Regulation of sterol and glycoalkaloid biosynthesis in potato (*Solanum tuberosum* L.) – Identification of key genes and enzymatic steps

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Abstract

Steroidal glycoalkaloids (SGA) are toxic secondary metabolites present in some members of the Solanaceae family, including potato and tomato. The SGA level in tubers of potato (*Solanum tuberosum* L.) depends on genetic factors, but can also increase in response to *e.g.* wounding and light exposure. An upper limit of 200 mg SGA/kg f.w. is recommended in tubers used for human consumption. The SGA biosynthesis and its regulation are not fully understood, although cholesterol is often suggested as a likely SGA precursor.

To gain more knowledge about the genetic regulation of SGA biosynthesis, a microarray study was performed during mechanical wounding or light exposure treatment of tubers from two potato cultivars. The results revealed six genes related to sterol and SGA biosynthesis as up-regulated during both treatments, and to be associated with increased SGA content. One of the genes, *StDWF1*, encoding a sterol Δ24-reductase similar to Arabidopsis *DWF1*, was further investigated in transgenic potato plants. Down-regulation of *StDWF1* lowered the level of cholesterol as well as of SGA, demonstrating an important role of this gene in SGA synthesis. Homeostatic regulation of cholesterol metabolism in plants was investigated by over-expression of mouse cholesterol hydroxylases. In Arabidopsis, increased levels of hydroxylated sterols altered sterol/steroid metabolism as well as reduced plant growth. Similar effects were not observed in corresponding potato transformants, indicating species differences in sterol metabolism. To evaluate cholesterol as a SGA precursor, deuterium-labeled cholesterol was applied to potato shoots. Using LC-MS, label was shown to be incorporated into SGA.

The work shows that increased SGA synthesis in potato tubers is mediated by the concerted action of at least six key genes, acting at different positions in steroid biosynthesis. Results also establish cholesterol as a SGA precursor in potato plants.

Keywords: *Solanum tuberosum*, *Arabidopsis thaliana*, secondary metabolites, glycoalkaloids, sterols, transcription profiling, GC-MS, LC-MS.

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Dedication

Dedicated to Mamma, Pappa, Samantha & Simon

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Nahar, N., Beste, L., Dutta, P., Jonsson, L., Sitbon, F. Transcript profiling of two potato cultivars during wounding or light exposure of tubers reveals key genes in sterol and glycoalkaloid biosynthesis. *Submitted for publication*

II Beste, L., Nahar, N., Arif, U., Dalman, K., Jonsson, L., Dutta, P., Sitbon, F. Decreased cholesterol and glycoalkaloid levels in transgenic potato plants by antisense expression of a sterol Δ^{24}-reductase gene. *Manuscript*

III Beste, L*., Nahar, N*., Dalman, K., Fujioka, S., Jonsson, L., Dutta, P., Sitbon, F. Synthesis of hydroxylated sterols in transgenic Arabidopsis plants alters growth and steroid metabolism. *Provisionally accepted for publication in Plant Physiology*

IV Nahar, N*., Petersson, E*., Dutta, P., Broberg, A., Åslund-Tröger, R., Jonsson, L., Sitbon, F. Cholesterol is a glycoalkaloid precursor in potato plants. *Manuscript*

* These authors contributed equally to the work

The contribution of Nurun Nahar to the papers included in this thesis was as follows:

I The respondent performed all the practical labwork and statistical analyses, except for the cDNA sequencing and gene phylogeny parts. Participation in the experimental design. Wrote sections of the manuscript.

II Sterol analyses of transgenic plants, using GC and GC-MS. Stress induction of tubers and sampling of materials for SGA analysis. qPCR analysis of gene expression.

III Sterol and hydroxysterol analyses by GC and GC-MS. GC-MS identification of Arabidopsis hydroxysterols. Planned and performed the stress tests. Wrote sections of the manuscript.

IV Experimental design of heavy-isotope feeding. Sterol analysis by GC and GC-MS. Joint first authors wrote the manuscript in collaboration.

Abbreviations

Acetyl-CoA	Acetyl-coenzyme A
BL	Brassinolide
BR	Brassinosteroid
BW	Body weight
CAS	Cycloartenol synthase
CH	Cholesterol hydroxylase
CPI	Cyclopropyl sterol isomerase
CS	Castasterone
CYP	Cytochrome P450
DIM/DWF1	DIMINUTO/DWARF1
DWF1-L	DWARF1-like
f.w.	Fresh weight
FPP	Farnesyl diphosphate
FPS	Farnesyl diphosphate synthase
GC-MS	Gas chromatography-mass spectrometry
GPP	Geranyl diphosphate
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HMGR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
HPLC	High-performance liquid chromatography
IPP	Isopentenyl diphosphate
LC-MS	Liquid chromatography-mass spectrometry
MVA	Mevalonic acid
MVDPD	Mevalonate diphosphate decarboxylase
MVK	Mevalonate kinase
PMVK	Phosphomevalonate kinase
qPCR	Quantitative real-time PCR
SGA	Steroidal glycoalkaloid
SGT1	Solanidine galactosyltransferase

SGT2	Solanidine glucosyltransferase
SGT3	Rhamnosyltransferase
SMO	Sterol-4α-methyl oxidase
SMT	Sterol methyltransferase
SPE	Solid-phase extraction
SQE	Squalene epoxidase
SQS	Squalene synthase
TLC	Thin layer chromatography

1 Introduction

1.1 The potato - one of the worlds most important food crops

The potato (*Solanum tuberosum* L.) is a dicotyledonous plant species that belongs to the large Solanaceae family. This family also includes other well-known cultivated crop species such as tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*) and pepper (*Capsicum annuum*) (Friedman and McDonald 1997). The origin of potato lays in South America, specifically the high Andean areas where the first domestication of wild potato started between 10,000 and 7,000 years ago (Bradshaw and Ramsay 2009). The native people in Andean highlands cultivated several types of tuber-bearing Solanaceous plant species, including the ancestors of our domestic potato. The cultivated tetraploid *S. tuberosum* has been proposed to be a hybrid between the wild diploid species *S. stentomum*, but could also be a hybrid between other wild species of potatoes. (Burton 1989). The parent stocks of our domestic potato species are natural hybrids that were selected by the cultivators because they suited the particular needs demanded at that time (Redcliffe 1985). The potato was reproduced either by vegetative propagation or/and by true seed. Current research suggests that South American civilizations were based on the production and consumption of seed crops such as maize and quinoa at lower altitudes, and potatoes and other tubers at higher altitudes. It is believed that crops were traded between farmers living in higher altitudes and farmers living in lower, and that this is how the potato moved to new ecological niches (Messer 2000). The Spanish explorers, who first reached South America around 1532 in their search of gold, also came across the potato and brought it back when they returned to Spain. The earliest record of potato growth in Europe is from 1565 in the Canary Islands. From there, the potato was sent around Europe,

first as exotic gifts and via herbalists, and later from farmer to farmer (Burton 1989). From Europe, this new type of crop was then spread all over the world. At first, superstitious farmers believed that the tubers were poisonous and they were rejected as "Devil´s apples" or "forbidden fruit of Eden". However, sailors were quick to appreciate potatoes as food, suitable to bring and eat on ocean voyages (Rodger 2007). In 1596 the Swiss botanist Caspar Bauhin wrote the first detailed description of the potato in Latin, and gave it the present botanic name *Solanum tuberosum* (McIntosh 1927). The global spread of potato started early in the 17th century when sailors, soldiers, missionaries, colonists and explorers brought tubers to Asia, Ireland, North America and South pacific as food and as a trading material (Horton 1987). Africa was the last continent to receive the potato; the earliest potato-growing story here dates back to 1830. Once potatoes were established in Europe, it benefited the whole society, especially the farmers. They could now produce more food quicker and on smaller plots than previously, making the population grow. In for example Ireland, the majority of the population was dependent on potato as the main food. Potato cultivation in Ireland became more and more intensive, resulting in a monoculture at the expense of other crops. However, the status of potato as a staple diet for the rural population resulted in a disaster when the potato crop failed (Burton 1989). In 1845 the potato fields were damaged by late blight, a disease caused by the oomycete *Phytophthora infestans*. The complete harvest was lost that year. The following two years, the weather was again favorable for *P. infestans*, and large amounts of the potato harvest was destroyed. These three disastrous years in a row caused what is now called the ′Great Irish Famine′, and resulted in one million deaths and was the immediate cause of the large-scale migration of people to America. This tragic event also led to the elimination of almost all Irish potato varieties grown at that time, but also initiated great efforts to develop more productive and disease-resistant varieties. Many of our modern varieties originate from that time. During the 20th century potato became established as a truly global food, and today it ranks as the world's fourth most important food crop, after maize, wheat and rice (Burton 1989).

1.1.1 Potato consumption today

To feed the world's growing population is of great concern, and strategies should be aimed at productive and nutritious food. The potato is ideally suited for this purpose. The years after World War II, potato production

increased in countries like Belarus, Poland, Germany, Britain and the former Soviet Union. From that time, potato production and consumption has significantly increased in the rest of the world, specifically in the developing countries. According to FAOSTAT data, the potato consumption and production in developing countries exceeded in year 2005 that of the developed countries. Today, 80 percent of the potato production takes place in Asia and Europe. China is the biggest potato producer in the world, and together with India accounts for one third of global production.

1.1.2 Nutritional value of potato tubers

Nutritionally, potato tubers are a good source of carbohydrates, vitamins, minerals, micronutrients and dietary fiber. In 100 g of the potato tuber 77 g is water and about 20 g is carbohydrates (Table 1). The tubers are also a good source of several minerals, such as potassium, phosphorus, magnesium, and rich in vitamin B and C. Potato tubers also contains folate, riboflavin, antioxidants and dietary fiber (Table 1) (Bradshaw and Ramsay 2009). These values apply provided the skin is not peeled off.

Table1. Nutritional composition of un-peeled potato tubers on a fresh-weight basis (adopted from Bradshaw and Ramsay (2009)).

