Biosynthesis of Steroidal Glycoalkaloids in *Solanum chacoense* Bitter

by

Alice Mutiti Mweetwa

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Dr. James G. Tokuhisa- Chairman

Dr. Richard E. Veilleux

Dr. John Jelesko

Dr. Chao Shang

Dr. Barry Flinn

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ABSTRACT

Steroidal glycoalkaloids (SGAs) are secondary metabolites produced by approximately 350 species in the Solanaceae family. SGAs are reported to be important for pest resistance and flavor enhancement at low concentrations but are toxic to humans and other mammals at high concentrations. Studies on sterol / SGA biosynthesis have implicated squalene synthase as a key regulatory enzyme because it catalyzes an irreversible step from the mevalonic acid pathway. However, the regulatory mechanisms of squalene synthase are not yet known. A study was conducted to elucidate the distribution pattern of SGAs and to clone the *squalene synthase* gene in order to determine a relationship between SGAs and gene expression levels. *Solanum chacoense,* a wild potato species was used as a model plant from which tissues were harvested at specified developmental stages and analyzed for SGA content. The results from the SGA analysis suggest a qualitative and quantitative tissue- and age-dependent accumulation of SGAs. Regenerative tissues such as, axiliary shoots, flowers and floral buds had the highest levels of 88, 49 and 63 µmole/g DW, respectively. The roots, stems and tubers showed the lowest amounts of SGAs of 1 to 8, 5 to 15 and 7 to 15 µmole/g DW, respectively. Stolons and tubers accumulated higher amounts of α-chaconine (59 to 67%) than α-solanine (61 to 64%) at all developmental stages analyzed. On the other hand, in young expanding, fully expanded, and old senescing leaves where leptine and leptinines tend to dominate, α-solanine and α-chaconine together accounted for only 8 to 15%, 7 to 15%, and 8 to 45%, respectively.

Plant organs that showed the highest biosynthetic activity for SGA production also had high levels of transcripts coding for genes of isoprenoid biosynthesis. The results from the cloning and characterization of *squalene synthase* suggest that the cloned cDNA fragment is a putative *S. chacoense squalene synthase* gene with an open reading frame / predicted protein precursor of 411 amino acids. The cloned cDNA has high similarity (68-100%) to known plant *squalene synthase* genes and contains six deduced peptide domains observed in other species. The 3' untranslated regions of floral buds, young leaves (early vegetative stage), and fully expanded leaves (anthesis) were different in length with, 249, 335, and 202 nucleotides, respectively. The Southern blot analysis suggests a single copy gene although the existence of a gene family cannot be ruled out.

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Table of Contents

List of Figures

List of Tables

1 Introduction

1.1 The Potato Crop

According to Food and Agriculture Organization (2008), potato is the number one non-grain food commodity in the world, with a record production in 2007 of 325 million tons. Among root and tuber crops, the temperate climate potato has a more world-wide cultivation than the more tropic adapted cassava, sweet potato, and yam (Hawkes, 1990). Recently, there have been major changes in the production and consumption of the potato crop globally. While most potatoes were grown and consumed in Europe, North America, and countries of the former Soviet Union, production and demand has increased in Asia, Africa, and Latin America since the 1990s.

Production quality of potato tubers encompasses a diverse array of features: texture, color of the raw and processed product, external and internal tuber morphology, general appearance and nutritive value. Important aesthetic qualities include the appearance of the tuber and freedom from defects and disorders, and therefore developmental and biochemical factors that contribute to the visual and organoleptic qualities and suitability for processing purposes are also important (Thompson *et al.*, 2008). A number of factors have been cited that lead to the development of internal defects namely, poor calcium nutrition, fluctuations in moisture availability, high temperatures, substandard nitrogen regimes, soil types,

poor planting rates, bad planting dates, disease, and finally, genetic factors (Sowokinos, 2007).

The use and economic significance of potato are derived from the tuber, which has approximately 82% of its dry matter as starch-rich carbohydrate. Potatoes are also a good source of vitamin C, which is present as both ascorbic acid and dehydroascorbic acid with typical concentrations of 15-25 mg/100 g FW (Storey and Davies, 1992). On the other hand, potatoes have low protein content on a fresh weight basis, with an average of 1.7-2.1 g per 100 g of fresh weight (Storey, 2007). For this reason, the potato is not considered as a protein source when compared with other foods. However, this amount of protein can make a significant contribution to the diet in places where there is a high consumption and limited availability of dietary protein (Storey and Davies, 1992). The low levels of the sulfur containing amino acids, methionine and cysteine limit the nutritive value of potatoes (Stiller *et al.*, 2007).

Apart from carbohydrates, proteins and vitamins, potato tubers also accumulate other compounds that affect food quality. Although potatoes are not normally considered a source of allergens, they can accumulate both heat-labile and heat-stable allergens such as patatin (Storey, 2007). Protease inhibitors and lecithins are examples of anti-nutritional compounds found in potatoes; heating (cooking) can reduce the negative effects of these compounds. Steroidal glycoalkaloids (SGAs), are produced by the potato and some other species in the *Solanum*

family; the presence of high levels of glycoalkaloids is undesirable for consumption because they have been associated with a bitter taste of potato tuber and are considered as human toxins when consumed at 2 mg / kg body weight (Storey and Davies, 1992, Smith *et al.*, 2008).

1.2 Steroidal glycoalkaloids

Steroidal glycoalkaloids (SGAs) are antinutritional secondary metabolites commonly produced by some members of the Solanaceae and Liliaceae families (Kaneko *et al.*, 1977, Valkonen *et al.*, 1996, Chen and Miller, 2000). SGAs found in the Solanaceae family are referred to as *Solanum* glycoalkaloids, while those in the Liliaceae family are termed *Veratrum* alkaloids. SGAs were first identified in potatoes in the early 19th century (Roddick, 1980, Smith *et al.*, 1996). Since then much effort has gone into the investigation of these compounds. Different types of SGAs have been isolated and characterized from more than 300 *Solanum* species (Lawson *et al.*, 1993, Friedman and McDonald, 1997). Examples of such members of the Solanaceae family include potatoes (both wild and cultivated), eggplants, peppers, and tomatoes.

1.2.1 Structure

In potato and tomato alone, at least 20 structurally different SGAs have been recognized, while 300 others have been found in other *Solanum* species (Friedman and Dao, 1992).

SGAs include a C_{27}-steroidal alkaloid whose structure consists of six rings- A, B, C, D, E, and F- and a nitrogen atom situated either in the E or F ring (Valkonen *et al*., 1996, Chen and Miller, 2000). The glycoside portion of SGAs consists of a polar tri- or tetra-saccharide attached by an ether linkage to C3 (in ring A) of the steroid (Ginzberg *et al.*, 2009). The oligosaccharides are specific combinations of different sugar monomers and include chacotriose (glucose, rhamnose, rhamnose), solatriose (galactose, glucose, rhamnose), lycotetraose (galactose, glucose, glucose, xylose) and commertetraose (galactose, glucose. glucose, glucose) (Table 1-1). The involvement of glycosyltransferases specific to trisaccharide-containing SGA formation and the sequential addition of monosaccharide units to the aglycone were first reported in 1991 (Zimowski, 1991). The enzymes UDP-galactose galactosyltranferase (SGT1) and UDP-glucose glucosyltranferase (SGT2) catalyze the addition of the UDP-galactose and UDP-glucose to C3 of the aglycone in the formation of gamma-forms of SGAs (Moehs *et al.*, 1997; McCue *et al.*, 2005,). The formation of beta-forms from gamma-forms is catalyzed by a rhamnosytranferase (SGT3) (McCue *et al.*, 2007). The enzyme(s) associated with the final sugar addition has not been characterized.

Cultivated potato, S. *tuberosum* L, contains α-chaconine and α-solanine which are derived from the aglycone solanidine (Table 1-1; (Ginzberg *et al.*, 2009)) although other SGAs such as α-solamarine, and β-solamarine derived from tomatidenol may also be present (Table 1-1). A-chaconine and α-solanine

constitute approximately 95% of the total SGA accumulation in S. *tuberosum* (Fewell and Roddick, 1997, Smith *et al.*, 1996, Fewell and Roddick, 1993).

In wild potato species, α-chaconine and α-solanine, plus other SGAs such as leptinines and leptines (referred to collectively as leptines), commersonine, dehydrocommersonine, and demissine may be present (Chen and Miller, 2000). The leptines are acetylated forms of α-chaconine and α-solanine, and their biosynthesis is suggested to involve an initial committed step of hydroxylation at C23 of solanidine to make leptinidine, which is then followed by an *O*-acetylation at the same carbon 23 to make acetylleptinidine. The so formed acetylleptinidine is then glycosylated to form the leptines (Lawson *et al*., 1993). While leptines are derived from aglycones which are modifications of solanidine, commersonine, and demissine are derived from demissidine (Lachman *et al.*, 2001). In the current study, the focus is the wild potato species *Solanum chacoense* Bitter, which has the capability to accumulate different combinations of all these compounds listed above in different tissues.

1.2.2 Significance

SGAs have been implicated in defense against herbivores, pests and pathogens (Bennett and Wallsgrove, 1994, Chen and Miller, 2000). They have been shown to be human toxins (Smith *et al.*, 2008) and are therefore an important factor to control in potato varietal development.

1.2.2.1 Potato flavor

SGAs if present at high concentrations impart a bitter taste to the potatoes; this bitter taste is associated with the alkaloid portion of the molecule. SGA contents higher than 20 mg/100 g fresh weight, can adversely affect the flavor of potatoes by causing bitterness (Sanford *et al.*, 1995). Below 20 mg / 100 g FW, SGAs enhance tuber flavor (Valkonen *et al.*, 1996, Gregory, 1984). Most potato tubers on the market contain 1-15 mg/100 g fresh weight, which does not affect culinary quality (Sinden *et al.*, 1984, Valkonen *et al.*, 1996).

1.2.2.2 Defense and Toxicity

SGAs are considered to be involved in the defense response against insect herbivores and pathogens (Chen and Miller, 2000, Cantwell, 1996). Adverse effects of SGAs on animals and humans, and microorganisms have been shown to be through a number of mechanisms.

The bitter/burning taste sensations humans experience may cause the pre-ingestive anti-feedant effects of SGAs (Smith *et al.*, 2001). Studies with snails have shown that both solanine and chaconine caused appreciable feeding inhibition at 1.0 mM, but chaconine was more effective; combinations of both compounds in differing ratios produced a synergistic inhibition of snail feeding (Smith *et al.*, 2001).

Disruption of cell membranes has been suggested to be the principal mechanism by which SGAs compromise the structural and functional integrity of cells / tissues of other organisms (Smith *et al*., 2001). Membrane disruption by SGAs has been demonstrated using simple systems such as phospholipid/sterol liposomes as well as complex intact systems such as the frog skin. At concentrations of 75 and 150 μM, α-chaconine has been shown to disrupt phosphatidylcholine/cholesterol liposomes resulting in 48 and 73% peroxidase leakage, respectively (Roddick and Rijnenberg, 1987). The extent of liposome disruption was assessed by determining peroxidase activity in the supernatant from a solution of SGAs mixed with liposome suspension. Experiments with liposomes containing cholesterol (the predominant sterol in animals), stigmasterol (the predominant sterol in plants), and ergosterol (the major fungal sterol) showed that 150 μM solarmargine disrupted membrane integrity resulting in more than 50% peroxidase leakage into solution (Roddick *et al*., 1992). These two experiments demonstrate that SGAs have the capability to disrupt membranes containing different kinds of sterol compounds. The impairment of membrane impermeability by glycoalkaloids has been suggested to result primarily from their ability to complex specifically with β-hydroxy sterols in the membranes (Roddick *et al*., 1992). Additionally, studies with frog embryos have suggested that SGAs depolarize membrane potential and therefore, have a direct effect on active transport of ions across the membranes through carriers and ion pumps. Frog skin mounted on an Ussing chamber have shown that frog skin actively

transports sodium from the pond side of the skin to the serosal side and that SGAs probably alter the entry channel for sodium found on the pond side of the frog membrane since they seem not to have any effect on the interstitial short-circuit current on the serosal-side membrane (Blankemeyer *et al.*, 1995, Blankemeyer *et al.*, 1998).

SGAs are also reported to be teratogenic; the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) has revealed that SGAs caused mortality and malformation of Xenopus embryos, with mixtures of an aglycone modified with chacotriose and solatriose having a synergistic effect compared with each SGA tested individually (Rayburn *et al.*, 1995, Rayburn *et al.*, 1994, Blankemeyer *et al.*, 1998). In these teratogenic studies mentioned, SGAs containing the chacotriose moiety have been shown to be more toxic than the solatriose derivative by a factor of 2 or more. The synergistic effects due to the interactions of different SGAs in foods can cause a problem estimating toxicity levels based on the toxicity of individual SGAs. The mechanism of action for teratogenicity in *Veratrum* alkaloid cyclopamine has been show to be through its inhibitory effects on the Hedgehog (Hh) signaling pathway causing holoprosencephaly (HPE) defects in diverse species including sheep, hamster, mouse, and zebra fish (Lipinski *et al.*, 2007), but this mechanism has not been shown for solanidine.

SGA toxicity to insects and mammals derive from their ability to inhibit acetylcholinesterase and butyrylcholinesterase (Roddick *et al.*, 2001). While

acetylcholinesterase is responsible for terminating cholinergic transmission at the neuromuscular junction and in the central nervous system (McGehee *et al*., 2000), the function of butyrylcholinesterase remains elusive (Roddick *et al.*, 2001). The potato SGAs, α-solanine and α-chaconine have been shown to directly inhibit esterase activity, having a greater potency and efficacy for butyrylcholinesterase than acetylcholinesterase (McGehee *et al*., 2000). The two potato SGAs are equally effective inhibitors of acetylcholinesterase and do not synergise in this action.

SGAs are also viewed as human toxins (Smith *et al.*, 2008). Acute poisoning may be experienced in humans if oral doses of 2 mg/kg body weight are taken. Symptoms resulting from SGA poisoning may include abdominal pain, vomiting, and diarrhea (Smith *et al.*, 2008, Arnqvist *et al.*, 2003). Because high levels of SGAs in potatoes result in bitter tasting tubers and potential toxicity to consumers, an upper limit of 20 mg/100 g fresh weight was suggested in 1924 by Bommer and Mattis (reviewed by Sinden *et al.*, 1984). This value has been generally accepted as a safe upper limit throughout the world.

Together, these works show that SGAs have anti-feedant activities, membrane disruption effects, anti-cholinerasterase activities and teratogenicity, through which they act to bring about toxicity in other organisms and thereby protect the plant from herbivores and microbial pests.

The potato plant is challenged with different pests; these include Colorado potato beetle, aphids, potato tuber moths, wireworms, pea leafminer, Andean potato weevils, potato psyllid, nematodes, and potato leafhopper (Raman and Radcliffe, 1992) . The potato crop is also prone to more than a hundred diseases caused either by bacteria, fungi, viruses or mycoplasmas (Hide and Lapwood, 1992). The major diseases include late and early blights, blackleg, and verticillium wilt (Hide and Lapwood, 1992). The control of these pests and diseases still remains a challenge and is reliant mostly on chemicals. Attention has been given to host plant resistance against pests and pathogens but has not been exploited to its full potential, which makes the incorporation of SGAs into new varieties as part of host plant resistance a very attractive avenue. Examples in which SGA deterrence of pests has been demonstrated are discussed below.

Colorado Potato Beetle: Of the pests, the CPB (*Leptinotarsa decemlineata*) is the most important insect pest of the potato crop in the USA. There are no commercial potato varieties that have significant resistance to CPB, although some wild species have been considered as important donors of genes to the potato for improving CPB resistance. SGAs found in *Solanum chacoense*, leptines and leptinines possess anti-feedant activities against CPB (Table 1-1&3; Lawson *et al*., 1993). Several studies have shown that leptines and leptinines are effective resistance factors against CPB (Coombs *et al.*, 2005, Cooper *et al.*, 2004, Coombs *et al.*, 2003, Jansky *et al.*, 1999, Jansky *et al.*, 2009). Studies with wild potato species producing other SGAs namely

commersonine and dehydrocommersonine as the major foliar SGAs have been shown to also have confer against CPB (Sinden *et al.*, 1980).

Aphids: On the other hand, SGA defense against aphids has not been particularly conclusive. While there was no significant effect of SGAs on *Macrosiphum euphorbiae* (Guntner *et al*., 1997), toxic effects of several SGAs were shown on the aphid *Schizapphis gramunum*, which does not normally settle on *Solanum* plants (Soule *et al.*, 1999).

Potato leafhopper: SGA-mediated defense against the potato leafhopper has also been demonstrated; a significant negative correlation ($r = -0.75$, $p = 0.01$) between foliar SGAs of a number of wild potato species and nymphal infestation by potato leafhopper was observed (Tingey *et al.*, 1978).

Fungi: A role for SGAs in resistance against fungi has been documented. Both α-solanine and α-chaconine have been shown to have a negative effect on fungal spore germination, normal hyphal morphology and growth ((Fewell and Roddick, 1997). At a concentration of 100 μM, α-chaconine inhibited *Alternaria brassiciola* and *Phoma medicaginis* spore germination by 100 and 50% respectively. Whereas α-solanine at the same concentration was inactive. In this study, a synergistic effect was observed when both compounds were at 50 μM (subactive level) and germination of spores of both fungal species was inhibited by 100% (Fewell and Roddick, 1997).

SGAs can therefore be considered to be involved in defense against insect herbivores and pathogens (Chen and Miller, 2000, Cantwell, 1996). The ability to act as defense compounds depends on the nature of the SGA (aglycone plus carbohydrate moiety), the interaction of different SGAs, and their concentrations in the plant.

1.2.3 Factors affecting distribution of SGAs in the plant

Factors affecting the levels of SGAs include the environment, genetics as well as the developmental stage and specific tissue of the plant. Environmental and genetic factors are the primary determinants of SGA levels and therefore their interaction determines the total levels (Ginzberg *et al.*, 2009, Maga, 1994). However, the ability to accumulate specific types of SGAs is determined by the genotype of the plant (Sanford *et al.*, 1995, Sinden *et al.*, 1984).

1.2.3.1 Plant developmental stage & organ

Glycoalkaloids are synthesized in all parts of the potato plant (Birch *et al.*, 2002) and organs that are important for survival and multiplication like flowers, unripe berries, young leaves, sprouts, and the area around potato eyes, tend to have more than twice the amounts of SGAs than other organs (Lachman *et al.*, 2001, Chen and Miller, 2000, Lawson *et al.*, 1993). In general, concentration has been shown to be higher in younger tissues or in tissues that have higher metabolic activities; this

could be attributed to the fact that chemical defense probably varies with maturity and that younger tissues are both valuable and vulnerable organs (McKey, 1974). A more detailed discussion of SGA distribution in the plant is given in section 1.3.1.

1.2.3.2 Environmental factors:

Light is an important factor affecting the levels of SGAs in the plant. The spectral profile, intensity, and duration of light have been shown to have an effect on the accumulation of SGAs in different plant organs. Blue, ultra-violet and infrared light have been cited as efficient elicitors of SGA biosynthesis (Valkonen *et al.*, 1996) while daylight has a greater effect than fluorescent light. Deahl *et al*. (1991) demonstrated the effect of light intensity on foliar SGA content; in a controlled environment experiment, plants grown at 250 μmol $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD) had increased SGA foliar (leptine) levels 2-4 fold compared to plants grown at 50 μmol $m^{-2}s^{-1}$ PPFD. In another study, different potato varieties were grown at PPFDs of 400 and 800 μmol $m^{-2}s^{-1}$ and potato tubers analyzed for SGA content (Nitithamyong *et al.*, 1999). In all varieties used, the total SGA content increased by an average of 16% in plants grown at the higher light intensity. In the same experiment, SGA levels were analyzed in tubers produced under different light durations; growth under constant light resulted in a 16% increase in SGA concentrations compared to growth under a 12 h photoperiod. In another study (Percival and Dixon, 1996), potato tubers exposed to continuous light (250 μmol-$m^{-2}s^{-1}$) for 144 h prior to SGA analysis accumulated significantly higher (1.5 to 2.0

times higher) amounts of SGAs than shaded controls. These studies demonstrate that high light intensity and longer exposure result in higher levels of SGAs.

SGA concentrations are also affected by low or high temperatures which favor accumulation. Nitithamyong et al. (1999) showed that SGA concentrations (significantly different at 95% confidence limit) were 0.43, 0.31, 0.57 and 0.70 mg/g DW, in tubers produced at 12, 16, 20, and 24^{o}C, respectively. In another study, potatoes grown during the summer (Mediterranean) accumulated five times more SGAs that those grown during the cool winter or spring seasons (Dimenstein *et al.*, 1997).

The effect of water availability on SGA content in tubers has been demonstrated; SGA contents in tubers harvested from drought-stressed plants of native Andean cultivars increased by 2–4 times relative to adequately watered control plants (Andre *et al.*, 2009). In the same study, increases in SGA content due to drought stress observed at harvest were also seen during storage, suggesting that the accelerated physiological age induced by drought stress may play a role in determining the postharvest levels of glycoalkaloids.

Soil fertility appears to influence SGA levels. Nitrogen application in potatoes at high rates (300 mg N/liter of water) significantly increased the levels of SGAs two to threefold when compared with a lower rate (100 mg N/liter of water) (Fragoyiannis *et al.*, 2000). Magnesium and molybdenum have been cited as

minerals having opposite effects on the levels of SGAs. Application of 40 and 100 pounds per acre of magnesium in the form of magnesium sulfate resulted in 42-43 mg/100 mg fresh weight and from a baseline of 8 mg/100 mg fresh weight total SGAs (Evans and Mondy, 1984). This study also demonstrated the year-to-year variation in SGA content when the same nutrient treatments were applied. The direct effects of sulfur were not tested; therefore it is not known whether or not this nutrient could be a confounding factor in such as an experiment. Reduced levels of SGAs with increased molybdenum application were observed (Mondy and Munshi, 1988).

Other factors affecting SGA levels are injury or damage to the plant or tuber, disease or insect attack, and rough handling during harvest or distribution. Injury brought about due to food preparation is an important factor to consider for SGA content (Maga, 1981). The study showed that long delays between slicing and subsequent processing resulted in increased SGA content; in this case, a two-fold increase was observed when potato slices were incubated at 20^{o}C for 12 h. Other wounding experiments have demonstrated that, depending on genotype, total SGA levels in wounded tubers increased by about 50% when compared to unwounded tubers stored under the same conditions (Percival and Dixon, 1996).

Taken together, these studies on the effects of several environmental factors suggest that extremes or stressful conditions result in higher SGA contents.

1.2.3.3 Genotype and breeding:

SGA content is a highly heritable trait (Sanford and Sinden, 1972). According to Sinden *et al*. (1984), single genes appear to control the biosynthesis of various sugar moieties found among potato SGAs. Sanford *et al*. (1996) have shown that genes responsible for controlling the synthesis of aglycones are mostly independent from those responsible for the glycosidic portion.

There are considerable differences in the types and amounts of SGAs among potato genotypes (Papathanasiou *et al.*, 1999, Papathanasiou *et al.*, 1998). The cultivated lines accumulate mostly α-chaconine and α-solanine while SGA types in wild potatoes are more diverse with as many as ten different types in some species (Distl and Wink, 2009, Deahl *et al.*, 1993).

Interspecific crosses of potato species can result in progeny that accumulate intermediate levels of SGAs and novel mixtures of SGAs relative to the parents. In an inheritance study of SGA levels in a hybrid cross between a high SGA producing wild potato, *Solanum caldasii* and a *S. tuberosum* cv., hybrid tubers accumulated approximately 10 times more SGAs than the *tuberosum* parent (Deahl *et al.*, 1993). Sanford *et al*. (1995, 1996) also demonstrated the segregation of leptines and other glycoalkaloids in *S. tuberosum* and *S. chacoense* crosses. Aglycone levels in the progeny from a hybrid cross between *S. brevidens* and *S. tuberosum* showed a wide range from 0.3-7.8 mg/g DW of solanidine, tomatidine, solanthrene and demissidine; these levels were intermediate between those of the parental lines

(Laurila *et al.*, 1996). In this study, Laurila *et al*. (1996) demonstrated that interspecific hybrids can produce SGAs, which did not occur in the parental genotypes. For example, *S. tuberosum* contained the aglycones solanidine and solanthrene, while *S. brevidens* was characterized by high contents of tomatidine and other unidentified aglycones, yet all the progeny accumulated demissidine, which was absent from either parent. This result has implications for use of wild species in the development of new potato cultivars. The fact that genotypes with novel or unpredictable concentrations of SGAs can be produced in interspecific crosses implies that careful selection of parental lines and thorough analyses of selected hybrids is imperative to maintain normal SGA levels.

1.2.4 Biosynthesis

The biosynthesis of SGAs begins in the mevalonic acid pathway, leads to sterol formation and then through the activities of novel enzymes, ends with the production and glycosylation of steroidal alkaloids. These biosyntheses are part of a complex isoprenoid metabolic network which also produces a large diversity of primary and secondary metabolites (Schaller, 2004).

Three molecules of acetyl-coenzyme A combine to yield 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) (Fig 1A). HMG-CoA is then reduced to mevalonic acid (MVA) by the action of HMG-CoA reductase (HMGR). After this reduction, mevalonate kinase and mevalonate 5-phosphate kinase phosphorylate MVA to form mevalonate 5-pyrophosphate, which then, after decarboxylation, yields isopentenyl diphosphate (IPP) and following isomerization, dimethylallyl

diphosphate (DMAPP) (Newman and Chappell, 1999). One molecule of DMAPP and two molecules of IPP are used to form farnesyl diphosphate (FPP). FPP is the direct substrate for sesquiterpene formation and through the activity of squalene synthase, triterpene biosynthesis (Piironen *et al.*, 2000).

Squalene is an immediate precursor for the cyclic triterpenoids, steroids and steroidal glycoalkaloids (Yoshioka *et al.*, 1999). Squalene is first converted into squalene 2, 3-epoxide in a reaction catalyzed by squalene epoxidase (Fig 1B). The cyclization of squalene epoxide results in the formation of the triterpenoid, cycloartenol. Cycloartenol is a precursor of the higher plant steroids. The path from cycloartenol can either lead to the formation of 24-methylenecycloartenol or cholesterol (Schaller, 2004). Radioactivity from labeled cycloartenol and lanosterol administered to *S. chacoense* was incorporated into solanidine as was labeled cholesterol into tomatidine in tomato, and solanidine in *S. tuberosum* (Heftmann, 1983). Arnqvist et al. (2003) have shown that over-expressing the enzyme involved in the biosynthesis of 24-methylenecycloartenol, sterol C24-methyltransferase (*SMT1)* leads to lower levels of cholesterol and glycoalkaloids in transgenic potato plants. These studies indicate that cholesterol has an important role in steroid alkaloid biosynthesis. The pathway for the biosynthesis of solanidine from cholesterol has been proposed on the basis of biosynthetic intermediates that have been observed in *Veratrum grandifolium*. According to this proposed pathway, cholesterol is hydroxylated to dormantinol, which is oxidized to dormantinone. In an acetylation step, dormantinone is converted into verazine, then with the closure

of rings F or E, etiolene and teinemine, and solanidine, solasodine or tomatidine are formed (Figure 1-2; Kaneko *et al.*, 1977). In *S. chacoense,* there are modifications following the formation of solanidine leading to the leptines. The formation of these aglycones is then followed by the sequential addition of monosaccharide units as discussed earlier (section 1. 2.1).

1.3 Project Justification

1.3.1 Plant development and tissue specificity as determinants of SGA accumulation

Because SGA accumulation is dependent upon the physiological/metabolic activities in the tissues, it is reasonable to assume that the concentration of SGAs in specific potato tissues might vary as the tissues age (Brown *et al.*, 1999). Total SGAs in *S. tuberosum* plants determined over the entire 10 growth stages showed that leaves of young plants had significantly higher contents (Kolbe and Stephan-Beckmann, 1997). The highest SGA levels (157 mg/plant) were reported to be at 60 d after emergence (flowering), which from then on declined to 66 mg/plant, by 120 d after emergence (senescence). Kolbe and Stephan-Beckmann (1997) also reported that SGA levels in stems varied with developmental stage of the plant and the highest levels (22 to 24 mg/plant) were reported between 60 to 70 d after emergence and lowest at senescence (9.6 mg/plant). However, this report does not demonstrate differences that might exist among leaves or stem portions of different physiological ages.

Studies to determine patterns of glycoalkaloid accumulation during tuber development showed that total SGA levels decreased between 65 and 135 d after planting as the mean fresh weight per tuber increased (Papathanasiou *et al.*, 1998). The rate of decrease in SGA levels was dependent on genotype. In another study, SGA concentration of *S. tuberosum* tubers decreased significantly from 70 mg/ 100 g FW when tubers were 2 mg FW to less than 20 mg/100 gFW as the tubers enlarged to 50 g FW (Papathanasiou *et al.*, 1999). Distribution of SGAs within the tubers is such that 83-96% of the SGAs are in the potato skin; 3-14% is in the cork, while 1% is in the cortex (Lachman *et al.*, 2001). Therefore, small tubers within a plant are expected to have higher SGA concentrations due to the higher surface to volume ratio whereas larger tubers have lower concentrations.

Because *S. tuberosum* accumulates mostly the SGAs α-chaconine and α-solanine, (Fewell and Roddick, 1997, Smith *et al.*, 1996, Fewell and Roddick, 1993), all the current data on SGA accumulation in leaves, stems, and tubers of a developing plant are based on totals or/ratios of α-chaconine and α-solanine only. Wild potato species, such as *S. chacoense* in addition to α-chaconine and α-solanine, may accumulate leptines and other types of SGAs (Ronning *et al.*, 1999, Deahl *et al.*, 1993) in an organ-specific manner (Ronning *et al.*, 1999, Sanford *et al.*, 1996, Mweetwa *et al.*, 2008). There are currently no known studies of the accumulation patterns of mixtures of SGAs during plant development that have been conducted with other genotypes (wild species).

1.3.2 Role of squalene synthase as a regulatory enzyme in SGA accumulation

Sterol biosynthesis is likely to be regulated at key enzymatic steps (Schaller *et al.*, 1995). Researchers have so far identified two regulatory steps in the formation of squalene; one involving the reduction of HMG-CoA into mevalonic acid and the other involving condensation of FPP into squalene.

HMG-CoA reductase (HMGR) is localized within the cell to the endoplasmic reticulum (ER) and anchored via transmembrane peptide domains (Campos and Boronat, 1995). *HMGR* genes constitute a family of three genes in *S. tuberosum* that are differentially regulated. Choi *et al*., (1992) used gene-specific probes for the three *HMGR* genes and were able to correlate wound-inducible steroid accumulation with the induction of *HMG1* gene expression and elicitor inducible accumulation of sesquiterpene phytoalexins with *HMG2* and *HMG3* gene expression. This is an example of metabolic changeover in which the regulation of end-product formation depends on coregulation of enzymes associated with each metabolic unit, as well as various posttranslational modifications modulating isoenzyme activity levels (Chappell, 1995).

The other putative regulatory point in this pathway is the tail-tail condensation of two molecules of farnesyl pyrophosphate (FPP) catalyzed by the action of squalene synthase in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and magnesium cofactors (Nakashima *et al.*, 1995). Squalene synthase is a microsomal protein anchored to the endoplasmic reticulum membrane (Kribii *et al.*, 1997). Squalene synthase has six highly conserved peptide domains that were

previously pointed out in fungi, humans and plants (Robinson *et al.*, 1993, Devarenne *et al.*, 1998, Huang *et al.*, 2007). The amino terminal region of squalene synthase has bee shown to be involved in substrate binding and catalytic activity of the protein while the carboxyl terminus is be involved in anchoring the protein to the ER (Huang *et al.*, 2007). Deletion of residues 388-411 in the carboxyl terminus (Domain VI) of the *Nicotiana tabacum* enzyme resulted in an active but soluble squalene synthase, confirming that the domain is associated with protein-lipid interactions (Devarenne *et al.*, 1998).

