Final Scientific Report

Cover Page

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Project Title: Identification and allelic variation of genes involved in the potato glycoalkaloid biosynthetic pathway

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Solanum, isoprenoid pathway, HMGR, squalene synthase, solanidine glycosyltransferase, *Solanum chacoense*, potato periderm, phytosterol.

Abbreviations commonly used in the report, in alphabetical order:

DW, dry weight; HMGR, 3-hydroxy-3-methylglutaryl coenzyme-A reductase; SGAs, steroidal glycoalkaloids; SGT, solanidine glycosyltransferase; SNP, single nucleotide polymorphism; SQS, squalene synthase.

Budget: IS: \$159,000

US: \$159,000

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Idit Ginzberg

Signature
Principal Investigator

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Authorizing Official, Principal Institution

Final Scientific Report

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	2			2
Submitted, in review, in preparation	2			2
Invited review papers				
Book chapters				
Books				
Master theses	1			1
Ph.D. theses	2			2
Abstracts	9	3		12
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Postdoct trainees in the lab of the Israeli PI

- Ufuk Demirel – passport TR-V 218328
- Akhilesh Kumar – ID: 0595049

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		1		1
Longer Visits (Sabbaticals)				

Description Cooperation:

The PIs met in the USA to summarize the data of the first two years of the project and to coordinate their workplan for the last research year.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

Abstract

Steroidal glycoalkaloids (SGAs) are secondary metabolites being part of the plant defense response. The two major SGAs in cultivated potato (*Solanum tuberosum*) are α -chaconine and α -solanine, which exhibit strong cellular lytic properties and inhibit acetylcholinesterase activity, and are poisonous at high concentrations for humans. As SGAs are not destroyed during cooking and frying commercial cultivars have been bred to contain low levels, and their content in tubers should not exceed 20 mg/100 g fresh weight. However, environmental factors can increase tuber SGA content above the safe level. The focus of the proposed research was to apply genomic approaches to identify candidate genes that control potato SGA content in order to develop tools for potato improvement by marker-assisted selection and/or transgenic approaches. To this end, the objectives of the proposal included identification of genes, metabolic intermediates and allelic variations in the potato SGA biosynthetic pathway.

The SGAs are biosynthesized by the sterol branch of the mevalonic acid/isoprenoid pathway. Transgenic potato plants that overexpress 3-hydroxy-3-methylglutaryl-CoA reductase 1 (*HMG1*) or squalene synthase 1 (*SQS1*), key enzymes of the mevalonic acid/isoprenoid pathway, exhibited elevated levels of solanine and chaconine as well as induced expression of genes downstream the pathway. These results suggest of coordinated regulation of isoprenoid (primary) metabolism and SGA secondary metabolism. The transgenic plants were further used to identify new SGA-related candidate genes by cDNA-AFLP approach and a novel glycosyltransferase was isolated. In addition, genes involved in phytosterol biosynthesis may have dual role and synthesize defense-related steroidal metabolites, such as SGAs, via lanosterol pathway. Potato lanosterol synthase sequence (*LAS*) was isolated and used to prepare transgenic plants with overexpressing and silencing constructs. Plants are currently being analyzed for SGA content.

The dynamics of SGA accumulation in the various organs of a potato species with high SGA content gave insights into the general regulation of SGA abundance. Leaf SGA levels in *S. chacoense* were 10 to 20-fold greater than those of *S. tuberosum*. The leptines, SGAs with strong antifeedant properties against Colorado potato beetles, were present in all aerial tissues except for early and mid-developmental stages of above ground stolons, and accounted for the high SGA content of *S. chacoense*. These results indicate the presence of regulatory mechanisms in most tissues except in stolons that limit the levels of α -solanine and α -chaconine and confine leptine accumulation to the aerial tissues.

The genomes of cultivated and wild potato contain a 4-member gene family coding for SQS. Three orthologs were cloned as cDNAs from *S. chacoense* and heterologously expressed in *E. coli*. Squalene accumulated in all *E. coli* lines transformed with each of the three gene constructs. Differential transcript abundance in various organs and amino acid sequence differences in the conserved domains of three isoenzymes indicate subfunctionalization of SQS activity and triterpene/sterol metabolism.

