Plant Sterol Metabolism with Emphasis on Glycoalkaloid Biosynthesis in Potato

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Abstract

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Glycoalkaloids are secondary metabolites present within species of the family Solanaceae. They are toxic to humans and animals, and it is important to keep the levels in potato (*Solanum tuberosum* L.) tubers below the safe limit for consumption. Glycoalkaloids are derived from sterols, which are important components of cell membranes. The sterol composition in plants is complex, and varies between species. For instance, cholesterol is a minor sterol in most plants, but a major one in the Solanaceae. Cholesterol has been suggested as a metabolic precursor to the glycoalkaloids in potato, although the biosynthetic pathway is largely unknown.

To investigate the role of sterols in glycoalkaloid biosynthesis, potato plants overexpressing a type 1 sterol methyltransferase (SMT1) were generated. *SMT1* plants displayed an increased level of alkylated sterols, while the level of the non-alkylated sterol cholesterol was decreased. Along with this there was a reduction of glycoalkaloid levels. To gain more insight into the sterol biosynthesis in plants, Arabidopsis lines were generated overexpressing *CYP710A1* and *CYP710A4*, encoding enzymes potentially involved in stigmasterol synthesis. Both transformants contained increased levels of stigmasterol and a decrease in the level of sitosterol. *CYP710A1* transformants also displayed increased levels of esterified sterols, suggesting that an increased stigmasterol level alone is sufficient to stimulate esterification of other sterols. The possibility of downregulating cholesterol and glycoalkaloid levels by increasing cholesterol catabolism was investigated in plants by expression of four mouse cDNAs encoding enzymes hydroxylating cholesterol. In Arabidopsis, plant growth and sterol/steroid levels were altered, indicating that the introduced hydroxysterol synthesis affected regulatory steps in steroid homeostasis. However, an increased level of hydroxylated cholesterol in potato had no effect on glycoalkaloid levels. A sterol Δ^{24}-reductase was downregulated in transgenic potato. Transformants displayed increased levels of Δ^{24}-sterols, while the levels of 24-saturated sterols such as cholesterol were decreased, as was their glycoalkaloid level. This reveals a new role for this type of enzymes in plant steroid metabolism.

Taken together, the results show that both cholesterol and glycoalkaloid biosynthesis can be downregulated in transgenic plants, and support the view of cholesterol as a metabolic precursor in glycoalkaloid biosynthesis.

Keywords: plant sterol, glycoalkaloid, *Solanum tuberosum*, *Arabidopsis thaliana*, secondary metabolism

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Dedikerad till Fredrick, Filippa och Fanny.

Abbreviations

BL	brassinolide
BLAST	basic local alignment search tool
Caco-2	human Caucasian colonic adenocarcinoma
CaMV	cauliflower mosaic virus
CAS	cycloartenol synthase
CH	cholesterol hydroxylase
CYP	cytochrome P450
FAD	flavine adenine dinucleotide
FPP	farnesyl diphosphate
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
HMGR	3-hydroxy-3-methylglutaryl coenzyme A reductase
HPLC	high-performance liquid chromatography
LCAT	lecitin:cholesterol acyltransferase
LDL	low-density lipoprotein
PSAT	phospholipid:sterol acyltransferase
RNAi	RNA interference
RT-PCR	reverse transcriptase PCR
SMT	sterol methyltransferase
TGA	total glycoalkaloids
TMS	trimethylsilyl
TLC	thin layer chromatography

Contents

Appendix

Paper I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Arnqvist, L., Dutta, P.C., Jonsson, L., Sitbon, F. 2003. Reduction of cholesterol and glycoalkaloid levels in transgenic potato plants by overexpression of a type 1 sterol methyltransferase cDNA. *Plant Physiol*. 131, 1792-1799.

II. Arnqvist, L., Persson, M., Jonsson, L., Dutta, P., Sitbon, F. 2007. Overexpression of CYP710A1 and CYP710A4 in transgenic *Arabidopsis* plants increases the level of stigmasterol at the expense of sitosterol. *Planta* DOI 10.1007/s00425-007-0618-8.

III. Arnqvist, L., Nahar, N., Dalman, K., Jonsson, L., Dutta, P., Sitbon, F. Synthesis of hydroxylated sterols in transgenic Arabidopsis plants alters growth and steroid metabolism. (manuscript).

IV. Arnqvist, L., Dalman, K., Jonsson, L., Dutta, P., Sitbon, F. Decreased glycoalkaloid level in transgenic potato plants by antisense expression of a sterol Δ^{24} -reductase. (manuscript).

Introduction

Importance of sterols

Early sterol research

Sterol research dates back to the year 1733 when glistening crystals from an alcoholic solution of gall stones was observed by Vallisneri. The work of Poulletier de la Salle, around year 1769, is referred to as the first close examination of a lipid from gall stones, soluble in alcohol and sedimenting as crystals (reviewed by Ellis 1918). In 1815, the lipid forming the crystals was shown not to be altered by saponification in the same way as other alcohol soluble/water insoluble biological compounds (Chevreul 1815). Four decades later, in 1859, the same compound (then referred to as cholestrine) was discovered to form esters, and was established as an alcohol (Berthelot 1859). The trivial name of this compound is today "cholesterol", which originates from the Greek words "chole" for "gall", and "steros" for "solid".

Cholesterol is the main sterol found in mammalian cells. Soon after it was discovered, the occurrence of cholesterol in various tissues from human and higher animals was investigated extensively (reviewed by Bills 1935). During the 20th century, cholesterol research became a key area of scientific investigation, (reviewed by Vance & Van den Bosch 2000). When sterols were first isolated from plants, the substances were confounded with cholesterol (Beneke 1862; Ritthausen 1863; Lindenmeyer 1863). In 1878 the term "phytosterol" (or "phytosterin") was used to describe sterol materials isolated from Calabar beans (*Physostigma venenosum* Balf.), and in the years that followed a number of papers were published reporting discoveries of plant sterols with melting points between 132 ºC and 137 ºC (Ellis 1918 and references therein). It became clear that "phytosterol" was not *one* sterol compound but a *mixture* of plant sterols.

Sterols in membranes of eukaryotes

Sterols are isoprenoid-derived lipids essential to eukaryotes. Sterols contain a non-polar water-insoluble hydrocarbon chain attached to one end of a steroid four-ring structure, together with a polar water-soluble OH-group at the opposite end of the ring structure (Staehelin & Newcomb 2000). This organisation gives sterols a water-amphipathic nature, and contributes to their anchoring in the lipid bilayers of the plasma membrane (Figure 1).

Eukaryotic plasma membranes are considered to be laterally sub-compartmented into discrete domains. "Lipid rafts" are plasma membrane microdomains, enriched in sterols and sphingolipids, and assumed to recruit and harbour specific proteins (Simons & Ikonen 1997). Sterol- and sphingolipid-rich detergent-insoluble membrane fractions have been isolated from animal- and yeast plasma membranes, and recently also from plasma membranes of plant cells (Peskan *et al.* 2000; Mongrand *et al.* 2004; Borner *et al*. 2005).

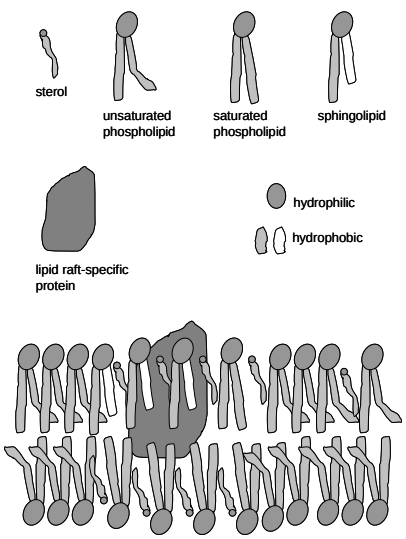


Figure 1. Sterols are components in the lipid bilayer of eukaryotic cell membranes. According to current models of plasma membranes, sterols are present throughout membranes but are enriched in so called "lipid rafts", together with sphingolipids, saturated phospholipids and specific proteins.

In many eukaryotes, the cell membrane contains a single main sterol, *e. g.* cholesterol in higher animals, and ergosterol in yeast (*Saccharomyces cerevisiae*) (Table 1). Cell membranes in plants, however, contain a mixture of several sterols. The most common ones are sitosterol, stigmasterol and 24-methylcholesterol. The precise sterol composition in plants varies between tissues and between species. For instance, cholesterol is a minor sterol in most plants, but a major sterol in members of the Solanaceae which includes important crop species such as tomato (*Lycopersicon esculentum*) and potato (*Solanum tuberosum* L.).

Table 1. Relative sterol composition in some model organisms, as percentages of the total of the major 4-desmethyl sterols.

Source	Sterol profile	Sterol metabolism	Reference
Solanum tuberosum Potato	Sitosterol 37%; Stigmasterol 20%; 24-methyl cholesterol 18%; Cholesterol 13%; Isofucosterol 12%	*De novo*	Paper I
Nicotiana tabacum Tobacco	Stigmasterol 38%; 24-methylcholesterol 23%; Sitosterol 17%; Cholesterol 13%; Isofucosterol 8%	*De novo*	Gondet *et al.* 1994
Arabidopsis thaliana Thale cress	Sitosterol 73%; 24-methylcholesterol 13%; Stigmasterol 7%; Isofucosterol 3%; Brassicasterol 2%; Cholesterol 2%	*De novo*	Schaeffer *et al*. 2001

Source	Sterol profile	Sterol metabolism	Reference
Hordeum vulgare Barley	Sitosterol 62%; Stigmasterol 23%; 24-methylcholesterol 15%	*De novo*	Rochester *et al.* 1987
Pinus pinea Mediterranean stone pine	Sitosterol 77%; 24-methylcholesterol 16%; Isofucosterol 7%	*De novo*	Nasri *et al.* 2007
Physcomitrella patens Moss	Stigmasterol 56%; 24-methylcholesterol 26%; Sitosterol 8%; Isofucosterol 7%; Cholesterol 3%	*De novo*	Arnqvist & Eklund 2007[1]
Chlamydomonas reinhardtii Green algae	Ergosterol; 24-ethylcholesta-5,7,22-trienol	*De novo*	Patterson 1991
Porphyra yenzuensis Red algae	Cholesterol	*De novo*	Patterson 1991
Saccharomyces cerevisiae Baker's yeast	Ergosterol	*De novo*	Parks *et al.* 1995
Glomus intraradices Endomycorrhizal fungus	24-methyl cholesterol 58%; Sitosterol 35%; Stigmasterol 3%; Isofucosterol 3%; Cholesterol 2%	*De novo*	Fontaine *et al.* 2001
Mus musculus Mouse	Cholesterol	*De novo*	
Danio rerio Zebrafish	Cholesterol	*De novo*	Hu *et al.* 2001
Drosophila melanogaster Fruitfly	Converts plant sterols to cholesterol	Dietary intake	Svoboda *et al.* 1991
Caenorhabditis elegans Nematode	Cholesterol	Dietary intake	Entchev & Kurzchalia 2005
Escherichia coli Bacteria	No sterols		Nes *et al.* 1980

1 Unpublished results, total desmethylsterols were extracted according to the method described in paper III.

A selection of model organisms listed in table 1 is compared with respect to their sterol composition. Bacteria are generally independent of sterols (Nes *et al.* 1980),

although several studies have reported low amounts of various sterols or sterol-like substances in cyanobacteria, methylotrophic and methanotrophic bacteria (reviewed by Volkman 2003). In animals cholesterol is the predominant sterol, and is either synthesised *de novo*, *e. g.* in vertebrates, or metabolised from sterols taken up from the diet, *e. g.* in insects and nematodes. Insects feeding on plants can dealkylate the side chain to make cholesterol from plant sterols (Svoboda *et al.* 1991) (see "24-des-, 24-methyl- and 24-ethylsterols", below). The sterol composition of nematodes reflects the sterol composition of the diet source (Chitwood 1991).

Ergosterol is the main sterol of higher fungi, while cholesterol, 24-methylene cholesterol and fucosterol are typical sterols of less advanced fungi (Parks & Weete 1991). The biosynthetic pathway of ergosterol synthesis in yeast has been studied extensively and all of the enzymes involved have been identified (Lees *et al.* 1995; Bard *et al.* 1996; Skaggs et al. 1996). The sterol composition of algae varies considerably between classes, families and species, and includes sterols different from the plant sterols (Patterson 1991).

Differences between organisms in sterol metabolism and sterol composition have been used for several practical purposes, such as targets in the control of pathogens, as markers to detect microorganisms, and to distinguish between extractions of oils. Ergosterol, present in many fungi but not in plants, has been used as an indicator to quantify fungal biomass in plant tissue (Gordon & Webster 1984). Moreover, antifungal substances blocking ergosterol synthesis are widely used to inhibit fungal pathogens (Mercer 1991). Sterols exclusively present in hazelnut oil can be used as markers to detect adulteration of virgin olive oil (Azadmard-Damirchi et al. 2005).

Sterol composition in plants

More than 250 sterols and sterol-related compounds have been isolated from plants (Akihisa *et al.* 1991), although a large fraction of these compounds are intermediates in sterol biosynthesis pathways and are only present at low levels. In addition, the natural occurrence of some sterols and sterol related compounds can be quite narrow, *e. g.* cimicifugenol in *Cimicifuga* species (Takemoto *et al.* 1970).

Depending on the context, plant sterols have been grouped according to different classification systems:

4-des-, 4-mono- and 4,4'-dimethylsterols

Sterols can be classified according to the number of methyl groups at the C4 position in the A ring (Figure 2), *i. e.* the presence or absence of C30 and C31 giving 4, 4'-dimethylsterols (with two methyl groups), 4-monomethylsterols (with one methyl group) and 4-desmethylsterols (without methyl groups) (Akihisa *et al.* 1991). Plants can demethylate sterols at the C4-position (Darnet & Rahier 2004), but are probably unable to carry out the reverse reaction. The 4,4'-dimethylsterols, *e. g.* cycloartenol and 24-methylene cycloartanol, are early plant sterol precursors,

while 4-monomethylsterols are intermediate plant sterols, *e. g.* 24-methylene lophenol and 24-ethylidene lophenol. Late precursors and the final end-product sterols constitute the desmethylsterols, which include the most common plant sterols, *e. g.* 24-methylcholesterol and sitosterol (Figure 3).

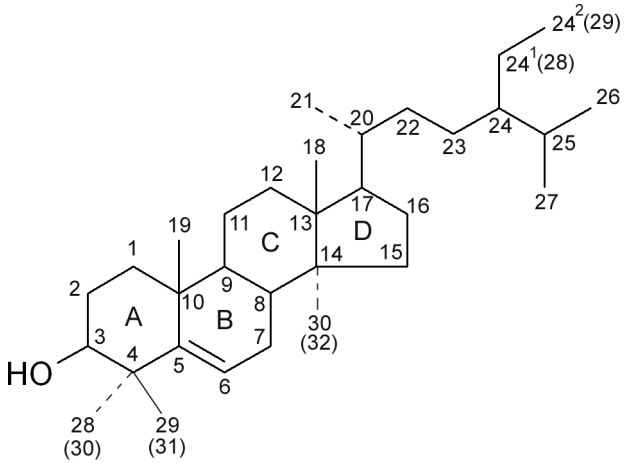


Figure 2. Sterol structure with carbon numbering according to 1989 IUPAC-IUB recommendations (Moss, 1989). The numbering according to earlier nomenclature is shown in parentheses. For convenience, the earlier numbering system is used in this thesis.

24-des-, 24-methyl- and 24-ethylsterols

Sterols are divided into three groups according to alkylations at the C24 position in the side chain: 24-desmethylsterols (without an alkyl group), 24-methylsterols (with one methyl group), and 24-ethylsterols (with one ethyl group) (Figure 2). Cholesterol is thus a 24-desmethylsterol, 24-methylene lophenol is a 24-methylsterol whereas the most abundant plant sterols sitosterol and stigmasterol are 24-ethylsterols (Figure 3). These sterol fractions can also be denoted C-8-, C-9- and C-10-sterols, referring to the number of carbons in the sterol side-chain. In addition, the 24-des-, 24-methyl- and 24-ethyl- 4-desmethylsterols are sometimes denoted C-27-, C-28- and C-29-sterols respectively, referring to the total number of carbons in the sterol molecule.

The precursor sterol cycloartenol is a 24-desmethylsterol. Two sequential side-chain methylations take place in the plant sterol biosynthetic pathway, and thus increase the side-chain by two carbon atoms. These reactions are catalysed by the sterol:methyltransferases SMT1 and SMT2 (Figure 3). The second methylation, ultimately leading to formation of the common plant sterols sitosterol and stigmasterol, is unique to plants. Plants are probably unable to catalyse the reverse reactions (demethylation of the side chain).