Squalene synthase activity in mammalian cells has been shown to increase or decrease in parallel with HMG-CoA reductase although the mechanism responsible for this regulation has not yet been elucidated (Robinson *et al*., 1993). In plants, examples of squalene synthase regulation have been limited. Elicitor mediated down-regulation of squalene synthase activity has been shown in *N. tabacum* cell suspension cultures (Vogeli and Chappell, 1988), although the mechanism is not yet clearly understood. Further work on elicitor mediated down-regulation of squalene synthase in *N. tabacum* cell suspension cultures demonstrated that squalene synthase activity declines over the first 6 h after elicitation and low levels of enzyme activity remained until 48 h after which recovery began (Threfall and Whitehead, 1988). Threlfall and Whitehead (1988) attributed the rapid inhibition of squalene synthase to the activity of low molecular weight compounds (e.g. phytoalexins) and macromolecules produced by cells in response to elicitation. The authors suggest a three-phase response following elicitation, beginning with the rapid inhibition of squalene synthase activity (first 6 h), accumulation of

phytoalexins and phenolic compounds, and finally a decline in the activities of enzymes synthesizing phytoalexins. Neither the work by Threlfall and Whitehead (1988) nor Vogeli and Chappell (1988) elucidated the cause for the reduced activity of squalene synthase. To show the relationship between squalene synthase and HMGR activities, inhibiting squalene synthase with squalestatin, an analog of FPP, did not result in compensatory response in squalene synthase activity; but instead resulted in a 2 to 4-fold increase in HMGR enzyme activity and gene transcript levels, and depletion of squalene-derived products in *N. tabacum* cell cultures without any change in *squalene synthase* transcript levels (Wentzinger *et al.*, 2002). These results indicate that inhibition of squalene synthase triggers an increased level of HMGR steady-state mRNA and enzyme activity, the arrest of sterol biosynthesis and depletion of squalene-derived products, which exerts a positive feedback effect on the transcription of HMGR (Wentzinger *et al.*, 2002). To investigate the regulatory mechanisms of sterol biosynthesis in *N. tabacum* cell cultures, Devarenne *et al*. (2002) also demonstrated suppression of squalene synthase activity upon elicitation (65-70% reduction between 8 and 14 h after elicitation). They demonstrated a significant reduction (80%) in *squalene synthase* transcripts within the first 14 h of elicitation and that enzyme activity can be partially correlated with the level of gene transcripts. On the other hand, over-expression of *squalene synthase* in Ginseng and *Eleutherococcus senticosus* (Lee *et al.*, 2004, Seo *et al.*, 2005) and *HMGR* (Schaller *et al.*, 1995) has been shown to lead to enhanced production of phytosterols and triterpenes. Current questions surrounding the regulation of squalene synthase focus on whether the amount of the

enzyme or the level of enzyme activity is regulated to bring about the different responses in the accumulation of sterols. Therefore, there are still areas that need further experimentation in order to fully understand the biological mechanisms underlying the actions of squalene synthase.

Expression patterns of *squalene synthase in planta* have been documented in several plants. Devarenne *et al*. (2002) reported predominant expression in shoot meristems, stems and leaf petioles of *N. tabacum* using a β-glucuronidase reporter gene linked to the *squalene synthase* promoter. Huang *et al*. (2007) showed that highest expression levels were observed in the roots of *Taxus cuspidata*. The predominant expression levels in the shoot apex and roots of licorice (Lee *et al.*, 2004) agree with those reported in tobacco and *Taxus cuspidata*,. Most recently, equal amounts of *squalene synthase* transcripts were reported in the stems and leaves, whereas 10 times less transcript was detected in roots of *Euphorbia tirucalli* (Uchida *et al.*, 2009). Clearly, there are differences in expression patterns of *squalene synthase* in different plants.

Whereas only single copies of the *squalene synthase* gene in *Euphorbia tirucalli* (Uchida *et al.*, 2009), *Taxus cuspidata (*Huang et al., 2007), yeast and human have been observed, more than one gene have been isolated from other species. For example, a cDNA with 87% identity at the amino acid level to *squalene synthase* from licorice was isolated (Hayashi *et al.*, 1999), and Lee *et al.* (2004) have shown that characterization of ginseng *squalene synthase* might be consistent with multiple genes. *Arabidopsis thaliana* (Kribii *et al*., 1997), *Nicotiana tabacum* (Devarenne *et al.*, 2002) and *Oryza sativa* possess two squalene *synthase genes* per genome. In

potato, a single copy of squalene synthase has been reported although the authors do not preclude the possibility of an additional squalene synthase gene (Yoshioka *et al.*, 1999). Thus, it is still unknown if there is more than a single copy of *squalene synthase* in potato and how this might affect the regulation of sterol biosynthesis in general and SGAs in particular. However, the existence of multiple copies of *squalene synthase* genes raises the question of the function of all the copies. For example, *SQS1* and *SQS2* Arabidopsis genes have an identical organization with respect to intron position and exon sizes and encode squalene synthase isoforms that show a high (84%) level of similarity. However, while *SQS1* mRNA is detectable in all plant tissues, *SQS2* is expressed at low levels in specific tissues or at particular developmental stages (Kriibi *et al*., 1997). Recently, *SQS2* has been shown to have no activity in vitro synthesizing squalene from FPP in the presence of NADPH and cofactors in Arabidopsis (Busquets *et al.*, 2008). In this experiment, the SQS2 protein expressed in *Saccharomyces cerevisiae erg9 mutants* lacking squalene synthase was unable to confer ergosterol prototrophy, an indication that SQS2 was not capable of synthesizing squalene from the substrates. With the existence of more than one copy in some of the plant genomes, it is important to test whether each gene copy is functional and it's potential role in regulating the sterol biosynthetic pathway.

1.3.3 Solanum chacoense Bitter as a model system for studying SGA biosynthesis

The genus *Solanum,* to which cultivated potato (*Solanum tuberosum*) belongs, contains the progenitors of cultivated potato as well as many wild potato species that can hybridize with *S. tuberosum*.

The distribution of the wild potato species includes much of the Americas; although no single wild species is found throughout a wide range, *S. colombiana*, *S. acaule*, and *S. chacoense* are remarkably widespread (Hawkes, 1990). *S. chacoense* is distributed in south Bolivia, north and central Argentina, Paraguay, Uruguay, and south Brazil where it grows as a weed in lowland pasture fields but can grow from sea level to about 2,350 m (Hawkes, 1990).

Solanum chacoense is divided into two subspecies, *chacoense* and *muelleri* (Hawkes, 1990). It is a diploid with 2*n* = 2x= 24 chromosomes (Hawkes and Hjerting, 1989). *S. chacoense* possesses a number of characteristics that have been of interest to breeders such as broad resistance, high starch and protein contents (Hawkes and Hjerting, 1989). The key characteristics of *S. chacoense* are outlined in detail in Table 1-2. The details of types of resistances and the lines of *S. chacoense* are listed in Table 1-3.

One characteristic of *S. chacoense* that has attracted attention is the capability to produce different types of SGAs in high amounts (Lawson *et al.*, 1993). S.

chacoense accumulates leptines and other types of SGAs in addition to the most common SGAs in commercial varieties, α-chaconine and α-solanine (Ronning *et al.*, 1999, Deahl *et al.*, 1993). This attribute makes it a suitable system in which to study the biosynthesis of SGAs.

1.4 Project goal

This study was undertaken to gain an understanding of the biosynthesis of the SGAs and the role of squalene synthase in regulating the accumulation of SGAs. The aim was to first establish a survey of SGA levels and composition during a time course of plant development and then identify those tissues that accumulate novel or different levels of SGAs. Secondly, because squalene synthase was suggested to be a key regulatory enzyme in pathways involving the biosynthesis of SGAs, the *squalene synthase* cDNA was characterized and compared to other plant *squalene synthase* sequences. A steady-state transcript analysis of *squalene synthase* and other isoprenoid and SGA biosynthesis genes was also conducted.

Taken together, these activities led to the development of an understanding of the distribution of SGAs in the *S. chacoense* and the characterization of a *squalene synthase* gene.

1.5 References

Andre CM, Schafleitner R, Guignard C, Oufir M, Aliaga CAA, Nomberto G, Hoffmann L, Hausman JF, Evers D, Larondelle Y (2009) Modification of the health-promoting value of potato tubers field grown under drought stress: emphasis on dietary antioxidant and glycoalkaloid contents in five native Andean Cultivars (*Solanum tuberosum* L.). Journal of Agricultural and Food Chemistry **57:** 599-609

Arnqvist L, Dutta PC, Jonsson L, Sitbon F (2003) Reduction of cholesterol and glycoalkaloid levels in transgenic potato plants by overexpression of a type 1 *sterol methyltransferase* cDNA. Plant Physiology **131:** 1792-1799

Bennett RN, Wallsgrove RM (1994) Secondary metabolites in plant defense-mechanisms. New Phytologist **127:** 617-633

Birch ANE, Geoghegan IE, Griffiths DW, McNicol JW (2002) The effect of genetic transformations for pest resistance on foliar solanidine-based glycoalkaloids of potato (*Solanum tuberosum*). Annals of Applied Biology **140:** 143-149

Blankemeyer JT, Atherton R, Friedman M (1995) Effect of potato glycoalkaloids alpha-chaconine and alpha-solanine on sodium active-transport in frog-skin. Journal of Agricultural and Food Chemistry **43:** 636-639

Blankemeyer JT, McWilliams ML, Rayburn JR, Weissenberg M, Friedman M (1998) Developmental toxicology of solamargine and solasonine glycoalkaloids in frog embryos. Food and Chemical Toxicology **36:** 383-389

Brown MS, McDonald GM, Friedman M (1999) Sampling leaves of young potato (*Solanum tuberosum*) plants for glycoalkaloid analysis. Journal of Agricultural and Food Chemistry **47:** 2331-2334

Busquets A, Keim V, Closa M, del Arco A, Boronat A, Arro M, Ferrer A (2008) *Arabidopsis thaliana* contains a single gene encoding squalene synthase. Plant Molecular Biology **67:** 25-36

Campos N, Boronat A (1995) Targeting and topology in the membrane of plant 3-hydroxy-3-methylglutaryl coenzyme a reductase. Plant Cell **7:** 2163-2174

Cantwell M (1996) A review of important facts about potato glycoalkaloids. Perishables Handling Newsletter **25:** 171-196

Chappell J (1995) The biochemistry and molecular-biology of isoprenoid metabolism. Plant Physiology **107:** 1-6

Chen Z, Miller AR (2000) Steroidal alkaloids in Solanaceous vegetable crops. Horticultural Reviews **25:** 171-196

Choi D, Ward BL, Bostock RM (1992) Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl coenzyme-a reductase genes in response to *Phytophthora infestans* and to its elicitor arachidonic-acid. Plant Cell **4:** 1333-1344

Coombs JJ, Douches DS, Cooper SG, Grafius EJ, Pett WL, Moyer DD (2005) Combining natural and engineered host plant resistance mechanisms in potato for Colorado potato beetle: Choice and no-choice field studies. Journal of the American Society for Horticultural Science **130:** 857-864

Coombs JJ, Douches DS, Li WB, Grafius EJ, Pett WL (2003) Field evaluation of natural, engineered, and combined resistance mechanisms in potato for control of

Colorado potato beetle. Journal of the American Society for Horticultural Science **128:** 219-224

Cooper SG, Douches DS, Grafius EJ (2004) Combining genetic engineering and traditional breeding to provide elevated resistance in potatoes to Colorado potato beetle. Entomologia Experimentalis Et Applicata **112:** 37-46

Deahl KL, Sinden SL, Young RJ (1993) Evaluation of wild tuber-bearing *Solanum* accessions for foliar glycoalkaloids level and composition. American Potato Journal **70:** 61-69

Devarenne TP, Ghosh A, Chappell J (2002) Regulation of squalene synthase, a key enzyme of sterol biosynthesis, in tobacco. Plant Physiology **129:** 1095-1106

Devarenne TP, Shin DH, Back K, Yin SH, Chappell J (1998) Molecular characterization of tobacco *squalene synthase* and regulation in response to fungal elicitor. Archives of Biochemistry and Biophysics **349:** 205-215

Dimenstein L, Lisker N, Kedar N, Levy D (1997) Changes in the content of steroidal glycoalkaloids in potato tubers grown in the field and in the greenhouse under different conditions of light, temperature and daylength. Physiological and Molecular Plant Pathology **50:** 391-402

Distl M, Wink M (2009) Identification and quantification of steroidal alkaloids from wild tuber-bearing *Solanum* species by HPLC and LC-ESI-MS. Potato Research **52:** 79-104

Evans D, Mondy NI (1984) Effect of magnesium fertilization on glycoalkaloid formation in potato tubers. Journal of Agric. Food Chem **32:** 465-466

Fewell AM, Roddick JG (1993) Interactive antifungal activity of the glycoalkaloids alpha-solanine and alpha-chaconine. Phytochemistry **33:** 323-328

Fewell AM, Roddick JG (1997) Potato glycoalkaloid impairment of fungal development. Mycological Research **101:** 597-603

Flinn B, Rothwell C, Griffiths R, Laguë M, De Koeyer DL, Sardana R, Audy P, Goyer C (2005) Potato Expressed Sequence Tag Generation and Analyses Using Standard and Unique cDNA Libraries Plant Molecular Biology **59:** 407-433

Fragoyiannis DA, McKinlay RG, D'Mello JPF, Bcpc B (2000) Effects of different nitrogen sources and aphid herbivory on foliar glycoalkaloid production in two potato cultivars. *In* International Conference on Pests and Diseases. British Crop Protection Council, Brighton, England, pp 919-924

Friedman M, Dao L (1992) Distribution of glycoalkaloids in potato plants and commercial potato products. Journal of Agricultural and Food Chemistry **40:** 419-423

Friedman M, McDonald GM (1997) Potato glycoalkaloids: chemistry, analysis, safety, and plant physiology. Critical Reviews in Plant Sciences **16:** 55-132

Ginzberg I, Tokuhisa JG, Veilleux RE (2009) Potato steroidal glycoalkaloids: Biosynthesis and genetic manipulation. Potato Research **52:** 1-15

Gregory P (1984) Glycoalkaloid composition of potatoes:diversity and biological implications. American Potato Journal **61:** 115-121

Hawkes JG (1990) The potato: evolution, biodiversity, and genetic resources. Belhaven Press, Oxford

Hawkes JG, Hjerting JP (1989) The potatoes of Bolivia: Their breeding value and evolutionary relationships. Clarendon, Oxford

Hayashi H, Hirota A, Hiraoka N, Ikeshiro Y (1999) Molecular cloning and characterization of two cDNAs for *Glycyrrhiza glabra* squalene synthase. Biological & Pharmaceutical Bulletin **22:** 947-950

Heftmann E (1983) Biogenesis of steroids in Solanaceae. Phytochemistry **22:** 1843-1860

Hide GA, Lapwood DH (1992) Disease aspects of potato production. *In* PM Harris, ed, The Potato Crop. Chapman and Hall, London, pp 403-475

Huang ZS, Jiang KJ, Pi Y, Hou R, Liao ZH, Cao Y, Han X, Wang Q, Sun XF, Tang KX (2007) Molecular cloning and characterization of the yew gene encoding squalene synthase from *Taxus cuspidata*. Journal of Biochemistry and Molecular Biology **40:** 625-635

Jansky S, Austin-Phillips S, McCarthy C (1999) Colorado potato beetle resistance in somatic hybrids of diploid interspecific *Solanum* clones. Hortscience **34:** 922-927

Jansky SH, Simon R, Spooner DM (2009) A test of taxonomic predictivity: resistance to the Colorado potato beetle in wild relatives of cultivated potato. Journal of Economic Entomology **102:** 422-431

Kaneko K, Tanaka MW, Mitsuhashi H (1977) Dormantinol, a possible precursor in solanidine biosynthesis, from budding *Veratrum grandiflorum*. Phytochemistry **16:** 1247-1251

Kolbe H, Stephan-Beckmann S (1997) Development, growth and chemical composition of the potato crop (*Solanum tuberosum* L.) .1. Leaf and stem. Potato Research **40:** 111-129

Kribii R, Arro M, DelArco A, Gonzalez V, Balcells L, Delourme D, Ferrer A, Karst F, Boronat A (1997) Cloning and characterization of the *Arabidopsis thaliana SQS1* gene encoding squalene synthase - Involvement of the C-terminal region of the enzyme in the channeling of squalene through the sterol pathway. European Journal of Biochemistry **249:** 61-69

Lachman J, Hamouz K, Orsak M, Pivec V (2001) Potato glycoalkaloids and their significance in plant protection and human nutrition - Review. Series Rostlinna **47:** 181-191

Laurila J, Laakso I, Valkonen JPT, Hiltunen R, Pehu E (1996) Formation of parental-type and novel glycoalkaloids in somatic hybrids between *Solanum brevidens* and S. *tuberosum*. Plant Science **118:** 145-155

Lawson RD, Veilleux RE, Miller AR (1993) Biochemistry and genetics of *Solanum chacoense* steroidal alkaloids: Natural resistance factors to the Colorado potato beetle. Current Topics in Bot. Research **1:** 335-352

Lee MH, Jeong JH, Seo JW, Shin CG, Kim YS, In JG, Yang DC, Yi JS, Choi YE (2004) Enhanced triterpene and phytosterol biosynthesis in *Panax ginseng* overexpressing squalene synthase gene. Plant and Cell Physiology **45:** 976-984

Lipinski RJ, Dengler E, Kiehn M, Peterson RE, Bushman W (2007) Identification and characterization of several dietary alkaloids as weak inhibitors of Hedgehog signaling. Toxicological Sciences **100:** 456-463

Maga JA (1981) Total and individual glycoalkaloid composition of stored potato slices. Journal of Food Processing and Preservation **5:** 23-29

Maga JA (1994) Glycoalkaloids in Solanaceae. Food Reviews International **10:** 385-418

McCue KF, Allen PV, Shepherd LVT, Blake A, Maccree MM, Rockhold DR, Novy RG, Stewart D, Davies HV, Belknap WR (2007) Potato glycosterol

rhamnosyltransferase, the terminal step in triose side-chain biosynthesis. Phytochemistry **68:** 327-334

McCue KF, Shepherd LVT, Allen PV, Maccree MM, Rockhold DR, Corsini DL, Davies HV, Belknap WR (2005) Metabolic compensation of steroidal glycoalkaloid biosynthesis in transgenic potato tubers: using reverse genetics to confirm the in vivo enzyme function of a steroidal alkaloid galactosyltransferase. Plant Science **168:** 267-273

McKey D (1974) Adaptive patterns in alkaloid physiology. The American Naturalist **108:** 305-320

Moehs CP, Allen PV, Friedman M, Belknap WR (1997) Cloning and expression of solanidine UDP-glucose glucosyltransferase from potato. Plant Journal **11:** 227-236

Mondy NI, Munshi CB (1988) Effect of molybdenum fertilization on the nitrogenous constituents of potatoes. Journal of Food Science **53:** 1901-1910

Mweetwa AM, Poe R, Tokuhisa JG (2008) Biosynthesis and regulation of steroidal glycoalkaloids in wild potato, *Solanum chacoense* bitter. *In* 105th Annual Conference of the American-Society-for-Horticultural-Science, Orlando, FL, pp 1287-1287

Nakashima T, Inoue T, Oka A, Nishino T, Osumi T, Hata S (1995) cloning, expression, and characterization of cDNA encoding *Arabidopsis thaliana* squalene synthase. Proceedings of the National Academy of Sciences of the United States of America **92:** 2328-2332

Newman JD, Chappell J (1999) Isoprenoid biosynthesis in plants: Carbon partitioning within the cytoplasmic pathway. Critical Reviews in Biochemistry and Molecular Biology **34:** 95-106

Nitithamyong A, Vonelbe JH, Wheeler RM, Tibbitts TW (1999) Glycoalkaloids in potato tubers grown under controlled environments. American Journal of Potato Research **76:** 337-343

Papathanasiou F, Mitchell SH, Harvey BMR (1998) Glycoalkaloid accumulation during tuber development of early potato cultivars. Potato Research **41:** 117-125

Papathanasiou F, Mitchell SH, Harvey BMR (1999) Variation in glycoalkaloid concentration of potato tubers harvested from mature plants. Journal of the Science of Food and Agriculture **79:** 32-36

Percival G, Dixon GR (1996) Glycoalkaloid concentrations in aerial tubers of potato (*Solanum tuberosum* L). Journal of the Science of Food and Agriculture **70:** 439-448

Piironen V, Lindsay DG, Miettinen TA, Toivo J, Lampi AM (2000) Plant sterols: biosynthesis, biological function and their importance to human nutrition. Journal of the Science of Food and Agriculture **80:** 939-966

Raman KV, Radcliffe EB, eds (1992) Pest aspects of potato production: Part 2 Insect pests, Ed Second. Chapman and Hall, London

Rayburn JR, Bantle JA, Friedman M (1994) Role of carbohydrate side-chains of potato glycoalkaloids in developmental toxicity. Journal of Agricultural and Food Chemistry **42:** 1511-1515

Rayburn JR, Friedman M, Bantle JA (1995) Synergistic interaction of glycoalkaloids alpha-chaconine and alpha-solanine on developmental toxicity in *Xenopus* embryos. Food and Chemical Toxicology **33:** 1013-1019

Robinson GW, Tsay YH, Kienzle BK, Smithmonroy CA, Bishop RW (1993) Conservation between human and fungal squalene synthetases - similarities in structure, function, and regulation. Molecular and Cellular Biology **13:** 2706-2717

Roddick JG (1980) A sterol-binding assay for potato glycoalkaloids. Phytochemistry **19:** 2455-2457

Roddick JG, Rijnenberg AL (1987) Synergistic interaction between the potato glycoalkaloids alpha-solanine and alpha-chaconine in relation to lysis of phospholipid sterol liposomes. Phytochemistry **26:** 1325-1328

Roddick JG, Rijnenberg AL, Weissenberg M (1992) Alterations to the permeability of liposome membranes by the solasodine-based glycoalkaloids solasonine and solamargine. Phytochemistry **31:** 1951-1954

Roddick JG, Weissenberg M, Leonard AL (2001) Membrane disruption and enzyme inhibition by naturally-occurring and modified chacotriose-containing Solanum steroidal glycoalkaloids. Phytochemistry **56:** 603-610

Ronning CM, Stommel JR, Kowalski SP, Sanford LL, Kobayashi RS, Pineada O (1999) Identification of molecular markers associated with leptine production in a population of *Solanum chacoense* Bitter. Theoretical and Applied Genetics **98:** 39-46

Sanford LL, Deahl KL, Sinden SL, Kobayashi RS (1995) Glycoalkaloid content in tubers of hybrid and backcross populations from a *Solanum tubersoum* x S. *chacoense* cross. American Potato Journal **72:** 261-271

Sanford LL, Deahl KL, Sinden SL, Kobayashi RS (1996) Segregation of leptines and other glycoalkaloids in *Solanum tuberosum* (4x) x S. *chacoense* (4x) crosses. American Potato Journal **73:** 21-33

Schaller H (2004) New aspects of sterol biosynthesis in growth and development of higher plants. Plant Physiology and Biochemistry **42:** 465-476

Schaller H, Grausem B, Benveniste P, Chye ML, Tan CT, Song YH, Chua NH (1995) Expression of *Hevea brasiliensi* 3-hydroxy-s-methylglutaryl coenzyme A reductase in tobacco results in sterol overproduction. Plant Physiology **109:** 761-770

Seo JW, Jeong JH, Shin CG, Lo SC, Han SS, Yu KW, Harada E, Han JY, Choi YE (2005) Overexpression of squalene synthase in *Eleutherococcus senticosus* increases phytosterol and triterpene accumulation. Phytochemistry **66:** 869-877

Sinden SL, Sanford LL, Osman SF (1980) Glycoalkaloids and resistance to the Colorado potato beetle in *Solanum chacoense* Bitter. American Potato Journal **57:** 331-343

Sinden SL, Sanford LL, Webb RE (1984) Genetic and environmental control of potato glycoalkaloids. American Potato Journal **61:** 141-156

Smith DB, Roddick JG, Jones JL (1996) Potato glycoalkaloids: Some unanswered questions. Trends in Food Science & Technology **7:** 126-131

Smith DB, Roddick JG, Jones JL (2001) Synergism between the potato glycoalkaloids alpha-chaconine and alpha-solanine in inhibition of snail feeding. Phytochemistry **57:** 229-234

Smith SW, Giesbrecht E, Thompson M, Nelson LS, Hoffman RS (2008) Solanaceous steroidal glycoalkaloids and poisoning by *Solanum torvum*, the normally edible susumber berry Toxicon **52:** 667-676.

Soule S, Guntner C, Vazquez A, Argandona VH, Ferreira F, Moyna P (1999) Effect of Solanum glycosides on the aphid *Schizaphis graminum*. Journal of Chemical Ecology **25:** 369-374

Sowokinos JR (2007) Internal physiological disorders and nutritional compositional factors that affect market quality. *In* D Vreugdenhil, ed, Potato biology and biotechnology. Elsevier

Stiller I, Davis G, Hesse H (2007) Improving the nutritive value of tubers: Elevation of cystein and glutathione contents in the potato cultvar White Lady by marker-free transformation. Journal of Biotechnology **128:** 335-343

Storey RMJ (2007) The harvested crop. *In* D Vreugdenhil, ed, Potato biology and biotechnology: Advances and perspectives. Elsevier, Amstedam, pp 441-466

Storey RMJ, Davies HV (1992) Tuber quality. *In* PM Harris, ed, The potato crop-the scientific basis for improvement. Chapman and Hall, London

Thompson AL, Love SL, Sowokinos JR, Thornton MK, Shock CC (2008) Review of the sugar end disorder in potato (*Solanum tuberosum*, L.). American Journal of Potato Research **85:** 375-386

Threfall DR, Whitehead IM (1988) Coordination of *squalene synthetase* and induction of sesquiterpenoid phytoalexins biosynthesis in cultures of *Nicotiana tabacum*. Phytochemistry **27:** 2567-2580

Tingey WM, Mackenzie JD, Peter G (1978) Total foliar glycoalkaloids and resistance of wild potato species to *Empoasca fabae* (Harris) American Journal of Potato Research **55:** 577-585

Uchida H, Yamashita H, Kajikawa M, Ohyama K, Nakayachi O, Sugiyama R, Yamato KT, Muranaka T, Fukuzawa H, Takemura M (2009) Cloning and characterization of a *squalene synthase* gene from a petroleum plant, *Euphorbia tirucalli* L. Planta **229:** 1243-1252

Valkonen JPT, Keskitalo M, Vasara T, Pietila L (1996) Potato glycoalkaloids: A burden or a blessing? Critical Reviews in Plant Sciences **15:** 1-20

Wentzinger LF, Bach TJ, Hartmann M-A (2002) Inhibition of squalene synthase and squalene epoxide in tobacco cells triggers an up-regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Plant Physiology **130:** 1-13

Yoshioka H, Yamada N, Doke N (1999) CDNA cloning of sesquiterpene cyclase and squalene synthase, and expression of the genes in potato tuber infected with *Phytophthora infestans*. Plant and Cell Physiology **40:** 993-998

Zimowski I (1991) Occurance of a glucosyltransferase specific for solanidine in potato plants. Phytochemistry **30:** 1827-1831

1.6 Graphs and Tables

Table1- 1:Glycoalkaloids of *S. tuberosum* and wild potato species (adapted from Sinden et al., 1980, Sinden et al., 1988, and Lachman et al., 2001). (Unknown glycosides associated with the SGA are indicated by the hyphen)

SGA	Aglycone	Sugar Moeity
S. *tuberosum*		
α-solanine	solanidine	galactose + glucose + rhamnose
β-solanine	solanidine	galactose + (glucose or rhamnose)
γ-solanine	solanidine	galactose
α-chaconine	solanidine	glucose + rhamnose + rhamnose
β-chaconine	solanidine	glucose + rhamnose
γ-chaconine	solanidine	glucose
α-solamarine	tomatidenol	galactose + glucose + rhamnose
β-solamarine	tomatidenol	glucose + rhamnose + rhamnose
Wild potatoes		
Leptine I	O (23)-acetylleptidine	glucose + rhamnose + rhamnose
Leptine II	O (23)-acetylleptidine	galactose + glucose + rhamnose
Leptine III	O (23)-acetylleptidine	galactose + glucose + glucose + xylose
Leptine IV	O (23)-acetylleptidine	galactose + glucose + glucose + glucose
Leptinine I	leptinidine	glucose + rhamnose + rhamnose
Leptinine II	leptinidine	galactose + glucose + rhamnose
Leptinine III	leptinidine	-
Leptinine IV	leptinidine	-
Commersonine	demissidine	galactose + glucose + glucose + glucose
Demissine	demissidine	galactose + glucose + glucose + xylose
Dehydrocommersonine	solanidine	galactose + glucose + glucose + glucose

Table1- 2: Description of *S. chacoense* Bitter (Hawkes and Hjerting, 1989)

Plant Organ	Size / Shape	Other Characteristics
Stem	20-80 cm tall; glabrous	With decurrent lines or very narrow wings
Leaf	15-25 cm x 5-15 cm broad; sparcely pubescent or glabrous; ovate to ovate-lanceolate or oblong leaflets;	
Inflorescence	Peduncle forked once or twice; pedicels 15-30 mm long	Typically multi-flowered;
Calyx	4-6 mm long; usually glabrous; acumens 1-3 mm long	
Corolla	White, stellate, 25-35 mm in diameter	
Anthers	6-7 mm long; filaments about 1 mm long	
Style	12-14 mm long; exerted up to 6 mm above the anther; stigma varies from globose to clavate, up to 1.2 mm diameter	
Berries	Globose, from 10 to 25 mm diameter	

Table1- 3: Description of *S. chacoense* characters of interest for breeding potato cultivars (Hawkes and Hjerting, 1989)

Name	Comments
Phytophtora infestans	Resistance found in some lines
Synchytrium endobioticum	Resistance found in several lines
Streptomyces scabies	Resistance found in several lines
Spongospora subterranea	
Verticillium albo-atrum	Resistance found in several lines
Verticillium dahliae	Resistance found in some lines
Fusarium spp	Resistance noted to *F. solani* and *F. sulphureum*
Macrophomina pustulans	Resistance found in several lines
Alternaria solani	Resistance found in several lines
Oospora pustulans	Resistance found in some lines
Rhizoctonia solani	Resistance resistance found in some hybrids with *S. tuberosum*
Pseudomonas solanacerum	Resistance found in several lines *
Erwinia carotovera	Resistance found in several lines **
Carynebacterium sepedonicm	Unconfirmed resistance
Potato Virux X	Resistance found in some lines
Potato Virus Y	Resistance found in several lines ***
Potato Leaf Roll Virus	Low resistance

Name	Comments
Potato Mosaic Virus	Resistance found in several lines
Leptinotarsa decemlineata	Resistance found in several lines
	(Accessions with leptines)
Myzus persicae	Resistance found in some lines
Macrosiphum euphorbiae	Resistance found in some lines
Empoasca fabae	Resistance found in several lines
Epitrix cucumeris	Resistance found in several lines
Phthorimaea operculella	Resistance found in some lines
Globodera spp	Resistance found in several lines **
Meloidogyne incognita	Very high resistance
Ditylenchus destructor	Resistance found in several lines
-	Medium resistance
-	High resistance
-	Good tolerance
-	High starch (dry matter) and high protein

- Resistance effective in humid tropics ad elsewhere ** Resistance to several pathotypes, ***: Also resistant to PVA

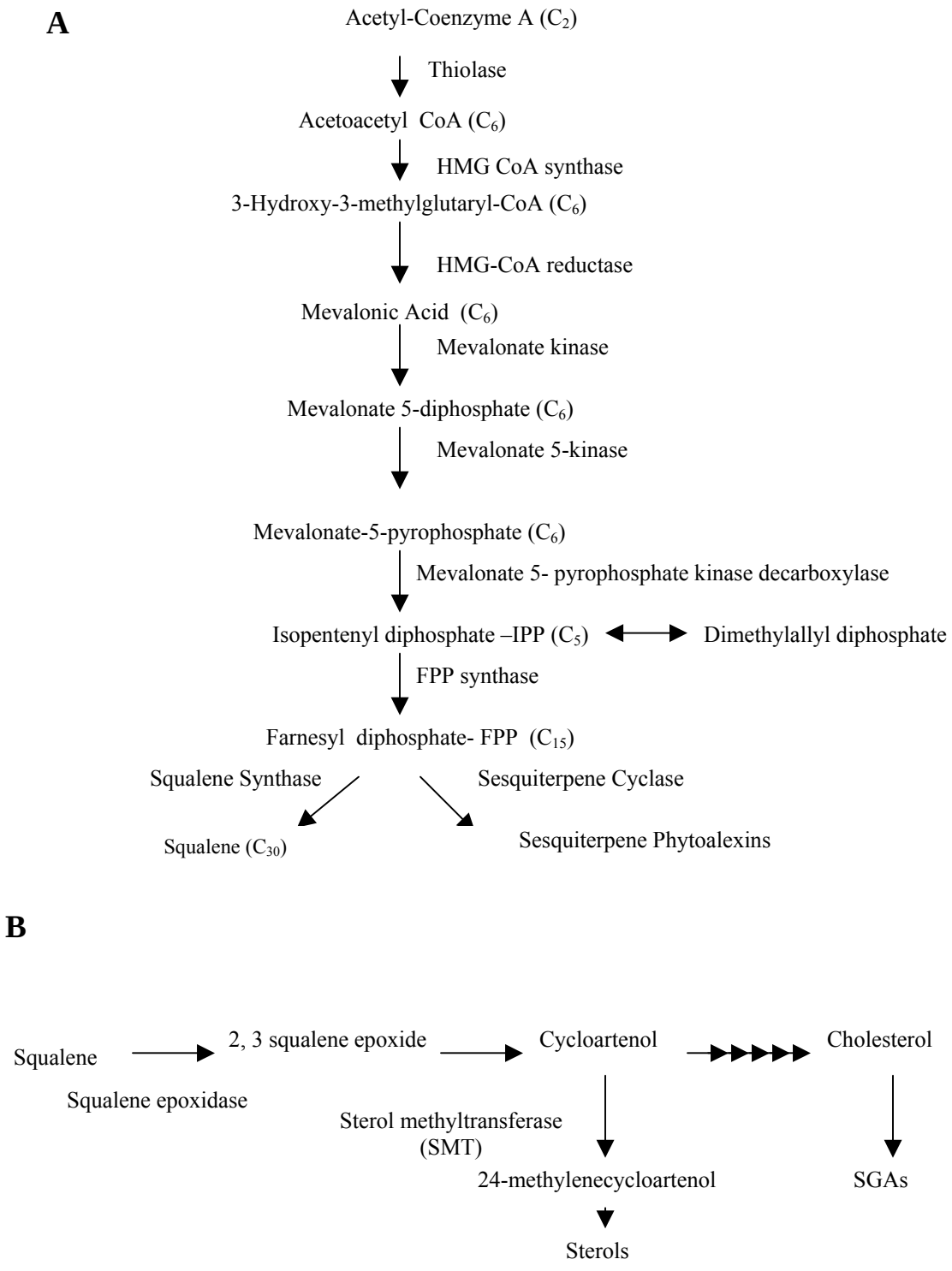


Figure 1-1 Outline of sterol and SGA biosynthesis. A. Synthesis of squalene and sesquiterpene phytoalexins from the mevalonic acid pathway in isoprenoid metabolism. B. Sterol and SGA biosynthesis from squalene.

