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The primary in vivo steroidal alkaloid glucosyltransferase from potato ☆

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Dedicated to Professor Rod Croteau on the occasion of his 60th birthday.

Abstract

To provide tools for breeders to control the steroidal glycoalkaloid (SGA) pathway in potato, we have investigated the steroidal alkaloid glycosyltransferase (*Sgt*) gene family. The committed step in the SGA pathway is the glycosylation of solanidine by either UDP-glucose or UDP-galactose leading to α-chaconine or α-solanine, respectively. The *Sgt2* gene was identified by deduced protein sequence homology to the previously identified *Sgt1* gene. SGT1 has glucosyltransferase activity in vitro, but in vivo serves as the UDP-galactose:solanidine galactosyltransferase. Two alleles of the *Sgt2* gene were isolated and its function was established with antisense transgenic lines and in vitro assays of recombinant protein. In tubers of transgenic potato (*Solanum tuberosum*) cvs. Lenape and Desirée expressing an antisense *Sgt2* gene construct, accumulation of α-solanine was increased and α-chaconine was reduced. Studies with recombinant SGT2 protein purified from yeast show that SGT2 glycosylation activity is highly specific for UDP-glucose as a sugar donor. This data establishes the function of the gene product (SGT2), as the primary UDP-glucose:solanidine glucosyltransferase in vivo.
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1. Introduction

Crop species produce a variety of commercially undesirable natural products. These are believed to have evolved as defense mechanisms against microbial and herbivorous pests. Steroidal glycoalkaloids (SGAs) are toxic metabolites found in potatoes (*Solanum tuberosum*) and other *Solanaceous* plants including tomato (*Lycopersicon esculentum*) and eggplant (*Solanum melongena*). The two predominant SGAs, α-solanine and (**6**) α-chaconine (**7**) accumulate in tubers and leaves (Fig. 1). The accumulation of SGAs can influence the quality of tubers in fresh and processed potatoes by introducing a bitter flavor. SGAs are a potential food safety hazard as the compounds are toxic and can inhibit acetylcholinesterase and disrupt cell membranes (Mensinga et al., 2005). Thus safety guidelines establish limits for SGA levels in commercial potato cultivars (Valkonen et al., 1996). As high levels of SGAs hamper breeding efforts to develop new and improved varieties of potatoes, we are exploring the use of transgenic approaches: (a) to understand the overall regulation of the biosynthetic pathway

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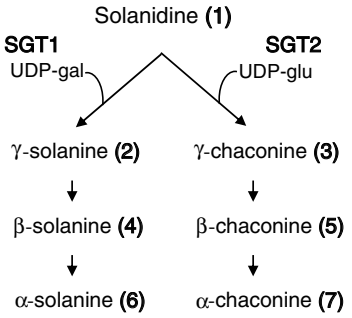

Fig. 1. Glycosylation steps of the SGA biosynthetic pathway. Solanidine (**1**) is the branch point for synthesis of the two predominant potato glycoalkaloids.

for SGAs and (b) to develop approaches that will assist the production of improved potato cultivars.

We are using members of the steroidal alkaloid glycosyltransferase (*Sgt*) gene family to downregulate SGA biosynthesis and thus reduce SGA accumulation in potato tubers. Potato SGAs contain either glucose (α-chaconine (**7**)) or galactose (α-solanine (**6**)) as the primary glycosyl residue (Fig. 1). The biosynthesis of γ-solanine (**2**) is catalyzed by UDP-galactose:solanidine galactosyltransferase (SGT1). A distinct enzyme UDP-glucose:solanidine glucosyltransferase (SGT2) catalyzes the biosynthesis of γ-chaconine (**3**) from UDP-glucose and solanidine (**1**) (Zimowski, 1990; Stapleton et al., 1991; Bergenstråhle et al., 1992). In crude protein extracts from potatoes, galactosyltransferase activities are unstable and present at low levels relative to glucosyltransferase activity (Stapleton et al., 1991; Bergenstråhle et al., 1992; Packowski and Wojciechowski, 1994). The difficulty in isolating pure SGT protein prevented the cloning of *Sgt* sequences via traditional antibody screening of expression libraries or by peptide sequencing and subsequent screening of cDNA libraries with synthetic DNA oligomers. The first potato *Sgt* cDNA (*Sgt1*) was cloned in yeast selected for their ability to detoxify the tomato SGA solasodine in the growth medium (Moehs et al., 1997). In vitro analysis showed that partially purified protein from yeast expressing SGT1 was capable of solanidine-dependent glucosylation activity (Moehs et al., 1997). When tested with potato and tomato aglycones recombinant SGT1 protein expressed in yeast was observed to have greater activity with UDP-galactose than with UDP-glucose as a substrate. The highest activity occurred with the tomato aglycone tomatidine as the substrate (McCue et al., 2005).

