

Reduction of Total Steroidal Glycoalkaloids in Potato Tubers Using Antisense Constructs of a Gene Encoding a Solanidine Glucosyl Transferase

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Abstract

Accumulation of steroidal glycoalkaloid (SGA) toxicants in potatoes affects food quality and safety. High levels of SGAs hamper breeding efforts to develop new varieties of potatoes with improved agronomic and post harvest properties. To speed breeding efforts for reducing SGAs in breeding lines and correcting flawed selections we are taking a molecular genetic approach to reduce SGAs. A cDNA encoding a solanidine glucosyltransferase (SGT) was isolated and used to construct antisense transgenes transcribed from either the Cauliflower Mosaic Virus 35S or a tuber-specific granule bound starch synthase promoter. Transgenic lines of potato (*Solanum tuberosum*) cultivar 'Lenape' expressing these transgenes exhibit phenotypes with significantly lower SGAs. Field trails over several years in Idaho and Wisconsin resulted in reproducible and statistically significant reductions of up to 40% of tuber SGAs. Reduction in SGAs is accompanied by expression of *Sgt* antisense RNA transcripts and a reduction in SGT protein levels. Analysis of transgenic cultivar 'Desiree' lines expressing an antisense *Sgt* revealed essentially complete down-regulation of solanine biosynthesis in several transgenic lines. Chaconine accumulation in these lines was not significantly affected. Additional transgenes encoding SGT and other SGA related sequences are currently being evaluated.

INTRODUCTION

Steroidal glycoalkaloids (SGAs) are ubiquitous secondary products produced by potatoes and many other Solanaceae. SGAs are believed to play a role in pest resistance (Fragoyiannis et al., 2001; Rangarajan et al., 2000) and are an important component of potato flavor (Maga, 1980). When overproduced SGAs confer bitterness to the taste of potatoes and in very high concentrations present a food safety issue (Maga, 1980; McWilliams et al., 2000; Nitithamyong et al., 1999). Because of this new potato selections are routinely screened to ensure SGAs in tubers remain below established limits of 20 mg/g fresh weight (FW) or 1.0 mg/g dry weight (DW) (Nitithamyong et al., 1999). Excessive levels of SGAs are an impediment to breeding programs trying to

introduce beneficial phenotypes from wild relatives. Some agronomically favorable cultivars such as ‘Lenape’ have been withdrawn from production due to a tendency to produce undesirable SGAs levels.

The structure and occurrence of potato SGAs was confirmed by mass spectroscopy (Chen et al., 1994). The biosynthetic pathways and demonstration of the in vitro activities of the individual enzymes is still being investigated by several labs (Griffiths et al., 2000; Kozukue et al., 2001). The two major SGAs in potato are solanine and chaconine. Both are triglycosylated steroidal alkaloids derived from solanidine and are structurally related to similar steroidal alkaloids found in tomato. Steroidal alkaloids accumulate in leaves and in tubers naturally, and their deposition is increased in response to wounding, light and cold storage (Maga, 1980).

Scientists and breeders have been seeking to lower SGAs and both conventional and non-conventional breeding can be used to produce lines with lower alkaloids (Esposito et al., 2002). We have chosen to use a direct genetic approach to lowering SGAs by manipulating the pathways responsible for their synthesis in a tuber specific manner.

A gene encoding a solanidine glucosyltransferase (*Sgt*) was cloned from a cDNA library expressed in yeast challenged to grow on solanidine. The enzymatic activity of the *Sgt* cDNA was confirmed in vitro (Moehs et al., 1997). The *Sgt* gene was used in a series of antisense constructs. These constructs are being tested for their efficacy in reducing levels of SGAs in round white chipping cultivars ‘Lenape’ and ‘Desiree’.

