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Swedish University of Agricultural Sciences  
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# **Oxysterols inhibit plant Sterol Methyl Transferase type-1 enzyme activity *in vitro***

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**Independent project in Biology, 30 hp, EX0565**

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# **Oxysterols inhibit plant Sterol Methyl Transferase type-1 enzyme activity *in vitro***

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## **Abstract**

Sterols are important structural components of eukaryotic cell membranes, where they influence membrane fluidity and permeability. Sterols contain a four-ring structure with a hydrocarbon side-chain. The sterol composition and structure differs among plants, mammals and fungi. Mammals and fungi contain a single main sterol such as cholesterol or ergosterol, respectively. In contrast, plants contain a mixture of several sterols with varying side-chain length such as sitosterol, stigmasterol and 24-methyl cholesterol. Side-chain length is regulated by the two enzymes, Sterol methyl transferase type-1 (SMT1) and type-2 (SMT2) which increase side-chain length by a methyl (SMT1) or ethyl (SMT2) group. Thus, regulation of SMT enzyme activity is crucial for sterol composition in plants.

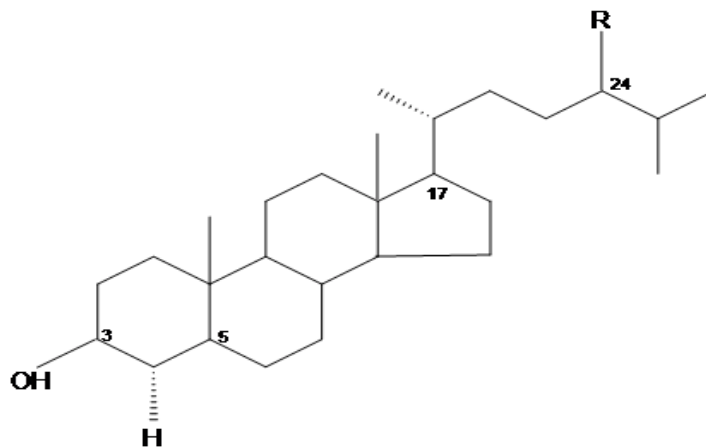
This project considers to what extent oxysterols, oxidized sterol forms, can regulate SMT1 activity. SMT1 activity was assayed in microsome preparations from three plant species, and different oxysterols were added to the enzyme reaction to investigate the effect on enzyme activity. Results showed that oxysterols inhibited SMT1 activity in all plant species tested. The inhibitory effect was stronger for side-chain oxysterols (24-OH and 25-OH cholesterol) than for ring hydroxylated ones ( $7\beta$ -OH cholesterol). The results suggest that oxysterols have an inhibitory effect on SMT1 activity in plants, and may be part of a feed-back regulation of sterol composition.

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## Introduction

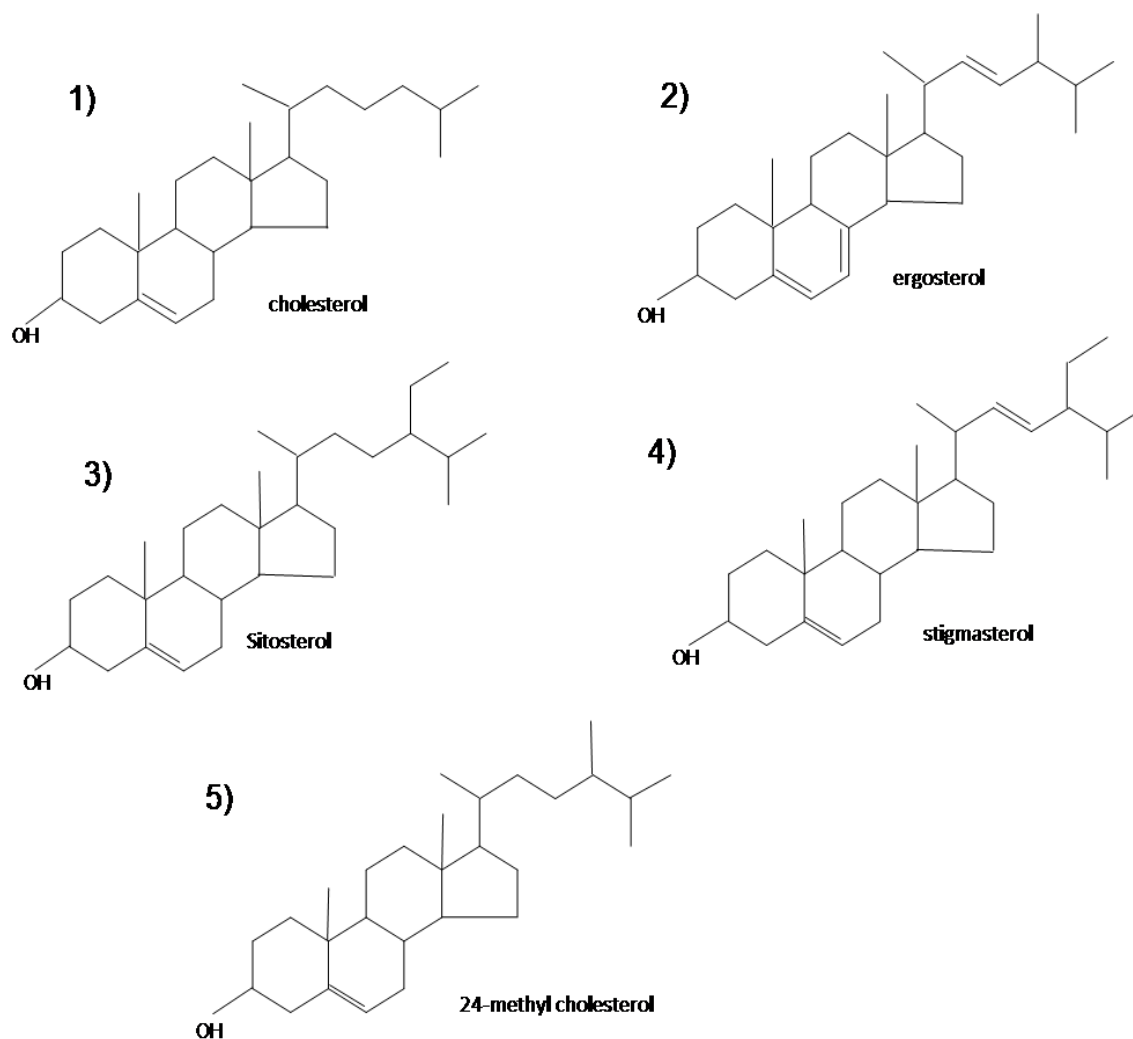
Sterols are essential compounds present as bulk lipids in all eukaryotic plasma membranes. Sterols influence important physical membrane properties such as fluidity and permeability. In addition, sterols may function as precursors to regulatory substances such as hormones, or alter the activity of certain regulatory proteins. A characteristic feature of functional sterols is a free 3 $\beta$ -hydroxyl group, a tetracyclic skeleton and an aliphatic side-chain with 8-10 carbon atoms (Benveniste 2004; Hartmann 1998) (Fig. 1).