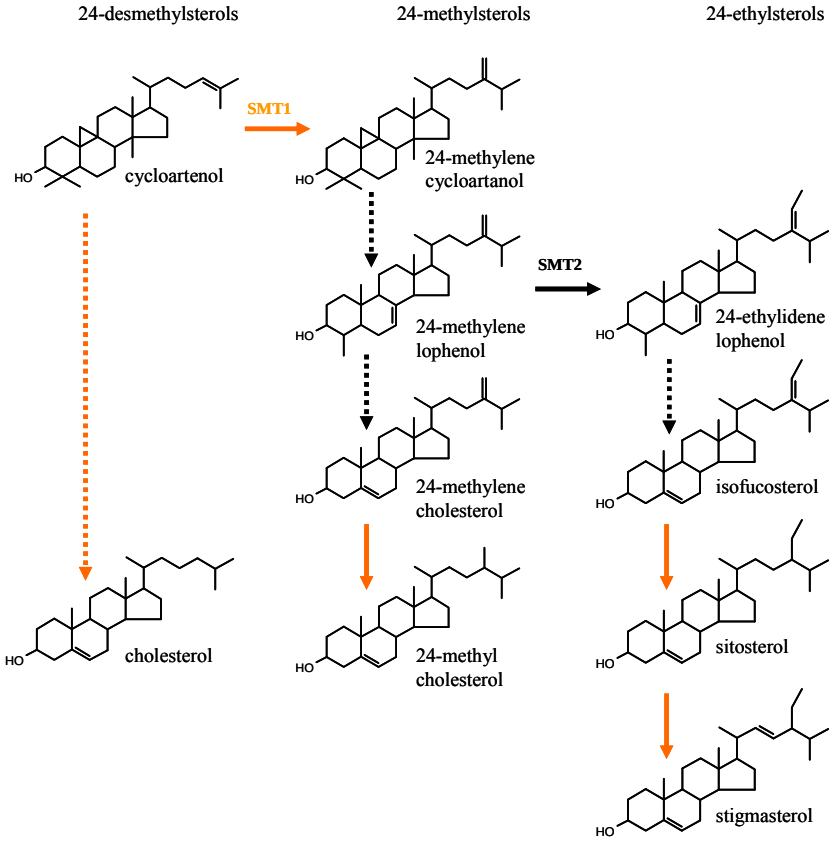


Figure 3. Sterols in plants presented in a simplified precursor-intermediate-end-product overview. Biosynthetic steps of special interest in this thesis are highlighted in orange. Dashed arrows indicate more than one step.

Free and conjugated sterols

Sterols are most commonly present in the free form, although anchored in the plasma membrane. Free sterols can be conjugated at the C3-hydroxy group with fatty acids to form steryl esters (Figure 4a), or sugars to form steryl glycosides (Figure 4b). Steryl glycosides can in turn also be esterified with a fatty acid to form acylated steryl glycosides (Figure 4c). The modification of free sterols into steryl esters with fatty acids is the most abundant conjugation form, and is of biological importance since it is connected with storage of sterols. Steryl glycosides and acylated steryl glycosides are, like free sterols, components of the plasma membranes, while steryl esters are not.

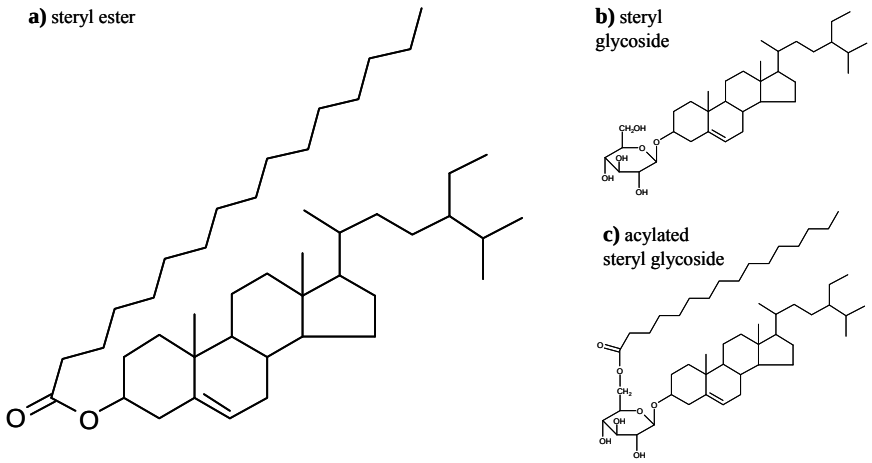


Figure 4. Plant sterol conjugates.

Sterol metabolism in plants

Plant sterols are biosynthesised in a branch of the isoprenoid pathway (Figure 5). The first step in this pathway specific to sterols is the cyclisation of 2,3-oxidosqualene to form the precursor sterol cycloartenol (Figure 6). The isoprenoid pathway also produces a large diversity of compounds necessary for various processes such as growth regulation, defence and photosynthesis. Some metabolic steps are compartmentalised into plastids, whereas the sterol pathway takes place in the cytosol.

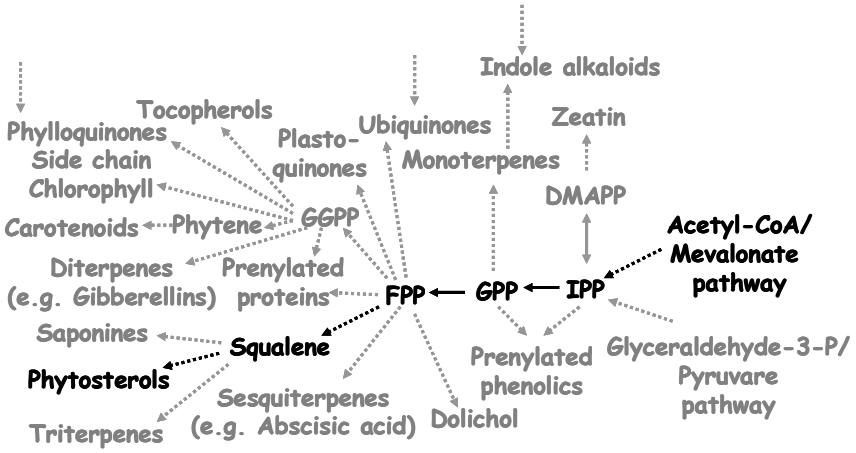


Figure 5. The isoprenoid pathway in plants. Plant sterols (or phytosterols) are biosynthesised in a branch of the cytoplasmic mevalonate pathway (adapted from Verpoorte 2000).

The first committed step in the synthesis of isoprenoids is regulated by the activity of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR),

which catalyses the reduction of 3-hydroxy-3-methylglutaryl coenzyme A to form mevalonic acid, an important isoprene precursor. A dramatic increase in sterol content was observed in transgenic tobacco overexpressing a HMGR (Schaller *et al.* 1995), while Arabidopsis with an antisense-mediated downregulation in HMGR activity showed reduced levels of sterols, triterpenes and sesquiterpenes (Godoy-Hernández *et al*. 1998). These, and other studies on HMGR expression and activity in *e. g.* potato plants (Yang *et al.* 1991; Choi *et al.* 1992; Oba *et al*. 1985), confirm the proposed key role for HMGR in the regulation of plant sterol biosynthesis (Bach 1986).

Squalene synthase is another sterol-biosynthetic enzyme with a putative key position in the regulation of sterol synthesis. This enzyme catalyses the formation of squalene from farnesyl diphosphate (FPP), at a branch point upstream of cycloartenol in the isoprenoid biosynthetic pathway (Figure 5) (Threlfall & Whitehead 1988; Vögeli & Chappell 1988; Chapell *et al.* 1989; Devarenne *et al.* 1998). Overexpression of squalene synthase in Siberian ginseng (*Eleutherococcs senticosus* Rupr. and Maxim.) enhanced the plant sterol and triterpene saponine biosynthesis, indicating a rate-limiting function of this enzyme (Seo *et al.* 2005). Moreover, chemical inhibition of squalene synthase in tobacco cells triggered an upregulation of HMGR at both the transcriptional and posttranslational levels (Wentzinger *et al.* 2002). Inhibition of squalene epoxidase (Figure 6) also triggered an upregulation of the HMGR activity, indicating a feedback regulation of HMGR in response to decreased sterol levels.

The biosynthesis of plant sterols has been extensively studied (reviewed by Bach & Benveniste 1997; Benveniste 2002; Benveniste 2004; Schaller 2004). The major end-product sterols, including cholesterol, are proposed to be synthesised in three parallel pathways (Figure 3) from the precursor sterol cycloartenol. Enzymes have been identified in Arabidopsis for every step in the synthesis of 24-methyl- and 24-ethyl plant sterols, although the "stigmasterol synthase" sterol C22-desaturase was isolated only recently (Morikawa *et al.* 2006; paper II) (Table 2). Twelve sterol-modifying steps are required to produce stigmasterol from cycloartenol (Table 2) (Figure 6c-n). However, the biosynthetic route to 4-desmethyl-24-desmethylsterols is less clear, presumably because these sterols are only minor ones in the model plant Arabidopsis, as well as in most other plant species. A tentative 24-desmethylsterol biosynthesis pathway, in which cholesterol is the "end-product" sterol, is illustrated in Figure 6. Analyses of the sterol composition of some plant sterol mutants and transgenic plants, indicate that the enzymes regulating 24-methyl- and 24-ethylsterols also are involved in the regulation of 24-desmethylsterols. For example *dim* Arabidopsis mutants, which display a reduced Δ24-reductase activity, did not only have decreased levels of sitosterol and 24-methylcholesterol, but also decreased levels of cholesterol (Klahre *et al.* 1998).

Table 2. Sterol regulating enzymes involved in the conversion of squalene to stigmasterol in plants.

Enzyme	Gene/ gene family	Step in Figure 6	Catalytic product	Yeast orthologue	Reference
Squalene epoxidase	*SQE*	a	2,3-Oxidosqualene	Erg1p	Rasbery *et al.* 2007
Cycloartenol synthase	*CAS1*	b	Cycloartenol	Erg7p	Corey *et al.* 1993
C24 methyltransferase	*SMT1/ CPH*	c	24-Methylene-cycloartanol	Erg6p	Bouvier-Navé *et al.* 1998
C4 demethylase[1]	*SMO1*	d	Cycloeucalenol	Erg25p	Darnet & Rahier 2004
Cyclopropyl isomerase	*CPI*	e	Obtusifoliol		Lovato *et al.* 2000
C14 demethylase	*CYP51*	f	4α-Methyl-ergostatrienol	Erg11p	Bak *et al.* 1997
Δ14 reductase	*FACKEL/ HYD2*	g	4α-Methyl-ergostadienol	Erg24p	Schrick *et al.* 2000
Δ8-Δ7 isomerase	*HYD1*	h	24-Methylene-lophenol	Erg2p	Souter *et al.* 2002
C28 methyltransferase	*SMT2/ CVP1*	i	24-Ethylidene-lophenol		Bouvier-Navé *et al.* 1997
C4 demethylase[1]	*SMO2*	j	Episterol Δ^7-Avenasterol	Erg25p	Darnet *et al.* 2001
C5 desaturase	*DWF7/ STE1*	k	5-Dehydroepisterol 5-Dehydro-avenasterol	Erg3p	Gachotte *et al.* 1995
Δ7 reductase	*DWF5*	l	24-Methylene-cholesterol Isofucosterol		Choe *et al.* 2000
Δ24 reductase	*DIM/ DWF1*	m	24-Methyl-cholesterol Sitosterol		Klahre *et al.* 1998
C22 desaturase	*CYP710A*	n	Brassicasterol/ Crinosterol Stigmasterol	Erg5p	Morikawa *et al.* 2006 Paper II

1. Additional enzymes; a decarboxylase and a 3-keto reductase, are probably also involved in the two sterol C4-demethylation steps. These enzymes are not yet cloned.

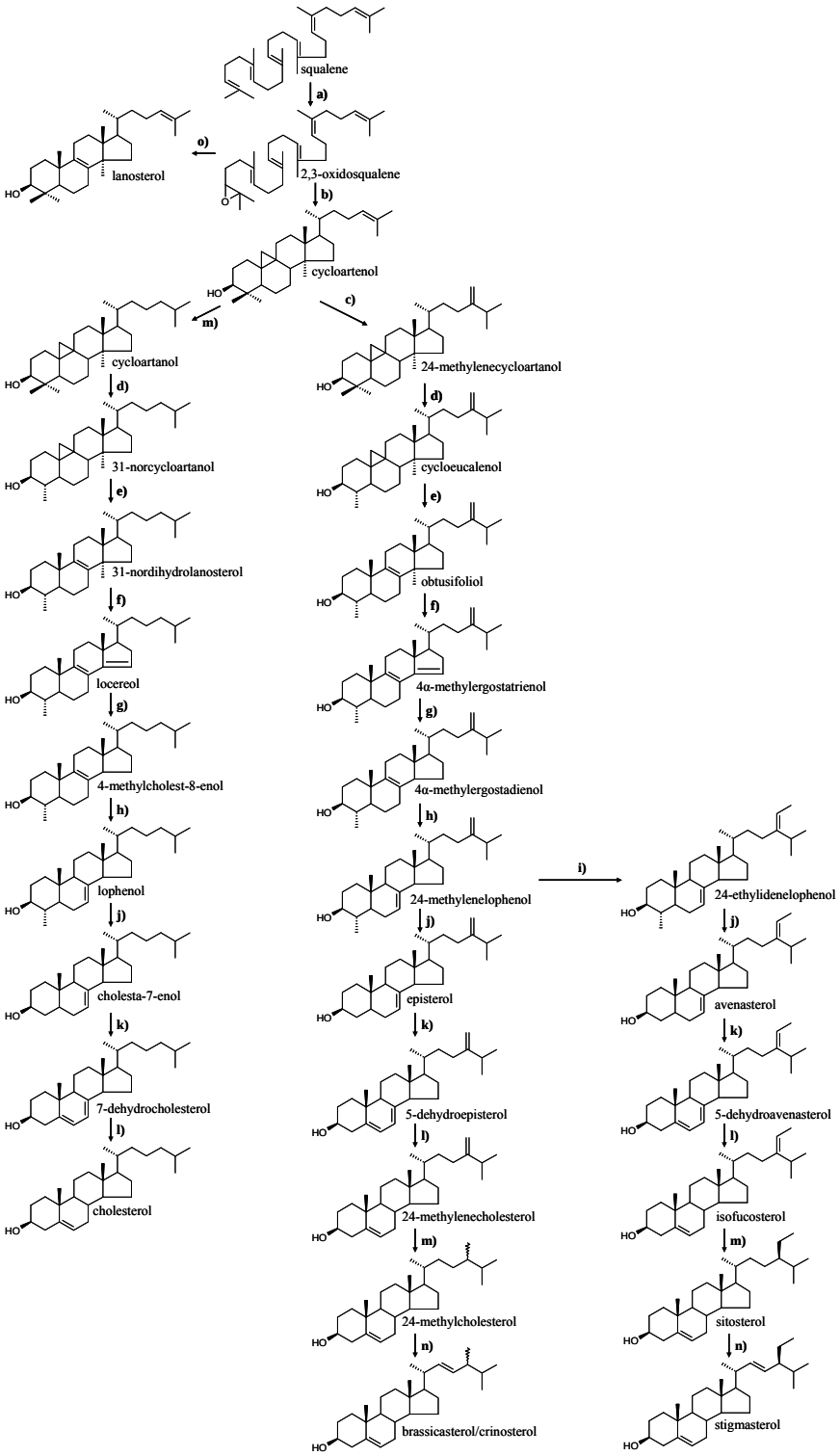

squalene
a)
2,3-oxidosqualene
o)
lanosterol
b)
cycloartenol
c)
m)
cycloartanol
24-methylenecycloartanol
d)
31-norcycloartanol
cycloeucalenol
e)
31-nordihydrolanosterol
obtusifoliol
f)
locereol
4α-methylergostatrienol
g)
4-methylcholest-8-enol
4α-methylergostadienol
h)
lophenol
24-methylenelophenol
i)
24-ethylidenelophenol
j)
cholesta-7-enol
episterol
avenasterol
k)
7-dehydrocholesterol
5-dehydroepisterol
5-dehydroavenasterol
l)
cholesterol
24-methylenecholesterol
isofucosterol
m)
24-methylcholesterol
sitosterol
n)
brassicasterol/crinosterol
stigmasterol

Figure 6. Hypothetical plant sterol biosynthetic pathway including 24-desmethyl sterols. Each arrow indicates a catalytic step regulated by a) squalene epoxidase; b) cycloartenol synthase; c) C24-methyltransferase; d) C4-demethylase; e) cyclopropyl isomerase; f) C14-demethylase; g) Δ14-reductase; h) Δ8-Δ7-isomerase; i) C28-methyltransferase; j) C4-demethylase; k) C5-desaturase; l) Δ7-reductase; m) Δ24-reductase; n) C22-desaturase; o) lanosterol synthase

All genes of the ergosterol biosynthetic pathway in yeast were cloned more than a decade ago, and genes encoding sterol-biosynthetic enzymes in Arabidopsis were initially identified by their sequence similarity to corresponding genes involved in the ergosterol synthesis in yeast, and characterised by functional expression in, or complementation of, yeast mutants defective in ergosterol synthesis. The Arabidopsis cycloartenol synthase (CAS) was expressed in the yeast mutant *erg7* lacking a lanosterol synthase (ERG7) (Corey *et al.* 1993) and found to catalyse cycloartenol synthesis. *SMT2,* encoding a sterol methyltransferase type 2, was expressed in *erg6* (Husselstein *et al.* 1996) and found to produce 24-ethylsterols. The sterol C-5-desaturase was identified by functional complementation of *erg3* (Gachotte *et al*. 1996). Later studies of Arabidopsis growth mutants led to the identification of additional genes in sterol synthesis. One of these, DIMINUTO/DWARF1 (DIM/DWF1)was shown to be absent in yeast. The DIM/DWF1 protein belongs to a class of FAD-dependent oxidoreductases (Choe *et al.* 1999a), and catalyses the reduction of the sterol Δ24[28] double bond in the synthesis of sitosterol and 24-methylcholesterol (Klahre *et al.* 1998). In yeast, this type of enzymatic function is performed by the Erg4p, a NADP-dependent oxidoreductase. Thus a similar biosynthetic step, a sterol C-24 reduction, is in yeast and plants catalysed by two different types of enzyme. Moreover, the isolation of DIM/DWF1 from plants rapidly led to the subsequent cloning of a human counterpart, seladin-1, which catalyses the last step in cholesterol synthesis (Greeve *et al.* 2000, Waterham *et al.* 2001). A considerable amount of work is now being carried out on this protein in order to understand its role in cholesterol homeostasis, and its relation to Alzheimer's disease. Thus, work on a dwarfed Arabidopsis sterol mutant had important implications for our understanding of human disease.