MATERIALS AND METHODS

Plant Materials

Round white potato (*Solanum tuberosum*) cultivars ‘Lenape’ and ‘Desiree’ were used in transgenesis experiments. Field evaluation of transgenic ‘Lenape’ lines was carried out in Aberdeen, Idaho in replicated plots (Coetzer et al., 2001). Additional materials for DNA, RNA and protein analysis grown in the glass house in Albany, California. Culture of transgenic ‘Desiree’ lines was carried out in a glass house in Invergowrie, Dundee, Scotland. Plant lines with reduced SGAs and growing true to type were selected from multiplication and replanting in subsequent years.

Transgene Constructs

Antisense transgenes with the *Sgt* gene were constructed using synthetic oligonucleotides that added BamHI sites the ends of the *Sgt* cDNA (Moehs et al., 1997) created by polymerase chain reaction and subsequent cloning into pCR2.1 (Stratagene). Transgenic ‘Lenape’ lines were initiated with antisense *Sgt* constructs under transcriptional control of either the 35S promoter or the potato granule bound starch synthase promoter (*Gss*) promoter (van der Steege et al., 1992) and the nopaline synthase (*Nos*) terminator (Beven et al., 1983), for (Lenape -35S-*Sgt*) LSS and (Lenape-*Gss-Sgt*) LGS plant lines, respectively, in the binary vector pCGN1547 (McBride and Summerfelt, 1990). A second generation of transgenic lines was produced with the *Gss* promoter, antisense *Sgt*, and the ubiquitin (*Ubi3*) terminator (Garbarino and Belknap, 1994) (*Gss-antisense Sgt-Ubi*) GaSU lines in cultivars ‘Lenape’ and ‘Desiree’, in the binary vector pBINPLUS/ARS a modified expression cassette containing the Ubi3 promoter and terminator (Garbarino and Belknap, 1994) in pBINPLUS (Van Engelen et al., 1995). Empty vector transformed controls include pCGN1547 in ‘Lenape’ (Len 8.1) and pBINPLUS/ARS in Desiree (pBPA). Potato transformation was carried out as previously described (Snyder and Belknap, 1993).

DNA and RNA Analysis

Whole tubers were measured, weighed and the outer 1-2 mm peeled and frozen in liquid nitrogen. Aliquots of frozen peel were ground into a powder in an Omni Mixer Homogenizer (Omni International). Frozen peel or powder was stored at -80 °C until

used for RNA or protein extraction. DNA was extracted from young meristems grown in the glass house and RNA was extracted from young fully expanded leaves from the glass house or tissue culture cabinet.

DNA was extracted from young meristems of glass house grown tubers (Draper and Scott, 1988). RNA was extracted from leaves of glass house plants or from previously frozen tuber peels (Verwoerd et al., 1989). Isolated DNA was digested with restriction enzymes as indicated. Nucleic acids were separated by agarose gel electrophoresis and blotted to positively charged membranes (Rickey and Belknap, 1991). The *Sgt* probe was isolated by BamHI digestion from pCR2.1 and the *NptII* probe was isolated by PmeI digestion of pBINPLUS/ARS. Probes were [³²P]-labeled using the Oligolabeling Kit (Pharmacia). Digoxigenin-labeled strand-specific RNA probes were generated using the Dig RNA Labeling Kit (Boehringer Mannheim)

Protein Analysis

Protein from previously frozen peels was extracted into aqueous solution and hybridized with antibody directed against SGT as previously described (Moehs et al., 1997).

Steroidal Glycoalkaloid Analysis

Total steroidal glycoalkaloids (TGAs) were determined colorimetrically from whole field grown tubers (Bergers, 1980). Analysis of variance was performed on TGA values when more than two replicates were analyzed.

Component glycoalkaloids, solanine and chaconine, were quantified from freeze-dried samples using HPLC (Hellenas, 1986). For Field tubers, the central 5mm longitudinal section, frozen in liquid nitrogen, and freeze-dried in a FreeZone Stoppering Tray Dryer (Labconco). For glass house grown minitubers, the whole minitubers were frozen and freeze-dried.