**Figure 1.** General sterol structure with a 3 $\beta$ -hydroxyl group, a tetracyclic skeleton, and an aliphatic side chain with 8-10 carbon atoms. R denotes a hydrogen, methyl or ethyl group. In plants and yeast, the sterol C-24 side-chain extension is catalyzed by sterol methyl transferase enzyme(s).

While these functional properties seem to be conserved in the eukaryotic kingdom, the sterol structure is more diverse. For instance, the main sterol in mammals is cholesterol which has eight carbon atoms in its side-chain (C8), whereas the main sterol in fungi is ergosterol having nine carbon atoms (C9) (Fig. 2). Further, the sterol composition in plants is characterized by a complex mixture of C8, C9 and C10 sterols (Fig. 2). Common main sterols in plants are sitosterol (C10), stigmasterol (C10) and 24-methylcholesterol (C9), whereas cholesterol (C8) levels are usually low. Moreover, the sterol composition in plants differs between species and even tissues (Benveniste 1986).

Animals only synthesize C8 sterols but plants can synthesize C9 or C10 sterols by adding one or two methyl groups to the C-24 position in the sterol side-chain. These added methyl groups are the only carbons of sterols that do not originate from the acetate-mevalonate pathway.



**Figure 2.** Diversity of the main end-product sterols in the eukaryotic kingdom. Mammals mainly contain cholesterol (1), fungi have ergosterol (2) whereas plants have a complex mixture of sterols *e.g.* sitosterol, stigmasterol and 24-methyl cholesterol (3, 4 and 5).

In plants and fungi, the sterol side-chain length is regulated by two distinct enzymes, sterol methyl transferases. Sterol methyl transferases are membrane bound enzymes present in the endoplasmic reticulum or mitochondria (Schaller 2003). Plants have two types of sterol methyl transferases; sterol methyl transferase type-1 (SMT1) and sterol methyl transferase type-2 (SMT2). SMT1 is responsible for adding a methyl group to the cycloartenol side-chain and yields a methylated cycloartenol; 24-methylene cycloartenol. SMT2 methylates 24-methylene lophenol to 24-ethylidene lophenol (Bouvier-Nave 1998).

Oxysterols are oxidized derivatives of cholesterol with a hydroxyl group on the side-chain or ring structure (Russell 2000). Oxysterols are produced by action of cholesterol hydroxylases (CHs) or

non-enzymatically by reactive oxygen species (Björkhem 2002). Oxysterols have a signaling role in sterol homeostasis especially in mammals and yeast.