Nutrient	Contents per 100 g
Carbohydrate	18 g
Fiber	1-2 g
Protein	0.6-2.1 g
Fat	200 mg
Potassium	280-564 mg
Phosphorus	30-60 mg
Calcium	5-18 mg
Iron	0.4-1.6 mg
Magnesium	14-18 mg
Thiamin B1	0.02-0.2 mg
Vitamin B6	0.13-0.44 mg
Vitamin C	8-54 mg
Vitamin E	0.1 mg
Total energy	364 kJ

1.1.3 Potato cultivars

Since its introduction to the world, many different potato varieties have been brought into cultivation. There are more than 4,100 different potato cultivars, which are grown in over 100 countries worldwide (Howard 1974). However, only a few hundred cultivars are of agricultural importance, largely depending on their demands and productivity in the environment were they are grown. The potato tuber quality depends on a number of factors including the physical appearance, disease resistance, tolerance to mechanical damage and rate of sprouting (Lisinska *et al*. 1989).

1.1.4 History and pedigree of 'Bintje' and 'King Edward'

The cultivar 'Bintje', originated in the Netherlands, bred by K. L. de Vries from a cross of 'Munstersen' x 'Jaune Dór' (Stevenson 1966). The cross was made in 1904 and it was marketed for first time in 1910. Initially, 'Bintje' was not very popular for human consumption because it was not mealy enough, and the cultivar was used mainly for feeding pigs. The popularity of 'Bintje' increased due to its high yield in combination with a shortage of food during the First World War. Later, 'Bintje' was introduced in France, and it quickly became popular due to its excellent quality for making French fries. The popularity continued to grow after the Second World War, and today, 'Bintje' is one of the most consumed varieties worldwide. 'Bintje' produces large oval tubers with smooth pale yellow skin and light yellow flesh. However, a disadvantage of 'Bintje', is it´s susceptibility to wart and to late blight. (Stevenson 1966).
The cultivar 'King Edward' was originally raised by a gardener in Northumberland in 1902, and was placed on a market 1910 (Redcliffe 1985). 'King Edward' is a cross of 'Magnum Bonum' and 'Beauty of Hebron', which both are derivatives of 'Rough Purple Chili' (Redcliffe 1985). The 'King Edward' became quickly popular due to its good shape and floury texture. This cultivar is still one of the most popular, but it has weaknesses when it comes to diseases. It is susceptible to potato cyst nematode and moderately susceptible to foliage blight and different environmental stresses, such as drought, mechanical damage and light exposure (Friedman 2006, Manuscript I).

1.1.5 Cultivation requirements

One main factor that has contributed to the success of the potato as a crop species is that it can be cultivated under most environmental conditions, *e.g.* under temperate, subtropical and tropical conditions. Potatoes can be grown in temperate zones in early spring, in warmer areas it is grown in late winter, and in hot tropical climates it is grown during the coolest months of the year. In some sub-tropical highlands, with mild temperatures and high solar radiation, potatoes can be grown throughout the year (Bradshaw *et al.* 2009). The potato can be grown in almost any soil type, except for saline and alkaline soils. The preferable soil-type should be loose, loamy (soil composed of sand, silt and clay), and sandy loam soil, which should be rich in organic substances. These soil types retain nutrients and water well and allow the air and water to flow freely.

1.2 Secondary metabolites in the Solanaceae

Plants synthesize a large number of organic compounds often classified as primary or secondary metabolites. Primary metabolites are compounds that have essential role, whereas the secondary metabolites are non-essential and function mainly as protective compounds against pathogens and herbivores, in communication processes, or as structural components. Potato produces diverse defense secondary metabolites that are toxic or biologically active in other organisms. These metabolites include steroidal glycoalkaloids (SGAs), calystegines, leptines and phenolic compounds (Friedman and McDonald 1997). Among these secondary metabolites, SGAs are the most studied, and also the main subject of this thesis. Other well-known crop species within the Solanaceae family that also produces SGAs are pepper (*Capsicum annuum*), eggplant (*Solanum melongena*), and tomato (*Solanum lycopersicum*) (Aubert *et al.* 1989). SGAs have also been found in other plant species *e.g.* in *Veratrum grandiflorum* which belong to the monocotyledonous *Liliaceae* family and thus is a very distant relative to the Solanaceae.

SGAs are nitrogen-containing steroidal glycosides, containing three different structural units: a non-polar C_{27} sterol ring skeleton, a water-soluble sugar unit with 1 to 3 monosaccharides attached to the 3-OH group of the sterol ring, and a nitrogen atom attaching E and F ring of the aglycone (Figure 1) (Tschesche and Hulpke 1967; Heftmann 1983). In cultivated potato, the

main SGAs are formed from two different aglycones, solanidine or spirosolane. Solanidine is the main aglycone, and leads to α-chaconine and α-solanine production, which accounts for 95% of total SGA content in most potato varieties. Solasodine and tomatidenol are alkaloids formed from the aglycone spirosolane (Friedman and McDonald 1997). However, other species within the Solanaceae have other types of SGA as the most predominant ones, such as solamargine and solasonine in eggplant (Aubert *et al.* 1989), and α-tomatine and dehydrotomatine in tomato (Friedman 2002).

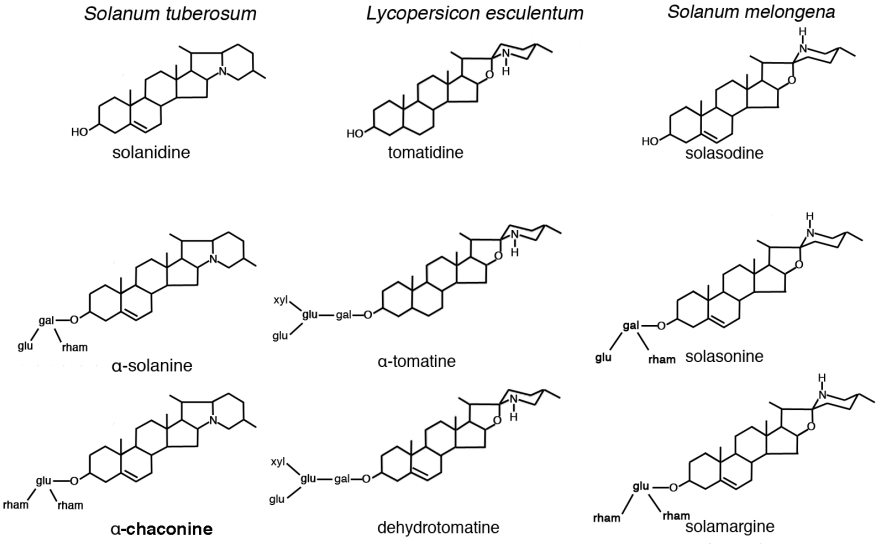

Figure 1. Chemical structure of the two most abundant glycoalkaloids and their aglycone in potato, tomato and eggplant.

1.2.1 SGA distribution within the potato plant

The synthesis of SGAs occurs in all parts of the plant and the SGAs distribution varies considerably between different parts and organs (Table 2). It has been stated that SGAs are not transported between different parts of the plants, and the presence of SGAs is hence thought to be tightly linked to its synthesis. The highest SGAs concentration has been detected in actively

growing tissues, such as young leaves, fruits, flowers and sprouts, whereas tubers contain relatively low amounts (Table 2). In tubers, the greatest concentration is in the phelloderm cell layers (Jonasson and Olsson 1994), a tissue just under the skin, while the flesh contains lower concentrations (Kozukue *et al.* 1987).

Table 2. Overview of total SGA contents in different parts of the potato plant.

Plant part	Total SGA content (mg/kg f.w.)	Reference
Roots	180-400	Lampitt *et al.* 1943
Stems	30-71	Kozukue *et al.* 1987
Leaves	925	Arnqvist *et al.* 2003
Flowers	3000-5000	Kozukue *et al.* 1987
Berries	380	Friedman *et al.* 1992
Sprouts	2000-4000	Wood *et al.* 1974
Skin	300-600	Wood *et al.* 1974
Peel	13-400	Kozukue *et al.* 1987
Flesh	120	Friedman *et al.* 2003
Peel + eye	300-500	Wood *et al.* 1974

The methods of quantification and cultivars analyzed differs between the studies.

1.2.2 Biosynthesis of SGAs

Despite the importance of SGAs for potato quality and safety, little is known about the SGA biosynthesis. SGAs are generally considered to originate from sterols, which in turn are formed via the mevalonic acid pathway. The first cyclic sterol, cycloartenol, is the precursor of common membrane sterols in plants, such as cholesterol, campesterol and sitosterol. These sterols are primary metabolites with a role in membrane function, but also serve as a starting material for synthesis of brassinosteroids (BR), and glycoalkaloids. The C-8 sterol, cholesterol has been suggested as a likely precursor of SGAs, but a conversion of cholesterol to SGAs has not yet been rigorously proven. Radioactive labelled cholesterol administrated to potato and tomato tissues showed an incorporation of radioactivity into SGA, which further led to the hypothesis of cholesterol as the starting compound (Heftmann *et al.* 1983 and references therein). Bergenstråhle *et al.* (1996) demonstrated the incorporation of (2-^{14}C)-mevalonate into cholesterol when applied to potato tuber discs. In a parallel study the same authors showed that SGA levels were decreased in tuber discs after inhibiting the cholesterol synthesis with

the inhibitor tridemorph. Down-regulation of cholesterol in transgenic potato plants led to a significantly decreased level of SGA (Arnqvist *et al.* 2003). The role of cholesterol as a SGA precursor was addressed in the present thesis, using deuterium-labelled cholesterol coupled to LC-MS/MS detection of labelled SGA (Manuscript IV). Incorporation of cholesterol-4-^{14}C into 26-hydroxycholesterol and cholest-4-en-3-one in etiolated potato sprouts performed by Heftmann *et al.* (1974) suggested these compounds as the first metabolites in conversion of cholesterol to solanidine.

