Effect of wounding and light exposure on sterol, glycoalkaloid, and calystegine levels in potato plants (*Solanum tuberosum* L. group Tuberosum)

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Abstract

Steroidal glycoalkaloids (SGA) are neurotoxic substances that are present in some members of the Solanaceae family, including crop species like potato (*Solanum tuberosum* L.) and tomato. The SGA level in the potato tuber is a genetic trait, but certain environmental factors such as wounding and light exposure can increase SGA levels several-fold, which may render tubers unsuitable for human consumption. There is little information about SGA biosynthesis. The sterol cholesterol is commonly regarded as a SGA precursor, but there is little evidence for this view.

To increase our understanding of the SGA biosynthesis and its molecular regulation, a microarray screen was performed using tubers from two potato cultivars subjected to wound and light treatments. Along with an alteration of sterol and SGA levels, the treatments were associated with an up-regulation of a small set of genes in sterol and SGA metabolism, including a gene encoding for the sterol reductase DWF1. *DWF1* genes were found in two differentially regulated subtypes; *DWF1* and *DWF1-like* (*DWF1-L*). Alteration of *DWF1* and *DWF1-L* expression in transgenic potato showed a role for these genes in sterol and SGA synthesis. Also up-regulated in the microarray study were three transaminase-like genes, and role of *StTAM1* in SGA synthesis was investigated by overexpression in transgenic potato. This resulted in elevated SGA levels, indicating the presence of a transamination in SGA synthesis. The genetic variation and stress responsiveness in Swedish potato cultivars regarding SGA and calystegine alkaloids (CA) level was determined by subjecting tubers to wounding, light exposure and elevated temperature. Only light and wounding increased SGA levels, and variation in the response was observed among the cultivars. CA levels were not stress-regulated, indicating that SGA and CA synthesis are not interrelated.

These results show that the SGA level in potato tubers are regulated by a concerted action of a small set of key genes acting at different steps in the sterol and SGA pathways. Results also demonstrate a genetic variation in stress responsiveness among Swedish potato cultivars, and have identified the most sensitive ones. Results could in the near future be used to improve post-harvest handling of potato cultivars.

Keywords: *Solanum tuberosum*, glycoalkaloids, sterols, calystegines, cholesterol, microarray.

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Dedication

To my parents and grandparents

As long as a word remains unspoken, you are its master; once you utter it, you become its slave.

Jabir Ibn Hayyan

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., Petersson, E., **Arif, U**., Beste, L., Dalman, K., Dutta, P., Jonsson, L., Sitbon, F. Transcript profiling of two potato cultivars during wounding and light exposure of tubers reveals key genes in sterol and glycoalkaloid biosynthesis. *Submitted for publication in Plant Physiology*

II Beste, L., **Arif, U**., Petersson, E., Nahar, N., Jonsson, L., Dutta, P., Sitbon, F. The potato sterol Δ24-reductase DWF1 has a key role in sterol and glycoalkaloid biosynthesis. *Manuscript*

III **Arif, U**., Petersson, E., Meijer, J., Sitbon, F. Overexpression of a transaminase-like cDNA in transgenic potato plants is correlated with an altered growth pattern and increased glycoalkaloid levels. *Manuscript*

IV Petersson, E[#]., **Arif, U**[#]., Schulzova, V., Krtkova, V., Hajslova, J., Meijer, J., Andersson, C., Jonsson, L., Sitbon, F. Glycoalkaloid and calystegine levels in table potato cultivars subjected to wounding, light, and heat treatments. *Accepted for publication in Journal of Agriculture and Food Chemistry.*

Joint first authors

Additional paper

Petersson, E/Nahar, N., Dahlin, P., **Arif, U**., Broberg, A., Åslund-Tröger, R., Dutta, P., Jonsson, L., Sitbon, F. Conversion of exogenous cholesterol into glycoalkaloids in potato shoots. *Submitted for publication*

The contribution of Usman Arif to the papers included in this thesis was as follows:

I Construction and verification of four sense and two antisense *35S:DWF1* Ti plasmids. Generation of the corresponding six transgenic DWF1 potato lines, and four Arabidopsis lines (platform transformation). Molecular characterization of all transgenic lines by RT-PCR. Summarized the transformant results. Participation in sterol and glycoalkaloid extractions concerning the potato transformants.

II Vector construction and generation of two antisense DWF1-L potato lines, plus molecular and functional screening of transformants. Summarized the transformant results. Participation in tuber stress treatments and subsequent glycoalkaloid extractions.

III All experimental work except LC-UV analysis. All bioinformatics work including phylogenetic analysis. Summarized the results and wrote the manuscript.

IV Participation in the outdoor growth of all potato cultivars. Responsible for stress experiments and compilation of the results. Participation/responsible in all glycoalkaloid extractions. Responsible for the collection of samples for calystegine analysis. Participation in writing of the manuscript. Shared first author.

Abbreviations

Acetyl-CoA	Acetyl-coenzyme A
BR	Brassinosteroids
CA	Calystegines
DIM/DWF1	Diminuto/DWARF1
DWF1-L	DWARF1-like
f.w.	Fresh weight
GC-MS	Gas chromoatography-mass spectrometry
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HMGR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
LC-MS	Liquid chromatography-mass spectrometry
LC-UV	Liquid chromatography-ultraviolet
PMT	Putescine methylransferase
RT-PCR	Reverse-transcriptase polymerase chain reaction
SGA	Steroidal glycoalkaloid
SGT1	Solanidine galactosyltransferase
SGT2	Solanidine glucocosyltransferase
SGT3	Rhamnosyltransferase
SMO	Sterol-4α-methyl oxidase
SMT	Sterol methyltransferase
SPE	Solid-phase extraction
SQS	Squalenesynthase
TRI	Tropninone reductase I
TRII	Tropinone reductase II

1 Introduction

1.1 The origin and history of the potato as a crop species

The potato (*Solanum tuberosum* L.) is today the most important non-grain food crop species worldwide, and is as such central to global food security. The potato is a dicotyledonous plant species belonging to the Solanaceae family that also contains other important crop species such as tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum* L.), sweet peppers (*Capsicum annum*) and eggplant (*Solanum melongena*) (Friedman *et al*., 1997). The potato is an annual plant species that can grow up to 30-100 cm high and form storage tubers below ground. The commercially grown potato is an auto-tetraploid that is generally propagated by vegetative means from tubers, but can also be cross-pollinated to form true seeds. Potato is a cool weather vegetable and the best time for growth is early spring. Potato finds itself successful because of its ability to grow under different environmental conditions, requiring very little but water and a slightly acidic soil.

The potato originated in the South American Andes of Peru and Bolivia, where it was domesticated between 10 000 - 7000 years ago (Spooner *et al*., 2005; Bradshaw and Ramsay, 2009). During the Spanish conquest of the Inca Empire in the 16th century, the potato was introduced into the Canary Islands (Francis, 2006). From Spain, over the next five centuries, it spread throughout Europe, Asia, North America, the Middle East, and Africa (Van deer Zaag, 1984; Burton, 1989; Rodger, 2007). Soldiers taking part in war during the 19th century introduced potatoes in Sweden. The cultivated potato (*S. tuberosum*) came into existence as a result of hybridization events between closely related species (Rodrigues *et al*., 2010).

However, the potato crop can be damaged significantly by pathogens such as the oomycete *Phytophthora infestans,* causing the late blight. The ´Great Irish Famine´ is well-known example on severe late blight consequences when

entire Irish potato harvest was lost for three successive years (1845-1847). This resulted in one million deaths from starvation due to the near monoculture of potatoes at that time. Thanks to more resistant varieties and better growth methods potato cultivation and production has been revolutionary during the 20th century, and the potato is now the world´s fourth most important crop after maize, wheat and rice (Burton, 1989).

1.1.1 Role of potatoes in global food requirement

Today, the potato is cultivated in all continents except the Antarctica (Hijmans, 2003). The world production of potatoes in year 2010 was 324 million tons (FAOSTAT) or about 50 kg per person on Earth, with China being the largest potato-producing country (Wang and Zhang, 2010). Potato production has in recent years observed a shift from the developed world towards the developing world that now accounts for about 80% of total potato production (FAOSTAT). Potato breeding is of great importance to develop new varieties with higher yield and more efficient use of water and nutrients, as well as better flavor and nutrient contents. In order to meet the increasing food requirements from a growing population, the potato is well-suited thanks to its rapid growth and food production. To acknowledge the role of the potato in global food production, the year 2008 was declared as the International year of Potato (IYP, 2008).

The potato tuber is nutritious because it provides important nutrients such as carbohydrates, vitamins, minerals, micronutrients and dietary fiber (Rodger, 2007). The tubers are derived from underground stem parts known as stolons. The upper part of stolons swells and develops into a tuber. Potato tubers provide different minerals like magnesium, potassium and phosphorus. Potato starch is a carbohydrate that can be metabolized by humans to get energy and in edible tubers ranges between 15-20g per 100g of potato tuber fresh weight (Bradshaw and Ramsay, 2009).

1.1.2 Potato varieties

The potato crop has flourished over the years through breeding practices and there are today more than 4000 potato varieties in commercial use across the globe, with some cultivars having greater agricultural importance than others (Roach, 2001). High productivity, disease resistance, resistance against insects and pests, tuber appearance and taste are some factors that make a cultivar suitable for cultivation and human consumption (Lisinska *et al.* 1989). In Sweden, over 100 varieties are grown for commercial use. There are no official statistics on the harvest or economic importance of the different varieties, but an estimate can be made from the production of certified seed potatoes

(Statistics Sweden, 2012). This shows that three cultivars (King Edward, Asterix and Bintje) together account for more than 50% of all certified potatoes. ´King Edward´, alone accounts for almost 25% of total production, an astonishing fact considering that the cultivar was introduced more than 100 years ago. It is reasonable to assume that this general picture is true also for total production of potatoes in Sweden.

