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Toxic Secondary Metabolite Production in Genetically Modified Potatoes in Response to Stress

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Potatoes produce a number of toxic secondary metabolites, which are divided into two groups: the sesquiterpenes and the glycoalkaloids (PGAs): whereas PGAs are largely preformed and present in toxic quantities in both the foliage and "green" potatoes, it is well documented that the levels of PGAs and sesquiterpenes are effected by many biotic an abiotic stresses. The development of genetically modified potato varieties has made it prudent to ascertain whether there may be changes in the amounts or types of these secondary metabolites either as a direct effect of the transgene or due to its interactions with environmental variables. Transgenic potato lines were exposed, along with nontransgenic lines, to a range of biotic and abiotic stresses and a range of environmental conditions in the field and store. Following stressing, a comparison was made of levels of potato glycoalkaloid and sesquiterpene levels between the two groups. Significant differences were observed in the levels of both glycoalkaloid and sesquiterpene levels between transgenic and control material and between infected and noninfected material.

KEYWORDS: ***Solanum tuberosum*; genetic modification; potato glycoalkaloids; sesquiterpene phytoalexins; human toxicity**

INTRODUCTION

Glycoalkaloids are antinutritional, secondary metabolites commonly found in *Solanum* species. Cultivated potato tubers are commonly known to be intrinsically mildly toxic, whereas the aerial parts of the plant are more profoundly toxic, due mainly to the presence of glycoalkaloids. If normally subterranean parts of the plant, such as the tubers, become exposed to light, these too will potentially become dangerously toxic. Fortunately, this eventuality is often accompanied by green coloration associated with chlorophyll production.

Glycoalkaloids. Potatoes naturally contain several types of alkaloids that are primarily associated with resistance to disease and predation. The most important group of alkaloids in commercial potato varieties is the glycoalkaloids (GA), a sugar molecule (usually a trisaccharide) linked to the steroidal alkaloid solanidine. The total glycoalkaloid content (TGA) of potato tubers varies widely. Values between 2 and 410 mg/100 g of fresh weight (FW) have been found (*1*), but in most cases the TGA concentration in whole tubers is between 10 and 150 mg/100 g of FW (*2*). Of the total glycoalkaloids in potato tubers, 95% comprises α-chaconine and α-solanine.

Glycoalkaloids are not evenly distributed within the tubers, but are present in higher concentrations at the periphery (reviewed in ref *3*). For this reason, tuber size is important to the GA level. Large and often unpredictable variations in GA levels can arise from differences in variety, locality, season, cultural practice, and stress factors. Today, the widely accepted safety limit for the level of total glycoalkaloids (TGA) in tubers is 200 mg/kg of FW (*3*, *4*). Glycoalkaloids are not destroyed during cooking or frying. Severe glycoalkaloid poisoning causes symptoms ranging from gastrointestinal disorders through confusion, hallucination, and partial paralysis to convulsions, coma, and death (*3*). Evidence suggests that human susceptibility to glycoalkaloid poisoning is high and very variable: oral doses in the range of 1−5 mg/kg of body weight are marginally to severely toxic to humans (*5*), whereas 3−6 mg/kg of body weight can be lethal (*6*).

Sesquiterpenes. Another group of compounds that are associated with defense is the phytoalexins, which are defined as a chemically diverse group of low molecular weight antimicrobial compounds that are produced after infection. Examples include simple phenylpropanoid derivatives, flavonoid- and isoflavonoid-derived phytoalexins, sesquiterpenes, and polyketides. Phytoalexins are synthesized from the cytosolic acetate/mevalonate pathway in isoprenoid biosynthetic system of plants; they may be biosynthetically derived from one or several primary biosynthetic pathways. Polyketides such as 6-methoxymellein and sesquiterpenes such as capsidiol are derived from the acetate−malonate and acetate−mevalonate pathways, respectively. In potato, tubers synthesize antifungal sesquiterpenoid phytoalexins in response to fungal infection or

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Table 1. Components of the T-DNA of Plasmid PFW14000

abbreviation	definition	source	size (bp)	function
RB	right border	*A. tumefaciens*	25	T-DNA integration
NPT II	neomycin phosphotransferase II	transposon TN5	2000	kanamycin resistance
PS3/27P	patatin promoter	potato	3994	confines expression to tuber tissue
Inv	invertase	potato	1836	inhibits expression of invertase
NOS TERM	nopaline synthase terminator	*A. tumefaciens*	272	stop signal
LB	left border	*A. tumefaciens*	25	T-DNA integration

Table 2. Components of the T-DNA of Plasmid PATC05034

abbreviation	definition	source	size (bp)	function
RB	right border	*A. tumefaciens*	25	T-DNA integration
NPT II	neomycin phosphotransferase II	transposon TN5	2000	kanamycin resistance
KNT1 PRO	KNT promoter	potato	890	promoter that up-regulates transcription at nematode feeding sites
maize RIP	maize ribosome inactivating protein	maize	756	enzymatic cleavage of ribosomes
NOSTERM	nopaline synthase terminator	*A. tumefaciens*	272	stop signal
LB	left border	*A. tumefaciens*	25	T-DNA integration

arachidonic acid elicitation. The activity of the early pathway enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) has been shown to increase rapidly and then decrease in response to these stimuli.

Rishitin, lubimin, and solavetivone were found to be the major sesquiterpenes present in 46 cultivars and breeding selections of potato investigated in ref *7*. These authors found that concentrations of total sesquiterpenes were low or undetectable in untreated tuber slices, but ranged from 5 to 101 μg/g of FW tissue 4 days after treatment with arachidonic acid. Sesquiterpene cyclase and squalene synthase are key branch point enzymes in the isoprenoid pathway for the synthesis of sesquiterpenoid phytoalexins and glycoalkaloids, respectively.

Although related compounds such as the sesquiterpene lactones and the picrotoxins are well documented for their extreme human toxicity, no limits have been set for the consumption of sesquiterpene phytoalexins, and indeed the present authors were unable to find any literature concerning their safety or otherwise in the human diet for sequiterpenes derived from potatoes or related species.