Previously, transgenic plant lines were generated with the *Sgt1* coding sequence in antisense orientation to test the in vivo affect of reducing SGT1 enzyme activity (McCue et al., 2003; McCue et al., 2005). Transgenic tubers from field trials displayed stable chemotypes with total SGA content ranging from 30% higher to 40% lower than in the wild type control (McCue et al., 2003). However, in these lines there was little correlation between *Sgt1* transcript level and total SGA level suggesting that the observed variation in SGA levels may be due to somaclonal variation rather than targeted downregulation of SGT1. Analysis of additional *Sgt1* antisense lines revealed a subset where α-solanine (**6**) biosynthesis was inhibited and the level of total SGAs was compensated for by increased accumulation of α-chaconine (**7**), which is more toxic than α-solanine (**6**) (McCue et al., 2005). The total level of SGAs was not altered. Despite its ability to serve as both a glucosyl- and galactosyl-transferase in vitro, the in vivo results assign the primary function of SGT1 as the solanidine galactosyl transferase involved in solanine (**6**) biosynthesis (McCue et al., 2005).

Additional SGT encoding sequences were identified in the potato EST database by their homology to the deduced SGT1 sequence. In this paper, we describe the function of the gene SOLtu:Sgt2.1 (*Sgt2*) with in vitro biochemical assays of recombinant protein and the analysis of the SGA content of transgenic potatoes expressing an antisense construct.

2. Results and discussion

2.1. Identification and cloning of Sgt2

The *Sgt2* sequence was identified in the TIGR EST database searching for protein homology with the deduced SGT1 sequence. Nine *Sgt2* cDNA fragments of 1036 or 1066 bp of the amino-terminal coding region were isolated using PCR with synthetic oligos. An antisense vector was constructed from the longer fragment and was used to transform potatoes. cDNAs for the carboxyl-terminal coding region and UTR were obtained in separate rounds of PCR. Eight clones with unique length poly(A) tails were obtained. Two ESTs with three UTRs of different lengths are present in the TIGR database; indicating multiple polyadenylation sites. No clones containing 5-prime upstream UTR sequences for *Sgt2* longer than the existing ESTs in the TIGR database were obtained from the cDNA library.

The composite cDNA containing the entire coding sequence was designated SOLtu:Sgt2.1 (*Sgt2*, GenBank Accession No. DQ218276). It was 1659 bp in length and contained an open reading frame encoding a 489 residue polypeptide (Fig. 2). The consensus *Sgt2* cDNA isolated from *S. tuberosum* cv. Lemhi showed a 99% identity to the *Sgt2* tentative consensus (TC) assemblage in the TIGR database. Comparing the coding region of *Sgt2* to that of *Sgt1*, the previously identified potato sterol alkaloid galactosyltransferase, revealed a 64% nucleic acid identity. This suggests that an antisense approach to silence one gene would not affect the other. Protein alignment of SGT2 to SGT1 showed an overall 73% conservation and 63% amino acid identity (Fig. 2). SGT2 also exhibited high identity and homology to other glycosyltransferases in several regions including the putative substrate binding recognition portion (amino acids #s 107–149), and the UDP-sugar binding region (amino acid #s 348–403), including the potential active site histidine residues (#360, #369) (Nawloka et al., 2003).