Of particular interest in sterol synthesis are the two types of sterol-C24-metehyltransferases, SMT1 and SMT2. These are key regulatory enzymes in the sense that they direct the carbon flow into the two pathways leading to 24-methyl- and 24-ethylsterols respectively (figure 3), and the 24-methyl- to 24-ethylsterol ratio is strictly regulated in plants (Schaller 2003), although the ratio varies between species.

Sterol synthesis is also important for the synthesis of brassinosteroids, a small class of sterol-derived growth hormones. A number of Arabidopsis mutants, denoted *dwf1* to *dwf7,* display a dwarf phenotype characterised by small, round, dark-green leaves, short stems, pedicels, and petioles (Choe *et al.* 2000). These mutants have been identified as being deficient in the brassinosteroid biosynthetic pathway. Brassinosteroids are synthesised from 24-methylcholesterol (see below), and among the *dwf1* to *dwf7* mutants, some have deficiencies within the sterol biosynthesis pathway; *dwf1/dim* (Choe *et al.* 1999a), *dwf5* (Choe *et al*. 2000) and

dwf7/ste1 (Gachotte *et al.* 1995; Gachotte *et al.* 1996; Husselstein *et al.* 1999; Choe *et al.* 1999b) (table 2), while others, such as *dwf2/bri1/cbb2, dwf3/cpd/cbb3, dwf4* and *dwf6/det2* are blocked in the brassinosteroid biosynthesis downstream of 24-methylenecholesterol (Asami & Yoshida 1999).

Lanosterol is the sterol precursor in animals and yeast, differing from cycloartenol by having a double bond at C8, and by not bearing a 9β,19-cyclopropan ring (Figure 6) Recently it was also shown that plants have the ability to synthesise lanosterol by the activity of a lanosterol synthase (Suzuki *et al.* 2006; Kolesnikova *et al.* 2006; Sawai *et al.* 2006). From these findings it was suggested that plant sterols might be synthesised by two biosynthetic routes, either *via* lanosterol or *via* cycloartenol, although firm evidence for this is lacking at the present time.

Steryl esters are thought to be a storage form of sterols, and to contribute to the homeostasis of free sterols in cell membranes. The interconversion of conjugated sterols and free sterols is regulated by the activities of a sterol acyltransferase and a steryl esterhydrolase, which thus either form esters from free sterols, or hydrolyse esters back to the free sterol form, respectively (Bouvier-Navé & Benveniste 1995). Several studies suggest that sterol ester formation is an active process in plants, and steryl ester levels are known to vary during plant development (Dyas & Goad 1993). Sterol over-producing tobacco mutants (Schaller *et al.* 1993) have increased levels of steryl estersforming lipid droplets in the cytoplasm (Gondet *et al.* 1994). In the present work, increased levels of steryl esters were also found in stigmasterol over-producing transgenic Arabidopsis (paper II). A phospholipid:sterol acyltransferase (PSAT), regulating the esterification (or acylation) of plant sterols to form steryl esters, was recently identified in Arabidopsis (Banas *et al.* 2005) from its homology to the lecithin:cholesterol acyltransferase (LCAT) in animals. The latter enzyme esterifies sitosterol almost as efficiently as cholesterol (Temel *et al.* 2003).

Functions of sterols in plants

The mixture of sterols in plants brings an additional dimension to the topic "functional roles of sterols", namely the importance of sterol homeostasis or action with respect to several individual sterol species. A dependency on sterol homeostasis for several events in plant growth and development have been revealed by studies on plant mutants and transformants defective in sterol biosynthesis (reviewed by Clouse 2002; Schaller 2003). In addition, dysfunctions in plants caused by changes in the plant sterol composition have been studied using sterol inhibitors, originally used as antifungal drugs targeting the ergosterol biosynthetic pathway (Hartmann 1998). Altered sterol composition at various stages of physiological maturity, or in different parts of a plant (Bush & Grunwald 1972; Grunwald 1975; Grunwald 1978), indicates that the sterol composition is of importance during the plant's life cycle. Changes in the plant sterol composition as a response to fluctuating environmental conditions, such as changes in sunlight conditions (Izzo & Navari-Izzo 1981), temperature (Guye 1989), water access (Navari-Izzo *et al.* 1989), salinity (Mansour *et al.* 1994) and ozone levels (Hellgren *et al.* 2001), indicate that the sterol composition is also important for the

survival of the plant in its environment. Moreover, studies on the properties of synthetic membranes (liposomes), following the application of different plant sterols *in vitro*, has been used as an approach to gain insight into the functions of sterols.

The precursor role of sterols in plants

Sterols in plant cell membranes serve as precursors or intermediates in biosynthetic routes leading to formation of the steroid hormones brassinosteroids and ecdysteroids, and the formation of the secondary compounds cardenolides and glycoalkaloids. In animals and humans the main sterol cholesterol serves a similar precursor role in the synthesis of steroid hormones such as estrogen and testosterone.

Brassinosteroids are plant steroid hormones biosynthesised from 24-methylcholesterol (Fujioka & Yokota 2003) in the 24-methylsterol pathway (Figure 3). Brassinosteroids stimulate cell elongation, cell division, vascular differentiation and are involved in other events in plant development as well (Altmann 1998).

Plants can use sterols for the synthesis of defence substances toxic to insects and animals. Several plant species are able of synthesising ecdysteroids from cholesterol (Costet-Corio *et al.* 1993), similar to the ones naturally made in insects. The phyto-ecdysteroids interfere with insect molting, and can be seen as a defence system at the steroid hormone level.

Cardenolides are secondary metabolites, present in *Digitalis* and some other plant genera, and it has been suggested that they are biosynthesised from stigmasterol (Kreis *et al.* 1998). The cardenolides digitonin and digitoxin have an important medical use in treatment of human heart insufficiency, and their levels have recently been increased in *Digitalis minor* by metabolic engineering of the steroid biosynthetic pathway (Sales *et al.* 2007).

Steroidal glycoalkaloids are secondary metabolites in plants of the Solanaceae, it has been proposed that they are biosynthesised from cholesterol. The physiology and biochemistry of glycoalkaloids in the cultivated potato is further described below, as part of the aims of this thesis. High levels of glycoalkaloids are toxic to humans, and their levels are kept low in the breeding of new potato varieties. In the present work glycoalkaloid levels have been lowered in transgenic potato plants (paper I and IV).

The regulation of cell membrane fluidity and permeability

Sterols modulate the ability of cell membrane components to flow, and influence the permeability of membranes to water and other molecules. The fluidity of a cell membrane is precisely regulated, and depends to a large part on its sterol levels. The rigidity of the plate-like ring structures of sterols tends to make membranes less fluid. Sterols increase the viscosity of the membrane but at low temperatures sterols can also prevent crystals forming between phospholipids, and thereby

lower the temperature for phase transition (the freeze point) of the membrane. Sitosterol, 24-methylcholesterol and cholesterol have been shown to reduce the plant cell membrane permeability to water and other small molecules, while stigmasterol has only slight, or no such effect (Schuler *et al.* 1991).

Lipid rafts

Sterol-rich lipid rafts are considered to be involved in a variety of cellular processes (reviewed by Bhat & Panstruga 2005). These include protein sorting and signal reception or transduction. An investigation at the proteomic level of detergent-resistant membranes from tobacco cells (Morel *et al.* 2006) showed that proteins involved in signalling, biotic- and abiotic stress-responses, cellular trafficking and cell wall metabolism were over-represented in lipid rafts, suggesting that these microdomains serve as signalling platforms for certain biological processes. Besides being constituents of lipid rafts, plant sterols are proposed to play a role in the formation and localisation of lipid rafts in the plasma membrane. In leek (*Allium porrum*) seedlings treated with a sterol biosynthesis inhibitor, fenpropimorph, some lipids important for lipid-raft-formation did not reach the plasma membrane, and most detergent-resistant membranes were located in the Golgi apparatus instead of in the plasma membrane, as they were in untreated seedlings (Laloi *et al.* 2007).

Regulation of plant development

Alterations in the sterol composition of plants have been shown to influence several events in their development. The mechanisms behind their function in plant development is not fully understood, but regulation of gene expression (He *et al.* 2003), modulation of the activity of enzymes in the plasma membrane, protein positioning (Willemsen *et al.* 2003) and involvement in hormone perception and signalling (Souter *et al.* 2002) have all been suggested. Sterols have also been proposed to act as signal molecules themselves.

Phenotypic characteristics of a number of Arabidopsis mutants, defective in different steps of the sterol biosynthetic pathway, illustrate well the importance ascribed to sterols in various aspects of plant development (Table 3). Mutations early in the sterol biosynthetic pathway (Figure 6), *i. e.* upstream of the methylation step converting 24-methylenelophenol to 24-ethylidenelophenol, causes severe embryonic defects, *e. g.* the *smt1* (Diener *et al.* 2000), *ell/fackel* (Jang *et al.* 2000; Schrick *et al.* 2000) and *hydra1* (Souter *et al.* 2002) mutants. *cvp1* (*cotyledon vascular pattern 1*) (Carland *et al.* 2002) and *frl1* (Hase *et al.* 2000) are two mutants defective in SMT2 with increased 24-methylcholesterol to sitosterol ratios. These mutants showed vascular patterning defects and serrated flowers (sepals and petals) respectively. Reduced growth, increased branching and low fertility are additional effects connected to an increased 24-methylcholesterol to sitosterol ratio, observed in transgenic Arabidopsis with reduced SMT2-1 expression (Schaeffer *et al.* 2001). The HMGR mutant *hmg1* showed an overall decrease in the levels of sterols and triterpenoids, had normal embryogenesis but displayed dwarfing, early senescence, and sterile phenotypes (Suzuki *et al.* 2004). The *sqe1* mutant, also mutated upstream of cycloartenol, had growth defects in

roots and developing seeds (Rasbery *et al.* 2007). None of these mutants could be rescued by the exogenous application of brassinosteroids. It cannot be excluded though, that the *hmg1* and *sqe1* mutant phenotypes could also be affected by changes in the levels of other isoprenoids or triterpenoids respectively.

Table 3. Phenotypes displayed by Arabidopsis mutants defective in sterol biosynthesis. These phenotypes could not be rescued by exogenous application of brassinosteroids.

Phenotype	Arabidopsis mutant	Reference
Disturbed embryonic patterning.	*smt1*	1
	ell/fackel	2,3
	hydra1	4
Disarranged and misshapen vascular cells.	*cvp1* (*smt2*)	5
Disturbed cell polarity and auxin efflux.	*orc (smt1)*	6
Early senescence, sterility and dwarfism.	*hmg1*	7
Disturbed cellulose synthesis and defective cell wall building.	*smt1* *fackel* *hydra1*	8
Ectopic endoreduplication causing serrated petals.	*frl1* (*smt2*)	9
Growth defects in roots and developing seeds.	*sqe1*	10

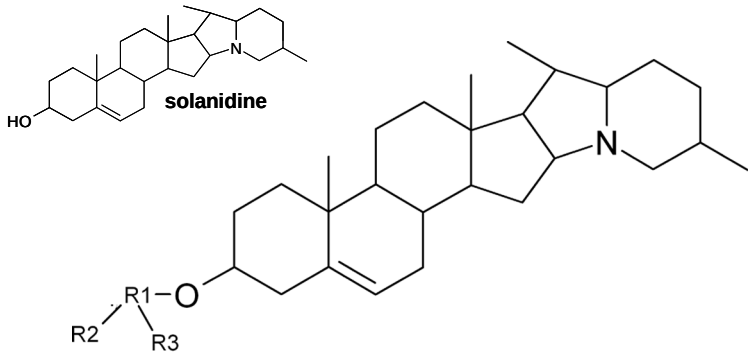
References: 1. Diener *et al.* 2000; 2. Jang *et al.* 2000; 3. Schrick *et al.* 2000; 4. Souter et al. 2002; 5. Carland *et al.* 2002; 6. Willemsen *et al*. 2003; 7. Suzuki *et al.* 2004; 8. Schrick *et al.* 2004; 9. Hase *et al*. 2005; 10. Rasbery *et al.* 2007.

Glycoalkaloids in potato plants

Steroidal glycoalkaloids are secondary metabolites present mainly within species of the family Solanaceae. Some examples of these substances are solamargine and solasonine in aubergine (*Solanum melongena* L.) (Aubert *et al.* 1989), α-tomatine and dehydrotomatine in tomato (*Lycopersicon esculentum*) (Friedman 2002), and α-chaconine and α-solanine in potato (Kuhn & Löw 1954). Due to the toxic properties of glycoalkaloids their synthesis and content in plants used for consumption may represent a serious problem to the consumer. The greatest concern in this respect has been about glycoalkaloid levels in edible potato tubers. The glycoalkaloids in cultivated potato, α-chaconine and α-solanine, are two glycosylated forms of the steroidal aglycon solanidine (Figure 7). Some cultivars of common potato, as well as other Solanum species, also contain glycoalkaloids based on other alkaloid aglycones (Sinden & Sanford 1981; Osman *et al.* 1976).

Glycoalkaloids are considered to contribute to a plants resistance to various pathogens. High glycoalkaloid content has been connected to an increased resistance against viral and microbial diseases, *e. g. Erwinia* soft rot (bacteria) (Austin *et al.* 1988), *Fusarium* species (Percival *et al.* 1998) and other fungi (Fewell & Roddick, 1997). They also give protection against attacks by insects, *e. g.* Colorado potato beetle, *Leptinotarsa decemlineata*, (Deahl *et al.* 1991; Sanford *et al.* 1997), potato leafhopper, *Empoasca fabae*, (Sanford *et al.* 1992) and wireworm, larvae of *Agriotes obscurus* L., (Jonasson & Olsson 1994). However, studies on the biological function of glycoalkaloids have seldom been conducted in the same cultivar, and genetic factors other than glycoalkaloid content may

influence results in this type of study. Transgenic plants with differences in glycoalkaloid content may represent an improved model system to analyse the role of glycoalkaloids more precisely.



	R1	R2	R3
α-chaconine	D-glucose	L-rhamnose	L-rhamnose
α-solanine	D-galactose	D-glucose	L-rhamnose

Figure 7. Structure of the glycoalkaloids in cultivated potato (*Solanum tuberosum* L.). α-chaconine and α-solanine are glycosylated derivatives of the aglycone solanidine (Adapted from Bergenstråhle 1995).

Glycoalkaloids are unevenly distributed within the potato plant, and accumulate in the water soluble fraction (Roddick 1977) of cells from various organs and tissues during development (Deahl *et al.* 1991; Eltayeb *et al.* 1997; Friedman & Dao 1992; Valkonen *et al.* 1996). The level per fresh weight is highest in flowers and fruit, then decreases in the order sprouts > leaves > stems > tuber.

Potato glycoalkaloids have toxic effects on animals and humans at levels from ~1 mg kg^{-1} body weight (Slanina 1990), and a lethal dose may be 3-6 mg kg^{-1} body weight (Morris & Lee 1984). The toxicity levels may depend on the α-chaconine/α-solanine ratio, since α-chaconine is the more toxic of the two (Friedman 2006), and also because α-chaconine and α-solanine can act synergistically at certain ratios (Roddick *et al.* 1988).