The limited information relating to sesquiterpenes derived from other species suggests that studies in potato would be of interest. Toxicity, for example, was noted for vallapianin derived from *Heritiera lottoralis* and used as a fish and spearhead poison (*8*), and two sesquiterpenes from the sponge were found to be toxic to brine shrimp (*9*). The fungal subdivision *Basidiomycotina* produces toxic sesquiterpenes, many of which are derived from the protoilludane skeleton; many such compounds have been examined for potential medicinal use. Antitumor activity, for example, was found in the case of illudin and its derivatives derived from *Clitocybe illudens* (*10*).

Genetic Manipulation and Potato Toxicity. Among the public, a number of concerns have been raised generally about the safety of genetically modified crops including that the new genes may have unforeseen effects, that the transgenes may transfer to other organisms, and that the transgenic crops may cause disease, be parasitic, or become a vector or reservoir for human, plant, or animal disease (for example, see http://www.greenpeace.org/international/campaigns/genetic-engineering). The development of genetically modified varieties such as those with altered starch/sugar metabolism or enhanced resistance to predation raises the questions as to whether there could be changes in the amounts or types glycoalkaloids and sesquiterpene phytoalexins produced within the edible portion of the plant, the tuber. In addition, the biotic and abiotic stress thresholds for eliciting a response and the stability of the genetic transformations under these conditions need to be examined and quantified in these new varieties. For the purposes of this study, potato lines were produced containing either a tuber-expressed anti-invertase gene (leading to reduced low-temperature reducing-sugar levels), a maize-derived ribosome inactivating protein driven by a promoter up-regulated at nematode feeding sites (leading to enhanced resistance to the potato cyst nematode), or both of these constructs.

Antisense Invertase. These lines contained, among other components, a potato-derived invertase sequence in antisense orientation driven by a patatin promoter and subtended by the *Agrobacterium*-derived nopaline synthase terminator. Expression of the antisense invertase gene leads to a reduction in the amount of invertase enzyme in the tubers resulting in a reduction in the accumulation of reducing sugars at low temperatures and subsequent browning problems during processing, in particular, cooking at high temperature.

Maize Ribosome-Inactivating Proteins (RIPs). The maize-derived ribosome-inactivating protein used in this study is a type 1 RIP. RIPs have been detected in more than 170 angiosperm plant species and are broadly divided into two types: type 1, comprising a single A chain; and type 2, which comprise both A and B chains (*11*). Both types have the same activity, whether intracellular or in cellfree systems, in that they inactivate ribosomes by cleaving the ribosomal RNA at specific loop structures. However, type 1 RIPs do not have lectin activity, which facilitates binding and uptake by cells, and are therefore up to a million times less toxic than type 2 RIPs. In this study, this gene was driven by a promoter (KNT 1) derived from tobacco, which is up-regulated at the site of root knot nematode feeding. This causes the RIP to be expressed in these sites, resulting in the localized death of the root cells, and prevents colonization by the potato cyst nematode.

A fuller description of the behavior of lines derived from these constructs is available in the Food Standards Agency final report of Project G01015 available by e-mail request to library&info@foodstandards.gsi.gov.uk.

MATERIALS AND METHODS

Vector Construction. cDNAs were isolated corresponding to the coding sequence of the potato acid invertase gene. This was subcloned into the pBin19-derived binary plasmid, pFW14000, in the antisense orientation to give the vector pFW14272. pFW14272 also contains a class I patatin promoter and a nopaline synthase terminator fragment.

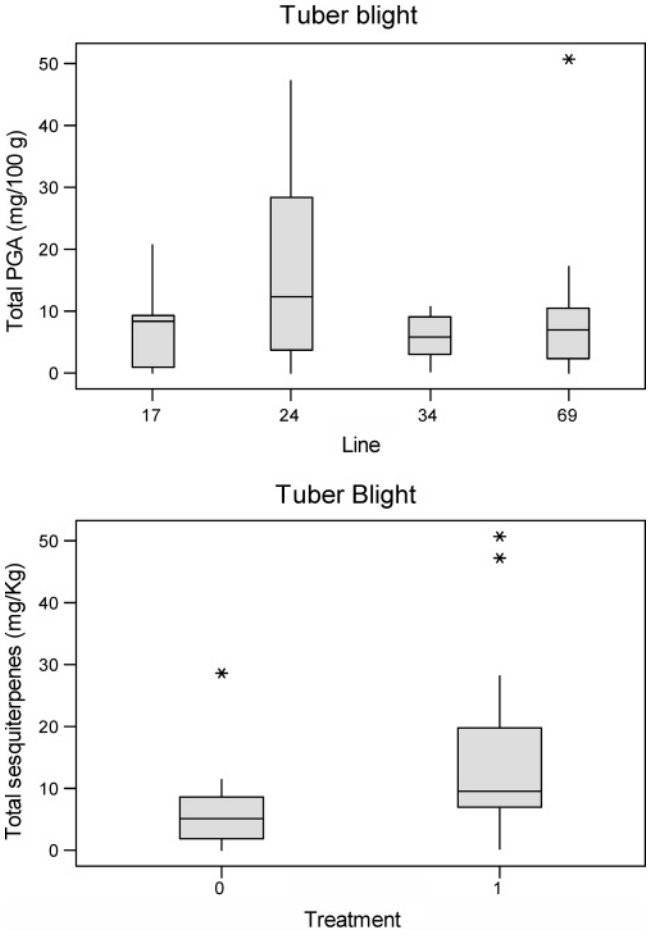

Figure 1. Box plots of PGA levels in anti-invertase lines grown in 2001 and subjected to tuber blight: (lines 24, 34, and 69) cv. Record, antisense invertase; (line 17) cv. Record (NCC); (treatment 0) control; (treatment 1) infected.