It has been shown that also plants have oxysterols such as  $7\alpha$ -hydroxyl and  $7\beta$ -hydroxyl of cholesterol, campesterol and sitosterol (Beste *et al.* 2011). Oxysterol composition in plants varies between species and tissues, as does the sterols. Compared to the homeostatic function of oxysterols in mammals, a corresponding biological activity in plants is not obvious. However, oxysterol production was recently associated with altered sterol metabolism, growth and development in *Arabidopsis* (Beste *et al.* 2011). In that study, mainly steps in sterol side-chain metabolism were affected by the presence of oxysterols. This included a decrease of C10 side-chain sterol, and an increase of C8 and C9, indicating that SMT activity may be influenced by the oxysterols.

Moreover, it has been shown that positive charges at position C-25 in the sterol side-chain or related nitrogen-containing compounds have an inhibitory effect on the SMT1 enzyme (Nes 2000). This brings into a question if side-chain oxysterols may have a similar inhibitory effect as the side-chain modifications by nitrogen groups or a positive charge.

To gain more knowledge about sterol methyl transferase enzyme and its regulation, SMT1 activity from *Arabidopsis* (*Arabidopsis thaliana*), potato (*Solanum tuberosum*) and sunflower (*Helianthus annuus*) was analyzed with or without oxysterols in the reaction mixture. Results show that oxysterols such as 24-OH or 25-OH cholesterol decrease the sterol methyl transferase activity.

## Aims

- 1) Characterize how oxysterols influence SMT1 activity
- 2) Characterize *SMT1* gene expression
- 3) Gain general knowledge about SMT1 regulation

## Material and methods

### *Plant materials*

The plant species used for SMT1 activity assays were sunflower (*H. annuus*) cultivar 'Sanbro', potato (*S. tuberosum*) cultivar 'King Edward' and Arabidopsis (*A. thaliana*) ecotype 'Columbia'. The leaves of two week-old plants grown in green house were used to prepare microsomes for SMT1 assays. Transgenic CH24 and CH25 Arabidopsis lines were those described by Beste *et al.* (2011).

### *Microsome preparation from plant leaves*

All steps of microsome preparations were performed in a cold room (4°C) with ice-cold solutions.

Microsomal fractions were isolated from 5 g of young expanding leaves by homogenization on ice using mortar and pestle in a homogenization buffer (100 mM potassium phosphate, 0.3 M mannitol, 1 mM EDTA, 4 mM MgCl<sub>2</sub>, 0.1 % bovine serum albumin, and 10 mM β-mercaptoethanol, pH 7.5). The homogenate was filtered through a miracloth sheet and centrifuged at 1,000 g for 5 min (Sorvall SS34), The supernatant was transferred to a new centrifuge tube and centrifuged at 10,000 g for another 5 min. A microsomal pellet was obtained by ultracentrifugation at 140,000 g for 90 min (Beckmann RPS55T). The pellet was homogenized in a resuspension buffer (100 mM potassium phosphate buffer, 20 % glycerol, 1 mM EDTA, 4 mM MgCl<sub>2</sub> and 1 mM reduced glutathione pH 7.5) and stored at -70 °C before analyzing enzyme activity, as described (Sitbon and Jonsson 2001).

Protein content was measured using the Bradford method and with bovine serum albumin as standard.

### *SMT1 enzymatic assay*

The natural SMT1 substrate cycloartenol (CA) was used at a concentration of 125 μM. Different sterols (cholesterol and sitosterol) and oxysterols (7β-OH cholesterol, 24-OH cholesterol and 25-OH-cholesterol) were added (or not) at various concentrations to the substrate, and 0.26 μM *S*-adenosyl-1-(methyl-<sup>14</sup>C) Met (Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, UK) was added as a methyl donor. The standard assay used a molar ratio of 1:1 between CA and the sterols/oxysterols. The mixture was incubated in a water-bath at 30°C for 90 min. The reaction



