Biosynthesis of Phytosterol Esters: Identification of a Sterol O-Acyltransferase in Arabidopsis[OA]

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Fatty acyl esters of phytosterols are a major form of sterol conjugates distributed in many parts of plants. In this study we report an Arabidopsis (Arabidopsis thaliana) gene, AtSAT1 (At3g51970), which encodes for a novel sterol O-acyltransferase. When expressed in yeast (Saccharomyces cerevisiae), AtSAT1 mediated production of sterol esters enriched with lanosterol. Enzyme property assessment using cell-free lysate of yeast expressing AtSAT1 suggested the enzyme preferred cycloartenol as acyl acceptor and saturated fatty acyl-Coenzyme A as acyl donor. Taking a transgenic approach, we showed that Arabidopsis seeds overexpressing AtSAT1 accumulated fatty acyl esters of cycloartenol, accompanied by substantial decreases in ester content of campesterol and β-sitosterol. Furthermore, fatty acid components of sterol esters from the transgenic lines were enriched with saturated and long-chain fatty acids. The enhanced AtSAT1 expression resulted in decreased level of free sterols, but the total sterol content in the transgenic seeds increased by up to 60% compared to that in wild type. We conclude that AtSAT1 mediates phytosterol ester biosynthesis, alternative to the route previously described for phospholipid-sterol acyltransferase, and provides the molecular basis for modification of phytosterol ester level in seeds.

Plant sterols, known generally as phytosterols, are integral components of the membrane lipid bilayer (Demel and De Kruyff, 1976; Schuler et al., 1991). Unlike animal systems in which cholesterol is most often the lone final product of sterol synthesis, each plant species has its own characteristic distribution of phytosterols, with the three most common phytosterols in nature being β-sitosterol, campesterol, and stigmasterol (Benveniste, 1986, 2004). In addition to free sterol form, phytosterols are also found in the form of conjugates, particularly fatty acyl sterol esters (SEs). SEs can be found in lipid bodies in the cytoplasm and are present at a substantial level in seeds. In canola (Brassica napus), for example, phytosterols constitute about 0.5% of seed oil (Sabir et al., 2003), 35% of which is in the form of SEs (Harker et al., 2003). The biochemical process of sterol acylation is believed to play a role in maintaining the free sterol content of cell membranes at their physiological levels (Schaller, 2004).

The effectiveness of phytosterols as a dietary component to lower serum cholesterol level in humans has been well documented in medical research for more than half a century (Moghadasian et al., 1997, 1999; Hendriks et al., 1999; Sierksma et al., 1999; Piironen et al., 2000; van Rensburg et al., 2000; Awad et al., 2001, 2003; Bouic, 2001). It also has been suggested that the average diet of the western world has a phytosterol intake far below prehistoric levels (Yankah and Jone, 2001). These studies led to recommendations of consumption of phytosterol-enriched food products by the general population and particularly mildly hypercholesterolemic subjects. However, limited quantities of phytosterols are currently a major barrier in satisfying the demands for such functional foods. Based on a firmly established concept that 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) plays a rate-limiting role in the flux control of sterol biosynthesis (Bach, 1986; Gondet et al., 1992, 1994; Chappell et al., 1995), efforts aimed at increasing phytosterol content have been concentrated on enhancing the activity of HMGR through introducing modified HMGR that is resistant to regulation (Chappell et al., 1995; Re et al., 1995; Schaller et al., 1995; Harker et al., 2003; Hey et al., 2006). Nonetheless, evidence suggesting the existence of other regulatory step(s) in the flux control of sterol biosynthesis can be found in some studies, particularly that of Gondet et al. (1994) who studied a sterol-overproducing tobacco (Nicotiana tabacum) mutant LABI-4, with HMGR levels approximately 3-fold higher than normal (Gondet et al., 1994). LABI-4 had a 10-fold stimulation of sterol content in calli compared to wild type, mostly in the form of SEs, but a mere 3-fold stimulation in the regenerated leaves. Intriguingly, both type of tissues had the same stimulation factor of HMGR (3-fold). Therefore, there appears to be other
contributing factor(s), independent of HMGR, participating in regulating total sterol content.