Solanum tuberosum is an autotetraploid species with 48 chromosomes. The basic chromosome number is 12. A milestone in potato research was reached in year 2011 when the potato genome sequence was released (Xu *et al*., 2011). The genome sequence was derived from the double haploid genotype of the *S. phureja* group, and from a diploid *S. tuberosum* breeding line. 39,031 protein-coding genes were annotated with 2,642 genes being specific to this large angiosperm clade. The availability of the potato genome sequence is a breakthrough in breeding as well as in genetics. The importance of potato tubers from a food perspective makes it imperative to understand metabolic and developmental processes occurring during different growth stages, and the potato genome will be critical to unfold genetic mechanisms underlying these processes. The use of potato is not restricted only for dietary purposes but potato application on industrial scale has flourished over the years especially due to starch production. Potato starch is a versatile polymer that can be used as renewable raw material and as a source of energy after conversion into ethanol. The starch can also be used as delivery vehicles that protect pharmaceutically active proteins from digestion (Jobling, 2004).

1.2 Plant secondary metabolism

Plant cells contain far more compounds than are needed for their growth and development. These extra compounds are known as secondary metabolites. Primary metabolites have thus functions that are essential for survival, whereas secondary metabolites are not critical for survival, but can be useful for the plant as a whole.

1.2.1 Structural diversity and functions of secondary metabolites

There has for long been debate over the function of secondary metabolites in the plant. According to one view ´´they are waste or detoxification products´´ (Williams *et al*., 1989). However, many agree on that common roles for these secondary metabolites where a function has been shown are to participate in plant defense and in the interaction with various organisms (Kimura *et al*., 2001). Additional roles are in symbiosis and allelopathic processes. The impact

of secondary metabolites on humans is huge with roles as pharmaceuticals, agrochemicals, and ingredients in cosmetics as well as food additives.
In plants, there is an immense structural diversity among secondary metabolites. This is controlled by well-defined biosynthetic pathways (Hartmann, 1996). Secondary metabolites are classified according to their biosynthetic origin, and some of the classes include polyketoids, phenylpropanoids, amino acids and peptides, terpenoids and alkaloids. This classification has its limitation due to the fact that some compounds use building blocks from multiple biosynthetic pathways, and some compounds might look similar enough, but are derived from different pathways (Gershenzon *et al*., 2012). However the majority of secondary metabolites are still recognized as having no known function.

1.2.2 What are Alkaloids?

The alkaloids constitute one of the most diverse groups of secondary metabolites in living organisms and have an array of structure types, biosynthetic pathways, and pharmacological activities (Osbourn and Lanzotti, 2009). A simple definition of an alkaloid has been suggested by Pelletier (1983): ´an alkaloid is a cyclic compound containing nitrogen in a negative oxidation state that is of limited distribution in living organisms´. About 27000 alkaloid structures have been characterized, of which 21000 are present in plants. Alkaloids carry one or more nitrogen atoms as primary, secondary or tertiary amines, and this confers alkaline properties to the molecule. However, the degree of basicity varies depending upon the structure of the alkaloid molecules and the presence or location of other functional groups (Dewick, 2011). Alkaloids are often classified according to the nitrogen-containing structure, *e.g.* pyrrolidine, piperidine, quinoline, isoquinoline, indole. Although, structural complexities expand rapidly to generate new subdivisions. The biosynthesis of alkaloids often involves building blocks from the acetate, shikimate, or methylerythritol phosphate pathways. The principal nitrogen donors involved in alkaloid biosynthesis are ornithine, lysine, tyrosine, tryptophan, and histidine (Dewick, 2011; Kaneko *et al*., 1976). The nitrogen is acquired via a transamination process, incorporating only the nitrogen atom from the amino acid, whereas the rest of the alkaloid molecule is derived from other building blocks (Prabhu and Hudson, 2010).

1.2.3 Alkaloid function in Plants and Animals

Alkaloids are part of an elaborate system of chemical defense in plants. The plants sequester alkaloids to use as a passive defense mechanism by acting as feeding deterrents for predating insects (Bentley *et al*., 1984). The bitter taste

of many alkaloids may be of importance in this aspect, and some alkaloids, *e.g.* quinine, strychnine, and brucine, are extremely bitter. There are, however, little data available on the ecological role of bitterness in plant-animal relationships. Although, it has been suggested that the bitterness of alkaloids is a universal feeding deterrent in plant foodstuffs (Molyneux and Ralphs, 1992). In addition to bitter taste, alkaloids may have a variety of toxic effects on animals. Toxic alkaloids are also found as part of conspicuous, often violent, insect defense systems, and these may be synthesized by the insect or acquired as part of their diet. Similar to plants, use of alkaloids as defense chemicals can be seen in marine ecosystems. Marine sponges may produce deterrent alkaloids such as latruculine and petrosins, which are assumed to be part of a chemical defense system (Proksch, 1994). Also algae (blue, red, green) produce alkaloids.

1.2.4 Alkaloid effects on Humans

Alkaloid-containing plants are an intrinsic part of diet and their presence in food chain can affect mammals including humans The pyrrolizidine alkaloids can cause acute and chronic liver toxicity (Koleva *et al*., 2012). Piperidine alkaloids can cause gastric mucosal injury, if consumed at much higher dose (Myers *et al*., 1987). Quinolizidine alkaloids present in lupin beans showed toxicity in humans who suffered from blurry vision, dry mouth and confusion (Di Grande *et al*., 2004). Ergot alkaloids interact with monoamine receptors such as dopamine receptors and adversely affect the cardiovascular, nervous and immune system of humans and animals (Horvath et al., 2004). Several of our most commonly used drugs are alkaloids from natural sources, and new alkaloid drugs are still being developed for clinical use. Alkaloids have many pharmacological activities including anticancer actions (Crag and Newman, 2005). Antibiotic activities are common for alkaloids and some are even used as antiseptic in medicine. However, it is difficult to know to which extent alkaloids give antimicrobial protection in the plant.

1.3 Alkaloids in the Solanaceae family

Some plant families are particularly rich in alkaloids. These families include the Apocynaceae, Rubiaceae, and Solanaceae. The Solanaceae alkaloids can be divided in two main classes, the steroidal and the tropane alkaloids. In addition to the SGAs, potato produces a range of other biologically active secondary metabolites including calystegine alkaloids (CA), protease inhibitors, lectins and phenolic compounds (Friedman *et al*., 1997). Steroidal alkaloids are triterpene-derived metabolites, often glycosylated to yield steroidal glycoalkaloids (SGAs) (Ginzberg *et al*, 2009). Tropane alkaloids and

glycoalkaloids are important alkaloids among the Solanaceae family members. Both these classes of alklaloids are produced naturally as a defense mechanism against insects, predators and pathogens, but can also have detrimental effects on humans if ingested orally (Friedman *et al*., 2003). Considerable interest in these substances comes from both medical use, and the agronomic impact in Solanceous food crops.

1.3.1 Potato glycoalkaloids

There are more than 80 different SGAs identified in potato species. The main SGAs in the cultivated potato are α-solanine and α-chaconine, which together constitute about 95% of total SGAs present in cultivated potato. The SGA level varies between tissues, cultivars and growing conditions (Ginzberg *et al*, 2009). Other members of the Solanaceae family that produce SGAs are tomato, eggplant and pepper. The toxicity varies between different forms of SGAs, but the only SGAs recorded as causing human deaths are those produced by potato (Morris and Lee, 1984).

The potato SGAs can be found in most of the tissues of plant but there is no evidence showing the transport of SGAs between different plant parts that means biosynthesis and catabolism of SGAs are regulated at tissue or organ level. The highest level has been reported to be present in floral and sprout tissues of potato plants (Smith *et al*., 1996; Friedman and McDonald, 1997). Inside the potato tubers, the highest levels of SGAs have been detected in the 1.5 mm of tissue just below the skin (Valkonen *et al*., 1996). Levels then decline towards the center, forming a gradient that is different between genotypes.

1.3.2 Structure of glycoalkaloids

The major *Solanum* alkaloids are steroidal alkamines, which possess the C27 steroidal skeleton of cholestane (Friedman *et al*., 1997). They represent one of the five structural groups including solanidanes, spirosolanes, 22,26-epiminocholestane, α-epiminocyclohemiketals, and 3-aminospirostanes (Schreiber, 1968). The SGAs found in potato species are of the solanidane and spirosolane type. The major SGAs present in cultivated potato species, α-solanine and α-chaconine, both are derived from the same aglycon; solanidine, but differ in their carbohydrate structure (Kuhn *et al*. 1955a, b). Two spirosolanes, solasodine and tomatidine, are the aglycons that give rise to SGAs in other *Solanum* family members. Solasodine is the aglycon of α-solasonine and solamargine SGAs, whereas tomatidine is the aglycon of α-tomatine and dehydrotomatine (Friedman *et al*., 1997).