Because *S. chacoense* and *S. phureja* differ so greatly for presence and accumulation of SGAs, we selected four candidate genes from different points along the biosynthetic pathway to determine if *chc* or *phu* specific alleles were associated with SGA expression in a segregating interspecific diploid population. For two of the four genes (*HMG2* and *SGT2*) F₂ plants with *chc* alleles expressed significantly greater total SGAs compared with heterozygotes and those with *phu* alleles. Although there are other determinants of SGA biosynthesis and composition in potato, the ability of allelic states at two genes to affect SGA levels confirms some of the above transgenic work where *chc* alleles at two other loci altered SGA expression in Desiree.

Present results reveal new opportunities to manipulate triterpene/sterol biosynthesis in more targeted ways with the objective of altering SGA content for both human health concerns and natural pesticide content without disrupting the essential metabolism and function of the phytosterol component of the membranes and the growth regulating brassinosteroids.

Achievements

1.1 Identification of SGA-related biosynthetic genes and potential regulation points

Previous studies indicated of a correlation between SGA levels and the abundance of transcript coding for *HMG1* and *SQS1* in potato tissues and potato genotypes varying in SGA content. At the present proposal, *Solanum tuberosum* cv. Desirée (low SGA producer) was transformed with a gene construct containing the coding region of either *HMG1* or *SQS1* of *Solanum chacoense* Bitt. clone 8380-1, a high SGA producer. SGA levels in transgenic HMG-plants were either greater than (in eight of 14 plants) or no different from untransformed controls, whereas only four of 12 SQS-transgenics had greater SGA levels than control, as determined by HPLC. Quantitative real-time PCR was used to estimate relative steady-state transcript levels of isoprenoid-, steroid-, and SGA-related genes in leaves of the transgenic plants compared to nontransgenic controls. HMG-transgenic plants exhibited increased transcript accumulation of downstream genes such as *SQS1*, sterol C24-methyltransferase type1 (*SMT1*) and solanidine glycosyltransferase 2 (*SGT2*), while SQS-transgenic plants had consistently lower transcript levels of *HMG1*. These results indicate of coordinated regulation of HMGR and SQS, and support previous reports of negative-feedback regulation of HMGR activity by high concentrations of end-product sterols and pathway intermediates, i.e. high sterol level in the SQS-transgenic plants led to downregulated levels of endogenous *HMG1*. HMG-transgenic plants exhibited changes in transcript accumulation for some sterol biosynthetic genes as well. Taken together, the data suggest coordinated regulation of isoprenoid metabolism and SGA secondary metabolism.

The branch point that separates the SGA-specific pathway from primary metabolism has not yet been verified. SGAs are derived from cholesterol, which in animals and fungi requires the activity of lanosterol synthase (LAS). Plants were considered to lack LAS and therefore the formation of cholesterol and phytosterols was thought to involve only cycloartenol synthase (CAS). LAS has recently been identified in plants and implicated by gene expression profiling in the chemical defense response. We have isolated the potato LAS coding region and promoter to clarify the role of LAS in sterol metabolism. Desirée plants (low SGA) were transformed with overexpression and silencing constructs of the endogenous potato *LAS1* and *CAS1* genes, and are currently being analyzed for SGA levels.

In addition, HMG-transgenic plants with altered high levels of SGAs were used to identify new SGA-related genes. Using cDNA-AFLP approach where RNA samples of transgenic plants were compared to non-transgenic control plants resulted with the isolation of an unknown glycosyltransferase (GT). Transgenic plants containing silencing construct of the GT were prepared and are currently being analyzed for solanine and chaconine levels.