Intermediate steps in the biosynthesis leading to solanidine have been proposed in several studies. Petersen *et al.* (1993) proposed that the biosynthesis of solanidine occurs via hydroxylation of cholesterol first at C-22, followed by another hydroxylation at C-26, to produce dormantinol and dormantinone. In the following steps an oxidation at C-22 was proposed before the introduction of an amino molecule at C-26. In their hypothesis, E-ring closure of solanidine occurs before the F-ring closure. A study made by Kaneko *et al.* (1976) suggests that cholesterol is the starting point of solanidine in *Veratrum grandiflorum*. A conversion of cholesterol to solanidine likely involves at least 3 different reaction steps (Kaneko *et al.* 1971, 1976, 1977). The synthesis of solanidine was proposed to start with a hydroxylation process of cholesterol to produce dormantinol, which would further be oxidized at C-22 to dormantinone. The terminal hydroxyl group at C-26 is probably replaced by an amino group by an exchange reaction involving the amino acid L-arginine as a nitrogen donor molecule to produce verazine (Kaneko *et al.* 1976). The incorporation of an N-atom is necessary for the F-ring closure. Prior to the E-ring closure, a hydroxyl group at C-16 has to be introduced to produce solanidine. Introduction of hydroxyl groups at different positions are likely to be catalyzed by different cytochrome P450 enzymes. This suggestion of alkaloid synthesis might also be true for other species including potato. In potato, the final steps in the biosynthesis of SGA involves addition of three sugar moites to C3 by three different glycosylating enzymes; solanidine galactosyltransferase (SGT1), solanidine glucosyltransferase (SGT2), and rhamnosyltransferase (SGT3). *SGT1*, encoding the enzyme galactosyltransferase, catalyzes γ-solanine formation from solanidine (McCue *et al.* 2003). Solanidine glucosyltransferase, encoded by *SGT2,* coverts solanidine to γ-chaconine (McCue *et al.* 2005). *SGT3,* encoding a rhamnosyltransferase, catalyzes the final formation of α-chaconine and α-solanine from their respective β forms (McCue *et al.* 2007). Interestingly, high expression levels of *SGT1* and *SGT3* were associated with a high SGA levels in wounded and light

exposed tuber. This indicates the a rate limiting function of the corresponding enzymes in stress-induced SGA synthesis in potato tubers (Manuscript I).

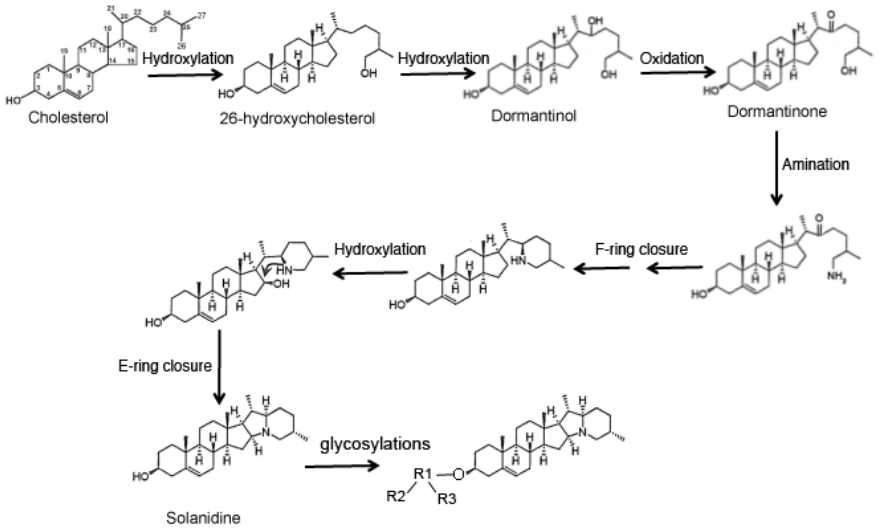

Figure 2. A hypothetical biosynthetic pathway of SGA from cholesterol in *Solanum tuberosum* (a modified version of Kaneko *et al.* 1976, Tschesche 1980, Petersen *et al.* 1993).

1.2.3 Potato SGAs contribute to plant resistance against pathogens

The potato SGAs are regarded as having a role in defense processes against pathogens and predators including fungi, viruses, bacteria, insects, and worms, but the exact role in the resistance is in many cases still undefined (Friedman and McDonald 1997). Studies on the interaction between SGAs and different pathogens often show unclear or conflicting results on SGA-based resistance. A somatic hybrid between wild (*Solanum brevidens*) and cultivated potato (*Solanum tuberosum*), which contained high amount of SGAs (Laurila *et al.* 2001), showed increased resistance against *Erwinia* soft rot (bacteria) (Austin *et al.* 1988). Percival *et al. (1998)* showed that light-exposed potato tubers with increased SGAs had decreased *Fusarium* fungal invasion. The SGAs have protective properties against attacks by insects, *e.g.* Colorado potato beetle, *Leptinotarsa decemlineata*, (Deahl *et al.* 1991) and potato leafhopper, *Empoasca fabae*, (Sandford *et al.* 1990; Sandford *et al.*

1992). McKee (1959) showed that the spore growth of *Fusarium caeruleum* was inhibited in presence of solanidine. A somatic hybrid between *S. tuberosum* and the wild type species *Solanum circaeifolium*, which contained high concentrations of SGA, were found to be resistant to both *Phytophthora infestans* and *Globdora pallida* (Mattheij *et al.* 1992). Experiments with wireworms, *Agriotes obscurus* feeding on four potato cultivars discs differing in their basal SGA levels revealed that the cultivars with low levels were more susceptible to attack, and that cultivars with high SGA were avoided (Jonasson and Olsson 1994).
From these studies it is clear that SGA is a key factor in resistance to different pathogens and attackers. However, different pathogen and predators seem to have evolved different strategies to overcome the toxicity of the SGAs, probably by converting them into less toxic compounds. Moreover, the high level of SGAs in flowers and seeds suggests also a function related to seed survival.

1.2.4 SGA effects on human and animals

SGAs have toxic effects on both humans and animals, and 1-2 mg/kg body weight (BW) is enough to induce toxic effects. The poisoning symptoms in human of SGAs include nausea, vomiting, diarrhea, stomach and abdominal cramps, headache, fever, rapid and weak pulse, rapid breathing, hallucination and in serious cases coma and death. It has been estimated that 2-5 mg/kg BW is a toxic dose of potato SGA, whereas 3-6 mg/kg BW dose may be fatal (Chen and Miller 2001; Friedman and McDonald 1997; Valkonen *et al.* 1996). Due to the toxic effects of SGA, an upper limit of 200 mg/kg fresh weight (f.w.) has been established by leading authorities, including the Swedish National Food Administration (LIVSFS 1993:36). In general, α-chaconine is more toxic than α-solanine, but synergistic effects occur when they are mixed at different ratios (Roddick *et al.* 1988). The aglycone solanidine seems to have a less toxic effect, indicating a role of the carbohydrate moiety on SGA toxicity (Rayburn *et al.* 1994).

1.2.5 The biological activities of SGAs

The two main biological effects of potato SGAs are caused by disruption of cell membranes in the digestive system, and by inhibition of acetylcholinesterase and butyrylcholinesterase activity in the central nervous

system (Chen and Miller 2001). It is considered that SGAs exert their effect at least in part by binding to membrane sterols, resulting in membrane disruption and leakage of the cell contents. The first toxic effect of SGAs could be caused by a complex formation between membrane sterol and SGA, a model system suggested by Keukens *et al.* (1995). The proposed mode of action probably involves an interaction of the aglycone part of the SGA to the membrane sterols in a 1:1 ratio, creating a hydrogen bonding dependent on the sugar moieties (Edwards 1997). This complex formation is followed by a rearrangement of the membrane structure, in which the membrane sterol is blocked from its interaction with other important components, resulting in leakage of cell contents. This gives symptoms of abdominal cramps, vomiting and diarrhea (Friedman and McDonald 1997). More serious toxic symptoms of SGA poisoning are associated with dysfunction of the central nervous system, leading to confusion, rapid and weak pulse, fever, low blood pressure, breathing difficulties and rapid respiration (McMillan *et al.* 1979). These symptoms are caused by SGA inhibition of acetyl- and butyrylcholinesterases. Both of these enzymes are necessary for catalyzing the hydrolysis of the neurotransmitter acetylcholine at synapses in the central nervous system.

1.2.6 Factors influencing SGA contents in tubers

Both genetic and environmental factors have been shown to affect SGA levels in potato tubers. The basal SGA content varies between genotypes, and is a genetically inheritable (Friedman and McDonald 1999; Sinden *et al.* 1984). In addition to this genetic control, several external factors during growth, or during the post-harvest period, can increase the levels further. For example, extreme temperatures, dry or wet growing seasons, and hail can increase the SGA concentrations during plant growth. Post-harvest factors including light exposure of the tubers, mechanical injury, temperature and storage time, can all increase the SGA level (Friedman and McDonald 1999; Percival *et al.* 1999). The tuber size can indirectly have an effect on the SGA levels. Smaller potatoes have higher SGA concentration than larger tubers, due to a higher surface area to volume ratio (Friedman and McDonald 1997; Smith *et al.* 1997). Excessive SGA levels have led to a withdrawal of the cultivars ′Lenape′ and ′Magnum Bonum′ from the US and Swedish markets, respectively (Hellenäs *et al.* 1995; Sinden and Webb 1974)

1.2.7 Leptines and calystegine alkaloids in Solanaceous species

Leptines constitute class of closely SGA-related compunds (Friedman 2006; Ginzberg et *al.* 2009). There are two types of leptine glycoalkaloids, leptinine and leptine. The leptine glycoalkaloids have an acetyl group at the C-23 position and are derived from the aglycon 23-acetylleptinidine, whereas leptinine glycoalkaloids have leptinindine (23-hydroxysolanidine) as their aglycone (Friedman and McDonald 1997) (Figure 3). Relatively few details are known about the leptines, and the biosynthetic pathway remains to be solved. The compounds are present in leaves but have not been detected in potato tubers. Further research is needed to determine why some Solanaceous plants produce glycoalkaloids only in leaves and not in both leaves and tubers.