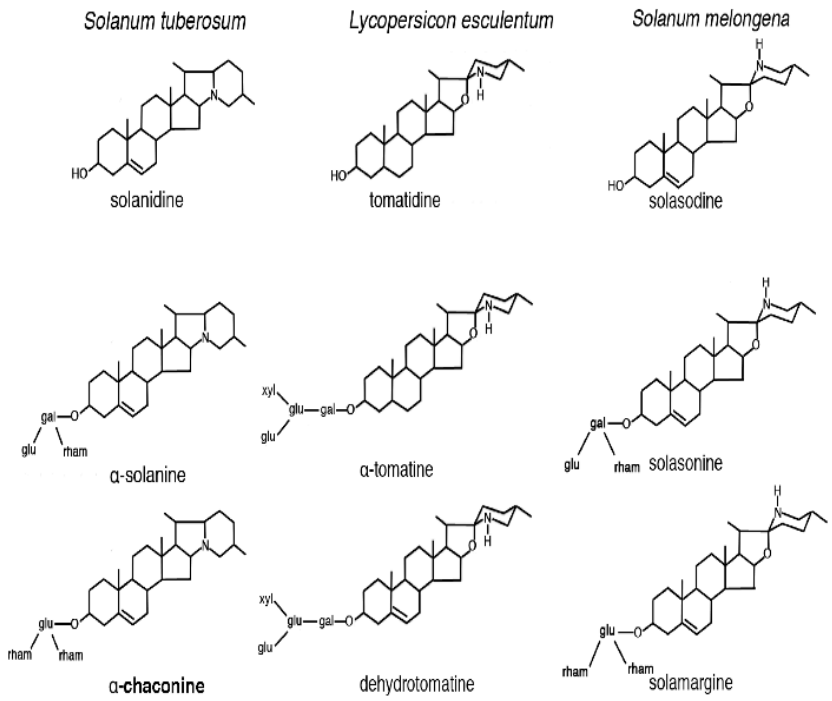

Figure 1. Chemical structures of major glycoalkaloids and their corresponding aglycones in some Solanceae family member (Nahar, 2011).

Aglycons have both polar and non-polar characteristics; a hydroxy group at the C-3 position makes their structure polar, while the rest of the C27 steroidal skeleton is non-polar (Heftmann, 1983). The saccharides attached to the 3-hydroxy position of aglycones can be found in different combinations. These saccharides include D-glucose, D-galactose, D-xylose and L-rhamnose in various combinations. In cultivated potato, one (γ-form), two (β-form), or three (α-form) sugar molecules are attached to the 3-hydroxy position of solanidine. D-glucose, D-galactose and R-rhamnose are the three sugar molecules attached to form α-solanine, while one D-glucose and two R-rhamnose sugar molecules are attached to make α-chaconine. This saccharide combination is known as solatriose and chacotriose for α-solanine and α-chaconine respectively. There are level of aglycons with di or monosaccharides (Osman and Sinden, 1977). In tomato, four saccharides are attached to the 3-hydroxy position of α-tomatine. These include D-xylose along with the above mentioned three saccharides, and the structure is named as lycotetraose (Kuhn *et al*., 1956). The SGAs present in egg plant, solasonine and solamargine carries the same three

saccharides as the cultivated potato, but the steroidal skeleton is different (Figure 1).

1.3.3 Glycoalkaloid biosynthesis

The potato is an important crop from an agronomic point of view, but there is still very little known about the SGA biosynthesis in potatoes and other solanaceous crop species. The biosynthesis of SGA is generally considered to originate from sterols (Heftmann, 1983). In potato and other plant species, the end-product sterols include cholesterol, campesterol and sitosterol, which have important functions in regulating the fluidity and permeability of membranes. Cholesterol is a minor sterol in most plant species, but occurs at high levels in SGA-producing species including potato, tomato and eggplants. Cholesterol has been suggested as the most likely precursor of SGAs (Heftmann, 1983), but firm evidence to prove this are still lacking. The incorporation of radioactivity in the SGA fraction using radioactively labeled cholesterol subjected to potato and tomato tissues led to the view of cholesterol as a SGA precursor (Heftmann, 1983). Some studies have since supported a precursor role for cholesterol. The inhibition of cholesterol synthesis with the inhibitor tridemorph led to reduced levels of SGAs in tuber discs. Along with this, incorporation of (2-^{14}C)-mevalonate into cholesterol when applied to potato discs also further indicated a possible role of cholesterol in SGA formation (Bergenstråhle *et al*., 1996). Transgenic potato plants with reduced levels of cholesterol displayed significantly reduced levels of SGAs (Arnqvist *et al*., 2003). Recently, the feeding of potato tissues with deuterium-labeled cholesterol resulted in the presence of deuterium-labeled SGAs (Nahar, 2011), strongly indicating a precursor role for cholesterol. Whether cholesterol is the only precursor, or just one precursor, remains to be investigated.

However, little is known about how cholesterol would be converted into SGA. Based on radioactive incorporation studies, Heftmann and Weaver (1974) proposed that the first metabolite of cholesterol was 26-hydroxy cholesterol, although validity of the analytical methods was questionable. In one model for SGA synthesis, the conversion of cholesterol into SGAs is activated by hydroxylation at C-22, followed by another hydroxylation at C-26 to produce dormantinol and dormantinone. A further oxidation step at C-22 was proposed before addition of nitrogen at C-26 (Petersen *et al*., 1993). In another study, cholesterol was suggested to be the starting point of solanidine also in *Veratrum grandiflorum*, a SGA-containing monocotyledoneous species.

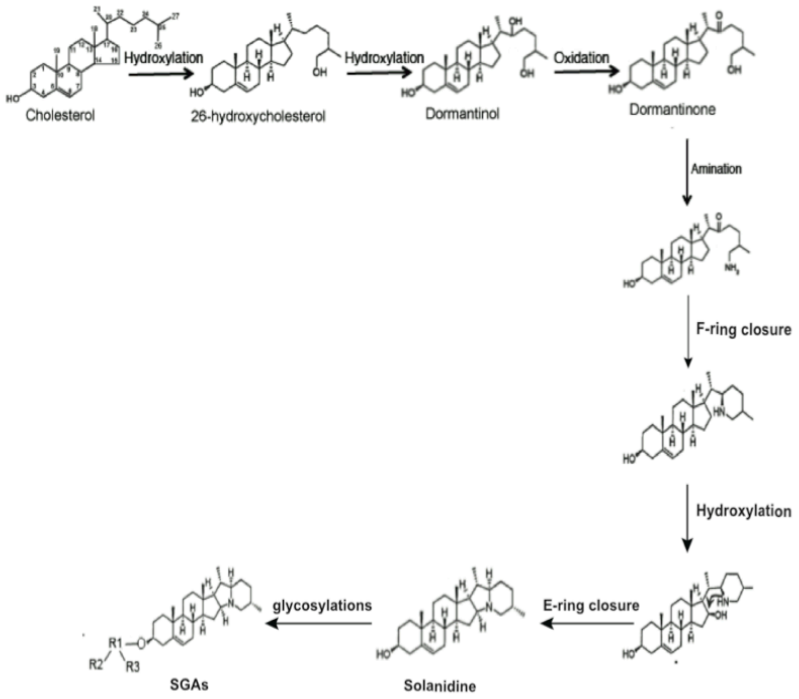

Figure 2. A schematic representation of SGA biosynthesis from cholesterol in *Solanum tuberosum* (modified form of Kaneko *et al*. 1976, Petersen *et al*. 1993, Nahar, 2011).

The conversion of cholesterol to solanidine was proposed to involve hydroxylations of cholesterol to produce dormantinol, followed by oxidation at C-22 to synthesize dormantinone. An amino group then replaces the hydroxyl group at C-26 by a transamination reaction that involves the amino acid arginine to produce verazine (Kaneko *et al*., 1976, 1977). The role of transamination in SGA biosynthesis was investigated in the present thesis (**III**). Whereas the metabolism of cholesterol to solanidine is rather unknown, the further metabolism of solanidine to SGA has been well investigated. In potato, the final steps involved in SGA biosynthesis include the addition of sugar molecules to the C-3 hydroxyl group of solanidine by three different glycosylating enzymes; solanidine galactosyltransferase (SGT1), solanidine glucosyltransferase (SGT2) and rhamnosyltransferase (SGT3). SGT1 catalyzes the initial formation of γ-solanine , whereas SGT2 catalyzes the corresponding formation of γ-chaconine. SGT3 then, catalyzes the final synthesis of α-

chaconine and α-solanine from their respective β-forms (McCue *et al*., 2005, 2006, 2007). The tomato SGT1-like enzyme GAME1 was demonstrated to be necessary for glycosylation of the aglycon tomatidine to synthesize α-tomatine. By down-regulation of GAME1, a 50% reduction in α-tomatine was observed. This showed the role of glycosylating enzymes as rate-limiting steps in the tomato SGA biosynthesis (Itkin *et al*., 2011).

1.3.4 Glycoalkaloid toxicity

SGAs are natural toxins and thought to have evolved as protective compounds in response to tissue invasion (Nema, 2008). The potato SGAs can improve taste of tubers, but can also become toxic at higher levels. The aglycon solanidine is less toxic than the corresponding glycosides that can be extremely poisonous. α-Chaconine is more toxic than α-solanine, but together they show synergestic effects (Roddick *et al.* 1988). To avoid problems of toxicity, 200mgSGA/kg of fresh weight of tuber is recommended as an upper safe limit. However, under certain environmental conditions, the SGA synthesis is enhanced, and levels can go upto higher than safe limit. A lethal dose in humans has been estimated to 2-6 mg kg^{-1} BW (Chen and Miller, 2001). SGAs at higher levels can also interfere with function of the central nervous system via inhibiting cholenestrase enzyme activity (Friedman *et al*., 1997). Toxic effects of SGAs give symptoms like gastrointestinal disorder, confusion, convulsions, coma or even death. SGAs are stable at even higher temperature so cooking and potato processing does not destroy them. SGAs also exhibit a long residence period in the human body so long-term consumption of potato can also lead towards poisonous effects (Friedman, 2006). To overcome this toxicity, cultivars with SGAs about 5-6mg/100g of fresh tubers are highly recommended (Parnell *et al*. 1984).