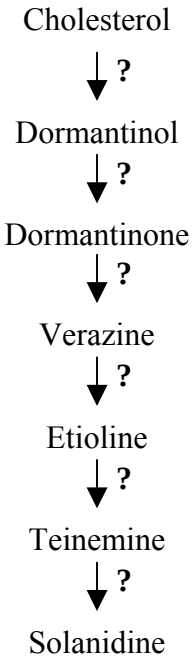


Figure 1-2 Proposed pathway from cholesterol to solanidine (Kaneko et al., 1977)

2 Introduction Developmental Time Course Investigation of SGA Distribution in *S. chacoense*

Abstract

Steroidal glycoalkaloids (SGAs) are secondary metabolites produced by approximately 350 species in the Solanaceae family. SGAs are reported to be important for pest resistance and flavor enhancement at low concentrations but maybe toxic to humans and other mammals at high concentrations. Studies on sterol/SGA biosynthesis have implicated 3-hydroxy-3-methylglutaryl CoA (HMGR) and squalene synthase as key regulatory enzymes. Three glycosyltransferases have so far been identified to be involved in the final steps of SGA biosynthesis. A study was conducted to elucidate the distribution pattern of SGAs and to determine a relationship between SGAs and gene expression levels of the key enzymes. *Solanum chacoense,* a wild potato species was used as a model plant from which tissues were harvested at specified developmental stages and analyzed for SGA content. The results from the SGA analysis suggest a qualitative and quantitative tissue- and age-dependent biosynthesis of SGAs. Regenerative tissues such as auxiliary shoots, flowers and floral buds had the highest levels of 88, 49 and 63 μmole/g DW, respectively. The roots, stems and tubers showed the lowest amounts of SGAs of 1 to 8, 5 to 15 and 7 to 16 μmole/g DW, respectively. Stolons and tubers accumulated higher proportions of α-chaconine (59 to 67 and 61 to 64%, respectively) than α-solanine at all developmental stages analyzed. On the other hand, in young expanding, fully expanded, and old senescing leaves where leptine-type tended to dominate, α-solanine and α-chaconine together accounted for only 8 to 15%, 7 to 15%, and 8 to 45%, respectively. Plant organs that showed the highest biosynthetic activity for SGA production also had high levels of transcripts of coding for genes of isoprenoid biosynthesis.

2.1 Introduction

Steroidal glycoalkaloids (SGAs) are a class of secondary metabolites produced by some members of the Solanaceae and Liliaceae families (Kaneko *et al.*, 1977, Valkonen *et al.*, 1996, Chen and Miller, 2000). Different types of SGAs have been isolated and characterized from more than 300 *Solanum* species (Lawson *et al.*, 1993, Friedman and McDonald, 1997) including potato (both wild and cultivated), eggplant, pepper, and tomato. In potato and tomato alone, at least 20 structurally different SGAs have been recognized, while 300 others have been found in other *Solanum* species (Friedman and Dao, 1992). SGAs are glycosides of heterocyclic steroid derivatives. The aglycone is a C_{27}-steroidal alkaloid structure consisting of six rings- A, B, C, D, E, and F- and a nitrogen atom situated either in the E or F ring (Chen and Miller, 2000; Valkonen et al., 1996). The glycoside portion of SGAs consists of a tri- or tetrasaccharide attached by an ether linkage to C3 (in ring A) of the steroid (Ginzberg et al., 2009). The oligosaccharides are combinations of specific sugar monomers and include chacotriose (glucose, rhamnose, rhamnose), solatriose (galactose, glucose, rhamnose), lycotetraose (galactose, glucose, glucose, xylose) and commertetraose (galactose, glucose. glucose, glucose).

SGAs have been implicated in toxicity to humans and other mammals (Smith et al., 2008) and are therefore an important factor in potato varietal development. When present at concentrations of more than 20 mg/100 g, SGAs impart a bitter taste to potatoes (Sanford et al., 1995). This bitter taste is associated with the alkaloid portion of the molecule (Croteau et al., 2000). Symptoms resulting from SGA poisoning may include abdominal pain, vomiting,

diarrhea, tachycardia, hypotension, and neurological disorders (Smith *et al.*, 2008, Arnqvist *et al.*, 2003).

SGAs have been implicated in plant chemical defense against herbivores, pests and pathogens (Bennett and Wallsgrove, 1994, Chen and Miller, 2000). Leptine glycoalkaloids found in *Solanum chacoense* have been shown to impart resistance to Colorado potato beetle through reduced feeding and increased mortality (Sinden *et al.*, 1986, Deahl *et al.*, 1991, Pelletier *et al.*, 1999). *Solanum* genotypes accumulating commersonine and dehydrocommersonine have also been shown to be resistant to CPB (Sinden et al., 1980). SGAs have also been implicated in resistance against the potato leafhopper (Tingey et al., 1978).

There are several toxic properties of SGAs. Disruption of cell membranes has been suggested to be the principal mechanism by which SGAs compromise the structural and functional integrity of cells/tissues of other organisms. SGAs are also reported to be teratogenic and have the ability to inhibit acetylcholinesterase and butyrylcholinesterase (Roddick et al., 2001).

The levels of SGAs in potato are modulated by environmental factors, plant organ and the developmental stage, genotype, and post-harvest handling (Maga, 1994 ; Ginzberg *et al.*, 2009). The ability to form specific types of SGAs is determined by the genotype of the plant (Sinden *et al.*, 1984, Sanford *et al.*, 1995,). Glycoalkaloids are synthesized in all parts of the potato plant (Birch et al., 2002) and organs that are important for survival and multiplication like flowers, unripe berries, young leaves, sprouts, and the area around potato eyes, tend to have more than twice the amounts of SGAs than other organs (Lawson *et al.*, 1993, Chen and

Miller, 2000, Lachman *et al.*, 2001). In general, concentration has been shown to be higher in younger tissues or in tissues that have higher metabolic activities. Because SGA accumulation is correlated with the physiological/metabolic activities in tissues, it is reasonable to assume that the concentration of SGAs in specific potato tissues might vary as tissues age (Brown et al., 1999).

The commercial importance of *S. tuberosum* has focused research on the SGAs commonly found in the tubers. Because *S. tuberosum* accumulates only two SGAs, α-chaconine and α-solanine, that differ only in the trisaccharide moiety (Fewell and Roddick, 1993, Smith *et al.*, 1996, Fewell and Roddick, 1997), our general understanding of SGA content and composition in the aerial part of the developing plant is limited and is based on totals or ratios of α-chaconine and α-solanine. Wild potato species such as *S. chacoense* accumulate higher levels of SGAs and in addition to α-chaconine and α-solanine, leptines, leptinines and other types of SGAs (Deahl *et al.*, 1993, Ronning *et al.*, 1999,) in an organ-specific manner (Sanford *et al.*, 1995, Ronning *et al.*, 1999, Mweetwa *et al.*, 2008). There are currently no known studies of the accumulation patterns of mixtures of SGAs during plant development that have been conducted with other genotypes (wild species) and how such changes can contribute to the overall defense capabilities of the plant.

The biosynthesis of SGAs begins with the mevalonic acid pathway, continues with sterol formation and then through the activities of novel enzymes, leads to the production of steroidal alkaloids and their glycosylation. These biosyntheses are part of a complex isoprenoid metabolic network which also produces a diversity of primary and secondary metabolites

(Schaller, 2004). Studies on sterol or SGA biosynthesis have implicated 3-hydroxy-3-methylglutaryl CoA (HMGR) and squalene synthase as key regulatory enzymes and cholesterol as a central metabolite (Chappell, 1995, Devarenne *et al.*, 2002, Devarenne *et al.*, 1998, Piironen *et al.*, 2000). Three glycosyltranferases have been shown to be involved in the final steps of SGA biosynthesis (Moehs *et al.*, 1997, Stapleton *et al.*, 1991, McCue *et al.*, 2002, McCue *et al.*, 2005, McCue *et al.*, 2006a, McCue *et al.*, 2006b, McCue *et al.*, 2007). A strong correlation between transcript levels of *HMGR1* and SGA content in leaves and tubers has recently been shown (Krits et al., 2007). However, expression levels of *squalene synthase* were partially but not consistently correlated with SGA content in this study.

With an overall goal of gaining an understanding of the biosynthesis of the SGAs in *Solanum chacoense*, we describe here: (i) a time course survey of SGA levels and composition in the developing plant, (ii) the identification of tissues that accumulate novel or differential levels of SGAs, and (iii) gene expression levels associated with key isoprenoid biosynthetic enzymes. With this information, further studies in potato could focus on establishing points within the SGA biosynthetic pathway that could be manipulated using molecular breeding techniques in order to enhance resistance to insects while reducing levels in the tubers.

2.2 Materials and Methods

2.2.1 ***Chemicals:***

Alpha-solanine (Cat. #045K7350) and alpha-chaconine (Cat. # 089H7032) standards (99% purity, from potato sprouts) were obtained from Sigma-Aldrich (St. Louis, MO). All primers

used were obtained from Integrated DNA Technologies (Coralville, IA, Table 2-1). Primers sequences were designed based on the 3' regions of the published of *S. tuberosum* genes.

Table 2- 1 The primers used in the study for semi-quantitative PCR

Primer	Target Gene	Direction	Sequence 5'-3'	GeneBank Reference Source	Expected Fragment Length (bp)
1 HMG1	*hmgr1*	+	TCTTGTGGAGCTGAACATGC	GI: 169484	267
2 HMG1	*hmgr1*	-	AAGCAAGCTGACTGTGATGC		267
1 HMG2	*hmgr2*	+	TTGTTCGTGAAGGTCCGTG	GI: 12082119	245
2 HMG2	*hmgr2*	-	TTATGTCTTTGCTATGTTAG		245
1 HMG3	*hmgr3*	+	TGTGTCAAGGTGGATGACC	GI: 7415989	249
2 HMG3	*hmgr3*	-	TAAGCACCACTGCATACCAG		249
1 SGT1	*SGT1*	+	TCTACAACGAGAAGGTAGTC	GI: 82802846	250
2 SGT1	*SGT1*	-	TGAGAGCAGTGAGATTGTTC		250
1 SGT2	*SGT2*	+	ATGCAGTGGAAGAAGGTGGG	GI: 78191093	240
2 SGT2	*SGT2*	-	ACATACCGAAACTTGAATCG		240
1 ActStur S	*Actin*	+	TGAGTTACCAGATGGTCAGG	GI: 21533	259
2 ActStur T	*Actin*	-	TCCTTGCTCATACGATCAGC		259
2 SQS ex9ex8	*SQS*	-	ATAGCACATAGCTAATGTCCC	*S. chacoense* cDNA	295
1 SQS ex8ex9	*SQS*	+	CGAGGAGAACTCGGTTAAGG		295

Table 2- 2 *S. chacoense* tissues harvested at different stages during plant development stages. DAT: days after transplanting. Numbers in parenthesis indicate the number of plants harvested for each biological replication.

	DAT	Tissues harvested									
Stage		Roots	Tubers	Leaves			Stolons		Floral buds	Flowers	Stems
				Senescing	Expanded	Young	Below ground	Above ground			
Early Vegetative	30 (3)	*		*		*					*
Mid Vegetative	44 (3)	*		*		*	*				*
Late Vegetative	58 (1)	*	*	*	*	*	*				*
Anthesis	71 (1)	*	*	*	*	*	*	*	*	*	*
25 d post anthesis	96 (1)	*	*	*	*	*	*		*		*
65 d post anthesis	136 (1)	*	*	*	*	*	*	*			*

2.2.2 ***Plant material***

Stolon cuttings of *Solanum chacoense* USDA diploid clone 8380-1 (*chc*80-1, PI 458310) were established in Sunshine Mix # 1 (SunGro, Bellevue, WA) and transferred to 10.3 cm pots after 30 d. Plants were grown in a controlled environment chamber (Conviron, Winnipeg) set to 60% relative humidity, a 10 h photoperiod and day/night temperatures of 24 and 19^{o}C, respectively. At 65 days after establishment, the photoperiod was adjusted to 14 h to induce flowering. Light intensity was maintained at approximately 275 μmol $m^{-2}s^{-1}$ (PAR). Plants were treated biweekly with MiracleGro All Purpose Fertilizer (15:30:15 N: K: P, Scotts, Marysville, OH) at a rate of 1g per L of water.

Tissue was harvested at six different growth stages approximately representing the entire life cycle of the plant: (1) early vegetative, (2) mid vegetative, (3) late vegetative (4) anthesis (flowering), (5) 25 d post-anthesis, and (6) 65 d post-anthesis. These stages and the type of tissues harvested are summarized in Table 2-2. The number of plants representing each biological replication varied with developmental stage (Table 2-2) but there were at least three biological replications for each sample. Whole plants were harvested at the same time of the day, and dissected into various tissues. In order to analyze SGAs in different parts of a developing stem, stems portions representing the older (upper), middle and younger (lower parts) of the stems were harvested at the late vegetative stage. After tissue harvest, fresh weights were determined and samples lyophilized (Labconco) for 72 h. Freeze-dried samples were ground in a mortar and pestle using liquid nitrogen to enhance the grinding efficiency.

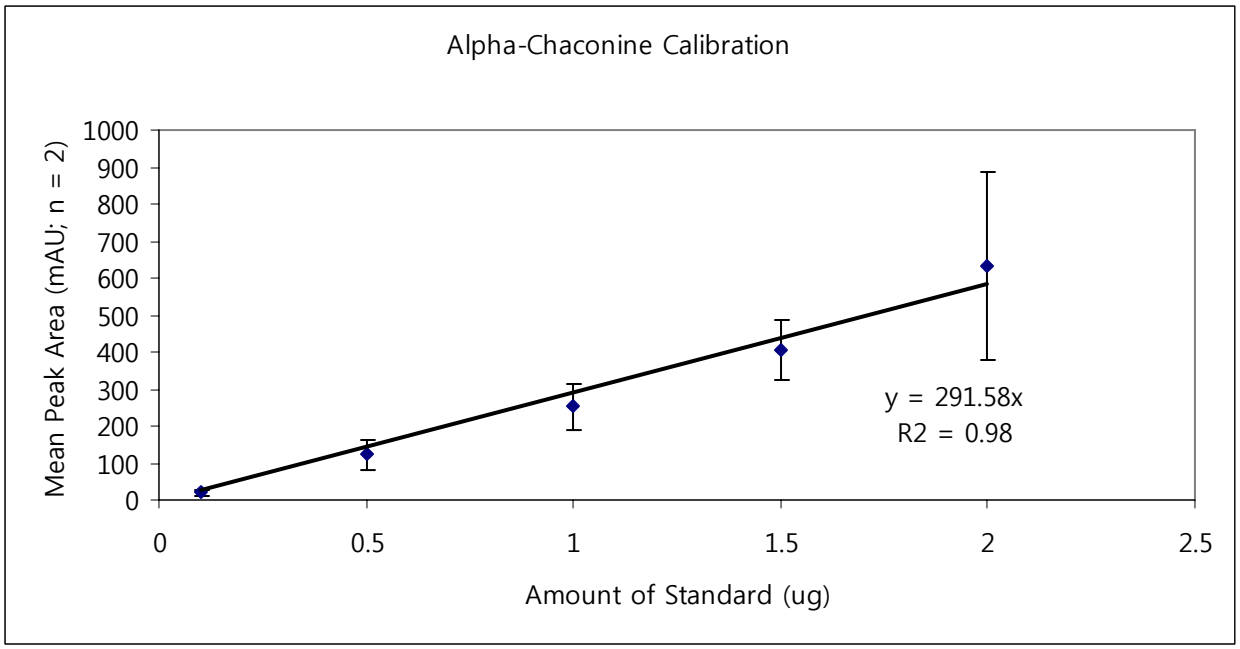
2.2.3 ***SGA extraction and analysis***

Extraction and profile analysis using high performance liquid chromatography (HPLC) followed the procedure of Edwards and Cobb (1996). In brief, the glycoalkaloids were extracted with aqueous acetic acid, concentrated, and cleaned up by solid phase extraction using C_{18} cartridge columns (Waters, Millford, MA).

2.2.4 SGA analysis: quantification and standard calibration curve determination

SGAs were separated and quantified by HPLC (Agilent HP 1200 Series) fitted with a C-18 reverse-phase column (Agilent Eclipse XDB-C18, 5 μm pore size and 4.6 x 150 mm) and a photodiode array detector. SGAs were eluted using a linear gradient of Solvent A, acetonitrile/ 0.01 M Tris-HCl (30:70, v/v pH 8.0), and Solvent B, acetonitrile/0.01 M Tris-HCl (80:20, v/v, pH 8.0) at a flow rate of 1 mL/min at 25^{o}C column temperature. The gradient elution was as follows: 0-10 min, 0-40% B; 10-13 min, 40-100% B; 13-15 min, 100% B; 15-15.5 min, 100-0% B; 15.5-20 min, 0% B. Eluent was monitored by diode array detection at 202 nm. Calibration curves for α-solanine and α-chaconine were generated with purified standards (Figure 2-1). Due to the lack of purified standards for leptines and leptinines, there were no calibration curves generated for these compounds.

Statistical differences between total SGAs and ratios were determined using the SAS program (version 9.1) by analysis of variance (ANOVA) using the Ryan-Einot-Gabriel-Welch multiple range test (REGWQ) values at α=0.05.



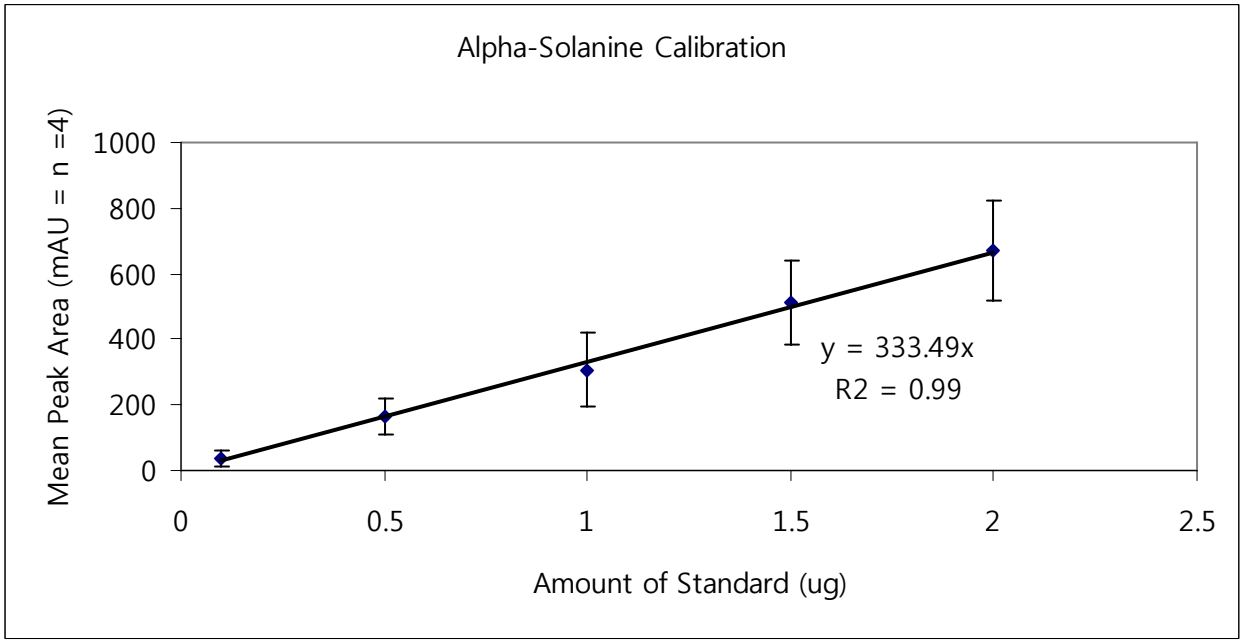


Figure 2-1: Peak area calibration with alpha-solanine and alpha-chaconine external standards.

2.2.5 SGA Identification: LC/MS

SGA preparations were fractionated on the analytical column described above with a binary linear gradient, consisting of 5mM ammonium acetate (Solvent A) and acetonitrile (Solvent B). The gradient consisted of 0.01 min, 30% solvent B; 0.01-2.00 min, 30% solvent B; 2.00-10.00 min, 30-60% solvent B; 10-15 min, 60% solvent B; 15-15.01 min, 60-30% solvent B; 15.01-17 min, 30% solvent B at flow rate of 0.5 mL/min at 50^{o}C. The eluent was directly analyzed by mass spectrometry using a Linear Ion Trap Quadrupole LC/MS/MS Spectrometer-3200 QTRAP INTRO (Applied Biosystems). The mass spectrometer was equipped with a turbo spray interface for electro spray ionization. Enhanced MS of positively charged ions in the positive mode was performed. The source was operated using a source temperature of 300^{o}C, an ion spray voltage of 5.5 kV, declustering potential of 30 V, and a collision cell entry potential of 18.5 V.

2.2.6 RNA extraction and cDNA synthesis

Tubers, stolons, roots, leaves, stems, flowers and floral buds were obtained at the anthesis stage of *S. chacoense* and treated with liquid nitrogen before storage at –80^{o}C until use. Total RNA was extracted from the tissues using TRIzol reagent (Carlsbad, CA) according to the provided instructions. RNA was quantified by spectrophotometry and assessed by formardehyde-denaturing agarose gels. First-strand cDNA was generated from 2 μg of total RNA using 200 U of M-MLV reverse transcriptase SuperScript II (Carlsbad, CA), using oligo $(dT)_{20}$ as a primer.

2.2.7 Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR):

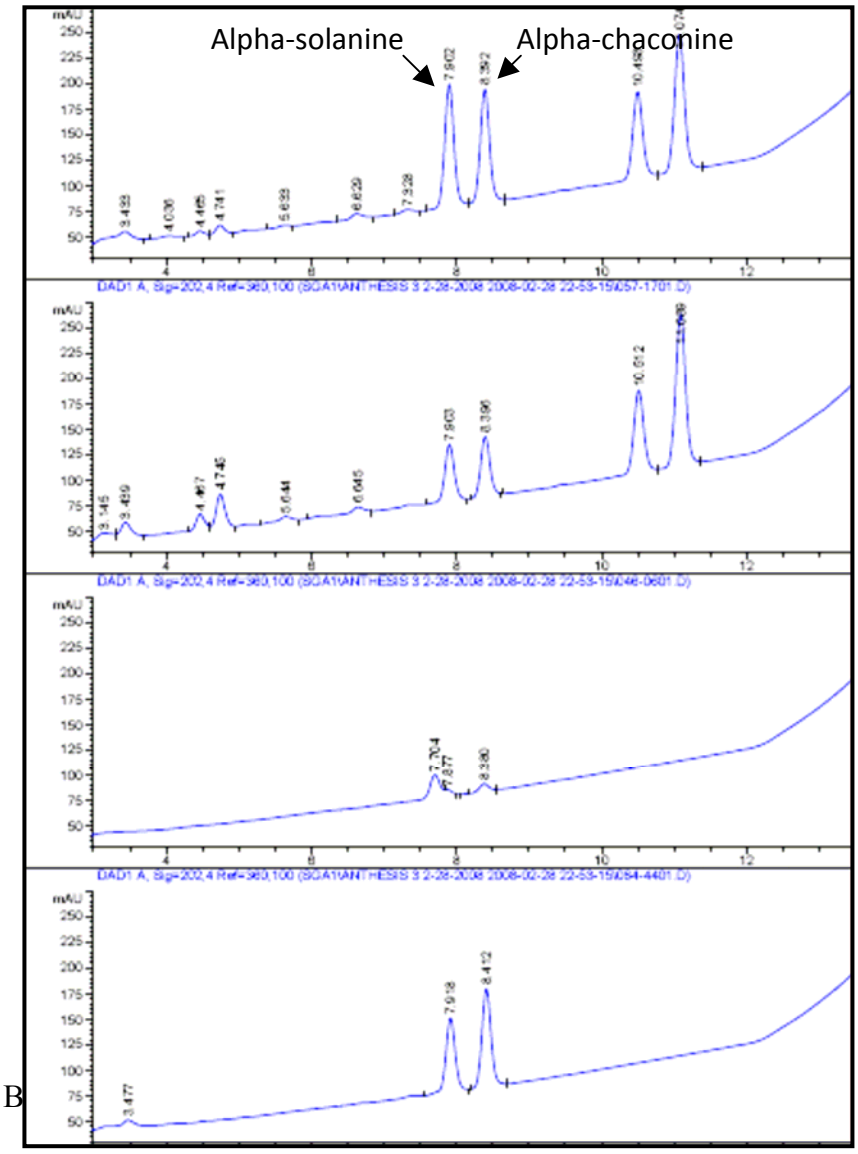
Differences in *squalene synthase, hmgr1, hmgr2, hmgr3, solanidine galactosyltransferase (sgt1),* and *solanidine glucosyltransferase (sgt2)* expression patterns in different tissues of *S. chacoense* at the anthesis stage were measured using semi-quantitative RT-PCR. Actin was used as an internal control for normalization. One microliter of diluted first-strand cDNA was used as template. Primers used to quantify gene transcript levels are listed in Table 2-2. The cycle conditions were an initial denaturation at 95 ^{o}C for 3 min, and 27 cycles at 94 ^{o}C for 30s, 58^{o}C for 30s 72^{o}C for 1 min, followed by a 5 min incubation at 72^{o}C. Shorter and longer cycles (25, 30, 35 and 40) were tested to ensure that the PCR had not plateaued at 27 cycles. The PCR products were fractionated by 1% agarose gel electrophoresis and the ethidium bromide-stained products visualized using a Kodak Gel logic 200 Imaging System (Kodak, USA).

2.3 Results

2.3.1 SGA identification

HPLC fractionation of the *S. chacoense* 80-1 tissue extracts revealed the presence of $A_{202\ nm}$ peaks that eluted at 7.9 and 8.4 minutes retention times (RT) with the α-solanine and α-chaconine standards, respectively. Additional peaks were detected in the extracts that contributed to three distinct $A_{202\ nm}$ chromatogram profiles (Figure 2-2). Extracts of aerial tissues including flower and leaf had six major peaks of which two had the same retention time as α-solanine and α-chaconine standards, and several minor peaks. Apart from α-solanine and α-chaconine, the major peaks in the aerial tissue extracts eluted at 4.4, 4.7, 10.6, and 11.0 minutes into the run, while the minor peaks at 5.8, 6.6, and 7.3 minutes. Extracts from roots had a simpler trace consisting of two major peaks, α-solanine and α-chaconine,

and a peak which was only partially resolved from α-solanine at 7.7 minutes RT. Tuber extracts contained only detectable levels of α-solanine and α-chaconine. In order to determine the types of SGAs in stolons, SGAs were extracted from branching, emergent shoots with chlorophyll, shoots with expanding leaves, and shoots with expanded leaves.



B

Figure 2-2 HPLC chromatograms of *S. chacoense* SGAs in flowers, leaves, roots, and tubers

Branching stolons accumulated only α-solanine and α-chaconine, while stolons at other stages of development accumulated additional compounds (Figure 2-3). To identify the additional peaks and to confirm the identity of the established peaks, root and aerial extracts were analyzed by LC/MS. MS confirmed the presence of α-solanine and α-chaconine.