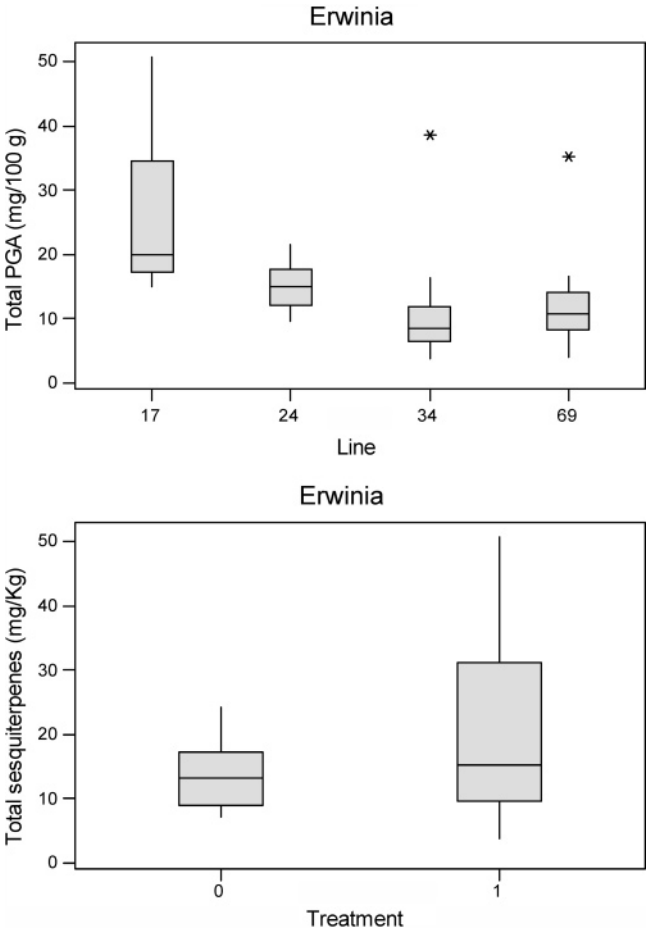

Figure 2. Box plots of PGA levels in anti-invertase lines grown in 2001 and subjected to *Erwinia*: (lines 24, 34, and 69) cv. Record, antisense invertase; (line 17) cv. Record (NCC); (treatment 0) control; (treatment 1) infected.

The recombinant gene for the maize RIP is constructed from the KNT1 promoter in a transcriptional fusion with the cDNA for maize ribonuclease inhibitory protein and the nopaline synthase terminator. The recombinant gene is subcloned into a modified pBin19 vector to give the pATC05034 binary vector). **Tables 1** and **2** list the components of the plasmids in more detail.

The control line is non-cocultivated material that has otherwise been through the same tissue culture and regeneration process as the other lines. The controls were treated in this way to allow for any effects that the tissue culture process might have in addition to that of the genetic transformation.

Potato Lines. The antisense invertase lines pFW14272-34, -24, and -69 were derived from the cultivar Record and are part of an ongoing commercial development program. These lines are therefore the culmination of several years of selection for normal growth and development and other characters representative of the originating variety. The cultivar Record is, however, more difficult to transform and regenerate than many other potato cultivars, and so the remainder of the lines used in this study were produced by transforming the cultivar Hermes.

Double Cocultivations. Leaf disks of the cultivar Hermes were cocultivated with a mixture of *Agrobacterium* cultures containing either the pFW14272 or pATC05024 plasmid; shoots were removed from leaf disks following regeneration on medium containing kanamycin. Regenerated plantlets were analyzed by PCR for the presence of the transgenes: pFW14272 alone, pATC05034 alone, and pFW14272 + pATC05034 combined (data not shown). Expression of the antisense genes was confirmed by assaying reducing sugar levels, whereas transcripts of the maize RIP genes were detected by RT PCR (data not shown).

Selected were five lines that contained the antisense invertase gene, three lines that contained the maize RIP gene, and eight lines that contained both. There were in excess of 70 plants per line, which gave a yield on the order of 200 minitubers per line.

Exposure of Genetically Modified Potatoes to Biotic and Abiotic Stresses. Potato plants of lines NCC 17 (non-cocultivated control), pFW14272-34, -24, and -69 (all unstacked anti-invertase lines) were subjected to biotic and abiotic stress in a series of field and pot experiments carried out between July and September 1999. All seed stocks had been multiplied together from the in vitro stage. Plants or tubers were exposed to blight (*Phyptohthora infestans*), potato leaf roll virus (PLRV), potato virus Y (PVY), white potato cyst nematode (*Globodera pallida*, pathotype Pa_{2-3}), bacterial wet rot (*Erwinia carotovora*), gangrene (*Phoma foveata*), gray field slugs and Budapest slugs (*Deroceras reticulatum* and *Milax budapestensis*, respectively), drought, and mechanical damage. In the following sections, only those treatments resulting in a significant change in PGA or sesquiterpene levels will be described further.

Blight in Tubers, Bacterial Wet Rot, Gangrene, Mechanical Damage, and Slug Damage. Field plots consisted of 4 rows of 15 plants and supplied tubers for several experiments. The lines were replicated in five randomized blocks.

Blight in Tubers. While the plants were still actively growing, a proportion of each plot was harvested, and 50 tubers were sprayed with a suspension of zoospores of *P. infestans*. A second sample was taken from each plot and held under the same conditions during the incubation period. After a week, tuber blight infection was assessed prior to biochemical assays of infected and uninfected samples. Following haulm destruction and harvest, the tubers were stored and samples taken for bacterial wet rot, gangrene, damage, and slug experiments.