1.3.5 Glycoalkaloids role in plant defense processes

Several studies have shown that *Solanum* SGAs are significant in conferring plant resistance against bacteria, fungi and insects. Phytopathogenic fungi in tomato need to break resistance caused by α-tomatine and phytoalexine for pathogen ingress and virulence. Some SGAs have a low antifungal activity when tested alone, but combinations of different steroidal compounds display pronounced synergistic effects (Roddick *et al*., 1988; Fewell and Roddick, 1993). Solanum SGAs play a significant role in resistance against the Colorado potato beetles (*Leptinotarsa decemlineata*) and the resistance is usually associated with higher levels of foliar potato glycoalkaloids (Lorenzen *et al*., 2001). Potato tubers stressed with Colorado potato beetle, accumulated higher levels of SGAs than unstressed tubers, to impart resistance (Hlywka *et al*.,

1994). Both solasonine and solamargine in mixture have been found to kill snails (Alzérecca and Hart, 1982), whereas α-solanine and α-chaconine alone deters feeding of snails (Smith *et al*., 2001). It has also been shown that there is direct correlation between potato leafhopper resistance and higher foliar solanidine glycoside contents (Sanford *et al*., 1990). According to Friedman (2002), increased accumulation of α-tomatine on tomato leaves, infected with bacteria *Clavibacter michiganense*, is an indication of defence initiation. Allopathic effects of SGAs were also shown on plant root growth (Sun *et al*., 2010).

It is obvious that *Solanum* SGAs are important for plant resistance, but there are studies that do not agree with any clear role of SGAs in plant resistance, for example resistance against *P. infestans* and potato leaf hopper (*Empoasca fabae*) (Tingey and Sinden, 1982). Moreover, many organisms have found ways to cope with SGA-induced resistance by having hydrolytic enzymes that convert SGAs into less toxic compounds (Osbourn, 1996).

1.3.6 SGA degradtion

It has been shown that potato tubers and sprouts contain hydrolytic enzymes; *e.g.* rhamnosidase, galactosidase, and glucosidase, which cleave their corresponding sugar from glycoalkaloids (Swain *et al.*, 1978). These enzymes may help plants to avoid autotoxicity, but their activity is very limited in intact mature tubers. The rhamnosidase involved in hydrolysis of the rhamnose moieties from α-chaconine has been isolated and purified from potato peel (Bushway *et al*., 1988). Hydrolysis of α-solanine into solanidine was observed when incubated with *P. infestans* (Holland and Taylor, 1979). Similar enzymatic activities were observed in extracts of the fungus *Gibberella pilicaris* that infect solanaceous plants (Weltring *et al*., 1997). These fungi may contain hydrolytic enzymes to avoid the antibiotic action of SGAs.

1.3.7 Positive effects of SGAs

In addition to toxic effects in humans, Solanum SGAs may also have beneficial effects. In tomato, α-tomatine has been shown to play a vital role in nutrition (Friedman, 2002) and to lower the levels of plasma cholesterol and triglycerids (Friedman *et al*., 2000). It has been shown that α-chaconine is involved in destroying human cancer cells HT-29 by inducing apotopsis (Lee *et al*., 2004; Yang *et al*., 2006). Other SGAs like α-solanine proved to be antiallergic against nightshade and cereals (Glubeva, 1966). In addition, α-solanine can induce hyperglycemic effects. Solamargine has anti-inflammatory effects, and act against herpes virus and cancer cells (Delporte *et al*., 1998). Hence,

Solanum SGAs may be used for human benefits to destroy cancer cells as well as in plants for antibiotic activities.

1.3.8 Factors involved in enhanced SGA levels

The SGA levels in leaves and tubes have been shown to be affected both by genetic and by environmental factors. It was shown that SGA contents in individual cultivars is genetically controlled, and that synthesis of higher levels of SGAs was heritable similar to other alkaloids (Sanford and Sinden, 1972). In addition to genetic factors, several environmental factors during growth or post harvest stages can influence SGA levels and it was proposed that any stress factor could alter glycoalkaloids contents (Sinden *et al*., 1984). Exposure of post harvest tubers to light for prolonged period of time can dramatically increase SGA levels (Dale *et al*., 1993). It was initially speculated that there is direct relationship between light-induced chlorophyll and glycoalkaloid accumulation in potato tubers, but later studies proved that these are independent processes without metabolic links (Edwards *et al*., 1998). Wounding or mechanical injury, temperature and storage time and conditions can also alter SGA levels (Friedman and McDonald 1999). In Sweden, the potato cv. Magnum Bonum was removed from the market because of high SGA levels, which were associated with environmental factors (Hellenäs *et al.,* 1995). Likewise, potato cv. Lenape was removed from US markets because of higher SGA levels (Sinden and Webb 1974).

1.3.9 Biological activity

The two major toxic properties of SGA are the ability of SGA to bind with membrane 3β-hydroxysterols to disrupt membrane function, and an anticholinesterase activity (Keukens *et al*., 1995). Both α-solanine and α-chaconine cause changes in sodium active transport and in membrane potential on frog skin, and α-chaconine proved to be more toxic than α-solanine (Blankemeyer *et al* 1998). The SGA-cholesterol interaction is an important mechanism involved in membrane disruption. It has been proposed that membrane disruption is due to the binding ability of SGAs with membrane sterols (Roddick, 1978). Many SGAs like α-tomatine, α-chaconine and α-solanine form complexes with cholesterol and other phytosterols resulting in membrane disruption, with α-tomatine being the more potent (Keukens *et al*., 1995). SGA also inhibit acetylcholinesterase enzymes, which catalyze hydrolysis of the neurotransmitter acetylcholine at synapses in the central nervous system. This inhibition may cause weak pulse, fever, rapid respiration and breathing problems (McMillan *et al*., 1979). Both α-solanine and α-

chaconine have equal potency in the *in vitro* inhibition of acetycholinesterase activity (Roddick, 1989).

1.3.10 Other SGAs in Solanaceae family members

In addition to α-solanine and α-chaconine found in the cultivated potato, there are other glycoalkaloids present in potato species. Two groups of closely related SGAs in the solanidane class are leptines and leptinines (Ginzberg *et al*., 2009). These glycoalkaloids differ from solatrioses and chacotriose by O-acetylation and hydroxylation at C-23, respectively (Sinden *et al* 1986). Leptines and leptinines are found to be distributed in a few Argentinian accessions of *Solanum chacoense* (Ronning *et al*., 1999). The leptines are important for plant resistance against the Colorado potato beetle (*L. decemlineata*) and usually associated with higher foliar SGA contents (Sinden *et al*., 1986).

1.4 Calystegine alkaloids

In addition to the SGAs, potato also contains other forms of alkaloids. Calystegines (CA) are polyhydroxylated nortropane alkaloids that were first identified in the transformed roots of *Calystegia sepium, Convulvulus arvensis,* and *Atropa belladonna* (Tepfer *et al*., 1988). CA have since then been found to be widely distributed in the plant kingdom, including the Solanaceae, Convolvulaceae, Maraceae and Brassicaceae (Bekkouche *et al*., 2001; Schimming *et al*., 1998; Brock *et al*., 2006). CA were originally thought to be present only in roots, but later on they have been identified in essentially all plant parts. However, it is still unclear whether CA are synthesized in all tissues, or if they are transported from roots towards aerial part of plants. CA have also been reported to be present in tubers (peel, flesh), leaves and sprouts, with the highest concentrations being measured in sprouts (Dräger *et al*., 1995). A considerable variation in CA contents was observed in flesh and peel, with higher levels in peels (Friedman *et al*., 2003).

1.4.1 Chemical structure and biosynthesis of CAs

CAs have three common structural features: a nortropane ring system, a tertiary hydroxyl group at the bicyclic ring bridgehead, and two to four additional hydroxyl groups at different positions (Andersson, 2002). Based on the number of hydroxyl groups, the CAs are grouped into three types denoted A, B and C, having three, four or five hydroxyl groups, respectively (Goldmann *et al*., 1996). The first CA structures to be elucidated were those of CA A_3, B_1 and B_2 (Goldmann *et al*., 1990). In potato, the CA A_3 and B_2 were

identified in tubers, while B_2 was also found in leaves of some potato cultivars (Nash *et al*., 1993) In addition, dihydroxylated nortropanes have also been detected in Solanceous plants (Asano *et al*., 2001). There are also some CA that deviate from the above-mentioned common structural features. One of those is CA N_1 that carries an amino substituent instead of a hydroxyl group on C-1 of the nortropane structure. Other uncommon forms of CA are the two N-methylated calystegines N-methyl-calystegine B_2 and N-methyl-calystegine C_1, both isolated from roots of *Lycium chinense* (Asano *et al*., 1997).