RESULTS AND DISCUSSION

Transgenic Lenape lines carrying the LSS and LGS antisense *Sgt* constructs were grown over a three year period in Idaho. The field grown tubers were harvested and analyzed for TGAs colorimetrically (Fig. 1). The greatest variation in TGAs was observed in 1997, the first year field trial using glass house produced mintuber seed. For the next two years TGAs were at or below WT levels. The only line with a statistically significant reduction was LSS8. This occurred over the entire three year period. However, lines LSS7, LSS11 and LGS12 remained consistently lower than controls in each of the last two years in replicated plots using field grown seed. During this period, additional lines containing the LSS and LGS transgene constructs were generated in California and Wisconsin and introduced into the fields in Idaho and Wisconsin for preliminary analyses and seed multiplication. In 2000, tubers from replicated field plots in Idaho were analyzed for TGAs and yield (Fig. 2). Line LGS 102 showed 72% reduction in TGAs compared to 'Lenape' WT tubers, however this line suffered unacceptable yield loss (32%). Thirteen lines in 2000 trials gave statistically significant reductions ($P > .01$) of 20 to 30% TGAs compared to 'Lenape' controls (26.5 mg/100g FW) (Series 1). Lines with greater than 30% reduction suffered lower yields. However, lower TGA accumulation may be the result of poor overall vigor. The remaining lines analyzed in 2000 (Series 2) had a range of yield values but TGAs were not significantly different than 'Lenape' WT. With the exception of line 102, none of the transgenic lines exceeded 50% TGA reduction.

To confirm the transformation of newly initiated lines, tubers were sprouted and young leaves harvested for RNA extraction. The isolated RNA was blotted to membrane and hybridized with a probe to the *NptII* selectable marker gene on the T-DNA. LGS and GaSU lines only show active antisense transcription from the *Gss* promoter in tubers. Fig. 3 shows the RNA blot that confirms active transcription of the *NptII* transcript in a subset of the GaSU lines. The LSS lines will express the antisense *Sgt* constitutively throughout the plant. Transformation of LSS lines was confirmed by analysis of RNA from young

leaves of tissue culture transplants. Fig. 4 shows the RNA blot analysis using a non-radioactive single stranded probe for the antisense *Sgt* transcript in selected LSS plant lines.

Total protein was extracted from tuber peels of LSS and LGS lines selected for normal or low TGAs and the WT and empty vector-transformed Lenape controls. Protein was blotted and probed with polyclonal sera directed to the SGT protein (Fig. 5). Relative TGA levels correspond with the observed intensity for SGT protein visualized by the antibody. LSS 8 that consistently has significantly reduced TGAs showed no visible SGT protein in the blot.

Antisense RNA levels were examined in total RNA extracted from tuber peels and probed with a non-radioactive single-stranded probe to the antisense *Sgt* transcript. Fig. 6 shows the relative abundance of the various antisense transcripts in selected transgenic lines for the three transgene constructs. The two LSS lines (8 and 27) show strong transcript levels. Transcripts from the LGS lines 11-49 show much weaker signals and have two different sized transcripts. Transcripts are not visible for the GaSU lines. Sense and antisense transcripts were visualized by hybridization of total RNA with a double-stranded radioactive probe (Fig. 7). Patterns of RNA expression are similar as seen for the antisense-specific probe with the addition of weak signals for the GaSU lines and the 'Lenape' WT control.

The transgene insertion patterns for selected lines containing the three different constructs is shown in Fig. 8. Because of the complicated pattern DNA blot hybridization using *Sgt* as a probe (due to high heterogeneity and multiple genes/pseudogenes, data not shown) we can only prove with the selectable marker. The BamHI/XbaI digest cuts the T-DNA strand in half. The NptII selectable marker is next to the Left border, expected only for full T-DNA insertion and will have a minimum size >3.0 kbp. Direct repeats of T-DNA inserts would be expected to produce bands at 6.4, 6.7 and 6.2 kbp for LSS, LGS and GaSU, respectively. As seen in Fig. 8 most lines have 1 or 2 insertions with the most complex pattern observed in GaSU 122.