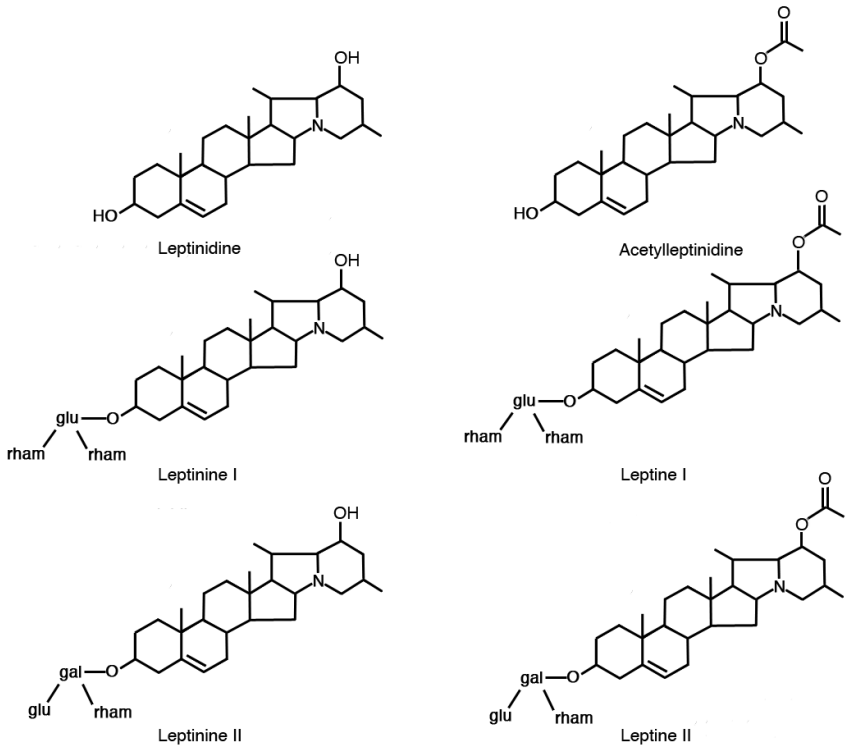

Figure 3. Chemical structures of leptine I and leptinine II and their respective aglycone.

A new class of potato alkaloids; calystegine alkaloids, was discovered by Nash et *al.* (1993). Calystegines are nitrogen-containing polyhydroxylated nortropane alkaloids and were first identified in plant roots, where they probably act as nutritional mediators between the plant and its symbiotic bacteria (Bekkouche et *al.* 2001). Later, they have also been identified in other parts of the plant, for example in potato tubers. Calystegines are divided into four different groups; A, B, C and N. Group A, B and C calystegines carries three, four and five hydroxyl groups, respectively, at various positions in the molecule (Figure 4). The fourth group of calystegines, N, contains an amino group. The biosynthesis of calystegines is assumed to originate from tropane alkaloids where putrescine is the precursor (Biastoff and Dräger 2007*;* Dräger 2003). The exact toxic effect of calystegines is unknown but as other tropanes they have a glycosidase-inhibitory activity by blocking the active site of the sugar moiety (Andersson 2002).

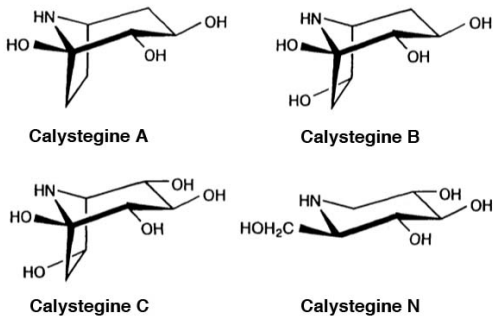

Figure 4. Chemical structure of potato calystegine A, B, C, and N (Andersson 2002).

1.3 Plant sterol metabolism

1.3.1 Sterols in general

In all eukaryotes, sterols are essential isoprenoid–derived lipids that have diverse functions. They are essential components of the cell membrane, but also act as precursors of steroid hormones or secondary metabolites. Sterols are mainly based on a steroid skeleton with four-rings, A, B, C and D, with a methyl substitution at the C-10 and C-13 positions. Attached to the D-ring end, the sterol molecules have a non-polar water-insoluble hydrocarbon chain, and at the opposite end in the A-ring they have a polar,

water-soluble OH-group (Hartmann 1998). This gives the molecule amphiphatic (*i.e.* both polar and non-polar) properties. Most organisms contain only one type of end-product sterol. For example, the major sterol in vertebrates is cholesterol, and the major sterol in fungi is ergosterol. In contrast, plants contain a mixture of various sterols referred to as phytosterols, including sitosterol, stigmasterol, campesterol, isofucosterol and cholesterol. The first four differ from cholesterol mainly in that they have an alkylated side chain. Insects do not synthesize sterols, instead they receive sterols or sterol precursors from food (Benveniste 2004).

1.3.2 Sterols are important membrane components

Sterols are a part of the cell membrane and have an amphipathic chemical structure; a polar hydrophilic head and a hydrophobic hydrocarbon chain. In the membrane, the C-3 hydroxyl group is facing the water interface and the hydrophobic side chain is bound to fatty acyl chains of the phospholipids and proteins facing the hydrophobic part of the membrane. This interaction between and fatty acyl chains restricts the movement of sterols in the membrane bilayer, thereby modulating membrane permeability and fluidity (Figure 5).

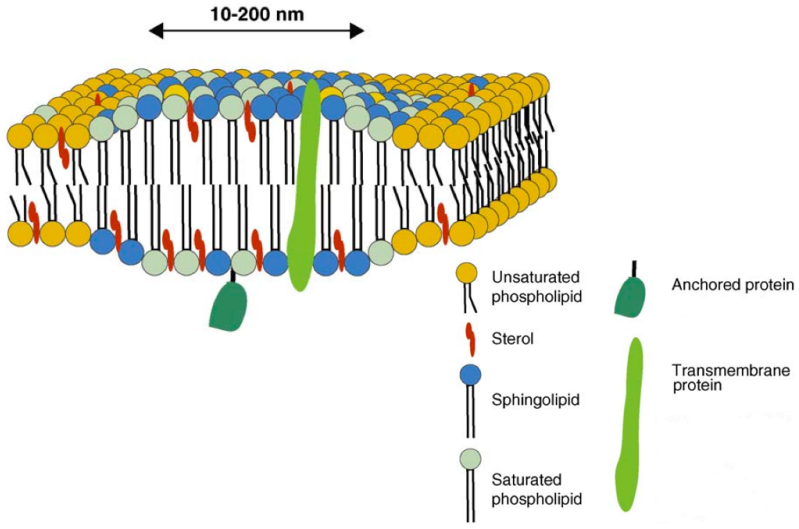

Figure 5. Sterols are important component of the membrane bilayer in eukaryotic cells with, consisting of a polar hydrophilic head and a hydrophobic hydrocarbon tail. Other important component of the membrane are saturated and unsaturated phosphlipids, sphingolipids and anchored and tramsmembrane protein. (Reprinted with permission from Boutté *et al.* 2009)

1.3.3 Classification and nomenclature of plant sterols

In general, plant sterols are classified in three groups depending on the number of methyl groups attached to the C-4 position in the A-ring: 4-desmethylsterols (without any methyl group bound to C-4) also sometimes referred to as regular sterols, 4α-monomethylsterols (with one methyl group) and 4,4-dimethylsterols (with two methyl groups attached) (Akihisa *et al.* 1991). Desmethylsterols, such as cholesterol, campesterol and sitosterol are the final end-product sterols. Sterols are also classified according to the presence of alkyl groups at C-24 in the side chain of sterols. These can be defined as 24-desmethylsterols (without an alkyl group attached), 24-methylsterol, and 24-ethylsterols. These sterols can also be defined as C8-, C9- or C10-sterols depending on the length of the sterol side chain, or C_{27}, C_{28}, C_{29} depending on the total number of carbon atoms (Figure 6).

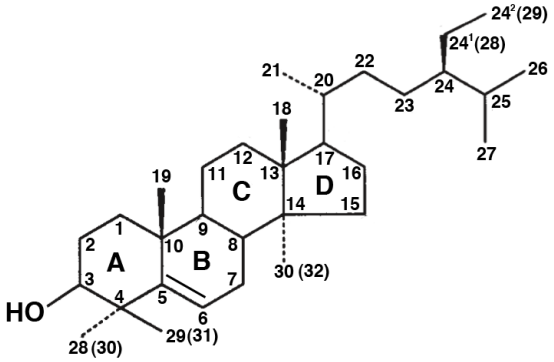

Figure 6. The basic plant sterol structure with carbon numbering according to the 1989 IUPAC-IUB recommendations (Hartmann 1998). The numbers in parentheses indicates the former nomenclature system which is used in this thesis.

Table 3. The most common 4-desmethylsterol, 4α-monomethylsterol and 4,4-dimethylsterols in the two model plants used in this thesis.

4-desmethylsterols	4α-monomethylsterols	4,4-dimethylsterols
Cholesterol	Lophenol	Cycloartenol
24-methylenecholesterol	Cycloeucalenol	Cycloartanol
Campesterol	Obtusifoliol	24-methylenecycloartanol
Isofucosterol	24-methylenelophenol	
Sitosterol	24-ethylidenelophenol	
Campesterol		

1.3.4 The sterol biosynthesis in plants

The biosynthesis of sterols occurs through four major stages: (1) synthesis of mevalonic acid from acetate, (2) conversion of mevalonic acid to squalene, (3) cyclization of squalene to give cycloartenol and (4) conversion of cycloartenol to end-product sterols (Grundwal 1975; Benveniste 2004). Key steps of plant sterol biosynthesis are summarized in figure 7 and 8.