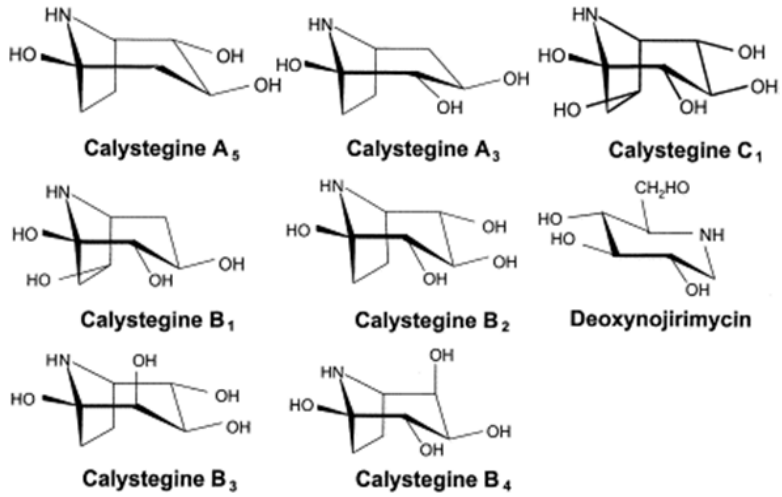

Figure 3. Chemical structure of different types of calystegines in potato

Calystegines have been reported to be synthesized via the tropane alkaloid pathway, starting from putrescine, which is derived from ornithine (Teuber *et al*., 2007). Putrescine is further methylated (Hashimoto, *et al*., 1989) by putrescine methyl transferase and subsequent oxidation reactions lead to form tropinone, that in turn act as a branch point of tropane alkaloid metabolism. Two tropinone reductases (TRI and TRII) have been characterized (Hashimoto *et al*., 1992) that act on tropinone to yield different products (Figure 4). TRI reduces tropinone to tropine, and ultimately leads to the synthesis of the highly toxic products (S)-hyoscyamine and (S)-scopolamine, whereas TRII reduces tropinone to pseudotropine which act as precursor to the calystegines (Dräger *et al*., 1994).

1.4.2 Impact of calystegines on food quality and safety

CAs are structurally related to the extremely toxic alkaloids hyoscyamine and scopolamine. Thus, since calystegines are present in potato, tomato and eggplants, which all are important components of human diet, it is imperative

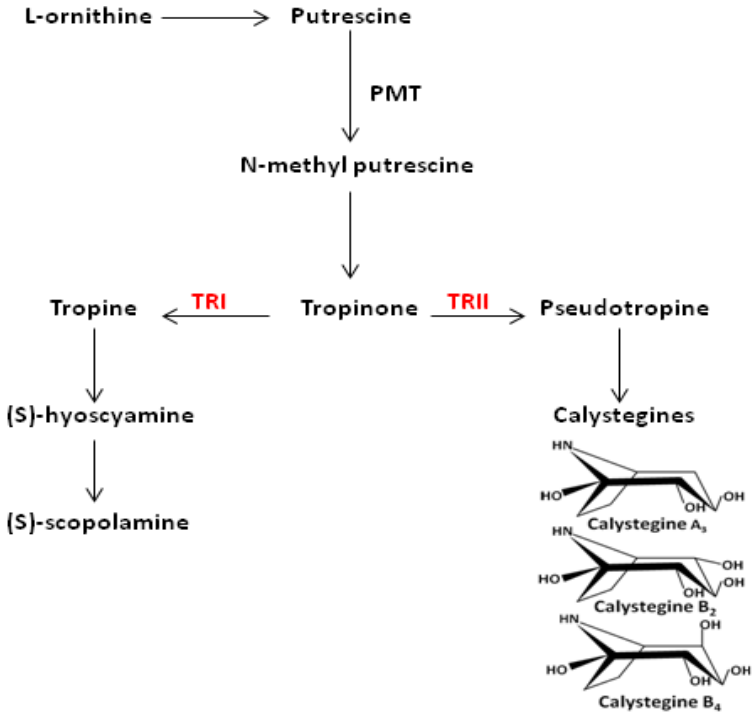

Figure 4. Schematic illustration of calystegine biosynthesis. Both TRI and TRII enzymes act on the substrate tropinone but only TRII leads to the synthesis of calystegines.

to get a clear understanding about the potentially toxic effects caused by CAs. Although it is clear that the acute toxicity of CAs can not be compared to that of hyoscyamine, CAs have been shown to inhibit glucosidases and galactosidases in the liver of mammals, a property that may cause symptoms related to lysosomal storage diseases (Asano *et al*., 1997). Feeding grazing animals with plants producing CAs have revealed symptoms of toxicity, but it is unclear whether this was due to CAs or other substances in the plants (Molyneux *et al*., 1995).

The ´crazy cow syndrome´ is also supposed to occur due to CA, but strong evidence is lacking. CA are present in lesser amounts in potato tubers, but over use of potato in the diet can hamper gastrointestinal processes. Thus, CA may in fact cause the gastrointestinal symptoms commonly interpreted as toxic SGA effects. Thus, there are at present no strong evidence for acute toxic effects of CA in humans, although more studies are needed particularly regarding long term exposure or synergistic effects with SGA.

The biological role of CAs within the plant has not been fully elucidated, but CA are generally considered as defence metabolites. In line with this is that

they are often found together with structurally related defence alkaloids such as cocain, atropine, and nicotine, all of which have N-methylputrescine as a common biosynthetic precursor. The interaction of CAs with the rhizosphere suggests that they are not involved in nodulation, but rather may be an exclusive source of carbon and nitrogen for certain soil bacteria having genes for CA catabolism (Griffiths *et al*., 2008). Allelopathic effects of CAs have also been reported (Goldmann *et al*., 1996).

1.5 Sterols in eukaryotes

Sterols are isoprenoid-derived molecules present in all eukaryotic cells, and have been assigned both general and species-specific cellular functions. The sterols function as bulk membrane lipid components, but also constitute precursors for a variety of additional metabolites such as the steroidal hormones occurring in mammals, plants and insects (Parish and Nes, 1997). The sterols may also regulate gene expression by interacting with regulatory proteins. This binding can be either covalent or non-covalent, depending upon on the species and regulatory proteins. The eukaryotic cell membrane is made of a phospholipid bilayer, which is reinforced by sterols (Bloch, 1983; Demel and De Kruyff, 1976).

The sterol composition in the membrane differs between organisms. For instance, cholesterol is the main sterol in vertebrates whereas ergosterol is the predominant sterol in fungi. In higher plants, the sterol composition is rather characterized by a complex mixture often termed as ´´phytosterols´´. This mixture generally includes cholesterol, campesterol, sitosterol and stigmasterol, which share a cholestane skeleton but differ in their side-chain structures (Nes and McKean, 1977). Sitosterol and stigmasterol often account for around 60% of total sterols in plants, with campesterol constituting most of the remainder (Schaeffer *et al*., 2001). Cholesterol levels are usually low, *e.g*. Arabidopsis contains only 1-2%. However, in some plant species including the potato, cholesterol may constitute between 10% and 20% of total (Table1). Insects do not have the ability to synthesize sterols *de novo* and must obtain the required sterols from their diet. However, insects have the ability to metabolize sterols to a certain degree, *e.g*. dealkylating the sterol side chain to synthesize cholesterol from plant sterols such as sitosterol (Svoboda *et al*., 1991).

1.5.1 Chemical structure and classification of sterols

Sterols are composed of three distinct structural parts; a tetracyclic ring structure (rings denoted A, B, C and D), a hydroxyl group at the C-3 position

on ring A, and an open aliphatic side chain attached to ring D. The C-3 hydroxylation and side-chain confers polar as well as non-polar characteristics,

Table 1. The sterol composition in different model plants. The potato contains higher levels of cholesterol than most other plants making potato a suitable model plant to study cholesterol metabolism and SGA biosynthesis.

Organism	Sitosterol	Campesterol	Stigmasterol	Cholesterol	Isofucosterol	Brassicasterol	References
Potato	37%	18%	20%	13%	12%		Arnqvist *et al*, 2003
Tobbaco	17%	23%	38%	13%	8%		Gondet *et al*, 1994
Arabidopsis	73%	13%	7%	2%	3%	2%	Shaeffer *et al*, 2001
Barley	62%	15%	23%				Rochestor *et al*, 1987
Moss	8%	26%	56%	3%	7%		Morikava *et al* 2009
Pinus pinea	77%	16%			7%		Nasri *et al*, 2007

respectively. Plant sterols are often classified on the basis of the number of methyl groups attached to the C-4 position. The first fully tetracyclic sterol precursor, cycloartenol, is a 4,4´-dimethyl sterol with two methyl groups at the C-4 position. During the synthesis of end product sterols, these methyl groups are successfully removed by the action of two distinct sterol methyl oxidases (SMO1 and SMO2) to yield 4-monomethyl sterols and the end-product 4-desmethyl sterols; *e.g.* cholesterol, campesterol, sitosterol and stigmasterol. Sterols are also classified into three categories depending on the alkylation at C-24; 24-desmethylsterols (without any alkylation at C-24), 24-methylsterols and 24-ethylsterols. The sterols are also attributed as C8, C9 and C10 side-chain sterols. The relative abundance of these different sterol forms varies between plant species.

Sterols are generally found in free form, anchored in the plasma membrane, but can also be present in conjugated forms with other molecules. Sterols thus occur as steryl esters, steryl glycosides and acylated steryl glycosides. Acylated sterols are esterified with fatty acids at the C-3 hydroxyl group, while sterol glycosides bind sugar molecules at the same position. Acylated steryl glycosides are formed by further esterification with long fatty acids. Steryl glycosides and acylated steryl glycosides are components of plasma membranes in a similar way as free sterols, while steryl esters are sterol storage forms without having a role in the plasma membrane.

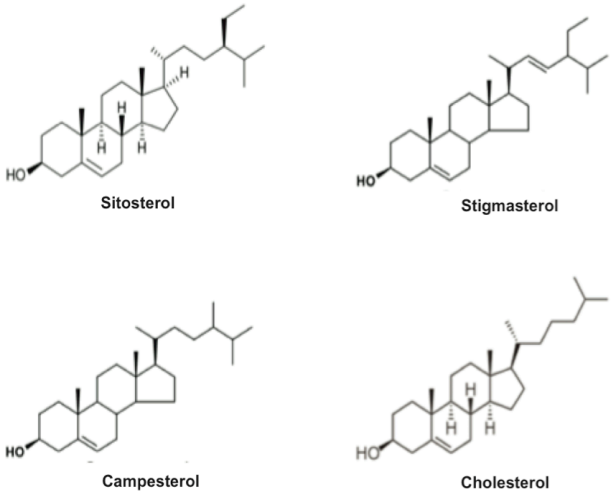

Figure 5. The chemical structure of major end-product sterols in plants.