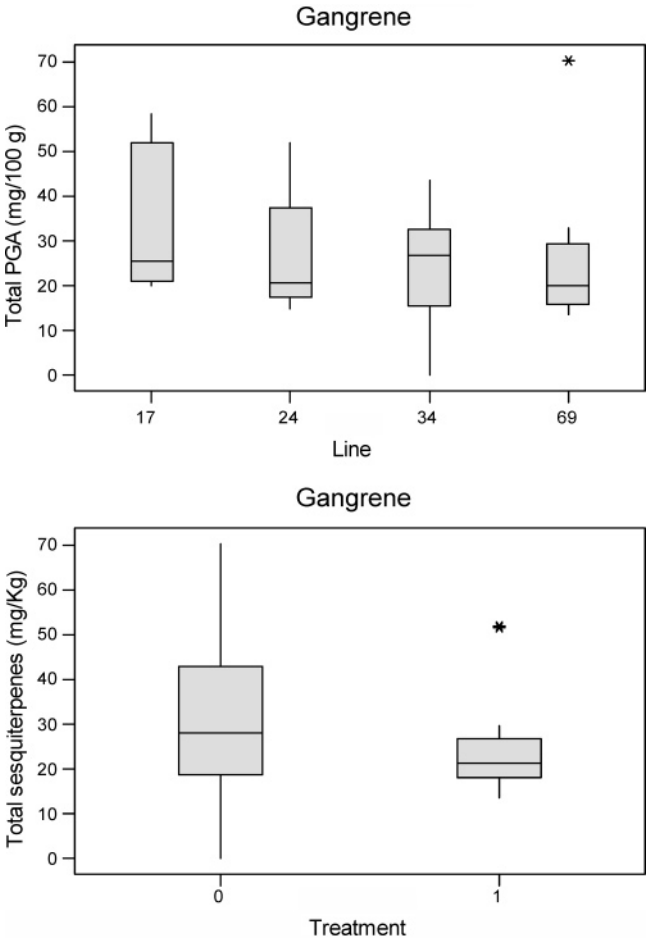

Figure 3. Box plots of PGA levels in anti-invertase lines grown in 2001 and subjected to gangrene: (lines 24, 34, and 69) cv. Record, antisense invertase; (line 17) cv. Record (NCC); (treatment 0) control; (treatment 1) infected.

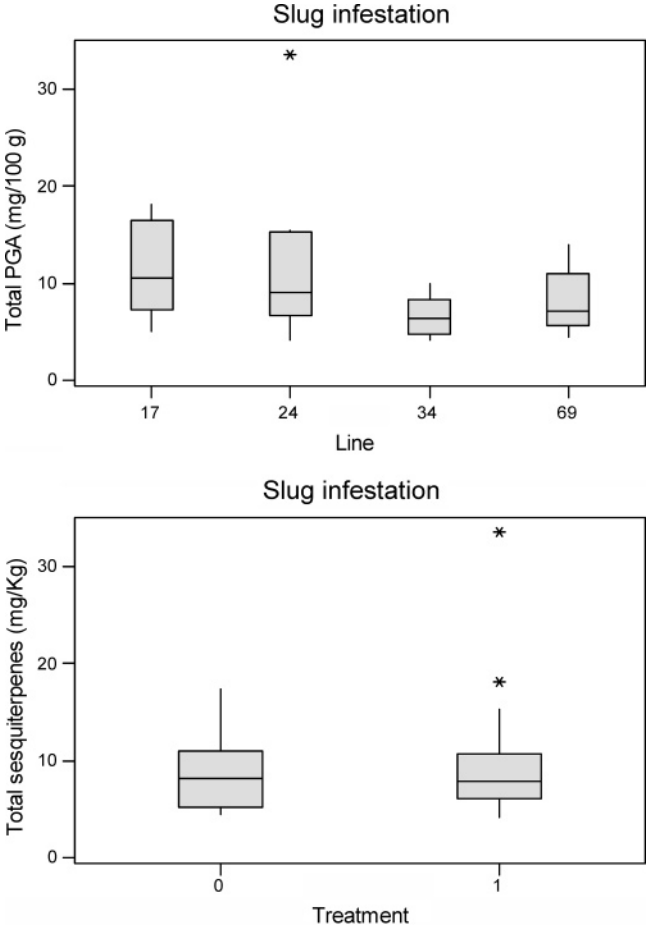

Figure 4. Box plots of PGA levels in anti-invertase lines grown in 2001 and subjected to slug infestation: (lines 24, 34, and 69) cv. Record, antisense invertase; (line 17) cv. Record (NCC); (treatment 0) control; (treatment 1) infected.

Bacterial Wet Rot. Twenty tubers from each field plot were placed in plastic trays on benches with wet capillary matting at 25 °C and covered with black polythene. Each tuber was pierced by a needle to a depth of 1 cm and the cavity filled with 50 μL of a suspension of *E. carotovora* subsp. *atroseptica*. After 7 days, infected tubers and uninfected control tubers were taken for biochemical assays. Control tubers were treated identically except that distilled water was added rather than *Erwinia* suspension.

Gangrene and Mechanical Damage. Forty tubers from each plot were rolled in a cement mixer with *P. foveata* inoculum. A second sample was rolled in a cement mixer without inoculum, and a third sample was left undamaged and kept in the same environment as the other samples throughout the incubation. The samples were assessed for gangrene and damage, respectively. Subsamples of tubers, which were homogeneous for disease and damage levels, were taken for biochemical assay.

Slug Damage. Twenty tubers were placed in plastic boxes (300 × 300 × 160 mm) boxes lined with damp absorbent paper and covered with muslin. The boxes were covered with black polythene and kept in a greenhouse maintained at ~20 °C. Slugs where collected from the field and five placed in each box (four *D. reticulatum* and one *M. budapestensis*). Later these numbers were quadrupled. The samples were assessed for the severity of feeding damage, and damaged and undamaged control tubers were taken for biochemical assays.

White Potato Cyst Nematode. Single plants were grown in 230 mm diameter pots with soil amended with cysts. The lines, with and without cysts, were replicated in 10 randomized blocks. Plant growth was poor and the progeny insufficient for replicated biochemical assays. Tubers from replicate pots were bulked into two replicates to give the necessary quantity of tubers.

Analysis of Potato Tuber Secondary Metabolite Content. *Preparation of Samples for Chemical Analysis.* Tubers were brushed in warm water to remove adhering soil and then patted dry with paper towels. As a general rule tubers measuring <1.5 cm in diameter were excluded from analysis as they were expected to show unrepresentative development and glycoalkaloid levels; of the remaining tubers, the required numbers, as stated above, were chosen at random. Each tuber was cut into equal fourths, and one-fourth was selected at random from each. Each fourth was then cut into ~1 cm cubes and transferred to a Tecator 1094 homogenizer. Approximately 100 mL of liquid nitrogen was added to the diced potatoes, which were allowed to freeze as the liquid nitrogen boiled away. The Tecator was then run at high speed until the material was finely macerated. The macerated samples were then transferred to a 500 mL container and stored at −20 °C.