1.3.5 First stage of sterol biosynthesis: conversion of acetate to mevalonic acid

Acetyl-coenzyme A (acetyl-CoA) is the precursor for the sterol biosynthesis and is converted to acetoacetyl-CoA. A condensation reaction between acetoacetyl-CoA and a second acetyl-CoA creates the product 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The reduction of HMG-CoA into mevalonic acid (MVA) is considered to be a rate-limiting step, and HMG-CoA is therefore an important isoprenoid precursor. This step is catalyzed by the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR). In the plant kingdom, HMGR is encoded by a small multigene family. This is in contrast to mammals, which only have a single HMGR gene (Suzuki and Muranaka 2006). The number of genes encoding HMGR in plants varies depending on the species. In Arabidopsis, HMGR is encoded by two distinct genes, *HMG1* and *HMG2* and the expression of these two genes differs; *HMG1* is expressed in all tissues, whereas the expression of *HMG2* is restricted to young seedlings, roots and inflorescences (Benveniste 2002). Tomato has four HMGR genes, while potato contains at least three HMGR genes that are regulated differently in response to stress stimuli (Choi *et al.* 1992). Over-expression of *HMG1* in transgenic tobacco plants resulted in a 6-fold increase of total sterols, including end-product sterols, demonstrating that the enzyme catalyzes a limiting step (Schaller *et al.* 1995). The characterization of *hmg1* and *hmg2* mutants in Arabidopsis revealed an altered phenotype of the *hmg1* plants, while *hmg2* plants showed no morphological phenotypes. The *hmg1* mutants were dwarfed and displayed earlier senescence and male sterility. The sterol levels in this mutant were reduced, supporting an important role of HMGR in Arabidopsis sterol biosynthesis (Suzuki *et al.* 2004). These results implicate a role of sterols in cell elongation, senescence, and fertility.

1.3.6 Second stage of the sterol biosynthesis: conversion of mevalonic acid into squalene

The six-carbon compound MVA is phosphorylated to mevalonate 5-phosphate by the action of mevalonate kinase (MVK). In contrast to HMGR, little is known about MVK and its regulation. The expression of MVK is particularly abundant in Arabidopsis roots and inflorescences (Lluch *et al.* 2000). Phosphomevalonate kinase (PMVK) catalyses an additional phosphorylation of mevalonate 5-phosphate into mevalonate 5-diphosphate (Benveniste 2002). Decarboxylation of mevalonate 5-diphosphate into isopentenyl diphosphate (IPP), which is the basic five-carbon isoprene unit, is catalyzed by the enzyme mevalonate diphosphate decarboxylase (MVDPD). IPP, and its isomer dimethylallyl diphosphate (DMAPP), are the active building blocks for all isoprenoids. In the following step, DMAPP and IPP are condensated in a head-to-tail fashion to generate geranyl diphosphate (GPP). There are two genes encoding IPP isomerases in Arabidopsis and expression of these genes are mainly found in roots (Campell *et al.* 1997). Farnesyl diphosphate (FPP) is formed by addition of a second IPP molecule to GPP by the action of the enzyme farnesyl diphosphate synthase (FPS). FPP is an essential compound, since it is the branch point of a large variety of isoprenoid end products, such as sesquiterpenes (C15), diterpenes (C20), triterpenes (C30) and tetraterpenoids (C40) (Figure 7). Arabidopsis contains two farnesyl diphosphate synthase genes, *FPS1* and *FPS2*, which encode three different isoforms (FPS1S, FPS1L, and FPS2). The expression of FPS1S is found in all tissues and throughout the entire plant development, suggesting that FPS1S is crucial for basic plant cell functions. In contrast, expression of FPS2 has only been detected during the early stages of flower development, suggesting a more specific and specialized function of FPS2 (Masferrer *et al.* 2002; Suzuki and Muranaka 2006). The enzyme squalene synthase (SQS) is then responsible for the condensation of two FPP molecules in a tail-to-tail/head-to-head fashion to create squalene, which is the first dedicated step in sterol biosynthesis. Over-expression of SQS in Siberian ginseng (*Eleutherococcus senticosus*) resulted in a 2- to 2.5-fold enhanced production of phytosterol and triterpene saponin in the transgenic lines compared to the control (Seo *et al.* 2005). This suggests that SQS might have a rate-limiting function in triterpene biosynthesis.

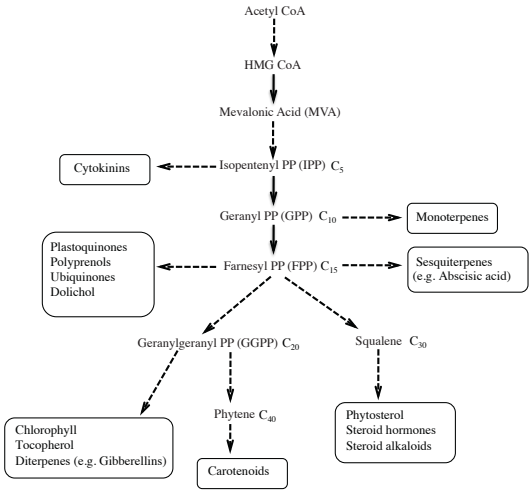

Figure 7. Schematic overview of the plant cytosolic isoprenoid biosynthesis.

1.3.7 Third stage of plant sterol biosynthesis: cyclization of squalene to cycloartenol

Cyclization of squalene to 2,3-oxidosqualene is catalyzed by squalene epoxidase (SQE), a step that counts as the first oxidation step in sterol biosynthesis. Characterization of Arabidopsis sqe1 mutants revealed severe developmental defects, including a shorter root, reduced hypocotyl elongation and smaller plants with unviable seeds. In Arabidopsis, six putative SQE genes have been identified, and *SQE1* seems to be the one essential for normal plant development, especially seed development (Rasbery *et al.* 2007). The cyclization of 2,3-oxidosqualene to form cycloartenol is catalysed by the enzyme cycloartenol synthase 1 (CAS1) in Arabidopsis (Corey *et al.* 1993). In plants this type of enzyme can also convert 2,3-oxidosqualene to a great variety of other triterpenes such as α-amyrin. The Arabidopsis weak allele mutant, *cas1-1* had accumulated level of 2,3-oxidosqualene, the substrate of CAS1. The *cas1-1* mutant developed albino inflorescence shoot and had low amounts of carotenoids and chlorophylls. The stronger mutants *cas1-2* and *cas1-3,* were defective in their male gametes and had abnormal leaf growth. These results implicate a CAS1 function in male gametophyte differentiation as well as in chlorophyll synthesis (Babiychuk *et al.* 2008).

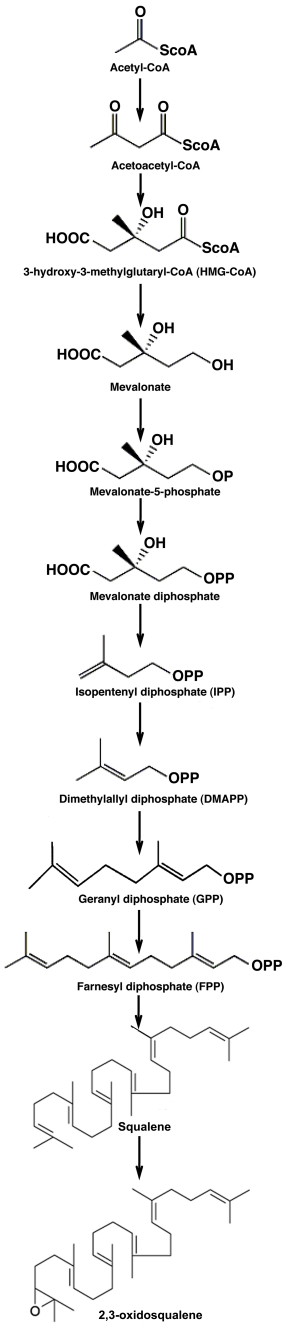

Figure 8. Overview of the three first stages of the sterol biosynthesis.

1.3.8 Fourth stage of plant sterol biosynthesis: cycloartenol to end-product sterols

Cycloartenol is the first fully cyclic plant sterol, and serves as the precursor to all end-product sterols. The synthesis of phytosterols takes place in three parallel pathways; the C8, C9 and C10 sterol pathways. Cholesterol is synthesized by the C8 pathway, campesterol by the C9, and isofucosterol, sitosterol and stigmasterol by the C10 pathway. In most plant species, Arabidopsis included, cholesterol is a minor sterol, often accounting for 1-2% of total sterol levels. Presumably, this underlies the general lack of knowledge concerning the cholesterol synthesis in plants. Cholesterol synthesis in human originates from lanosterol, making this pathway difficult to extrapolate to plants. Plants differ in their sterol composition from vertebrates by the presence of both 24-methyl (C9) and 24-ethyl (C10) sterols; sterols with a methyl or ethyl group present at carbon-24 in their side chain. In the initial step, S-adenosyl-L-methionine-sterol-C-methyltransferase 1 (SMT1) adds a methyl group to cycloartenol at position 24, resulting in 24-methylene cycloartanol (Bouvier-Navé *et al.* 1998). Several studies have demonstrated the importance of this step for subsequent sterol synthesis in Arabidopsis as well as other plant species. Over-expression of soybean *GmSMT1* in transgenic potato plants led to an increased total sterol level compered to controls, largely due to increased levels of isofucosterol and sitosterol. The levels of cholesterol and SGAs as a consequence were reduced in the transgenic lines. These results demonstrate the critical role of the SMT1 for sterol canalization, but also support the idea of cholesterol as a precursor of potato SGAs (Arnqvist *et al.* 2003). Conversely, Arabidopsis *smt1* mutant had an increased level of cholesterol at the expense of sitosterol. These mutants displayed a poor growth and reduced fertility, an abnormal root system, and a defective embryo morphogenesis (Diener *et al.* 2000; Schaller 2003).