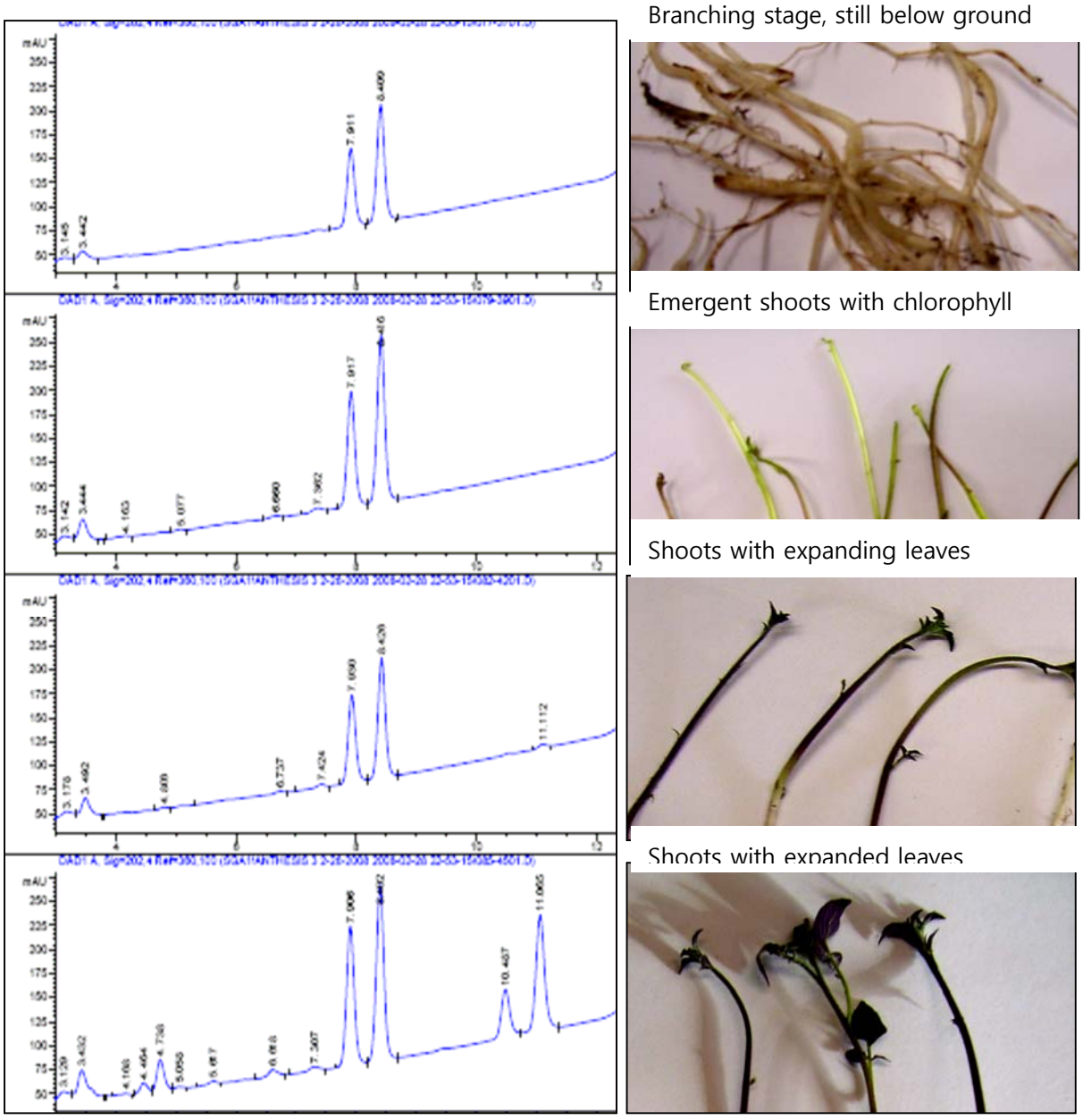


Figure 2-3 Variation of SGAs composition in developing stolons. Stolons at different developmental stages were harvested and tissues analyzed for SGA content. Types of SGAs accumulated were dependent upon stage of stolon development**.**

The MS profiles included molecular ions with m/z ratios of 852.5 and 868.4 as well as fragment ions representing the serial loss of 146/146/162 (chacotriose: glucose-rhamnose-rhamnose) and 146/162/162 (solatriose: galactose-glucose-rhamnose) amu, respectively (Table 2-3). The HPLC fractions of aerial extracts containing additional peaks generating parent ion m/z ratios of 910.4 and 926.4 with serial mass losses in the fragment ions of either 146/146/162 (chacotriose) and 146/162/162 (solatriose) amu (Table 2-3), respectively, and 868.4 and 884.4 with serial losses of 146/146/162 and 146/162/162, respectively. SGA-containing fractions from root extracts had molecular ion m/z ratios of 852.5, 868.4, and 1046.4 with mass loss series in the fragment ions of either 146/146/162 and 146/162/162 or 162/162/162/162 (commertetraose: galactose-glucose-glucose-glucose) amu (Table 2-3). The aglycones related to each of these compounds and the compound identifications are shown in Table 2-3.

The total ion current chromatogram in Figure 2-4 A shows that the root extract had two major peaks; the peak eluting at 9.48 min was that of the compound with m/z 1046 which we confirmed to be dehydrocommersonine by looking at published data (Distl and Wink, 2009). The peak eluting at 10.05 min was a combination of α-solanine and α-chaconine. The total ion current chromatogram of SGA extracts from aerial tissues, showed the presence of four, poorly resolved peaks eluting at 7.81, 7.98, 9.70 and 9.87 min. These retention times correspond to the elution of leptinines (7.81 and 7.98 min), leptines (9.7 min) and α-solanine and α-chaconine (9.87 min). In all these cases, the glycoalkaloid in a pair with the chacotriose carbohydrate moiety eluted later than the solatriose partner (Table 2-3). The

identities of the leptines and leptinines were deduced by comparing published data from Stobiecki *et al* (2003) and our data.

Table 2- 3 Fragmentation of SGAs found in *S. chacoense* plant extracts

Mass to charge ratio (m/z) of terminal ion	Pattern of fragmentation	Corresponding m/z losses	Mass to charge ratio of parent ion	Possible number of sugars	Tentative identification	Retention Time (min)
398*	398,560,706,852	162,146,146	852	3	Alpha-chaconine	10.2
398	398,560,722,868	162,162,146	868	3	Alpha-solanine	9.87
398	398,560,706,868	162,146,162	868	3	Alpha-solanine	9.87
398	398,560,706	162,146	706	2	Beta-chaconine	9.80
398	398,560,722	162,162	722	2	Beta-solanine	8.80
398	398,560,722,884,1046	162,162,162,162	1046	4	Dehydrocommersonine	9.48
456**	456,618,764,910	162,146,146	910	3	Leptine I	9.70
456	456,618,764,926	162,146,162	926	3	Leptine II	9.60
414***	414,576,722,868	162,146,146	868	3	Leptinine I	8.00
414	414,576,722,884	162,146,162	884	3	Leptinine II	7.80

* solanidine
** acetylleptinidine
*** leptinidine

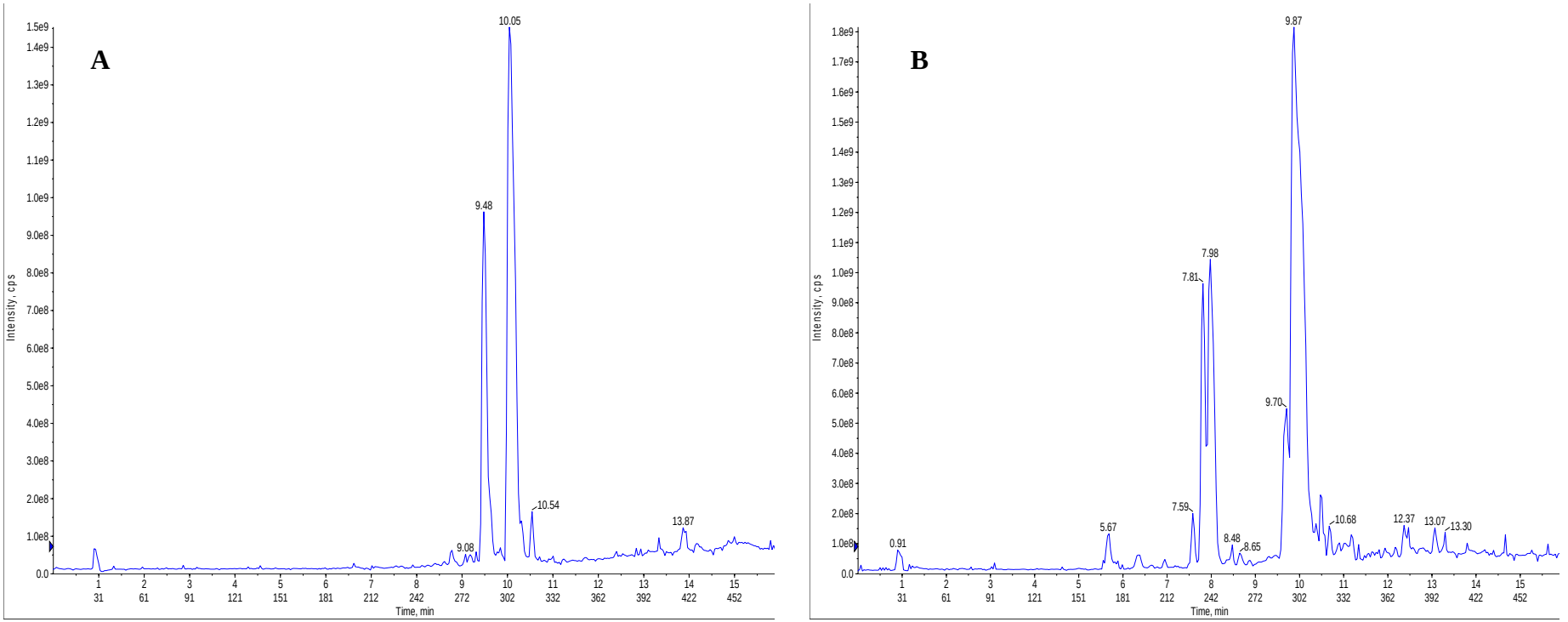


Figure 2-4 LC-MS chromatogram showing total ion current of SGAs present in roots (A) and aerial (B) extracts.

2.3.2 Variation in total SGA content

To better understand the relationship between plant organ and SGA content, SGAs were extracted from different organs, separated using HPLC, and quantified using peak areas and the α-chaconine conversion factor described in Figure 2-1 above. Total SGA levels varied with both tissue type and developmental stage. Regenerative tissues such as axiliary shoots (top / young expanding leaves), flowers and floral buds had the highest levels of 20 to 88, 49 and 63 μmole/g DW, respectively. The roots, stems and tubers showed the lowest amounts of SGAs at 1 to 8, 5 to 15 and 7 to 15 μmole/g DW, respectively (Table 2-4).

2.3.3 Roots

Roots accumulated the lowest total SGA levels at all the developmental stages, with a range of 1 to 8.25 μmole/g DW. There were significant differences ($\alpha=0.05$, $p<0.0015$) in total SGA accumulating at different developmental stages (Figure 2-5). The greatest accumulation of total SGAs was observed at anthesis, while the lowest was at the early and late vegetative stages. In general, there was no particular pattern of SGA accumulation with time. Total SGA levels were contributed by α-solanine, α-chaconine, and dehydrocommersonine.

2.3.4 Tubers and stolons

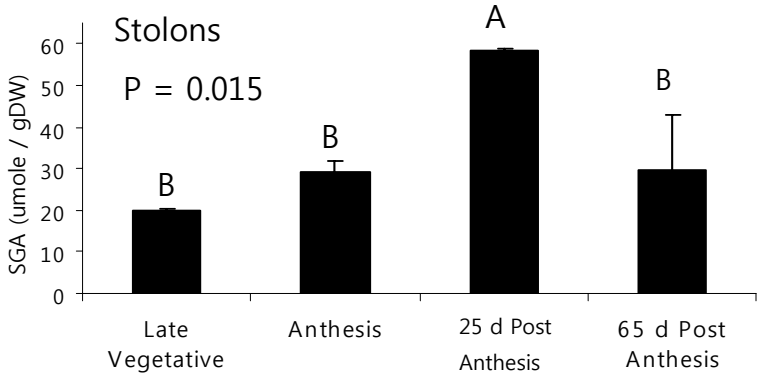
Tuberization (appearance of tubers) was first observed at the late vegetative stage and total SGA levels in tubers were monitored from that stage onwards.

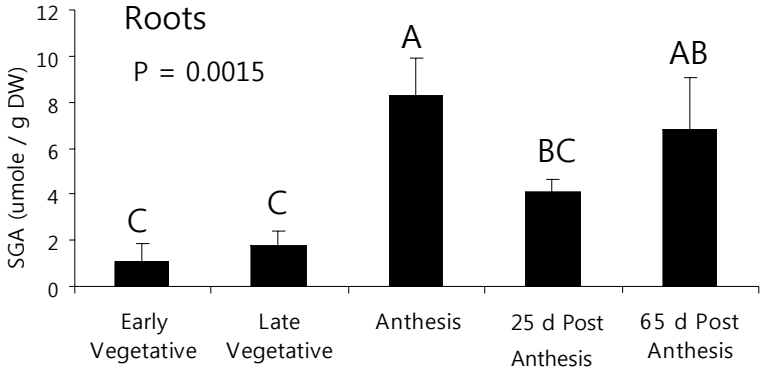
Table 2- 4 Total SGA concentrations in tissues of *S. chacoense* 80-1 during the vegetative, anthesis, and post-anthesis developmental stages. Means ± standard deviation, N=3. DAT: days after transplanting. Means followed by the same letter within a column are not significantly different.

	DAT	Total SGA (µmole/g DW)							
Stage		Roots	Tubers	Leaves			Stolons	Flowers	Floral buds
				Senescing	Expanded	Young			
Early Vegetative	30 (3)	1.1±0.8c	-	27.7±2.6b	-	24.5±2.8c	-	-	-
Mid Vegetative	44 (3)	1.0±0.6c	-	25.4±0.6b	-	25.5±0.4c	-	-	-
Late Vegetative	58 (1)	1.8±0.6c	6.9±0.0a	31.8±3.6ab	22.8±2.7c	33.6±2.0c	19.7±1b	-	-
Anthesis	71 (1)	8.3±1.7a	9.7±3.5a	21.7±2.3b	29.5±0.4c	45.8±2.1b	29.4±2.3b	49.3±2.1	62.7± 1.0
25 d post-anthesis	97 (1)	4.1±0.5bc	15.2±1.7a	30.2±1.5ab	49.3±2.5b	77.4±4.5a	58.5±0.5b	-	-
65 d post-anthesis	136 (1)	7±2.2ab	11.9±1.0a	44.2±10.3a	65.6±3.4a	87.9±5.6a	29.6±13.0b	-	-
P value		0.0015	0.41	0.068	0.0001	0.0001	0.015		

The results show that total SGA levels ranged from 6.95 to 15.16 μmole/g DW. Figure 2-5 shows a steady increase from the late vegetative stage to 14 d post anthesis and then a decline to 11.87 μmole /g DW at 65 d post anthesis. However, these changes between developmental stages were not significantly different at α=0.05 (P= 0.41). Compared to roots, tubers accumulated total SGA levels at all developmental stages sampled.

In contrast, it was observed that belowground stolons (rhizomes) accumulated three times more SGAs than tubers (Figure 2-5). The figure shows similar patterns of total SGA accumulation in tubers and stolons with plant development. Stolons and tubers both accumulated only α-solanine and α-chaconine; however, the proportions of α-chaconine to α-solanine tended to change with time. Alpha-chaconine accounted for 61 to 64% of the total SGAs in stolons and 59 to 67% in the tubers (Figure 2-7 A-B). These contributions translate into ratios of 1.56 to 1.8 and 1.4 to 2.0 of α-chaconine: α-solanine in stolons and tubers, respectively. There appeared to be an increase in the proportion of α-chaconine and a corresponding decrease in α-solanine as stolons and tubers developed from the late vegetative to 65-d post anthesis stages. However, these changes were statistically significant at α=0.05 (P = 0.74 and P = 0.21, for tubers and stolons, respectively).





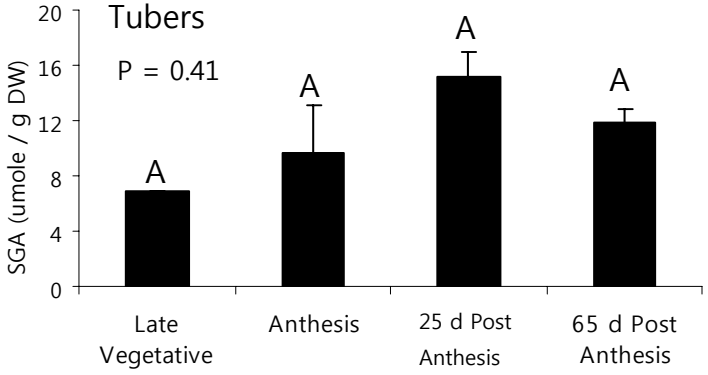
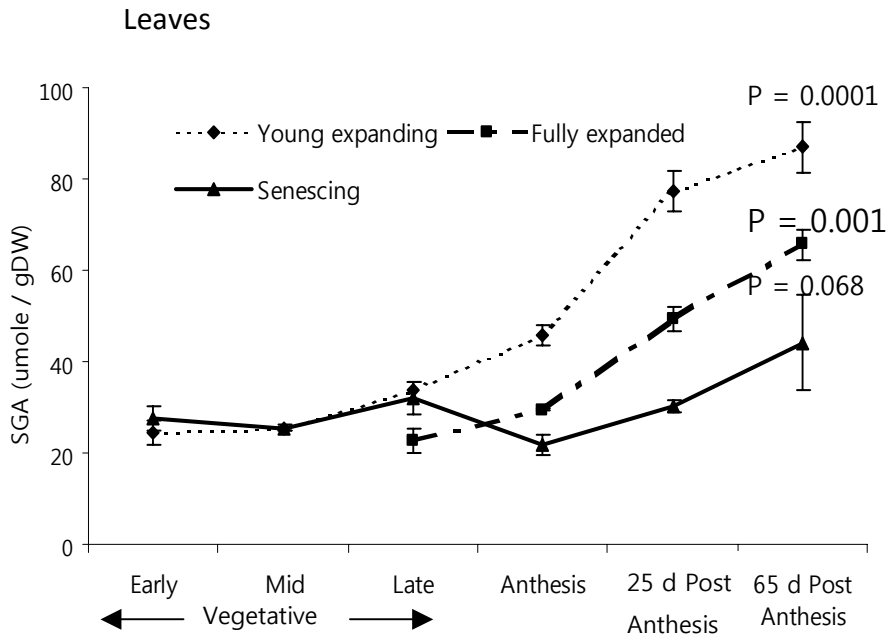


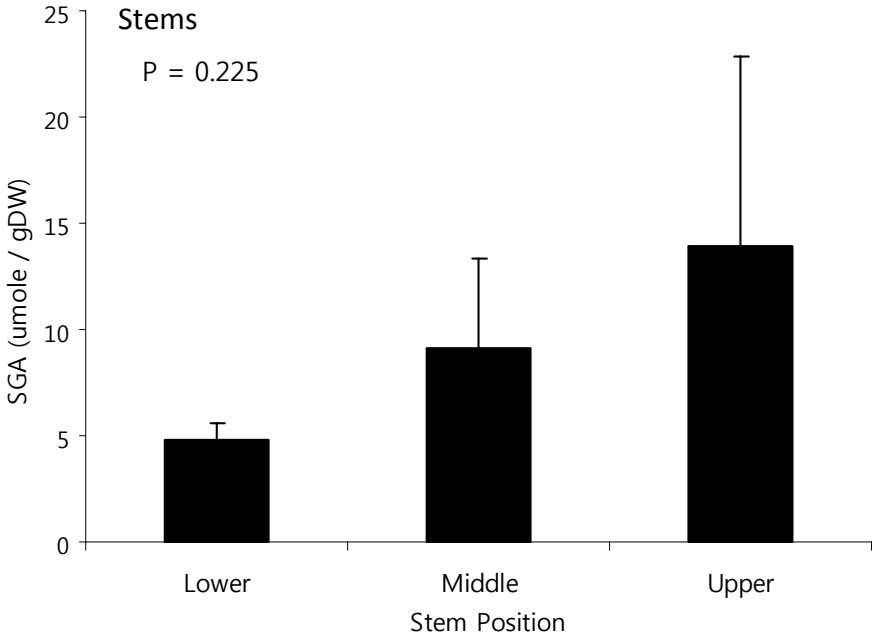
Figure 2-5 Changes in total SGA accumulation in belowground tissues of *S.chacoense* 80-1 during plant development. Plants were harvested at the early vegetative, mid vegetative, late vegetative, anthesis, 25 d post anthesis and 65 d post anthesis stages. SGAs were extracted from roots harvested at different stages of development and analyzed with HPLC. Each bar represents a mean (n = 3) and its standard deviation. Bars within each tissue with the same letter are not significantly different.

2.3.5 Leaves

At the early and mid vegetative stages, the size of the plant limited sampling to only the young expanding and bottom senescing leaves. Assignment of leaves to the different categories at harvest was dependent upon their position on the plant. At all developmental stages, only leaves on the main stem were harvested for SGA analysis. The young expanding leaves category also included the shoot meristems. The pattern of SGA accumulation in the leaves of *S. chacoense* was dependent not only upon the age of the leaves, but also on the stage of development of the entire plant. The results show that at these developmental stages, there was neither a difference in the levels of total SGAs accumulated in the young and senescing leaves, nor a difference between the two developmental stages (Figure 2-6). With plant development from the vegetative stage to anthesis, there was an accompanying increase in SGA levels in young expanding leaves (top) and fully expanded leaves (middle) but not in the senescing leaves. After anthesis, SGA levels continued to increase in all leaves until 65d post anthesis; levels increased by 240%, 300% and 140% in young, fully expanded and senescing leaves, respectively. The statistical analysis showed that there were no significant changes in total SGAs levels in the senescing leaves during plant development ($P = 0.068$), while the changes were significant in the young expanding and the fully expanding leaves at $\alpha = 0.05$ ($P = 0.0001$ and $P = 0.001$, respectively).

Alpha-solanine and alpha-chaconine together accounted for only 8 to 15%, 7 to 15%, and 8 to 45% of the total SGAs in young expanding, fully expanded, and old senescing leaves, respectively. The increase in the proportions of other SGAs with plant development was brought about by the quantitative increase in the leptine-type of SGAs (Figures 2- 7 C-E).





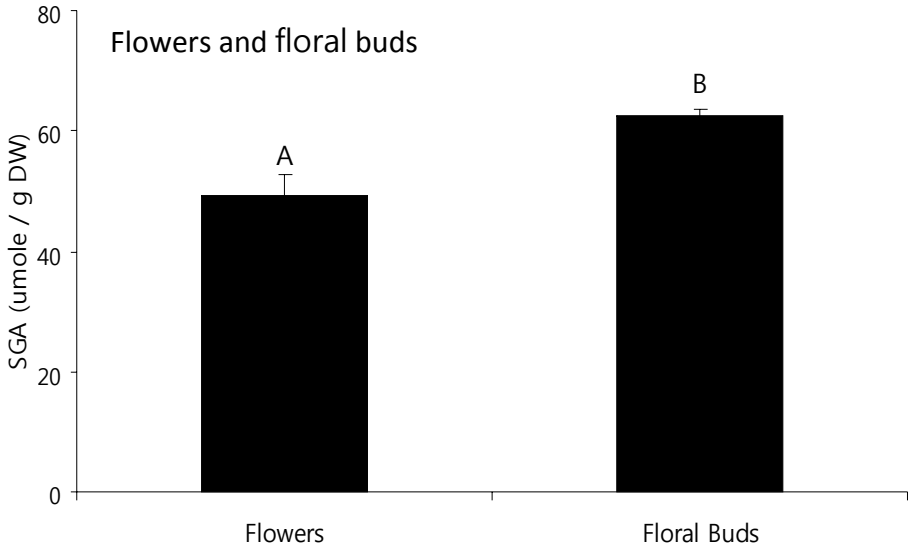


Figure 2-6 SGA accumulation in leaves, stems, flowers and floral buds of *S.chacoense* 80-1. Each bar represents a mean (n = 3) and its standard deviation.

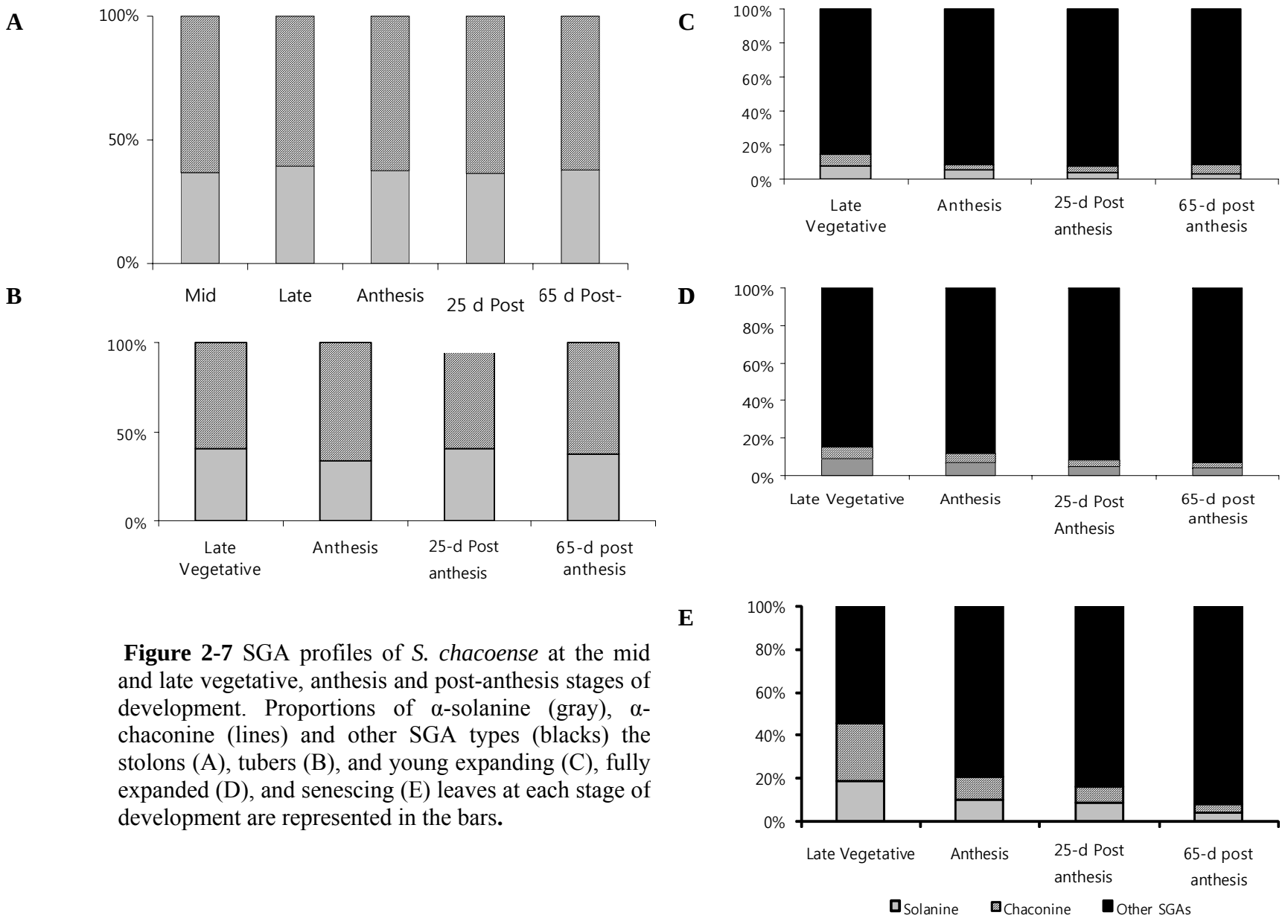


Figure 2-7 SGA profiles of *S. chacoense* at the mid and late vegetative, anthesis and post-anthesis stages of development. Proportions of α-solanine (gray), α-chaconine (lines) and other SGA types (blacks) the stolons (A), tubers (B), and young expanding (C), fully expanded (D), and senescing (E) leaves at each stage of development are represented in the bars**.**

The observed increases in the proportions of other SGA in all the leaf types were statistically significant at $\alpha=0.05$ with $p < 0.0001$ in all cases.

2.3.6 Stems

The analysis of SGAs in stems was slightly different from that of other organs; instead of following SGA accumulation in the stems over plant development, SGAs were analyzed from stem lower, middle and upper portions, representing the oldest, the older and the younger stem portions. In aboveground tissues, stems had the lowest total SGA levels. The results (Figure. 2-6) show that older basipetal portions of the stem had lower levels of SGA compared with younger portions. However this variation was not statistically significant at $\alpha = 0.05$ ($P = 0.225$).

2.3.7 Flowers and floral buds

Total SGA levels in flowers and floral buds were determined only at the anthesis stage, although buds and flowers both continued to appear at later stages of development from the side shoots. The results show high levels of SGAs, with 49.31 and 62.69 μmole/g DW for flowers and floral buds respectively (Figure 2-6).

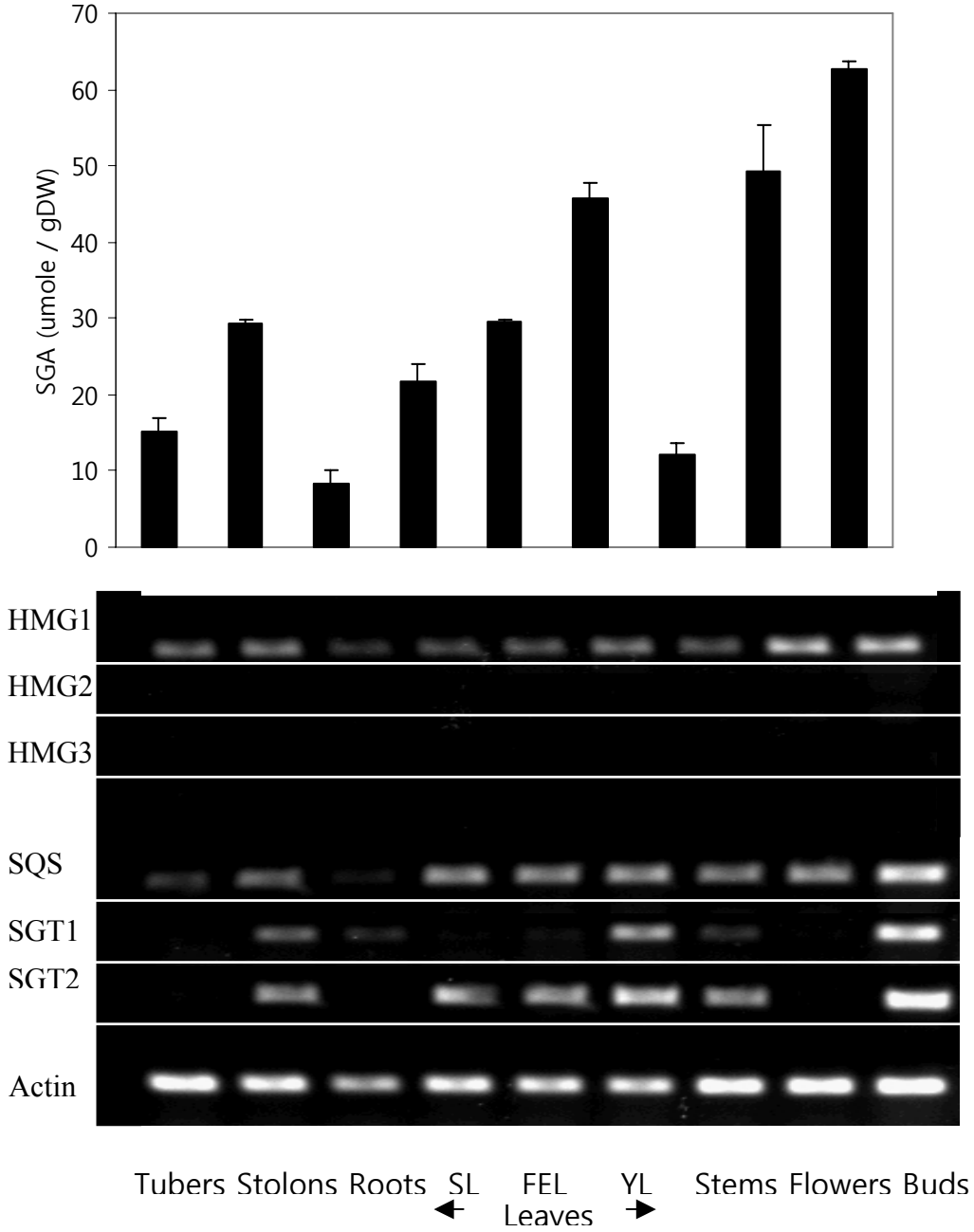


Figure 2-8 Transcript levels in various tissues of isoprenoid and SGA biosynthetic genes. A. SGA concentrations in different tissues of *S. chacoense* at anthesis. B. Isoprenoid (*hmgr* and *squalene synthase*) and SGA biosynthetic genes (*sgt1* and *sgt2*). Fragments were generated using cDNA libraries from tissues harvested at anthesis (27 PCR cycles). SL: senescing leaves; FEL: fully expanded leaves, and YL: young leaves.

