STEROL METHYLTRANSFERASE 1 Controls the Level of Cholesterol in Plants

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The side chain in plant sterols can have either a methyl or ethyl addition at carbon 24 that is absent in cholesterol. The ethyl addition is the product of two sequential methyl additions. Arabidopsis contains three genes—sterol methyltransferase 1 (SMT1), SMT2, and SMT3—homologous to yeast ERG6, which is known to encode an S-adenosylmethionine-dependent C-24 SMT that catalyzes a single methyl addition. The SMT1 polypeptide is the most similar of these Arabidopsis homologs to yeast Erg6p. Moreover, expression of Arabidopsis SMT1 in erg6 restores SMT activity to the yeast mutant. The smt1 plants have pleiotropic defects: poor growth and fertility, sensitivity of the root to calcium, and a loss of proper embryo morphogenesis. smt1 has an altered sterol content: it accumulates cholesterol and has less C-24 alkylated sterols content. Escherichia coli extracts, obtained from a strain expressing the Arabidopsis SMT1 protein, can perform both the methyl and ethyl additions to appropriate sterol substrates, although with different kinetics. The fact that smt1 null mutants still produce alkylated sterols and that SMT1 can catalyze both alkylation steps shows that there is considerable overlap in the substrate specificity of enzymes in sterol biosynthesis. The availability of the SMT1 gene and mutant should permit the manipulation of phytosterol composition, which will help elucidate the role of sterols in animal nutrition.

INTRODUCTION

Sterols are ubiquitous among eukaryotic organisms and serve both as bulk membrane lipid components and as precursors for additional metabolites such as mammalian steroid hormones, plant brassinosteroid hormones, and insect ecdysteroids (Parish and Nes, 1997).

The major sterols of plants and fungi contain an alkyl substitution at carbon 24 that is absent in cholesterol, the dominant sterol of virtually all animals (Nes and McKean, 1977). The methyl and ethyl substituents at C-24 are the only sterol carbons not derived from the acetate–mevalonate pathway and are absent in the first sterol intermediate of plants (cycloartenol) as well as animals and fungi (lanosterol) (Nes and McKean, 1977).

The carbons of the alkyl group at C-24 are added by way of an S-adenosylmethionine (SAM)-dependent transmethylation (Parks, 1958). The methyl (C1) or ethyl (C2) substituent at C-24 is the product of either a single carbon addition or two sequential single carbon additions. The alkylation product of the first transmethylation, a C-24 methylene (C1) sterol, is the substrate for the second transmethylation reaction to generate a C-24 ethylenic (C2) sterol (Castle et al., 1963).

The first and second C-methyl additions are performed as distinct steps in phytosterol synthesis (Figure 1) by the enzyme SMT. However, the C-24 SMT, like other enzymes in sterol biosynthesis, can be somewhat promiscuous. In photosynthetic organisms, cycloartenol is thought to be the substrate of the first methylation (Figure 1, step 1) because 24-methylene cycloartenol, the earliest possible product of transmethylation, is a frequently detected minor (and presumably intermediate) sterol in plant extracts (Guo et al., 1995). Moreover, cycloartenol is the best in vitro substrate for C-24 methyl addition, compared with other potential sterol intermediates (Nes et al., 1991, 1998b). 24-Methylene lophenol is often considered the optimal substrate for the second transmethylation (Figure 1, step 7) (Rahier et al., 1986).

In the fungus Saccharomyces cerevisiae, the transmethylation that produces the dominant 24-methyl sterol, ergosterol, is performed by a SAM-dependent C-24 SMT, Erg6p (Bard, 1972). The erg6 mutant, isolated for its resistance to the polyene antibiotic nystatin, was the first recognized yeast mutant with a deficiency in ergosterol biosynthesis.
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(Molzahn and Woods, 1972). Mutants defective in late steps of sterol biosynthesis show reduced sensitivity to polyenes such as nystatin that disrupt the membranes, although the connection between nystatin resistance and the defect in ergosterol biosynthesis is not completely understood.

The erg6 defect in ergosterol biosynthesis leads to a broad spectrum of phenotypes that can be attributed to a lack of membrane integrity. For example, erg6 mutants are hypersensitive to hypertonic shock with various metal salts (Bard et al., 1978), hypersensitive to Na\(^+\) and Li\(^+\) (Welihinda et al., 1994), supersensitive to recovery from arrest by nalidixic acid (Prendergast et al., 1995), deficient in active tryptophan uptake (Gaber et al., 1989), and more readily absorbent of the dye crystal violet than is the wild type (Bard et al., 1978). Because the erg6 mutation increases permeability, it is useful for studying small molecules such as brefeldin A that fail to enter wild-type cells efficiently (Shah and Klausner, 1993).

The SMTs from other fungi and plants have been identified by either their sequence similarity to yeast Erg6p or their ability to suppress defects of the yeast erg6 mutant (Hardwick and Pelham, 1994; Husselstein et al., 1996; Shi et al., 1996; Jensen-Pergakes et al., 1998). The SMTs from diverse organisms are clearly related and fall into three classes, based on sequence relatedness: a distinct class of fungal genes and two classes of plant genes. The fungal ERG6 genes from S. cerevisiae, Candida albicans, and Schizosaccharomyces pombe share at least 52% identity to each other and somewhat less (no more than 43% identity) to any of the plant SMT genes (Jensen-Pergakes et al., 1998). Members of the first class of plant SMTs are at least 69% identical to each other and no more than 47% identical to sequences in the two other classes. A second class of plant SMTs shares at least 71% identity among its members and has no more than 42% identity to members in the other two classes (Bouvier-Nave et al., 1998).

The two sequence-related classes of plant SMT proteins are thought to reflect their roles in distinct enzymatic steps (Grebenok et al., 1997; Bouvier-Nave et al., 1998). Because the first class shares more sequence conservation with the fungal ERG6 genes (which exclusively produce methylated sterols, or C\(_1\) addition) than does the second class, the first class of plant SMTs is proposed to be responsible, in planta, for the first SMT step. The members of the second class of plant SMTs, which are more distantly related to ERG6, are proposed to perform in planta the second SMT step responsible for C\(_2\) substitution (Husselstein et al., 1996; Bouvier-Nave et al., 1998). The available set of plant SMT sequences discounts a phylogenetic significance for the two classes.