1.5.2 The role of sterols in plants

Phytosterols have diverse functions in a range of metabolic and developmental processes (Schaller, 2003). In common with the role of sterols in other eukaryotes, phytosterols play a vital role in plants by influencing physical properties of membrane (Hartmann, 1998). As a consequence, sterols probably play a role in the adaptation to temperature, and temperature-induced alteration of the sterol composition has been demonstrated in tobacco (Grunwald, 1970). In the membrane, the hydrophobic side chain interacts with fatty acyl chains of phospholipids and proteins, while the 3-hydroxyl is facing the aqueous phase. This interaction restricts the movement of sterols in the membrane bilayer to maintain fluidity and permeability of membranes (Piironen *et al*., 2000).

In plants, sterol levels influence membrane integrity that in turn can affect plant growth, and a number of sterol-biosynthetic mutants with altered growth and development has been described. Presence of plant sterols in an appropriate ratio was proposed as a key factor for membrane channeling and an appropriate growth pattern (Schaller, 2003).

Phytosterols also constitute precursors to a class of steroidal growth hormones, the brassinosteroids (BRs). In Arabidopsis, it has been shown that campesterol acts as a main BRs precursor, whereas in tomato this role is fulfilled by cholesterol (Schaeffer *et al*., 2001; Yokot *et al*., 1997). The BRs regulate many different aspects of plant growth and development. BRs are known to stimulate cell elongation, division and are also involved in vascular system differentiation, plant reproduction, and stress responses (Altmann, 1998, 1999; Clouse and Sasse, 1998). It has been shown that mutants defective in either BR

biosynthesis or signaling display altered developmental phenotypes including dwarfism, reduced fertility, and abnormal vasculature (Clouse and Feldmann, 1999). One class of BR mutants includes dwarfs defective in BR biosynthesis. As plants produce BR using sterols as precursors, some sterol-biosynthesis mutants also display a BR-related dwarf phenotype. The exogenous application of BRs can recover normal phenotype in this case. In Arabidopsis, *dwf7* and *dwf5* mutants are defective in the Δ^7 sterol C-5 desaturation step and Δ^7 reduction, respectively. Both mutants showed a reduced growth and dwarf phenotype that was recovered by exogenous BR treatment (Choe *et al*., 1999 and 2000). The *dwf1* mutant of Arabidopsis was demonstrated to be defective in isomerization and reduction of the sterol $\Delta^{24(28)}$ bond, which is a necessary step in plant sterol biosynthesis. This step likely constitutes a limiting step also in BRs biosynthetic pathway by directly influencing campesterol levels. The *dwf1* mutant showed a severe dwarf phenotype with greatly reduced fertility, but this phenotype was rescued by exogenous application of BRs (Klahre *et al*., 1998). Another class of mutants is defective in BR signaling, and is characterized by the fact the dwarf phenotype cannot be recovered by exogenous application of BRs. For instance, the *brassinosteroid insensitive 1* (*bri1*) mutant, defective in the BR receptor, displayed a dwarf phenotype but was resistant to exogenously applied BRs (Clouse *et al*., 1996).

Phytosterols also contribute to defense by incurring resistance in plants against predators and pathogens. Sterols produce different secondary metabolites that have roles in resistance. The withanolide class of such sterol-derived defense substances produced in plants, and probably derived from campesterol (Lockley *et al*., 1976). Withanolides act as deterrents against feeding insect larvae and other herbivores (Ascher *et al*., 1980). Similarly, cardiac glycosides present in many species are probably also derived from a sterol, such as stigmasterol, and also contribute to resistance against grazing animals by interfering directly with the electrolyte balance within heart muscles. Other secondary defense metabolites derived from sterols include as previously outlined the SGAs, which have been proposed to be derived from cholesterol (Heftman *et al*, 1983).

Besides a role as signalling molecules and important membrane components phytosterols are important also in other processes. The ratio of campesterol to sitosterol has been proposed to modulate growth in Arabidopsis (Schaeffer *et al*., 2001). A role for phytosterols in plant innate immunity against bacterial pathogens was shown by silencing squalene synthase (SQS), a key gene in sterol metabolism, through regulating nutrient efflux into the apoplast (Wang *et al*., 2012). RNAi-mediated disruption of squalene synthase (SQS) has also been found active in improving drought tolerance and yield in

rice (Manavalan *et al*., 2012). In general, sterol synthesis varies between cell types, and it is evident that sterol levels are high in proliferating and actively growing tissues while the levels decline in mature tissues. The involvement of structural sterols was observed in both the initiation and tip growth of root hairs in Arabidopsis (Ovečka *et al*., 2010).
In certain other organisms, humans included, have a role as signaling molecules regulating various metabolic reactions by binding to regulatory receptor proteins (Farese and Herz, 1998; Edwards and Ericsson, 1999). However, whether this is the case also in plants is not known, although the occurrence of domains with similarity to sterol-binding proteins in plant transcription factors has been observed (Edwards and Ericsson, 1999). With the present information available related to plant sterol homeostasis, it is apparent that the level of sterols is genetically controlled and of great importance for correct plant growth and development

1.5.3 Sterol biosynthesis in plants

Sterols in plants are almost completely derived from acetyl-coenzyme A (acetyl-CoA). Acetyl-CoA is dimerized to acetoacetyl-CoA that then forms 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by a condensation reaction. HMG-CoA is reduced to mevalonic acid by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), a rate-limiting step in human isoprenoid biosynthesis. *HMGR* over-expression in transgenic tobacco plants resulted in increased levels of total sterols, indicating that HMGR is a limiting enzyme also in plant sterol biosynthesis (Schaller *et al*., 1995).

The next step in sterol synthesis is to form squalene from mevalonic acid, and this is performend by a series of reactions where the final step is catalysed by the squalene synthase enzyme (SQS). Also this step is potentially rate limiting, as over-expression of *SQS* enhanced synthesis of phytosterols (Seo *et al*., 2005). The first fully cyclized sterol intermediates, cycloartenol and lanosterol, are made from squalene in a series of reactions. Cycloartenol then serves as common precursor for all end-product sterols in three parallel pathways leading towards cholesterol, campesterol, sitosterol (Benvineste, 2002). The role of lanosterol in plants is unclear. An increase in sterol side-chain length occurs by two alkylation reactions. Cycloartenol is methylated by the enzyme sterol methyltransferase type-1 (SMT1) at C-24. S-adenosyl methionine acts as the methyl donor in this process, thus introducing one non-acetyl-CoA carbon atom in the plant sterol structure. Transgenic potato plants overexpressing a *SMT1* cDNA displayed an increase in total sterols, especially in isofucosterol and sitosterol as compared to controls.

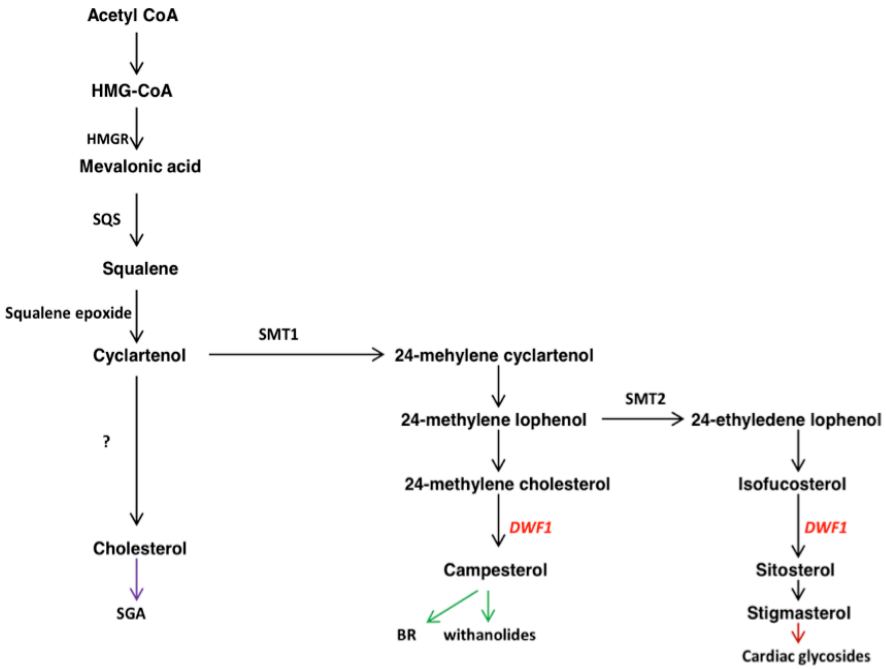

Figure 6. Model for biosynthesis of sterols and sterol-derived compounds in plants. Cyclartenol acts as common precursor of all end-product sterols. DWF1 catalyzes the final step in campesterol and sitosterol synthesis, whereas the corresponding reaction in the cholesterol biosynthesis is largely unknown. Cholesterol leads to form toxic steroidal glycoalkaloids (SGA), campesterol is the precursor for brassinosteroid hormones, and also the withanolides. Cardiac glycosides are thought to be synthesized from stigmasterols.