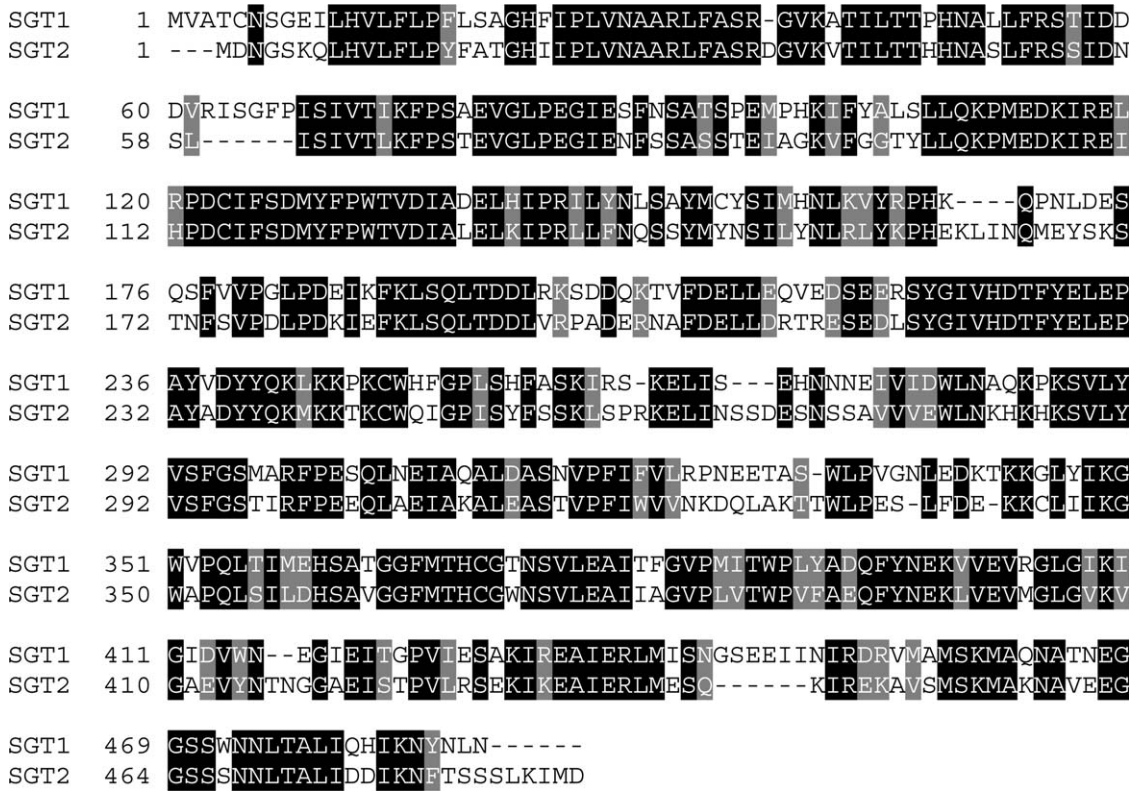

Fig. 2. Alignment of SGT2 and SGT1 deduced amino acid sequences showing regions of identity (black) and similarity (gray).

2.2. Occurrence of the Sgt2 locus in S. tuberosum

The approximate copy number and allelic variation of *Sgt2* in *S. tuberosum* was evaluated using genomic DNA blot analysis. Fig. 3 shows the banding patterns after digestion with 4 different restriction endonucleases. *Bam*HI was not predicted to cut within the *Sgt2* cDNA whereas *Eco*RI, *Hin*DIII and *Xba*I were all predicted to cut once within the coding region towards the 3-prime end of the probe. This favors stronger hybridization to the 5-prime half of the gene. Each digest resulted in 1–2 strong bands and 2–4 lighter bands. Digests with more than 2 lighter bands may represent restriction polymorphisms distal to the 3-prime end in some of the alleles. *Bam*HI was not predicted to cut the *Sgt2* cDNA sequence, yet two weak bands are present, so two or more weakly hybridizing bands may represent truncated pseudogenes, unknown introns or unrelated sequences. The relatively small number of bands, and expected presence of up to four alleles in tetraploid *S. tuberosum* suggests that *Sgt2* is present as a single copy per haploid genome.

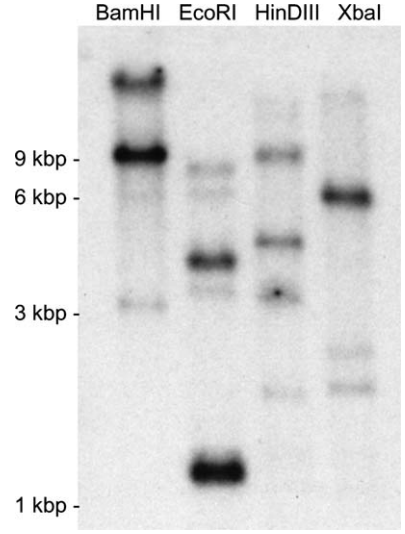

Fig. 3. Occurrence of *Sgt2* in the potato genome. Genomic analysis using 10 μg DNA from *S. tuberosum* cv. Lenape cut with the restriction endonucleases as indicated and probed with the 1066 bp amino terminal fragment of *Sgt2*.