Toxicity at low levels is due to the formation of complexes between glycoalkaloids and cholesterol, and causes cell membrane disruption in cells lining the gastrointestinal tract (Keukens *et al.* 1996). This gives symptoms of vomiting, diarrhoea and abdominal pain (Morris & Lee 1984). Cell membrane integrity in other organs can also be affected. The expression of several genes involved in sterol biosynthesis was upregulated in a Caco-2 intestinal epithelial cell line exposed to α-chaconine. The authors resoned that upregulated cholesterol biosynthesis could be a part of the mechanism of glycoalkaloid toxicity causing the cell membrane disruption (Mandimika *et al.* 2007).

At high levels glycoalkaloids inhibit acetylcholine esterase enzymes, and thereby affect the central nervous system. Such symptoms involve drowsiness, confusion, disturbances of vision, fever, rapid weak pulse, low blood pressure and rapid

respiration (McMillan & Thompson 1979). More serious symptoms involve breathing difficulties, loss of consciousness and in extreme cases may even lead to death.

Potato cultivars are considered safe for consumption, in terms of glycoalkaloid content, if levels in tubers do not exceed 200 mg kg^{-1} fresh weight (Valkonen *et al.* 1996). According to this a child with a bodyweight of 15 kg can only eat up to 75 mg of a safe potato variety without any risk for poisoning. Investigations on glycoalkaloid contents in potato report on levels far below the safety limit for consumption for most cultivars sold for human consumption, ~75 ± 50 mg kg^{-1} fresh weight in tubers (Friedman *et al.* 2003; Concon 1988). However, Parnell *et al.* (1984) and other authors (*e. g.* Korpan *et al.* 2004) have concluded that the safety limit for glycoalkaloid content in potato tubers should be revised (lowered) since it only relates to sporadic outbreaks of poisoning and not to possible chronic effects. It should also be remembered that both α-solanine and α-chaconine are heat-stable and therefore not reduced by cooking.

The total level of glycoalkaloids (TGA) and the α-chaconine/α-solanine ratio in potato is determined by genotype (Sanford & Sinden 1972), but is also regulated by external and internal factors. TGA accumulation is triggered by exposure to light (Dale *et al.* 1993; Percival *et al.* 1996) and mechanical wounding (Sinden 1972; Salunkhe & Wu 1979), and is downregulated by the plant hormone ethylene (Bergenstråhle *et al.* 1992). TGA levels vary considerably among potato varieties and plant organs or tissues (Friedman & Dao 1992), but conditions during growth, harvest- and post-harvest handling *e. g.* grading (Olsson 1986) and storage (Griffiths *et al.* 1998) also impact on the levels in tubers. TGA levels tend to be higher in organically grown potato tubers (not exposed to pesticides) compared to conventionally grown ones (Hajslova *et al.* 2005). This is in line with the view of glycoalkaloids as molecules giving resistance against pests and predators.

Glycoalkaloid biosynthesis

Genes, enzymes and intermediates in the glycoalkaloid pathway, and external and internal factors involved in regulating TGA levels and α-chaconine/α-solanine ratios, have been pursued to some extent. In the proposed biosynthetic pathway (Figure 8) α-chaconine and α-solanine are formed from cholesterol via the common precursor solanidine (Tschesche & Hulpke 1967; Canonica *et al.* 1977; Heftmann 1983). As described above SMT1 regulates the cholesterol level in plants, and in paper I the capacity of SMT1 to also regulate TGA levels was tested.

Three genes downstream of solanidine have been cloned and characterised (Figure 8). Sgt1, encoding the galactosyltransferase catalysing the conversion of solanidine to γ-solanine (McCue *et al.* 2003), was cloned in 1997 by isolation of yeast cells becoming capable to detoxify the aglycone solasodine in the growth medium after transformation with a potato cDNA library (Moehs *et al.* 1997). Sgt2 was isolated based on its homology to Sgt1, and was shown to encode a glucosyltransferase catalysing the synthesis of α-chaconine from solanidine in

vivo (McCue *et al.* 2006). Similarly, Sgt3, encoding the rhamnosyltransferase catalysing the terminal step in both α-solanine and α-chaconine synthesis, was cloned only recently (McCue *et al.* 2007). Interestingly, both Sgt1 and Sgt2 co-express with genes upsteam in the isoprenoid pathway (Krits *et al.* 2007), and newly identified TGA-regulating genes might be found among additional genes co-expressing with Sgt1 and Sgt2. However, Sgt1 was shown not to be induced by light or wounding, and thus is not likely to represent a limiting factor in stress-induced TGA synthesis (our unpublished results).

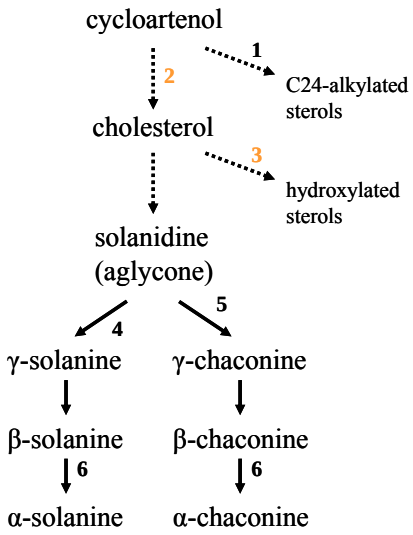


Figure 8. Glycoalkaloid biosynthetic pathway in potato. Numbering indicates identified (black) and tentative (orange) regulation points catalysed by **1.** sterol C24-methyl transferase type 1 (paper I); **2.** ΔC24-reductase (paper IV); **3.** cholesterol hydroxylases (paper III); **4.** solanidine galactosyltransferase (McCue *et al.* 2003); **5.** solanidine glucosyltransferase (McCue *et al.* 2006); **6.** glycosterol rhamnosyltransferase (McCue *et al.* 2007). Three strategies to regulate TGA levels by genetic engineering are tested in the present work: increased metabolic flux into competitive pathways (**1.**); decreased flux by reduction of an enzyme upstream in the pathway (**2.**); increased catabolism of a precursor compound (**3.**).

Some phospholipids have been shown to modulate steroid glucosyltransferase activity (Pazkowski *et al.* 2001), suggesting that the lipid environment is of importance in glycoalkaloid synthesis.

Glycoalkaloids in plant breeding

The American potato cultivar Lenape, with "high starch" and "crisp chip" traits and excellent for chipping and storage (Akeley 1968), was shortly after its introduction to the market subjected to sale restrictions due to high glycoalkaloid content (Zitnak & Johnston 1970). Similarly, the well-established Swedish cultivar, Magnum Bonum, was withdrawn from the market (Hellenäs *et al.* 1995), despite having many desirable properties. These examples are instructive in terms of the problems that high glycoalkaloid levels may cause, and underscore the importance of considering glycoalkaloids in potato breeding programs. However, what is generally considered within breeding are the basal TGA levels in tubers at the time for harvesting, not the stress-induced ones which may be several-fold higher. Since glycoalkaloid levels are influenced by environmental factors, metabolic engineering may be one method to keep their levels constantly low, without being responsive to stress factors.

Considering the proposed positive effects of glycoalkaloid levels in the human diet (reviewed by Friedman 2006), *e. g.* anti-carcinogenic and anti-viral effects, and the enhanced flavour/bitter taste (Sinden *et al.* 1976) that is a desire for some consumers, a low constant level of glycoalkaloids levels might be optimal: a suggestion that could be in conflict with the arguments claiming chronic "accumulating" negative effects of glycoalkaloids on human health mentioned above.

Whether involving transgenic plants or not, it will in relation to all aspects of potato breeding be prudent to identify and characterise the genes in TGA synthesis and their regulation.

Aims of the work

The overall aim of this work has been to increase knowledge on the regulation of sterol homeostasis in plants, with special reference to the importance of sterols as precursors for the synthesis of glycoalkaloids in potato. Towards this end, different approaches were investigated to reduce glycoalkaloid levels in potato by genetic engineering.

Specific aims were:

- to study the relation between SMT1 expression and glycoalkaloid levels in potato, and the effect of channeling the carbon flux into parallel sterol-biosynthetic pathways competitive to the cholesterol biosynthetic pathway.
- to identify the enzymes catalysing the biosynthesis of Δ^{22}-desaturated plant sterols, and to study whether an increased stigmasterol/sitosterol ratio influences plant growth or sterol metabolism.
- to study if increased levels of hydroxylated sterols influences plant growth or steroid metabolism, and to test if glycoalkaloid levels in potato can be reduced by hydroxylation of the precursor metabolite cholesterol.
- to identify enzymes with a role in the Δ^{24}-reduction step in cholesterol biosynthesis, and to decrease the synthesis of cholesterol and glycoalkaloids in potato by reducing sterol Δ^{24}-reductase expression.

Materials and methods

Arabidopsis and potato have been used in this work as model systems to study sterol metabolism and glycoalkaloid regulation. Various cDNAs have been expressed in these plants in sense or antisense orientation behind a constitutive promoter, and the steroid composition and visible phenotype of genetically modified plants have been analysed. The materials and methods used include cloning of genes, generation of transgenic Arabidopsis lines and potato clones, analyses of gene expression, enzymatic assays, extraction, quantification and

identification of plant sterols by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS), TGA and brassinosteroid analyses, hormone and steroid treatments of plants, sequence alignments and phylogenetic tree constructions. This is described in papers I-IV, but some aspects on plant materials, transformation methods and analytical methods, are discussed here in a broader perspective.

Plant materials and transformation methods

Arabidopsis thaliana

Arabidopsis (*Arabidopsis thaliana*) is a highly inbreeding diploid weed, easy to cross, and with a short generation cycle. The species is easily mutated and transformed, and lines (genotypes) can conveniently be kept and stored as seeds. Over 1000 ecotypes are available and can be used as "background" wild types in plant genetic studies (Koornneef *et al.* 2004). Due to these features, Arabidopsis is today the most commonly used model species for plant research (Somerville & Koornneef 2002). As described earlier, this has also greatly contributed to the current understanding of plant sterol metabolism, and mutants defective in sterol metabolism, and transformants with up- or downregulated expression of sterol regulating genes, have been studied extensively. However, it is of interest to study sterol metabolism, and its genetic basis, more extensively in plants other than Arabidopsis, bearing in mind the large variation in many aspects of sterol metabolism among different plant species.

Arabidopsis has a rich complement of secondary metabolites (reviewed by D'Aurea & Gershenzon, 2005), although other plant taxa have their own characteristic sets of secondary metabolites as well. The use of Arabidopsis as a model organism for studying plant secondary metabolism is however, for obvious reasons, limited to the biosynthetic pathways that are present in this model plant. The absence of glycoalkaloids in Arabidopsis, but their presence in potato, is a good example of this fact.

The floral dip method

Arabidopsis thaliana ecotype Columbia was transformed using the "floral dip" method according to a protocol by Clough & Bent (1998), modified from the "*Agrobacterium* vacuum infiltration" method described by Bechtold *et al.* (1993). These methods involve *Agrobacterium tumefaciens*-mediated gene transfer, in the case of the floral dip method simply by dipping floral tissues into a solution containing the *Agrobacterium* strain. The method allows efficient plant transformation without the need for tissue culture, and even severe effects of a transformation on the performance and viability of the genetically modified material can be monitored directly in the first generation of transgenic plants. This is not always the case when transgenic plants are generated from tissue culture, since the transformed cells must be capable of correct morphogenesis to generate a transgenic plant, and hence are largely selected for normal growth.

Solanum tuberosum

Cultivated potato (*Solanum tuberosum* L.) is also a model plant, although in a much more narrow aspect than Arabidopsis. *S. tuberosum* is well studied because it is an agronomically important plant, and represents a model for plants with a starch-storing sink tissue, *i. e.* the below ground tubers used for human consumption. Protocols for genetic studies of potato are available, *e. g.* transformation protocols, as well as cDNA databases and potato cultivars suitable for scientific research. The species is a self-infertile autotetraploid with a high level of heterozygosity. It is regenerated vegetatively and clones can be kept in tissue- or green-house culture (Figure 9), and can be stored as tubers. The cultivar Désirée was used in our transformation experiments since it is fast in setting shoots from tissue culture, and tubers of this cultivar do not need a long overwintering. Désirée originates from the Netherlands, and is a popular and important cultivar thanks to its resistance properties to various pests and diseases. Due to the short regeneration cycle, and its responsiveness in *in vitro* experiments, Désirée is often used in genetic experiments (Beaujean *et al.* 1998).

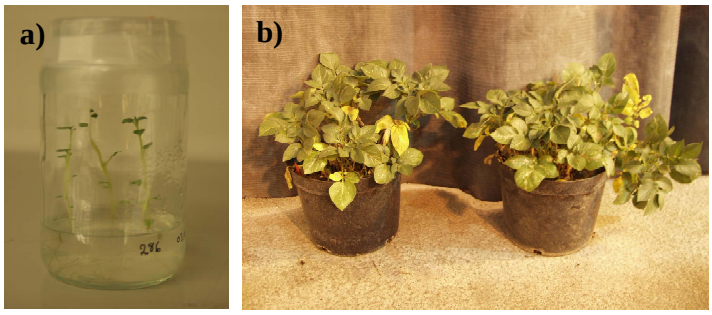


Figure 9. Transgenic and wild type *Solanum tuberosum* L. cv. Désirée a) kept under axenic conditions on Murashige-Skoog medium or b) in soil in plastic pots in a green house.

Agrobacterium-mediated transformation of potato

Transgenic potato plants were generated by *Agrobacterium tumefaciens*-mediated transformation of leaf-discs and internodal explants (Knapp *et al.* 1988; Beaujean *et al.* 1998). The procedure involves tissue culturing, *i. e.* shoot formation from transgenic micro-calli selected for a co-transferred antibiotic resistance trait. The variability of transgene expression among transformants, probably due to position effects from random transgene integration, and endogenous differential expression, was analysed by RNA blot experiments. The selectable marker kanamycin used in the initial experiments was shown to give more reproducible results compared to hygromycin, and was thus used in all subsequent studies.

Analytical methods

Sterols

Sterols were prepared from leaves and tubers using extraction methods designed according to the chemical properties of free and esterified sterols described above.

Free sterols and the sterol moieties from esterified sterols were extracted by a direct saponification of crushed leaf samples followed by an hexane extraction of the solution containing unsaponifiables. The method was used for screening large sets of leaf samples since it is fast and convenient. This direct saponification method turned out not to be as sensitive and accurate for measuring sterols present at low concentrations as other methods used in this work.

Free and esterified sterols were separated by running total lipids on a thin layer chromatography (TLC) plate with dichloromethane as developing solvent, prior to the saponification of steryl esters.

The 4-des-, 4-mono- and 4,4´dimethylsterol fractions could also be separated by TLC. The polarity of these compounds decreases as 4-desmethylsterols>4-monomthylsterols>4,4´-dimethylsterols.

Since the free sterol pool reflects the composition of sterols in the plasma membrane, while the free + esterified sterols reflects the amount of sterols synthesised and stored, it was of interest to analyse both fractions. When the free and esterified sterol fractions are compared, proportional differences in the sterol composition are observed (Table 4).

Table 4. Relative proportions in potato leaves of sterols in the free and esterified form, expressed as percentages of total desmethylsterols. F+E=Free + Esterified

	Relative proportion (%)				
Sterol fraction	Chole-sterol	24-Methyl-cholesterol	Isofuco-sterol	Sito-sterol	Stigma-sterol
Free	15	13	15	36	21
Esterified	7	33	8	39	12
F+E	13	19	12	37	19

Sterols were identified by gas chromatography-mass spectrometry (GC-MS), and quantified using gas chromatography (GC). Trimethylsilyl (TMS) ether derivatives of sterols were prepared to stabilise sterols and increase resolution in MS analysis.

Glycoalkaloids

Glycoalkaloids were extracted from plant materials and determined by high-performance liquid chromatography (HPLC) using an assay modified from Hellenäs (1986), according to the Swedish National Food Administration standards, Ref. NMKL 13.4, or using the Association of Official Analytical

Chemists (AOAC) official method 997.13. (Rita Svensson, SW Seed, pers. comm.). These methods are recommended for the routine analyses of glycoalkaloids, and were accurate and sensitive enough for our requirements. The analyses were performed by The Swedish Cereal Laboratory AB (Svalöv) or SW Seed, Svalöf Weibull AB.

Results and discussion

Reduction of cholesterol and glycoalkaloid levels in transgenic potato plants

Previous work indicated a key role for SMT1 activity in regulating the level of cholesterol and glycoalkaloids in the potato (Bergenstråhle *et al.* 1992). These observations were further investigated here by a direct overexpression of SMT1 in transgenic plants, and by analysis of SMT1 gene expression in relation to treatments known to affect the SMT1 enzymatic activity or the synthesis of glycoalkaloids.