Analysis of component glycoalkaloids was performed on lines generated in 2001 with the GaSU construct in the cultivar 'Desiree'. Minitubers from green house-grown transgenic lines were analyzed for solanine, chaconine and TGAs by HPLC (Fig. 9). In component analysis performed on the first generation of LSS and LGS lines, both solanine and chaconine were equally reduced in lines with lower TGAs (data not shown). In 'Desiree' lines 2.5, 3.5 and 11.2, with high, low and normal alkaloids respectively, dramatic reductions in solanine with only modest increases in chaconine are observed. This chemotypical difference may be due to additional genes present in cultivar 'Lenape' or by a different in vivo function than that demonstrated in vitro (solanidine glucosyl-transferase) for the *Sgt* gene product (Moehs et al., 1997).

It is also possible that 'Lenape' lines selected for lowered alkaloids were the result of somaclonal variation such as that observed by (Esposito et al., 2002), and that despite expression of antisense transcripts, the variation in SGT protein and TGA levels was not or only partially due to antisense activity. Analysis of 'Desiree' lines indicate that a range of TGA levels are observed which mask the greater effect of the antisense transgene in these lines which appears to cause large reductions in solanine levels. Analysis of additional transgenic lines and transgenes will be required for confirmation of the source variation and in vivo function of *Sgt*.