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**Figure 1. Phytosterol Biosynthetic Pathway.**

Cycloartenol, the first polycyclic sterol intermediate, is converted into the dominant C5-sterols: cholesterol, campesterol, and sitosterol. The color code distinguishes the degree of C-24 alkylation (blue for the C\(_1\) addition and red for the C\(_2\) addition) on the black carbon skeleton of the sterol intermediates or products. For the names of the products and some intermediates, the color indicates whether the sterol has no addition (black), C\(_1\) addition (blue), or C\(_2\) addition (red) at C-24. The kinetically favored pathway for biosynthesis of cholesterol (black), campesterol (blue), and sitosterol (red) is also indicated with colored arrows. The enzymatic steps are numbered as follows: 1, S-adenosyl-L-methionine:C24(25) C-methyltransferase; 2, C-4 methyl oxidase/C-3 dehydrogenase/C-3 keto reductase; 3, cycloeucalenol-obtusifoliol isomerase; 4, C-14 demethylase; 5, C14 reductase; 6, C8-C7 isomerase; 7, S-adenosyl-L-methionine:C24(28) C-methyltransferase; 8, C-4 methyl oxidase/C-3 dehydrogenase/C-3 keto reductase; 9, C5 desaturase; 10, C7 reductase; and 11, C24(25) reductase.
Sterol Methyltransferase 1

because the genomes of both monocot and dicot plants contain members of both SMT groups.

Despite this categorization, recent biochemical analysis of plant SMT activities indicates that the functional distinction may not be so clearly defined (Bouvier-Nave et al., 1998). Members from either SMT class are capable of performing both C$_1$ and C$_2$ additions but can differ with respect to substrate affinities, substrate reaction rates, or allosteric modulation. Heterologous expression of either class one or class two enzymes from tobacco (Ntsmt1-1 or Ntsmt2-1) in the erg6 yeast mutant leads to the accumulation of both C$_1$ (ergosterol) and C$_2$ (24-ethylidene-24,25-dihydrolanosterol) alkylated sterols, whereas the erg6 mutant alone accumulates only sterols lacking the C-24 methyl group (Bouvier-Nave et al., 1998).

The Arabidopsis genome contains at least three genes related to ERG6. Two of the genes, SMT2 and SMT3, have been cloned and expressed in yeast. Each is capable of promoting both C$_1$ and C$_2$ addition when expressed in erg6 yeast cells (Bouvier-Nave et al., 1997; Schaller et al., 1998). Also, SMT2 can perform both C-methyl additions stereoselectively when expressed in Escherichia coli (Tong et al., 1997). These data suggest that the plant enzymes are ordinarily capable of both the C$_1$ and C$_2$ additions.

In this report we identify the function of the third Arabidopsis SMT gene SMT1 by the expression of the cloned gene and the analysis of mutants lacking this enzyme. Until now, the functional importance for the C-24 alkylation of sterols in plants has been appraised only indirectly (Bloch, 1983; Hartmann, 1998). Here, we describe the phenotypic defects associated with the diminished sterol C-24 alkylation resulting from the absence of the Arabidopsis class one C-24 SMT.

RESULTS

smt1 Mutant

A screen of Ac-mutagenized seed pools for plants displaying abnormal root system formation yielded four independent strains with very similar root growth defects (James et al., 1995). All four mutants were also indistinguishable with respect to the other phenotypes we observed. Subsequent analysis showed that all four mutations are in a gene encoding an SMT. The mutants, as shown in Figure 2, have shorter petioles and smaller, rounder leaves, which make their rosettes more compact than that of the wild-type SMT1 plant; moreover, the stems of smt1 plants are shorter than those of SMT1. Although the smt1 flowers appear normal, the siliques that develop are stunted.

Two of the alleles, smt1-2 and smt1-3, were shown to segregate as single, recessive Mendelian traits (Table 1). Crosses of either smt1-2 or smt1-3 to wild-type Wassilewski (Ws) or Columbia ecotype, respectively, yielded 18% F$_2$ progeny with the mutant phenotype of the original smt1 parent. The lower than expected frequencies of Smt$^+$ plants in one of the crosses may be attributable to a selective disadvantage of the smt1 gametes.
smt1 mutants were initially identified on agar plates for their stunted and disfigured roots with swelling in the epidermis and cortex, as seen in Figure 3. In the figure, the meristems of the more severely distorted roots are arrested. In the differentiation zone, in which wild-type root cells in the cortex and epidermis are rectangular and elongated, the same cells in smt1 roots are rounded and stunted (Figures 3F and 3G). Along the root length, the cell files in the smt1 root lack their characteristic order and delineation, which may be responsible for the disordered pattern of root hair growth on the epidermis.

Surprisingly, smt1 plants grown in soil had normal-looking roots that were indistinguishable from the roots of wild-type SMT1 plants. The aberrant root phenotype on agar medium was not accentuated or rescued by changing various growth parameters (see Methods). However, after ~2 weeks of growth, the roots of smt1-3 plants shown in Figure 3D are indistinguishable from those of the wild type shown in Figure 3C. One explanation for this behavior is that some component of the agar medium that is inhibitory to the roots of smt1 seedlings is detoxified over time so that the smt1 roots can expand normally.

A test of the various components of the plant nutrient agar growth medium (PNG; see Methods for composition) revealed that the smt1-1 strains were sensitive to the calcium salt concentration. Decreasing the plant nutrient salt concentration by 10-fold led to marked improvement in the growth of smt1-1 and smt1-3 roots, whereas alteration of other medium components had no effect. To identify the inhibitory ion or ions, we tested the growth of the mutant on PNG in which each component salt (see Methods) was diluted 10-fold. Decreasing the calcium salt gave a noticeable improvement in root growth: a longer primary root and improved overall appearance. The same change in calcium salt concentration had no effect on wild-type roots. Because this sensitivity was apparent with medium containing either CaCl₂ or Ca(NO₃)₂, we conclude that the mutant is hypersensitive to calcium ions. The fact that the mutant phenotype could not be completely suppressed by lowering the calcium concentration suggests that other inhibitory factors are also present in the agar medium.

**smt1 Plants Have Low Fertility**

smt1 plants produce normal flowers but have severely reduced fertility, yielding at most one to two viable seeds per silique. The drastic reduction in fertility results from aborted embryos, not reduced fertilization. The pollen produced by smt1 plants covers the stigmas of these smt1 plants. Moreover, microscopic inspection of the smt1 pollen tubes indi-
icates that these tubes appear to grow into the stigma and to the ovules in a manner indistinguishable from wild-type pollen tubes. In addition, in smt1 plants with good overall growth, most ovules are fertilized. Inspection of immature siliques, cleared and dissected, revealed that 85% of ovaries from smt1 plants—412 ovaries from 16 siliques were examined—contained developing embryos, whereas in self-fertilized wild-type plants, 94% of all ovaries—374 ovaries from eight siliques were examined—contained developing embryos. This slight difference in fertilization does not explain the much lower seed yield of smt1 plants.