The role of SMT1

Overexpression of SMT1 in transgenic potato plants

Potato was transformed to express a cDNA encoding the soybean (*Glycine max*) type 1 sterol methyltransferase, GmSMT1, from the constitutive CaMV 35S-derived promoter pTET1 (paper 1). The *GmSMT1* cDNA was chosen because the enzymatic specificity of the encoded protein had already been established (Shi *et al.* 1996), and the use of a heterologous cDNA would minimise the risk of co-suppression in transgenic potato plants. An enzyme assay using microsomal preparations from *GmSMT1*-potato, incubated with the preferred SMT1 substrate cycloartenol, showed that the transformants had about a 10-fold increase in the level of SMT1 activity compared to wild-type plants. The transgenic potato clones displayed an up to 360% increase in the total sterol levels, mainly due to increased levels of isofucosterol and sitosterol (24-ethylsterols). However, the level of free cholesterol (a 24-desmethylsterol) was decreased. Concurrent with the altered sterol composition, the transgenic plants also displayed reduced levels of glycoalkaloids, down to 41% (in leaves) and 63% (in tubers) of the wild-type levels. The transformants did not display any visible difference in their growth and development compared with wild-type controls. These results are well in line with cholesterol as a glycoalkaloid precursor, and were the first to show that glycoalkaloids can be manipulated by genetic transformation.

Analysis of potato SMT gene expression after ethrel treatment

The plant hormone ethylene has previously been shown to inhibit the accumulation of glycoalkaloids in wounded potato tubers, at least partly mediated by an increased SMT activity (Bergenstråhle *et al.* 1992). To further analyse this

finding, the capacity of ethylene to influence potato *SMT* (*StSMT*) expression at the mRNA level was examined. Wild type potato plants grown on Murashige Skoog medium (Figure 9a) were treated with 100µM, 1mM, 10mM and 100mM levels of ethrel, an ethylene-releasing substance. RNA was prepared from leaves and stems, and *StSMT* transcript levels were analysed by Northern blot using a potato specific *StSMT* probe. Ethrel, at concentrations of 1mM or higher, clearly increased *SMT* mRNA levels in potato (Figure 10). The probe used did not discriminate between SMT1 and SMT2 genes, and the increase may therefore be due to higher expression of either gene, or both. However, the ethylene-mediated increase in SMT1 enzymatic activity in potato (Bergenstråhle *et al.* 1992) suggests that the increase in SMT mRNA levels is at least partly due to an increased level of SMT1.

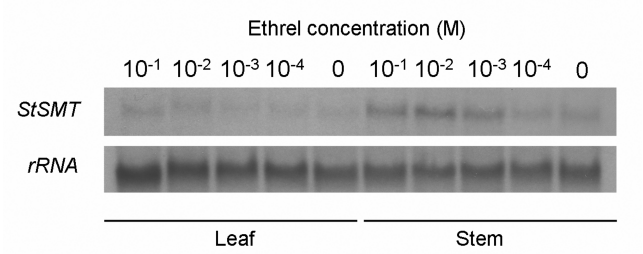


Figure 10. Ethrel treatment causes an up-regulation of *StSMT* transcript levels. Wild-type potato plantlets were treated with different concentrations of ethrel and *StSMT* transcript levels in leaves and stems were analysed by Northern blot using a probe specific to potato *SMT*.

Additional ways of reducing glycoalkaloid levels

Antisense expression of a potato Δ^{24}–reductase

A search for Arabidopsis sterol synthesis mutants with reduced levels of cholesterol, revealed such alterations in the *dim1* mutant, deficient in the sterol Δ^{24}–reductase enzyme DIMINUTO/DWARF1 (DIM/DWF1) (Klahre *et al.* 1998). While a role of DWF1 in plant cholesterol synthesis is not known, the human counterpart seladin-1 has been shown to convert desmosterol to cholesterol (Waterham *et al.* 2001). To analyse a role of DWF1 in plant cholesterol synthesis, a BLAST search of The Institute for Genomic Research (TIGR) potato EST database using the Arabidopsis sterol Δ^{24}-reductase DIM/DWF1 as the query, revealed clones that grouped under three tentative concensus (TC) sequences in the DFCI *Solanum tuberosum* Gene Index (StGI). The cDNAs (*StDWF1*s) encoded proteins with high similarity to DIM/DWF1. Constructs with these cDNAs in sense and antisense direction relative to the CaMV 35S promoter were used to transform potato plants, and the transformants then were screened for an altered sterol composition and glycoalkaloid level (Table 4). No clear-cut changes in the sterol composition were observed in either of the sense transformants. However, when the potato *StDWF1* expression was down-regulated in antisense transformants Δ24-sterols accumulated, and the levels of sitosterol, 24-methyl cholesterol and cholesterol were reduced. Interestingly, the glycoalkaloid level

was concomitantly reduced by over 50% with the reduced cholesterol level (Paper IV).

Table 4. Glycoalkaloid composition in tubers from separate *35S:StDWF1* sense and antisense transgenic potato clones. n=3 for wt. TGA, total glycoalkaloids, mg kg^{-1}

Genotype	α-solanine	α-chaconine	TGA
Désirée wt	56±9	163±23	219±32
StDWF1-1-1 sense	43	137	180
StDWF1-1-2 sense	45	139	184
StDWF1-1 antisense	25	70	95
StDWF1-2-1 sense	50	144	194
StDWF1-2-2 sense	53	167	220
StDWF1-2-3 sense	40	125	165
StDWF1-2-4 sense	47	156	203
StDWF1-2-5 sense	43	140	183

Hydroxylation of cholesterol in potato

To explore if an increased cholesterol catabolism in potato could be associated with a reduced glycoalkaloid content, four mouse cDNAs encoding cholesterol hydroxylases (CHs), catalysing the hydroxylation of cholesterol at the C-7, C-24, C-25, or C-27 position (Russel, 2000), were expressed in transgenic potato plants. In addition, to investigate the corresponding effects on sterol homeostasis in a plant species normally containing little cholesterol, the same constructs were also used to transform Arabidopsis (paper III, see below). The expected products, 7α-hydroxycholesterol and 25-hydroxycholesterol, were identified in the CH7- and CH25 transformants respectively (Figure 12a and 12c). The levels were quantified in two clones per type of transformant, showing 2.4 ± 0.23 mg kg^{-1} and 3.5 ± 0.21 mg kg^{-1} 7α-hydroxycholesterol and 6.6 ± 1.3 mg kg^{-1} and 8.5 ± 2.8 mg kg^{-1} 25-hydroxycholesterol. Low amounts of unidentified compounds were present in the fraction of hydroxylated sterols from CH24 transformants, while no hydroxysterols were observed in CH27 transformants or in wild-type. In addition, CH25 transformants also contained about 15 additional compounds tentatively identified as 25-hydroxylated sterols, at levels of about 4.5 mg kg^{-1} and lower, seen as peaks in the GC-MS chromatogram (Figure 11c).

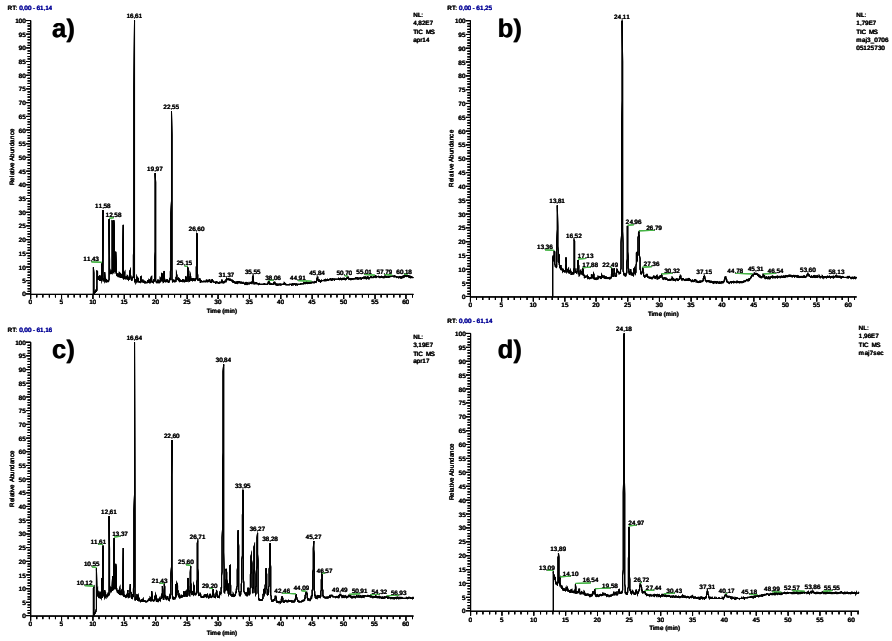


Figure 11. GC-separation of hydroxylated sterols extracted from leaves from a) CH7 potato, peak identity at Rt 16,61 = 5α-cholestane (internal standard), 22,55 = 19-hydroxycholesterol (internal standard), 19,97 = 7α-hydroxycholesterol; b) CH24 potato, peak identity at Rt 24,11=19-hydroxycholesterol (internal standard); c) CH25 potato, rt 16,64 = 5α-cholestane (internal standard), rt 22,60 = 19-hydroxycholesterol (internal standard), rt 30,84 = 25-hydroxycholesterol; d) CH27, rt 24,18 = 19-hydroxycholesterol. Hydroxylated sterols were extracted using the method described in paper III.

The sterol composition was investigated in a large set of transgenic CH7-, CH24-, CH25- and CH27 clones. However, in contrast to the case of Arabidopsis, expressing CH24 and CH25 (paper III), no clear-cut alterations in sterol profiles were observed in CH24- and CH25 for potato, although a certain variation was observed among the clones that needs to be analysed further. Neither CH7- nor CH27-potato plants displayed any significant changes in the sterol composition compared with wild-type, but this was also the case for CH7- and CH27 Arabidopsis plants (paper III).

Clones with dwarf-like phenotypes and misshapen leaves appeared frequently among the CH7- (two out of five) and CH25- (11 out of 24) potato clones, while all CH24- and CH27 potato plants displayed a wild-type morphology (Figure 11b). However, the severity of the phenotypic alteration could not be linked to a strong CH-transcript level, as analysed by Northern blot and RT-PCR (Figure 11a), *i. e.* did the transgenic CH7-clones no. 2, 3 and 4 have normal growth, while clones no. 1 and 5 showed the dwarf phenotype. The phenotypes might be the result of a higher degree of somaclonal variation in the transgenic clones.

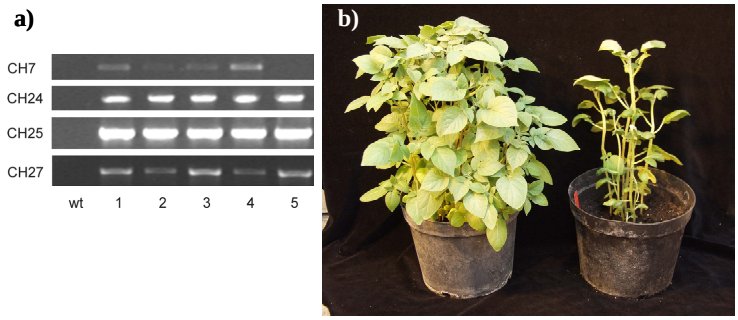


Figure 13. a) Transcript levels of transgenic potato expressing cholesterol hydroxylases, measured by rtPCR. b) The visible phenotype of a wild-type potato plant (left) and a CH7-potato clone (clone 1) showing reduced growth and misshapen leaves (right).

An increased hydroxylation of cholesterol in potato led to clearly detectable levels of hydroxysterols, but seemingly without a corresponding alteration in the level of cholesterol or other sterols. This suggests that this plant species, where cholesterol levels are normally high, is able to compensate for the loss of cholesterol caused by hydroxylation. This is presumably mediated by an increased rate of synthesis, thus maintaining sterols at normal levels. This suggests that increased degradation of cholesterol not will be an efficient way to reduce glycoalkaloid levels in transgenic plants. An analysis on the glycoalkaloids in the CH-expressing potato confirmed this (not shown).

Sterol metabolism and homeostasis

The likely precursor role of a sterol (cholesterol) in glycoalkaloid synthesis suggests that a detailed knowledge of sterol synthesis pathways, as well as the homeostatic mechanisms maintaining sterols at physiologically appropriate levels, is necessary to understand the metabolic partitioning of carbon into various sterol forms or into glycoalkaloids. To this end, we investigated the synthesis of stigmasterol, a sterol whose synthesis was not clear and where the postulated Δ^{22}-desaturase involved in its synthesis had not yet been described (paper II). In addition, we analysed effects on the general sterol metabolism in transgenic Arabidopsis plants where the out-put of cholesterol had been upregulated by hydroxylation (paper III).

Stigmasterol synthesis in plants

PCR cloning of CYP710A1 and CYP710A4

We hypothesised that synthesis of stigmasterol in plants is catalysed by a sterol C-22 desaturase, similar to the Erg5p-catalysed synthesis of ergosterol in yeast. A BLAST search with the yeast sterol C22-desaturase Erg5p revealed four sequences, denoted *CYP710A1*, *-A2*, *-A3*, and *-A4*, in the Arabidopsis genome, all of which encoded putative cytochrome P450 proteins with 24-25% identity to Erg5p (Table 5). Two members, *CYP710A1* and *CYP710A4* were overexpressed in

transgenic *Arabidopsis* to investigate the function of the corresponding proteins (paper II).

Table 5. Amino acid homology (%) between *Arabidopsis thaliana* CYP710A proteins and Erg5p from *Saccharomyces cerevisiae*. Values are from pairwise alignments.

	Homology (%)			
Enzyme	CYP710A1	CYP710A2	CYP710A3	CYP710A4
CYP710A2	82			
CYP710A3	77	74		
CYP710A4	76	74	93	
Erg 5p	25	24	24	25

Sterol C22-desaturase activity

Overexpression of *CYP710A1* in transgenic Arabidopsis plants led to an up to sevenfold increase in the total stigmasterol level (Figure 12), compared with wild-type plants. Concomitant with this was a decrease in the sitosterol level, whereas the levels of other sterols were unaltered. A similar effect, although much less pronounced, was seen in plants overexpressing *CYP710A4*. These results confirm and extend the recent findings regarding the function of CYP710A1 in stigmasterol synthesis (Morikawa *et al.* 2006).

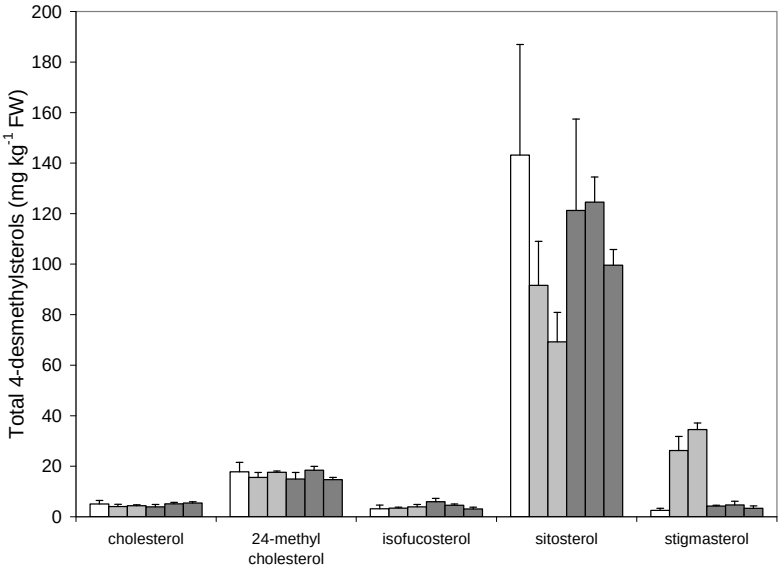


Figure 12. Total 4-desmethylsterol (free + esterified) levels in leaves from wild-type (white columns) and transgenic *CYP710A1* (light grey columns) and *CYP710A4* (dark grey columns) Arabidopsis plants. Mean value ± range from wild-type (n=10) and transgenic (n=3) lines.

Accumulation of steryl esters

The increased stigmasterol content in *CYP710A1* was due to increases of both free and esterified stigmasterol. When esterified sterols were extracted separately, an increased esterification of cholesterol, 24-methylcholesterol and isofucosterol was also observed, despite the fact that their levels were not increased by the transformation. This suggests that an increased stigmasterol level alone is sufficient to stimulate esterification of other major sterols, and provides insight into potential feedback mechanisms in sterol metabolism.

The identification of stigmasterol synthase probably represents one of the last clonings in Arabidopsis of a gene encoding a sterol-synthesising enzyme. The completion of sterol gene isolation in this model plant should now increase our understanding on the regulation of sterol metabolism.

Transgenic Arabidopsis plants with increased hydroxysterol synthesis

To explore the possible function of hydroxylated cholesterol in plants, the four mouse cDNAs encoding cholesterol hydroxylases (CHs) described above were expressed in Arabidopsis from the CaMV 35S promoter. Transgenic lines were selected on kanamycin-containing medium and the transgenic expression was verified by Northern blot- and RT-PCR-experiments in the T3 generation. Analyses on the hydroxysterol fractions from these plants showed that the transgenic CH7-, CH24-, and CH25 lines contained compounds determined as 7α-hydroxy-, 24-hydroxy- and 25-hydroxycholesterol respectively. CH24 lines also contained a compound determined as 24-hydroxy-24-methylcholesterol and CH25 lines contained 11 additional compounds determined as 25-hydroxysterols, including compounds tentatively identified as 25-hydroxy-24-methylenecholesterol, 25-hydroxy-24-methylcholesterol, 25-hydroxy-isofucosterol and 25-hydroxy-stigmasterol. In contrast, no 27-hydroxysterols were observed in CH27 plants. These results were well in line with the observations from the parallel study of these genes expressed in potato, although the levels of 7α-hydroxycholesterol in CH7 potato plants were 20-fold to 30-fold higher than they were in CH7 Arabidopsis plants, indicating that cholesterol, as a substrate for the CHs, might be limiting in Arabidopsis.