Despite the ubiquitous presence of phytosterol esters in plant cells, the metabolic process of sterol acylation remains poorly understood. Sterol esterification is suggested to take place using several acyl donors including phospholipid, diacylglycerol, or triacylglycerol (Zimowski and Wojciechowski, 1981a, 1981b; Dyas and Goad, 1993). A recent report on the phospholipid:sterol O-acyltransferase gene from Arabidopsis (Arabidopsis thaliana; AtPSAT) established a route of transacylation between phospholipids and sterols in plant SE synthesis (Banas et al., 2005). AtPSAT is a lecithin:cholesterol acyltransferase protein family member homologous to human soluble lecithin:cholesterol acyltransferase. In this study, we took a functional complementation approach using a yeast (Saccharomyces cerevisiae) strain in which two sterol O-acyltransferase-encoding genes, ARE1 and ARE2, are disrupted. We identified a novel Arabidopsis sterol O-acyltransferase AtSAT1, which is structurally related to the acyl-CoA cholesterol acyltransferase (ACAT) in animal systems. We present evidence that overexpression of AtSAT1 alters SE synthesis in Arabidopsis, providing elevated levels of commercially desirable phytosterols.

RESULTS

Cloning of AtSAT1 from Arabidopsis

Taking a yeast functional complementation strategy (Bach and Benveniste, 1997), we focused our search of a plant sterol O-acyltransferase to the superfamily of membrane-bound O-acyltransferases (MBOATs; Hofmann, 2000), because the common biochemical functionality of the MBOAT family proteins is to mediate the transfer of organic acids in ester or thioester forms to hydroxyl groups of membrane-embedded acyl acceptors.

The yeast mutant SCY059 produces only a residual amount of SE because the two principle sterol O-acyltransferase genes, ARE1 and ARE2, were interrupted (Yang et al., 1996). Employing this strain, we introduced members of the Arabidopsis MBOAT family to test functional complementation. Fifteen cDNAs corresponding to MBOAT family candidate genes (listed in “Materials and Methods”) were cloned into the yeast expression vector pYES2.1 under the control of GAL1 promoter. The yeast transformants were subjected to GAL1 induction condition and harvested for neutral lipid analysis. When separated by thin-layer chromatography (TLC), SCY059 harboring At3g51970 produced an additional band in the neutral lipid extraction (Fig. 1A). HPLC analysis also revealed similar results from these cells (Fig. 1B). Both the TLC Rf value and the HPLC retention time of the novel products were consistent with that of SE.

To verify the identity of the product, the HPLC fraction was collected and subjected to alkaline hydrolysis (saponification). The saponification extract was derivatized with N,O-bis(trimethylsilyl)-trifluoroacetamide + 1% trimethylchlorosilane (BSTFA + 1% TMCS) and detected as trimethylsilyl (TMS) derivatives by gas chromatography (GC)-mass spectrometry (MS). GC profile revealed four major peaks from the sterol fraction (Fig. 2A). All the GC fractions were identified by searching the National Institute of Standards and Technology (NIST) 2.0 mass spectra library. The first peak, also present in the control strain, corresponded to squalene. The other three peaks displayed mass spectra identical to that of ergosterol, lanosterol, and 4,4-dimethyl-8,24-cholestadienol (also known as 4,4-dimethylzymosterol), respectively (Fig. 2A). The five peaks from the fatty acid fraction were found to be 14:0, 16:1, 16:0, 18:1, and 18:0 fatty acids (Fig. 2B).
These results confirmed that expression of At3g51970 conferred SCY059 an ability to produce SE and At3g51970 was then tentatively designated as AtSAT1.

AtSAT1 Is Structurally Related to Acyl-CoA Sterol Acyltransferases of Yeast and Animal Origins

AtSAT1 is predicted to be an integral membrane protein with eight transmembrane domains, and has a putative signal peptide with a cleavage site at the C terminus of AtSAT1. This conserved His residue was suggested to be a part of the active site (Hofmann, 2000). However, when compared with other ACATs, AtSAT1 protein is a shorter polypeptide, lacking the extended N-terminal region found in other sterol acyltransferases. Moreover, two conservative motifs, FYxWDWN and H/YSF previously suggested as being crucial for ACAT activity (Cao et al., 1996; Oelkers et al., 1998), were also absent in AtSAT1.

