thr	
	45 50 55	
15	AGC TTC TAT GAG TAT GGT TGG GGT GAA TCC TTC CAC TTT GCT CAC AGA	302
	Ser Phe Tyr Glu Tyr Gly Trp Gly Glu Ser Phe His Phe Ala His Arg	
	60 65 70 75	
	TGG AAT GGA GAA TCC TTA CGT GAA AGC ATC AAG CGA CAT GAG CAT TTT	350
	Trp Asn Gly Glu Ser Leu Arg Glu Ser Ile Lys Arg His Glu His Phe	
20	80 85 90	
	CTT GCC CTG CAA CTT GGT TTG AAA CCA GGA ATG AAG GTT TTA GAT GTG	398
	Leu Ala Leu Gln Leu Gly Leu Lys Pro Gly Met Lys Val Leu Asp Val	
	95 100 105	
	GGC TGT GGA ATA GGT GGA CCA CTG AGA GAA ATT GCA AGA TTT AGC TCA	446
25	Gly Cys Gly Ile Gly Gly Pro Leu Arg Glu Ile Ala Arg Phe Ser Ser	
	110 115 120	
	ACT TCA GTT ACC GGA TTG AAT AAC AAC GAA TAC CAG ATA ACC AGG GGA	494
	Thr Ser Val Thr Gly Leu Asn Asn Asn Glu Tyr Gln Ile Thr Arg Gly	
	125 130 135	
30	AAG GAG CTC AAC CGT TTA GCA GGA ATT AGT GGA ACA TGT GAT TTT GTC	542
	Lys Glu Leu Asn Arg Leu Ala Gly Ile Ser Gly Thr Cys Asp Phe Val	
	140 145 150 155	

	AAG GCG GAC TTC ATG AAG ATG CCG TTC GAT GAC AAC ACT TTT GAT GCT	590
	Lys Ala Asp Phe Met Lys Met Pro Phe Asp Asp Asn Thr Phe Asp Ala	
	160 165 170	
5	GTT TAC GCC ATT GAG GCA ACA TGT CAT GCA CCT GAT CCA GTT GGT TGC	638
	Val Tyr Ala Ile Glu Ala Thr Cys His Ala Pro Asp Pro Val Gly Cys	
	175 180 185	
	TAC AAG GAG ATA TAT CGT GTG TTG AAG CCT GGC CAG TGC TTT GCC GTG	686
	Tyr Lys Glu Ile Tyr Arg Val Leu Lys Pro Gly Gln Cys Phe Ala Val	
	190 195 200	
10	TAC GAG TGG TGC ATT ACG GAT CAC TAT GAT CCT AAC AAT GCA ACC CAC	734
	Tyr Glu Trp Cys Ile Thr Asp His Tyr Asp Pro Asn Asn Ala Thr His	
	205 210 215	
	AAA AGG ATC AAG GAT GAA ATT GAG CTT GGC AAT GGC CTG CCA GAT ATC	782
15	Lys Arg Ile Lys Asp Glu Ile Glu Leu Gly Asn Gly Leu Pro Asp Ile	
	220 225 230 235	
	AGA AGC ACT CGG CAA TGT CTC CAG GCA GTA AAA GAC GCC GGG TTT GAG	830
	Arg Ser Thr Arg Gln Cys Leu Gln Ala Val Lys Asp Ala Gly Phe Glu	
	240 245 250	
20	GTT GTT TGG GAT AAG GAT CTT GCT GAA GAT TCT CCC TTG CCT TGG TAC	878
	Val Val Trp Asp Lys Asp Leu Ala Glu Asp Ser Pro Leu Pro Trp Tyr	
	255 260 265	
	TTG CCC TTG GAT CCA AGC CGA TTC TCC CTG AGT AGC TTC CGT TTG ACC	926
	Leu Pro Leu Asp Pro Ser Arg Phe Ser Leu Ser Ser Phe Arg Leu Thr	
	270 275 280	
25	TCT GTG GGA CGC ATG ATT ACC CGC ACA ATG GTC AAG GCC CTG GAG TAC	974
	Ser Val Gly Arg Met Ile Thr Arg Thr Met Val Lys Ala Leu Glu Tyr	
	285 290 295	
	GTT GGT CTT GCT CCG CAG GGC AGT GAG AGG GTC TCT AAT TTC CTG GAG	1022
30	Val Gly Leu Ala Pro Gln Gly Ser Glu Arg Val Ser Asn Phe Leu Glu	
	300 305 310 315	
	AAG GCT GCA GAA GGG CTG GTA GAG GGC GGA AAG AAG GAG ATC TTC ACG	1070
	Lys Ala Ala Glu Gly Leu Val Glu Gly Gly Lys Lys Glu Ile Phe Thr	
	320 325 330	