HPLC Determination of Glycoalkaloid Content of Potato Tubers. Approximately 5 g of frozen macerated sample was weighed into a beaker and the weight recorded to an accuracy of 0.001 g. The tissue samples were then extracted in a solution comprising 0.02 M heptanesulfonic acid containing 1 mg/mL sodium bisulfate made up in 10% aqueous acetic acid (extractant solution) in an industrial blender. Debris was removed by centrifugation and the supernatant recovered. The extracts were made up to a constant volume and stored at −20 °C until required.

Prior to HPLC the extracts were cleaned and concentrated in 6 mL of C18 (end capped) SPE columns and finally eluted in hot methanol/0.1 M HCl (1:1 v/v). The eluates were then evaporated to dryness and redissolved in MeOH/0.1 M HCl. All solutions were degassed, prior to preparing the HPLC apparatus, by filtering under vacuum through a 0.2 μm 47 mm nylon membrane filter. The HPLC columns [Phenomenex ODS2 Sphericlone (5 μm) 250 × 4.6 mm column] were conditioned by running methanol through the system at 1.5 mL/min

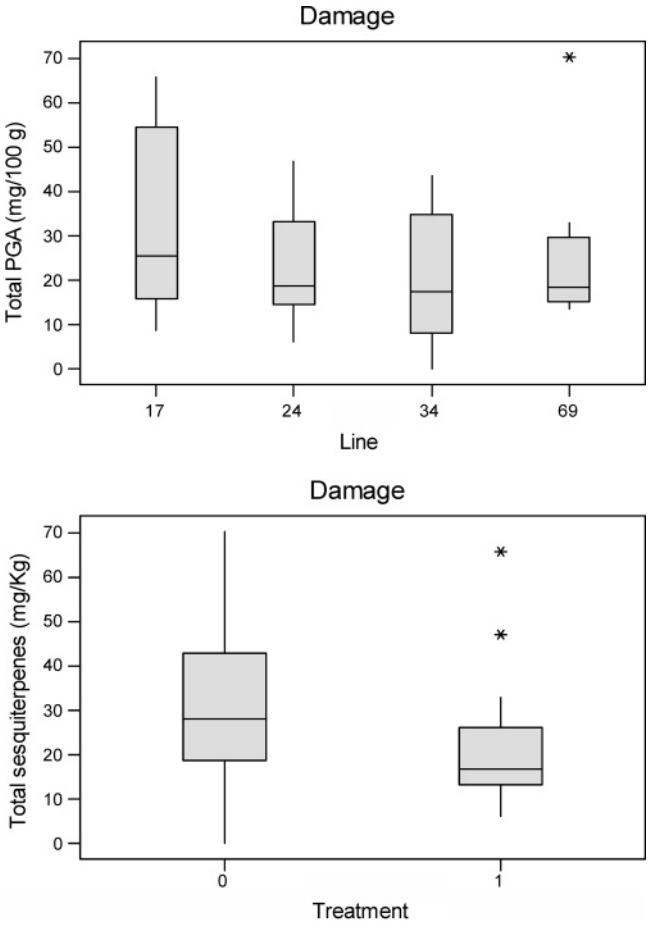

Figure 5. Box plots of PGA levels in anti-invertase lines grown in 2001 and subjected to mechanical damage: (lines 24, 34, and 69) cv. Record, antisense invertase; (line 17) cv. Record (NCC); (treatment 0) control; (treatment 1) infected.

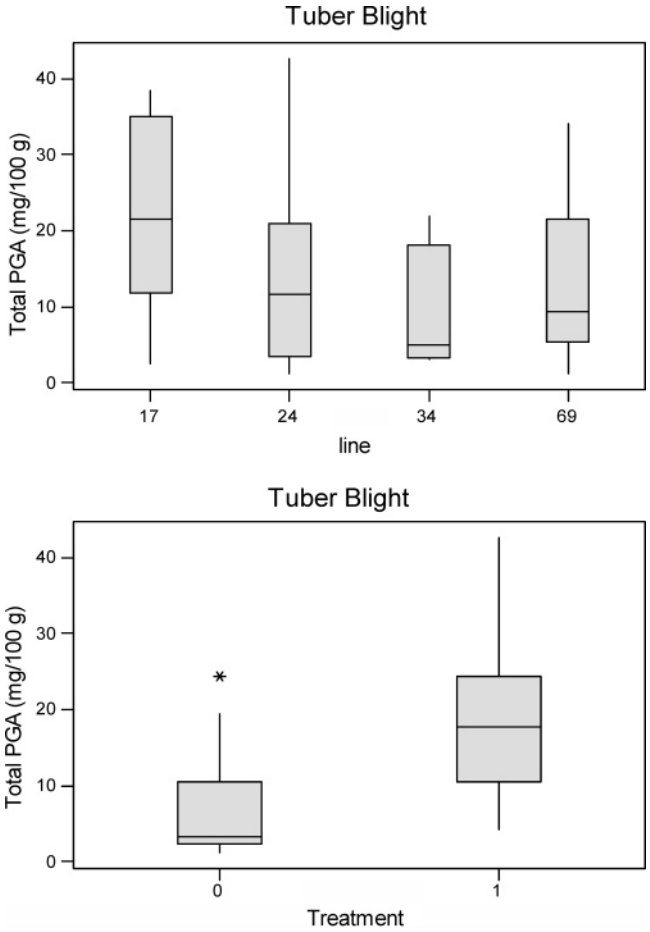

Figure 6. Box plots of PGA levels in antisense invertase lines grown in 2002 and subjected to tuber blight: (lines 24, 34, and 69) cv. Record, antisense invertase; (line 17) cv. Record (NCC); (treatment 0) control; (treatment 1) infected.

for 10 min followed by 50% methanol, 50% Tris-HCl buffer (0.01 M Tris-HCl, pH 7.8) at 1.5 mL/min for 10 min.