Heterologous Expression of AtSAT1 in Yeast Produces Mainly Lanosterol Esters

We estimated SE content of the yeast strains by quantifying sterols released through alkaline hydrolysis. As reported previously (Yang et al., 1996), the vector-only transformant of SCY059 still produced a trace amount of SE (0.027 μmol/g dry weight [DW] of yeast cells). The same strain heterologously expressing AtSAT1 increased SE production by 10-fold to 2.7 μmol/g DW, though still lower than 9.4 μmol/g DW of parental wild-type strain SCY062. The levels of free sterols in SCY059, SCY059/AtSAT1, and SCY062 were found at 18.1 μmol/g DW, 14.9 μmol/g DW, and 13.2 μmol/g DW, respectively.

Considering SCY059 still retained a low level of SE synthesis capacity, there was the possibility of AtSAT1 being a facilitator, enhancing the residual SE synthesis activity in the mutant strain. If this were true, SCY059 harboring AtSAT1 would likely produce SE with a profile of sterol or fatty acid moiety similar to that of the vector-alone transformant. This was experimentally proven untrue. As shown in Figure 4A, the molar composition of the fatty acid species saponified from the SE collected from the vector-only strain and the SEs produced by SCY059/AtSAT1 were different. Likewise, the sterol composition of the SE also displayed major differences due to the expression of AtSAT1 (Fig. 4B). The residual SE in SCY059/empty vector consisted of mainly ergosterol. On the other hand, expression of AtSAT1 resulted in production of SE containing chiefly lanosterol, which contributed to 88.1% of total sterol moiety. Thus, AtSAT1 appeared to preferentially acylate lanosterol when expressed in yeast.

Substrate Preference of AtSAT1

In light of substrate preferences of ACAT-related enzymes reported to date, we examined fatty acyl donor preferences of AtSAT1 using cell-free lysate of yeast with various fatty acyl-CoAs. Lipids extracted from in vitro reactions were separated by TLC and the incorporation of [3H]lanosterol into SE was measured. Because the cell-free lysate was expected to contain certain amount of acyl-CoA, reactions without added acyl-CoA were set as blank controls for enzyme activity calculation. As shown in Figure 5A, AtSAT1 had a substrate preference in the order of 16:0 > 18:0 >
16:1 > 18:1. 18:2 was found to negatively affect sterol O-acyltransferase activity.

We used stigmasterol, β-sitosterol, and cycloartenol as well as two sterols of yeast origin, lanosterol and ergosterol, to assess [14C]16:0-CoA acylation. Assessment of sterol substrate preferences entailed a methyl-β-cyclodextrin wash to extract free sterols from the preparations (Rodal et al., 1999), due to the concern that endogenous sterols may interfere with substrate specificity assessment. However, there apparently was still a substantial amount of endogenous sterols remaining, because the background value in the absence of a methyl-β-cyclodextrin wash was much higher than expected. Therefore, we used a specialized assay method to assess substrate specificity, as described by Rodal et al. (1999).

Figure 3. Sequence alignment of AtSAT1 with other reported sterol acyltransferases. Alignment was performed with CLUSTALV from the DNASTAR package run with default multiple alignment parameters (gap opening penalty: 10, gap extension penalty: 10). Accession numbers of proteins are: AtSAT1 (Arabidopsis, AAQ65159); HsACAT1 (Homo sapiens, NP_003092); HsACAT2 (H. sapiens, NP_003569); MmACAT1 (Mus musculus, Q61263); MmACAT2 (M. musculus, NP_666176); RnACAT1 (Rattus norvegicus, O70536); RnACAT2 (R. norvegicus, NP_714950); ScARE1 (yeast, P25628); and ScARE2 (yeast, P53629). Residues boxed in dashed or solid lines were previously reported as conserved functional motifs in ACATs. The black bar on the bottom marks a shared domain, and the arrow indicates the His likely to be an active-site residue (Hofmann, 2000).
of added sterol substrate was nonetheless high. We also attempted acetone extraction of the membrane fractions to reduce the endogenous sterol levels. Unfortunately the acetone treatment essentially eliminated the enzyme activity (data not shown). This led us to use reactions without exogenous sterol additions as control experiments. Under our assay conditions, the yeast strain harboring empty vector displayed a reduced 16:0 acylation activity in the presence of exogenous sterol. Addition of ergosterol to the yeast lysate expressing \( \text{AtSAT1} \) also gave rise to a negative value on SE formation. Such a negative effect of exogenous sterol addition on sterol \( \text{O-acyltransferase} \) activity was previously reported for animal ACAT (Tavani et al., 1982; Yang et al., 1997) and the Arabidopsis \( \text{AtPSAT1} \) assays (Banas´ et al., 2005). Despite these results, we could consistently detect SE synthesis activity with most of the sterol substrates when \( \text{AtSAT1} \) was expressed. The highest activity was found with cycloartenol, followed by \( \beta \)-sitosterol, lanosterol, and stigmasterol (Fig. 5B).