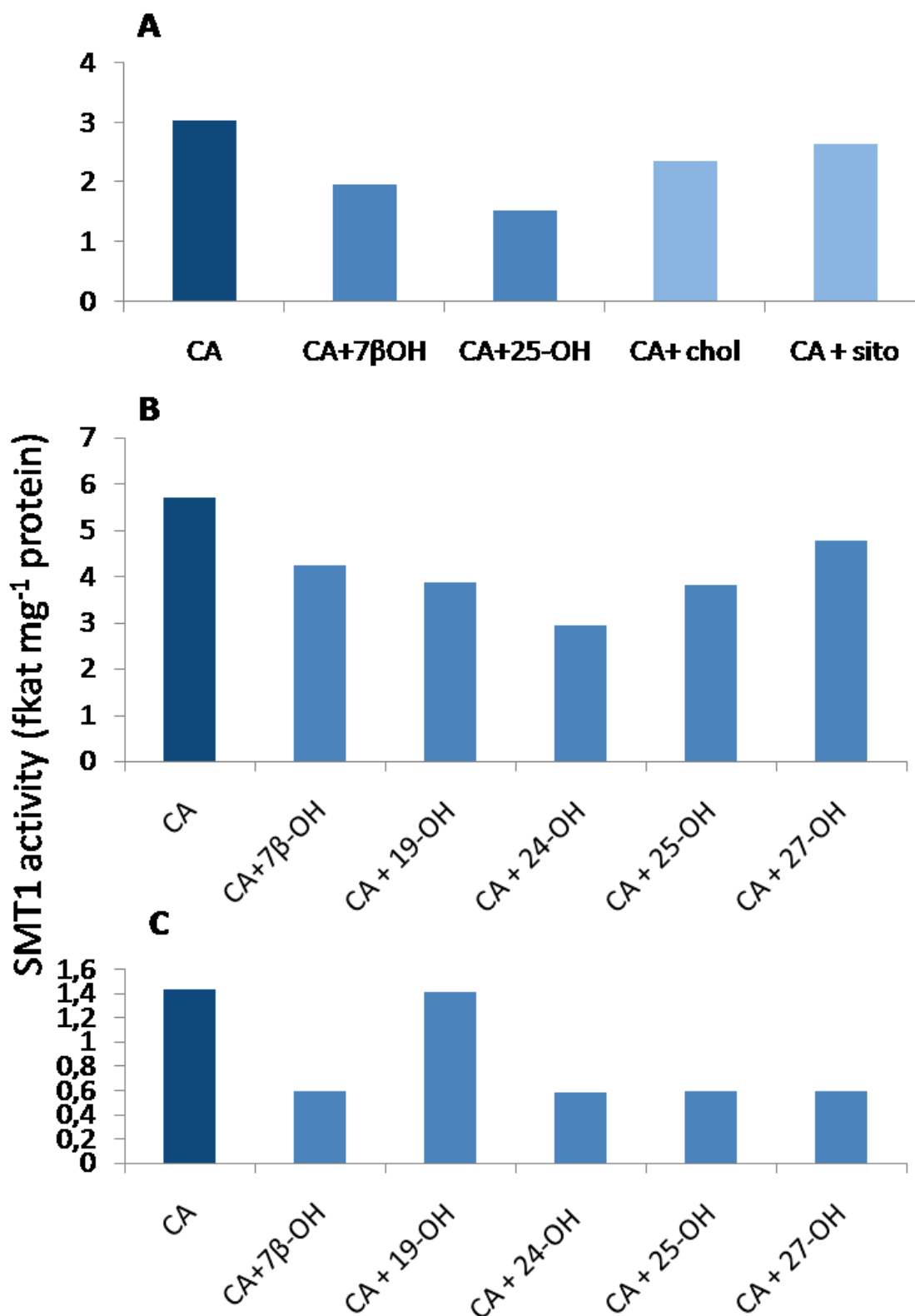
was terminated by addition of 100  $\mu$ l of 2 M potassium hydroxide. A mixture of sterol carriers (lanosterol and cholesterol) was added before extracting sterols with *n*-hexane. Sterols were separated by two TLC runs using dichloromethane as the developing solvent, visualized by berberine staining, and fractions of di-methyl sterols (SMT1 activity) were scraped directly into a liquid scintillation cocktail (Arnqvist *et al.* 2003). Radioactivity was determined by liquid scintillation. Radioactivity counts per minute (CPM) was converted to enzyme activity by considering the total substrate concentration in the assay, and expressed as  $\mu$ kat  $\text{mg}^{-1}$  protein. Protein quantification was done using the Bradford assay (BioRad) and a serial dilution of bovine serum albumin as the reference.

Genevestigator database ([www.genevestigator.com](http://www.genevestigator.com)) was used to study SMT1 transcript regulation.

## Results

### *Oxysterols inhibit SMT1 enzyme activity in plant microsome preparations*

The influence of different oxysterols on SMT1 activity with the native substrate CA was assayed in microsome preparations from three plant species belonging to different families; potato (Solanaceae), sunflower (Asteraceae) and Arabidopsis (Brassicaceae). Among the oxysterols tested were sterols hydroxylated in the A-ring (7 $\beta$ -OH cholesterol), and in the side-chain (19-OH, 24-OH, 25-OH, 27-OH cholesterol). Addition of oxysterols to the standard SMT1 assay in potato microsomes showed an inhibitory effect in potato microsomes, the strongest one being for 25-OH cholesterol, whereas non-hydroxylated sterols (cholesterol, sitosterol) were less active in this aspect (Fig. 3A).