In subsequent steps, 24-methylene cycloartanol is demethylated at C-4 by the action of sterol-4α-methyl oxidase 1 (SMO1) to create cycloeucalenol, which is isomerized to obtusifoliol by the enzyme cyclopropyl sterol isomerase (CPI) (Lovat *et al.* 2000). The obtusifoliol 14α-demethylase (CYP51) that belongs to the cytochrome P450 family converts obtusifoliol to 4α-methylergostatrienol (Bak *et al.* 1997). Antisense expression of *obtusifoliol 14α-demethylase* in Arabidopsis gave semi-dwarf plants during the early growth period, which had a longer life span and increased amounts of obtusifoliol (Kushiro *et al.* 2001). A sterol C-14 reductase enzyme, encoded

by the gene *FACKEL*, is required for the conversion of 4α-methylergostatrienol to 4α-methylfecosterol, as has been revealed by the Arabidopsis *fackel* mutant. This mutant had an increased number of cotyledons, multiple shoot meristems, and shorter roots. At the same time, the amount of 4α-methylergostatrienol was 10-fold higher in the *fackel* mutant compared to the control (Jang *et al.* 2000). Souter *et al.* (2002), demonstrated that the *HYDRA1* gene encodes the subsequent Δ8-Δ7 sterol isomerase, converting 4α-methylfecosterol to 24-methylenelophenol. In a second methylation reaction, 24-methylene lophenol is converted to 24-ethylidene lophenol by action of the enzyme C-24 sterol methyltransferase 2 (SMT2). Over-expression of *SMT2* in Arabidopsis resulted in accumulation of sitosterol at the expense of campesterol, and thus disturbed the balance between the 24-methyl and 24-ethyl sterol levels. These plants exhibited reduced stature and growth, both of which could be rescued by brassinosteroid (BR) treatment. The rescue by BR indicates that the SMT2 over-expresser was defective in BR synthesis, since campesterol a precursor of BRs (Schaller 2003). The co-suppression of *SMT2* showed the opposite effect on sterol composition, with high levels of campesterol and a reduced content of sitosterol. These plants showed a complex developmental phenotype, including a reduced plant size with increased branching of secondary stems, altered flower morphology and low fertility. In contrast to the *SMT2* over-expressing lines, these phenotypes could not be rescued by BR treatment. Together, these results demonstrate that plants require a proper balance of campesterol and sitosterol for normal growth (Schaeffer *et al.* 2001). Thereafter, the sterol pathway goes through a bifurcation resulting in two parallel pathways leading to the formation of campesterol or sitosterol. The branchpoint enzyme sterol-4α-methyl oxidase 2 (SMO2) removes the second methyl group from the C-4 of the 24-methylenelophenol and 24-ethylidenelophenol to create episterol and Δ^7-avenasterol (Darnet *et al.* 2001). This was revealed by the down-regulation of *SMO2* in *Nicotiana benthamiana*, which led to accumulation of 24-methylenelophenol and 24-ethylidenelophenol (Darnet and Rahier 2004).

The enzyme, C-5-desaturase encoded by *DWF7/STE1* is responsible for the introduction of a double bond at C-5 in episterol (C9) and Δ^7-avenasterol (C10) to obtain 5-dehydroepisterol and 5-dehydroavenasterol (Gachotte *et al.* 1995). In the following step Δ7-reductase encoded by *DWF5* catalyzes the reduction of Δ7-double bond of 5-dehydroepisterol and 5-dehydroavenasterol to generate 24-methylene cholesterol (C9) and isofucosterol (C10) (Choe *et al.* 2000). In the next step, both 24-methylene

cholesterol and isofucosterol are first isomerized at C-24(25), and then the double bond is reduced to produce campesterol and sitosterol by the same enzyme, a Δ24-reductase encoded by the gene DIM/DWF1 (Klahre *et al.* 1998). It is clear that, most of the final enzymatic steps are shared between the C9 and C10-sterol patways. The enzymes C5-desaturase, Δ7-reductase, and Δ24-reductase play critical roles in plant sterol metabolism by being active in the steps directly upstream of campesterol, which is a precursor of growth hormone brassinosteroids. Mutations of the corresponding genes *DWF7/STE1*, *DWF5* and *DWF1/DIM* in Arabidopsis block successive steps in the conversion of episterol to campesterol. The mutant phenotypes of the homozygote mutants are dwarfs with dark-green and epinastic leafs, they have a prolonged life span and are partially sterile. Exogenously application of BR improves the growth of these mutants, indicating that these plants are defective in BR synthesis (Clouse 2002, Schaller 2004). Interestingly, the Arabidopsis *dwf1/dim* mutant also had slightly decreased level of cholesterol (Klahre *et al.* 1998), indicating a role of DWF1 in cholesterol synthesis (Manuscript II). Morikawa *et al.* (2006) showed that *Arabidopsis* contains two different sterol C-22 desaturases, catalyzing the synthesis of stigmasterol and brassicasterol. At the same time, Arnqvist *et al.* (2007) showed that overexpression of the C-22 desaturases in *Arabidopsis* increased levels of free stigmasterol and decreased levels of sitosterol. Furthermore, the transgenic lines displayed higher levels of esterified stigmasterol, cholesterol, 24-methylcholesterol and isofucosterol, respectively, indicating feed-back regulation of sterol levels.

Figure 9. Model for plant sterol biosynthesis (Arnqvist 2007; Benveniste 2004).

1.3.9 Sterol composition in potato and Arabidopsis

The sterol levels and composition in plants displays a considerable degree of variation, both between tissues and between plant species. For instance, cholesterol is a minor sterol in most plants, but a major sterol in the Solanaceae, including tomato and potato. This sterol variation is illustrated by the relative desmethylsterol composition in potato and Arabidopsis leaves (Table 4).

Table 4. The relative composition of 4-desmetylsterols in potato and Arabidopsis leafs.

Plant species	Sterol content	(% of total sterols)	Reference
Solanum tuberosum	Cholesterol	13%	Arnqvist *et al.* 2003
Potato	Campesterol	18%	
	Isofucosterol	12%	
	Sitosterol	37%	
	Stigmasterol	20%	
Arabidopsis thaliana	Cholesterol	2%	Schaeffer *et al.* 2001
Thale cress	Campesterol	13%	
	Brassicasterol	2%	
	Isofucosterol	3%	
	Sitosterol	73%	
	Stigmasterol	7%	

1.3.10 Conjugated forms of phytosterols

Sterols can be conjugated to different molecules in plants. The most common form of sterol conjugates found in plants are steryl esters, steryl glycosides and acylated steryl glycosides. Steryl esters are formed by attachment of fatty acids to the C3-hydroxyl group of the sterol. Steryl glycosides instead have a sugar molecule conjugated to the C3-hydroxyl group. Further esterification of steryl esters with long fatty acids create acylated steryl glycosides. Dependent on the plant species, age, tissue and physiological status, distinctive concentrations of individual conjugates can be found. Such conjugated forms of sterols are considered as storage forms of free sterols (Wojciechowski 1991).

1.3.11 Brassinosteroids, a class of plant growth hormones

Brassinosteroids (BRs) are plant growth hormones with different regulatory functions. BRs are synthesized from campesterol in a complex pathway involving side chain hydroxylations and oxidations of the B-ring. Brassinolide (BL) and its immediate precursor castasterone (CS) are the most biologically active BRs in plants. Examples on BR functions include promotion of cell elongation and division, differentiation of the vascular system, and reproduction and stress responses. BRs are also involved in developmental processes including photomorhogenesis and skotomorphogenesis (Altmann 1998; Clouse and Sasse 1998; Clouse 2002; Fujioka and Sakurai 1997). BR mutants defective both in perception/signaling and biosynthesis have been identified in Arabidopsis as well as other plant species. Both biosynthesis and signaling mutants display a characteristic phenotype, including short and robust stature (dwarfs), dark-green round and curled leaves, short petioles and pedicles. Most of these mutants have reduced fertility and a prolonged lifespan. (Noguchi *et al.* 1999)

2 Aims

Considering the importance of SGAs for potato quality and food safety, the aim of this thesis has been to identify key genes and enzymes that are part of the biosynthesis of potato SGAs and their sterol precursors. Special emphasis was given to the proposed role of cholesterol as the principal SGA precursor.

The specific aims were:

• to identify genes involved in sterol and SGA biosynthesis in potato tubers during wounding stress or light exposure.

• to characterize the role of one of the identified genes, *StDWF1,* encoding a potato sterol Δ^{24}-reductase, in sterol and SGA metabolism.

• to investigate the homeostatic regulation of cholesterol and other sterols in plants.

• to evaluate cholesterol as a SGA precursor in potato plants.

3 Materials and methods

3.1 Choice of model plants

Arabidopsis (*Arabidopsis thaliana* (L.) Heynh) is an annual diploid flowering plant, usually rising to 20–25 cm tall. At the stem base the leaves are forming a rosette surrounding a central stem with smaller leaves. The primary roots are simple in structure, and later produces smaller lateral roots. The entire life cycle of Arabidopsis can be completed within six weeks and an individual plant can produce a large number of seeds. Arabidopsis has a small genome size, and easy to cross, mutate and transform. Due to these criteria, Arabidopsis has become the prime organism for plant research and was for instance the first plant species to have its genome sequenced. Sterol metabolism in Arabidopsis has been extensively studied and the current knowledge is to a large part based on a large collection of mutants defective in sterol metabolism. A great numbers of Arabidopsis transformants with up- or down- regulated expression of sterol-biosynthetic genes has also contributed to our current knowledge of plant sterol metabolism in general.

Potato (*Solanum tuberosum* L.) is a perennial plant that can grow about 60 cm high, and in the stem the inflorescences are formed, the flowers are pentamerous. The potato is a self-infertile autotetraploid, which is mostly cross-pollinated by insects. The fertilized flowers develop into berry type fruits that can contain up to 300 seeds. Potato tubers are a swollen part of the stem set below the ground, and constitute a storage organ of the plant. The potato is amenable for genetic manipulation methods, and suitable to a wide range of various tissue-culture techniques. The cultivar 'Désirée' is the most frequently used in transformation experiments because it is very responsive *in vitro*, and is relatively fast in setting shoots from tissue culture

(Beaujean *et al.* 1998). For these reasons this cultivar was used in the transformation experiments of the present study. For stress treatments, common food potatoes such as 'Asterix', 'Bintje', 'Ditta', 'King Edward' and 'Sava' were investigated. The history of 'Bintje' and 'King Edward' cultivars is described in the 1.1.4 section.

3.2 Gene expression analyses by microarray and quantitative real-time PCR (qPCR)

The microarray technology is a widely used technique in molecular biology to study global gene expression in a certain tissue. In a cDNA microarray, single stranded DNA sequences (usually many thousands) are spotted onto a solid surface. The cDNA of two different samples are labeled with a red and a green fluorescent dye and then applied to the microarray slide. The single strands of cDNA hybridize to their complementary sequences on the array and the intensity of fluorophores is scanned. The signal intensity of the sample is then compared to that of a control in the same spot. The relative intensities of the fluorophores are then used to quantify the differential expression of genes between the two samples. The major advantage of microarray technique is that the expression level of a great number of genes can be studied simultaneously.