Rather, most embryos of smt1 abort. From the appearance of dissected seeds, the abortion does not seem to occur at a particular developmental stage. At early stages of embryogenesis, regions of the normally colorless embryo sac may appear darkened and presumably necrotic, usually including regions closest to the funicle attachment, the chalazal nucellus, as seen in Figures 4G and 4I to 4M.

The infertility caused by smt1 appears to be a recessive maternal defect. The SMT1/smt1 heterozygote is completely fertile and produces nearly a quarter of the embryos formed in the germinated seedlings revealed various defects. The most remarkable feature of the smt1 seedlings is the variable number and shape of their cotyledons. Representative seedlings with aberrant cotyledon structures are shown in Figures 4P and 4Q, along with the usual dicot seedling for comparison.

To examine this defective embryogenesis, we compared the developing embryos from the wild type shown in Figures 4A to 4E and smt1-3 plants shown in Figures 4F to 4J. The smt1 embryos from all four smt1 alleles have difficulty forming the gross structures that distinguish the different stages of embryogenesis, for example, the projections in the heart stage or torpedo stage that give rise to the cotyledons. In comparison with the wild type, the cell mass in an smt1 embryo has a flattened or dulfed shape.

Observations suggest that the aberrant shape of the smt1/smt1 embryos is not a maternal defect. The 18% smt1/smt1 progeny obtained from self-fertilized SMT1/smt1 heterozygotes frequently possess an aberrant number of cotyledons and a shape similar to the smt1/smt1 progeny of self-fertilized smt1/smt1 homozygotes. As described below, the expression of a transgenic SMT1 cDNA partially complemented smt1-3, restoring fertility but not normal embryo morphogenesis. This dichotomy suggests that these two phenotypes are the outcomes of two distinct processes.

In addition, smt1 seedlings have an apparent requirement for an exogenous carbon energy source such as glucose or sucrose after germination (as described in Methods). The amounts of major seed storage proteins and triacylglycerols contained in smt1-1 and smt1-3 seeds are comparable with those of the wild type (A.C. Diener, unpublished results), making it unlikely that diminished stores of these compounds are the basis of the carbon source requirement.

**smt1 Mutants Are the Result of DNA Rearrangements**

Two of the four mutants, smt1-1 and smt1-2, possessed an Ac transposon linked to the mutant phenotype. By DNA gel blot analysis, linkage of the mutant phenotype and Ac element was observed in the bulked mutant offspring from an SMT1/smt1-1 heterozygous plant. The genomic sequence flanking the Ac insertion in smt1-1 was isolated by inverse polymerase chain reaction (PCR), as described in Methods. As shown in Figure 5A, in either of the two mutants smt1-1 or smt1-2, a single Ac transposon insertion and the resulting DNA restriction fragment length polymorphisms are located in a genomic region, bacterial artificial chromosome (BAC) clone MSH12 (Genbank accession number AB006704), previously sequenced by the international Arabidopsis Genome Initiative (AGI). These two insertions fall within the same predicted gene, MSH12.10, that is highly homologous to a previously characterized soybean C-24 SMT (Shi et al., 1996).

All four mutants have alterations in the SMT1/MSH12.10 open reading frame that are likely to result in the complete absence of SMT1 function. The smt1-1 allele results from an insertion of an Ac element in the second intron of SMT1. The Ac element in smt1-1 is presumably immobilized by a small deletion of its 5’ end. In addition, the Ac element in smt1-1 is flanked by an unusual 222-bp genomic duplication.

The smt1-2 allele results from an Ac insertion in exon 12. The transposon is flanked by an 8-bp genomic duplication that is typical of the targets of Ac insertion. The smt1-2/smt1-2 plants are unstable and give rise to revertant progeny with a wild-type phenotype. The instability of homozygous smt1-2 strains is best explained by the presence of an active Ac transposon in the mutant. Upon further testing, some wild-type revertants among the progeny of smt1-2/smt1-2 plants proved to be heterozygous for smt1 (SMT1/ smt1-2) but remained homozygous for the linked hygromycin-resistance marker. The faint band in the smt1-2 DNA lane in Figure 5A, corresponding to the wild-type-size genomic fragment, is molecular evidence for the active Ac transposition, which is presumably responsible for reversion events. The overall growth of soil-grown smt1-2/smt1-2 plants tends to be better than that of the other three mutants—probably because of frequent somatic excision of the Ac allele to yield wild-type sectors.

**smt1 Embryogenesis Is Aberrant**

Of those few seeds obtained from self-fertilized smt1 plants, roughly three-quarters germinated on agar plates. However, the embryos formed in the germinated seedlings revealed various defects. The most remarkable feature of the smt1 seedlings is the variable number and shape of their cotyledons. Representative seedlings with aberrant cotyledon structures are shown in Figures 4P and 4Q, along with the usual dicot seedling for comparison.

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Observations suggest that the aberrant shape of the smt1/smt1 embryos is not a maternal defect. The 18% smt1/smt1 progeny obtained from self-fertilized SMT1/smt1 heterozygotes frequently possess an aberrant number of cotyledons and a shape similar to the smt1/smt1 progeny of self-fertilized smt1/smt1 homozygotes. As described below, the expression of a transgenic SMT1 cDNA partially complemented smt1-3, restoring fertility but not normal embryo morphogenesis. This dichotomy suggests that these two phenotypes are the outcomes of two distinct processes.

In addition, smt1 seedlings have an apparent requirement for an exogenous carbon energy source such as glucose or sucrose after germination (as described in Methods). The amounts of major seed storage proteins and triacylglycerols contained in smt1-1 and smt1-3 seeds are comparable with those of the wild type (A.C. Diener, unpublished results), making it unlikely that diminished stores of these compounds are the basis of the carbon source requirement.
Figure 4. smt1 is Defective in Embryogenesis.

(A) to (J) The embryos of wild-type SMT1 ([A] to [E]) and smt1 ([F] to [J]).
(B) and (F) Early globular stage.
(C) and (G) Midglobular stage.
(D) and (H) Late globular stage.
(E) and (I) Heart stage.
(F) and (J) Late heart stage.
(K) to (O) Examples of awry embryogenesis in smt1 siliques.
(K) and (L) Extensive necrosis and cellular swelling of embryo sac with early globular stage embryo.
(M) Extensive necrosis of embryo sac with heart stage embryo.
(N) A massive globular embryo lacking any of the form of wild-type embryos.
(O) Late heart stage embryo with three cotyledons forming at the apex.
The smt1-3 allele contains no Ac insertion; instead, a short sequence duplication and a single base pair insertion in exon 10 shift the reading frame and give rise to a nonsense codon (TGA) close to the site of the mutation. The DNA lesion in smt1-3 is likely to have arisen from excision of Ac. The smt1-4 allele is a complex DNA rearrangement (deletion and insertion) that is also likely to be the aftermath of an Ac excision (Figure 5B). All four alleles lead to a similar phenotype, probably the null phenotype, because each is likely to lead to complete loss of function.