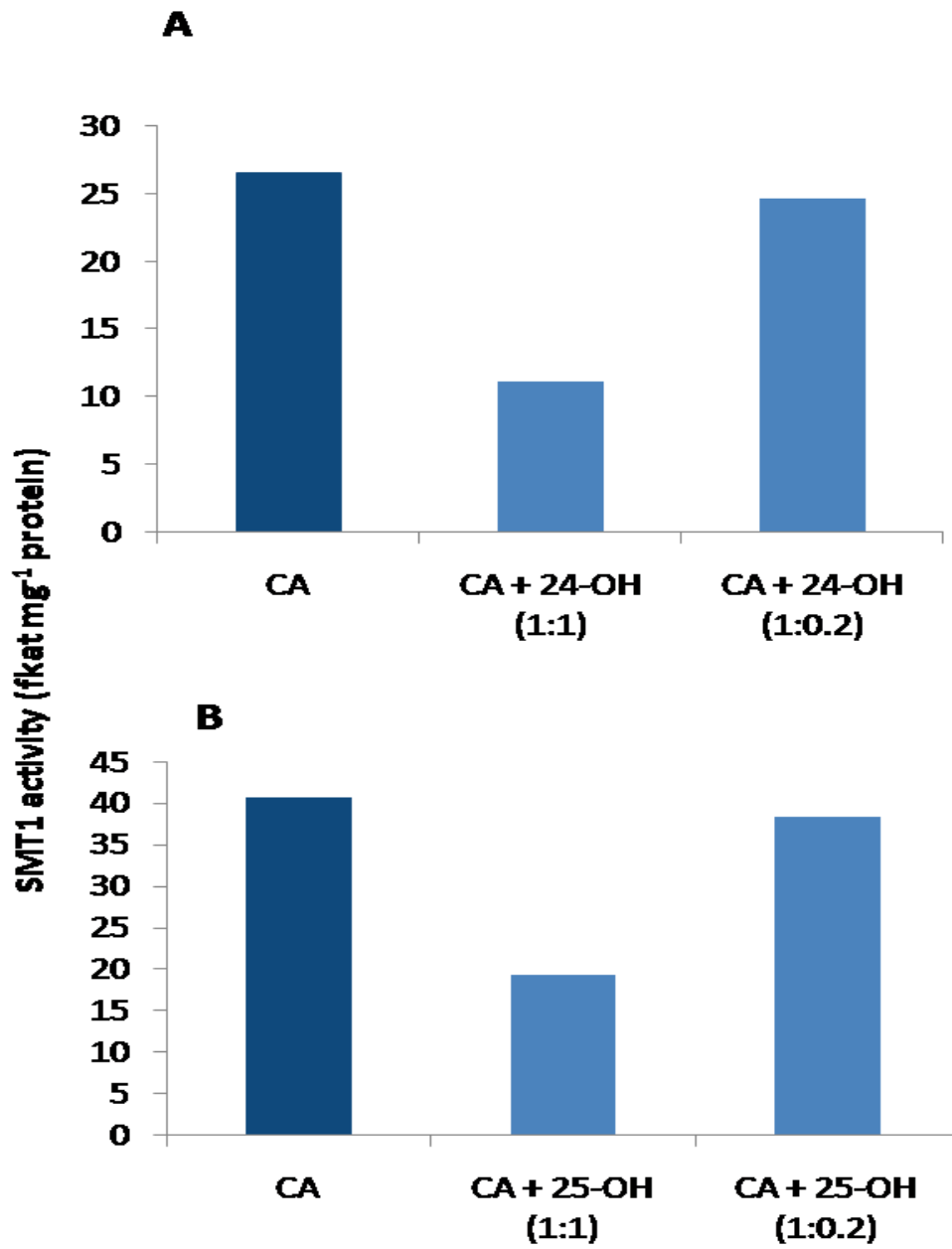


**Figure 3.** Oxysterols inhibit SMT1 activity *in vitro*. SMT1 enzyme activity was assayed in potato (A), sunflower (B) and Arabidopsis (C) microsomes preparations with the natural substrate cycloartenol (CA), with or without the addition of different oxysterols (7 $\beta$ -OH, 19-OH, 24-OH, 25-OH and 27-OH cholesterol), or sterols (cholesterol and sitosterol), using a molar ratio of 1:1 between CA and oxysterols or sterols.

Likewise, an inhibitory effect of oxysterols was observed in sunflower (Fig.3B) and Arabidopsis (Fig. 3C). Collectively, the results show that 24-OH and 25-OH cholesterol have the strongest inhibitory effect on SMT1 activity.