Quantitative real-time PCR (qPCR) is commonly used for validation of microarray results. In qPCR a use of fluorescent DNA-binding dye is applied to the sample mixture and the fluorescence signal is measured at each PCR cycle that is proportional to the amount of PCR product. Because fluorescence of the dye increases as the dye binds to the double-stranded DNA, the synthesized DNA can be monitored as an increased fluorescent signal. During qPCR two amplification phases can be observed; an exponential phase, followed by a plateau phase. During the exponential phase, the amounts of PCR products double in each cycle. During the plateau phase, the reaction slows down due to limited amount of reaction components. The part of amplification curves that is used for analysis is the exponential phase. The cycle number at which enough amplified product is accumulated to give a detectable fluorescent signal is called threshold value (Ct-value). This Ct-value can be used to calculate the initial amount of the template.

3.3 Analytical methods

Plants contain numerous chemical compounds, and prior to their quantification/identification, appropriate methods are needed both during the sample preparation and the analysis step. Sample clean-up can be performed by separation with thin-layer chromatography (TLC) or solid-phase extraction (SPE). For separation during the analytical step, gas chromatography (GC) or liquid chromatography (LC) can be used depending on the chemical properties of the compound. GC is commonly used for non-polar volatile compounds, and LC for polar non-volatile compounds.

Chromatographic separation is based on the fact that chemical substances interact to different degrees with a stationary phase, which makes them move at different rates through the chromatographic system and thus become separated in time. In GC the mobile phase is a gas, and in LC a liquid. Both GC and LC can be coupled to mass spectrometry (MS) if reliable identification of the substance mass is desired.

3.4 Sterol analysis

Sterols and hydroxysterols were extracted from leaves and tubers with chlorofom/methanol, and subjected to clean-up with TLC for sterols, or SPE for hydroxysterols. The different sterol fractions were then derivatised to TMS ether, and analysed with GC and identified with GC-MS.

3.5 Glycoalkaloid analysis

Briefly, SGAs were extracted with acidified metanol solution, and purified with SPE according to Distl *et al.* (2009). Ratios of isotopes were determined by the selective LC-MS/MS technique. SGA levels were quantified by LC-UV, which is not affected by the ion suppression phenomenon present in LC-MS, and thereby gives more reliable quantification.

4 Results and Discussion

4.1 Transcript profiling of two potato cultivars during wounding or light exposure of tubers reveals key genes in sterol and glycoalkaloid biosynthesis (I)

The SGA biosynthesis and its regulation are mainly unknown processes. To unravel these processes, SGA accumulation after a wounding stress or light exposure treatment was compared in five common Swedish potato cultivars. This showed that SGA accumulation differed significantly among the cultivars. The cultivars 'King Edward' (responsive to both types of stress) and 'Bintje' (tolerant to both types of stress), were chosen for further analyses. Tubers from both cultivars were subjected to time-course experiments, where samples were taken during 48 h following wounding, or 96 h following light exposure. The samples were analyzed for sterol and SGA levels, as well as for parallel changes in transcript profiles. The temporal response of SGA accumulation after wounding was characterized by a slight but significant increase in 'Bintje' and by a stronger increase in 'King Edward'. In contrast, within 96 h of light exposure, only 'King Edward' displayed a significant increase of SGA. Following wounding, a general increase of desmethyl sterols was observed in both cultivars. This included an increase of cholesterol, which was more pronounced in 'King Edward' than in 'Bintje'. Following light exposure, cholesterol increased 2-fold specifically in 'King Edward'. No other significant desmethyl sterol alteration was observed in either cultivar.

To identify wound- or light-regulated genes underlying the SGA accumulation, RNA was extracted from the same tuber materials, and subjected to microarray gene expression profiling. Clustering analyses

revealed that a total number of 1048 and 972 genes where differentially expressed following wounding stress in 'Bintje' and in 'King Edward', respectively. The corresponding result following light exposure was 278 and 438 differentially expressed genes in 'Bintje' and 'King Edward', respectively.

Genes that were differentially expressed in both cultivars following the two treatments were studied in more detail. Following wounding, a statistical overrepresentation of the functional categories ′cellular structure′, ′cellular metabolism′ and ′stress responses′ was demonstrated in both cultivars. Following light exposure, a statistical overrepresentation was shown in both cultivars for genes involved in ′photosynthesis′ and ′stress-responses′. The functional class 'cellular metabolism', was further investigated. This revealed that genes with a role in amino acid and secondary metabolism were overrepresented in both cultivars following wounding. In addition, an overrepresentation of genes involved in sterol metabolism was observed in 'King Edward'. A combination of microarray and qPCR analyses of sterol and SGA biosynthetic genes revealed at least six genes (*HMGR1*, *PSS1*, *SMO1-1*, *DWF1-L*, *SGT1* and *SGT3)* to be more strongly induced by wounding and light exposure in 'King Edward' than in 'Bintje'. Further, a specific up-regulation of *CYP51* after wounding, and of *HYD1* after light exposure, was found in 'King Edward'. This indicates a distinct role for these genes in the respective stress response. Importantly, these eight genes have regulatory functions at different points in the isoprenoid pathway. *HMGR1* and *PSS1* act at the pre-cycloartenol part of the pathway, *SMO1-1*, *DWF1-L*, *CYP51*, *HYD1* at the post-cycloartenol part, and *SGT1* and *SGT3* at post-solanidine part in SGA biosynthesis. These eight genes might thus be candidate key genes for the regulation of sterol and SGA levels in potato.

Two of the up-regulated genes, *DWF1-L* and *SMO1-1*, were closely related to other genes on the array (*DWF1* and *SMO1-2*), but whose level of expression was not altered by the treatments. Comparison of the deduced amino acid sequence of potato DWF1 and DWF1-L revealed a 79% aminio acid identity. Notably, between three and four hydrophobic amino acid residues present at the N-terminal of DWF1-L were absent in DWF1. A phylogenetic analysis of DWF1 and DWF1-L showed that Solanaceous plant species that produce SGA contained genes encoding the two divergent DWF1 proteins (DWF1 and DWF1-L), whereas plant species not producing SGA contained only genes for the DWF1 type of protein.

In summary, these studies show that the degree of SGA induction in potato tubers after a stress differs both between cultivars and the type of stress. The general increase of end-product desmethyl sterols after wounding suggests a need of sterols for membrane repair and synthesis, as well as for SGA production. The increased levels of cholesterol found in association with increased levels of SGA, supports this sterol as a SGA precursor. Six genes acting at different levels in the triterpenoid biosynthesis pathway were up-regulated under conditions of increased SGA synthesis, and might have important regulatory functions for sterol and SGA synthesis. Taken together, these data provide novel insights into the molecular processes underlying SGA accumulation in potato tubers, and allow for the selection of candidate genes for future functional studies.

4.2 Decreased cholesterol and glycoalkaloid levels in transgenic potato plants by antisense expression of a sterol Δ^{24}-reductase gene (II)

In Arabidopsis, the Δ24-reduction step in the biosynthesis of campesterol and sitosterol is catalyzed by the DIMINUTO/DWARF1 (DIM/DWF1) protein. (Klahre *et al.* 1998). The corresponding reaction in cholesterol synthesis is unknown.

Potato contains two distinct *DWF1* types, which encode proteins denoted DWF1 and DWF1-L1, -L2, and -L3 (Manuscript I). Up-regulation of DWF1-L during conditions of increased SGA synthesis indicated a role in this process. To investigate the function of potato *DWF1*-related genes in potato sterol and SGA synthesis, transgenic potato plants were raised expressing *StDWF1* and *StDWF1-L* cDNAs in both antisense and sense orientation from the CaMV 35S promoter.

Compared to the wild type, the StDWF1 and StDWF1-L sense transformants showed an about 2-fold increase of campesterol and sitosterol, whereas no alteration in the cholesterol level was observed. Potato *DWF1* antisense lines showed a clear reduction in levels of cholesterol, campesterol and sitosterol, wheras the corresponding levels of campesterol and sitosterol precursors were increased. These results are well in line with what has been shown in Arabidopsis, rice and pea *dwf1* mutants (Hong *et al.* 2005; Klahre *et al.* 1998; Nomura *et al. 1*999,) and sense overexpressors, and strongly suggest that StDWF1 and StDWF1-L are DIM/DWF1 orthologues. In

addition, 4-monomethylsterol and 4,4´-dimethylsterols were increased in the transformants, indicating that *DWF1* might influence other steps in sterol synthesis. The decreased level of cholesterol, campesterol and sitosterol in antisense transformants could be linked with a significant decrease in SGA. Further, wounding and light exposure of antisense *StDWF1* tubers showed a reduced SGA accumulation compared to the wild type, indicating an important role of *DWF1* in SGA biosynthesis. The results show a role of StDWF1 protein in cholesterol and SGA biosynthesis, and further support cholesterol as a glycoalkaloid precursor.

4.3 Synthesis of hydroxylated sterols in transgenic Arabidopsis plants alters growth and steroid metabolism (III)

To explore the homeostatic regulation of cholesterol and other sterols in plants, cholesterol turnover was increased by over-expression of four mouse cDNA encoding cholesterol hydroxylases (CHs) in both Arabidopsis and potato plants. These four cDNA encode CHs that catalyze the hydroxylation of cholesterol at the C-7, C-24, C-25 or C-27 positions.

In CH7 and CH25 potato transformants the expected hydroxylated cholesterol metabolites, 7-hydroxycholesterol and 25-hydroxycholesterol, respectively, were identified by GC-MS. However, no additional hydroxysterol species could be indentified in the CH27 transformants. Most of the potato CH transformants displayed a normal growth without any major alteration in their sterol profile compared with the wild type. However, the level of cholesterol was reduced to some extent in CH25 transformants, although without any alteration in SGA levels as determined by HPLC analysis.
In contrast to the situation in CH potato transformants, overexpression of CHs in Arabidopsis led to different phenotypic effects, the strongest ones being observed in CH25 lines. The CH25 transgenic plants were dark-green dwarfs with delayed flowering and malformed flowers compared with wild type plants. The phenotype was more severe in the homozygous plants than in heterozygous. CH7, CH24 and CH27 transformants were phenotypically similar to the wild type, although a number of CH24 lines were somewhat smaller in size.