**SMT1 Transcript and Protein Sequence**

The nucleotide sequence of the open reading frame in the SMT1 cDNAs was identical to the sequence reported for MSH12.10 by AGI. The exon and intron boundaries of SMT1/MSH12.10 were as predicted by AGI except for an additional intron and exon, containing an untranslated sequence, at the 5' end. SMT1 encodes the same polypeptide motifs common to SMT2, SMT3, ERG6, and all previously reported SMTs. However, as shown in Figure 6, the polypeptide sequence of SMT1 resembles the class one SMTs more than do the other two Arabidopsis SMTs, SMT2 and SMT3. As is the case with other SMTs that catalyze the first methyl-transfer reaction preferentially to the second methyl-transfer reaction, SMT1 is more related to the yeast SMT ERG6 than to the two other Arabidopsis SMTs, which preferentially generate the 24-ethyl sterol side chain.

**SMT1 Transgenes Complement smt1-3**

Transgenic smt1-3/smt1-3 plants containing constructs derived either from a genomic clone of SMT1 or from an SMT1 cDNA have a wild-type phenotype. One construct contained a transgene with the SMT1 locus on the HindIII genomic fragment, depicted in Figure 5B. This genomic segment of SMT1 completely complemented the smt1-3 allele in homozygous smt1-3/smt1-3 plants, as explained in Methods. The AGI annotation, in addition to our own sequence analysis, suggests that this genomic sequence lacks any open reading frame other than smt1 with significant length or homology to Genbank database entries. The other transgene contains an SMT1 cDNA inserted in front of the 35S cauliflower mosaic virus (CaMV) promoter in a standard expression construct. Homozygous smt1-3/smt1-3 plants containing the SMT1 cDNA transgene were essentially wild type in appearance and fertility, except for the persistence of a defect in embryogenesis. This defect in embryogenesis could be a consequence of the 35S CaMV promoter, which might lead to abnormal expression of SMT1 during embryogenesis. Alternatively, intron sequence might be critical for the proper embryonic SMT1 expression pattern.

**SMT1 Encodes SMT Activity**

Several in vivo and in vitro assays suggest that the Arabidopsis SMT1 gene encodes an SMT. SMT1 suppresses the defects of a yeast mutant (erg6) that lacks SMT activity, suggesting that the Arabidopsis gene performs a step in sterol biosynthesis analogous to that performed by Erg6p. Yeast erg6 strains have several phenotypes such as sensitivity to lithium salt and cycloheximide and resistance to nystatin. In Figure 7, expression of the Arabidopsis SMT1 cDNA from the ADH1 promoter in a yeast erg6 strain partially relieves the sensitivities to lithium acetate and cycloheximide and diminishes the resistance to nystatin. Extracts of erg6::HIS3 yeast strains that express SMT1 have three prominent 24-methyl sterols, one of which is ergosterol (see Methods).

E. coli strains expressing the SMT1 cDNA produced SMT activity in crude homogenates, whereas strains lacking the plant gene did not. Using cycloartenol as the sterol substrate, we measured apparent $K_m$ and $V_{max}$ values of 42 $\mu$M and 5.2 pmol min$^{-1}$ mg$^{-1}$ protein in Figure 8A for the conversion to 24-methylene cycloartenol (Figure 1, step 1). Whereas 24(28)-methylene cycloartenol was not a suitable substrate, the SMT1-containing extracts did convert 24(28)-methylene lophenol to the 24-ethylidene sterol (Figure 1, step 7, and Figure 8B). However, because the second transmethylation in the latter reaction was performed with ~10-fold less efficiency than the first transmethylation with cycloartenol, accurate measurement of kinetic parameters of the first transmethylation reaction was precluded.

**smt1-1 Plants Accumulate Cholesterol**

The role of SMT1 in Arabidopsis sterol biosynthesis was determined by comparing the sterol composition of 1-month-old smt1-1 with wild-type SMT1 plants (Table 2). The smt1-1 plants had 25% more mass of sterol per gram of plant material than did the wild-type SMT1 plants. The most noteworthy difference in the relative content of major sterols is that in smt1-1 plants, cholesterol is the most abundant sterol.

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**Figure 4.** (continued).

(P) and (Q) smt1 seedlings germinated on plant nutrient agar (PNA) and left for 1 month.

(P) Monocot, dicot, and tricot (from left to right).

(Q) Crown cotyledon (above) and ringed cotyledon (below).
and accumulates at the expense of sitosterol (24-ethyl cholesterol), which ordinarily is the most abundant sterol in Arabidopsis. Surprisingly, though, the campesterol (24-methyl cholesterol) content is relatively unaffected in smt1-1. Other than the greater than fourfold increase in cholesterol and reduction in sitosterol, the relative amounts of the other sterols are similar in smt1-1 and SMT1 plants. Moreover, the overall content of the dominant C5 sterols (where C5 refers to the desaturation at carbon 5 in the sterol nucleus, a double bond between carbons 5 and 6)—cholesterol, campesterol, sitosterol, and 24-methylene-cholesterol, which all have the same polycyclic ring structure—is the same 80% in both smt1-1 and SMT1 wild type. Nevertheless, some minor sterol intermediates, including cholesta-7-enol and 14-methylcholesta-8-enol, do show low but unusual accumulation (Table 2).

**smt1 Seedlings Are Sensitive to Brassinosteroid**

The smt1 plants have short hypocotyls, a phenotype typical of brassinosteroid-deficient mutants such as det2 and dwf1 (Fujioka et al., 1997; Klahre et al., 1998; Choe et al., 1999a). Given smt1-1’s defect in sterol biosynthesis, we compared smt1 with det2 and dwf1 to determine whether these mutants had similar responses to exogenous brassinosteroid on the hypocotyl and primary root. In Figure 9, epibrassinolide (100 nM) stimulated hypocotyl length of the det2 or dwf1 dwarf mutant to increase 5.5- or 4.5-fold, respectively, whereas the hypocotyl length of smt1-3 showed only a modest 1.8-fold increase. However, the epibrassinolide stimulation in smt1-3 was still greater than that seen for hypocotyl elongation in the wild type (a 1.2-fold increase). Similar effects on hypocotyl growth were observed for etiolated seedlings of the mutants and wild type on medium containing epibrassinolide (up to 500 nM).