### *The inhibitory effect of oxysterols is concentration-dependent*

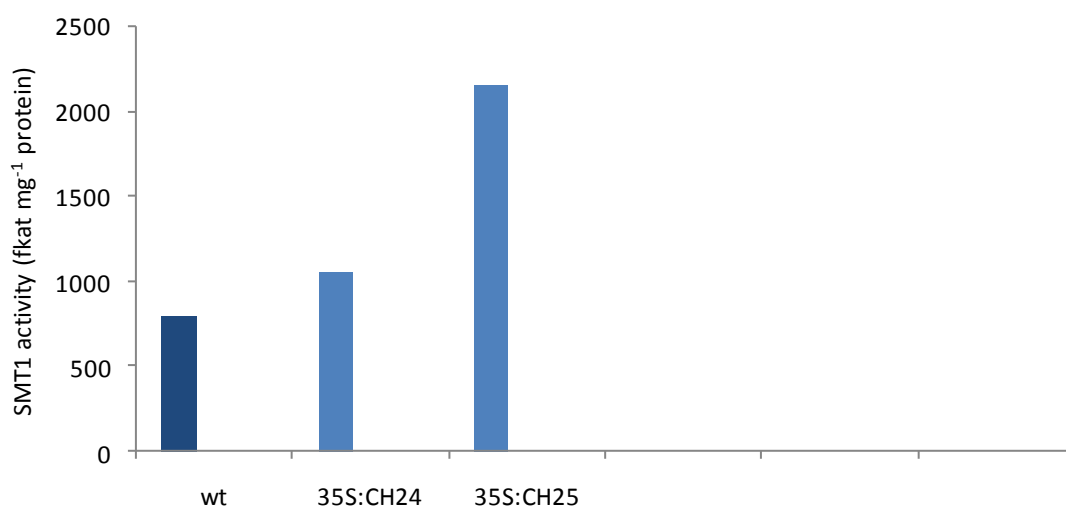
To test the specificity of oxysterol inhibition, oxysterols were added at different concentrations to SMT1 assays in sunflower microsomes. For both 24-OH cholesterol and 25-OH cholesterol, the inhibition was greater at a CA:oxysterol ratio of 1:1 than at 1:0.2 (Fig. 4). At a CA:oxysterol ratio of 1:1, SMT1 activity was about 40 % of the activity with CA only, whereas the activity was 95 % when the proportion of CA was increased (1:0.2) (Fig. 4A). A similar result was obtained with 25-OH cholesterol (Fig. 4B). This suggests that the inhibitory effect of both 24-OH and 25-OH cholesterol was specific for the oxysterols, and not related to other components in the mixture, such as substances used to solubilize oxysterols.



**Figure 4.** *Inhibitory effect of oxysterols is concentration-dependent.* SMT1 activity was assayed in sunflower microsomes with the natural substrate cycloartenol (CA) and different concentrations of the oxysterols 24-OH cholesterol (A) and 25-OH cholesterol (B).

## *SMT1 enzyme activity in transgenic oxysterol-producing Arabidopsis plants*

To investigate if oxysterols inhibit SMT1 activity also under *in vivo* conditions, SMT1 activity was assayed in microsomes prepared from transgenic Arabidopsis plants overproducing 24-OH and 25-OH cholesterol from expression of mouse cDNA encoding the cholesterol hydroxylases CH24 (CYP46A1, AF094479) and CH25 (Ch25h, NM\_009890) expressed from the cauliflower mosaic virus 35S promoter. Oxysterol analysis of transgenic plants showed that they have a higher level of 24-OH and 25-OH cholesterol compared to the undetectable levels wild type (Beste *et al.* 2011). Unexpectedly, SMT1 activity in microsomes prepared from transgenic plants was increased about 32 % for CH24 and 171 % for CH25 compared to the wild type (Fig. 5).



**Figure 5.** Increased SMT1 activity in oxysterol-overproducing transgenic plants. Enzymatic activity of SMT1 in microsome preparations from wild type Arabidopsis plants and CH24 and CH25 transgenic oxysterol-overproducing Arabidopsis plants.

## *SMT1 gene regulation*

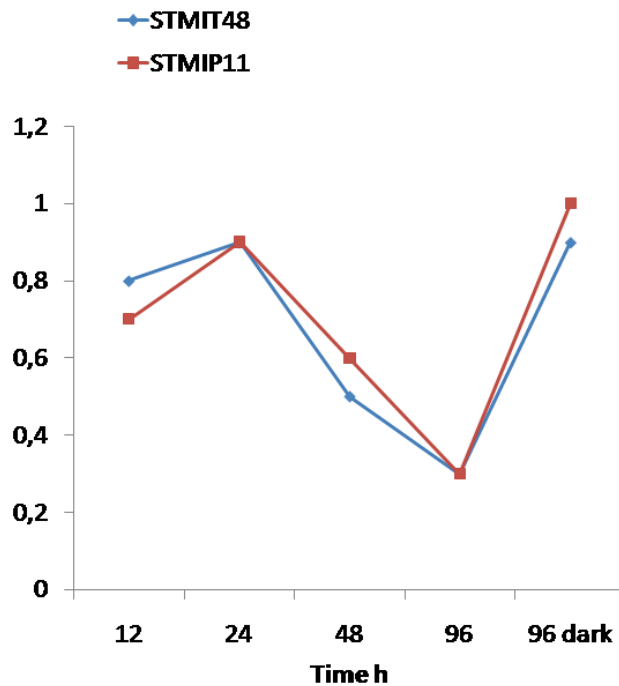
The SMT1 activity might also be regulated at the gene expression level. To investigate this possibility, the Genevestigator database was mined for treatments that would significantly alter *SMT1* expression in Arabidopsis. This showed that some factors may have a regulatory effect on *SMT1* expression. For instance, drought, darkness at 4°C and syringolin showed lower *SMT1* transcript levels suggesting downregulated gene expression. On the other hand, hypoxia,

germination (48 h) and stratification (48 h) can upregulate the gene expression. Several factors, *e.g.* ozon, potassium chloride or indole-3-acetic acid (IAA), do not seem to have any effect on *SMT1* gene expression (Table 1).

**Table 1.** *SMT1* gene expression in Arabidopsis. Data retrieved from Genevestigator. Cut-off values were +100% or -50 % relative to controls.