Analyses of hydroxysterol profiles in these transgenic lines demonstrated the expected hydroxylated cholesterol forms in CH7, CH24 and CH25 transformants, while no 27-hydroxycholesterol could be detected in CH27 lines. CH25 plants contained 11 additional hydroxysterol species, whereas there was one extra hydroxysterol in CH24 plants, determined as 24-hydroxy-24-methylcholesterol. The only hydroxysterol found to increase in CH7 lines was 7α-hydroxycholesterol, suggesting a higher substrate specificity of the CH7 enzyme compared with CH24 and CH25. Analysis of larger quantities of wild-type Arabidopsis leaves revealed trace levels of the α and β forms of 7-hydroxycholesterol, 7-hydroxycampesterol and 7-hydroxysitosterol.

Interestingly, the sterol composition in CH24 and CH25 plants was drastically altered compared to the wild type, mainly due to a considerable increase in the level of 24-methylenecholesterol and campesterol (C9) with an associated decrease in the level of sitosterol (C10). Unexpectedly, the cholesterol (C8) content was increased in these transgenic lines compared to the control. A significant increase of 4,4´-dimethylsterols, such as cycloartenol and 24-methylene cycloartanol, and almost all 4-monomethylsterols analyzed was also observed. The increase or decrease of specific sterols was less pronounced in CH24 lines than in the CH25 lines. No significant variation in the levels of sterols in CH7 and CH27 plants could be observed compared to the control plants. A parallel analysis of enzyme activities in CH24 transformants revealed 1.6-fold and 3-fold higher SMT1 and SMT2 activity, respectively. The same enzymes showed 3-fold and 7-fold higher activity in the CH25 transgenic lines.

An investigation of BRs in CH25 lines revealed a decrease of several BRs in CH25 plants compared to the control. The reduction was more pronounced in the homozygous plants than in the heterozygous. The growth defects of CH25 heterozygous plants could be rescued completely by the application of castasterone (CS) and brassinolide (BL), although the homozygous plants could not be rescued fully. These results implicate that the growth defects of CH25 plants are caused by a reduced synthesis of endogenous BRs. In addition, investigation of CH24 and CH25 plants to various physiological stress treatments revealed a higher drought tolerance of CH25 line compared to the control plants.

The altered sterol composition in CH24 and CH25 lines suggests that a hydroxylation of the sterol side chain has a stronger biological effect than

hydroxylation of the sterol skeleton as in CH7 lines. Altered sterol levels could be caused by a hydroxysterol-mediated inhibition or activation of rate-limiting enzymes in the sterol pathway. The higher activity of the enzymes SMT1 and SMT2 observed in CH24 and CH25 lines could be a compensatory mechanism to overcome the altered sterol metabolism. The enhanced drought tolerance observed in CH25 lines could be caused by BR deficiency, causing a constant cellular stress activating stress tolerance genes, and/or enhancing the activities of relevant enzymes.
These results also implicate that potato plants are capable of compensating for an increased turnover of cholesterol, possibly via feed-back regulation of key enzymatic steps. This would mean that either cholesterol itself, or a hydroxylated cholesterol species could participate in a feed-back regulation earlier in the biosynthesis pathway. In potato, such system might sustain a high level of endogenous cholesterol at the expense of C9 and C10 sterols.

4.4 Cholesterol is a glycoalkaloid precursor in potato plants (IV)

The presence of SGA in potato plants has been known since 1826 (Baup 1826) but the biosynthetic pathway has not yet been completely elucidated. Some studies have indicated cholesterol as the main SGA precursor, but firm evidence is lacking. Based on radioactive cholesterol feeding, a hypothetical biosynthetic pathway of SGA in potato proceeds via the following steps: cholesterol → solanidine → α-chaconine and α-solanine (Heftmann 1983). Clear evidence for this model is lacking, partly because of the weaknesses of the analytical methods applied so far. Today, incorporation of heavy isotope-labeled precursor into a product coupled to its identification by MS analysis offers a more rigorous evidence for a substrate-product relationship. Considering the role of cholesterol as a likely precursor to SGA, potato plants were fed with deuterium-labeled cholesterol (D_5-cholesterol) for a period of three weeks. The uptake of labeled cholesterol and subsequent formation of labeled SGA were analyzed by GC-MS and LC-MS/MS, respectively.

GC-MS analyses of endogenous and D_5-cholesterol showed a variation in the uptake and transport of added D_5-cholesterol. Of the 200 μg labeled cholesterol applied, 2.9% was recovered in the free form, with 2.15 % in the old stem, 0.013% in young stem, 0.68% on old leaves and 0.014% in young leaves. For all D_5-cholesterol treated tissues, label was incorporated in both

α-chaconine and α-solanine. The turnover from labeled cholesterol to labeled SGA was greater in the more metabolically active young parts than in the older parts of the plant. The turnover from D_5-cholesterol into D_5-SGA was found to be around 22% in young leaves, 11.5% in young stem, 3% in old leaves and 0.2 % in old stem. The results indicate that the metabolic conversion of cholesterol into SGA decreases in the following order; young leaves → young stem → old leaves→ old stem. The fact that rather low total amounts of labeled D_5-cholesterol was found in the plant materials suggests that the excess amount of cholesterol might have been saved in conjugated forms, such as steryl esters. The excess D_5-cholesterol might also have been used for synthesize of other compounds than SGA, such as oxysterols and BR.

In conclusion, the study unequivocally establishes cholesterol as a precursor to both α-chaconine and α-solanine in the potato. This confirms earlier studies where radioactively labeled cholesterol applied to potato and tomato tissues showed incorporation into the SGA fraction (Heftmann *et al*. 1967; Heftmann 1983). The results also lend credibility to other studies suggesting cholesterol as a precursor for other types of defense metabolites such as diosgenin, saponin, and phytoecdysones.

5 Conclusions

- The relative increase of SGA levels in potato tubers in response to wounding and light exposure varies among potato cultivars. 'King Edward' was shown to be a particularly responsive cultivar in this aspect, whereas 'Bintje' was more tolerant.
- Induction of SGA accumulation by wounding and light exposure is due to the concerted action of at least six genes acting at different positions in the sterol/SGA biosynthesis pathway. Wounding and light increase SGA levels largely by activating the same set of genes, although certain stress-specific differences do exist.
- The potato *StDWF1* and *StDWF1-L* genes constitute a small multigene family, orthologous to the Arabidopsis *DIM/DWF1*. Expression of *StDWF1-L*, but not of *StDWF1*, was strongly up-regulated by wounding and light exposure. The occurrence of *StDWF1-L* in plants was seemingly restricted to species containing SGA, indicating an important role in SGA biosynthesis.
- Altered expression of *StDWF1* and *StDWF1-L* in transgenic potato plants indicated a similar role of the corresponding proteins in sterol and SGA biosynthesis. Increased expression of the gene(s) is likely necessary, but not sufficient, for elevated SGA after a stress.
- Hydroxysterols were identified by GC-MS as naturally occurring sterol metabolites in Arabidopsis plants. Increased sterol turnover by hydroxysterol production in Arabidopsis transformants altered growth and sterol/BR metabolism, indicating a biological role for hydroxysterols in plants.
- Incorporation of deuterium-labeled cholesterol into deuterium-labeled α-chaconine and α-solanine was shown using LC-MS/MS. This confirms previous radiochemical investigations, and clearly establishes cholesterol as a SGA precursor in the potato.

6 Future Perspectives

- In order to study the regulation of sterol and SGA biosynthesis at the transcriptional level, a comparative promoter analysis of sterol and SGA-regulating genes could help to identify common elements, and eventually also the gene regulatory network(s) in function. Promoters sharing a common function, *e.g.* promoters responsive to wounding, could clarify co-transcription of genes involved in sterol and SGA regulation. Doubtlessly, the sequencing of the potato genome will in the near future simplify this type of studies.
- Of particular interest for SGA synthesis is the insertion of the nitrogen atom into cholesterol derivatives, which most likely involves an amino acid such as glycine, alanine or arginine as a donor molecule. Incorporation studies with ^{15}N-labeled glycine, alanine or arginine administrated to potato shoots could clarify the origin of nitrogen atom in potato SGA. Further, potato genes encoding enzymes likely to catalyze the introduction of the nitrogen atom into SGA, could be isolated. Their functional role in SGA biosynthesis could be tested using either overexpression or down-regulation in transgenic plants. The same approach could be applied for genes encoding enzymes that might be involved in the hydroxylation process of intermediate metabolites of solanidine. In addition, deuterium-labeled hydroxycholesterol derivatives could be characterized in potato shoots after feeding with deuterium-labeled cholesterol. The combination of feeding experiments and genetically engineering techniques may eventually clarify the entire SGA biosynthesis pathway in potato plants.
- The transgenic Arabidopsis CH25 plants with increased levels of hydroxysterols and altered end-product sterols showed enhanced drought tolerance. In the future it would be interesting to see if these plants are tolerant also to other stresses, *e.g.* various pathogens. Since abiotic stresses

can affect a complex network of processes it would be interesting to investigate the mechanism behind the drought tolerance, for instance using a microarray study.

- In order to gain more insight on the occurrence of endogenous hydoxylated sterol species in Arabidopsis, a time-course feeding experiment with labeled sterol precursors could be applied to elucidate the hydroxysterol synthesis in this plant. To further explore the function of hydroxysterols in plants one could exogenously apply hydroxysterols to wild type plants and evaluate the rate limiting enzyme activity in sterol biosynthesis, and/or global transcript changes.
- To gain more knowledge about the cholesterol synthesis in potato plants, antisense expression of the key gene at the branch point, *SMT1,* could help to investigate the rate limiting steps involved in cholesterol pathway. Alternatively, Arabidopsis *smt1* mutants could also be used as a model to identify the rate limiting steps in this pathway.
- The identification of key genes in SGA synthesis could be used to test or predict the stress sensitivity among different potato cultivars, thereby facilitating the potato breeding process.

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