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Figures

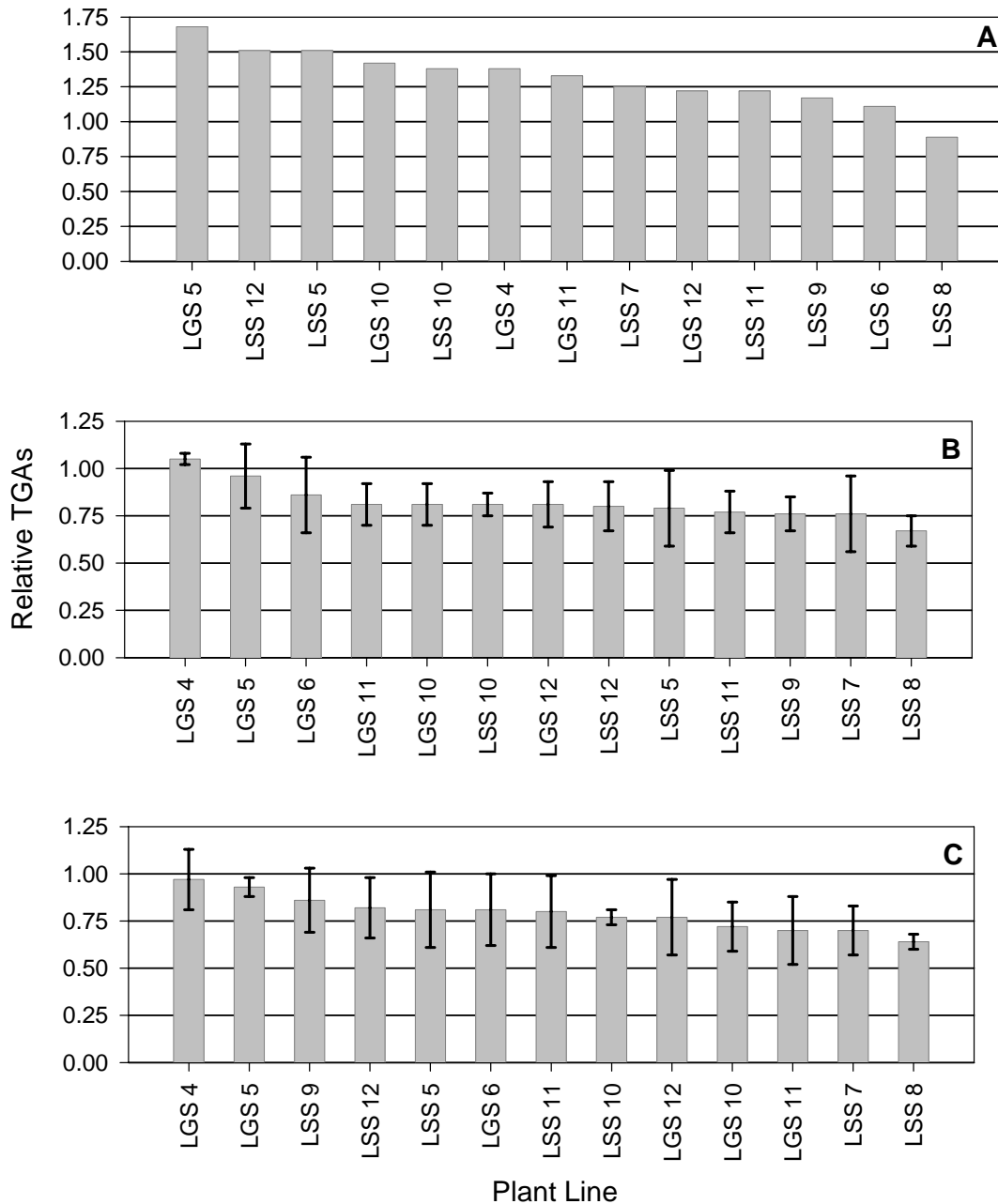


Fig. 1. Variation of TGAs from field grown tubers in lines containing the *Sgt* antisense transgene. Values represent the average TGAs of multiple samples relative to the Lenape WT controls of 30.4, 23.5 and 42.0 (mg/100 g FW) for 1997, 1998 and 1999, respectively. A. Average of two field-grown tubers in 1997 from glass house produced mini tubers. B. Triplicate analysis of field grown tubers from 1998. C. Triplicate analysis of field grown tubers from 1999. Bars represent the means of samples and error bars represent +/- one standard deviation.

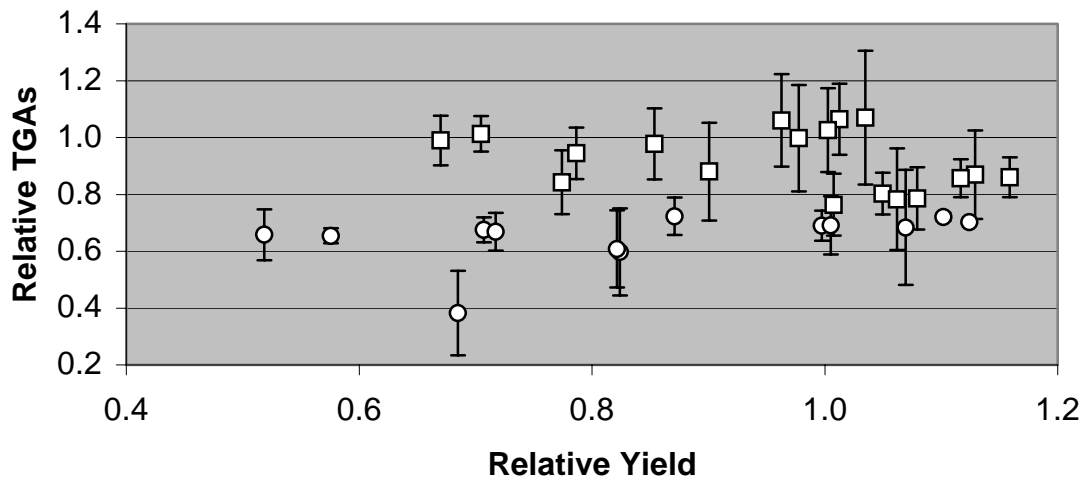


Fig. 2. Relationship of TGAs versus yield in LSS, LGS and GaSU transgenic lines from replicated field plots grown in Idaho in 2000. Relative TGAs represent the mean for one tuber from each of 4 replicated plots. Series 1 [\circ] represents lines with mean TGAs significantly reduced compared to Lenape WT controls. Series 2 [\square] lines not significantly different. Error bars represent +/- one standard deviation. Relative Yields represent a single measurement of CWT/A compared to the average of two Lenape controls.