Because phosphatidylcholine (PC) has been previously implicated as an acyl donor for SE synthesis (Banás et al., 2005), the ability of \( \text{AtSAT1} \) to use this lipid as substrate was assessed using dipalmitoyl-PC with \( ^{14}\text{C} \) label in both the sn-1 and sn-2 fatty acyl moieties. We found that, while the PC could indeed sustain SE synthesis above the background of the control yeast strain, it was nonetheless at a level one-fiftieth of that when 16:0-CoA was provided. We also expressed \( \text{AtSAT1} \) in the \( \text{are1are2lor1dga1} \) quadruple yeast mutant devoid of triacylglycerol (Sandager et al., 2002). While the formation of triacylglycerols was not detectable, the quadruple yeast mutant expressing \( \text{AtSAT1} \) was still capable of producing SE. This result was significant in two respects: first, it suggested that \( \text{AtSAT1} \) did not require triacylglycerols (TAGs) as a fatty acyl donor for SE biosynthesis, and second, it could not acylate diacylglycerol to produce TAG.

### Expression Profile of \( \text{AtSAT1} \)

Based on transcript profile obtained from Genevestigator at [https://www.genevestigator.ethz.ch/](https://www.genevestigator.ethz.ch/), \( \text{AtSAT1} \) is expressed in all tissues examined, but particularly abundant in the elongation zone of roots followed by developing microspore and germinating seedlings.

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**Figure 4.** Fatty acid profile and sterol composition of SE in yeast strains. A, Mol % of fatty acid species released from SE of SCY059 expressing \( \text{AtSAT1} \) in comparison to that of the vector-only control and the parental strain (WT). B, Profiles of the sterol moieties of SE. The SE was saponified in methanolic-KOH for 2 h at 80°C. The fatty acids were transmethylated with methanolic HCl and free sterols were derivatized with BSTFA (1% TMCS).

**Figure 5.** Substrate preference assessment of \( \text{AtSAT1} \). A, Fatty acyl-CoA selectivity of \( \text{AtSAT1} \). Cell-free lysates were assayed for acylation of \( ^{1}\text{H} \)lanosterol. Fatty acyl-CoAs used for the assay were palmitoyl-CoA (16:0), palmitoleoyl-CoA (16:1), stearoyl-CoA (18:0), oleoyl-CoA (18:1), and linoleoyl-CoA (18:2). B, Sterol preference of \( \text{AtSAT1} \). Cell-free lysates were assessed for acylation of ergosterol, lanosterol, stigmasterol, \( \beta \)-sitosterol, and cycloartenol in the presence of \( ^{14}\text{C} \)palmitoyl-CoA. Reactions without exogenous sterol (or fatty acyl-CoA) served as control. Enzyme activity was calculated based on the difference of sterol acylation between reactions with added sterol (or fatty acyl-CoAs) substrates and reactions without sterols (or fatty acyl-CoAs). The negative values in some reactions were caused by high background sterol acylation in the control in the absence of exogenous sterol substrates.
Plant expression vector under the control of a seed-specific napin promoter (Josefsson et al., 1987). The transgenic plants showed no apparent developmental difference from wild-type plants. Real-time quantitative (qRT)-PCR revealed that \textit{AtSAT1} was approximately 20-fold overexpressed in developing siliques of most \textit{AtSAT1} transgenic lines (Fig. 6).

Seeds harvested from four wild-type plants and 12 transgenic plant (T1) lines, raised simultaneously in the same growth chamber, were selected for sterol analysis. \textit{AtSAT1} overexpression led to increases in SE by more than 2-fold and total sterol content by 64% in some transgenic lines (Table I). Overexpression of \textit{AtSAT1} in seed, as exemplified by one transgenic line shown in Fig. 7, also drastically altered the SE-sterol profile. In wild-type seeds, the two major SE species, $\beta$-sitosterol ester and campesterol ester, accounted for 80.4% and 17.5% of total SE, respectively. Cycloartenol ester was detected as a minor component at a distant third with 2.1%. Strikingly, the \textit{AtSAT1} transgenic seeds produced cycloartenol ester as the most prominent component, representing up to an average of 64.1% of total SE. On the other hand, the esters of $\beta$-sitosterol and campesterol were reduced to averages of 25.2% and 5.2%, respectively. We also found that 24-methylene cycloartenol was increased from non-detectable level in wild-type seeds to 5.6% in the transgenic line seeds. The enhanced SE overproduction also caused reduction of free sterol from 58.8% in wild type to 28.3% of total sterols in transgenic plants.