The HPLC was set to run each sample for 25 min: isocratic mode with 50% acetonitrile, 50% Tris-HCl buffer. Helium degas was set for 50 mL/min, and the glycoalkaloids were assessed by UV detection (Waters 486) at 202 nm. The chromatograms were integrated using Waters Millenium software. The glycoalkaloids in sample extracts were quantified by comparison with standard solutions of solanine and chaconine (500 mg/L in methanol). These standards are included with each batch of sample extracts examined with the HPLC. The recovery rate of glycoalkaloids in samples (mean recovery = 33.7%) was assessed by analyzing samples previously fortified with an aliquot of solanine and chaconine standard solutions to ~100 mg/100 g

$$\text{glycoalkaloids (mg/100g)} = \left(\frac{PA_{extr}}{PA_{std}} \times C_{std} \times \frac{0.25}{\text{sample}}\right)_{\text{solanine}} + \left(\frac{PA_{extr}}{PA_{std}} \times C_{std} \times \frac{0.25}{\text{sample}}\right)_{\text{chaconine}}$$

where PA_{extr} is the analyte peak area from sample extract chromatogram, PA_{std} is the analyte peak area from standard solution chromatogram, C_{std} is the concentration of analyte in standard solution (in mg/L), and sample is the quantity of sample taken for analysis (in g).

Determination of Sesquiterpene Content of Potato Tubers. Approximately 50 g of frozen macerated sample was weighed into a beaker and the weight recorded to an accuracy of 0.001 g. An aliquot of 50 mL of acetone and 50 mL of diethyl ether was added to the beaker and swirled gently to mix. The beaker was then clamped onto an Ultra Turax top-drive homogenizer such that the blades of the homogenizer were just above the bottom of the beaker. The homogenizer speed was gradually increased until a vigorous mixing action was observed and then maintained for a minimum of 5 min.

Approximately 25 g of Celite was added to the homogenate and the homogenizer run for a further minute. The extract was filtered under vacuum into a Büchner flask and the filter cake returned to the beaker. The filtrate was dried over sodium sulfate and transferred to a Kuderna−Danish apparatus. A 100 mL aliquot of diethyl ether was added to the filter cake and homogenized for a further 2 min. This second extract was filtered and dried as before and then added to the first extract in the Kuderna−Danish apparatus.

Antibumping granules were added to the extract, and a 250 mL receiving vessel was connected to the bottom of a Snyder fractionating column attached to the Kuderna−Danish apparatus. The Kuderna−Danish apparatus was heated from a steam bath and the solvent fractionated up the Snyder column. Once the extract was reduced to <5 mL in the tube, the steam bath was lowered to the point that the extract and vapor merely refluxed in the receiving vessel and the base of the Snyder column. The extract was refluxed for 2 min and then allowed to cool. The receiver and tube were removed from the Snyder column, and the receiver was washed with a small portion of solvent, which was allowed to drain into the tube. The tube was then removed from the vessel and stoppered. A 1 mL aliquot of ethyl acetate was added to the tube and the volume reduced to 1 mL by use of a gentle stream of dry nitrogen blown onto the surface of the solvent from a Pasteur pipet. The extract was then transferred to a 1 mL screw-cap chromatography vial.

Detection and Quantification by Gas Chromatography (GC). Potato sesquiterpenoids were separated on a Carlo Erba temperature programmable gas chromatograph. The following temperature program was found to give separation of all potato sesquitepenes when using a CPSil5 25 m × 0.32 mm × 0.25 μm column and splitless injection

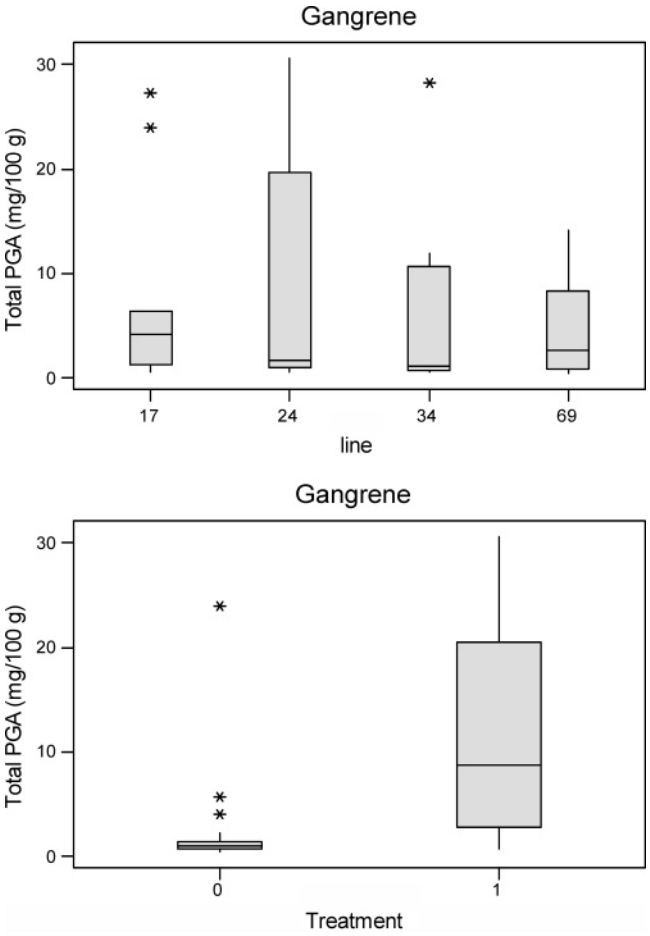

Figure 7. Box plots of PGA levels in antisense invertase lines grown in 2002 and subjected to gangrene: (lines 24, 34, and 69) cv. Record, antisense invertase; (line 17) cv. Record (NCC); (treatment 0) control; (treatment 1) infected.