The CH24- and CH25 transgenic Arabidopsis lines showed alterations in the sterol composition, mainly due to increased levels of 24-methylenecholesterol, 24-methylcholesterol and isofucosterol, and decreases in the level of sitosterol. The strongest effects on the sterol homeostasis were seen in the CH25-lines, which even contained increased levels of cholesterol. Increases of 4,4´-di- and 4-monomethylsterols were observed in CH25 lines. Sterol analyses of CH7- and CH27 lines did not reveal any alterations compared to the composition of sterols in wild-type plants.

Certain CH25 plants displayed a stunted growth, had dark-green leaves and a dwarf phenotype resembling brassinosteroid-related mutants. The general phenotype of CH7-, CH24- and CH27 plants was similar to that of control lines, but several CH7 and CH24 lines were somewhat smaller in size. An analysis on

the brassinosteroid levels in CH25 lines showed decreased levels of the brassinolide (BL) precursors indicating a blocking/down-regulation of the BL biosynthetic pathway in CH25 plants. They were responsive to BL treatment in both root and shoot, indicating that reduced endogenous level of brassinosteroids probably is the prime cause of the CH25 dwarf phenotype.

The interpretation of these results must consider the fact that they are the outcomes from two changed parameters in connection with the sterol hydroxylation process; (1) the output of sterols, and (2) the production of hydroxysterols. These parameters could be studied separately by increasing the output of sterols in another way than by hydroxylation, *e. g.* by increasing the sterol conjugation to fatty acids, or to apply hydroxysterols to the plants exogenously. In addition, the altered growth of Arabidopsis CH25 plants could be a consequence of the presence of either (or several) of the hydroxysterols produced in these plants. To test if the presence of 25-hydroxycholesterol as such influences growth, Arabidopsis wild-type seeds were germinated on a 25-hydroxycholesterol containing medium. The plants revealed a normal phenotype and sterol composition suggesting that either the synthesis of other metabolites than 25-hydroxycholesterol or the output of plant sterols causes the phenotype of CH25 plants.

Trace levels of the compound determined as 7α-hydroxycholesterol were observed in wild-type Arabidopsis as well as in CH24-, CH25- and CH27 transformants. This is an interesting observation since small quantities of exogenously applied 7-hydroxycholesterol and 7-hydroxysitosterol have previously been shown to induce resistance against fungal pathogens in Barley (*Hordeum vulgare* L.) (Schabdach *et al.* 1995; König & Seifert 1998), and the transformants could hence be used to test these aspects in more detail.

Conclusions and applications

The results presented in this thesis provide increased knowledge about the genes and regulatory mechanisms in plant sterol metabolism, and their roles in glycoalkaloid biosynthesis in cultivated potato. Furthermore, genetic engineering has been successfully used as a tool to control glycoalkaloid levels in potato. The findings are valuable for the understanding of sterol homeostasis, and might become useful in biotechnological aspects of plant breeding, *e. g.* genetic engineering, as well as in other aspects, *e. g.* screening for natural genetic variation in the expression of genes in sterol and glycoalkaloid biosynthesis, in present and future crop varieties.

The specific conclusions and tentative applications from the results presented in this thesis are as follows.

- *SMT1* overexpression reduces the cholesterol and glycoalkaloid levels in transgenic potato. Transgenic plants with reduced glycoalkaloid levels can now be used to study the biological function of glycoalkaloids, for

instance the effects of a reduced glycoalkaloid level on the growth or survival of potato plants in the field. Overexpression of SMT1 might be used as a tool to create genetically modified potato varieties with consistently low glycoalkaloid levels.

- *SMT1* overexpression increases the total sterol content in potato, mainly due to increased levels of isofucosterol and sitosterol. These plant sterols are of interest in human nutrition because they have a structure similar to cholesterol and can lower human plasma cholesterol and LDL cholesterol by competing for intestinal absorption (Piironen et al. 2000 and references therein). Genetic engineering of SMT1 expression in oil crops could increase the plant sterol levels and decrease the cholesterol level in oils used for human consumption.
- CYP710A1 and CYP710A4 regulate the biosynthesis of stigmasterol in plants. An increased stigmasterol/sitosterol ratio does not influence the visual plant morphology of Arabidopsis, and the increase of stigmasterol alone is concurrent with an upregulated sterol esterification. The cloning of the genes encoding sterol Δ^{22}-desaturases will be useful to clarify the function of stigmasterol and brassicasterol in plant biological processes. Cloning and expression of CYP710A genes in *Digitalis* species can clarify the role of stigmasterol as the precursor of cardenolides and it may be possible to enhance the cardenolide production by a metabolically engineered CYP710A activity in cardenolides-producing crops. A modulation of the sterol composition towards increased stigmasterol content in oil crops could be another application.
- Increased levels of hydroxylated sterols alters steroid metabolism and influences plant growth in Arabidopsis. The transgenic plants with increased levels of hydroxysterols may be used to elucidate a possible role for these compounds as signal molecules in *e. g.* plant defence against predators.
- The *StDWF1* gene is a potato *DIM/DWF1* orthologue. Down-regulation of *StDWF1* expression in potato increases Δ^{24}-sterols at the expense of cholesterol, 24-methylcholesterol and sitosterol, and lowers glycoalkaloid levels as well. This suggests that this enzyme may have a role in cholesterol synthesis that has not so far been recognised.

Suggested research

In relation to the results presented within this thesis, the following future lines of research are suggested:

- Genes involved in sterol and glycoalkaloid biosynthesis could be investigated by microarray analysis of potato materials with different glycoalkaloid levels. Such materials could, for instance, be light-induced or wounded tubers, or just genotypes differing in their basal glycoalkaloid level.
- To further study a role of stigmasterol, *e. g.* in defence against pathogens, plants with increased or repressed sterol Δ^{22}-desaturase activity could be

tested for increased/decreased resistance after exposure to biotic and abiotic stresses. In the case of creating plants with decreased stigmasterol levels, it would be most interesting to use plant materials with naturally high levels of stigmasterol, *e. g.* cultivated potato and the moss *Physcomitrella patens*. The repression of sterol C22-desaturase activity can be carried out by cloning and silencing (by antisense expression or RNAi) of CYP710A homologues within these plants. Furthermore, the role of stigmasterol as a precursor to cardenolides in *Digitalis minor* can be investigated by overexpressing the activity of CYP710A genes in this species.

- To clarify the mechanisms behind the altered sterol homeostasis in Arabidopsis CH24- and CH25 transformants, the expression of genes in sterol synthesis could be studied by PCR or microarray. In order to investigate the occurrence of hydroxylated sterols as endogenous signal compound in plants, oxysterol fractions should be extracted from larger quantities of starting material, ~100 g fresh weight plant tissue, to allow detection of compounds present in very low quantities. The function of hydroxysterols in plants could be further explored by studies of responses to exogenously applied hydroxylated sterols. An identification of plausible endogenous sterol hydroxylases in plants, apart from those involved in brassinosteroid synthesis, would help to clarify these questions. However, hydroxylated sterols might also be produced by autooxidation, and genes in their synthesis are thus not a necessity.
- The biosynthesis of cholesterol and glycoalkaloids in plants is still not clear. One approach to identify genes in cholesterol synthesis could be to investigate the expression of genes within species that contains high cholesterol levels at certain developmental stages, or in certain tissues, *e. g.* in the case of early apical development of *Brassica campestris* (Hobbs *et al.* 1996). Furthermore, one could look for accumulating sterol intermediates in the 4,4´-dimethyl- and 4-monomethylsterol fractions in plants overexpressing HMGR, or alternatively, with reduced expression of SMT1, and perhaps thereby identify rate limiting steps in the cholesterol biosynthetic pathway in plants. The conversion of cholesterol and other intermediates to glycoalkaloids could be established by feeding with heavy-isotope-labeled substrates, coupled to HPLC-MS analysis.
- To gain more insight into the regulation of plant sterol and glycoalkaloid biosynthesis on the transcriptional level, a survey of the promoter regions of steroid regulating genes might lead to the identification of targets for transcription factors within these sequences. Important mechanisms for coordinating co-transcription of genes involved in sterol and glycoalkaloid regulation might be revealed in this way.
- The preservation of the genetic diversity found in primitive cultivars and wild relatives of crops is important for sustainable agriculture. This diversity constitutes a genetic resource in the breeding for new cultivars, resistant/tolerant to present and future biotic and abiotic stresses. There are about 225 tuber-bearing species in the *Solanum* genera (Mullins *et al.* 2006) and it would be prudent to explore variability in sterol and glycoalkaloid regulation among the wild and semi-wild *Solanum* species

as they may become, and some already are, used in breeding programs. Moreover, the unconscious selection of certain genotypes during the evolution of the cultivated potato probably involved selection for low glycoalkaloid levels in the tubers. Furthermore, wild diploid *Solanum* species could become useful for making knockout mutants with deficiencies in sterol or glycoalkaloid biosynthesis. Analyses on steroid composition and gene expression in tuber bearing *Solanum* species could tell us more about ”natural” targets for glycoalkaloid regulation, and the evolution of glycoalkaloid-regulating genes.

References

Akeley, R.V., Mills, W.R., Cunningham, C.E. & Watts, J. 1968. Lenape: a new potato variety high in solids and chipping quality. *Am. Potato J.* 45, 142-145

Akihisa, T., Kokke, W. & Tamura, T. 1991. Naturally occurring sterols and related compounds from plants. In *Physiology and biochemistry of sterols.* Edited by G.W. Patterson & W.D. Nes. The American Oil Chemists Society, Champaign. Illinois. 172-228. pp.

Altmann, T. 1998. A tale of dwarfs and drugs: brassinosteroids to the rescue. *Trends Genet.*14, 490-495

Asami, T. & Yoshida, S. 1999. Brassinosteroid biosynthesis inhibitors. *Trends Plant Sci.* 4, 348-353

Aubert, S., Daunay, M.C. & Pochard, E. 1989. Saponins and steroidal alkaloids of eggplant (*Solanum melongena*). Food interest, analytical methods, and localization in fruit. *Agronomie* 9, 641-651

Austin, S., Lojkowska, E., Ehlenfeldt, M.K., Kelman, A. & Helgeson, J.P. 1988. Fertile interspecific somatic hybrids of *Solanum*: A novel source of resistance to *Erwinia* soft rot. *Phytopathology* 78, 1216-1220

Azadmard-Damirchi, S., Savage, G.P. & Dutta, P.C. 2005. Sterol fractions in hazelnut and virgin olive oils and 4,4´-dimethylsterols as possible markers for detection of adulteration of virgin olive oil. *J. Am. Oil Chem. Soc.* 82, 717-725

Bach, T.J. 1986. Hydroxymethylglutaryl-CoA reductase, a key enzyme in phytosterol synthesis? *Lipids* 21, 82-88

Bach, T.J. & Benveniste, P. 1997. Cloning of cDNAs or genes encoding enzymes of sterol biosynthesis from plants and other eukaryotes: heterologous expression and complementation analysis of mutations for functional characterization. *Prog. Lipid Res.* 36, 197-226

Bak, S., Kahn, R.A., Olsen, C.E. & Halkier, B.A. 1997. Cloning and expression in Escherichia coli of the obtusifoliol 14α-demethylase of Sorghum bicolor (L.) Moench, a cytochrome P450 orthologous to the sterol 14α-demethylases (CYP51) from fungi and mammals. *Plant J.* 11, 191-201

Banas, A., Carlsson, A.S., Huang, B., Lenman, M., Banas, W., Lee, M., Noiriel, A., Benveniste, P., Schaller, H., Bouvier-Navé, P. & Stymne, S. 2005. Cellular sterol ester synthesis in plants is performed by an enzyme (phospholipid:sterol acyltransferase) different from the yeast and mammalian acyl-CoA:sterol acyltransferases. *J. Biol. Chem.* 280, 34626-34634

Bard, M., Bruner, D.A., Pierson, C.A., Lees, N.D., Biermann, B., Frye, L., Koegel, C. & Barbuch, R. 1996. Cloning and characterization of *ERG25*, the *Saccharomyces cerevisiae* gene encoding C-4 sterol methyl oxidase. *Proc. Natl. Acad. Sci. USA* 93, 186-190

Beaujean, A., Sangwan, R.S., Lecardonnel, A. & Sangwan-Norreel, B.S. 1998. *Agrobacterium*-mediated transformation of three economically important potato cultivars

using sliced intermodal explants: an efficient protocol of transformation. *J. exp. bot.* 49, 1589-1595

Bechtold, N., Ellis, J. & Pelletier, G. 1993. In planta Agrobacterium mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. *C.R. Acad. Sci. Paris Life Sci.* 316, 1194-1199

Beneke, F.W. 1862. Cholesterin im Pflanzenreich aufgefunden. *Ann. chem. pharm.* 122, 249-255.

Benveniste, P. 2002. Sterol metabolism. In.*The Arabidopsis Book*. Edited by C. Somerville & E. Meyerowitz. The American Society of Plant Biologists. Rockville. Maryland.

Benveniste, P. 2004. Biosynthesis and accumulation of sterols. *Annu. Rev. Plant Biol.* 55, 429-457

Bergenstråhle, A. 1995. Glycoalkaloid Synthesis in potato tubers. PhD thesis, Swedish University of Agricultural Sciences. SLU/Repro, Uppsala. Sweden

Bergenstråhle, A., Tillberg, E. & Johnsson, L. 1992. Regulation of glycoalkaloid accumulation in potato tuber discs. *J. Plant Physiol.* 140, 269-275

Berthelot. 1859. Sur plusieurs alcools nouveaux. Combinaisons des acids avec la cholestérine, l'éthal, le camphre de Bornéo et la méconine. *Ann. chim. phys*. 56, 51-98

Bhat, R.A. & Panstruga, R. 2005. Lipid rafts in plants. *Planta* 223, 5-19

Bills, C.E. 1935. Physiology of the sterols, including vitamin D. *Physiol. rev.* 15, 1-97

Borner G.H.H., Sherrier, D.J., Weimar, T., Michaelson, L.V., Hawkins, N.D., MacAskill, A., Napier, J.A., Beale, M.H., Lilley, K.S. & Dupree, P. 2005. Analysis of detergent-resistant membranes in Arabidopsis. Evidence for plasma membrane lipid rafts. *Plant Physiol.* 137, 104–116

Bouvier-Navé, P. & Benveniste, P. 1995. Sterol acyl transferase and steryl ester hydrolase activities in a tobacco mutant which overproduces sterols. *Plant Sci.* 110, 11-19

Bouvier-Navé, P., Husselstein, T. & Benveniste, P. 1998. Two families of sterol methyltransferases are involved in the first and second methylations steps of plant sterol biosynthesis. *Eur. J. Biochem.* 256, 88-96

Bouvier-Navé, P., Husselstein, T., Desprez, T. & Benveniste, P. 1997. Identification of cDNAs encoding sterol methyl-transferases involved in the second methylation step of plant sterol biosynthesis. *Eur. J. Biochem*. 246, 518-529

Bush, P.B. & Grunwald, C. 1972. Sterol changes during germination of *Nicotiana tabacum* seeds. *Plant Physiol.* 50, 69-72

Canonica, L., Ronchietti, F., Russo, G. & Sportoletti, G. 1977. Fate of the 16β-hydrogen atom of cholesterol in the biosynthesis of tomatidine and solanidine. *J. C. S. Chem. Comm.* 286-287

Carland, F.M., Fujioka, S., Takatsuto, S., Yoshida, S. & Nelson, T. 2002. The identification of *CVP1* reveals a role for sterols in vascular patterning. *Plant Cell* 14, 2045-2058

Chappell, J., Vonlanken, C., Vögeli, U. & Bhatt, P. 1989. Sterol and sesquiterpenoid biosynthesis during a growth-cycle of tobacco cell suspension cultures. *Plant Cell Rep.* 8, 48-52

Chevreul, M.E. 1815. Recherches chimiques sur plusieurs corps gras et particulièrement sur leurs combinaisons avec les alkalis. *Ann. Chim.* 94, 80-107, 113-144.

Chitwood, D.J. 1991. Nematode sterol biochemistry. In *Physiology and biochemistry of sterols.* Edited by G.W. Patterson & W.D. Nes. The American Oil Chemists Society, Champaign. Illinois. 257-293. pp.