We further analyzed the profile of the fatty acid component of the SEs in seeds overexpressing \textit{AtSAT1} (Table II). In comparison to wild-type seeds, there was a drastic increase in the molar percentage of 16:0, from

![Table I.](http://www.plantphysiol.orgwarts/145/275/423/)

**Table I. Content of phytosterol esters and free sterols in wild-type and \textit{AtSAT1} transgenic seeds**

Arabidopsis (Columbia) plants were transformed with vector pSE129::\textit{AtSAT1}, in which \textit{AtSAT1} was under the control of a napin promoter. The four wild-type (WT) controls and 12 \textit{AtSAT1} overexpression (OE) lines were raised under identical conditions. Lipids were extracted in chloroform/methanol (2/1) from WT and transgenic seeds, followed by separation via normal-phase HPLC. SE fractions were collected and saponified in 7.5% methanolic KOH. Released free sterols were derivatized with BSTFA containing 1% TMCS and quantified by GC. The data were presented as averages of two analyses. Camp, Campesterol; Sito, sitosterol; Cyclo, cycloartenol; MethyCyclo, 24-methylene cycloartenol; nd, nondetectable.

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less than 15% to approaching 30%. Significant increases were also apparent in the composition of very long-chain fatty acids 20:0 and 20:1. The overall changes in fatty acid component of the SEs in the transgenic lines can be summarized as saturated and long-chain fatty acids replacing unsaturated fatty acids in wild-type seeds, particularly 18:2 and 18:3. The fatty acid composition of SE in AtSAT1-overexpressed seeds was consistent with the in vitro fatty-CoA specificity results.

DISCUSSION

In this study we identified AtSAT1 as a sterol O-acyltransferase from Arabidopsis that belongs to the MBOAT family. Our conclusion on the functional identity of AtSAT1 was based on the following lines of evidence: (1) heterologous expression of AtSAT1 enabled a yeast SE-deficient mutant SCY059 to synthesize a considerable amount of SE; (2) AtSAT1 mediated the synthesis of SE with sterol and fatty acid profiles distinctively different from that of the trace amount of SE synthesized in the yeast mutant strain; and (3) transgenic overexpression of AtSAT1 in Arabidopsis substantially increased SE content in seeds. Furthermore, since we did not find detectable levels of TAG when expressing AtSAT1 in the are1are2lro1dga1 quadruple yeast mutant, it appears that AtSAT1 could not acylate diacylglycerol for TAG biosynthesis.

Expression of AtSAT1 in yeast strain SCY059 resulted in the accumulation of SE consisting mainly of lanosterol, rather than the most abundant sterol being ergosterol, the end product of the sterol biosynthesis pathway in yeast (Zweytick et al., 2000), suggesting that AtSAT1 possesses a certain degree of sterol substrate specificity. The SE composition resulting from expression of AtSAT1 was clearly different from reports of overexpressing the ARE1 and ARE2 genes in SCY059, which produced SE enriched in zymosterol and ergosterol (Jensen-Pergakes et al., 2001). Thus, it can also be inferred that sterol preference of AtSAT1 is distinctively different from that of the yeast sterol O-acyltransferases. AtSAT1 also appeared to possess substrate preference toward fatty acyl donors, displaying the highest activity when saturated fatty acyl-CoAs (16:0 and 18:0) were provided. This is in contrast to the Arabidopsis phospholipid sterol O-acyltransferase AtPSAT, which discriminates against saturated fatty acids and was suggested to play a minor role in the synthesis of SE with saturated fatty acids (Banás et al., 2005). On this basis, it is important to note that previously published work has established that SE in some tissues of Brassica and Arabidopsis are highly enriched in saturated fatty acids (Hernández-Pinzón et al., 1999).