Epibrassinolide is extremely inhibitory to the smt1 roots as compared with the wild type (Figure 9). smt1-3 root growth was greatly inhibited at epibrassinolide concentrations (10 nM) that fail to inhibit the wild type. These results suggest that roots of smt1 are hypersensitive to epibrassinolide.

One explanation for the phenotype of smt1 roots is that they accumulate toxic amounts of brassinolides. However, attempts to reduce the endogenous brassinolide biosynthesis of smt1, by introducing the det2 mutation into the smt1 background, failed to improve the poor growth of smt1-3 roots. The smt1-3 det2 double mutant displayed the phenotypic attributes of both mutations: the same abnormal root cell shape and disordered cell files as smt1, and the more stunted root elongation of det2.

**SMT1::GUS Expression**

To visualize the spatial and relative activity of SMT1 transcriptional expression, we transformed plants with a con-
The polypeptide sequence of the three Arabidopsis SMTs and the Saccharomyces ERG6-encoded protein were aligned using MegAlign software (clustal method; DNASTAR). Any residue in ERG6 identical to an aligned residue in any other SMT is highlighted by white print on black. Seven motifs are boxed and labeled. S-Adenosylmethionine-dependent methyltransferases share three motifs: SAM I, SAM II, and SAM III. Two motifs, SMT I and SMT II (invariant), are found in C-24 SMTs. The class one C-24 SMTs in plants are distinguished from class two by two motifs, labeled class I A and class I B (Grebenok et al., 1997).
SMT1::GUS expression is conspicuously absent. The transcripts of the two other SMT genes in Arabidopsis, SMT2 (A.C. Diener, unpublished results) and SMT3, are also broadly expressed throughout the plant. No SMT1 transcript appears to accumulate in smt1-1 plants in which an Ac insertion sits near the 5' end of SMT1; a small amount of SMT1 transcript appears in smt1-3, in which a frameshift truncates the SMT1 after amino acid 209 by adding a single lysine residue to the C terminus. The greater amount of SMT3 RNA in smt1-1 plants than in smt1-3 plants, as seen in Figure 11, was observed in several different experiments. Although smt1-1 and smt1-3 are morphologically identical, RNA accumulation of SMT3 (and SMT2; A.C. Diener, unpublished results) is greater than in the wild type in plants with the smt1-1 allele but not in plants with the smt1-3 allele. The amount of expression of all three SMT genes in Arabidopsis is also unaffected in 3.5-week-old dwf1 plants, a dwarf mutant deficient in a enzymatic activity immediately downstream of the SMTs (A.C. Diener, unpublished results).

SMT Expression in Embryos

All three SMT genes have strong and spatially distinct expression in developing embryos, as detected by the in situ RNA hybridization shown in Figure 12. We could detect RNA for the SMT genes in both early (heart stage; W.J. Whorisky, unpublished results) and later stage embryos, in which expression domains for each were more clearly demarcated. SMT1 RNA is strongly expressed below the cotyledons and much less so in the cotyledons (Figures 12A to 12D). SMT2 RNA is strongly expressed throughout the developing embryo (Figures 12E to 12H). SMT3 RNA is expressed strongly in the hypocotyl and radicle primordium but is excluded from the central vasculature; in the cotyledons, SMT3 RNA expression is strongest at the apical tips (Figures 12I to 12L). Because of the strong embryonic phenotypes, we suspect that these RNA patterns reflect SMT1 protein expression in the embryos, despite the lack of detection in the carpels or embryo sac with the SMT1::GUS fusion.
DISCUSSION

There are three genes for C-24 SMT (SMT1, SMT2, and SMT3) in Arabidopsis. No other family members could be detected by PCR amplification with a set of degenerate primers to highly conserved motifs in SMT genes, by low stringency hybridization to genomic DNA gel blots with SMT1, SMT2, or SMT3 probes, or by sequence database searches (A.C. Diener, unpublished results). The SMT activity of SMT2 and SMT3 was previously analyzed by heterologous expression in yeast and in extracts of E. coli expressing a cDNA of SMT2 (Husselstein et al., 1996; Bouvier-Nave et al., 1997; Tong et al., 1997). The closely related SMT2 and SMT3 proteins have been proposed to perform in planta specifically the second C-methyl addition (Figure 1, step 7), based on the substrate preference of their enzymatic activity (Bouvier-Nave et al., 1998). Our analysis of the SMT1 gene suggests that SMT1 preferentially catalyzes the first methyl addition (Figure 1, step 1).

Evidence for the functional assignment of SMT1 comes from several experiments. For example, SMT1 suppresses many of the phenotypes associated with the yeast erg6 mutation. Moreover, expression of SMT1 in erg6 cells leads to the synthesis of 24-methyl sterols that are not present in the erg6 mutant when the Arabidopsis SMT1 gene is not included. In contrast, the predominant sterols when Arabidopsis SMT2 was expressed in erg6 were C-24 ethylated species. A first methyl addition by SMT1 is further supported by the observation that SMT1 activity expressed in E. coli acted to catalyze a SAM-dependent transmethylation of cycloartenol. The SMT1 protein obtained from E. coli showed ~10-fold less methyltransferase activity with 24-methylene lophenol than with cycloartenol. These data, supporting a preference for the first methyl addition activity, agree with earlier data obtained with the tobacco class one SMT Ntsmt1-1 (Bouvier-Nave et al., 1998).

The pattern of accumulation of sterols in smt1 is also consistent with a defect in C-24 transmethylation. In smt1-1 plants, cholesterol accounts for one-fourth of all sterol and accumulates at the expense of sitosterol; the amount of campesterol, however, is unaffected. Given that smt1 has lost methyltransferase activity, the accumulation of cholesterol, which lacks C-24 alklylation, is reasonable. However, the deficiency in sitosterol (a C-24 ethylated sterol) and the nearly normal accumulation of campesterol (a C-24 methylated sterol) are unexpected. Actually, if SMT1 were the only enzyme responsible in planta for the first transmethylation reaction of cycloartenol to produce 24-methylene cycloartenol, smt1-1 should be completely deficient for C-24 alklylation because the second transmethylation reaction occurs only after the first transmethylation reaction. Because the majority of sterols in smt1-1 plants are alkylated, SMT1 cannot be the only enzyme in Arabidopsis capable of the first C-24 methyltransferation step.

One explanation for the accumulation of methylated sterols in smt1 is that in the absence of SMT1, these methylations are performed by other enzymes that do not ordinarily perform this reaction in the wild type. The ability of enzymes to act as “imposters” has been described in the mitogen-activated protein kinase pathway of yeast and may be a more general phenomenon (Madhani et al., 1997). In the wild type, the protein interactions of the SMT1 enzyme may exclude the other potential SMTs, namely, SMT2 and SMT3, from target substrates. In smt1, however, the other enzymes, although with perhaps lower affinity, may now have access to these substrates and perform methylations—albeit at a different rate and with different affinities.