2.3. Wound-induced expression of Sgt2

Potato SGAs are knows to accumulate in tubers upon wounding (Bergenstråhle et al., 1992). To examine the effect of wounding on *Sgt2* mRNA abundance, total RNA was extracted from wounded leaves and tubers and probed with *Sgt2* or *Sgt1* (Fig. 4). As expected the expression of *Sgt1* and *Sgt2* is coordinately regulated in response to wounding in the tubers with nearly identical patterns of mRNA accumulation. Transcript levels of *Sgt1* or *Sgt2* in total mRNA decreased to undetectable levels 2 h after wounding, increased dramatically by 16 h and subsequently decreased to lower levels by 48 h. Although SGAs are also found in leaves, the pattern of expression is quite different. After wounding there is a large increase in steady state levels of *Sgt2* that is still strong at 16 h. Conversely, *Sgt1* does not show an increase in mRNA abundance after wounding.

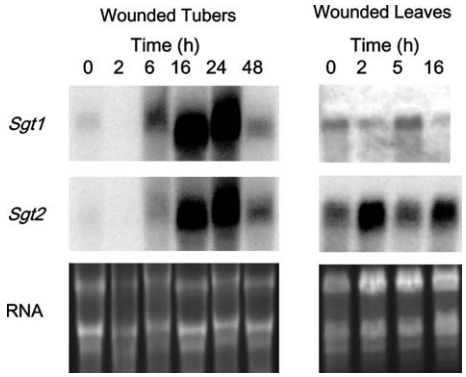

Fig. 4. Steady state levels of *Sgt1* and *Sgt2* RNA in wounded potato tubers and leaves. Potato cv. Lenape tubers and leaves were wounded and total RNA extracted at the times indicated. The RNA gels (20 μg/lane tubers, 10 μg/lane leaves) were blotted and probed with *Sgt1* and *Sgt2*. The ethidium bromide stained gels (bottom) show loading consistency and RNA integrity.

As our probes do not discriminate between the two *Sgt2* alleles it is not possible to discern whether both alleles are involved in induction in either tissue.

2.4. Steroidal glycoalkaloid accumulation

The SGA levels (α-solanine (**6**) and α-chaconine (**7**)) were measured in uniform slices of field-grown tubers of Lenape (Fig. 5(a)) or whole glasshouse-grown minitubers of Desirée (Fig. 5(b)). The range in total SGA levels (α-solanine (**6**) + α-chaconine (**7**)) for Lenape *Sgt2* antisense lines varies from 23% above to 39% below the wild type control. Similar variation was observed in the Desirée *Sgt2* antisense lines (50% above to 27% below). This variation has now been stable over three generations and is within the range attributable to somaclonal variation (Esposito et al., 2002). The variation in total SGA levels between lines and individual tubers makes it difficult to elucidate effects of antisense constructs on the accumulation of either α-solanine (**6**) or α-chaconine (**7**). However, an examination of the ratio of α-solanine (**6**) to α-chaconine (**7**) quickly highlights the plants with an effective antisense response. In Lenape lines 1605, 1608 and 1619, and Desirée lines 2–11, 2–17 and 2–18, the ratio of α-solanine (**6**) to α-chaconine (**7**) was noticeably altered compared to wild type. In these lines effective antisense *Sgt2* action modified the balance between glucosyl transferase and galactosyltransferase activity. In most of the affected lines α-chaconine (**7**) levels were reduced and there was increased α-solanine (**6**) accumulation. In lines 1608, 1619 and 2–18, the total SGA levels are increased (perhaps due to somaclonal variation), but the antisense *Sgt2* activity affects the ratio of α-solanine (**6**) to α-chaconine (**7**). The reductions in total SGAs and in α-chaconine (**7**) in Lenape line 1605 were both statistically significant ($P < 0.001$), as