2.3.8 Expression patterns of ScSQS

In order to determine the expression levels of *squalene synthase, hmgr, sgt1 and sgt2* in tissues with differential total SGA levels and types, semi-quantitative PCR (27 cycles) was performed. The results show that the expression levels of isoprenoid and SGA biosynthesis genes, and SGA were somewhat correlated with SGA levels (Figure 2-7). In floral buds, roots and tubers, SGA levels correlated well with *squalene synthase, sgt1, sgt2* and *hmg1* expression levels. Low expression levels of *sgt1* and *sgt2* were observed in the flowers of *S. chacoense* but were more detectable at higher PCR cycles such as 35 and 40 (data not shown).

2.4 Discussion

Glycoalkaloids are synthesized in all organs of the potato plant (Birch et al., 2002), and the organs that are important for survival and multiplication like flowers, unripe berries, young leaves, sprouts, and the area around potato eyes, tend to have more than twice the amounts of SGAs than other organs (Lawson *et al.*, 1993, Chen and Miller, 2000, Lachman *et al.*, 2001). The results from this study show that the highest concentrations of SGAs were concentrated in the youngest and reproductive plant organs. Flowers, floral buds and young leaves had the highest SGA levels, while roots, tubers and stems had the lowest levels. The young expanding leaves had the highest levels, ranging from 20 to 88 μmole/gDW. Fully expanding leaves accumulated 20 to 64 μmole/gDW, and senescing leaves had

amounts 20 to 40 µmole/gDW. Floral buds and flowers accumulated 63 and 49 µmole/gDW, respectively. The roots, stems and tubers accumulated the lowest total levels 1 to 8, 5 to 15, 7 to 16 µmole/gDW. This pattern of accumulation in *S. chacoense* is in agreement with already established patterns of SGA accumulation within the *S. tuberosum* plant (Chen and Miller, 2000, Valkonen *et al.*, 1996, Lawson *et al.*, 1993). Developmental time course analysis of SGA accumulation over the 10 growth and development stages in *S. tuberosum* also showed that leaves of young plants had significantly higher contents compared with older leaves (Kolbe and Stephan-Beckmann, 1997). In general, concentration has been shown to be higher in younger tissues or in tissues that have higher metabolic activities; and this could be attributed to the fact that chemical defense probably varies with maturity and that younger tissues are both valuable and vulnerable organs (McKey, 1974). According to the optimal defense theory, the distribution of defense is a function of relative fitness values of plant parts and their probability of being attacked. Work by Ohnmeiss and Baldwin (2000) has clearly demonstrated that younger tissues (leaves) of *Nicotiana sylvestris* have higher fitness value to the plant and therefore tend to be better defended by greater accumulation of nicotine compared with older leaves.

A comparison of SGA composition of different organs showed that aerial tissues had the highest diversity of individual compounds. Therefore, floral buds, flowers and leaves did not only have high amounts of total SGAs, but had accumulated α-solanine and α-chaconine common to all tissues and also the leptine-type SGAs. In foliage, the leptine-type (other) SGAs tended to dominate with percent

contributions as high as 92%. Earlier studies with the same *S. chacoense* accession, PI 458310 (Sanford et al., 1996), also showed that leaf SGAs were dominated by leptines, accounting for 90% of the total. This could reflect the need to maximize the defense potential of these organs by the accumulation of greater proportion of the more toxic SGA types. Several studies have reported that leptine-type SGAs *in S. chacoense* leaves are a resistance factor against CPB (Sinden *et al.*, 1980, Sinden *et al.*, 1986, Deahl *et al.*, 1991, Sanford *et al.*, 1997, Kowalski *et al.*, 1999). Additional studies indicate that while leptine-type SGAs may contribute to this resistance, they are not necessarily essential for the expression of resistance and that other SGAs such as commersonine and demissine maybe equally important for CPB resistance (Sinden et al., 1980). It has been suggested that leptine I is the most effective feeding deterrent against CPB followed by tomatine and demissine, and then solanine and chaconine (Tingey, 1984). The tubers and stolons accumulated only α-solanine and α-chaconine at all the developmental stages analyzed. The proportion of α-chaconine was 61 to 64% and 59 to 67% in tubers and stolons, respectively. In all cases both stolons and tubers accumulated more α-chaconine with ratios ranging from 1.56 to 1.8 and 1.4 to 2.0, respectively. However, lower α-chaconine: α-solanine ratios (0.78, 1.2) have been reported in the tubers of other *S. chacoense* genotypes (Sanford et al., 1995). In *S. tuberosum,* Osman (1979) and Maga (1980) reported a 60% proportion of α-chaconine while Jadhav et al. (1981) have reported ranges of α-chaconine proportions from 50 to 87%. In *S. juzepcukii*, tubers have been reported to accumulate as low as 30% α-chaconine (Osman, 1979). Several studies have reported synergism between chacotriose and

solatriose SGAs in their action against fungi (Fewell and Roddick, 1993) and snails (Smith et al., 2001), and in membrane disruption (Friedman *et al.*, 1991, Fewell *et al.*, 1994, Roddick and Rijnenberg, 1987, Roddick *et al.*, 1992), but when applied separately, chacotriose based SGAs tend to have more toxic effects (Blankemeyer *et al.*, 1998, Smith *et al.*, 1996). This suggests that the proportions of different types of SGAs are important for the overall toxicity effects. Experiments with different ratios of α-chaconine: α-solanine have shown that at some concentrations, the two compounds will synergise, while at others they will be additive (Rayburn et al., 1995). Such possibilities make it difficult to predict the toxicity of a mixture of SGAs. Therefore, the changes in the proportions of different SGAs with plant development might have some significance for plant defense.

As part of our objective to determine the qualitative differences in the spectra of SGAs of different tissues during critical developmental stages, we monitored SGA profiles in developing stolons. The results in Figure 2-3 show that there is a gradual shift in the profile of SGAs as the stolons develop from the branching stage to become shoots with expanded leaves. Our results show that branching stolons produced only α-solanine and α-chaconine; while stolons that had transitioned into leafy shoots acquired the capability to accumulate leptine-type SGAs in addition. Our results suggest that the ability to accumulate leptine-type SGAs was associated with the development of aerial shoots (stems and foliage) SGAs.

The results from this study also showed the presence of dehydrocommersonine in the roots; this SGA had first been reported in the root cell cultures from *S. chacoense* PI 320281 and in the stems, leaves and tubers of *S. spegazzinii* (Zacharius and Osman, 1977). It has since then been reported in the aerial parts of *S. commersonii* (Vazquez et al., 1997) and most recently in the leaves of *S. tuberosum* ssp. *andigena* (Distl and Wink, 2009). Belowground tissues namely, stolons and tubers, and the aerial tissues did not accumulate dehydrocommersonine but it was uniquely accumulated in the roots. These results are consistent with the findings of Zacharius and Osman (1977), who reported dehydrocommersonine only in the roots, and not in the tubers from where the roots were initiated. In addition to the findings reported by Zacharius and Osman (1977), our results show that *S. chacoense* 80-1 roots are capable of accumulating α-solanine and α-chaconine in addition to dehydrocommersonine. Both these findings suggest that roots are capable of synthesizing SGAs not found in other parts of the plant. Dehydrocommersonine and commersonine have been reported to be important for resistance to CPB. The *S. chacoense* genotypes that accumulated these types of SGAs were more resistant than those accumulating only solanine and chaconine (Sinden et al., 1980). Since dehydrocommersonine differs from solanine and chaconine only in the size and composition of the sugar group, it is possible that the number of sugar groups is as important or even more important than the aglycone present for defense. The presence of these compounds in the roots of *S. chacoense* raises the question of whether there is a particular requirement for enhanced defense against particular soil pests.

Levels of SGAs generally increased in all tissues during plant development and in some cases decreased after anthesis. In foliage the largest increase was observed between anthesis and 65-d post anthesis (Figure 2-5). During these last three stages, the youngest leaves had the highest SGA levels. Our observation of the increase in SGA levels with plant development were rather unexpected; developmental time course analysis in *S. tuberosum* suggested a decrease in the levels of only α-solanine and α-chaconine (Kolbe and Stephan-Beckmann, 1997). However, our study also shows a reduction in the levels of only α-solanine and α-chaconine in the leaves, but an increase in the levels of leptine-type SGAs. The continual increase in the levels of SGAs could be attributed to the fact that while the cultivated *S. tuberosum* grows as an annual plant, *S. chacoense* continues to grow as a perennial herb. This suggests that *S. chacoense* plants continue to increase defense in the leaves during development.

The expression levels of isoprenoid genes and SGAs were somewhat correlated with SGA levels (Figure 2-8). In floral buds, roots and tubers, SGA levels correlated well with *squalene synthase, sgt1, sgt2* and *hmg1* expression levels. These results agree with those reported in *S. tuberosum* where it has been shown that genotypes exhibiting high levels of SGAs in the leaves and tubers, also had high transcript levels of *hmg1* and *squalene synthase* (Krits et al., 2007). Moehs et al. (1997) reported that steady state transcript levels of both *hmg1* and *sgt2 in* meristems and developing leaves of S. *tuberosum* showed highest mRNA levels in meristematic tissue and decreased with leaf maturation. Our results also show that in all cases (except for flowers where they were both undetectable), the *sgt2*

transcript levels were higher than those of *sgt1*. Similar results were reported by Krits *et al*. (2007) who suggested a good correlation between the documented ratios of α-chaconine: α-solanine and the transcript ratio of *sgt2*: *sgt1*. The ratios of α-chaconine: α-solanine, and in particular the higher levels of α-chaconine in comparison to α-solanine (60:40) have been attributed to differential levels of activities of the enzymes SGT1 and SGT2. Crude extracts of potato sprouts were shown to have low SGT1 activity compared to SGT2 (Stapleton et al., 1991). SGT2 has also been demonstrated to have the extra capability to act as galactosyltransferase *in vitro* (Zimowski, 1991). However, SGT1 has also been shown to have glucosyltransferase activity *in vitro* but has a preference for UDP-galactose as the sugar donor *in planta* (McCue et al., 2005). Based our results and those of Krits et al. (2007), gene transcription might be another level at which the regulation of accumulation occurs. The *in vivo* assays using an antisense transgene of potato *sgt1* have shown that α-solanine levels can be appreciably reduced due to reduced levels of *sgt1* transcripts (McCue et al., 2005).

2.5 Conclusions

Solanum chacoense 80-1 accumulates qualitatively and quantitatively different SGAs in different tissues. SGA levels vary with type of plant organ and developmental stage, and there is a possible existence of independent pathways responsible for the biosynthesis of SGAs in different organs because each organ is capable of producing different amounts and types. Although tubers and stolons accumulate only alpha-solanine and alpha-chaconine, other parts of the plant also accumulate other types of SGAs. Roots accumulate dehydrocommersonine and aerial tissues accumulate leptine-type SGAs. Roots accumulated the least amounts

of SGAs while young leaves, flowers and floral buds accumulated the greatest. In aerial parts that accumulated leptine-type SGAs, alpha-solanine and alpha-chaconine contributed less than 30% of the total SGAs on average. Plant organs that showed the highest biosynthetic activity for SGA production also had high levels of transcripts of isoprenoid genes.

2.6 References:

Arnqvist L, Dutta PC, Jonsson L, Sitbon F (2003) Reduction of cholesterol and glycoalkaloid levels in transgenic potato plants by overexpression of a type 1 sterol methyltransferase cDNA. Plant Physiology **131:** 1792-1799

Bennett RN, Wallsgrove RM (1994) Secondary metabolites in plant defense-mechanisms. New Phytologist **127:** 617-633

Birch ANE, Geoghegan IE, Griffiths DW, McNicol JW (2002) The effect of genetic transformations for pest resistance on foliar solanidine-based glycoalkaloids of potato (*Solanum tuberosum*). Annals of Applied Biology **140:** 143-149

Blankemeyer JT, McWilliams ML, Rayburn JR, Weissenberg M, Friedman M (1998) Developmental toxicology of solamargine and solasonine glycoalkaloids in frog embryos. Food and Chemical Toxicology **36:** 383-389

Brown MS, McDonald GM, Friedman M (1999) Sampling leaves of young potato (*Solanum tuberosum*) plants for glycoalkaloid analysis. Journal of Agricultural and Food Chemistry **47:** 2331-2334

Chappell J (1995) The biochemistry and molecular-biology of isoprenoid metabolism. Plant Physiology **107:** 1-6

Chen Z, Miller AR (2000) Steroidal alkaloids in Solanaceous vegetable crops. Horticultural Reviews **25:** 171-196

Croteau R, Kutchan TM, Lewis GN, eds (2000) Natural products (secondary metabolites). American Society of Plant Physiologists, Rockville, MD

Deahl KL, Cantelo WW, Sinden SL, Sanford LL (1991) The effect of light intensity on Colorado potato beetle resistance and foliar glycoalkaloid concentration of four *Solanum chacoense* clones. American Potato Journal **68:** 659-666

Deahl KL, Sinden SL, Young RJ (1993) Evaluation of wild tuber-bearing *Solanum* accessions for foliar glycoalkaloids level and composition. American Potato Journal **70:** 61-69

Devarenne TP, Ghosh A, Chappell J (2002) Regulation of squalene synthase, a key enzyme of sterol biosynthesis, in tobacco. Plant Physiology **129:** 1095-1106

Devarenne TP, Shin DH, Back K, Yin SH, Chappell J (1998) Molecular characterization of tobacco squalene synthase and regulation in response to fungal elicitor. Archives of Biochemistry and Biophysics **349:** 205-215

Distl M, Wink M (2009) Identification and quantification of steroidal alkaloids from wild tuber-bearing *Solanum* species by HPLC and LC-ESI-MS. Potato Research **52:** 79-104

Fewell AM, Roddick JG (1993) Interactive antifungal activity of the glycoalkaloids alpha-solanine and alpha-chaconine. Phytochemistry **33:** 323-328

Fewell AM, Roddick JG (1997) Potato glycoalkaloid impairment of fungal development. Mycological Research **101:** 597-603

Fewell AM, Roddick JG, Weissenberg M (1994) Interactions between the glycoalkaloids solasonine and solamargine in relation to inhibition of fungal growth. Phytochemistry **37:** 1007-1011

Friedman M, Dao L (1992) Distribution of glycoalkaloids in potato plants and commercial potato products. Journal of Agricultural and Food Chemistry **40:** 419-423

Friedman M, McDonald GM (1997) Potato glycoalkaloids: chemistry, analysis, safety, and plant physiology. Critical Reviews in Plant Sciences **16:** 55-132

Friedman M, Rayburn JR, Bantle JA (1991) Developmental toxicology of potato alkaloids in the frog embryo teratogenesis assay *Xenopus* (FETAX). Food and Chemical Toxicology **29:** 537-547

Ginzberg I, Tokuhisa JG, Veilleux RE (2009) Potato steroidal glycoalkaloids: Biosynthesis and genetic manipulation. Potato Research **52:** 1-15

Kaneko K, Tanaka MW, Mitsuhashi H (1977) Dormantinol, a possible precursor in solanidine biosynthesis, from budding *Veratrum grandiflorum*. Phytochemistry **16:** 1247-1251

Kolbe H, StephanBeckmann S (1997) Development, growth and chemical composition of the potato crop (*Solanum tuberosum* L.) .1. Leaf and stem. Potato Research **40:** 111-129

Kowalski SP, Domek JM, Deahl KL, Sanford LL (1999) Performance of Colorado potato beetle larvae, *Leptinotarsa decemlineata* (Say), reared on synthetic diets supplemented with Solanum glycoalkaloids. American Journal of Potato Research **76:** 305-312

Krits P, Fogelman E, Ginzberg I (2007) Potato steroidal glycoalkaloid levels and the expression of key isoprenoid metabolic genes. Planta **227:** 143-150

Lachman J, Hamouz K, Orsak M, Pivec V (2001) Potato glycoalkaloids and their significance in plant protection and human nutrition - Review. Series Rostlinna **47:** 181-191

Lawson RD, Veilleux RE, Miller AR (1993) Biochemistry and genetics of *Solanum chacoense* steroidal alkaloids: Natural resistance factors to the Colorado potato beetle. Current Topics in Bot. Research **1:** 335-352

Maga JA (1994) Glycoalkaloids in Solanaceae. Food Reviews International **10:** 385-418

McCue KF, Allen PV, Rockhold D, Corsini DL, Shepherd LVT, Davies H, Moehs CP, Joyce P (2002) Reduction of total steroidal glycoalkaloids in potato tubers using antisense constructs of a gene encoding a solanidine glucosyl transferase. *In* RY Yada, ed, 26th International Horticultural Congress, Toronto, Canada, pp 77-86

McCue KF, Allen PV, Shepherd LVT, Blake A, Maccree MM, Rockhold DR, Novy RG, Stewart D, Davies HV, Belknap WR (2007) Potato glycosterol rhamnosyltransferase, the terminal step in triose side-chain biosynthesis. Phytochemistry **68:** 327-334

McCue KF, Allen PV, Shepherd LVT, Blake A, Rockhold DR, Novy RG, Stewart D, Davies HV, Belknap WR (2006) Manipulation and compensation of steroidal glycoalkaloid biosynthesis in potatoes. *In* DMBLGJ Spooner, ed, 6th International Solanaceae Conference, Madison, WI, pp 343-349

McCue KF, Allen PV, Shepherd LVT, Blake A, Whitworth J, Maccree MM, Rockhold DR, Stewart D, Davies HV, Belknap WR (2006) The primary in vivo steroidal alkaloid glucosyltransferase from potato. Phytochemistry **67:** 1590-1597

McCue KF, Shepherd LVT, Allen PV, Maccree MM, Rockhold DR, Corsini DL, Davies HV, Belknap WR (2005) Metabolic compensation of steroidal glycoalkaloid biosynthesis in transgenic potato tubers: using reverse genetics to confirm the in vivo enzyme function of a steroidal alkaloid galactosyltransferase. Plant Science **168:** 267-273

McKey D (1974) Adaptive patterns in alkaloid physiology. The American Naturalist **108:** 305-320

Moehs CP, Allen PV, Friedman M, Belknap WR (1997) Cloning and expression of solanidine UDP-glucose glucosyltransferase from potato. Plant Journal **11:** 227-236

Mweetwa AM, Poe R, Tokuhisa JG (2008) Biosynthesis and regulation of steroidal glycoalkaloids in wild potato, *Solanum chacoense* bitter. *In* 105th Annual Conference of the American-Society-for-Horticultural-Science, Orlando, FL, pp 1287-1287

Pelletier Y, Grondin G, Maltais P (1999) Mechanism of resistance to the Colorado potato beetle in wild *Solanum* species. Journal of Economic Entomology **92:** 708-713

Piironen V, Lindsay DG, Miettinen TA, Toivo J, Lampi AM (2000) Plant sterols: biosynthesis, biological function and their importance to human nutrition. Journal of the Science of Food and Agriculture **80:** 939-966

Rayburn JR, Friedman M, Bantle JA (1995) Synergistic interaction of glycoalkaloids alpha-chaconine and alpha-solanine on developmental toxicity in *Xenopus* embryos. Food and Chemical Toxicology **33:** 1013-1019

Roddick JG, Rijnenberg AL (1987) Synergistic interaction between the potato glycoalkaloids alpha-solanine and alpha-chaconine in relation to lysis of phospholipid sterol liposomes. Phytochemistry **26:** 1325-1328

Roddick JG, Rijnenberg AL, Weissenberg M (1992) Alterations to the permeability of liposome membranes by the solasodine-based glycoalkaloids solasonine and solamargine. Phytochemistry **31:** 1951-1954

Roddick JG, Weissenberg M, Leonard AL (2001) Membrane disruption and enzyme inhibition by naturally-occurring and modified chacotriose-containing *Solanum* steroidal glycoalkaloids. Phytochemistry **56:** 603-610

Ronning CM, Stommel JR, Kowalski SP, Sanford LL, Kobayashi RS, Pineada O (1999) Identification of molecular markers associated with leptine production in a population of *Solanum chacoense* Bitter. Theoretical and Applied Genetics **98:** 39-46

Sanford LL, Deahl KL, Sinden SL, Kobayashi RS (1995) Glycoalkaloid content in tubers of hybrid and backcross populations from a *Solanum tubersoum* X S. *chacoense* cross. American Potato Journal **72:** 261-271

Sanford LL, Deahl KL, Sinden SL, Kobayashi RS (1996) Segregation of leptines and other glycoalkaloids in *Solanum tuberosum* (4X) X S. *chacoense* (4X) crosses. American Potato Journal **73:** 21-33

Sanford LL, Kobayashi RS, Deahl KL, Sinden SL (1997) Diploid and tetraploid *Solanum chacoense* genotypes that synthesize leptine glycoalkaloids and deter feeding by Colorado potato beetle. American Potato Journal **74:** 15-21

Schaller H (2004) New aspects of sterol biosynthesis in growth and development of higher plants. Plant Physiology and Biochemistry **42:** 465-476

Sinden SL, Sanford LL, Cantelo WW, Deahl KL (1986) Leptine glycoalkaloids and resistance to the colorado potato beetle (coleoptera, chrysomelidae) in *Solanum chacoense*. Environmental Entomology **15:** 1057-1062

Sinden SL, Sanford LL, Osman SF (1980) Glycoalkaloids and resistance to the Colorado potato beetle in *Solanum chacoense* Bitter. American Potato Journal **57:** 331-343

Sinden SL, Sanford LL, Webb RE (1984) Genetic and environmental control of potato glycoalkaloids. American Potato Journal **61:** 141-156

Smith DB, Roddick JG, Jones JL (1996) Potato glycoalkaloids: Some unanswered questions. Trends in Food Science & Technology **7:** 126-131

Smith DB, Roddick JG, Jones JL (2001) Synergism between the potato glycoalkaloids alpha-chaconine and alpha-solanine in inhibition of snail feeding. Phytochemistry **57:** 229-234

Smith SW, Giesbrecht E, Thompson M, Nelson LS, Hoffman RS (2008) Solanaceous steroidal glycoalkaloids and poisoning by *Solanum torvum*, the normally edible susumber berry Toxicon **52:**667-676.

Stapleton A, Allen PV, Friedman M, Belknap WR (1991) purification and characterization of solanidine glucosyltransferase from the potato (*Solanum-tuberosum*). Journal of Agricultural and Food Chemistry **39:** 1187-1193

Stobiecki M, Matysiak-Kata W, Franski R, Skala J, Szopa J (2003) Monitoring changes in anthocyanin and steroid alkaloid glycoside content in lines of transgenic potato plants using liquid chromatography/mass spectrometry. Phytochemistry **62:** 959-969

Tingey WM (1984) Glycoalkaloids as pest resistance factors. American Potato Journal **61:** 157-167

Tingey WM, Mackenzie JD, Peter G (1978) Total foliar glycoalkaloids and resistance of wild potato species to *Empoasca fabae* (Harris) American Journal of Potato Research **55:** 577-585

Valkonen JPT, Keskitalo M, Vasara T, Pietila L (1996) Potato glycoalkaloids: A burden or a blessing? Critical Reviews in Plant Sciences **15:** 1-20

Vazquez A, Gonzalez G, Ferreira F, Moyna P, Kenne L (1997) Glycoalkaloids of *Solanum commersonii* Dun ex Poir. Euphytica **95:** 195-201

Zacharius RM, Osman SF (1977) Glycoalkaloids in tissue-culture of solanum species - dehydrocommersonine from cultured roots of *Solanum chacoense*. Plant Science Letters **10:** 283-287

Zimowski I (1991) Occurrence of a glucosyltransferase specific for solanidine in potato plants. Phytochemistry **30:** 1827-1831

3 Cloning and Characterization of a squalene synthase gene from *Solanum chacoense*

Abstract

Studies on sterol biosynthesis have implicated squalene synthase as a key regulatory enzyme because it catalyzes an irreversible step into the sterol biosynthetic pathway from the mevalonic acid pathway. Squalene synthase is represented in the yeast and human genomes as a single gene. In plants, squalene synthase appears to be encoded by one or two distinct genes. A study was undertaken to clone and characterize the *Solanum chacoense* gene coding for *squalene synthase* (*ScSQS1*) A cDNA was cloned that predicted a polypeptide of 411 amino acids with high similarity of 68-100% (at the amino acid level) to known plant *squalene synthase* genes and contains six deduced peptide domains observed in other species. The *ScSQS1* cDNA is represented by a variable 3' untranslated region. The genomic structure has 13 exons and 12 introns. The Southern blot analysis indicated a single-copy gene.

3.1 Introduction

Sterols are important structural and signaling molecules of eukaryotic organisms; they serve as membrane lipid components, precursors for additional metabolites such as mammalian steroid hormones, insect ecdysteroids, plant brassinosteroid hormones (Bakrim *et al.*, 2008, Olkkonen and Hynynen, 2009), and other steroid derivatives such as steroid alkaloids (Heftmann, 1983). The biosynthesis of sterols begins in the mevalonic acid pathway, a branch of the isoprenoid metabolism, which also produces a diversity of secondary metabolites (Schaller, 2004). The biosynthesis of sterols can be divided into two parts: the first part involves the formation of C_5 units and their subsequent condensation. During this part, three molecules of acetyl-coenzyme A (C_2) combine to yield 3-hydroxy-3-methylglutaryl CoA (HMG-CoA). HMG-CoA is then reduced to mevalonic acid (MVA) (C_6) by the action of HMG-CoA reductase (HMGR). After this reduction mevalonate kinase and mevalonate 5-phosphate kinase phosphorylate MVA to form mevalonate 5-pyrophosphate, which then, after decarboxylation, yields isopentenyl diphosphate (IPP) and following isomerization, dimethylallyl diphosphate (DMAPP) (Newman and Chappell, 1999). One DMAPP molecule and two of IPP molecules are used to form farnesyl diphosphate (FPP) (C_{15}). FPP is the direct substrate for sesquiterpene formation and through the activity of squalene synthase, triterpene biosynthesis (Piironen *et al.*, 2000). Squalene is an immediate precursor for the cyclic triterpenes, which lead to steroid and steroidal glycoalkaloid formation (Yoshioka *et al.*, 1999).

The second part of sterol biosynthesis involves the conversion of squalene into squalene 2, 3-epoxide in a reaction catalyzed by squalene epoxidase. The cyclization of squalene epoxide results in the formation of the triterpenoid, cycloartenol, which is the most rudimentary sterol (Ginzberg et al., 2009). Cycloartenol is a precursor of the higher plant steroids (Benveniste, 2004). The path from cycloartenol can either lead to the formation of 24-methylenecycloartenol or cholesterol, campesterol, and sitosterol (Schaller, 2004). Both parts of sterol biosynthesis are considered to be primary metabolism; the conversion of these sterols, for example, cholesterol into steroid alkaloids, is secondary metabolism.

Squalene synthase catalyzes the first enzymatic step committing carbon away from the central isoprenoid pathway into sterol biosynthesis (Devarenne et al., 2002). Due to the positioning of squalene synthase in the sterol biosynthetic pathway, it has been suggested to have a potential regulatory role (Devarenne *et al.*, 1998, Threfall and Whitehead, 1988, Vogeli and Chappell, 1988). However, the actual regulatory role of squalene synthase remains elusive.

Squalene synthases have been characterized in *Arabidopsis thaliana* (Kribii et al., 1997), *Oryza sativa* (Hata et al., 1997), *Nicotiana tabacum* (Devarenne et al., 1998), *Solanum tuberosum* (Yoshioka et al., 1999), *Lotus japonicus* (Akamine et al., 2003), *Panax ginseng* (Lee et al., 2004), *Centella asiatica* (Kim et al., 2005), *Taxus cuspidata* (Huang et al., 2007), *Euphorbia tirucalli* (Uchida et al., 2009), and other species (Lee and Poulter, 2008, Robinson *et al.*, 1993). Squalene synthase is a microsomal protein anchored to the endoplasmic reticulum membrane through the hydrophobic carboxyl terminus (Devarenne et al., 1998). Squalene synthase has six

highly conserved peptide domains that were identified in fungi, humans and plants (Robinson *et al.*, 1993, Devarenne *et al.*, 1998, Huang *et al.*, 2007). These conserved domains are believed to represent important regions for enzyme activity and localization to the smooth endoplasmic reticulum (ER). The amino terminal region of squalene synthase has been to be involved in substrate binding and catalytic activity of the protein.

The gene encoding the squalene synthase protein is arranged in a complex structure of 13 exons and 12 introns, which is highly conserved among the plant species. Single copies of the *squalene synthase* gene were found in *Euphorbia tirucalli* (Uchida *et al.*, 2009), *Taxus cuspidata (*Huang et al., 2007), yeast and human. However, two gene copies have been identified in other species. For example, a cDNA with 87% identity at the amino acid level to *squalene synthase* from licorice was isolated (Hayashi *et al.*, 1999), and Lee *et al* (2004) have shown that characterization of Ginseng *squalene synthase* might be consistent with multiple genes. *Arabidopsis thaliana* (Kribii *et al*., 1997), *Nicotiana tabacum* (Devarenne *et al.*, 2002) and *Oryza sativa* possesses two squalene *synthase genes* per genome. *Populus trichorpa* also posses two 90% similar *squalene synthase* gene copies per genome. In potato, a single copy of *squalene synthase* has been reported although the authors did not preclude the possibility of an additional *squalene synthase* gene (Yoshioka *et al.*, 1999).

Expression patterns of *squalene synthase* have been documented in several plants. Devarenne *et al*. (2002) reported predominant expression in shoot meristems, stems and leaf petioles of tobacco through *squalene synthase* promoter--β-glucuronidase

(GUS) expression. Huang *et al*. (2007) showed that the highest expression levels were observed in the roots of *Taxus cuspidata*. The predominant expression levels in the shoot apex and roots of licorice (Lee *et al.*, 2004) are consistent with those reported in tobacco and *T. cuspidata*, respectively. Most recently, equivalent levels of *squalene synthase* transcripts were reported in the stems and leaves of *Euphorbia tirucalli*, with 10 times less detected in roots (Uchida *et al.*, 2009). Clearly, there exist differences in the expression patterns of *squalene synthase* in different plants. Uchida *et al.* (2009) observed that the accumulation of *squalene synthase* transcripts in *Euphorbia tirucalli* was consistent with greater amounts of latex exudation from stems and petioles than roots. In *S. tuberosum,* high level of *squalene synthase* transcripts was shown to be associated with high SGA levels in the tubers (Krits et al., 2007)

Here, we report the molecular cloning and characterization of the *squalene synthase* gene from *Solanum chacoense.* The results show that the deduced *S. chacoense* polypeptide has high similarity to known plant squalene synthase polypeptides and that it has 411 amino acid residues. The genomic organization of the gene shows the presence of 13 exons and 12 introns.

3.2 Materials and Methods

3.2.1 Plant Materials and Chemicals:

Solanum chacoense (USDA diploid clone 8380-1 and the corresponding monoploid lines, 8380-1-3 and 8380-1-4 developed through anther cultures (Veilleux and Miller, 1998) and maintained by Dr R. E. Veilleux, Virginia Tech were established

from stolons in cell packs of Sunshine Mix # 1 (SunGro, Bellevue, WA), then transferred to 10.3 cm pots after 30 d. Plants were cultured in controlled environment growth chambers with settings of a 10 h photoperiod, and day and night temperatures of 24 and 19°C, respectively. To induce flowering, the photoperiod was adjusted to 14 h at 41 days after planting. The relative humidity was maintained at 60% throughout the study. Light intensity was maintained at approximately 275 μmol photons $m^{-2}s^{-1}$. Plants were treated with MiracleGro All Purpose Fertilizer (15:30:15 N: K: P, Scotts, Marysville, OH) at a rate of 1g per L of water biweekly.

All oligonucleotide primers were from Integrated DNA Technologies (Coralville, IA) and are shown in Table 3-1. Restriction enzymes were from New England BioLabs. Top 10 chemically competent *E. coli* cells (strain DH10B) were obtained from InVitrogen (Carlsbad, CA). The cloning vector pGEM T Easy was from Promega (Madison, WI). PCR fragments were purified using the NucleoSpin Extract II Kit (Macherey-Nagel, Bethlehem, PA).