These considerations bear directly on the relationship between the two sequence-related classes of plant SMT genes and the two SMT activities. The question is whether the division by sequence relatedness reflects a rigid functional difference. The ascomycete fungus S. cerevisiae produces only C-24 methylated sterols by the SMT Erg6p, a yeast protein for which the amino acid sequence is more closely related to that of the class one plant family of SMT genes than to the class two genes. On the basis of this relatedness, it has been argued that in planta, only the class one plant SMT genes perform the first methyl addition. However, the presumption that the more recent divergence of the Arabidopsis SMT1 and Saccharomyces Erg6p protein is related to the restriction of SMT activity to the first transmethylation step deserves some scrutiny. For instance, the Basidiomycete rust Uromyces phaseoli is capable of producing sterols with either a C1 or C2 addition (Bansal and Knoche, 1981). The inability to separate the first and second

Table 2. Major Sterol Content of Whole Plant

<table>
<thead>
<tr>
<th>Major Sterol</th>
<th>Fraction of Total Sterol&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SMT1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>48</td>
</tr>
<tr>
<td>Campesterol</td>
<td>20</td>
</tr>
<tr>
<td>Isofucosterol</td>
<td>10</td>
</tr>
<tr>
<td>Cycloartenol</td>
<td>10</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>6</td>
</tr>
<tr>
<td>24-Methylene-cholesterol</td>
<td>5</td>
</tr>
<tr>
<td>Fucosterol</td>
<td>1</td>
</tr>
<tr>
<td>24-Methylene-cycloartenol</td>
<td>Trace</td>
</tr>
<tr>
<td>Cholesta-7-enol</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cholestanol</td>
<td>ND</td>
</tr>
<tr>
<td>14-Methyl-cholesta-8-enol</td>
<td>ND</td>
</tr>
<tr>
<td>Sitostanol</td>
<td>Trace</td>
</tr>
<tr>
<td>Campestanol</td>
<td>Trace</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as a percentage.
<sup>b</sup>Wild-type SMT1 plants contained 179 µg/g fresh weight.
<sup>c</sup>Mutant smt1-1 plants contained 223 µg/g fresh weight. The smt1-1 plants are morphologically different from the wild type, and these tissue differences may influence the total sterol content.
<sup>d</sup>ND, not determined.
transmethylation reactions of *U. phaseoli* by chromatographic schemes led to the conclusion that both activities reside in the same protein complex; furthermore, both of the Uromyces SMT activities shared identical sensitivities to various salts and detergents. It is conceivable, therefore, that the progenitor of Saccharomyces ERG6 and the Arabidopsis SMT1 gene might have been responsible for both C1 and C2 alkyl additions. By this argument, the Saccharomyces ERG6 has since lost the ability to perform both steps and has evolved into an enzyme capable of only the first methyltransferase reaction. In support of this contention, a single conserved amino acid change in *ERG6* has been found to impart a C2 addition activity (Nes et al., 1999).

The phenotype of smt1 adds to the increasing knowledge about the biological importance of sterols in plants. smt1, like the other two Arabidopsis mutants specifically defective in sterol biosynthesis (dwf1/dim/cbb1 and ste1/dwf7) (Gachotte et al., 1995; Kauschmann et al., 1996; Klahre et al., 1998; Choe et al., 1999a, 1999b), results in small plants. However, smt1 does not share the distinctive dwarf appearance of the brassinolide-deficient dwf1 and dwf7. Moreover, the morphological phenotypes of dwf1 and dwf7 are rescued by brassinosteroid, whereas the smt1 defect is not (Choe et al., 1999a, 1999b).

The reduced hypocotyl length of smt1 is only modestly affected by exogenous epibrassinolide, as compared with the more dramatic effect of epibrassinolide on mutants det2 and dwf1. Actually, both smt1 and wild-type plants accumulate about the same amount of campesterol, the immediate sterol precursor of brassinolide. Of course, these observations discount only the possibility of an exclusive defect in brassinolide biosynthesis. Already in the developing embryo, strikingly distinct patterns of expression were seen for the three SMT RNAs, and again, in the rosette, we observed stronger SMT1 expression in leaves undergoing expansion. The mild restriction of growth of the leaves or stems may reveal a subtle and local deficiency for brassinosteroid.

Restricted accumulation of alkylated dominant sterols is the most dramatic effect of the smt1 mutation. The first SMT C1 reaction is the first biosynthetic step in sterol transformation (Figure 1), yet in smt1, most sterol accumulates as cholesterol, a natural but minor dominant sterol, and not as cycloartenol, the immediate precursor of the first SMT reaction. Compromised transmethylation in smt1 plants failed to restrict further sterol transformation (Benveniste, 1986; Bach and Benveniste, 1997). Irregular regulation of sterol synthesis by the skewed pattern of C-24 alkylation may explain the apparent 25% overabundance of sterols in smt1-1 plants. However, with the caveat that we have not ascertained the subcellular distribution of sterols in smt1 plants, the enumerated smt1 defects may be attributed specifically to a reduction in C-24 alkylation and not to the absence of the normally dominant C5-sterols, because 80% of sterols in both smt1 and wild-type plants have the normal C5 polycyclic ring structure.

Among sterol modifications, C-24 alkylation appears to be

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**Figure 9.** Sensitivity to Brassinosteroid.

(A) to (D) Different ecotypes of plants grown for 1 week at 22°C under yellow light on PNG medium supplemented with (from left to right) 0, 10, or 100 nM epibrassinolide. Triangle points mark the hypocotyl-root junction.

(A) det2.

(B) dwf1. Shown are two seedlings on 100 nM epibrassinolide.

(C) smt1-3.

(D) Ws ecotype.

(E) and (F) Hypocotyl and root lengths, respectively, for each mutant; values are an average of at least 8 seedlings. Error bars represent the standard deviation.
especially critical for sterol membrane function; its deficiency in smt1 is probably responsible for the altered membrane properties and the phenotypes associated with the mutation. We have found that smt1 roots are hypersensitive to calcium salts and epibrassinolide and that smt1 root growth is affected by some component or condition initially present in agar plates. Work with yeast ergosterol biosynthetic mutants suggests that the lack of C-24 alkylation in erg6 strains is more consequential to membrane integrity, as assessed by cation sensitivity and dye permeability, than are alterations in sterol B-ring structure affected by either the erg2 mutation (deficient in C8-C7 isomerization, equivalent to step 6 in Figure 1) or erg3 (deficient in C5-desaturase activity, equivalent to STE1/DWF7, step 9 in Figure 1) (Bard et al., 1978). The Arabidopsis ste1 mutant, deficient in an activity equivalent to that of yeast ERG3, has no visible morphological consequence, despite the fact that only 32% of accumulated sterols are the normally dominant C5-sterols, whereas the majority (68%) of sterols are the C5,7-sterols, which are normally intermediate (Gachotte et al., 1995). In the case of the stronger dwf7, allelic to ste1, virtually all of the mutant phenotype has been attributed to brassinosteroid deficiency because the dwarfism of the mutant can be rescued by supplementation with exogenous brassinosteroid (Choe et al., 1999b).