Choe, S., Dilkes, B.P., Gregory, B.D., Ross, A.S., Yuan, H., Noguchi, T., Fujioka, S., Takatsuto, S., Tanaka, A., Yoshida, S., Tax, F.E. & Feldmann, K.A. 1999a. The Arabidopsis dwarf1 mutant is defective in the conversion of 24-methylenecholesterol to campesterol in brassinosteroid biosynthesis. *Plant Physiol.* 119, 897-907

Choe, S., Noguchi, T., Fujioka, S., Takatsuto, S., Tissier, C.P., Gregory, B.D., Ross, A.S., Tanaka, A., Yoshida, S., Tax, F.E. & Feldmann, K.A. 1999b. The Arabidopsis dwf7/ste1 mutant is defective in the Δ^7 sterol C-5 desaturation step leading to brassinoid biosynthesis. *Plant Cell* 11, 207-221

Choe, S., Tanaka, A., Noguchi, T., Fujioka, S., Takatsuto, S., Ross, A.S., Tax, F.E., Yoshida, S. & Feldmann, K.A. 2000. Lesions in the sterol Δ^7 reductase gene of

Arabidopsis cause dwarfism due to block in brassinosteroid biosynthesis. *Plant J.* 21, 431-443
Choi, D., Ward, B.L. & Bostock, R.M. 1992. Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl Coenzyme A reductase genes in response to *Phytophtora infestans* and its elicitor arachidonic acid. *Plant Cell* 4, 1333-1344
Clough, S.J. & Bent, A.F. 2000. Female reproductive tissues are primary target of *Agrobacterium*-mediated transformation by the Arabidopsis floral-dip method. *Plant physiol.* 123, 895-904
Clouse, S.D. 2002. Arabidopsis mutants reveal multiple roles for sterols in plant development. *Plant Cell* 14, 1995-2000
Concon, J. 1988. Food toxicology: principles and concepts. Part A.N.Y., Marcel Dekker Inc. New York.
Corey, E.J., Matsuda, S.P.T. & Bartel, B. 1993. Isolation of an *Arabidopsis thaliana* gene encoding cycloartenol synthase by functional expression in a yeast mutant lacking lanosterol synthase by the use of a chromatographic screen. *Proc. Natl Acad. Sci. USA* 90, 11628-11632
Costet-Corio, M.F., Chapuis, L., Mouillet, J.F. & Delbecque, J.P. 1993. Sterol and ecdysteroid profiles of *Serratula tinctoria* L.: plant and cell cultures producing steroids. *Insect Biochem. Mol. Biol.* 23, 175-180
Dale, M.F.B., Griffiths, D.W., Bain, H. & Todd, D. 1993. Glycoalkaloid increase in *Solanum tuberosum* on exposure to light. *Ann. Appl. Biol.* 123, 411-418
Darnet, S., Bard, M. & Rahier, A. 2001. Functional identification of sterol-4α-methyl oxidase cDNAs from *Arabidopsis thaliana* by complementation of a yeast *erg25* mutant lacking sterol-4α-methyl oxidation. *FEBS Lett.* 508, 39-43
Darnet, S. & Rahier, A. 2004. Plant sterol biosynthesis: identification of two distinct families of sterol 4α-methyl oxidases. *Biochem. J.* 378, 889-898
D'Aurea & Gershenzon 2005. The secondary metabolism of *Arabidopsis thaliana*: growing like a weed. *Curr. Opin. Plant Biol.* 8, 308-316
Deahl, K.L., Cantelo, W.W., Sinden, S.L. & Sanford, L.L. 1991. The effect of light intensity on colorado potato beetle resistanceand foliar glycoalkaloid concentration of four *Solanum chacoense* clones. *Am. Potato J.* 68, 659-666
Devarenne, T.P., Shin, D.H., Back, K., Yin, S.H. & Chappell, J. 1998. Molecular characterization of tobacco squalene synthase and regulation in response to fungal elicitor. *Arch. Biochem. Biophys.* 349, 205-215
Diener, A.C., Li, H., Zhou, W., Whoriskey, W.J., Nes, W.D & Fink, G.R. 2000. Sterol methyl transferase 1 controls the level of cholesterol in plants. *Plant Cell* 12, 853-870
Dyas, L. & Goad, L.J. 1993. Steryl fatty acyl esters in plants. *Phytochemistry* 34, 17-29
Ellis, M.T. 1918. Contributions to our knowledge of the plant sterols. Part I. The sterol content of wheat (*Triticum sativum*). *Biochem. J.* 12, 160-172
Eltayeb, E.A., AlAnsari, A.S & Roddick, J.G. 1997. Changes in the steroidal alkaloid solasodine during development of *Solanum nigrum* and *Solanum incanum. Phytochemistry* 46, 489-494
Entchev, E.V. & Kurzchalia, V. 2005. Requirement of sterols in the life cycle of the nematode *Caenorhabditis elegans*. *Semin. Cell Dev.Biol*. 16, 175-182
Fewell, A.M. & Roddick, J.G. 1997. Potato glycoalkaloid impairment of fungal development. *Mycol. Res.* 101, 597-603
Fontaine, J., Grandmougin-Ferjani, A., Hartmann, M.-A. & Sancholle, M. 2001. Sterol biosynthesis by the arbuscular mycorrhizal fungus *Glomus intraradices*. *Lipids* 36, 1357-1363
Friedman, M. 2002. Tomato glycoalkaloids: role in the plant and in the diet. *J. Agric. Food Chem.* 50, 5751-5780
Friedman, M. 2006. Potato glycoalkaloids and metabolites: roles in the plant and in the diet. *J. Agric. Food Chem.* 54, 8655-8681
Friedman, M. & Dao, L. 1992. Distribution of glycoalkaloids in potato plants and commercial potato. *J. Agric. Food Chem.* 40, 419-423
Friedman, M., Roitman, J.N. & Kozukue, N. 2003. Glycoalkaloid and calystegine contents of eight potato cultivars. *J. Agric. Food Chem.* 51, 2964-2973

Fujioka, S. & Yokota, T. 2003. Biosynthesis and metabolism of brassinosteroids. *Annu. Rev. Plant Biol.* 54, 137-164

Gachotte, D., Husselstein, T., Bard, M., Lcroute, F. & Benveniste, P. 1996. Isolation and characterization of an *Arabidopsis thaliana* cDNA encoding a Δ^7-sterol-C-5-desaturase by functional complementation of a defective yeast mutant. *Plant J.* 9, 391-398

Gachotte, D., Meens, R. & Benveniste, P. 1995. An Arabidopsis mutant deficient in sterol biosynthesis: heterologous complementation by *ERG 3* encoding a Δ^7-sterol-C-5-desaturase from yeast. *Plant J.* 8, 407-416

Godoy-Hernández, G.C., Chappell, J., Devarenne, T.P., García-Pineda, E., Gueva-García, A.A. & Lozoya-Gloria, E. 1998. Antisense expression of *HMG*1 from *Arabidopsis thaliana* encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase, reduces isoprenoid production in transgenic tobacco plants. *J. Plant Physiol*. 153, 415-424

Gondet, L., Bronner, R. & Benveniste, P. 1994. Regulation of sterol content in membranes by subcellular compartmentation of steryl-esters accumulating in a sterol-overproducing tobacco mutant. *Plant. physiol.* 105, 509-518

Gordon, T.R. & Webster, R.K. 1984. Evaluation of ergosterol as an indicator of infestation of barley seed by *Drechslera graminea. Phytopathology* 74, 1125-1127

Greeve, I., Hermans-Borgmeyer, I., Brellinger, C., Kasper, D., Gomez-Isla, T., Behl, C., Levkau, B. & Nitsch, R.M. 2000. The human DIMINUTO/DWARF1 homolog seladin-1 confers resistance to Alzheimer's disease-associated neurodegeneration and oxidative stress. *J. Neurosci.* 20, 7345-7352

Griffiths, D.W., Bain, H. & Dale, F.B 1998. Effects of storage temperature on potato (*Solanum tuberosum* L.) tuber glycoalkaloid content and the subsequent accumulation of glycoalkaloids and chlorophyll in response to light exposure. *J. Agric. Food Chem.* 46, 5262-5268

Grunwald, C. 1975. Phytosterols in tobacco leaves at various stages of physiological maturity. *Phytochemistry* 14, 79-82

Grunwald, C. 1978. Shading influence on the sterol balance of *Nicotiana tabacum* L. *Plant Physiol.* 61, 76-79

Guye, M.G. 1988. Sterol composition in relation to chill-sensitivity in *Phaseolus* spp. *J. Exp. Bot.* 39, 1091-1096

Guye, M.G. 1989. Phospholipid, sterol composition and ethylene production in relation to choline-induced chill-tolerance in mung bean (*Vigna radiata* L. Wilcz.) during a chill-warm cycle. *J. Exp. Bot.* 40, 369-374

Hajslova, J., Schulzova, V., Slanina, P., Janne, K., Hellenäs, K.E. & Andersson, Ch. 2005. Quality of organically and conventionally grown potatoes: four-year study of micronutrients, metals, secondary metabolites, enzymic browning and organoleptic properties. *Food Addit. Contam.* 22, 514-534

Hartmann, M.-A. 1998. Plant sterols and the membrane environment. *Trends Plant Sci.* 3, 170-175

Hase, Y., Fujioka, S., Yoshida, S., Sun, G., Umeda, M. & Tanaka, A. 2005. Ectopic endoreduplication caused by sterol alteration results in serrated petals in *Arabidopsis. J. Exp. Bot.* 56, 1263-1268

Hase, Y., Tanaka, A., Baba, T. & Watanabe, H. 2000. FRL1 is required for petal and sepal development in Arabidopsis. *Plant J.* 24, 21–32.

He, J.-X., Fujioka, S., Li, T.-C., Kang, S.G., Seto, H., Takatsuto, S., Yoshida, S. & Jang, J.-C. 2003. Sterols regulate development and gene expression in Arabidopsis. *Plant Physiol.* 131, 1258-1269

Heftmann, E. 1983. Biogenesis of steroids in Solanaceae. *Phytochemistry* 22, 1843-1860

Hellenäs, K.-E. 1986. A simplified procedure for quantification of potato glycoalkaloids in tuber extracts by HPLC: comparison with ELISA and a colorimetric method. *J. Sci. Food Agric.* 37, 776-782

Hellenäs, K.-E., Branzell, C., Johansson, H. & Slanina, P. 1995. High levels of glycoalkaloids in the established Swedish potato variety Magnum Bonum. *J. Sci. Food Agric.* 68, 249-255

Hellgren, L.I., Selldén, G. & Sandelius, A.S. 2001. Effects of moderately enhanced levels of ozone on the acyl lipid composition and dynamical properties of plasma membranes isolated from garden pea (*Pisum sativum*). *Physiol. Plant.* 111, 165-171

Hobbs, D.H., Hume, J.H., Rolph, C.E. & Cooke, D.T. 1996. Changes in lipid composition during floral development of *Brassica campestris. Phytochemistry* 42, 335-339

Hu, M.-C., Chiang, E.F.-L., Tong, S.-K., Lai, W., Hsu, N.-C., Wang, L.C.-K. & Chung, B.-c. 2001. Regulation of steroidogenesis in transgenic mice and zebrafish. *Mol. Cell Endocrinol*. 171, 9-14

Husselstein, T., Gachotte, D., Desprez, T., Bard, M. & Benveniste, P. 1996. Transformation of *Saccharomyces cerevisiae* with a cDNA encoding a sterol C-methyltransferase from *Arabidopsis thaliana* results in the synthesis of 24-ethyl sterols. *FEBS Lett.* 381, 87-92

Husselstein, T., Schaller, H., Gachotte, D and Benveniste, P. 1999. Δ^7-sterol-C5-desaturase: molecular characterization and functional expression of wild-type and mutant alleles. *Plant Mol. Biol.* 39, 891-906

Izzo, R. & Navari-Izzo, F. 1981. Sterol composition and accumulation in *Glycine max* (L.) Merr. leaves under different filtered sunlight conditions. *Plant Physiol.* 67, 1073-1077

Jang, J.-C., Fujioka, S., Tasaka, M., Seto, H., Takatsuto, S., Ishii, A., Aida, M., Yoshida, S. & Sheen, J. 2000. A critical role of sterols in embryonic patterning and meristem programming revealed by the *fackel* mutants of *Arabidopsis thaliana*. *Genes Dev.* 14, 1485-1497

Jonasson, T. & Olsson, K. 1994. The influence of glycoalkaloids, chlorogenic acid and sugars on the susceptibility of potato tubers to wireworm. *Potato res.* 37, 205-216

Keukens, E.A.J., de Vrije, T., Jansen, L.A.M., de Boer, H., Janssen, M., de Kroon, A.I.P.M., Jongen, W.M.F. & de Kruijff, B. 1996. Glycoalkaloids selectively permeabilize cholesterol containing biomembranes. *Biochim. Biophys. Acta* 1279, 243-250

Klahre, U., Noguchi, T., Fujioka, S., Takatsuto, S., Yokota, T., Nomura, T., Yoshida, S. & Chua, N.-H. 1998. The Arabidopsis *DIMINUTO/DWARF1* gene encodes a protein involved in steroid synthesis. *Plant Cell* 10, 1677-1690

Knapp, S., Coupland, G., Uhrig, H., Starlinger, P. & Salamini, F. 1988. Transposition of the maize transposable element *Ac* in *Solanum tuberosum. Mol. Gen. Genet.* 213, 285-290

Kolesnikova, M.D., Xiong, Q., Lodeiro, S., Hua, L. & Matsuda, S.P.T. 2006. Lanosterol biosynthesis in plants. *Arch. Biochem. Biophys.* 447, 87-95

Koornneef, M., Alonso-Blanco, C. & Vreugdenhil, D. 2004. Naturally occurring genetic variation in *Arabidopsis thaliana*. *Annu. Rev. Plant Biol.* 55, 141-172

Korpan, Y.I., Nazarenko, E.A., Skryshevskaya, I.V., Martelet, C., Jaffrezic-Renault, N. and El'skaya, A.V. 2004. Potato glycoalkaloids: true safety or false sense of security? *Trends Biotechnol.* 22, 147-151

Kreis, W., Hensel, A. & Stuhlemmer, U. 1998. Cardenolide biosynthesis in foxglove. *Planta Med.* 64, 491-499

Krits, P., Fogelman, E. & Ginzberg, I. 2007. Potato steroidal glycoalkaloid levels and the expression of key isoprenoid metabolic genes. *Planta* DOI 10.1007/s00425-007-0602-3

Kuhn, R. & Löw, I. 1954. Die konstitution des solanins. *Angew. Chem.* 66, 639-640

König, M. & Seifert, K. 1998. Uptake and translocation of exogenously applied 7-oxosterols in barley. *Phytochemistry* 49, 1257-1263

Laloi, M., Perret, A.-M., Chantre, L., Melser, S., Cantrel, C., Vaultier, M-N., Zachowski, A., Bathany, K., Schmitter, J.-M., Vallet, M., Lessire, R., Hartmann, M.-A. & Moreau, P. 2007. Insight into the role of specific lipids in the formation and delivery of lipid microdomains to the plasma membrane of plant cells. *Plant Physiol.* 143, 461-472

Lees, N.D., Skaggs, B., Kirsch, D.R. & Bard, M. 1995. Cloning of the late genes in the ergosterol biosynthetic pathway of *Saccharomyces cerevisiae* - a review. *Lipids* 30, 221-226

Lindenmeyer, O. 1863. Beiträge zur Kenntniss des cholesterins. *J. prakt. chem.* 90, 321-331

Lovato, M.A., Hart, E.A., Segura, M.J.R., Giner, J.L. & Matsuda, S.P.T. 2000. Functional cloning of an *Arabidopsis thaliana* cDNA encoding cycloeucalenol cycloisomerase. *J. Biol. Chem.* 275, 13394-13397

Mandimika, T., Baykus, H., Poortman, J., Garza, C., Kuiper, H. & Peijnenburg, A. 2007. Induction of the cholesterol biosynthesis pathway in differentiated Caco-2 cells by the potato glycoalkaloid α-chaconine. *Food Chem. Toxicol.* 45, 1918-1927

Mansour, M.M.F., van Hasselt, P.R. & Kuiper, P.J.C. 1994. Plasma membrane lipid alterations induced by NaCl in winter wheat roots. *Physiol. Plant.* 92, 473-478

McCue, K.F., Allen, P.V., Shepherd, L.V.T., Blake, A., Maccree, M.M., Rockhold, D.R., Novy, R.G., Stewart, D., Davies, H.V. & Belknap, W.R. 2007. Potato glycosterol rhamnosyltransferase, the terminal step in triose side-chain biosynthesis. *Phytochemistry* 68, 327-334

McCue, K.F., Allen, P.V., Shepherd, L.V.T., Blake, A., Whitworth, J., Maccree, M.M., Rockhold, D.R., Stewart, D., Davies, H. & Belknap, W.R. 2006. The primary in vivo steroidal alkaloid glucosyltransferase from potato (*Solanum tuberosum*). *Phytochemistry* 67, 1590-1597