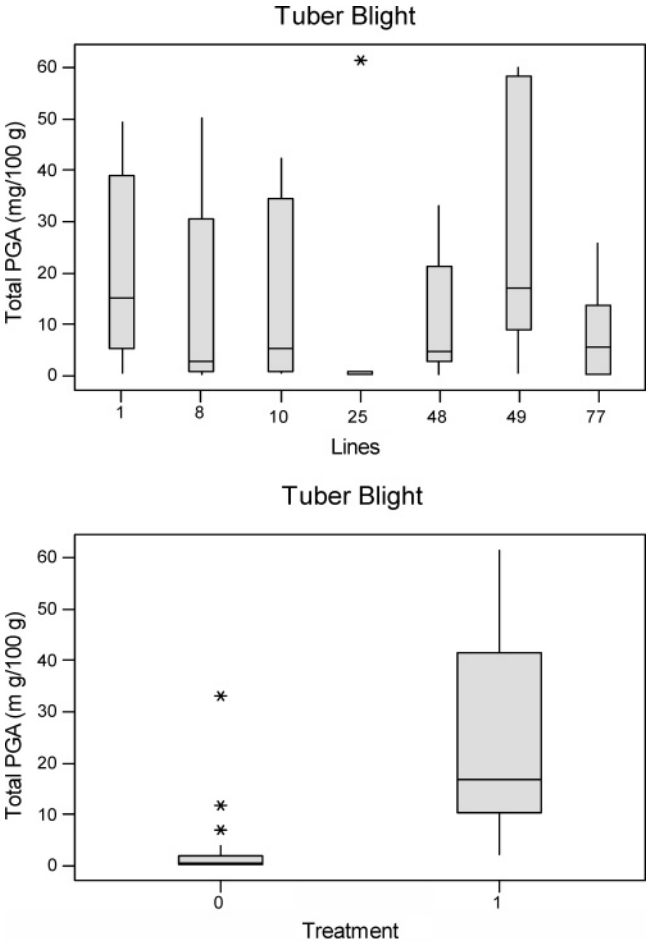

Figure 8. Box plots of PGA levels in maize RIP and stacked lines grown in 2002 and subjected to tuber blight: (lines 8, 10, 48, and 49) cv. Hermes, antisense invertase + maize-RIP; (lines 25 and 77) cv, Hermes, maize-RIP; (line 1) cv. Hermes (NCC); (treatment 0) control; (treatment 1) infected.

(split valve closed for 45 s, column flow ~2 mL/min): injector oven, 250 °C; detector oven, 250 °C; program, 70 °C for 1 min, ramp at ∝ to 120 °C, then at 10 °C/min to 210 °C. The GC was set up so that the gas mixture for the flame ionization detector (FID) was correct with air and hydrogen both at 30 psi.

Reference materials were generated by extracting potato tubers that were severely diseased. The diseased tubers were extracted in accordance with the general analytical protocol, but ethereal extracts were washed sequentially with 0.1 M HCl, 0.1 NaOH, and distilled water before the Kuderna−Danish concentration step. The concentrated extracts were assessed by GC-FID in accordance with the general analytical protocol. The chromatograms were compared with chromatograms for standard solutions prepared from commercially available Cedrol (Fluka). The same extracts and standard solutions were assessed by GC-MS under similar chromatographic conditions. The peaks identified by GC-MS were identified in the GC-FID traces by reference to their relative retention times and quantified with reference to the standard solutions prepared from the reference sample of Cedrol.

RESULTS

Sesquiterpene and Glycoalkaloid Levels. The following sesquiterpenes were identified: cedrol, diacetylphytuberin, ledol, sesquiterpene (unknown), solavetone, and rishitin. A figure showing the structures of these compounds can be found at http://www.scri.sari.ac.uk/TiPP/pps/Chart.pdf. In the following figures, the sum of these sesquiterpenes has been expressed as cedrol equivalents as this was a conveniently available standard to use.

The results are categorized according to year, metabolite, line, and treatment for the purposes of analysis. In the following text, "effects due to line" will be used to refer to differences both between transgenic and nontransgenic metabolite levels, whereas "effects of treatment" will refer to the effects of being infected with a pathogen or other stress.

A series of box plots for each of the factors were plotted (**Figures 1−15**). The box plots summarize the shape, dispersion, and center of the data and also reveal outliers. The bottom edge of the box represents the first quartile (Q1), whereas the top edge represents the third quartile (Q3). Thus, the box portion of the plot represents the middle 50% of the observations (the interquartile range, IQR). The line drawn through the box represents the median of the data. The lines extending from the box indicate the lowest and highest values in the data set, excluding outliers. Outliers, which are represented by asterisks, are values that lie outside the box by >1.5 times the IQR.

Two-way ANOVAs were carried out on the data, but because of the difficulty in testing ANOVA assumptions such as normality on samples of this kind, the data were also subjected to a Scheirer−Ray−Hare (S−R−H) test, although this test is more conservative than ANOVA, thereby increasing the likelihood of making a type II error. The threshold of statistical significance was taken to be at the usual 5% level. An extensive statistical appendix is available in the report on Project G01015 available from library&info@foodstandards.gsi.gov.uk.