<b>Inhibition</b>	Drought (- 70 %)	Darkness (- 50 %)	Syringolin (- 70 %)
<b>Neutral</b>	Ozon	KCl	IAA
<b>Stimulation</b>	Hypoxia (+ 200 %)	Germination (48 h) (+ 300 %)	Stratification (48 h) (+ 150 %)

The light response was investigated also in a potato tuber microarray study (Nurun *et al.* submitted). This showed a slight reduction compared to the controls (Fig. 6), well in line with increased cholesterol levels reported in the study.



**Figure 6.** Microarray SMT1 expression in potato 'King Edward' during light exposure. Effects at different time points are presented as relative expression levels.

## Discussion

It has been shown that mammals have oxysterols that are formed from cholesterol by action of specific cholesterol hydroxylases. For instance, in mouse the cholesterol hydroxylases can hydroxylate cholesterol at C-7, C-24, C-25 and C-27. One function of these hydroxyl cholesterol in mammals is in sterol homeostasis (Russell 2000). Specific oxysterols bind to a nuclear hormone receptor family called Liver X receptors (LXRs). LXR dimerizes with retinoic X receptor and the dimer binds to specific response elements of target genes in cholesterol homeostasis (Cummins 2006). Oxysterol signaling is also mediated by oxysterol-binding proteins (OSBPs). OSBPs can bind to oxysterols with high specificity and affect sterol homeostasis, although the mechanism is unknown (Olkkonen and Levine 2004).

König & Seifert showed that plants contain oxysterols as well (König and Seifert 1998).  $7\alpha$ -Hydroxy,  $7\beta$ -hydroxy and 7-keto forms of campesterol, sitosterol and stigmasterol were identified

in barley roots (Köning and Seifert 1998). Recently,  $\alpha$  and  $\beta$  forms of 7-hydroxycholesterol, 7-hydroxy-24methylcholesterol, 7-hydroxycampesterol and 7-hydroxysitosterol were demonstrated in *Arabidopsis* (Beste *et al.* 2011). Together this strongly suggests that oxysterols are natural sterol metabolites in plants. However, a role in sterol homeostasis has not been reported. Compared to what is known about regulation of sterol homeostasis in mammals, there is much less known about this process in plants, and no orthologues to the sterol homeostasis machinery in mammals have been found in plants (Beste *et al.* 2011). One possibility of sterol regulation, might be to influence the activity of key sterol-biosynthetic enzymes, and the present study was undertaken to investigate this possibility.

Plant SMT1 enzyme activity is regulated by a number of substances (Nes 2000). Charged aziridine steroid compounds and sulfur derivatives can inhibit SMT1 enzyme activity, especially if the charges are located on C25 in the side-chain of steroids. Aziridine can bind with its nitrogen atom to steroid carbons and form a new compound which the SMT enzyme cannot bind to, thus inhibiting SMT activity. Sulfur and ammonium compounds act in the same way as aziridine in inhibiting SMT enzyme activity (Kanagasabai *et al.* 2004). Certain steroid alkylations, like C26 or C27 methylation can inactivate the enzyme as well (Song and Nes 2007).

In the present study, the enzyme activity measurements showed that 25-OH cholesterol, 7 $\beta$ -OH cholesterol, sitosterol and cholesterol all decreased the SMT1 enzyme activity to a certain extent in microsomes prepared from three different plant species (Fig. 3). Comparing the effect of oxysterols, 25-OH and 24-OH cholesterol caused the strongest inhibition of SMT1 enzyme activity, showing up to a 50 % reduction of enzyme activity (Fig. 3). These oxysterols had the strongest inhibitory effect among the ones tested, possibly because they have an OH group in their side-chain close to C-25 position where the SMT1 enzyme will add a methyl group. Thus the OH group may occupy a space that prevents SMT enzyme activity. The inhibitory effect of unhydroxylated sterols (cholesterol and sitosterol) was not as strong as that of 25-OH cholesterol. At a CA:oxysterol ratio of 1:1 SMT1 activity was about 40 % of the activity with CA only, whereas the activity was 95 % when the concentration of CA was increased (1:0.2) (Fig. 4). This suggests that the inhibitory effect of both 24-OH and 25-OH was concentration dependent.

To study the inhibitory effect of oxysterols *in vivo*, transgenic plants were generated that can make 24-OH and 25-OH cholesterol. However, the SMT1 activity in these plants was quite opposite of what was observed *in vitro* (Fig. 5). The reason for this is not known. One explanation may be that oxysterols do have an inhibitory effect on SMT1 enzyme activity. Thus the enzyme activity in transgenic plants is inhibited compared to the wild type. To compensate for the reduced SMT1 enzyme activity in transgenic plants, the plants produce more SMT1 enzyme compared to the wild type. When the microsome fraction is prepared, the oxysterols may be lost from the microsomes due to their higher polarity. This means that the microsomes will have increased SMT1 enzyme levels, but lack compounds that inhibit the enzyme. Hence, when the enzyme activity is measured, it will be detected as an apparent increase compared to the wild type.