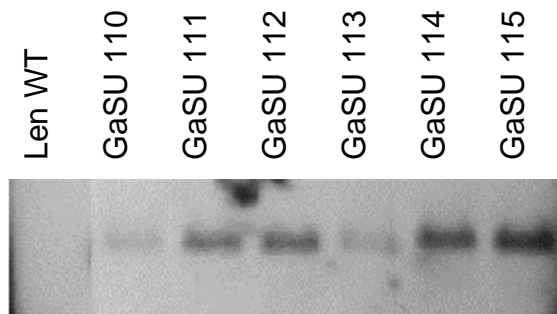


Fig. 3. Expression of selectable marker transcripts in young leaves. Lines from Lenape transformed with the GaSU construct and grown in Wisconsin in 1999. Each lane contains 15 μ g of total RNA hybridized to a [32 P]-labeled probe from the coding sequence of the *NptII* gene.

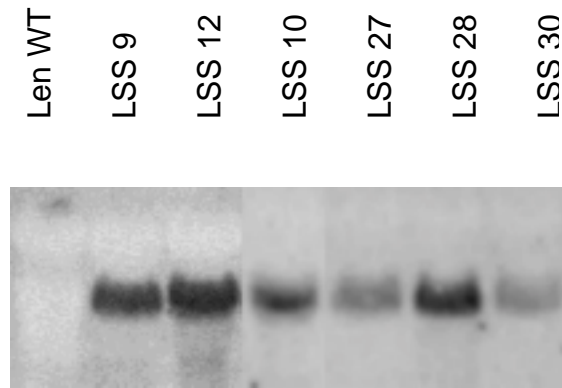


Fig. 4. Expression of Antisense *Sgt* transcripts in LSS lines from 1998. Antisense *Sgt* RNA was detected using a strand-specific digoxigenin-labeled probe on ~20 μ g of total RNA extracted from young expanded leaves of tissue culture transplants and young leaves of a Lenape WT plant.

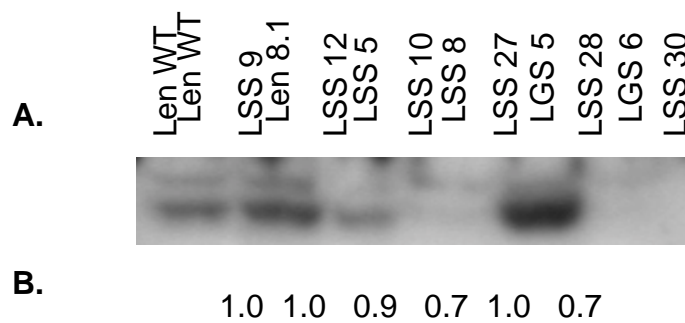


Fig. 5. Protein blot in control and antisense transgenics grown in the field in 1999. Total protein was extracted from peels and reacted with a polyclonal antibody directed towards SGT. A. Fluorograph of protein blot. B. Relative TGA levels compared to Lenape WT.