McCue, K.F., Corsini, D.L., Allen, P.V., Rockhold, D.R., Maccree, M.M., Shepherd, L.V.T., Moehs, C.P., Joyce, P., Davies, H. & Belknap, W.R. 2003. Reduction of total steroidal glycoalkaloids in potato tubers using antisense constructs of a gene encoding a solanidine glucosyl transferase. *Acta Hort*. 619, 77-86

McMillan, M. & Thompson, J.C. 1979. An outbreak of suspected solanine poisoning in schoolboys: examination of criteria of solanine poisoning. *Q. J. Med.* 48, 227-243

Mercer, E.I. 1991. Sterol biosynthesis inhibitors: Their current status and modes of action. *Lipids* 26, 584-597

Moehs, C.P., Allen, P.V., Friedman, M. & Belknap, W.R. 1997. Cloning and expression of solanidine UDP-glucose glucosyltransferase from potato. *Plant J.* 11, 227-236

Mongrand, S., Morel, J., Laroche, J., Claverol, S., Carde, J.-P., Hartmann, M.-A., Bonneu, M., Simon-Plas, F., Lessire, R. & Bessoule, J.-J. 2004. Lipid rafts in higher plant cells; purification and characterization of triton X-100-insoluble microdomains from tobacco plasma membrane. *J. Biol. Chem.* 279, 36277-36286

Morel, J., Claverol, S., Mongrand, S., Furt, F., Fromentin, J., Bessoule, J.-J., Blein, J.-P. & Simon-Plas, F. 2006. Proteomics of plant detergent-resistant membranes. *Mol. Cell Proteomics* 5, 1396-1411

Morikawa, T., Mizutani, M., Aoki, N., Watanabe, B., Saga, H., Saito, S., Oikawa, A., Suzuki, H., Sakurai, N., Shibata, D., Wadano, A., Sakata, K. & Ohta, D. 2006. Cytochrome P450 CYP710A encodes the sterol C-22 desaturase in Arabidopsis and Tomato. *Plant cell* 18, 1008-1022

Morris, S.C. & Lee, T.H. 1984. The toxicity and teratogenicity of *Solanaceae* glycoalkaloids, articularly those of potato (*Solanum tuberosum*): a review. *Food Technol. Aust.* 36, 118-124

Moss, G.P. 1989. The nomenclature of steroids: Recommendations by the IUPAC-IUB joint commission on biochemical nomenclature. *Eur.J. Biochem.* 186, 429-458

Mullins, E., Milbourne, D., Petti, C., Doyle-Prestwich, B.M. & Meade, C. 2006.Potato in the age of biotechnology. *Trends Plant Sci.* 11, 254-260

Nasri, N., Fady, B. & Triki, S. 2007. Quantification of sterols and aliphatic alcohols in mediterranean stone pine (*Pinus pinea* L.) populations. *J Agric. Food Chem.* 55, 2251-2255

Navari-Izzo, F., Quartacci, M.F. & Izzo, R. 1989. Lipid changes in maize seedlings in response to field water deficit. *J. Exp. Bot.* 40, 675-680

Nes, W.R., Alder, J.H., Frasinel, C., Nes, W.D., Young, M. & Joseph, J.M. 1980. The independence of photosynthesis and aerobiosis from sterol biosynthesis in bacteria. *Phytochemistry* 19, 1439-1443

Oba, K., Kondo, K., Doke, N & Uritani, I 1985. Induction of 3-hydroxy-3-methylglutaryl CoA reductase in potato tubers after slicing, fungal infection or chemical treatment, and some properties of the enzyme. *Plant Cell Physiol.* 26, 873-880

Olsson, K. 1986. The influence of genotype on the effects of impact damage on the accumulation of glycoalkaloids in potato tubers. *Potato Res.* 29, 1-12

Osman, S.F., Herb, S.F., Fitzpatrick, T.J. and Sinden, S.L. (1976) Commersonine, a new glycoalkaloid from two *Solanum* species. *Phytochemistry* 15, 1065-1067

Parks, L.W., Smith, S.J. & Crowley, J.H. 1995. Biochemical and physiological effects of sterol alterations in yeast-a review. *Lipids* 30, 227-230

Parks, L.W. & Weete, J.D. 1991. Fungal sterols. in *Physiology and biochemistry of sterols.* Edited by G.W. Patterson & W.D. Nes. The American Oil Chemists Society, Champaign. Illinois. 158-171

Parnell, A., Bhuva, V.S. & Bintcliffe, E.J.B. 1984. The glycoalkaloid content of potato varieties*. J. Natl. Inst. Agric. Bot.* 16, 535-541

Patterson, G.W. 1991. Sterols of Algae. In *Physiology and Biochemistry of Sterols.* Edited by G.W. Patterson & W.D. Nes. The American Oil Chemists Society, Champaign. Illinois. 118–157. pp.

Pazkowski, C., Kalinowska, M. & Wojciechowski, Z.A. 2001. Phospholipids modulate the substrate specificity of soluble UDP-glucose:steroid glucosyltransferas from eggplant leaves. *Phytochemistry* 58, 663-669

Percival, G.C., Dixon, G.R. & Sword, A. 1996. Glycoalkaloid concentrations of potato tubers following exposure to daylight. *J. Sci. Food Agric.* 71, 59-63

Percival, G.C., Karim, M.S. & Dixon, G.R. 1998. Influence of light enhanced glycoalkaloids on resistance of potato tubers to *Fusarium sulphureum* and *Fusarium solani* var. *coeruleum. Plant Pathol.* 47, 665-670

Peskan, T., Westermann, M. & Oelmüller, R. 2000. Identification of low-density triton X-100-insoluble plasma membrane microdomains in higher plants. *Eur. J. Biochem.* 267, 6989-6995

Piironen, V., Lindsay, D.G., Miettinen, T.A., Toivo, J. & Lampi, A.-M. 2000. Plant sterols: biosynthesis, biological function nd their importance to human nutrition. *J. Sci. Food Agric.* 80:939-966

Rasbery, J.M., Shan, H., LeClair, R.J., Norman, M., Matsuda, S.P.T. & Bartel, B. 2007. *Arabidopsis thaliana* squalene epoxidase 1 is essential for root and seed development. *J. Biol. Chem.* 282, 17002-17013

Ritthausen, H. 1863. Chemische Notitzen. IV. Cholesterin im fett des weizen. *J. prakt. chem.*. 88, 145-146

Rochester, C.P., Kjellbom, P., Andersson, B. & Larsson, C. 1987. Lipid composition of plasma membranes isolated from light-grown barley (*Hordeum vulgare*) leaves: identification of cerebroside as a major component. *Arch. Biochem. Biophys*. 255, 385-391

Roddick, J.G. 1977. Subcellular localization of steroidal glycoalkaloids in vegetative organs of *Lycopersicon esculentum* and *Solanum tuberosum*. *Phytochemistry* 16, 805-807.

Roddick, J.G., Rijnenberg, A.L. & Osman, S.F. 1988. Synergisticinteraction between potato glycoalkaloids α-solanine and α-chaconine in relation to destabilization of cell membranes: ecological implications. *J. Chem. Ecol.* 14, 889-902

Russel, D.W. 2000. Oxysterol biosynthetic enzymes. *Biochem. Biophys. Acta* 1529, 126-135

Sales, E., Muñoz-Bertomeu, J., Arrillaga, I. & Segura, J. 2007. Enhancement of cardenolides and phytosterol levels by expression of an N-terminally truncated 3-hydroxy-3-methylglutaryl CoA reductase in transgenic *Digitalis minor*. *Planta Med.* 73, 605-610

Salunkhe, D.K. & Wu, M.T. (1979) Control of postharvest glycoalkaloid formation in potato tubers. *J. Food Protection* 42, 519-525

Sanford, L.L., Deahl, K.L., Sinden, S.L. & Ladd, T.L.Jr. 1992. Glycoalkaloid contents in tubers from *Solanum tuberosum* populations selected for potato leafhopper resistance. *Am. Potato J*. 69, 693-703

Sanford, L.L. & Sinden, S.L. 1972. Inheritance of potato glycoalkaloids *Am. Potato J.* 49, 209-217

Sawai, S., Akashi, T., Sakurai, N., Suzuki, H., Shibata, D., Ayabe, S.-i. & Aoki, T. 2006. Plant lanosterol synthase: Divergence of the sterol and triterpene biosynthetic pathways in eukaryotes. *Plant Cell Physiol.* 47, 673-677

Schabdach, H., Johne, S., Steiner, U. & Seifert, K. 1995. Plant disease resistance inducing activity of 7-oxo- and 7-hydroxysterols *Z. Naturforsch.* 50c, 252-262

Schaeffer, A., Bronner, R., Benveniste, P. & Schaller, H. 2001. The ratio of campesterol to sitosterol that modulates growth in *Arabidopsis* is controlled by *STEROL METHYLTRANSFERASE 2;1*. *Plant J.* 25, 605-615

Schaller, H. 2003. The role of sterols in plant growth and development. *Prog. Lipid Res.* 42, 163-175

Schaller, H. 2004. New aspects of sterol biosynthesis in growth and development of higher plants. *Plant Physiol. Biochem.* 42, 465-476

Schaller, H., Grausem, B., Benveniste, P., Chye, M.L., Tan, C.T., Song, Y.H. & Chua, N.-H. 1995. Expression of the *Hevea brasiliensis* (H.B.K) Müll. Arg. 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 in tobacco results in sterol overproduction. *Plant Physiol.* 109, 761-770

Schrick, K., Fujioka, S., Takatsuto, S., Stierhof, Y.-D., Stransky, H., Yoshida, S. & Jürgens, G. 2004. A link between sterol biosynthesis, the cell wall, and cellulose in *Arabidopsis*. *Plant J.* 38, 227-243

Schrick, K., Mayer, U., Horrichs, A., Kuhnt, C., Bellini, C., Dangl, J., Schmidt, J. & Jürgens, G. 2000. FACKEL is a sterol C-14 reductase required for organized cell division and expansion in Arabidopsis embryogenesis. *Genes & Develop.* 14, 1471-1484

Schuler, I., Milon, A., Nakatani, Y., Ourisson, G., Albrecht, A.-M., Benveniste, P. & Hartmann, M.-A. 1991. Differential effects of plant sterols on water permeability and on acyl chain oredering of soybean phosphatidylcholine bilayers. *Proc. Natl. Acad. Sci. USA* 88, 6926-6930

Seo, J.-W., Jeong, J.-H., Shin, C.-G., Lo, S.-C., Han, S.-S., Yu, K.-W., Harada, E., Han, J.-Y. & Choi, Y.-E. 2005. Overexpression of squalene synthase in *Eleutherococcus senticosus* increases phytosterol and triterpene accumulation. *Phytochemistry* 66, 869-877

Shi, J., Gonzales, R., Bhattacharyya, M.K. 1996. Identification and characterization of an S-adenosyl-L-methionine: Δ^{24}-sterol-C-methyltransferase cDNA from soybean. *J. Biol. Chem.* 271, 9384-9389

Simons, K., Ikonen, E. 1997. Functional rafts in cell membranes. *Nature* 387, 569-572

Sinden, S.L. (1972) Effect of light and mechanical injury on the glycoalkaloid content of greening-resistant potato tubers. *Am. Potato J.* 49, 368

Sinden, S.L., Deahl, K.L. & Aulenbach, B.B. 1976. Effect of glycoalkaloids and phenolics on potato flavor. *J. Food Sci.* 41, 520-523

Sinden, S.L. & Sanford, L.L. 1981. Origin and inheritance of solamarine glycoalkaloids in commercial potato cultivars. *Am. Potato J.* 58, 305-325

Skaggs, B.A., Alexander, J.F., Pierson, C.A., Schweitzer, K.S., Chun, K.T., Koegel, C., Barbuch, R. & Bard, M. (1996) Cloning and characterization of the *Saccharomyces cerevisiae* C-22 sterol desaturase gene, encoding a second cytochrome P-450 involved in ergosterol biosynthesis. *Gene* 169, 105-109

Slanina, P. 1990. Assessment of health-risks related to glycoalkaloids ("solanine") in potatoes: a Nordic view. *Vår Föda* 43, suppl. 1:1-14

Somerville, C. & Koornneef, M. 2002. A fortunate choice: the history of *Arabidopsis* as a model plant. *Nat. Rev. Genet.* 3, 883-889

Souter, M., Topping, J., Pullen, M., Friml, J., Palme, K., Hackett, R., Grierson, D. & Lindsey, K. 2002. Hydra mutants of *Arabidopsis* are defective in sterol profiles and auxin and ethylene signaling. *Plant Cell* 14, 1017-1031

Staehelin, L.A. & Newcomb, E.H. 2000. Membrane structure and membranous organelles. In *Biochemistry and molecular biology of plants.* Edited by B.B. Buchanan, W. Gruissem & R.L. Jones. The American Society of Plant Physiologists, Rockville, Maryland. 2-50. pp.

Suzuki, S., Kamide, Y., Nagata, N., Seki, H., Ohyama, K., Kato, H., Masuda, K., Sato, S., Kato, T., Tabata, S., Yoshida, S. & Muranaka, T. 2004. Loss of function of *3-hydroxy-3-methylglutaryl coenzyme A reductase 1 (HMG1)* in *Arabidopsis* leads to dwarfing, early senescence and male sterility, and reduced sterol levels. *Plant J.* 37, 750-761

Suzuki, M., Xiang, T., Ohyama, K., Seki, H., Saito, K., Muranaka, T., Hayashi, H., Katsube, Y., Kushiro, T., Shibuya, M. & Ebizuka, Y. 2006. Lanosterol synthase in dicotyledonous plants. *Plant Cell Physiol.* 47, 565-571

Svoboda, J.A., Weirich, G.F., Feldlaufer, M.F. 1991. Recent advances in insect steroid biochemistry, in *Physiology and biochemistry of sterols* Ed by Patterson, G.W. & Nes, W.D. American Oil Chemists' Society, Champaign, IL, 294-323

Takemoto, T., Kusano, G. & Yamamoto, N. 1970. Studies on the constituents of Cimicifuga spp. VII. structure of cimicifugenol. *Yakugaku Zasshi* 90, 68-72

Temel, R.E., Gebre, A.K., Parks, J.S. & Rudel, L.L. 2003. Compared with Acyl-CoA:Cholesterol *O*-Acyltransferase (ACAT) 1 and Lecithin:Cholesterol Acyltransferase, ACAT2 displays the greatest capacity to differentiate cholesterol from sitosterol. *J. Biol. Chem.* 278, 47594-47601

Threlfall; D.R. & Whitehead, I.M. 1988. Coordinated inhibition of squalene synthetase and induction of enzymes of sesquiterpenoid phytoalexin biosynthesis in cultures of *Nicotiana tabacum*. *Phytochemistry* 27, 2567-2580

Tschesche, R. & Hulpke, H. 1967. Biogenese von solanidin aus cholestrin. *Z. Naturforsch.* 22b, 791

Valkonen, J.P.T., Keskitalo, M., Vasara, T. & Pietila, L. 1996. Potato glycoalkaloids: a burden or a blessing? *Crit. Rev. Plant Sci.* 15, 1-20

Vance, D.E. & Van den Bosch, H. 2000. Cholesterol in the year 2000. *Biochim. Biophys. Acta.* 1529, 1-8

Verpoorte, R. 2000. in *Metabolic engineering of plant secondary metabolism*. Edited by Verpoorte, R. & Alfermann, A.W. Kluwer, Dordrecht, The Netherlands. 1-29. pp.

Volkman, J.K. 2003. Sterols in microorganisms. *Appl. Microbiol. Biotechnol.* 60, 495-506

Vögeli, U. & Chappell, J. 1988. Induction of sesquiterpene cyclase and suppression of squalene synthetase activities in plant cell cultures treated with fungal elicitor. *Plant Physiol.* 88, 1291-1296

Waterham, H.R., Koster, J., Romeijn, G.J., Hennekam, R.C, Vreken, P., Andersson, H.C., FitzPatrick, D.R., Kelley, R.I. & Wanders, R.J. 2001. Mutations in the 3beta-hydroxysterol Delta24-reductase gene cause desmosterolosis, an autosomal recessive disorder of cholesterol biosynthesis. *Am. J. Hum. Genet.* 69, 685-694

Wentzinger, L.F., Bach, T.J. & Hartmann, M.-A. 2002. Inhibition of squalene synthase and squalene epoxidase in tobacco cells triggers an up-regulation of 3-hydroxy-3-methylglutaryl Coenzyme A reductase. *Plant Physiol.* 130, 334-346

Willemsen, V., Friml, J., Grebe, M., van den Toorn, A., Palme, K. & Scheres, B. 2003. Cell polarity and PIN protein positioning in Arabidopsis require *STEROL METHYLTRANSFERASE 1* function. *Plant Cell* 15, 612-625

Yang, Z., Park, H., Lacy, G.H. & Cramer, C.L. 1991. Differential activation of potato 3-hydroxy-3-methylglutaryl Coenzyme A reductase genes by wounding and pathogen challenge. *Plant Cell* 3, 397-405

Zitnack, A. & Johnson, G.R. 1970. Glycoalkaloid content of B5141-6 potatoes. *Am. Potato J.* 47, 256-260

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