1.2 Dynamics of SGA accumulation and squalene synthase expression in *S. chacoense*

A comprehensive time course analysis of SGA profile and content in all organs of *S. chacoense* Bitt. USDA 8380-1 was undertaken to define the dynamics of SGA accumulation during the life cycle of a wild potato species with high SGA content. *S. chacoense* contains α -solanine and α -chaconine, the SGAs of cultivated potato (*S. tuberosum* L.), and their abundance in all tissues except for stolons ranged from 5 to 15 $\mu\text{mol/g}$ dry weight (DW), levels 2 to 6-fold higher than in cultivated potato. Four additional SGAs, leptinines I and II, and leptines I and II, account for the high SGA content of wild potato with accumulation up to 62 $\mu\text{mol/g}$ DW. These novel SGAs only accumulate in leaves, flowers and other aerial tissues. However, the SGA content of stolons is distinct from all other tissues with α -solanine and α -chaconine at least 3-fold higher at 55 $\mu\text{mol/g}$ DW and with the appearance of leptinines and leptines only at the final stages of above ground stolon development. In general, SGA content was highest in the primordial tissues with progressively less in the expanding and mature tissues of each organ. Steady-state transcript levels were determined for representative genes of the mevalonate, C15 condensation, and final glycosylation phases of the SGA biosynthetic pathway. Only the primordial tissues had marker transcripts present for all three phases of the pathway. Together, these results implicate the primordial tissues (i.e. flower buds, apical meristems and leaf primordia) as major sites of SGA biosynthesis. Characterization of a gene coding for squalene synthase (SQS), a central enzyme of SGA biosynthesis, revealed new information about SQS in potato that will be the basis for a manuscript. A gene family was predicted from a DNA blot and a four-member family, the largest family of SQS in any species to date, was defined by a comprehensive search of the recently published genome sequence of the diploid *S. tuberosum* Group Phureja. Pseudochromosomes 1 and 10 each had one gene with 10 having an additional concatemer. Three cDNA orthologs were isolated from *S. chacoense* and expressed as IPTG-induced polypeptides (minus the coding region for the membrane binding domain) in *Escherichia coli* along with a gene construct coding for bacterial farnesyl diphosphate synthase (which produces the substrate for SQS). Gas chromatograms of lipid extracts of IPTG-induced *E. coli* revealed the accumulation of squalene with all three SQS expression constructs thus indicating the expression of functional enzymes. Squalene levels (SQS1>SQS2>SQS4) were inversely correlated with the number of nonconserved amino acids in the active site domains (68 amino acids from *S. tuberosum* SQS1): SQS1, SQS2, and SQS4 had 0, 3, and 14 nonconserved amino acids, respectively. Semiquantitative RT-PCR indicated distinct expression patterns for each of the gene transcripts in various tissues. The genes for SQS1 and SQS2 were expressed in most tissues whereas SQS4 transcript was found primarily in the floral buds. The three genes coding for SQS all have an intron 6-11 bases downstream of the stop codon. This unusual feature could play a role in the degradation of prematurely terminated

SQS transcripts lacking the exon coding for the membrane binding domain. Translation of such corrupted transcripts could produce active enzyme without a membrane anchor, causing the inappropriate accumulation of squalene in cytosolic membranes outside of an enzyme complex metabolon.

1.3 Allelic variation of SGA biosynthetic genes

A candidate gene approach was used to identify genetic factors related to biosynthesis and accumulation of SGAs. Partial coding regions at four candidate genes [3-hydroxy-3-methylglutaryl coenzyme A reductase 2 (*HMG2*); 2,3-squalene epoxidase (*SQE*); solanidine galactosyltransferase (*SGT1*); and solanidine glucosyltransferase (*SGT2*)] in the glycoalkaloid biosynthetic pathway were amplified, cloned, and sequenced in two potato species differing in SGA composition: *S. chacoense* clone USDA 8380-1 (*chc* 80-1) and *S. phureja* clone DH OT-B × N-B P5/AR2. An F₂ population between these plant lines, with 148 individuals in five families, was genotyped using intron size differences or tag SNP markers found in haplotypes at candidate genes. The Chi-square analysis showed agreement between the observed and expected F₂ genotypic segregation ratios for all genes. The concentrations of α-solanine, α-chaconine, leptine I, leptine II and total SGAs were estimated. The range of accumulation varied broadly from 0 to 84.9 µg/mg DW among the four compounds, and from 0 to 126.9 µg/mg DW for total SGAs. Regression analysis showed a positive correlation between α-solanine and α-chaconine accumulation ($n=140$, $r^2=0.84$, $P<0.001^*$) as well as for leptine I and leptine II ($n=38$, $r^2=0.86$, $P<0.001^*$). In relation with biosynthesis of SGAs, Chi square analysis confirmed a pattern of 15:1 presence/absence ratios of synthesis for α-solanine and α-chaconine, while a 3:1 presence/absence ratio for synthesis for leptine I and leptine II. In the genotyping analysis, F₂ plants with *S. chacoense* alleles for *HMG2* and *SGT2* showed significantly greater accumulation of SGAs; for leptine I and leptine II when any *HMG2 chc* 80-1 alleles were present ($P<0.03$ and 0.03), and for total SGAs in samples with homozygous alleles for *SGT2* ($P<0.05$). There was also a significant interaction between *HMG2* and *SGT2* with the greatest levels of total SGAs, α-solanine and α-chaconine in plants with homozygous *chc* 80-1 alleles at both loci. However, *SGT1* and *SQE* alleles did not affect SGA accumulation; neither showed any interaction with any other gene for all the response variables ($P<0.005$, 0.02 and 0.02). Finally, a whole genome SNP genotyping analysis of 43 F₂ plants led to the identification of new possible candidate genes. Loci at ten potato genome pseudochromosomes were associated with SGA accumulation (<http://solanaceae.plantbiology.msu.edu/cgi-bin/gbrowse/potato/>). Pseudochromosome 6 held the greatest number of superscaffolds with significant SNPs for most SGAs. In addition, superscaffolds on pseudochromosomes 10 and 11 had significant SNPs for α-solanine; pseudochromosome 11 was also significant for leptine I and leptine II. Sesquiterpene synthase 2, *HMG1*, UDP-rhamnose: rhamnosyltransferase (*SGT3*), and two copies of *SQS* were potential candidate genes found in close