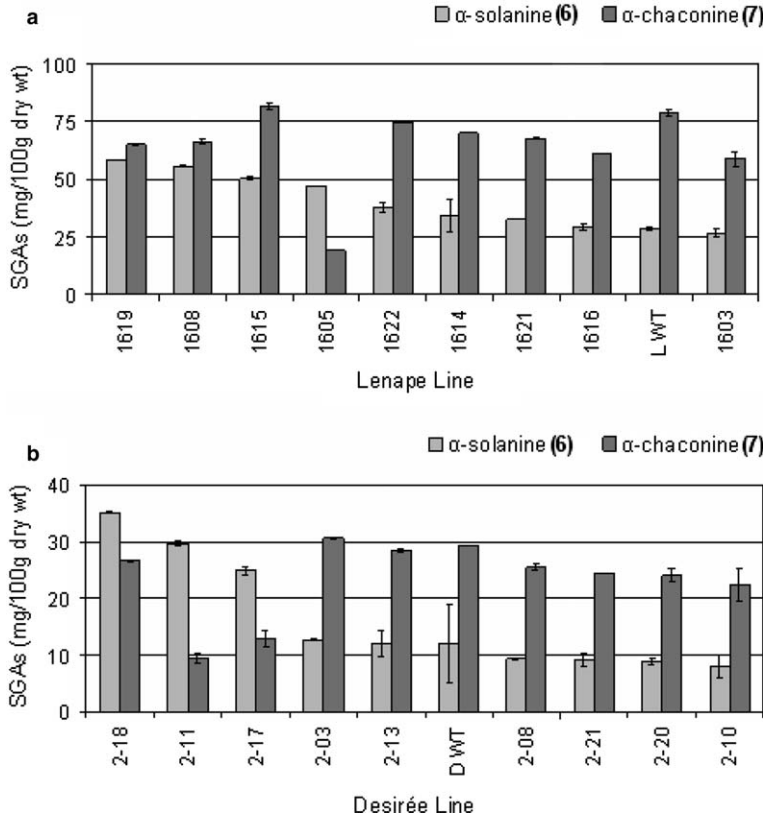

Fig. 5. SGA content of tubers from transgenic potatoes expressing the *Sgt2* antisense transgene. Levels of α-solanine (**6**) and α-chaconine (**7**) in selected transgenic and wild type (WT) control lines of (a) Lenape and (b) Desirée. The plant lines are arranged in the graphs sorted by decreasing α-solanine (**6**) levels. Values are the average of three slices from three field-grown tubers (Lenape) or twos glasshouse-grown minitubers (Desirée). Error bars show s.d.

was the reduction of α-chaconine (**7**) in Desirée lines 2–11 and 2–17 (both at $P < 0.001$).

2.5. Analysis of transgene integration

To examine the affect of transgene integration on antisense transgene expression, genomic DNA was examined for integration patterns by DNA blot analysis. *Hin*DIII digested DNA was probed with the *Npt*II selectable marker gene to eliminate background bands due to the endogenous *Sgt2* gene. *Hin*DIII is predicted to cut once within the TDNA construct and produce a fragment >1723 bp. The result was a simple pattern of bands with single copy inserts in lines 1603, 1616 and 1621 and two copies in lines 1614, 1615, and 1622. In lines 1605, 1608 and 1619 however, very dark banding patterns were observed suggesting multiple insertions (Supplementary Fig. 1).

2.6. Expression of antisense Sgt2

Steady state levels of *Sgt2* mRNA were examined by RNA blot analysis. To test for transgene efficacy in down-regulating *Sgt2* expression, RNA was isolated from transgenic tissue and was probed with the *Sgt2* sequence (Fig. 6a). Reduced steady state levels of *Sgt2* transcripts were observed in lines 1605 (almost completely lacking endogenous transcripts), 1608 and 1619, indicative of effective antisense transgenes where both sense and antisense message are degraded resulting in reduced activity of the SGT2 protein (Robert et al., 1989). The RNA blot was re-probed with the *Npt*II selectable marker (Supplementary Fig. 2(a)). This probe revealed strong bands in all lines with the exception of the control. The single copy insert lines resulted in the lowest levels of *Npt*II mRNA abundance; two copy insert lines had higher levels, and the multiple copy insert lines had the highest levels. The exception was line 1605 with multiple copy inserts did not have high levels of *Npt*II mRNA. This may be due to suppression of *Npt*II along with *Sgt2* or an absence of additional complete copies of *Npt*II in the complex integration. The ethidium bromide stained gel was used to confirm RNA quality and loading consistency (Supplementary Fig. 2(b)).

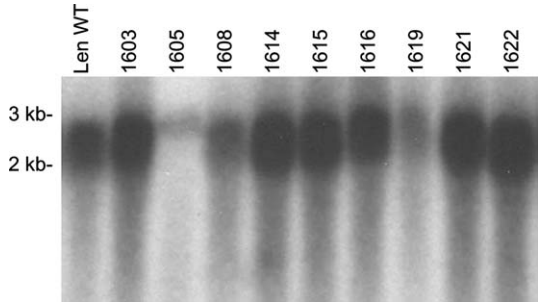

Fig. 6. Steady state RNA expression of *Sgt2* transcripts in Lenape lines. Analysis of steady state levels of total RNA (30 μg/lane) in control and transgenic lines of Lenape.