In parallel, those plants displayed reduced levels of cholesterol (Arnqvist et al., 2003). Conversely, the *smt1* mutant in Arabidopsis accumulates cholesterol and has less C-24 alkylated sterol contents (*e.g*. less campesterol, sitosterol and stigmasterol) (Diener *et al*., 2000). This indicates a pivotal role for SMT1 in the channeling of sterol synthesis between alkylated and non-alkylated side-chain sterols. A second methylation can further increase the sterol side-chain length. This step is catalyzed by sterol methyltransferase type-2 (SMT2), and the preferred substrate is 24-methylene lophenol. This leads towards increased synthesis of C10 side-chain sterols such as sitosterol, but at the expense of C8 and C9 sterols. The altered sterol profile in *smt2* mutant Arabidopsis lines showed higher levels of campesterol at the expense of sitosterol, which led to a reduced stature and growth but which could not be rescued by exogenous BR treatment (Schaffer *et al*., 2001). This work indicates that appropriate ratios of all sterols within the membrane are necessary for normal growth and development of plants, and that alterations in their sterol profile may cause

aberrant growth and development. Another important step in plant sterol synthesis is the reduction of the sterol side-chain double bond. The side-chain of both cycloartenol and lanosterol contains a C24(25) double bond, which has to be removed to give the saturated side-chain of a functional end-product sterol (Benveniste 2002). In the biosynthesis of the C9 and C10 side-chain sterols campesterol and sitosterol, this reduction occurs at the last biosynthetic step, and is catalysed by the DWARF1 (DWF1/DIM) enzyme. Substrates are both 24-methylene cholesterol and isofucosterol, respectively. The DWF1 enzyme is a FAD-containing and calmodulin-binding protein, and was originally identified in a study of the Arabidopsis *dwarf1* mutant (Klahre *et al.,* 1998). Later, *DWF1* homologues have been shown to be important also for development and sterol metabolism in rice and pea (Hong *et al*., 2005; Tao *et al*., 2004). Also a human DWF1 counterpart, 24-dehydrocholesterol reductase (DHCR24), has been identified and shown to be important for proper sterol synthesis, in this case in the synthesis of cholesterol (a C8 sterol) from desmosterol (Waterham *et al*., 2001). Failure to perform this step causes desmosterolosis, a genetic disorder that leads to defect brain development and in most cases is lethal. As desmosterol is not an endogenous sterol in plants, the side-chain reduction in plant cholesterol synthesis is likely occurring with another DWF1 substrate. This step is particularly interesting in solanaceous plants such as potato, which have comparatively high cholesterol levels, and uses this sterol in defense. Part of this thesis work covers the functional characterization of *DWF1* genes in potato (**I**), and their role in sterol and SGA biosynthesis (**II**).

2 AIMS

Despite the identification of SGAs almost 100 years ago, there is still not much information about the SGA biosynthesis and its regulation. The main work in this thesis has been focused on identifying genes relevant to the biosynthesis of sterols and SGAs. Part of the work was dedicated to evaluate stress metabolic responses among important potato cultivars from agronomic point of view, grown in Sweden. The specific aims were:

- To identify and characterize key genes involved in sterol and SGA biosynthesis
- To investigate the role of nitrogen in SGA biosynthesis
- To determine the variation among potato cultivars regarding SGA and CA levels after stress

3 Material and Methods

3.1 General considerations

This work was aimed to get insight into the biosynthesis of glycoalkaloids occurring in certain plants, and the cultivated potato (*Solanum tuberosum* L.) was used as the model species. The work included identification and functional characterization of genes encoding key metabolic enzymes in SGA biosynthesis. Using a broad combination of molecular techniques, key genes were identified and functionally characterized in transgenic potato plants by expression in both sense and antisense orientation from a constitutive promoter. Transgenic plants were analyzed for their sterol and SGA contents, as well as for growth and development. The molecular techniques included identification of genes, cloning of genes, generation of transgenic potato plants, gene expression analysis, phylogenetic tree construction and sequence alignment. Analytical techniques included sterol extractions and measurements using gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), as well as glycoalkaloid and calystegine quantifications using liquid chromatography and ultraviolet detection (LC-UV) and liquid chromatography coupled to mass spectrometry (LC-MS), respectively. Some details regarding selection of model plant, transformation and analytical methods are discussed in the following sections.

3.2 Plant materials

In order to screen table potato cultivars for their SGA and CA production after different forms of stress, potato tubers were purchased from certified producers and in some cases retail stores. The selected cultivars, including King Edward and Bintje, accounted for over 75% of the cultivars that are grown in central

Sweden. The potato tubers were propagated in pots in a greenhouse or under outdoor conditions depending on the experiment. While the propagation in pots may be seen as physiologically different from propagation in field experiments, this method enabled controlled growth of a large set of cultivars at a reasonable cost. Any difference between cultivars is thus likely to have a genetic basis.

3.3 *Agrobacterium*-mediated transformation of potato plants

Due to its agronomic importance, the potato has been extensively studied and used as model plant for *in vitro* experiments. Potato was the first major food crop where genetic manipulation was successfully applied (Bejaj and Sopory, 1986). The recent release of the potato genome, along with the availability of transformation methodology and suitable potato cultivars for research purposes, are a few factors that now support potato as a model plant for studies of a number of important plant processes including secondary metabolism. Desiree is the most common *S. tuberosum* cultivar that is used for transformation. Desiree initiate shoot formation early and requires short time for storage at cold temperature to break tuber dormancy. Due to its *in vitro* responsiveness and short propagation cycle, Desiree was used in current study for transformations as well.
Agrobacterium tumefaciens mediated transformation was done using internodal explants from Desiree (Beaujean *et al*., 1998). Shoots were generated from transgenic calli selected for the co-transferred kanamycin resistance gene *nptII*. Transgenic expression was monitored using reverse-transcription polymerase chain reaction (RT-PCR). Kanamycin resistance was used as selectable marker due to the high efficiencyrate obtained in previous experiments.

3.4 Stress treatment of potato tubers

Potato tubers from different potato cultivars were stress treated in order to increase SGA levels. Light, wound and heat were the stresses subjected to tubers, along with controls without any stress treatment. In order to wound potatoes, uniform tubers of almost equal size and shape were selected and cut transversely from middle of tuber to get potato discs which were further incubated for up to two days (48h) before freezing in liquid nitrogen. For light exposure, potato tubers were incubated for 8 days under white fluorescent light. Light intensity used was 110 μmoles m^{-2} s^{-1}, and the spectral distribution is shown in (**IV**). For heat treatment tubers were incubated for 7days in the dark at 34°C. Shorter treatment times were investigated in set-up experiment.

3.5 Sterol measurements

Sterols were extracted from leaves using an improved method developed within the research group. Sterols were extracted with a chloroform/methanol solution and, after drying under nitrogen gas, dissolved in hexane before solid phase extraction on a C18 column. Samples were washed with hexane and eluted with hexane:ethyl acetate solution. Eluted samples were further dried under N_2 and dissolved in hexane before analysis using GC-MS for identification and measurements of sterol fractions. The sterols measured were 4-desmethyl sterols in the free form, including cholesterol, sitosterol, campesterol and stigmasterol. Desmosterol were used as an internal standard in all sterol measurements.

3.6 Glycoalkaloid and calystegine quantifications

SGA were extracted from both leaves and tubers. Materials were crushed in an extraction buffer and homogenized into a slurry which was further centrifuged to obtain a clear supernatant. Cleaning-up was performed by solid phase extraction according to a protocol slightly modified from Hellenäs and Branzell (1997). Samples were eluted in 50% acetonitrile, before quantification of SGA using LC-UV. Solamargine was used as an internal standard for quantification. CA were analyzed by collaborators in the Czech Republic where further analysis were made. Calystegines (A_3, B_2, B_4) were quantified by LC-MS using purified calystegines for reference and quantification.

4 Results and discussion

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

In order to understand the SGA biosynthesis in greater detail, and to identify genes having roles in the sterol and SGAs biosynthetic pathways, tubers from the potato cultivars King Edward and Bintje were stressed by wounding and light treatment to increase SGA production. Wound stress was subjected for 48 h and light treatment was for 96 h. During wounding, both Bintje and King Edward showed increased levels of SGAs with a slightly more pronounced increase in King Edward. Under light treatment only King Edward showed a significant SGA induction. Having shown induction under both stresses, King Edward was concluded to be the more stress responsive cultivar. To identify genes involved in sterol and SGA biosynthesis, RNA was extracted from stressed tubers of both King Edward and Bintje and subjected to microarray gene expression profiling. Clustering analysis revealed over 100 up-regulated genes in King Edward and Bintje under both wound and light stress. Only those genes were selected to study in details which were more strongly up-regulated in King Edward and that had a tentative role in sterol or SGA metabolism. The microarray results were further confirmed by real-time PCR. This revealed at least 6 genes in sterol and SGAs biosynthesis (*HMGR1, PSS1, SMO1-1, DWF1-L, SGT1, SGT3*) to have a stronger induction in King Edward than in Bintje. *HMGR* and *PSS1* have a role in the pre-cyclartenol pathway, while *DWF-L* and *SMO1-1* act post-cyclartenol. *SGT1* and *SGT3* act at post-solanidine points and are responsible for the addition of sugars to the aglycone, solanidine. *DWF1-L* and *SMO1*-1 were strongly induced while the very similar *DWF1* and *SMO1-2* were not upregulated. *DWF1* and *DWF1-L* were studied in more detail to identify their function in sterol and SGAs biosynthesis

pathways. The deduced amino acid sequence of DWF1 and DWF1-L proteins showed 79% identity. A stretch of 3-4 amino acids hydrophobic residues was present in DWF1-L but absent in the DWF1 protein.

In order to characterize *DWF1* and *DWF1-L* genes functionally, transgenic Arabidopsis and potato plants were generated expressing both *DWF1* and *DWF1-L* genes in sense orientation from the CaMV 35S promoter. Both types of transformants showed increased levels of sitosterol and campesterol as compared to wild type plants, while cholesterol levels were not altered. Likewise, SGA levels were similar to controls both in leaves and stressed tubers, indicating that increased *DWF1-L* expression alone is not sufficient for increased SGA.