proximity to the highly significant SNPs on these three pseudochromosomes. Regarding synthesis of SGAs, significant SNPs clustered at the base of pseudochromosome 6 with another on pseudochromosome 1 associated with the synthesis of α -solanine and α -chaconine. Sterol desaturase and cytochrome P450 were potential candidate genes found on pseudochromosome 1, as well as cytochrome P450 71D7 on pseudochromosome 6. For leptine biosynthesis, significant SNPs on pseudochromosomes 2, 7 and 8 were found. A cytochrome P450 with steroid hydroxylase activity CYP72A58 was found on pseudochromosome 7. DWARF1/DIMINUTE and *HMG2* were tightly linked candidates, with BRASSINAZOLE-RESISTANT 1 protein, sterol-C5(6)-desaturase homolog, squalene epoxidase and N-acetyltransferase occurring downstream on pseudochromosome 2. Chromosome 8 harbored BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 and *SGT2* which was only 1.6 Mb from a significant SNP. The whole genome study offered by the SNP chip analysis allowed the identification of candidate genes that have been previously associated with sterol and SGA production and also confirmed the importance of *HMG2* and *SGT2*. These results could be used for molecular assisted breeding or genetic transformation to improve potato pest resistance.

Details of cooperation

The PIs worked cooperatively to fulfill the objectives of work-plan. Dr. Tokuhisa performed chemical analyses of leaf extracts of *HMG1* and *SQS1* transgenic plants that were sent to him by Dr. Ginzberg. Dr. Ginzberg used the same transgenic plants to analyze alteration in the expression of solanidine glycosyltransferase, *sgt1* and *sgt2*, as well as of isoprenoid key-genes suspected to be involved in determination of SGA levels. Dr. Veilleux identified allelic variations within the same genes by comparing their corresponding sequences in low SGA producer *S. phureja* and high SGA producer *S. chacoense*, as well as in segregating population of *chc* and *phu* intercrosses.

The PIs met in the USA at mid period of the project, published together two reviewed papers (another two in preparation), and presented the collaborative work in 11 scientific meetings by oral and poster presentations.

List of reviewed publications

Ginzberg I., Thippeswamy M., Fogelman E., Demirel U., Mweetwa A.M., **Tokuhisa J.** and **Veilleux R.** (2011). Induction of potato steroidal glycoalkaloid biosynthetic pathway by overexpression of cDNA encoding primary metabolism HMG-CoA reductase and squalene synthase. *Planta* 235:1341-1353

Mweetwa A.M., Hunter, D., Poe R., Harich K.C., **Ginzberg I.**, **Veilleux R.E.** and **Tokuhisa J.G.** (2011). Steroidal glycoalkaloids in *Solanum chacoense*. *Phytochemistry* 75:32-40

Appendix

Table of contents

Published papers	6
Participation in scientific meetings	6

Published papers

Ginzberg I et al., 2012

<http://www.springerlink.com/content/t6164q721462p450/>

Mweetwa et al., 2012

<http://www.sciencedirect.com/science/article/pii/S0031942211005577>

Pdf files are attached.