Data obtained from transgenic plants were generally in line with results of enzyme property assessment performed through heterologous expression of AtSAT1 in yeast. Consistent with the high enzyme activity detected with cycloartenol in our in vitro assays, enhanced AtSAT1 expression drastically advanced accumulation of cycloartenol esters in Arabidopsis seeds. Likewise, for the fatty acid components of the SEs, we observed increases in the molar ratio of saturated fatty acids, including 16:0, 18:0, and 20:0. Elevated sterol acylation with the long-chain monounsaturated eicosenoic acid was also evident. On the other hand, the polyunsaturated fatty acids were proportionally reduced.

Previous biochemical studies suggested that there are several potential acyl donors for SE biosynthesis in plants (Garcia and Mudd, 1978a, 1978b, 1978c; Zimowski and Wojciechowski, 1981a, 1981b), including acyl-CoA, phospholipids, diacylglycerol, and triglyceride. In the case of AtSAT1, triglyceride can be ruled out because TAG-deficient are1are2lro1dga1 quadruple yeast mutant expressing AtSAT1 produced SE. When PC was supplied as an acyl donor the detected sterol acylation was above residual level of the control, but far below that in the presence of acyl-CoA. It is unclear whether PC served as an immediate acyl donor or indirectly through mobilization of acyl group from PC to the fatty acyl-CoA pool. We suggest that AtSAT1 is an acyl-CoA:sterol acyltransferase. However, since the specific activity in our assay was lower than that of other reported ACATs (Macauley et al., 1986; Yang et al., 1997), and our in vitro assays with yeast cell lysate was complicated by a high background of steroid

Figure 7. GC profiles of sterols saponified from SE extracted from seeds of wild type (WT) and one representative AtSAT1 overexpression line. Lipids were extracted from seeds with chloroform:methanol (2:1, v/v) and separated through normal-phase HPLC. SE fractions were collected and saponified with 7.5% KOH in 95% methanol. The resulting free sterols were derivatized with BSTFA:pyridine (1:1, v/v). Identification of mass spectra and assignment of GC/MS peaks was carried out by a library search (NIST, version 2.0). 1, Cholesterol (internal standard); 2, campesteryl; 3, β-sitosterol; 4, cycloartenol; and 5, 24-methylene cycloartenol.
acylation, this supposition should be approached with caution. In light of the fact that AtSAT1 belongs to a large family of proteins in which some have promiscuous substrate preference, the substrate specificity of AtSAT1 requires further study.

SE has been long proposed to be the storage and transport form of sterols for their intracellular movement (Kemp et al., 1967; Janiszowska and Kasprzyk, 1977) and movement between tissues (Holmer et al., 1973). Radiolabeling experiments in Apium graveolens suspension cell culture indicated SE pool is under rapid turnover with esterification and hydrolysis process occurring concomitantly (Dyas and Goad, 1993). It was also proposed that esterification and hydrolysis should occur alternatively to newly synthesized sterol intermediates for orderly structural modification until end product 4-desmethyl sterols were formed. In our study, seed-specific overexpression of AtSAT1 in Arabidopsis resulted in the accumulation of cycloartenol esters, which otherwise occur only as a minor sterol component in wild-type seeds. Taken together with the in vitro sterol substrate preference results, the simplest explanation for such a metabolic outcome is that AtSAT1 selectively channeled cycloartenol into the ester pool (Fig. 8). Cycloartenol is the first tetracyclic sterol precursors in plant sterol biosynthesis pathway, and is considered an intermediate in the phytosterol biosynthesis pathway equivalent to lanosterol in yeast and animal systems (Benveniste 1986; Lovato et al., 2000; Kolesnikova et al., 2006; Suzuki et al., 2006). After cycloartenol, several major biochemical steps including methyltransferase, demethylation, and desaturation reactions need to take place before the final products of β-sitosterol and campesterol can be formed (Benveniste, 2004). The elevated expression of AtSAT1 could enhance the esterification of cycloartenol, thereby removing cycloartenol from the mainstream of sterol biosynthetic pathway into the formation of esters. This is consistent with our results in the transgenic seeds where the contents of β-sitosterol and campesterol, regardless if in the form of free or esterified forms, were reduced when compared to wild type. A question remains as to the physiological significance of such a role of AtSAT1 in plant metabolism and development. While highly speculative, we suggest that because cycloartenol has much better micellar solubility than the sterol end products, a role for AtSAT1 is to synthesize cycloartenol esters, which may serve as a significant form of transported sterol conjugates in certain developmental stages.