Maternal fertility and embryogenesis are particularly sensitive to the loss of SMT1 function. The dwf7 mutant also has reduced fertility unrelated to its brassinosteroid deficiency (Choe et al., 1999b); however, the nature of the dwf7 fertility problem awaits more careful examination. The aberrant morphogenesis of the smt1 embryo may be analogous to the inhibited growth of smt1 roots on agar: The appearance of the embryonic projections for cotyledons and the hypocotyl/root axis is muted, whereas in the case of the attenuated smt1 root, cells are short and rounded, failing to form regular files or their normal elongated, rectangular shape. When grown in soil, smt1 root cells can have normal shape and the roots look like wild-type roots. We speculate, by extension of what is known about the yeast erg6 mutant, that the altered membrane integrity of smt1 is responsible for its conditional root phenotype, calcium salt sensitivity of roots, and defects in embryo morphogenesis.

Two problems complicate the analysis of the steroid pathway by molecular genetic approaches. First, the sterol transformation pathway is not a simple linear pathway. For example, a defect in SMT1 alkylation fails to interrupt the further transformation of cycloartenol into the ordinarily dominant C5-sterols. Thus, it is difficult to determine the

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**Figure 10.** Expression of SMT1::GUS in Transgenic Plants.

Three-week-old SMT1::GUS plant indigo-stained with X-Gluc and cleared of endogenous pigments. 

(A) The aerial half of the plant. 

(B) The whole root system. 

(C) Newly formed lateral roots. 

(D) Lateral root primordia within root. 

(E) The root apical end.
normal substrates for each enzyme by using as an indication of the precursors those sterols that accumulate in a sterol biosynthetic mutant. Second, in mutants, many of the sterol biosynthetic enzymes may plausibly perform reactions that they do not perform in the wild type. For example, SMT2 and SMT3 may substitute, however poorly, for SMT1 activity in smt1. These secondary overlapping activities would be revealed if it were possible to construct strains with mutations in all three SMT genes.

The availability of both mutations and genes in the Arabidopsis sterol biosynthetic pathway raises the possibility of manipulating plant sterol biosynthesis. Alterations in sterol composition may lead to improved agricultural varieties and allow the impact of phytosterol intake on animal diet and nutrition to be addressed.

METHODS

Plant Strains and Growth Conditions

The smt1 mutations, which lack sterol methyltransferase (SMT)-encoding genes, originate from an Ac transgenic line in the Wassilewskija (Ws) ecotype. The plants were grown under continuous illumination to be addressed.

Figure 11. RNA Expression of SMT1 and SMT3.

RNA was size-separated and blotted onto nitrocellulose for RNA gel blot analysis. Ethidium bromide staining of rRNA in each gel accompanies the hybridization. In the first three lanes, rosette leaves were collected for RNA extraction from SMT1/SMT1, smt1-1/ smt1-1, and smt1-3/smt1-3 plants. In the remaining lanes, RNA was extracted from the aerial part indicated of 2-week-old soil-grown plants.

Plant Transformation

We selected on Luria-Bertani (LB) plates plus 100 mg/L spectinomycin (Sigma) Agrobacterium tumefaciens (GV3101) transformed by electroporation with PZP-based vectors (Hajdukiewicz et al., 1994). An initial 5 mL of 2 × LB liquid culture with spectinomycin selection was diluted into 600 mL of 2 × LB without spectinomycin selection, and the cells were grown to saturation. The Agrobacterium cells were pelleted and resuspended in 0.5 × Murashige and Skoog medium (Gibco BRL/Life Technologies) plus 5% sucrose. Secondary inflorescences of Columbia ecotype plants were dipped in Agrobacterium suspensions under vacuum for 5 min. Seeds from the transformed plants were selected with 25 mg/L kanamycin B (Sigma).

Nucleic Acids

Unless otherwise noted, all molecular biology protocols were derived from standard protocols (Ausubel et al., 1997). Plant genomic DNA was prepared using a preliminary protocol for genomic DNA extrac-
tion from plant leaves with Qiagen-tip (provided by Qiagen, Valencia, CA). Oligonucleotides were purchased from and DNA sequencing was performed by contract with Research Genetics (Huntsville, AL). RNA was extracted from plant material by using Tri Reagent (Molecular Research Center). For DNA gel blot analysis, 2 \( \mu \)g of restriction enzyme–digested DNA was loaded per lane. For RNA gel blot analysis, 5 \( \mu \)g of total RNA was loaded per lane. In situ hybridization was performed as described previously (Geng et al., 1999). The probes for \( \textit{SMT2} \) and \( \textit{SMT3} \) were derived from the expressed sequence tags 66F6T7 and ATTS3237, respectively, provided by the Arabidopsis Biological Resource Center.

**Library Subclones and Plasmid Constructions**

Genomic sequence flanking the \( \text{Ac} \) element in \( \text{smt1-1} \) was retrieved by inverse polymerase chain reaction (PCR): 5 \( \mu \)g of \( \text{smt1-1} \) DNA digested with HindIII in 20 \( \mu \)L of reaction medium and ligated in 400 \( \mu \)L. After glycojen coprecipitation, 1/10 of resuspended material served as template for PCR with the primers AD333 (5’-CGG-TTATACGATAACGGTCG-3’) and AD334 (5’-GTACGATGAAGT-GTTAGCC-3’). The PCR product of appropriate size was subcloned into Bluescript II vector (BS; Stratagene Inc.) that had been previously digested with EcoRV and treated for subcloning Taq-derived PCR products (Ausubel et al., 1997). This insert served as the probe for hybridization to both a lambda genomic library, in lambda GEM11 (a gift from J.T. Mulligan and R.W. Davis, Stanford University), and a lambda cDNA library, in lambda gt10 (a gift from C. Luschnig, member of Fink laboratory); both libraries were derived from Arabidopsis Columbia ecotype. The 8.4-kb HindIII fragment predicted by the Arabidopsis Genome Initiative (AGI) (depicted in Figure 5B) was subcloned from a lambda genomic clone into BS (BS/SMT/H3) and subsequently into the PZP212 binary vector (PZP/SMT/H3) for plant transformation. Of several cDNA library isolates, cSMT1.13 was completely sequenced and was designated \( \textit{SMT1} \) cDNA. The cSMT1.13 sequence consists of nucleotides 7 through 1242 of GenBank accession number AF195648 and was flanked by the linker sequence 5’-GAATTCCCGGGCAGGT-3’ at both ends. For plant expression of \( \textit{SMT1} \) cDNA, we digested BS/SMT1.13 with Xmal and ligated the cDNA into the Xmal site of PZP-35S, constructed by inserting the SacI/Nsil fragment containing the 35S promoter and 3’ nos terminator of pART7 into the T-DNA PstI/Sacl sites.