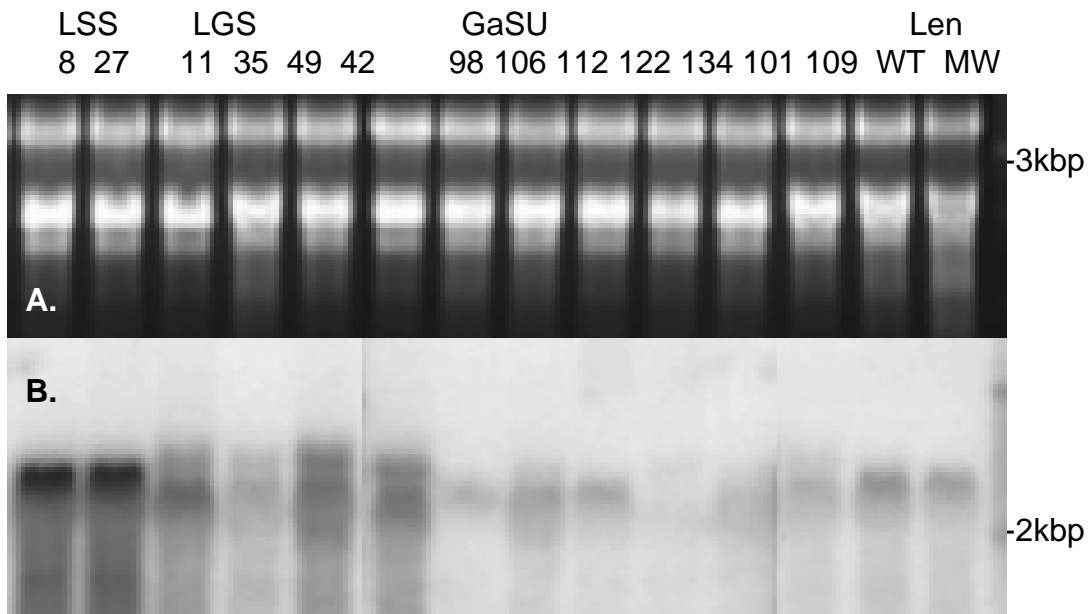


Fig. 6. Detection of *Sgt* transcripts in tuber peels of antisense lines grown in 2001 in the field in Idaho. Each lane was loaded with 12 μ g total RNA. A. Ethidium bromide stained RNA gel prior to membrane transfer. B. Autoradiograph using double stranded [32 P]-labeled probe from the *Sgt* cDNA. Lanes have been re-arranged to group lines by transgene construct.

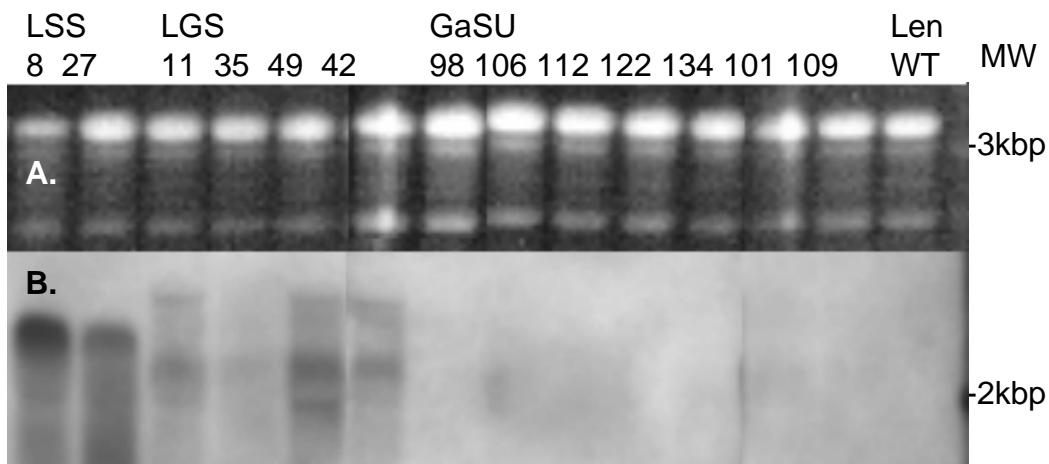


Fig. 7. Detection of antisense transcripts in tuber peels of antisense lines grown in 2001 in the field in Idaho. Each lane was loaded with 3 μ g total RNA. A. Ethidium bromide stained RNA gel prior to membrane transfer. B. Antisense-specific non-radioactive single stranded *Sgt* probe. Lanes have been re-arranged to group lines by transgene construct.