3.2.2 RNA extraction and cDNA synthesis:

Tubers, stolons, roots, leaves, stems, flowers and floral buds were harvested at different developmental stages of diploid *S. chacoense* and stored at –80°C before use. Total RNA was extracted from the tissues using TRIzol reagent according to the provided instructions (InVitrogen, Carlsbad, CA). RNA was quantified by spectrophotometry and assessed by formardehyde- denaturing agarose gels. First-strand cDNA was generated from 2 μg of total RNA using 200 U of M-MLV

reverse transcriptase SuperScript II (Invitrogen, Carlsbad, CA), and oligo $(dT)_{20}$ as a primer.

3.2.3 Isolation of cDNA for squalene synthase:

A pair of synthetic degenerate oligonucleotide primers (SQSSYN F, SQSSYN R; Table 3-1) was designed to two highly conserved regions of the squalene synthase gene with the sequence based on the alignment of SQS nucleotide sequences of *Datura innoxia* (GI: 62945916), *Capsicum annuum* (GI: 4426952), *Nicotiana tabacum* (GI: 1552716) and *Solanum tuberosum* (GI: 5360654). The PCR used the primer pair and an aliquot of the leaf cDNA preparation, and the following cycle conditions: 35 cycles for 95^{o}C for 3 min, 55^{o}C for 30 s, 72^{o}C for 1 min, followed by a final 5 min template extension at 72^{o}C. A 960 bp DNA fragment which was identified by gel electrophoresis, isolated and purified using the NucleoSpin Extract II Kit and cloned into pGEM T Easy vector following the manufacturer's instructions, and sequenced (Virginia Bioinformatics Institute).

The entire open reading frame coding for SQS was isolated from leaf cDNA preparations using primers SQS.1A F and SQS.2A R (Table 3-2) based on the SQS nucleotide sequence from *S. tuberosum* (GI: BAA82093) and the PCR cycle conditions described above. The PCR product was cloned and sequenced as above. To isolate the 3'-untranslated region (3'UTR) of the SQS gene, we used an anchored PCR strategy as described by Huang et al (2007). In brief, a PCR was prepared using 1SQSEX9EX10 (9F) (Table 3-2), a forward primer with a nucleotide sequence at the 3' end of the SQS ORF, a reverse primer RP-oligo-dT 1

(Table 3-2) and various cDNA preparations. The PCR cycle conditions were as described above. PCR products were purified and cloned as described above.

The 5'-untranslated region (5'UTR) of the SQS gene was isolated using the high efficiency thermal asymmetric interlaced (TAIL) PCR procedure of Liu and Chen (2007). In brief, 50 ng of genomic DNA from *S. chacoense* was used as the first PCR template in a series of nested PCRs. All primers used for the pre-amplification, primary and secondary TAIL PCR are listed in Table 3-1(Liu and Chen, 2007). The forward primers were random primers LAD1-1. LAD1-2, LAD1-3, LAD1-4 and AC1, while the reverse primers were gene specific, SQS-0a, SQS-1a, and SQS-2a. The PCR products were purified and then sequenced as described above.

3.2.4 Gene Sequence Analysis and Characterization:

All nucleotide and amino acid sequence analyses were done with Lasergene 7 (DNASTAR Inc, Madison, WI). All Sequence alignments, phylogenetic trees and percent similarity tables were generated using Clustal W multi-alignment (Gap penalty: 10.00, Gaplength penalty: 0.20).

3.2.5 Genomic DNA Extraction and Southern Blot Analysis:

Genomic DNA was extracted from leaves of the diploid and the monoploid lines using the hexadecyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle**,** 1987). The DNA blot was prepared and probed as described below by Lofstrand Labs Ltd. (Gaithersburg, MD). Twenty microgram DNA aliquots were

digested with the *Xba*I, EcoRI, *EcoR*V, and *Hind* III restriction enzymes (New England BioLabs). Approximately 7.5 µg of the digested DNA was fractionated by agarose gel electrophoresis (0.7%) at 22 V for 18 h and then transferred to Nytran supercharge membranes (Schleicher and Schuell BioScience, Keene, NH). The hybridization probe was generated by the incorporation of P^{32} into randomly primed DNA by PCR using the 960 bp cDNA as a template. Hybridization of the probe to the membrane was conducted at 68^{o}C for 27 h overnight. The low stringency wash was done in 2X SSC+0.1% SDS at 55^{o}C, with a three buffer change over period of 60 minutes. After a low stringency wash, the membrane was autoradiographed for 2.5 d and then a high stringency wash was done at 68^{o}C for 90 minutes. The membrane was then autoradiographed for 4 d using an intensifier screen.

3.2.6 Compilation of the genomic sequence:

PCR was performed using primers based on exon sequences of the cDNA (Table 3-3) to amplify the twelve intron regions. Each PCR product was generated using 2 µg of leaf genomic diploid and monoploid DNA using the PCR program described above. Each fragment was isolated, purified and sequenced as described above. For each fragment, forward and reverse sequencing was performed; at least two independent PCR products were used in each instance. All the individual sequences were combined and a consensus *squalene synthase* genomic sequence obtained.

3.2.7 Expression profile determinations by real time PCR:

Real-time PCR was carried out using the ABI7300 (Applied Biosystems, Carlsbad, CA) as singleplex assays. Reactions were set up in a final volume of 25 µl containing 12.5 µl of SYBR (R) Green PCR Master Mix (ABI, Foster City, CA).) 10 pmole of forward and reverse primers and 1 µl of cDNA preparation. The thermal cycling conditions consisted of 3 stages, Stage 1: 50 ^{o}C for 2 min., Stage 2: 95 ^{o}C for 10 min, Stage 3-1: 95 ^{o}C for 15 sec., Stage 3-2: 55 ^{o}C for 20 sec, and Stage 3-3: 60 ^{o}C for 1 min. C_t and slope data were collected at Stage 3-3, which is the dissociated stage. Primers designed for the *squalene synthase* gene (1SQSEX8EX9, 1SQSEX9EX8; Table 3-1) and for an actin gene (actin gene family 4) (Actin F, Actin R; Table 3-1) generated products of 168 and 143 bp, respectively. The amount of the SQS transcript was related to that of the reference gene (*actin*) by the method described by (Pfaffl, 2001). Each sample was tested in triplicate and each tissue sample consisted of three independent replications.

3.3 Results and Discussion

3.3.1 cDNA cloning of S. chacoense squalene synthase (ScSQS1)

The oligonucleotide primers designed for PCR cloning from *S. chacoense* were based on nucleotide sequences coding for squalene synthase from solanaceous species in *Capsicum, Datura, Nicotiana* and *Solanum*, which have 96% identity (data not shown). The degenerate oligonucleotides (SQSSSYN F, SQSSSYN R) mapped to highly conserved regions of SQS. From the initial RT/PCR using the leaf total RNA, a 960 bp DNA fragment was isolated. Direct sequencing of the

amplified fragment revealed that it was a continuous ORF and when aligned with other known plant *squalene synthase* genes showed 74-97% identity. The 960 bp sequence was used to design other primers to amplify the 3'UTR, and genomic sequences. The open reading frame (ORF) was obtained with primers SQS 1A.F and SQS 2A R, at the starting methionine and at the stop codon. A single 1236 bp fragment was obtained, which codes for 411 residues (Figure 3-1) with a theoretical molecular weight of 42.03 kDa and an isoelectric point value of 7.3.

A comparison of the *S. chacoense* squalene synthase with other plant species revealed the presence of the six highly conserved domains I-VI (Figure 3-1). Among these domains, VI and I were hydrophobic, while domains II and IV contained aspartate rich motifs. In an attempt to find regulatory sites within the ORF, we identified three sites. Of these, the casein kinase II phosphorylation site located at Thr^{78} within the ScSQS1 (Figure 3-1). PEST sites, characterized by the presence of the amino acid residues proline, glutamate, serine, and threonine, were located at amino acids 79 – 90 within the sequence. Both the phosphorylation site and the PEST sequence were located in Domain II of ScSQS1 (Figure 3-1). The DYLED sequence is characterized by the presence of the amino acid residues aspartate, tyrosine, leucine, and glutamate, was located in Domain IV (Fig 3-1). The conserved domains of known *squalene synthase* proteins are believed to represent important regions for enzyme activity and localization to the smooth endoplasmic reticulum. Domain II is an aspartate (D) rich domain, which is believed to be involved in the catalytic activity of the enzyme (Devarenne et al., 2002). Aspartate rich motifs have been proposed to coordinate and or facilitate the farnesyl

pyrophosphate binding through a magnesium ion requirement (Ashby et al., 1990). Casein kinase II is a protein that has unclear cellular functions although it is known to catalyze the phosphorylation of serine and threonine residues in many different proteins (Kuenzel and Krebs, 1985). Devarenne *et al*. (2002) have demonstrated that a fragment of the protein containing this site can be readily phosphorylated *in vitro*. However, it is not known whether the phosphorylation at this site might regulate enzyme activity *in vivo,* and most of the presently known casein kinase II-catalyzed reactions are not accompanied by apparent functional changes in the substrates (Kuenzel and Krebs, 1985). In plants, examples of squalene synthase regulation have been limited; elicitor mediated down-regulation of squalene synthase has been shown (Vogeli and Chappell, 1988), although the mechanism is not yet clearly understood. One would speculate that squalene synthase might possibly be regulated through phosphorylation at Thr^{78} or another site, or it might be regulated through another process such as degradation. The amino acid residues PEST (proline, glutamic acid, serine and tyrosine) located in domain II around the casein kinase phosphorylation site (79-90) have been previously identified as being involved in targeting proteins for degradation (Rogers et al., 1986). However, it is not known whether or not this motif contributes to turnover of plant squalene synthase enzymes.

The tail-tail condensation of two molecules of farnesyl pyrophosphate occurs in two steps: the formation of presqualene pyrophosphate (PSPP) by the action of squalene in the presence of magnesium, and the conversion of PSPP to squalene in the presence of NADPH and magnesium. In the first reaction, the motif DYLED

(aspartate, tyrosine, leucine, glutamic acid, aspartate) in squalene synthase is required to bind the diphosphates of FPP via bridging magnesium ions (Pandit et al., 2000). The DYLED motif required for this activity is located in conserved domain IV as already mentioned. Substitutions of aspartate to glutamate in the first or last positions through site-directed mutagenesis have resulted in a 90-fold reduction or no effect on the activity of the enzyme, respectively showing that the first aspartate in this domain is the key residue for catalysis (Marrero et al., 1992). Squalene synthase is a microsomal protein that has been shown to possess C-terminal hydrophobic residues that anchor it to the endoplasmic reticulum membrane (Kribii et al., 1997). Domain VI has been proposed to be the putative membrane anchoring part of the protein (Devarenne et al., 2002). Robinson et al. (1993) and Devarenne et al. (2002) have shown that deletion of the most carboxyl terminal of this polypeptide converted both the *S. cerevisiae* and *N. tabacum* squalene synthase proteins from membrane-bound to soluble enzymes. Both of these studies establish that this is the only transmembrane domain of the protein. To define the 3-UTR, 'we used the primers 1SQSEX9EX10 (9F) and RP-OdT1, and cDNA preparations from several different tissues to PCR amplify and clone products of various lengths of approximately 700 bp. The variation in length was identified by sequence analysis (Figure 3-2) to be associated with the length of the 3'-UTR. The longest 3'-UTR of 335 bases was cloned from cDNA prepared from young leaves while shorter UTRs of 202 and 249 bases were cloned from cDNAs from fully expanded leaves and floral buds, respectively (Figure 3-2). In order to determine whether these differences in the length of the 3'UTR were tissue specific, a PCR using another forward primer 1SQSEX10EX11 (10F) and RP-OdT1 and

cDNA from tubers, stolons, roots, leaves, stems, flowers and floral buds as templates was performed using the conditions described above. The results after the gel (2%) electrophoresis of the PCR products showed the presence of more than one band: one smaller band of approximately 650 bp and a larger fainter band of about 750 bp (Figure 3-2 B).

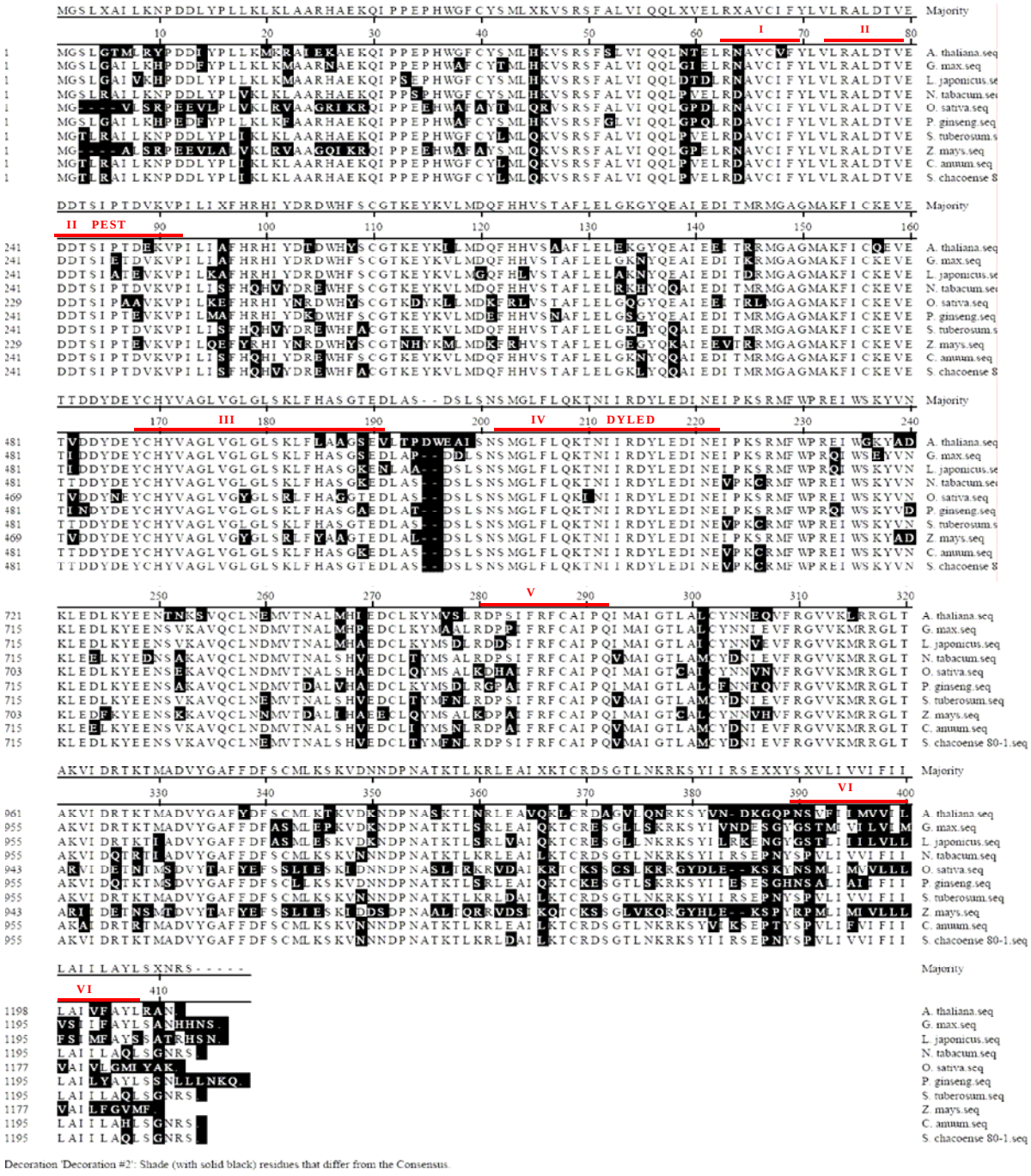


Figure 3-1 Multiple alignment of plant squalene synthase polypeptides. The sequences are from *Arabidopsis thaliana* (BAA06103, Nakashima et al., 1995), *Glycine max* (BAA22559, Hata et al., 1997), *Lotus japonicus* (BAC56854, Akamine et al., 2003), *Nicotiana tabacum* (AAB08578, Devarenne et al., 1998), *Oryza sativa* (BAA22558, Hata et al., 1997), *Panax ginseng* (BAD08242, Lee et al., 2004), *Solanum chacoense* 80-1, *Solanum tuberosum* (BAA82093, Yoshioka et al., 1999). Six known highly conserved peptide regions are underlined. The PEST and DYLED peptide domains are also indicated.

To determine if these PCR products were based on *ScSQS*1, a restriction digest with *Mse*I was conducted and the results showed that the products had digested as expected (results not shown).

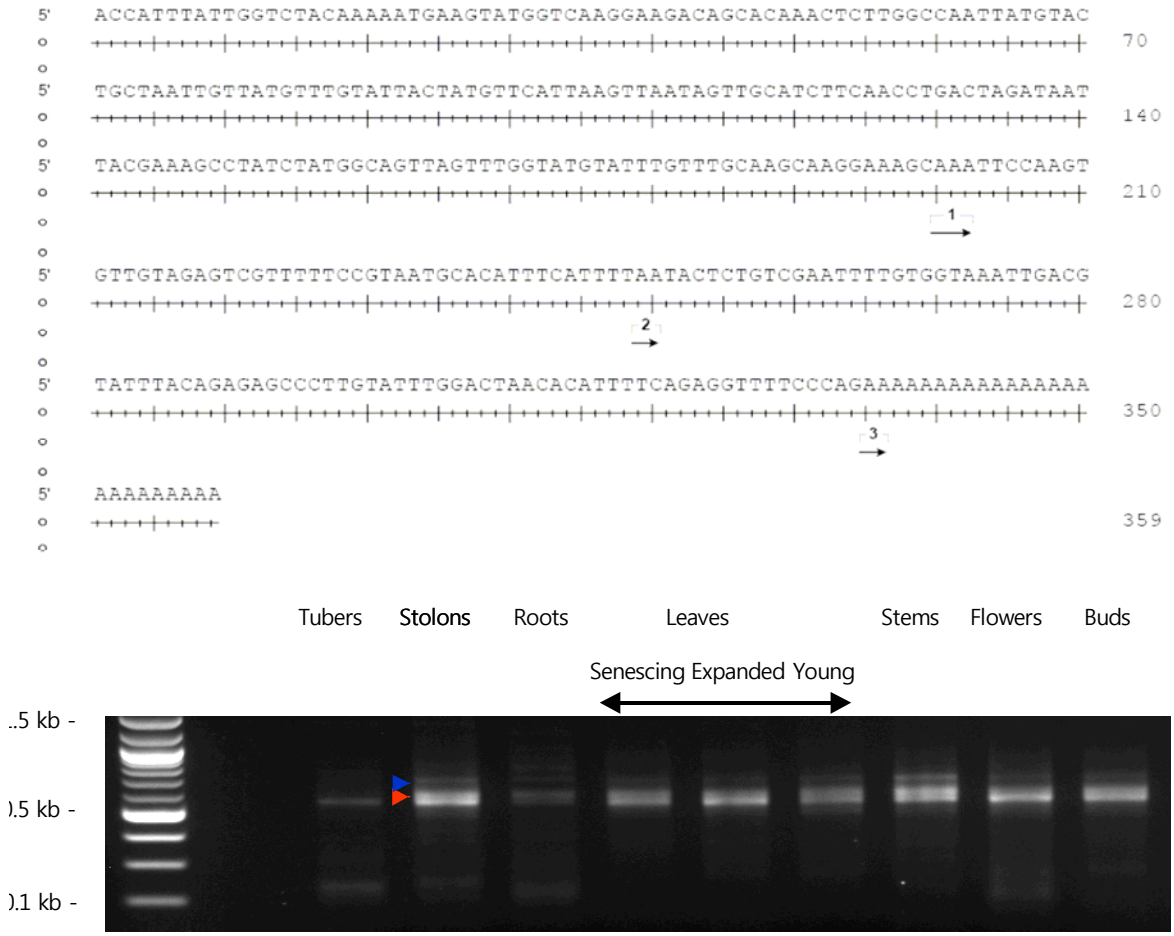


Figure 3-2 A. 3' untranslated regions sequence of *S. chacoense* showing the start of the poly A tails for the fully expanded leaves (1: 202 bp), floral buds (2: 249 bp) and young expanding leaves (3: 335 bp). **B**. Gel electrophoresis of PCR products of cDNA from exon 10 to the PolyA tail. Arrows show presence of multiple products with different sizes. Red arrow: ~ 650 bp, blue arrow, ~ 750 bp products.

The results from our study revealed that the longer 3'UTR observed in young leaves (335 bp) was also documented in the tuber cDNA characterized by Yoshioka et al (1999). We observed that the longest cDNA 3'UTR sequence was the same as that

of the genomic sequence (data not shown). Further analysis of *S. chacoense* cDNA revealed that the differences in the 3'UTR were not tissue specific but could be due to the presence of multiple mRNA species. This observation was supported by the fact that an earlier characterization of several tuber *S. tuberosum* squalene synthase cDNA clones also revealed differences in the 3'UTR (Yoshioka et al., 1999). These differences in the 3'UTR observed by both our studies and those of Yoshioka et al (1999) could be attributed to multiple species generated by alternative polyadenylation (APA) events of the same transcript. APA has been suggested to play an important role in gene expression regulation and the presence of multiple poly (A) sites has been shown in more than half of the human genes and over 25% of Arabidopsis genes (Shen et al., 2008). Further experimentation is required to understand the role of polymorphic 3'UTR in *squalene synthase*.

In order to obtain the 5' end of the gene, TAIL PCR was performed using a nested approach with four random forward primers and three gene specific reverse primers (Table 3-1). Single products of about 170 bp from the primary reaction were cleaned up and sequenced. The analysis of the sequence revealed a 165 bp sequence upstream of the starting methionine; BLAST results of the sequence revealed a 97% similarity between the first 133 bp upstream of the starting methionine of *S. chacoense* and that of *S. tuberosum* published 5'UTR sequence (Yoshioka et al., 1999). Therefore, the isolated fragment was taken to represent the equivalent 5'UTR of the cDNA.

3.3.2 Comparison of ScSQS1 with other squalene sequences

The deduced amino acid sequence for *S. chacoense* squalene synthase predicts a polypeptide of 411 amino acid residues, a molecular mass of 47.02 kDa and an isoelectric point at pH 7.3. This size of the protein falls within the range of published plant *squalene synthase* proteins which is 403-414 amino acid (Nakashima *et al.*, 1995, Hata *et al.*, 1997, Devarenne *et al.*, 1998, Akamine *et al.*, 2003, Kim *et al.*, 2005, Huang *et al.*, 2007). In terms of amino acid sequence comparisons, ScSQS1 showed a range of 68-100% sequence identity with the most dissimilar comparison with the monocots *Zea mays* and *Oryza sativa* (about 70%), 76-81% identity with distantly related dicots and 95-100% with species in the Solanaceae (Figure. 3-3 A & B). The dicot and monocot enzymes formed two distinct groups. An interesting feature of the alignment is that the enzymes from *S. tuberosum* and *S. chacoense* had a few synonymous single nucleotide polymorphisms resulting in a 100% similarity between the two enzymes. These results suggest that squalene synthase is a highly conserved protein or that te species are closely related. Considering that *S. tuberosum* and *S. chacoense* have the capability to accumulate low and high levels of the steroidal glycoalkaloids, which are derived from squalene as a precursor, the similarity in the enzymes might suggest that levels of these sterol compounds may be regulated by either transcriptional or posttranslational mechanisms if squalene synthase is a key regulatory enzyme in this process.

A

Percent Identity

	1	2	3	4	5	6	7	8	9	10		
1		79.7	77.3	75.1	68.3	77.0	75.6	67.7	75.3	75.6	1	A. thaliana.seq
2	23.7		88.6	81.8	72.0	84.3	82.3	70.6	82.3	82.3	2	G. max.seq
3	27.1	12.3		81.3	72.3	82.6	80.8	69.7	80.8	80.8	3	L. japonicus.seq
4	30.4	20.9	21.6		70.0	82.0	95.4	67.7	94.9	95.4	4	N. tabacum.seq
5	41.1	35.0	34.6	38.2		72.5	70.8	85.3	71.0	70.8	5	O. sativa.seq
6	27.5	17.7	19.8	20.6	34.2		81.3	71.9	81.8	81.3	6	P. ginseng.seq
7	29.6	20.3	22.2	4.8	37.0	21.6		68.4	95.9	100.0	7	S. tuberosum.seq
8	42.2	37.2	38.8	42.2	16.4	35.2	40.9		68.7	68.4	8	Z. mays.seq
9	30.0	20.3	22.2	5.3	36.6	20.9	4.2	40.5		95.9	9	C. anuum.seq
10	29.6	20.3	22.2	4.8	37.0	21.6	0.0	40.9	4.2		10	S. chacoense 80-1
	1	2	3	4	5	6	7	8	9	10		

Divergence

B

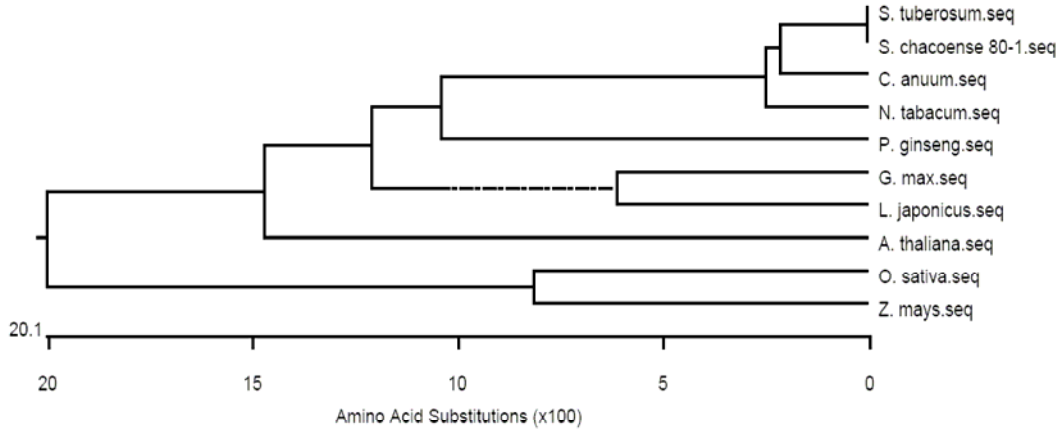


Figure 3-3 Percent identity and amino acid substitutions among various plant squalene synthase polypeptides constructed using MegAlign (ClustalW). **A**. Pair-wise comparisons show that similarities range from 68 to 100%. **B.** Phylogenetic tree showing the amino acid substitutions indicates the evolutionary relationships among the polypeptides from the different species.

3.3.3 Genomic organization of the ScSQS1 gene

The genomic organization of the *S. chacoense* squalene synthase gene revealed the presence of 13 exons and 12 introns with a total of 8.3+ kb in length from the translation start site to the stop site (Figure 3-4 A). The exon sizes ranged from 42 to 195 bp, while that of introns ranged from 72 to 2500+ bp.

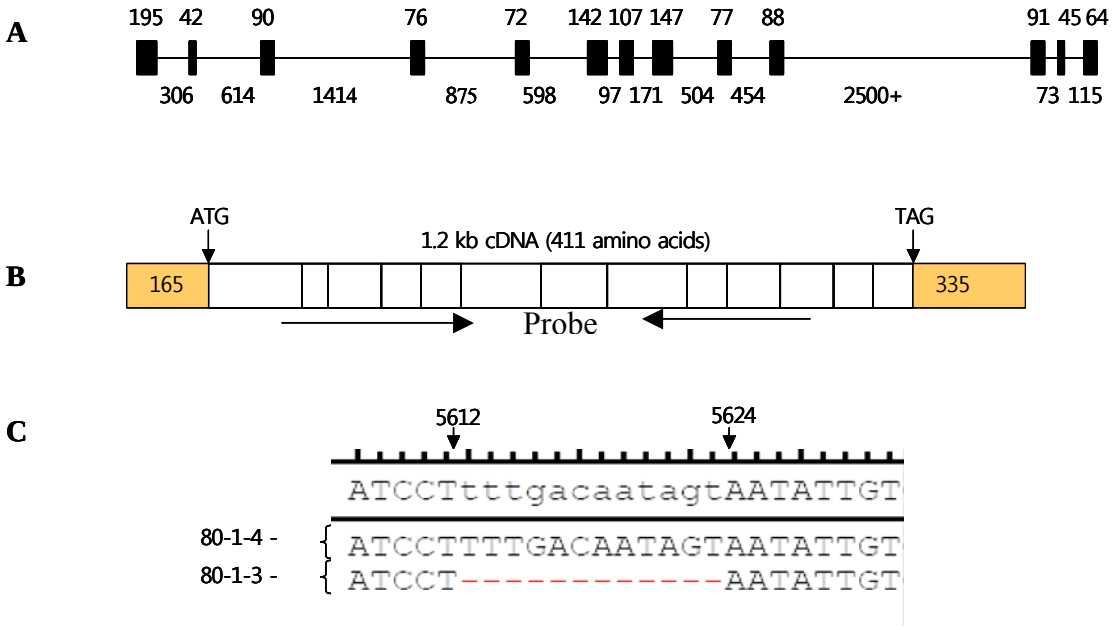


Figure 3-4 Organization of (**A**) *ScSQS1* gene showing exon (white boxes) and intron (lines) localization and length (bp) and (**B**) full length cDNA showing 5' and 3' untranslated regions (grey boxes) and the ORF. (**C)** Polymorphic region in intron 9 of *S.chacoense* monoploids, 80-1-3 and 80-1-4, 12 bp extra found between nucleotides 5612 –5 624 in the 80-1-4 genomic sequence. The numbering is relative to the genomic sequence starting at 5607 bp. Monoploid DNA was used for sequencing introns 9 and 10, the rest of the introns were sequenced using diploid DNA.

In general, the *S. chacoense squalene synthase* gene is organized in a complex structure with small exons and large introns, a characteristic that is common to mammalian genes (Xianzhi and Naihu, 2002). In comparison to other plant *squalene synthases*, *S. chacoense* had relatively large introns 3 and 10. Due to the large size of intron 10, the complete sequence is not yet available. Intron 9 was also interesting in that we were able to identify a polymorphic site within the sequence between the two alleles of the gene. Analysis of monoploids 80-1-3 and 80-1-4 DNA sequences revealed that the 80-1-4 possessed 12 nucleotides more than 80-1-3 between the nucleotides 5612 and 5624 (Figure 3-4C). The significance of this polymorphism was not established considering that it falls in an intronic region.