2.7. Isolation of Sgt2 coding sequences and expression vector construction

Recombinant SGT2 protein was prepared by expressing the intact coding sequence isolated from the wounded tuber cDNA library by PCR. The PCR resulted in 14 isolates identical to the original *Sgt2* amino-terminal fragment and 5 isolates 99.7% identical with a 21 bp deletion (SOLtu:Sgt2.2, GenBank Accession No. DQ218277). A TC in the TIGR database that is distinct from the original *Sgt2* and contains the same 21 bp deletion has since been generated. Extensive homology between the two alleles suggests that both alleles would be simultaneously downregulated in plants expressing effective antisense genotypes. Both proteins were expressed and tested for glucosyltransferase activity in situ in yeast (data not shown). Only the more abundant SGT2 (based on our coding region amplification results and TIGR EST abundance) was purified and assayed in vitro.

2.8. Biochemical analysis of SGT2

The observation that the major effect of antisense *Sgt2* was to reduce α-chaconine (**7**) levels suggested that it served as the primary solanidine glucosyltransferase. This was confirmed by in vitro studies on the activity of recombinant SGT2 protein with Solanaceous SGAs and either UDP-[^{3}H]glucose or UDP-[^{3}H]galactose as the sugar donor (Table 1). The recombinant SGT2 was readily capable of utilizing UDP-glucose as a substrate in vitro with all three Solanaceous aglycones (solanidine (**1**), solasodine and tomatidine) with maximum activity obtained with the potato aglycone solanidine. No activity was observed with UDP-galactose as a substrate for any of the aglycones tested. The threshold for activity was set at 2-fold the reaction blank.

Inhibition studies were performed using a separate SGT2 enzyme preparation (activity of 410 ± 22 nkat mg^{-1}) (Table 2). The addition of UDP-galactose had no competitive effect on SGT2 activity. This is contrary to a previous report that showed inhibition by UDP-galactose when a mixture of partially purified SGT2 was used in the presence of SGT1 (Bergenstråhle et al., 1992). The addition of 1000 μM α-solanine (**6**) caused 39% inhibition while,

Table 1
SGT2 UDP-sugar and aglycone preference

Substrate	UDP-[^{3}H]Glucose (nkat mg^{-1})	UDP-[^{3}H]Galactose (nkat mg^{-1})
Solanidine	1060 ± 25	NA
Solasodine	880 ± 29	NA
Tomatidine	930 ± 152	NA

Glycosyltransferase activity of the recombinant SGT2:his fusion protein purified from yeast. Values represent the average of duplicate assays ± s.d (nkat mg^{-1} = moles of product per second per mg protein × 10^{-9}). NA = no activity, for reactions with dpm counts less than 2-fold the assay blank.

Table 2
Effect of UDP-galactose or triose end products, α-solanine (**6**)or α-chaconine (**7**), on recombinant SGT2 glucosyltransferase activity

Substrate concentration (μM)	UDP-galactose (nkat mg^{-1})	α-Solanine (**6**) (nkat mg^{-1})	α-Chaconine (**7**) (nkat mg^{-1})
33	380 ± 7.4	410 ± 102	490 ± 4.8
100	500 ± 84	430 ± 33	520 ± 10.6
1000	390 ± 23	250 ± 31	NA

Glucosyltransferase activity of the recombinant SGT2:his fusion protein purified from yeast in the presence of added UDP-galactose or steroidal alkaloid triose end products. Values represent the average of duplicate assays ± s.d. NA = no activity, for reactions with dpm counts less than 2-fold the assay blank.

1000 μM α-chaconine (**7**) completely inhibited SGT2 activity. This demonstrates weak feedback inhibition of the primary glucosylation step by both downstream triose end products.

3. Conclusions

The two *Sgt2* sequences (*Sgt2.1* and *Sgt2.2*) isolated from the *S. tuberosum* cDNA library likely represent two major alleles expressed in the heterozygous tetraploid genome. Subsequent to our isolation of these genes, the addition of more ESTs to the *S. tuberosum* Gene Index has resulted in the assembly of two independent TC sequences representing each allele. The presence of no more than two major bands in the genomic DNA blot analysis indicates that *Sgt2* is a low copy gene in *S. tuberosum*. This in combination with two distinct coding sequences from PCR and in the EST database suggests that there are only two major active alleles.

The two solanidine glycosyltransferases represent the committed steps for carbon flow into the SGA pathway. Downregulation of either *Sgt1* or *Sgt2* tends to cause an increase in the accumulation of the end product of the other branch, α-chaconine (**7**) or α-solanine (**6**), respectively. The reduction and compensation is greater in plant lines expressing effective antisense *Sgt1* than in plant lines expressing effective antisense *Sgt2*. This may be due to the subtle differences in the substrate specificities for these two enzymes. SGT2 is very specific for UDP-glucose as a sugar donor completely lacking any galactosyltransferase activity. SGT1 has a marked preference for UDP-galactose, but does have some glucosyltransferase activity (Moehs et al., 1997; McCue et al., 2005), and may partially function in vivo in the absence of normal SGT2 activity.