Taken together, results show that *DWF1* and *DWF1-L* genes encode proteins orthologous to the DWF1 sterol side-chain reductase in Arabidopsis and that both proteins act at the final biosynthetic step of the end-product sterols biosynthesis of sitosterol and campesterol. The normal SGA levels in DWF1 and DWF1-L overexpressors show that these genes are important, but not sufficient for increased SGA biosynthesis. This points towards the presence of other key genes acting downstream of cholesterol. On the other hand, the reduced levels of both sterols and SGA in the *DWF1* antisense transformants show a role of DWF1 in SGA biosynthesis, and further strengthened the precursor role of sterols in SGA biosynthesis.

4.2 The potato sterol Δ24-reductase DWF1 has a key role in sterol and glycoalkaloid biosynthesis

Transgenic plants expressing *DWF1* and *DWF1-L* genes in sense orientation showed an increase in sitosterol and campesterol (**I**). To further study and identify the role of *DWF1* and *DWF1-L* genes in sterol and SGA biosynthesis, transgenic potato plants were raised expressing also *DWF1-L* genes in antisense orientation from the CaMV 35S promoter. However, this did not alter sterol levels in the transformants, likely due to a redundant function of DWF1.

The desmethyl sterol profile of DWF1 antisense plants was shown to be similar to that of the Arabidopsis *dwf1* mutant, and included an increase of the known DWF1 substrates 24-methylcholesterol and isofucosterol. This strongly indicates that the potato DWF1 genes encode proteins that are orthologous to the Arabidopsis DWF1. However, unlike *dwf1*, the antisense DWF1 potato lines also showed an increase in 4-monomethyl and 4,4-dimethylesterols. In particular one monomethyl sterol was significantly increased, indicating the DWF1 in potato may have an additional unknown substrate, accumulating upstream of the antisense block. This may be the case also in the Arabidopsis sterol metabolism, but is probable below the level of detection since the

cholesterol levels are much lower than in potato. Moreover, the lower levels of sterol/cholesterol in antisense *DWF1* transformants attenuated the SGA induction in tubers after both wound and light stress treatments, confirming a role for *DWF1* also in the stress-induced biosynthesis of SGA.

4.3 Overexpression of a transaminase-like cDNA in transgenic potato plants is correlated with an altered growth pattern and increased glycoalkaloid level

The history of SGA presence in potato plants dates back to 1826 (Baup, 1826), but there is still very little information available about how exactly SGA are synthesized. Cholesterol is generally considered as a possible precursor of SGA synthesis in potato plants (*e.g.* Heftmann, 1983, Arnqvist *et al*., 2003). In particular the metabolic steps in the conversion of cholesterol into the aglycone, solanidine, are not well known. Although it is clear that they must involve an incorporation of a nitrogen atom.

Aiming at the identification of genes involved in the amination of SGA, the microarray study performed in (**I**) was re-analysed for stress-regulated genes with a potential role in nitrogen metabolism. This revealed an up-regulation of three potato transaminase-like genes (*StTAM1, StTAM2, StTAM3)*, which showed high similarities to known γ-aminobutyric acid-transaminases (GABA-transaminases) in tomato (Clarke *et al*., 2009) and pepper (Lang *et al*., 2009). Other transaminase-like genes were not altered. Interestingly, the *StTAM* genes were co-regulated with *DWF1-L* and other potential key genes in SGA synthesis identified in (I). To evaluate the function of *StTAM* genes in SGA biosynthesis, transgenic potato plants were generated expressing *StTAM1* gene in sense orientation from the CaMV 35S promoter.

The potato *35S:StTAM1* transformants were identified using RT-PCR. Compared to control plants, the transformants exhibited an altered phenotype with a slightly reduced growth rate, fewer stems per plant, and dark-green epinastic leaves. The SGA level in a group of transformants was significantly higher than that in control plants. A high SGA level was well correlated with the transgene expression..

The results showed that *StTAM1* is part of small multigene family of stress-regulated GABA transaminase-like genes with a vital role in SGA biosynthesis. Based on the structural similarity of *StTAM1* to GABA transaminases, GABA may be a nitrogen donor in a transamination process leading to solanidine formation. However, at the present state of research, it cannot be excluded that the elevated SGA levels in the transformants are an indirect consequence of stress effects from altered phenotype.

4.4 Glycoalkaloid and calystegine levels in table potato cultivars subjected to wounding, light, and heat treatments

The SGA and CA are the toxic substances present in the members of Solanaceae family members including potato, tomato and eggplant. The SGA are present in all parts of potato plants and the highest levels are found in flowers, berries, sprouts and other actively growing tissues, whereas SGA level in potato tubers are lower. The SGA level in healthy unstressed potato tubers depends on genotype, but certain environmental factors including wounding, light exposure and post-harvest handling can increase SGA level several-fold compared to the basal levels. A high level of SGA can be toxic in humans by causing gastrointestinal abnormalities and affecting the central nervous system. The upper safe limit in potato tuber recommended for human consumption is 200 mg kg^{-1} fresh weight (f.w.). The CA can inhibit various glycosidases that increases with the degree of hydroxylation of the molecule. There is not much information available about calystegine levels in potato and the mechanism of their toxicity is also unclear. However, the interplay in potato between these agronomically important alkaloids has seldom been investigated, and is essentially unknown with regard to stress responses in potato tubers.

To increase the knowledge about the genetic variation in stress responsiveness among potato cultivars, 21 different potato cultivars grown in central Sweden were screened for their sensitivity against environmental stresses regarding SGA and CA levels. The potato plants were planted in large pots, grown, harvested and stored at the same time to attain uniform conditions and to reveal cultivar differences. The potato tubers were subjected to post-harvest stresses, including wounding, light treatment and elevated temperature. The results indicated large variation of SGA levels among the different cultivars. Some potato cultivars showed increased level of SGA under both treatments of light and wounding, whereas other cultivars displayed higher SGA levels either under wound treatment or light exposure. Some genotypes did not show strong SGA induction under any stress. Heat treatment did not show any effect on SGA levels. In general, there were increased ratios of α-solanine to α-chaconine after the stress exposure, which indicates a stronger induction of SGT1 under stress treatment than of SGT2.
CA levels were measured from the same tuber materials as were used for the SGA extraction. The CA A_3, B_2 and B_4 were analyzed, and results indicated that there was variation among different potato cultivars but there was no signification difference on CA levels under stress exposure.
Taken together, the results suggest that SGA levels are induced by light and wounding, but with clear variations between cultivars. By contrast, heat is

likely not a trigger of SGA synthesis. CA levels in tubers are not induced by stress exposure. Hence, the metabolism of SGA and CA is not interrelated in potato tubers.

5 Conclusions

Increased SGA biosynthesis in potato tubers occurs as a response to both wounding and light exposure, and is mediated by a concerted induction of at least six key genes acting in the synthesis of sterols and downstream SGA precursors. The three key genes that had a role in sterol synthesis were present in two subtypes; one stress-regulated and one stress-tolerant. This may be a way for the plant to separate the use of sterols for housekeeping functions or for stress-regulated defence metabolism.

A role for *DWF1* in potato SGA synthesis was demonstrated in tubers from antisense plants, both regarding the basal tuber SGA metabolism, and the increased SGA synthesis after stress. In antisense DWF1 plants, an accumulation of the recognized DWF1 desmethylsterol substrates along with certain monomethyl sterols, indicates that DWF1 may have additional sterol substrates to the already known ones.

The transaminase-like *StTAM1* gene was shown to be important for SGA metabolism. The apparent co-regulation of *StTAM1* with the key sterol/SGA genes, further strengthens the stress-regulated SGA metabolism as a concerted activation of an entire biosynthetic pathway. This may be used to mine for other genes acting in the pathway.

There is a significant variation of stress responsiveness regarding SGA and CA levels in Swedish table potato cultivars, indicating that a high or low SGA production after stress is not a genetic trait that has been bred for. The different stress-induced alterations of SGA and CA levels, showed that biosynthesis of these metabolites are not interrelated. The variation of light responsiveness among the potato cultivars suggests that this trait should be considered to a greater extent in the post-harvest treatment of potato tubers.

6 Future Perspectives

- To gain more knowledge about the regulation of sterols and SGA biosynthesis at the transcription level, a comparative promoter analysis of sterol and SGA regulating genes could identify common elements that may lead to identification of gene regulatory network. The availability of potato genome facilitated the analysis at sequence level. The availability of *DWF1* and *DWF1-L* GUS: expression in potato plants would help to see responsiveness to stress by treating plants with different hormones and stress treatments.
- The pathway leading to sitosterol and campesterol biosynthesis is known but pathway for cholesterol synthesis is still not clear and one of the future goals could be to unravel cholesterol synthesis in potato. The role of *SMT1* could help to understand rate-limiting step in cholesterol synthesis.
- In order to understand SGA biosynthesis, role of genes encoding enzymes involved in the hydroxylation process of intermediate metabolites of solanidine is vital. The identification and characterizations of these genes in potato plants can help to get better understanding about SGA biosynthesis.
- Incorporation of nitrogen is one of the important steps in SGA biosynthesis and characterization of genes involved in transamination clearly enhances the knowledge about SGA biosynthesis. To get even more clear picture, analysis of aglycone; the solanidine, will be crucial to clarify role of transaminases in SGA biosynthesis.
- The combination of feeding experiments and genetically engineered techniques may eventually unravel the entire SGA biosynthesis and precursor role of cholesterol in SGA biosynthesis.

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