Incorporating phytosterols into the diet may be an effective approach to lower total and LDL cholesterol levels in humans, but free phytosterols are difficult to incorporate into commercial foods because of their low solubility. Fortunately, phytosterol esters can be dissolved in vegetable oil at a concentration 10 times higher than that of the free phytosterols. In this study, we found that overexpression of AtSAT1 could lead to enhanced total sterol content, primarily due to the increase in cycloartenol ester level. Cycloartenol is particularly enriched in oryzanol derived from rice (Oryza sativa) bran oil (Rukmini and Raghuram, 1991). It has been suggested that cycloartenol is the effective ingredient in rice bran oil for lowering plasma non-HDL cholesterol level (Wilson et al., 2007). We believe that the engineering outcome of the current research is likely to be repeated in crops species, particularly canola, offering a novel genetic engineering target for enhanced phytosterol content in vegetable oil. We must note, although the increase in seed oil SE content was significant, increase in total sterol content was still modest. Further studies on the regulation of phytosterol biosynthesis are clearly required to improve phytosterol content in seeds.

### Table II. Fatty acid composition of SEs in wild-type (WT) and AtSAT1 overexpression (OE) plants

WT and transgenic seeds were from the same lines as that in Table I. SE purified through HPLC was transmethylated in methanolic HCl. The resultant fatty acid methyl ester products were quantified by GC for fatty acid mole ratio calculation. The data were presented as averages of two analyses.

<table>
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<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
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The aqueous layer was neutralized with HCl and extracted twice with hexane to obtain free fatty acids. The fatty acid fraction was derivatized with BSTFA containing 1% TMCS and sterol derivatives were obtained in a mixture of BSTFA and pyridine (1:1, v/v) at room temperature for 1 h, and separated on a Hewlett-Packard 6890 GC with a capillary column DB-5 (Hewlett-Packard HP5, 35 m length × 0.25 mm diameter × 0.25 μm thickness). GC-MS analysis was accomplished using an Agilent 5973 mass selective detector coupled to an Agilent 6890 N gas chromatograph. The mass-selective detector was run under standard electron impact conditions (70 eV), scanning an effective mass-to-charge ratio range of 40 to 700 at 2.26 scans/s. The mass spectra were compared with entries in the NIST mass-spectral database, version 2.0.

**Quantitative Analysis of SE**

After HPLC separation of neutral lipid, SE fraction was collected, dried under N2 stream, and saponified in 7.5% KOH in 95% methanol. Separation and quantification of sterol species was performed on a Hewlett-Packard 6890 series GC with a capillary column DB-5 and flame ionization detector. The peaks of each sterol species were compared to the cholesterol (internal standard) peak to determine the amount of each sterol present. Fatty acid methyl esters were separated on GC with DB23 column. The quantification was performed in triplicate with three different batches of yeast cultures. The content of SE was expressed as micromoles of sterol moiety saponified from SE on a dry yeast cell weight basis.

**Sterol O-Acyltransferase Activity Assay Using Cell-Free Lysates**

AtSAT1 expression induction in yeast cells was performed according to manufacturer’s protocol for pYES2.1 TOPO TA expression kit (Invitrogen). The cells, resuspended in 1 mL cell wall-breaking buffer, were shaken vigorously in the presence of acid-washed glass beads. The resultant homogenate was centrifuged at 1,500 g for 5 min at 4°C. The supernatant was removed, aliquoted, and stored at −76°C. For sterol substrate specificity determination, the supernatant was washed twice with 80% methyl-β-cycloexetrin (Sigma) for removal of free sterols embedded in endoplasmic reticulum membrane (Rodal et al., 1999). Protein concentration was measured using Bio-Rad Protein Assay kit for final AtSAT1 activity calculation. Substrate specificity was determined by a previously described method (Yang et al., 1997) with slight modification. For fatty acid-CoA substrate specificity, a reaction mixture containing 200 μg micromolar protein, 10 μg unlabeled lanosterol, 200 μg bovine serum albumin, and 30 μg reduced glutathione in a final volume of 400 μL of 0.1 M potassium phosphate buffer, pH 7.4, was sonicated for 5 min. One microliter of [3H]lanosterol (1 μCi/μL, 10 mCi/mmol, American Radiolabeled Chemicals) was added and incubated at 30°C with 100 rpm shaking for 30 min to allow incorporation of sterols into the membrane. Sterols were suspended in reaction buffer with the help of Triton-X100 at a ratio of 30:1 (Tritus/sterol, w/v). The reaction was initiated by adding 50 mL 0.4 mM acyl-CoA (C16:0, C16:1, C18:0, C18:1, and C18:2, respectively). Reaction without exogenous acyl-CoA was used as control. The reaction mixture for sterol substrate preference determination was essentially the same as that for acyl-CoA specificity assays except that [14C]palmitoyl-CoA (50 mL, 10mCi/nmol, 180 μCi) was utilized to measure SE production. Reactions were terminated by the addition of 3 mL of chloroform/methanol (2:1, v/v). Cholesterol oleate was added as a carrier. The chloroform layer containing lipid was removed and dried under a N2 stream, redissolved in 100 μL chloroform, and then applied to a J.T. Baker S250 PTLC silica plate gel. After visualization with iodine, the bands corresponding to SE and cholesterol were quantitated. The specificity of AtSAT1 activity was determined as picomoles of acyl-CoA or lanosterol converted to SE per milligram protein per minute (pmol mg−1 min−1).