The effective Lenape antisense lines show a consistent correlation with multiple T-DNA inserts, reduction in steady state levels of *Sgt2* RNA, and either reduced α-chaconine (**7**) and/or an increased α-solanine (6) accumulation. All of this is consistent with the conclusion that SGT2 is the primary in vivo solanidine glucosyltransferase in a dedicated branch of SGA biosynthesis specific for the formation of α-chaconine (**7**). Based on these data, we assign the function of the gene product SGT2 as E.C. 2.4.1.173, a UDP-glucose 3-β-sterol glucosyltransferase. Future research will focus on the combined downregulation of *Sgt1* and *Sgt2* in concert to reduce the accumulation of both of these toxic compounds in the tubers of commercial potato cultivars.

4. Experimental

4.1. Plant materials

Potato (*S. tuberosum* L.) cv. Lenape (Akeley et al., 1968) was grown in a glasshouse in Albany, CA for collection of meristems for DNA isolation. For SGA and mRNA analyses, Lenape tubers were harvested from field plots (Coetzer et al., 2001) in Aberdeen, ID, USA and Desirée tubers were harvested from glasshouse-grown plants in Invergowrie, Dundee, UK.

4.2. Sgt2 cDNA Isolation

Identification of a putative SGT2 coding sequence was accomplished by screening the TIGR expressed sequence tag (EST) database of expressed potato genes. The EST database was searched for sequences whose predicted protein translation contained homology to the known SGT1 (Moehs et al., 1997) (GenBank Accession No. U82367). The candidate TC sequence was then checked for the frequency of occurrence and tissue distribution of the component ESTs as compared to *Sgt1*. The most homologous sequence was TC10074 (1178 bp). *Sgt2* cDNA sequences were amplified by PCR from a wound-induced tuber cDNA library prepared from *S. tuberosum* cv. Lemhi (Garbarino et al., 1992). Internal primers (forward: #1384 CTTCCTTCCTTACTTCGCC; and #1386 GGATAACGGGAGCAAGC; and reverse: #1414 GATGGTTAGTTGCGGTGC) were used for the amplification of *Sgt2* sequences of 1036 and 1066 bp, respectively. An additional outward facing primer was matched with M13 vector primers to obtain *Sgt2* cDNA 3-prime terminal regions (#1453 TACCGTCCCTTTCATTTGG) for cloning into pCR2.1 (TA cloning vector, Invitrogen).

4.3. Transgene construction

The antisense transgene was constructed with a 1011 bp *Hin*cII/*Eco*RV sub-fragment of the *Sgt2* amino terminal fragment including 18 bp of vector on the *Eco*RV end. The fragment was ligated in antisense orientation downstream of a 1206 bp potato *GBSS6* promoter (van der Liej et al., 1991), for tuber-specific transcription (van der Steege et al., 1992), followed by a 404 bp potato *Ubi3* polyadenylation signal (Garbarino and Belknap, 1994). The transgene is inserted in a modified pBINPLUS binary vector (van Engelen et al., 1995), pBINPLUS/ARS, for mobilization into potato varieties Lenape and Desirée via *Agrobacterium*-mediated transformation (Snyder and Belknap, 1993). The pBINPLUS/ARS vector is identical to pBINPLUS except that control sequences for the *Npt*II plant selectable marker (nopaline synthase promoter and terminator pBINPLUS) have been

replaced by potato *Ubi3* promoter and polyadenylation signal (Garbarino and Belknap, 1994) sequences in pBINPLUS/ARS.

4.4. Expression vector construction

For expression of SGT2 protein in yeast, additional full length SGT2 coding sequences were amplified by PCR directly from the *S. tuberosum* cv. Lemhi cDNA library (Garbarino et al., 1992). Amplification was performed with primers directed to the 5-prime and 3-prime ends of the longest open reading frame (ORF) in *Sgt2* (forward: #1618 GGTACCATGGATAACGGGAGCAAGCC, and reverse: #1619 GGTACCGTTGTTAATCCATGATCTTCAATG, or #1623 CTCGAGATCCATGATCTTCAATGAAGAAG). The amplified fragments were cloned into the pYES 2.1 V5/TOPO expression vector (Invitrogen). A *Kpn*I site addition to the Met start codon provides an optimal Kozak start consensus for the recombinant protein (#1618). An *Xho*I replacement of the native stop (#1623) gives the construct a readthrough fusion to the V5 antibody epitope and 6× His C-terminal tag in the pYES2.1/V5-His TOPO cloning vector (Invitrogen). Yeast was transformed with the recombinant vector and grown under inducing conditions. Protein was extracted according to manufacturer's recommendations (Invitrogen).