Participation in scientific meetings

Tokuhisa, J., Mweetwa, A., Manrique Carpintero, N., Wadlington, W., Ginzberg, I., Veilleux, R. (2009). The Steroidal Glycoalkaloids of *Solanum chacoense*. 49th Annual Phytochemical Society of North America Meeting and Symposia. Towson, USA (8-12 Aug) (Speaker)

Ginzberg, I., Tokuhisa, J., Veilleux, R., Muddarangappa, T., Demirel, U., Fogelman, E., Zoldan, D. (2009). Regulation of glycoalkaloids synthesis in potato. FAV HEALTH 2009; 3rd International Symposium on Human Health Effects of Fruits and Vegetables. Avignon, France (18-21 Oct.) (Speaker)

Ginzberg, I., Tokuhisa, J., Veilleux, R., Muddarangappa, T., Demirel, U., Fogelman, E., Zoldan, D. (2009). Regulation of glycoalkaloids synthesis in potato. SOL2009; The 6th Solanaceae Genome Workshop 2009. New Delhi, India (8-13 Nov.) (Speaker)

Tokuhisa, J., Mutiti Mweetwa, A., Manrique Carpintero, N., Wadlington, W., Ginzberg, I., Veilleux, R. (2010). The formation of steroidal glycoalkaloids in the wild potato species *Solanum chacoense*. 2nd Banff Conference on Plant Metabolism. Banff, Canada (24-28 June.)

Tokuhisa, J., Mutiti Mweetwa, A., Manrique Carpintero, N., Wadlington, W., Veilleux, R. (2010). Steroidal glycoalkaloids in the wild potato species *Solanum chacoense*. Plant Biology 2010 Annual meeting of the American Society of Plant Biologists. Montreal, Canada (31 Jul-4 Aug.)

- Manrique Carpintero, N., Piovano, S.M., Tokuhisa, J., Ginzberg, I., Veilleux, R.E. (2010). SNP Discovery at Candidate Genes in the Glycoalkaloid Biosynthetic Pathway of Potato. 94th Annual meeting of the Potato Association of America. Corvallis, USA
- Ginzberg, I., Tokuhisa, J., Veilleux, R., Muddarangappa, T., Demirel, U., Kumar A. and Fogelman, E. (2011). Regulation of steroidal glycoalkaloids in potato. The 18th Triennial Conference of the European Association for Potato Research (EAPR). Oulu, Finland (24-29 Jul.) (Speaker)
- Tokuhisa, J., Mweetwa, A., Manrique Carpintero, N., Wadlington, W., Hunter, D., Veilleux, R. (2011) How do plants make cytotoxins without crippling themselves? Steroidal glycoalkaloid biosynthesis in wild potato. 28th Mid-Atlantic Plant Molecular Biology Society Meeting. Patuxent Research Refuge, USA (15-16 Aug.)
- Manrique Carpintero, N., S. Piovano, J. Tokuhisa, I. Ginzberg, R. Veilleux (2011) SNP Discovery at candidate genes in the glycoalkaloid biosynthetic pathway of potato, Plant & Animal Genomes XIX Conference, San Diego (15-19 Jan.)
- Manrique Carpintero, N., S. Piovano, J. Tokuhisa, I. Ginzberg, R. Veilleux (2011) SNP Discovery at candidate genes in the glycoalkaloid biosynthetic pathway of potato, 8th Solanaceae and 2nd Cucurbitaceae Joint Conference, Kobe, Japan (28 Nov.-1 Dec.)
- Tokuhisa, J.G., Mweetwa, A.M., Veilleux, R.E., Wadlington, W., Wolberg, C.G. (2012) Squalene synthase and the accumulation of steroidal glycoalkaloids in potato. 51st Annual meeting of the Phytochemical Society of North America. London, Canada (11-15 Aug.) (Speaker)
- Manrique Carpintero, N.C., S.M. Piovano, J.G. Tokuhisa, I. Ginzberg, R.E. Veilleux (2012) SNP marker analysis at candidate genes in the glycoalkaloid biosynthetic pathway in a *Solanum phureja* × *S. chacoense* segregating population, SOL 2012: 9th Solanaceae Conference, Neuchatel, Switzerland (26-30 Aug.) (Speaker)