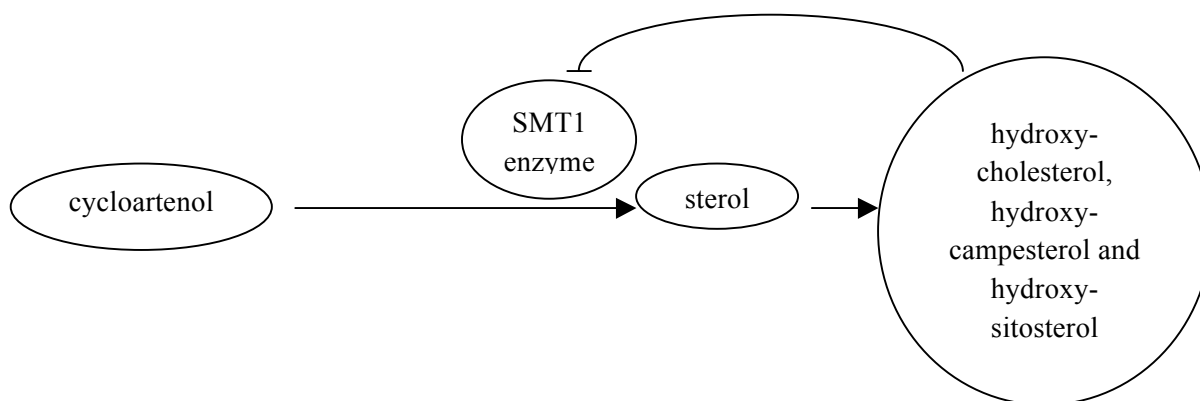
Some factors have a regulatory effect on SMT1 activity at the gene expression level. For instance, drought lowers sterol and lipid levels in plant cells. The lipid decrease could reflect a need for down-regulated lipid biosynthesis that leads to a greater stability of cell membranes (Yordanov *et al.* 2000). Reduced lipid amounts under drought conditions are well-matched with inhibitory effect of darkness on *SMT1* transcript levels (Table 1). Another factor that downregulates *SMT1* transcript levels is light. Plants chloroplast control the degree of CO<sub>2</sub> incorporated into sterols. In darkness the amount of chloroplast will decrease and as a consequence the CO<sub>2</sub> incorporation into sterols will decrease as well. Moreover, it has been shown that plants sterols during light exposure are mostly sitosterol and campesterol whereas during dark exposure it is cholesterol (Grunwald 1975). This could be due to the downregulated *SMT1* enzyme activity during darkness (Grunwald 1975). These studies agreed well with databases searches and *SMT1* expression (Table 1) and (Fig. 6). This hypothesis could be tested by hydroxysterol analysis of microsomes fractions, together with a western blot analysis of *SMT1* protein levels using *SMT1*-specific antibodies.

It is not clear how hypoxia might upregulate *SMT1* expression in plants. However, the effect of hypoxia on sterol metabolism has been studied in mammals and fungi. This has shown that mammals and fungi have an adapted gene for hypoxia conditions. Sterol regulatory element-binding proteins (SREBPs) in the yeast *Schizosaccharomyces pombe* has been shown to adapt cells to hypoxia conditions. SREBPs are highly conserved membrane bound transcription factors found in mammals and fungi as a regulatory factor for sterol and lipid metabolism. SREBP (Sre1) and sterol cleavage activating protein (SCAP, Scp1), act together to measure the oxygen level. Sre1 has been adapted to hypoxia and it is induced more than two times in hypoxia condition (Willger 2008). Thus under hypoxia conditions SREBPs are induced and that lead to increased sterol synthesis. However, SREBPs are lacking in plants but it cannot be excluded that other proteins fulfill a similar function.

Another factor that stimulates *SMT1* gene expression is germination. During germination sterol compositions in plants changes (Bush and Grunwald 1972). Stigmasterol, campesterol and sitosterol increase during germination (Bush and Grunwald 1972) indicating that *SMT* enzyme activity is increased. This studies are compatible with the database results in table 1.

## Conclusion

SMT1 is an important enzyme in plants, but there is at present little information about its regulation at protein and RNA levels. Based on results shown in this study, I speculate that oxysterols can regulate the SMT1 enzyme activity. Once the amount of sterols is increased, oxysterols will increase as well either from sterol oxidation by molecular oxygen or from action of unknown enzymes, *e.g.* CH-like CYPs. Increased oxysterols have an inhibitory effect on SMT1 enzyme activity and down-regulate its activity, leading to less sterol produced and/or an altered sterol composition with an increase in the proportion of cholesterol (Fig 7). This may be particularly relevant for plant species using cholesterol as a precursor for defense substances, *e.g.* glycoalkaloids in potato.



**Figure 7.** Model for feed-back regulation of SMT1 enzyme activity. When the amount of oxysterols is increased it will have a negative feed-back on SMT1 enzyme activity.

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