4.5. Protein purification and analysis

Protein purification was carried out using the His protein isolation system (Sigma) according to manufacturer's instructions. Purification was assessed via SDS PAGE and staining with Coomassie blue. Elution of recombinant proteins was monitored by Western blot analysis using the anti-V5 epitope antibody (Invitrogen). Solanidine glycosyltransferase assays were carried out using 100 μL of column eluate after buffer exchange and concentration. Assays were run for 60 min at 37 °C. SGAs and UDP-sugars were provided at 33 μM unless otherwise indicated. Radioactive UDP-sugar stock solutions were prepared to contain $\sim 5 \times 10^5$ dpm UDP-[^{3}H]glucose or UDP-[^{3}H]galactose per reaction. Inhibitor studies included the addition of α-solanine (**6**) or α-chaconine (**7**) dissolved in DMSO to the final concentrations as indicated. Control reactions had the addition of an equal volume of DMSO in the absence of inhibitor. Products were separated using anion exchange resin (Stapleton et al., 1991). No activity is reported for reactions with less than 2-fold the average dpm counts of the blank reactions. Values are the mean of duplicate assays.

4.6. Steroidal glycoalkaloid determinations

Levels of SGAs were quantified from field-grown Lenape or glasshouse-grown Desirée. Freeze dried material was produced from Lenape and Desirée tubers as described by McCue et al. (2005) and Defernez et al. (2004).

Glycoalkaloid levels were determined by an LC–MS method on a Thermo LCQ-DECA (Hemel Hempstead, UK) LC–MS. Glycoalkaloids were separated on a C_{18} Synergi Hydro column (2 mm × 150 mm; Phenomenex, Macclesfield, UK). Chromatographic conditions were as follows; Flow rate, 200 μl min^{-1}; 1 min at 0.2% HCO_2H in dH_2O then a linear gradient to 0.2% HCO_2H in CH_3CN–dH_2O (9:1) over 20 min, followed by re-equilibration at 0.2% HCO_2H in dH_2O for 5 min.

Glycoalkaloid analyses employed electrospray ionization-mass spectrometry (ESI-MS) with the following MS conditions; sheath gas (N_2) 70 psi, auxiliary gas 15 psi, spray voltage 4500 V and capillary temp. 250 °C. α-Solanine (**6**) and α-chaconine (**7**) were quantified using calibration curves constructed within the analytical run and employed the following ions specific for calibration and estimation; α-solanine (**6**) – m/z 868.8 $[M+H]^+$ and α-chaconine (**7**) – m/z 852.7 $[M+H]^+$. Five calibration points were repeated four times, as were the tuber analyses. Statistical analysis of data was performed by One Way ANOVA (SigmaStat 3.1 for Windows 2004. Systat Software, Inc.) for triplicate Lenape data and by Dunnett's test (SAS Institute Inc. 2004. SAS OnlineDoc® 9.1.3. Cary, NC: SAS Institute Inc.) for duplicate Desirée data, using unequal variances (Littell et al., 2002).

4.7. RNA blots

Total RNA was prepared from tuber peels obtained using a hand-held vegetable peeler. Peels were frozen in liquid nitrogen, ground and extracted for RNA as previously described (Verwoerd et al., 1989). RNA was isolated and fractionated by agarose gel electrophoresis, and transferred to a charged nylon membrane (Roche) (Rickey and Belknap, 1991). RNA blots were hybridized with random primed (GE Healthcare) double stranded probes of the *Sgt1* cDNA (1582 bp) (Moehs et al., 1997), *Sgt2* cDNA (1066 bp), or the *Npt*II (803 bp) selectable marker isolated with a *Pme*I digest of pBINPLUS/ARS.

4.8. DNA blots

DNA was isolated from young shoot tips frozen in liquid nitrogen and extracted as previously described (Draper and Scott, 1988). DNA was digested with restriction enzymes as indicated in the figure legends, separated by agarose electrophoresis, blotted to charged nylon membranes, and hybridized with the double stranded *Sgt2* fragment (1066 bp) or the *Npt*II (803 bp) selectable marker isolated with a *Pme*I digest of pBINPLUS/ARS.

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Appendix A. Suppplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2005.09.037.

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