3.3.4 Southern Blot Analysis and multiple gene copy analysis

As part of the process to investigate the possibility of the existence of multiple gene copies of *squalene synthase* in *S. chacoense*, we first determined the similarities

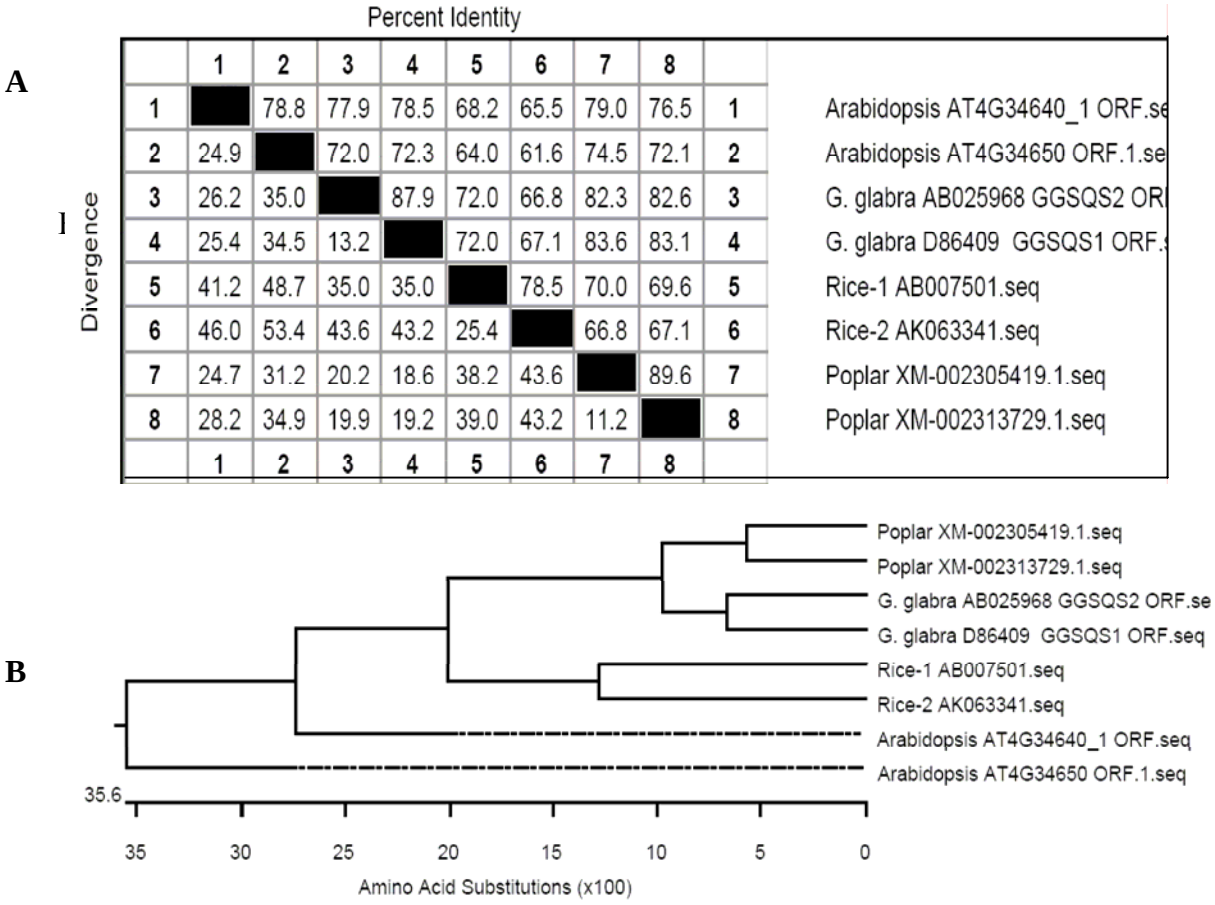


Figure 3-5 Percent identity (**A**) and amino acid substitutions (**B**) of squalene synthase peptides from species with confirmed multiple gene copies.

that exist among copies of the same gene in *Arabidopsis thaliana*, *Oryza sativa*, *Populus trichorma.,* and *Glycyrrhiza glabra.* The results in Figure 3-5 A revealed that there is 79-90% amino acid similarity between the gene copies, with the highest similarity being found in poplar (90%) followed by licorice (88%), Arabidopsis (79%) and rice (78%), in that order. Additionally, each pair of gene copies is

closely related within each genome, except for that of the *Arabidopsis thaliana* (Figure 3-5 B). The divergence observed between the two gene copies in Arabidopsis was shown to be due to differences in the c-terminal region of the gene (Kribii et al., 1997).

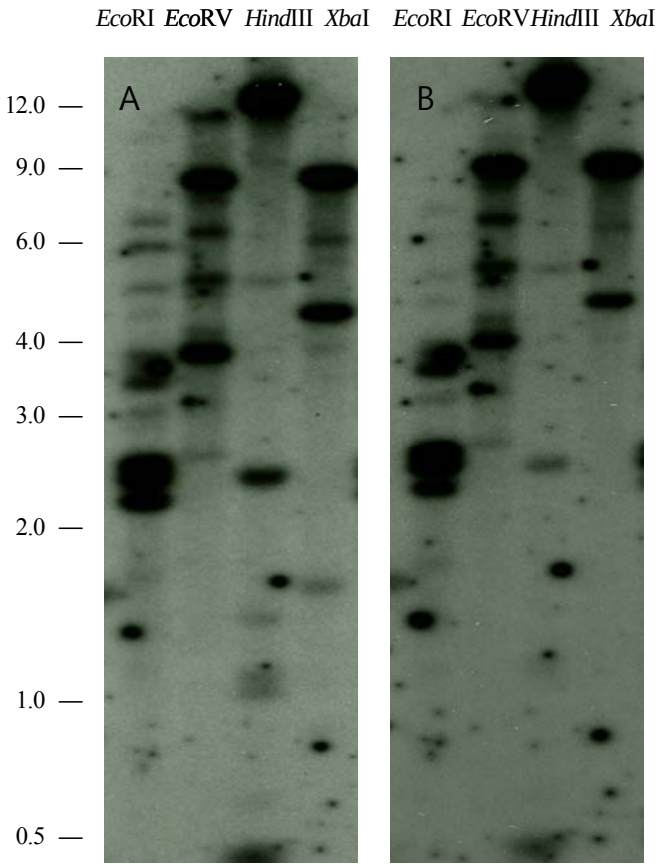


Figure 3-6 Southern blot analysis of total monoploid 80-1-3 DNA using SQS cDNA probe. Total DNA isolated from *S. chacoense* was digested with the restriction enzymes indicated at the top of the panel, electrophoresed, blotted onto a nytran membrane, and subjected to hybridization using SQS cDNA as a probe. The positions of the molecular weight markers are indicated on the left of the panel **A.** Low stringency wash, **B** High stringency wash

In order to determine the gene family size of *squalene synthase* in *S. chacoense*, a Southern blot analysis was performed. *S. chacoense* genomic (diploid and monoploid) DNA digested with *Xba*I, *Eco*RI, *Hind*III, and *Eco*RV, and then probed with a randomly primed 960-bp internal *squalene synthase* cDNA fragment, several hybridizing fragments were seen for each digestion treatment under low and high

stringency conditions (Figure 3-6). At least two intense bands and several faint bands were observed for each enzyme.

Table 3- 1Expected fragment sizes of hybridizing fragments bp

Enzyme	Expected Fragment Sizes bp
Eco RI	3313+, 2323, 1484+
Eco RV	3055+, 21, 421, 3623+
Hind III	547+, 2485, 1563, 384, 2141+
Xba I	1036+, 4596, 1488+

Based on the restriction maps for the genomic sequence (Table 3-1; Supplemental Figure 3-8), and the relative hybridization intensities, the DNA blot is consistent with at least one copy of the gene in genome. The results from the analysis do not preclude of the existence of a second gene copy. This result and that of Yoshioka et al (1991) suggest that it is still unknown if there is more than a single copy of *squalene synthase* in potato. However, the existence of multiple copies of *squalene synthase* genes raises the question of the function of all the copies. With the existence of more than one copy in some of the plant genomes, it is important to test whether each gene copy is functional and define the specific role for each gene copy in the sterol biosynthetic pathway.

3.3.5 Expression patterns of ScSQS1

In order to determine the expression pattern of *squalene synthase* in the tissues of *S. chacoense*, real time PCR was performed. The results of the real time PCR revealed that relative gene expression of *squalene synthase* was similar in the roots, leaves, and buds, but was lower in tubers and flowers (Figure 3-7). Roots, leaves and buds

accumulated levels of transcripts ~1 unit while tubers, flowers and stems accumulated less levels corresponding to 0.07, 0.2 and 0.6 units, respectively. The levels of SGAs are also shown (data from chapter 2 of this thesis); except for the tubers and buds, there was no correlation between the relative gene expression levels of *squalene synthase* and SGA accumulation in the different plant tissues.

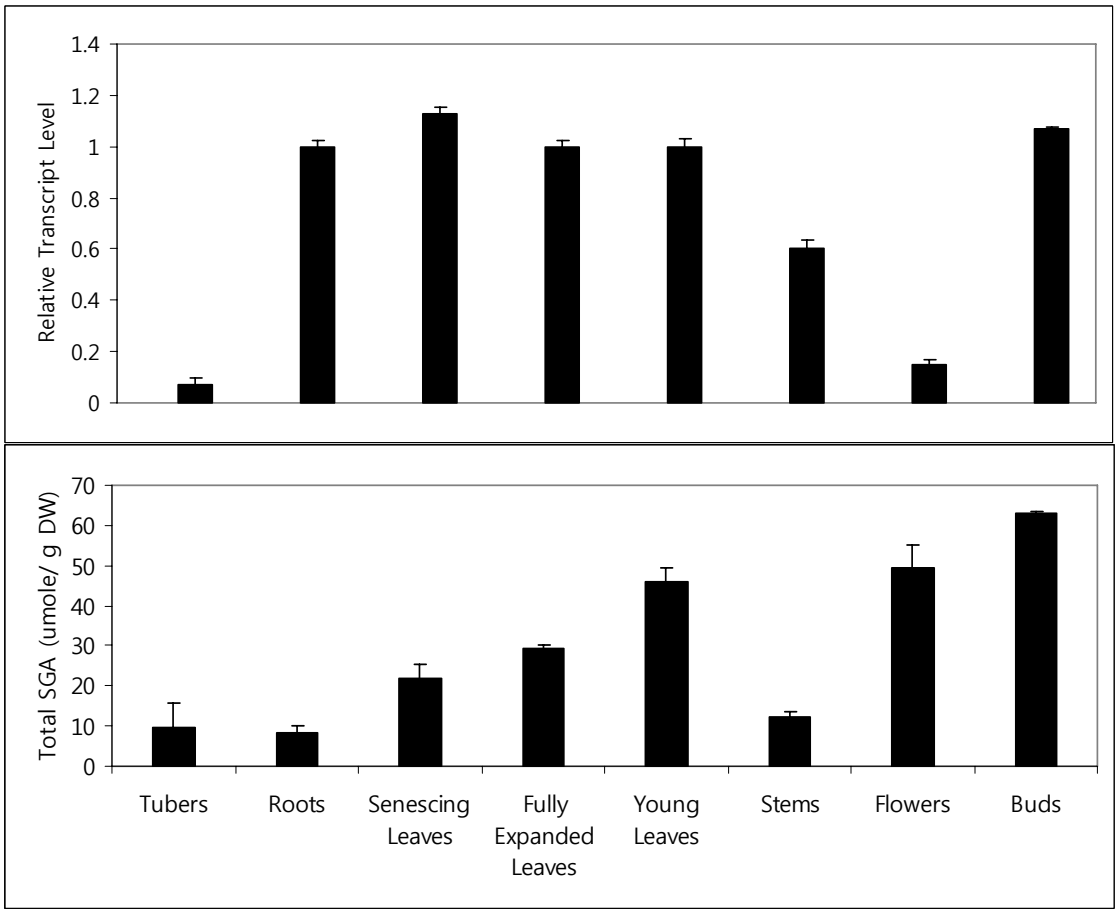


Figure 3-7. Relative **e**xpression of *ScSQS1* in different tissues of S. *chacoense* including tubers, roots, leaves, stems, flowers and floral buds. Actin was used as an endogenous control. Each bar is represented by 9 observations (three biological and three technical replications).

Although our results show that the low levels of SGAs in tubers corresponded with low expression levels of *squalene synthase*, a similar relationship was not true of

the leaves or flowers. In leaves of *S. tuberosum (Desiree and LT-7), S. phureja (1-3)*, *S. chacoense* (80-1 and 55-1), and a hybrid between *S. chacoense* and *S. phureja* (CP-2), Krits et al. (2007) suggested a correlation between *squalene synthase* mRNA expression levels and SGA content. However, the association between SGA levels and *squalene synthase* transcripts in the tuber tissues, phelloderm and parenchyma, was rather weak. Therefore, our observations that SGA levels and *squalene synthase* transcript levels are not always correlated agree with the findings of Krits et al. (2007). Both of these observations might suggest that if squalene synthase has a role in determining the accumulation of SGAs, the regulation is beyond the transcriptional level. Therefore, studies of squalene synthase translation, enzyme activity, and/ or stability (degradation) are necessary to establish the role of squalene synthase on SGA accumulation. Additionally, while we do not observe a correlation between transcript and SGA levels, it is possible that the observed transcript levels are correlated with the levels of other sterols. For example, in *E. tirucalli* (Uchida et al., 2009), *M. truncatula* (Suzuki et al., 2002), *P. ginseng* (Lee et al., 2004), and *L. japonicus* (Akamine et al., 2003), the high expression of levels of *squalene synthase* in roots was associated with the accumulation of substantial amounts of sterol and tri-terpene saponins. The expression of levels *squalene synthase* transcripts has previously been linked with high levels of cell multiplication (Uchida et al., 2009). Studies by Devarenne et al. (2002) have also shown that the gene is expressed predominately in the meristematic regions of the stems of *N. tabacum*.

3.4 Conclusions

The cloned sequence is a putative *squalene synthase* gene, with 411 amino acids and high similarity to other known *squalene synthase* genes. The cDNA has polymorphic 3'UTR possibly due to the presence of multiple mRNA species brought about through alternative polyadenylation. *S. chacoense squalene synthase* genomic sequence is complex with 13 exons and 12 introns, ranging in size from 42-195, and 73-2500 bp, respectively. Based on the probe used for the Southern blot, the *S. chacoense* genome has at least one copy of squalene synthase, although the possibility of another gene copy cannot be precluded. The accumulation of transcripts in various tissues of the plant showed lower levels in tubers, flowers and stems compared to roots, leaves and floral buds. The lack of consistent correlation between SGA levels and *squalene synthase* transcript levels suggest that if squalene synthase is determining SGA accumulation, this process is regulated at some post-transcriptional level, or that squalene synthase might be correlated with sterol accumulation in general as has been shown on other species. Therefore, experiments establishing these relationships in *S. chacoense* would be worthwhile.

3.5 References

Akamine, S., Nakamori, K., Chechetka, S.A., Banba, M., Umehara, Y., Kouchi, H., Izui, K. & Hata, S. (2003) cDNA cloning, mRNA expression, and mutational analysis of the *squalene synthase* gene of *Lotus japonicus*. *Biochimica Et Biophysica Acta-Gene Structure and Expression,* **1626,** 97-101.

Andre, C.M., Schafleitner, R., Guignard, C., Oufir, M., Aliaga, C.A.A., Nomberto, G., Hoffmann, L., Hausman, J.F., Evers, D. & Larondelle, Y. (2009) Modification of the health-promoting value of potato tubers field grown under drought stress: Emphasis on dietary antioxidant and glycoalkaloid contents in five native Andean

cultivars (*Solanum tuberosum* L.). *Journal of Agricultural and Food Chemistry,* **57,** 599-609.

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

Ashby, M.N., Spear, D.H. & Edwards, P.A. (1990) Molecular Biology of Atherosclerosis. (ed A. D. Attie). Elsevier, Amsterdam.

Bakrim, A., Maria, A., Sayah, F., Lafont, R. & Takvorian, N. (2008) Ecdysteroids in spinach (*Spinacia oleracea* L.): Biosynthesis, transport and regulation of levels. *Plant Physiology and Biochemistry,* **46,** 844-854.

Bennett, R.N. & Wallsgrove, R.M. (1994) Secondary metabolites in plant defense-mechanisms. *New Phytologist,* **127,** 617-633.

Benveniste, P. (2004) Biosynthesis and accumulation of sterols. *Annual Review of Plant Biology,* **55,** 429-457.

Birch, A.N.E., Geoghegan, I.E., Griffiths, D.W. & McNicol, J.W. (2002) The effect of genetic transformations for pest resistance on foliar solanidine-based glycoalkaloids of potato (*Solanum tuberosum*). *Annals of Applied Biology,* **140,** 143-149.

Blankemeyer, J.T., Atherton, R. & Friedman, M. (1995) Effect of potato glycoalkaloids alpha-chaconine and alpha-solanine on sodium active-transport in frog-skin. *Journal of Agricultural and Food Chemistry,* **43,** 636-639.

Blankemeyer, J.T., McWilliams, M.L., Rayburn, J.R., Weissenberg, M. & Friedman, M. (1998) Developmental toxicology of solamargine and solasonine glycoalkaloids in frog embryos. *Food and Chemical Toxicology,* **36,** 383-389.

Brown, M.S., McDonald, G.M. & Friedman, M. (1999) Sampling leaves of young potato (Solanum tuberosum) plants for glycoalkaloid analysis. *Journal of Agricultural and Food Chemistry,* **47,** 2331-2334.

Busquets, A., Keim, V., Closa, M., del Arco, A., Boronat, A., Arro, M. & Ferrer, A. (2008) *Arabidopsis thaliana* contains a single gene encoding squalene synthase. *Plant Molecular Biology,* **67,** 25-36.

Campos, N. & Boronat, A. (1995) Targeting and topology in the membrane of plant 3-hydroxy-3-methylglutaryl coenzyme a reductase. *Plant Cell,* **7,** 2163-2174.

Cantwell, M. (1996) A review of important facts about potato glycoalkaloids. *Perishables Handling Newsletter,* **25,** 171-196.

Chappell, J. (1995) The biochemistry and molecular-biology of isoprenoid metabolism. *Plant Physiology,* **107,** 1-6.

Chen, Z. & Miller, A.R. (2000) Steroidal alkaloids in Solanaceous vegetable crops. *Horticultural Reviews,* **25,** 171-196.

Choi, D., Ward, B.L. & Bostock, R.M. (1992) Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl coenzyme-a reductase genes in response to *Phytophthora infestans* and to its elicitor arachidonic-acid. *Plant Cell,* **4,** 1333-1344.

Coombs, J.J., Douches, D.S., Cooper, S.G., Grafius, E.J., Pett, W.L. & Moyer, D.D. (2005) Combining natural and engineered host plant resistance mechanisms in potato for Colorado potato beetle: Choice and no-choice field studies. *Journal of the American Society for Horticultural Science,* **130,** 857-864.

Coombs, J.J., Douches, D.S., Li, W.B., Grafius, E.J. & Pett, W.L. (2003) Field evaluation of natural, engineered, and combined resistance mechanisms in potato for control of Colorado potato beetle. *Journal of the American Society for Horticultural Science,* **128,** 219-224.

Cooper, S.G., Douches, D.S. & Grafius, E.J. (2004) Combining genetic engineering and traditional breeding to provide elevated resistance in potatoes to Colorado potato beetle. *Entomologia Experimentalis Et Applicata,* **112,** 37-46.

Croteau, R., Kutchan, T.M. & Lewis, G.N. (2000) Natural products (secondary metabolites). *Biochemistry and Molecular Biology of Plants* (eds B. B. Buchanan, W. Gruissem & R. L. Jones). American Society of Plant Physiologists, Rockville, MD.

Deahl, K.L., Cantelo, W.W., Sinden, S.L. & Sanford, L.L. (1991) The effect of light intensity on Colorado potato beetle resistance and foliar glycoalkaloid concentration o four *Solanum chacoense* clones. *American Potato Journal,* **68,** 659-666.

Deahl, K.L., Sinden, S.L. & Young, R.J. (1993) Evaluation of wild tuber-bearing *Solanum* accessions for foliar glycoalkaloids level and composition. *American Potato Journal,* **70,** 61-69.

Devarenne, T.P., Ghosh, A. & Chappell, J. (2002) Regulation of squalene synthase, a key enzyme of sterol biosynthesis, in tobacco. *Plant Physiology,* **129,** 1095-1106.

Devarenne, T.P., Shin, D.H., Back, K., Yin, S.H. & Chappell, J. (1998) Molecular characterization of tobacco squalene synthase and regulation in response to fungal elicitor. *Archives of Biochemistry and Biophysics,* **349,** 205-215.

Dimenstein, L., Lisker, N., Kedar, N. & Levy, D. (1997) Changes in the content of steroidal glycoalkaloids in potato tubers grown in the field and in the greenhouse under different conditions of light, temperature and daylength. *Physiological and Molecular Plant Pathology,* **50,** 391-402.

Distl, M. & Wink, M. (2009) Identification and quantification of steroidal alkaloids from wild tuber-bearing *Solanum* species by HPLC and LC-ESI-MS. *Potato Research,* **52,** 79-104.

Doyle, J. J. & J. L. Doyle**.** 1987**.** A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19**:** 11-15**.**

Evans, D. & Mondy, N.I. (1984) Effect of magnesium fertilization on glycoalkaloid formation in potato tubers. *Journal of Agric. Food Chem,* **32,** 465-466.

Fewell, A.M. & Roddick, J.G. (1993) Interactive antifungal activity of the glycoalkaloids alpha-solanine and alpha-chaconine. *Phytochemistry,* **33,** 323-328.

Fewell, A.M. & Roddick, J.G. (1997) Potato glycoalkaloid impairment of fungal development. *Mycological Research,* **101,** 597-603.

Fewell, A.M., Roddick, J.G. & Weissenberg, M. (1994) Interactions between the glycoalkaloids solasonine and solamargine in relation to inhibition of fungal growth. *Phytochemistry,* **37,** 1007-1011.

Fragoyiannis, D.A., McKinlay, R.G., D'Mello, J.P.F. & Bcpc, B. (2000) Effects of different nitrogen sources and aphid herbivory on foliar glycoalkaloid production in two potato cultivars. *International Conference on Pests and Diseases*, pp. 919-924. British Crop Protection Council, Brighton, England.

Friedman, M. & Dao, L. (1992) Distribution of glycoalkaloids in potato plants and commercial potato products. *Journal of Agricultural and Food Chemistry,* **40,** 419-423.

Friedman, M. & McDonald, G.M. (1997) Potato glycoalkaloids: chemistry, analysis, safety, and plant physiology. *Critical Reviews in Plant Sciences,* **16,** 55-132.

Friedman, M., Rayburn, J.R. & Bantle, J.A. (1991) Developmental toxicology of potato alkaloids in the frog embryo teratogenesis assay *Xenopus* (FETAX). *Food and Chemical Toxicology,* **29,** 537-547.

Ginzberg, I., Tokuhisa, J.G. & Veilleux, R.E. (2009) Potato steroidal glycoalkaloids: Biosynthesis and genetic manipulation. *Potato Research,* **52,** 1-15.

Gregory, P. (1984) Gkycoalkaloid composition of potatoes: diversity and biological implications. *American Potato Journal* **61,** 115-121.

Hata, S., Sanmiya, K., Kouchi, H., Matsuoka, M., Yamamoto, N. & Izui, K. (1997) cDNA cloning of squalene synthase genes from mono- and dicotyledonous plants, and expression of the gene in rice. *Plant and Cell Physiology,* **38,** 1409-1413.

Hawkes, J.G. (1990) The potato: evolution, biodiversity, and genetic resources. Belhaven Press, Oxford.

Hawkes, J.G. & Hjerting, J.P. (1989) The potatoes of Bolivia: Their breeding value and evolutionary relationships. Clarendon, Oxford.

Hayashi, H., Hirota, A., Hiraoka, N. & Ikeshiro, Y. (1999) Molecular cloning and characterization of two cDNAs for *Glycyrrhiza glabra squalene synthase*. *Biological & Pharmaceutical Bulletin,* **22,** 947-950.

Heftmann, E. (1983) Biogenesis of steroids in solanaceae. *Phytochemistry,* **22,** 1843-1860.

Hide, G.A. & Lapwood, D.H. (1992) Disease aspects of potato production. *The Potato Crop* (ed P. M. Harris), pp. 403-475. Chapman and Hall, London.

Huang, Z.S., Jiang, K.J., Pi, Y., Hou, R., Liao, Z.H., Cao, Y., Han, X., Wang, Q., Sun, X.F. & Tang, K.X. (2007) Molecular cloning and characterization of the yew gene encoding squalene synthase from *Taxus cuspidata*. *Journal of Biochemistry and Molecular Biology,* **40,** 625-635.

Jansky, S., Austin-Phillips, S. & McCarthy, C. (1999) Colorado potato beetle resistance in somatic hybrids of diploid interspecific *Solanum* clones. *HortScience,* **34,** 922-927.

Jansky, S.H., Simon, R. & Spooner, D.M. (2009) A test of taxonomic predictivity: resistance to the Colorado potato beetle in wild relatives of cultivated potato. *Journal of Economic Entomology,* **102,** 422-431.

Kaneko, K., Tanaka, M.W. & Mitsuhashi, H. (1977) Dormantinol, a possible precursor in solanidine biosynthesis, from budding Veratrum-grandiflorum. *Phytochemistry,* **16,** 1247-1251.

Kim, O.T., Seong, N.S., Kim, M.Y. & Hwang, B. (2005) Isolation and characterization of squalene synthase cDNA from *Centella asiatica* (L.) Urban. *Journal of Plant Biology,* **48,** 263-269.

Kolbe, H. & StephanBeckmann, S. (1997) Development, growth and chemical composition of the potato crop (*Solanum tuberosum* L.) .1. Leaf and stem. *Potato Research,* **40,** 111-129.

Kowalski, S.P., Domek, J.M., Deahl, K.L. & Sanford, L.L. (1999) Performance of Colorado potato beetle larvae, *Leptinotarsa decemlineata* (Say), reared on synthetic diets supplemented with *Solanum* glycoalkaloids. *American Journal of Potato Research,* **76,** 305-312.

Kribii, R., Arro, M., DelArco, A., Gonzalez, V., Balcells, L., Delourme, D., Ferrer, A., Karst, F. & Boronat, A. (1997) Cloning and characterization of the *Arabidopsis thaliana SQS1* gene encoding squalene synthase - Involvement of the C-terminal region of the enzyme in the channeling of squalene through the sterol pathway. *European Journal of Biochemistry,* **249,** 61-69.

Krits, P., Fogelman, E. & Ginzberg, I. (2007) Potato steroidal glycoalkaloid levels and the expression of key isoprenoid metabolic genes. *Planta,* **227,** 143-150.

Kuenzel, E.A. & Krebs, E.G. (1985) A synthentic peptide substrate specific for casien kinase II. *Proceedings of the National Academy of Science,* **82,** 737-741.

Lachman, J., Hamouz, K., Orsak, M. & Pivec, V. (2001) Potato glycoalkaloids and their significance in plant protection and human nutrition - Review. *Series Rostlinna,* **47,** 181-191.

Laurila, J., Laakso, I., Valkonen, J.P.T., Hiltunen, R. & Pehu, E. (1996) Formation of parental-type and novel glycoalkaloids in somatic hybrids between *Solanum brevidens* and S. *tuberosum*. *Plant Science,* **118,** 145-155.

Lawson, R.D., Veilleux, R.E. & Miller, A.R. (1993) Biochemistry and genetics of *Solanum chacoense* steroidal alkaloids: Natural resistance factors to the Colorado potato beetle. *Current Topics in Bot. Research,* **1,** 335-352.

Lee, M.H., Jeong, J.H., Seo, J.W., Shin, C.G., Kim, Y.S., In, J.G., Yang, D.C., Yi, J.S. & Choi, Y.E. (2004) Enhanced triterpene and phytosterol biosynthesis in *Panax ginseng* overexpressing squalene synthase gene. *Plant and Cell Physiology,* **45,** 976-984.

Lee, S. & Poulter, C.D. (2008) Cloning, solubilization, and characterization of squalene synthase from *Thermosynechococcus elongatus* BP-1. *Journal of Bacteriology,* **190,** 3808-3816.

Lipinski, R.J., Dengler, E., Kiehn, M., Peterson, R.E. & Bushman, W. (2007) Identification and characterization of several dietary alkaloids as weak inhibitors of Hedgehog signaling. *Toxicological Sciences,* **100,** 456-463.

Liu, Y.G. & Chen, Y. (2007) High-efficiency thermal asymmetric interlaced PCR for amplification of unknown flanking sequences. *BioTechniques,* **43,** 649-+.

Maga, J.A. (1981) Total and individual glycoalkaloid composition of stored potato slices. *Journal of Food Processing and Preservation,* **5,** 23-29.

Maga, J.A. (1994) Glycoalkaloids in Solanaceae. *Food Reviews International,* **10,** 385-418.

Marrero, P.F., Poulter, C.D. & Edwards, P.A. (1992) Effects of site-directed mutagenesis of the highly conserved aspartate residues in domain-ii of farnesyl diphosphate synthase activity. *Journal of Biological Chemistry,* **267,** 21873-21878.

McCue, K.F., Allen, P.V., Rockhold, D., Corsini, D.L., Shepherd, L.V.T., Davies, H., Moehs, C.P. & Joyce, P. (2002) Reduction of total steroidal glycoalkaloids in potato tubers using antisense constructs of a gene encoding a solanidine glucosyl transferase. *26th International Horticultural Congress* (ed R. Y. Yada), pp. 77-86. Toronto, Canada.

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

McCue, K.F., Allen, P.V., Shepherd, L.V.T., Blake, A., Rockhold, D.R., Novy, R.G., Stewart, D., Davies, H.V. & Belknap, W.R. (2006) Manipulation and compensation of steroidal glycoalkaloid biosynthesis in potatoes. *6th International Solanaceae Conference* (ed D. M. B. L. G. J. Spooner), pp. 343-349. Madison, WI.

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

McCue, K.F., Shepherd, L.V.T., Allen, P.V., Maccree, M.M., Rockhold, D.R., Corsini, D.L., Davies, H.V. & Belknap, W.R. (2005) Metabolic compensation of steroidal glycoalkaloid biosynthesis in transgenic potato tubers: using reverse genetics to confirm the in vivo enzyme function of a steroidal alkaloid galactosyltransferase. *Plant Science,* **168,** 267-273.

McKey, D. (1974) Adaptive patterns in alkaloid physiology. *The American Naturalist,* **108,** 305-320.

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

Mondy, N.I. & Munshi, C.B. (1988) Effect of molybdenum fertilization on the nitrogenous constituents of potatoes. *Journal of Food Science,* **53,** 1901-1910.

Mweetwa, A.M., Poe, R. & Tokuhisa, J.G. (2008) Biosynthesis and regulation of steroidal glycoalkaloids in wild potato, *Solanum chacoense* Bitter. *105th Annual Conference of the American Society for Horticultura -Science*, pp. 1287-1287. Orlando, FL.

Nakashima, T., Inoue, T., Oka, A., Nishino, T., Osumi, T. & Hata, S. (1995) Cloning, expression, and characterization of cDNAs encoding *Arabidopsis thaliana* squalene synthase. *Proceedings of the National Academy of Sciences of the United States of America,* **92,** 2328-2332.

Newman, J.D. & Chappell, J. (1999) Isoprenoid biosynthesis in plants: Carbon partitioning within the cytoplasmic pathway. *Critical Reviews in Biochemistry and Molecular Biology,* **34,** 95-106.

Nitithamyong, A., Vonelbe, J.H., Wheeler, R.M. & Tibbitts, T.W. (1999) Glycoalkaloids in potato tubers grown under controlled environments. *American Journal of Potato Research,* **76,** 337-343.

Olkkonen, V.M. & Hynynen, R. (2009) Interactions of oxysterols with membranes and proteins. *Molecular Aspects of Medicine,* **30,** 123-133.

Pandit, J., Danley, D.E., Schulte, G.K., Mazzalupo, S., Pauly, T.A., Hayward, C.M., Hamanaka, E.S., Thompson, J.F. & Harwood, H.J. (2000) Crystal structure of human squalene synthase - A key enzyme in cholesterol biosynthesis. *Journal of Biological Chemistry,* **275,** 30610-30617.

Papathanasiou, F., Mitchell, S.H. & Harvey, B.M.R. (1998) Glycoalkaloid accumulation during tuber development of early potato cultivars. *Potato Research,* **41,** 117-125.

Papathanasiou, F., Mitchell, S.H. & Harvey, B.M.R. (1999) Variation in glycoalkaloid concentration of potato tubers harvested from mature plants. *Journal of the Science of Food and Agriculture,* **79,** 32-36.

Pelletier, Y., Grondin, G. & Maltais, P. (1999) Mechanism of resistance to the Colorado potato beetle in wild *Solanum* species. *Journal of Economic Entomology,* **92,** 708-713.

Percival, G. & Dixon, G.R. (1996) Glycoalkaloid concentrations in aerial tubers of potato (Solanum tuberosum L). *Journal of the Science of Food and Agriculture,* **70,** 439-448.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research,* **29**.

Piironen, V., Lindsay, D.G., Miettinen, T.A., Toivo, J. & Lampi, A.M. (2000) Plant sterols: biosynthesis, biological function and their importance to human nutrition. *Journal of the Science of Food and Agriculture,* **80,** 939-966.

Raman, K.V. & Radcliffe, E.B. (1992) Pest aspects of potato production: Part 2 Insect pests. *The potato crop: The scientific basis for improvement* (ed P. M. Harris). Chapman and Hall, London.

Rayburn, J.R., Bantle, J.A. & Friedman, M. (1994) Role of carbohydrate side-chains of potato glycoalkaloids in developmental toxicity. *Journal of Agricultural and Food Chemistry,* **42,** 1511-1515.