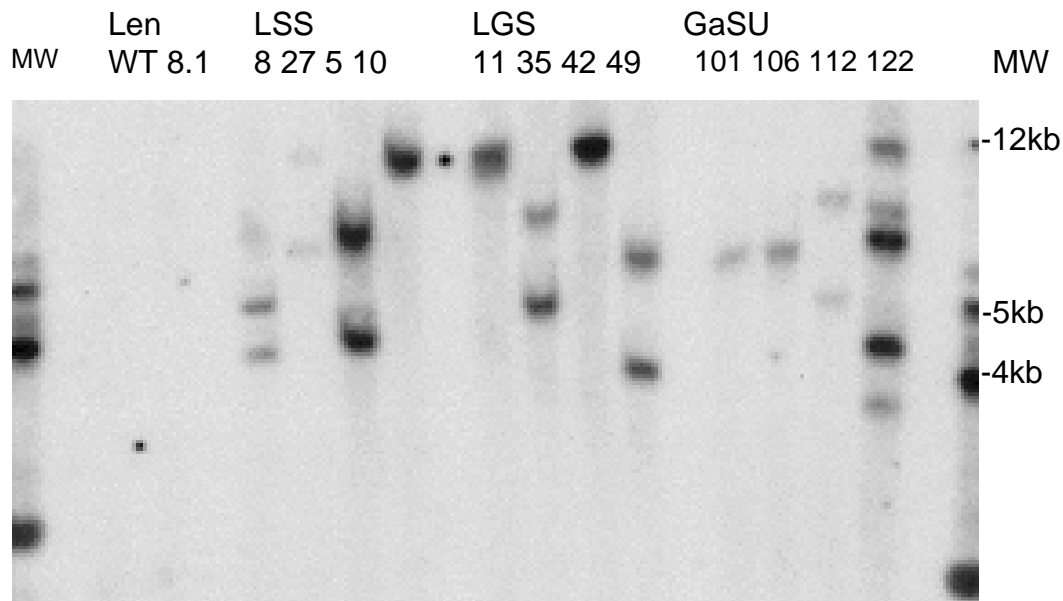


Fig. 8. Analysis of transgene insertion patterns in *Sgt* antisense Lenape lines grown in 2001. Genomic DNA was double-digested with XbaI and BamHI and hybridized to a double stranded [³²P]-labeled probe from the *NptII* coding region of the T-DNA.

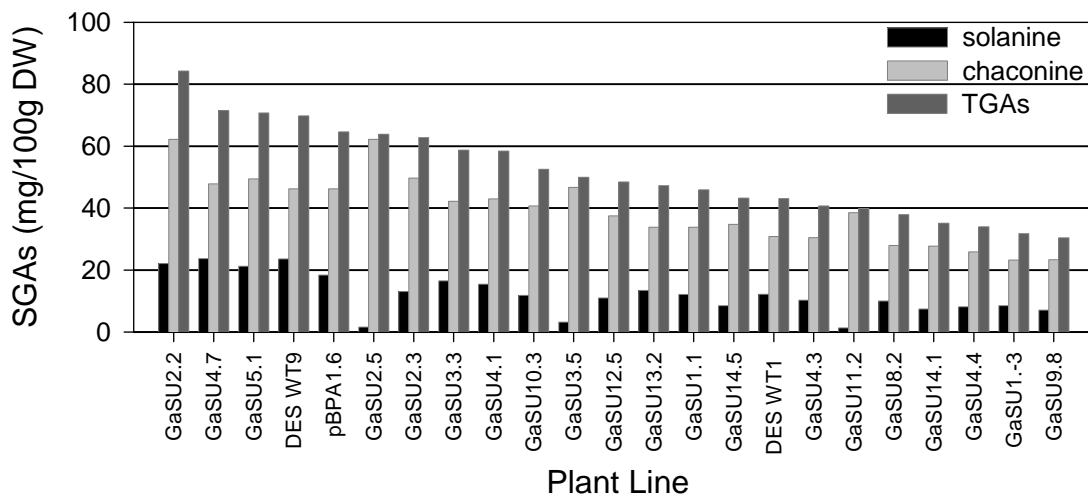


Fig. 9. HPLC analysis of component SGAs solanine, chaconine and TGAs in glass house-grown minitubers of Desiree transformed with the GaSU construct. Lines are presented by decreasing TGAs. Data includes samples for two Desiree WT and one pBINPLUS/ARS-transformed control.