CCA ATG TAC TTC TTT CTT GTT CGG AAG CCT CTT CTG GAA TGA 1112
 Pro Met Tyr Phe Phe Leu Val Arg Lys Pro Leu Leu Glu *
 335 340 345

GCTCTTGGAT CACCTTTTCA GAGAGAGAAG GCAAGTGGTC ATTTCAAGA AGCCGAGGAG 1172

5 AGGGAACCTG GAATCAAGAA AACCTTCAGC TCTCCTGTGT AGGAGGAAAG TTAACGAACA 1232

GTGTAGTAAC TGTTCACTT TGTGTTTATT CAGTTGTTTT GCTGCTTGAG GTTATTCGTT 1292

TCTAGGTGGG GGTTGGAATC CTTTTCGCCA TAAACCTCTC AGTGGCATAA ATAAGATGGT 1352

TTGCATAAAA AAAAAAAAAA AAAAAAAAAA A 1383

(2) INFORMATION FOR SEQ ID NO:2:

- 10 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 345 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- 15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ser Lys Ser Gly Ala Leu Asp Leu Ala Ser Gly Leu Gly Gly Lys
 1 5 10 15

Ile Asn Lys Val Glu Val Lys Ser Ala Val Asp Glu Tyr Glu Lys Tyr
 20 25 30

20 His Gly Tyr Tyr Gly Gly Lys Glu Glu Ala Arg Lys Ser Asn Tyr Thr
 35 40 45

Asp Met Val Asn Lys Tyr Tyr Asp Leu Ala Thr Ser Phe Tyr Glu Tyr
 50 55 60

25 Gly Trp Gly Glu Ser Phe His Phe Ala His Arg Trp Asn Gly Glu Ser
 65 70 75 80

Leu Arg Glu Ser Ile Lys Arg His Glu His Phe Leu Ala Leu Gln Leu
 85 90 95

Gly Leu Lys Pro Gly Met Lys Val Leu Asp Val Gly Cys Gly Ile Gly
 100 105 110

30 Gly Pro Leu Arg Glu Ile Ala Arg Phe Ser Ser Thr Ser Val Thr Gly
 115 120 125

Leu Asn Asn Asn Glu Tyr Gln Ile Thr Arg Gly Lys Glu Leu Asn Arg
 130 135 140

Leu Ala Gly Ile Ser Gly Thr Cys Asp Phe Val Lys Ala Asp Phe Met
 145 150 155 160

5 Lys Met Pro Phe Asp Asp Asn Thr Phe Asp Ala Val Tyr Ala Ile Glu
 165 170 175

Ala Thr Cys His Ala Pro Asp Pro Val Gly Cys Tyr Lys Glu Ile Tyr
 180 185 190

10 Arg Val Leu Lys Pro Gly Gln Cys Phe Ala Val Tyr Glu Trp Cys Ile
 195 200 205

Thr Asp His Tyr Asp Pro Asn Asn Ala Thr His Lys Arg Ile Lys Asp
 210 215 220

Glu Ile Glu Leu Gly Asn Gly Leu Pro Asp Ile Arg Ser Thr Arg Gln
 225 230 235 240

15 Cys Leu Gln Ala Val Lys Asp Ala Gly Phe Glu Val Val Trp Asp Lys
 245 250 255

Asp Leu Ala Glu Asp Ser Pro Leu Pro Trp Tyr Leu Pro Leu Asp Pro
 260 265 270

20 Ser Arg Phe Ser Leu Ser Ser Phe Arg Leu Thr Ser Val Gly Arg Met
 275 280 285

Ile Thr Arg Thr Met Val Lys Ala Leu Glu Tyr Val Gly Leu Ala Pro
 290 295 300

Gln Gly Ser Glu Arg Val Ser Asn Phe Leu Glu Lys Ala Ala Glu Gly
 305 310 315 320

25 Leu Val Glu Gly Gly Lys Lys Glu Ile Phe Thr Pro Met Tyr Phe Phe
 325 330 335

Leu Val Arg Lys Pro Leu Leu Glu *
 340 345