Rayburn, J.R., Friedman, M. & Bantle, J.A. (1995) Synergistic interaction of glycoalkaloids alpha-chaconine and alpha-solanine on developmental toxicity in *Xenopus* embryos. *Food and Chemical Toxicology,* **33,** 1013-1019.

Robinson, G.W., Tsay, Y.H., Kienzle, B.K., Smithmonroy, C.A. & Bishop, R.W. (1993) Conservation between human and fungal squalene synthetases - similarities in structure, function, and regulation. *Molecular and Cellular Biology,* **13,** 2706-2717.

Roddick, J.G. (1980) A sterol-binding assay for potato glycoalkaloids. *Phytochemistry,* **19,** 2455-2457.

Roddick, J.G. & Rijnenberg, A.L. (1987) Synergistic interaction between the potato glycoalkaloids alpha-solanine and alpha-chaconine in relation to lysis of phospholipid sterol liposomes. *Phytochemistry,* **26,** 1325-1328.

Roddick, J.G., Rijnenberg, A.L. & Weissenberg, M. (1992) Alterations to the permeability of liposome membranes by the solasodine-based glycoalkaloids solasonine and solamargine. *Phytochemistry,* **31,** 1951-1954.

Roddick, J.G., Weissenberg, M. & Leonard, A.L. (2001) Membrane disruption and enzyme inhibition by naturally-occurring and modified chacotriose-containing *Solanum* steroidal glycoalkaloids. *Phytochemistry,* **56,** 603-610.

Rogers, S., Wells, R. & Rechsteiner, M. (1986) Amino acid sequences common to rapidly degraded proteins: the pest hypothesis. *Science,* **234,** 364-368.

Ronning, C.M., Stommel, J.R., Kowalski, S.P., Sanford, L.L., Kobayashi, R.S. & Pineada, O. (1999) Identification of molecular markers associated with leptine production in a population of *Solanum chacoense* Bitter. *Theoretical and Applied Genetics,* **98,** 39-46.

Sanford, L.L., Deahl, K.L., Sinden, S.L. & Kobayashi, R.S. (1995) Glycoalkaloid content in tubers of hybrid and backcross populations from a *Solanum tubersoum* X S. *chacoense* cross. *American Potato Journal* **72,** 261-271.

Sanford, L.L., Deahl, K.L., Sinden, S.L. & Kobayashi, R.S. (1996) Segregation of leptines and other glycoalkaloids in *Solanum tuberosum* (4x) x S. *chacoense* (4x) crosses. *American Potato Journal,* **73,** 21-33.

Sanford, L.L., Kobayashi, R.S., Deahl, K.L. & Sinden, S.L. (1997) Diploid and tetraploid Solanum chacoense genotypes that synthesize leptine glycoalkaloids and deter feeding by Colorado potato beetle. *American Potato Journal,* **74,** 15-21.

Schaller, H. (2004) New aspects of sterol biosynthesis in growth and development of higher plants. *Plant Physiology and Biochemistry,* **42,** 465-476.

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

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

Shen, Y.J., Ji, G.L., Haas, B.J., Wu, X.H., Zheng, J.T., Reese, G.J. & Li, Q.Q. (2008) Genome level analysis of rice mRNA 3'-end processing signals and alternative polyadenylation. *Nucleic Acids Research,* **36,** 3150-3161.

Sinden, S.L., Sanford, L.L., Cantelo, W.W. & Deahl, K.L. (1986) Leptine glycoalkaloids and resistance to the Colorado potato beetle (Coleoptera, Chrysomelidae) in *Solanum chacoense*. *Environmental Entomology,* **15,** 1057-1062.

Sinden, S.L., Sanford, L.L. & Osman, S.F. (1980) Glycoalkaloids and resistance to the Colorado potato beetle in *Solanum chacoense* Bitter. *American Potato Journal,* **57,** 331-343.

Sinden, S.L., Sanford, L.L. & Webb, R.E. (1984) Genetic and environmental control of potato glycoalkaloids. *American Potato Journal,* **61,** 141-156.

Smith, D.B., Roddick, J.G. & Jones, J.L. (1996) Potato glycoalkaloids: Some unanswered questions. *Trends in Food Science & Technology,* **7,** 126-131.

Smith, D.B., Roddick, J.G. & Jones, J.L. (2001) Synergism between the potato glycoalkaloids alpha-chaconine and alpha-solanine in inhibition of snail feeding. *Phytochemistry,* **57,** 229-234.

Smith, S.W., Giesbrecht, E., Thompson, M., Nelson, L.S. & Hoffman, R.S. (2008) Solanaceous steroidal glycoalkaloids and poisoning by *Solanum torvum*, the normally edible susumber berry. *Toxicon,* **52,** 667-676.

Soule, S., Guntner, C., Vazquez, A., Argandona, V.H., Ferreira, F. & Moyna, P. (1999) Effect of *Solanum* glycosides on the aphid *Schizaphis graminum*. *Journal of Chemical Ecology,* **25,** 369-374.

Sowokinos, J.R. (2007) Internal physiological disorders and nutritional compositional factors that affect market quality. *Potato Biology and Biotechnology* (ed D. Vreugdenhil). Elsevier.

Stapleton, A., Allen, P.V., Friedman, M. & Belknap, W.R. (1991) Purification and characterization of solanidine glucosyltransferase from the potato (*Solanum tuberosum*). *Journal of Agricultural and Food Chemistry,* **39,** 1187-1193.

Stiller, I., Davis, G. & Hesse, H. (2007) Improving the nutritive value of tubers: Elevation of cystein and glutathione contents in the potato cultvar White Lady by marker-free transformation. *Journal of Biotechnology,* **128,** 335-343.

Storey, R.M.J. (2007) The harvested crop. *Potato biology and biotechnology: Advances and perspectives* (ed D. Vreugdenhil), pp. 441-466. Elsevier, Amstedam.

Storey, R.M.J. & Davies, H.V. (1992) Tuber quality. The potato crop-the scientific basis for improvement (ed P. M. Harris). Chapman and Hall, London.

Suzuki, H., Achnine, L., Xu, R., Matsuda, S.P.T. & Dixon, R.A. (2002) A genomics approach to the early stages of triterpene saponin biosynthesis in *Medicago truncatula*. *Plant Journal,* **32,** 1033-1048.

Thompson, A.L., Love, S.L., Sowokinos, J.R., Thornton, M.K. & Shock, C.C. (2008) Review of the sugar end disorder in potato (Solanum tuberosum, L.). *American Journal of Potato Research,* **85,** 375-386.

Threfall, D.R. & Whitehead, I.M. (1988) Coordination of *squalene synthetase* and induction of sesquiterpenoid phytoalexins biosynthesis in cultures of *Nicotiana tabacum*. *Phytochemistry,* **27,** 2567-2580.

Tingey, W.M. (1984) Glycoalkaloids as pest resistance factors. *American Potato Journal,* **61,** 157-167.

Tingey, W.M., Mackenzie, J.D. & Peter, G. (1978) Total foliar glycoalkaloids and resistance of wild potato species to *Empoasca fabae* (Harris) *American Journal of Potato Research* **55,** 577-585.

Uchida, H., Yamashita, H., Kajikawa, M., Ohyama, K., Nakayachi, O., Sugiyama, R., Yamato, K.T., Muranaka, T., Fukuzawa, H. & Takemura, M. (2009) Cloning and characterization of a squalene synthase gene from a petroleum plant, Euphorbia tirucalli L. *Planta,* **229,** 1243-1252.

Valkonen, J.P.T., Keskitalo, M., Vasara, T. & Pietila, L. (1996) Potato glycoalkaloids: A burden or a blessing? *Critical Reviews in Plant Sciences,* **15,** 1-20.

Vazquez, A., Gonzalez, G., Ferreira, F., Moyna, P. & Kenne, L. (1997) Glycoalkaloids of *Solanum commersonii* Dun ex Poir. *Euphytica,* **95,** 195-201.

Veillux RE, Miller AR (1998) Hybrid breakdown in the F1 between *Solanum chacoense* and *S. phureja* and gene transfer for leptine biosynthesis. Journal of the American Society for Horticultural Science 123, 854-858.

Vogeli, U. & Chappell, J. (1988) Induction of sesquiterpene cyclase and suppression of squalene synthetase in plant cell cultures treated wth fungal elicitor. *Plant Physiology,* **88,** 1291-1296.

Wentzinger, L.F., Bach, T.J. & Hartmann, M.-A. (2002) Inhibition of squalene synthase and squalene epoxide in tobacco cells triggers an up-regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Plant Physiology,* **130,** 1-13.

Xianzhi, X. & Naihu, W. (2002) Introns in higher plant genes. *Chinese Science Bulletin,* **47,** 1409-1415.

Yoshioka, H., Yamada, N. & Doke, N. (1999) CDNA cloning of sesquiterpene cyclase and squalene synthase, and expression of the genes in potato tuber infected with Phytophthora infestans. *Plant and Cell Physiology,* **40,** 993-998.

Zacharius, R.M. & Osman, S.F. (1977) Glycoalkaloids in tissue culture of *Solanum* species - dehydrocommersonine from cultured roots of *Solanun chacoense*. *Plant Science Letters,* **10,** 283-287.

Zimowski, I. (1991) Occurrence of a glucosyltransferase specific for solanidine in potato plants. *Phytochemistry*, **30,** 1827-1831.

3.6 Tables and Supplemental Figures

Table 3- 2: Primers used for cDNA cloning

Primer	Direction	Sequence 5'-3'	Region
SQSSYN F	+	TGCTCTCGTCATTCAACAGC	5'
SQSSYN R	-	TCCCGATCTCTGCAAGTTT	3'
SQS.1A F	+	ATGGGAACATGAGGGCGA	5'
SQS.2 A R	-	ACCAATAAATGGTCTAAGATCG	3'
1SQSEX9EX10 (9F)	+	ATAGCACATAGCTAATGTCCC	3'
1SQSEX10EX10 (10F)	+	GGTCATTGACCGGACCAAGA	3'
RP-Oligo-dT1	-	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT	Poly A
SQS-0a	-	CGACAGGAAGCTGTTGAATGACGAGAGCAAAAC	5'
SQS-1a	-	ACGATGGACTCCAGTCCGGCCCCAATGTGGCTCAGGCGGGATCTGCTTTTC	5'
SQS-2a	-	CTGGATTCTTCAGAATCGCCCTCAATGTTC	5'
LAD1-1	+	ACGATGGACTCCAGAGCGGCCGC(G/C/A)N (G/C/A)NNNGGAA	Random
LAD1-2	+	ACGATGGACTCCAGAGCGGCCGC(G/C/T)N (G/C/A)NNNGGTT	Random
LAD1-3	+	ACGATGGACTCCAGAGCGGCCGC(G/C/A) (G/C/A)(N (G/C/A)NNNCCAA	Random
LAD1-4	+	ACGATGGACTCCAGAGCGGCCGC(G/C/T) (G/C/T)N (G/C/T)NNNCGGT	Random
AC1	+	ACGATGGACTCCAGAG	Random
1SQSEX8EX9	+	CGAGGAGAACTCGGTTAGG	3'
1SQSEX9EX9	-	ATAGCACATAGCTAATGTCCC	3'
Actin F	+	ATGGCAGACGGAGAGGATATTC	
Actin R	-	CCTGTGGACAATAGAAGGAC	

Table 3- 3: Primers used for genomic DNA sequence compilation

Primer	Direction	Sequence 5'-3'	Region
1SQS1EX1EX2	+	CTCCCTTTGGCTTTCTGGAT	Exon 1
1SQS1EX2EX3	+	GGTCCTTCGAGCACTGGACA	Exon 2
1SQS1EX3EX4	+	CCAGCATACCCACCGATGTT	Exon 3
1SQS1EX4EX5	+	GGAGTACAAGGTTCTCATGG	Exon 4
1SQS1EX5EX6	+	GGACATTACCATGAGGATGG	Exon 5
1SQS1EX6EX7	+	TATGTAGCTGGGCTTGTTGG	Exon 6
1SQS1EX7EX8	+	GCCCCGTGAGATTTGGAGTA	Exon 7
1SQS1EX8EX9	+	CGAGGAGAACTCGGTTAAGG	Exon 8
1SQS1EX9EX10	+	GGGACATTAGCTATGTGCTAT	Exon 9
1SQS1EX10EX11	+	GGTCATTGACCGGACCAAGA	Exon 10
1SQS1EX11EX12	+	TGAAGAGGCTTGACGCCATC	Exon 11
1SQS1EX12EX13	+	GCCTAATTACAGTCCAGTTCT	Exon 12
1SQS2EX2EX1	-	TGTCCAGTGCTCGAAGGACC	Exon 2
1SQS2EX3EX2	-	AACATCGGTGGGTATGCTGG	Exon 3
1SQS2EX4EX3	-	CCATGAGAACCTTGTACTCC	Exon 4
1SQS2EX5EX4	-	CCATCCTCATGGTAATGTCC	Exon 5
1SQS2EX6EX5	-	CCAACAAGCCCAGCTACATA	Exon 6
1SQS2EX7EX6	-	TACTCCAAATCTCACGGGGC	Exon 7
1SQS2EX8EX7	-	CCTTAACCGAGTTCTCCTCG	Exon 8
1SQS2EX9EX8	-	ATAGCACATAGCTAATGTCCC	Exon 9
1SQS2EX10EX9	-	TCTTGGTCCGGTCAATGACC	Exon 10
1SQS2EX11EX10	-	AGGATGGCGTCAAGCCCTT	Exon 11
1SQS2EX12EX11	-	AGAACTGGACTGTAATTAGGC	Exon 12
1SQS2EX13EX12	-	CTAAGATCGGTTGCCAGAA	Exon 13

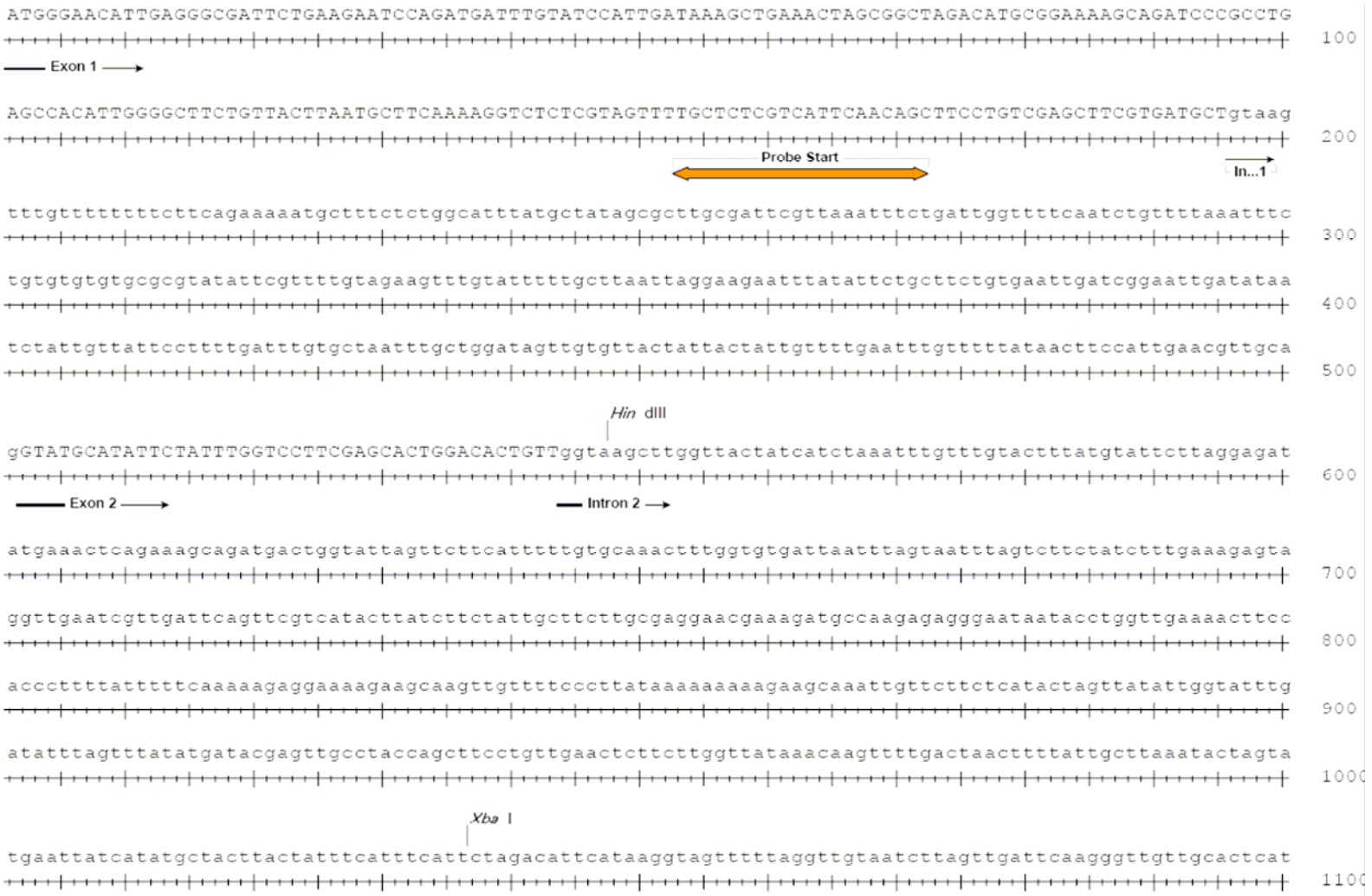


Figure 3-8 Whole genomic sequence of *S. chacoense* squalene synthase. Upper case letters represent the exonic nucleotides, while the lower case represent the intronic nucleotides. (Incomplete sequence for intron # 10 indicated as 'n').

Figure 3-8 Whole genomic sequence of *S. chacoense squalene synthase* (Cont'd)

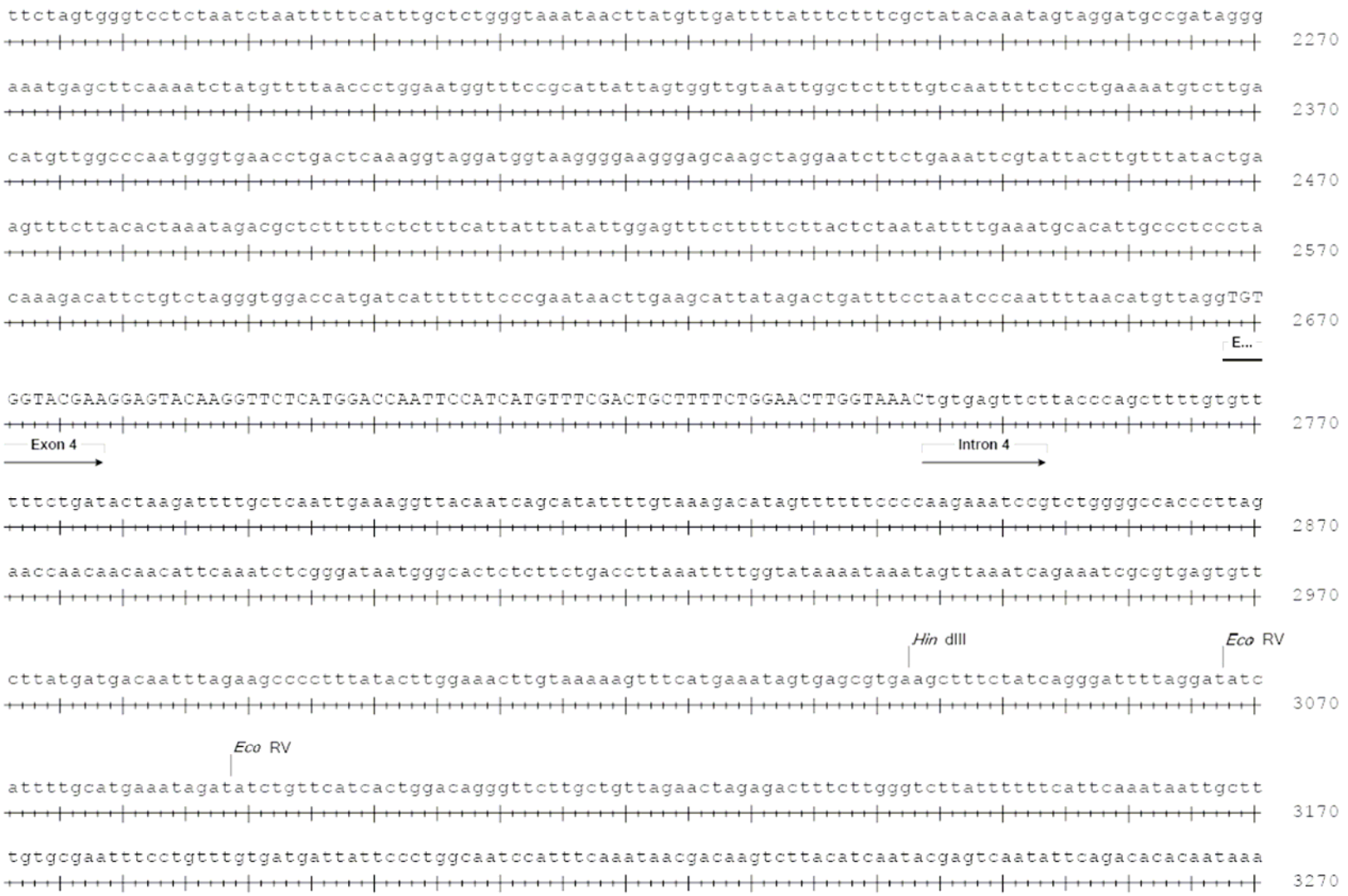


Figure 3-8 Whole genomic sequence of *S. chacoense* squalene synthase (Cont'd)

Figure 3-8 Whole genomic sequence of *S. chacoense* squalene synthase (Cont'd

Figure 3-8 Whole genomic sequence of *S. chacoense* squalene synthase (Cont'd

Figure 3-8 Whole genomic sequence of *S. chacoense* squalene synthase (Cont'd

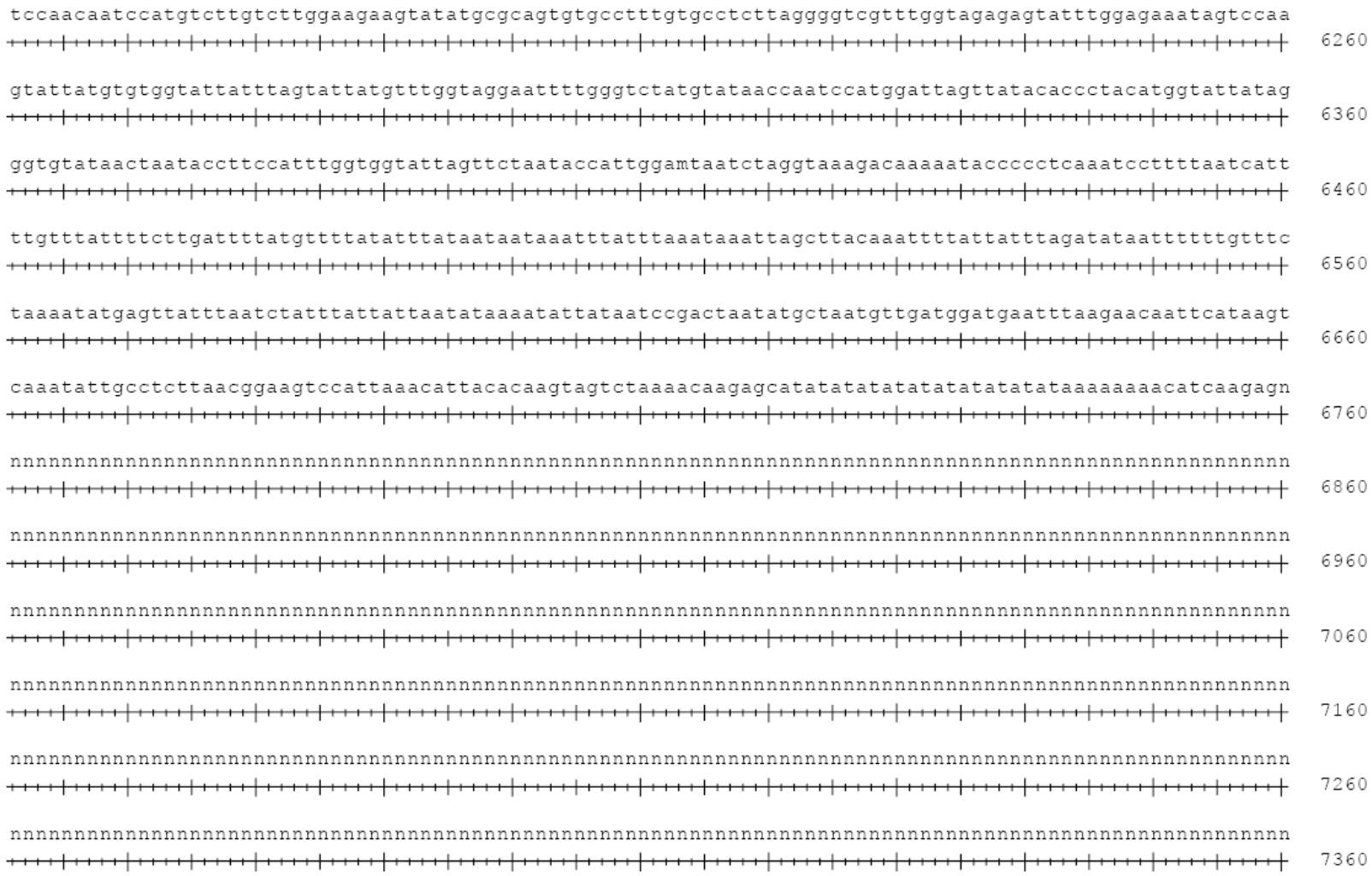


Figure 3-8 Whole genomic sequence of *S. chacoense* squalene synthase (Cont'd

Figure 3-8 Whole genomic sequence of *S. chacoense* squalene synthase (Cont'd

4 Conclusions

The goal of this study was to gain an understanding of the biosynthesis of SGAs in *Solanum chacoense*. The first objective was to understand the location and timing of enzyme activities necessary for the accumulation of various SGA compounds. To accomplish this objective, a time course survey on SGA levels and composition in a developing plant was established and plant tissues that accumulate novel or differential levels of SGAs identified. *S. chacoense* 8380-1 plants were grown and harvested at the early, mid, and late vegetative, anthesis, and post-anthesis stages and SGAs monitored. The results showed that *S. chacoense* has the capability to accumulate different levels and types of SGAs in a developmental and tissue specific manner. The LC/MS and HPLC analyses allowed us to identify α-solanine, α-chaconine, dehydrocommersonine, leptines I & II, and leptinine I & II. While all the tissues had the capability to accumulate α-solanine, and α-chaconine, dehydrocommersonine accumulation was restricted to the roots, and the leptines and leptinines to the aerial tissues. It can be concluded that *S. chacoense* tissues might have independent biosynthetic pathways that enable them to accumulate unique SGAs types in the different tissues. However, there is still a need to isolate the various compounds (especially the leptines and leptinines) observed in the aerial tissues to confirm their identities using different techniques such as the nuclear magnetic resonance.

The levels of total SGA generally increased with plant development although levels in tubers, stolons and roots decreased after anthesis. Young leaves, flowers and floral buds

tended to accumulate the greatest levels of total SGAs; this shows SGAs accumulate readily in tissues with high metabolic activities.

The second objective was to establish alternative modes of squalene synthase regulation in *S. chacoense*. In order to accomplish this objective, the squalene synthase gene was cloned and characterized. The results showed that the cloned cDNA was a putative *squalene synthase* gene encoding a protein with 411 amino acid residues and high sequence similarity (68-100%) to other known plant squalene synthases. The cDNA has polymorphic 3'UTR possibly due to the presence of multiple mRNA species brought about through alternative polyadenylation. *S. chacoense squalene synthase* genomic sequence showed a complex organization with 13 exons and 12 introns, ranging in size from 42-195, and 73-2500 bp, respectively. The Southern blot analysis revealed that *S. chacoense* genome has at least one copy of squalene synthase, although the existence of a second copy cannot be precluded.

In order to establish a relationship between SGA levels and expression of isoprenoid genes, the gene expression levels were monitored using both semi-quantitative and real time PCRs. The results from the semi quantitative PCR showed that the expression levels of *squalene synthase, sgt1, sgt2* and *hmg1* correlated well with SGA levels in floral buds, roots and tubers. However, the results from the real time PCR did not confirm this correlation between relative gene expression levels of *squalene synthase* and SGAs in the same tissues. With these results, it is not possible to establish the direct role of squalene synthase in determining the levels of SGAs. Because regulation of squalene synthase

could be at the posttranscriptional level or beyond, further experimentation is imperative. Such experiments would seek to establish the relationship between sterol and SGA accumulation and squalene synthase enzyme expression and activity.

Appendices: Analysis of variance tables

Analysis of variance for total SGAs in **roots** different developmental stages of *S. chacoense* 80-1 ($\alpha = 0.05$)

Source of Variance	Degrees of freedom	Sums of Squares	Mean Square	F-Value	Probability
Developmental stage	5	171.2	34.2	8.6	0.0015
Error	11	43.6	4		
Total	16	214.79			

Analysis of variance for total SGAs in **stolons** different developmental stages of *S. chacoense* 80-1 ($\alpha = 0.05$)

Source of Variance	Degrees of freedom	Sums of Squares	Mean Square	F-Value	Probability
Developmental stage	3	2531.7	853.9	6.5	0.0154
Error	8	1039.06	129.9		
Total	11	3570.8			

Analysis of variance for total SGAs in **tubers** different developmental stages of *S. chacoense* 80-1 ($\alpha = 0.05$)

Source of Variance	Degrees of freedom	Sums of Squares	Mean Square	F-Value	Probability
Developmental stage	3	38.8	12.9	1.2	0.41
Error	4	42.8	10.7		
Total	7	81.6			

Analysis of variance for total SGAs in different **stem** positions at the late vegetative developmental stage of *S. chacoense* 80-1 ($\alpha = 0.05$)

Source of Variance	Degrees of freedom	Sums of Squares	Mean Square	F-Value	Probability
Position	2	125.4	62.7	1.9	0.23
Error	6	195.1	32.5		
Total	8	320.5			

Analysis of variance for ratios of α-chaconine: -solanine in the **tubers** at different developmental stage of *S. chacoense* 80-1 (α = 0.05)

Source of Variance	Degrees of freedom	Sums of Squares	Mean Square	F-Value	Probability
Developmental stage	3	0.24	0.08	0.43	0.74
Error	4	0.75	0.19		
Total	7	1.00			

Analysis of variance for ratios of α-chaconine: -solanine in the **stolons** at different developmental stage of *S. chacoense* 80-1 (α = 0.05)

Source of Variance	Degrees of freedom	Sums of Squares	Mean Square	F-Value	Probability
Developmental stage	3	0.08	0.03	1.83	0.21
Error	8	0.11	0.01		
Total	11	0.18			

Analysis of variance for levels of other SGAs in the **young expanding leaves** at different developmental stage of *S. chacoense* 80-1 ($\alpha = 0.05$)

Source of Variance	Degrees of freedom	Sums of Squares	Mean Square	F-Value	Probability
Developmental stage	3	5179.2	1726.4	875.6	<0.0001
Error	8	15.8	2.0		
Total	11	5194.9			

Analysis of variance for levels of other SGAs in the **fully expanded leaves** at different developmental stage of *S. chacoense* 80-1 ($\alpha = 0.05$)

Source of Variance	Degrees of freedom	Sums of Squares	Mean Square	F-Value	Probability
Developmental stage	3	3362.3	1120.8	14684.1	<0.0001
Error	8	0.6	0.1		
Total	11	3362.9			

Analysis of variance for levels of other SGAs in the **senescing leaves** at different developmental stage of *S. chacoense* 80-1 ($\alpha = 0.05$)

Source of Variance	Degrees of freedom	Sums of Squares	Mean Square	F-Value	Probability
Developmental stage	3	1101.3	1726.4	603476	<0.0001
Error	8	0.0	0.00		
Total	11	1101.3			