**Figure 12. RNA Expression of SMTs in Late-Stage Embryos.**

Micrometer sections of siliques with developing embryos were hybridized with antisense RNA probes of cDNA subclones of \( \textit{SMT1} \) (A to D), \( \textit{SMT2} \) (E to H), or \( \textit{SMT3} \) (I to L). The blue color is a fluorescent nuclear stain, and the red color is signal reflected by silver grains. (A), (B), (E), (F), (I), and (J) Approximately longitudinally sections through the embryos. (C), (D), (G), (H), (K), and (L) Cross-sectional cuts through hypocotyl and cotyledons. The coat of the embryo sac produces a strong background signal. Sense-strand SMT probes failed to produce marked signals or signal patterns.
of PZP212. For the translational fusion of SMT1 to the uidA reporter gene, the 4-kb HindIII/Sall fragment, containing the SMT1 intergenic region and first half of SMT1 from the BS/SMT1/H3, was subcloned into HindIII- and Sall-digested PZP212/GUS1 vector in which the EcoRI/HindIII T-DNA insert of pBI101.1 was subcloned into EcoRI/HindIII of PZP212. The Ac DNA used for a hybridization probe was the PstI insert in pAc3 (a gift from N. Federoff, Pennsylvania State University). For yeast expression, the SMT1 cDNA was removed from BS/cSMT1.13 with Xmal. We amplified ERG6 by PCR from yeast genomic DNA by using the primers AD421 (5'-CGAATTCGGGCGACCATAGATTGGAACCGG-3') and AD422 (5'-CGAATTCGGGCGGTTATATACCGTGCGCTT-3') and digested the product with Xmal. We then subcloned ERG6 or SMT1 cDNA into Xmal between the ADH1 promoter and terminator in the pAD4M 2-μm plasmid (with LEU2 marker) for yeast expression (Ballester et al., 1989).

Yeast Strains and Growth Conditions

Unless otherwise stated, yeast strains were grown in standard conditions and analyzed by established techniques (Guthrie and Fink, 1991). We created a 153-bp deletion and hemizygous for kanamycin resistance, the Smt phenotype segregated in the pAD4M 2-μm plasmid (with LEU2 marker) for yeast expression (Ballester et al., 1989).

smt1-3 Complementation with Transgenes

The smt1 mutation is tightly linked to the hygromycin resistance marker from the transgene that introduced the Ac element, which we confirmed in the smt1-3 backcross F2 population. Both transgenes made for complementation-conferring kanamycin resistance to Columbia ecotype after transformation. Therefore, in crosses, we monitored the expression of smt1-3 and transgenes by assessing resistance to hygromycin (16 mg/L) and kanamycin (25 mg/L), respectively. In lines homozygous for hygromycin (smt1-3) resistance and hemizygous for kanamycin resistance, the Smt phenotype segregated with Smt· segregating with kanamycin resistance.

Staining of SMT1::β-Glucuronidase Transgenic Plants

After 3 weeks of growth on PNA plates, SMT1::β-glucuronidase (GUS) transgenic plants were submerged in GUS staining buffer plus 0.2 mg/L X-Gluc (Rose Scientific Ltd.), the tissues were infiltrated with staining buffer under vacuum and incubated overnight at 36°C (Jefferson et al., 1987). After rinses with water, we cleared the tissue with ethanol:acetic acid (9:1 [v/v]) and followed this with washes with 90, 70, and 50% diluted ethanol. The tissues were then left in 50% glycerol for later photography.

Light Microscopy

Whole plants were observed and photographed under an MSA Wild Heerbrugg (Leica, Englewood, CA) microscope equipped for photography. Siliques were fixed overnight in an ethanol:acetic acid (9:1) solution, rinsed in 90 and 70% ethanol, and cleared with chloral hydrate:glycerol:water (8:1:2 [v/v]) solution. The cleared siliques were opened on a microscope slide, and the seeds were teased out of the siliques. Embryos were visualized by using a Zeiss Axioshot (Carl Zeiss, Thornwood, NY) microscope equipped for photography.

Sterol Enzymology and Analysis

Expression of the SMT1 cSMT1.13 cDNA in Escherichia coli and subsequent biochemical analysis of protein extracts were performed as previously described (Nes et al., 1991, 1998a; Tong et al., 1997). The conditions of the experiments were as follows. A fixed concentration of [methyl-14C]BAM (50 μM) was added, along with various concentrations of cycloartenol (30, 25, 50, 75, 100, 150, and 200 μM) in Tween 80 (0.1% v/v), to a homogenate preparation of enzyme derived from E. coli, pH 7.5. Each incubation was performed at 30°C for 45 min. The protein concentration in each homogenate suspension was ~1.5 mg/mL. The SMT-generated product, 24-ethylidene lophenol, was established by HPLC radiocounting (C18-Whatman column eluted with methanol at ambient temperature), which indicated a single peak of radioactivity coinciding with a mass peak corresponding to a known specimen of 24-ethylidene lophenol. The combined nonsaponifiable lipid fraction from a preparative-scale incubation (300 assays) was diluted with 50 μg of 24-ethylidene lophenol to serve as carrier, and the sample was injected into a C18-Zorbax (Hewlett Packard, Palo Alto, CA) semipreparative column eluted with methanol. The HPLC conditions are not sufficiently sensitive to distinguish the geometry of the 24(28)-ethylidene bond. The sterols in the SMT1-expressing erg6 strain described above were analyzed as previously described (Nes et al., 1999). Three gas–liquid chromatographic peaks corresponded to 24-methylated sterols; based on their mass spectra, these were ergosterol, 24(28)-methylene-24,25-dihydrolanosterol, and 4,4-dimethylcholesta-8,14,24(28)-trienol. The structure of the sterols from Arabidopsis plants was established by comparison with authentic specimens (Guo et al., 1995).

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