**Generation of Transgenic Plants**

A cDNA of AtSAT1 was PCR amplified with primers FP-Xhel (5’-CAAGAATCTAGATGGCAGTTCATCAACAGGCA-3’) and RP-KpnI (5’-GATCACCTCGATTACACACGGCAGGTTA-3’), and ligated into the Xhel
and *Knyt* sites of pSE129 vector. The *AtSAT1* sequence and the construct integrity were verified by sequencing. The resulting gene expression cassette in the transformation vector contained a canola (*Brassica napus*) seed-specific napin promoter and *Agrobacterium* NOS terminator flanking the 5’ and 3’ ends of the *AtSAT1* cDNA, respectively. A single colony of *Agrobacterium* (GV3101) carrying napin/*AtSAT1* transformation vector was cultured in Luria-Bertani medium containing 50 mg mL⁻¹ kanamycin and 25 mg mL⁻¹ gentamycin. Plant transformation was performed through vacuum infiltration. Selection of *T₅* transgenic seeds was carried on media containing one-half Murashige and Skoog, Gamborg medium, 0.8% (w/v) phytagar, 3% (w/v) Suc, 50 mg mL⁻¹ kanamycin, and 50 mg mL⁻¹ timentin.

**qRT-PCR and Data Analysis**

To quantify transcript levels, total RNA was extracted from siliques of wild-type and transgenic *T₅* plants using Qiagen NEasy Plant Mini kit. One microgram of DNase I-treated RNA was used as template for cDNA synthesis (Invitrogen superscript First-Strand Synthesis kit). qRT-PCR was performed on Applied Biosystems StepOne Real-Time PCR system in triplicates. The β-actin-8 gene (AT1G49240) was used as endogenous standard for expression level calculation. The cDNA samples were 50 times diluted for amplification of SYBR green-labeled PCR fragments (Power SYBR Green PCR Master mix, Applied Biosystems) by using gene-specific primers (forward 5’-CTTGA-GATCATCCTTGCCGCACAGC-3’ and reverse 5’-AGTGATGTCGCTATCGG-GATCATCCTTGCCGCACAGC-3’) for *AtSAT1*. The expression of β-actin-8 was assayed using forward primer 5’-AACACGACACCGGAAATTTGCTGAGA-3’ and reverse primer 5’-TGGAGGAGCTGGTTTTCGAGGT-3’. The corresponding amplicon size of β-actin-8 and *AtSAT1* are 101 and 99 bp, respectively. The cycling parameters included an initial DNA denaturation step at 95°C for 10 min, followed by 40 cycles of PCR with DNA denaturation at 95°C for 15 s, and primer annealing and extension at 60°C for 1 min. qRT-PCR reactions with RNA templates (minus RT) were used as controls to check for DNA contamination or amplification of nonspecific products. Controls with no added template were included for each primer pairs to ensure primer dimerization was not interfering with amplification detection. qRT-PCR results were captured and analyzed using software StepOne (version 1.0). The cycle number at which the fluorescence passed the cycle threshold (*Ct*), automatically determined by the software StepOne (version 1.0), was used for *AtSAT1* expression level quantitation. *AtSAT1* relative expression levels in each cDNA sample were obtained through normalization to β-actin-8 by the formula 2⁻ⁿδCt (ATSAT1 - Cβ-actin).

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