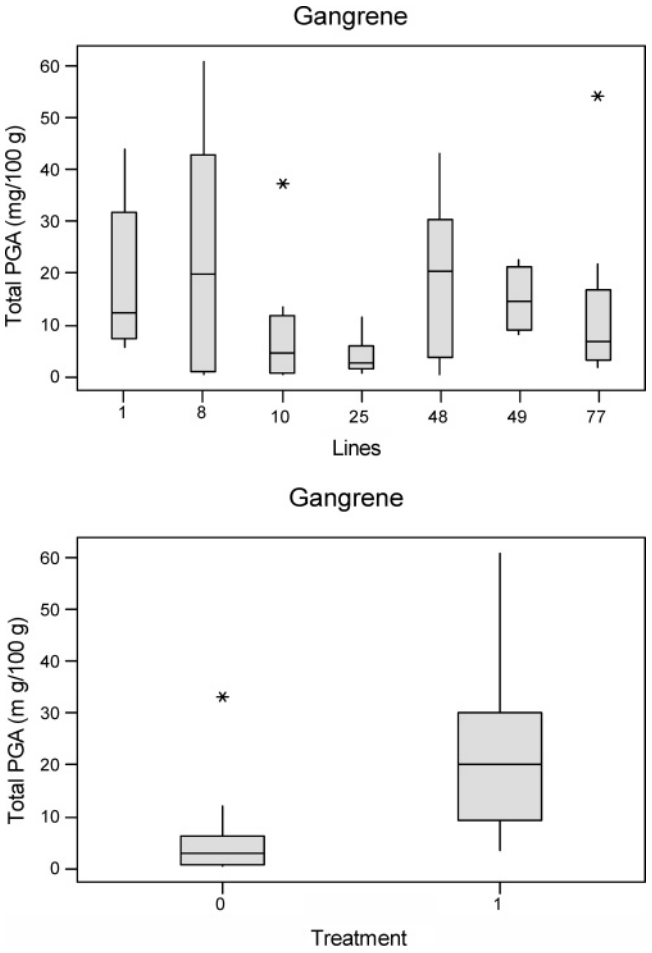

Figure 9. Box plots of PGA levels in maize RIP and stacked lines grown in 2002 and subjected to gangrene: (lines 8, 10, 48, and 49) cv. Hermes, antisense invertase + maize-RIP; (lines 25 and 77) cv, Hermes, maize-RIP; (line 1) cv. Hermes (NCC); (treatment 0) control; (treatment 1) infected.

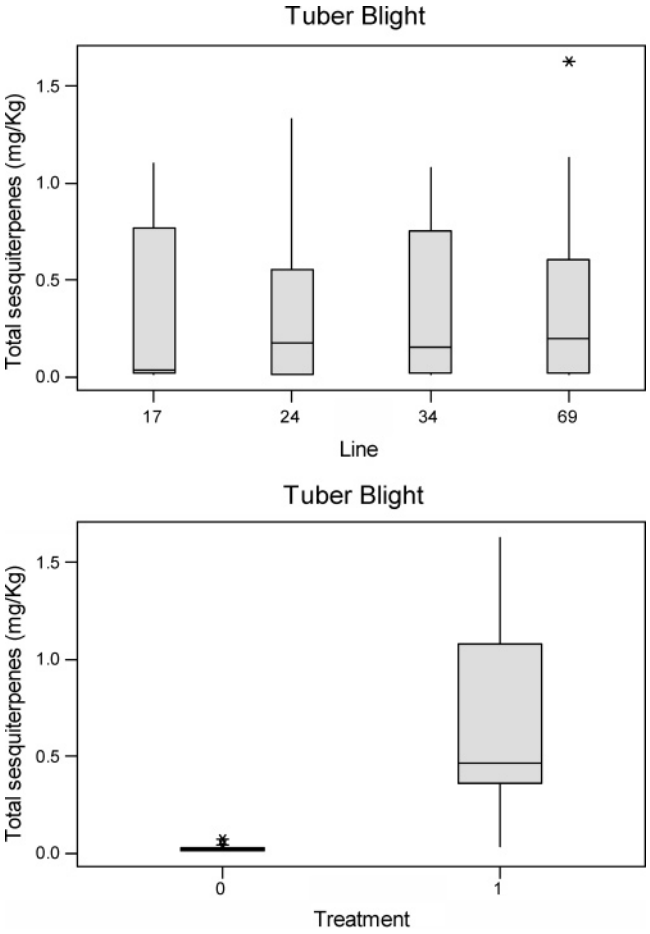

Figure 10. Box plots of sesquiterpene levels in the antisense invertase lines grown in 2001 and subjected to tuber blight: (lines 24, 34, and 69) cv. Record, antisense invertase; (line 17) cv. Record (NCC); (treatment 0) control; (treatment 1) infected.

Tuber Levels of Potato Glycoalkaloids: Antisense Invertase Lines Grown in 2001. The box plots (**Figures 1**−**5**) clearly show how the variation is apportioned among the factors: PGA levels are lower in each of the anti-invertase lines in the gangrene, damage, and *Erwinia*-treated material. In slug-damaged material, both the control (line 17) and line 24 are above the mean for all observations, whereas lines 34 and 69 are below it. In the material infected with tuber blight, lines 17 and 34 are below the overall mean, whereas line 24 is significantly above it. Line 69 was near the overall mean. The effect of slug infestation, *Erwinia*, and tuber blight infection was to increase the levels of PGA observed in the tubers. This was reversed in the material subjected to physical damage and gangrene, where the PGA levels were significantly decreased by the treatment.

The ANOVA results showed a statistically significant effect due to infection with tuber blight ($p = 0.013$) and gangrene ($p = 0.003$), whereas differences due to physical damage achieved a p level of 0.085. There was a significant effect due to the transformation in lines infected with *Erwinia* ($p = 0.006$). Similar results were observed in the S−R−H test with significant differences occurring between noninfected material and material subjected to tuber blight ($p = 0.009$) and physical damage ($p = 0.024$); differences due to gangrene infection were not found to be significant in this test. In concordance with the ANOVA results, a significant reduction in PGA levels was observed in transgenic lines as compared with the nontransgenic controls in treatments infected with *Erwinia* ($p = 0.006$). Differences in PGA levels between transgenic and nontransgenic lines as a result of slug infestation achieved a p level of 0.073.

Tuber Levels of Potato Glycoalkaloids: Antisense Invertase Lines Grown in 2002. The box plots (**Figures 6** and **7**) show that the mean PGA levels of the transgenic lines were suppressed in the material subjected to tuber blight. In material subjected to gangrene, lines 34 and 69 were suppressed with respect to the control, whereas line 24 was slightly increased. All treatments showed a strong increase in PGA levels as a result of pathogen infection. The ANOVA revealed that the differences were significant only in the case of infected versus noninfected material subject to gangrene ($p = 0.002$) and tuber blight ($p < 0.001$); this was also supported by the S−R−H test, where differences in PGA levels between infected and noninfected were significant at $p < 0.001$ for both tuber blight and gangrene. The differences observed between transgenic and nontransgenic material in the box plots were not found to be statistically significant in this test.

Tuber Levels of Potato Glycoalkaloids: Maize RIP and Stacked Lines Grown in 2002. The box plot (**Figure 8**) shows greater variability in PGA levels among the lines than was observed in the antisense invertase material, with some lines (8, 48, and 49) showing increased levels with respect to the controls, whereas the remainder were lower. The ANOVA confirmed that the differences observed among the lines and between infected and uninfected materials were highly signifi-

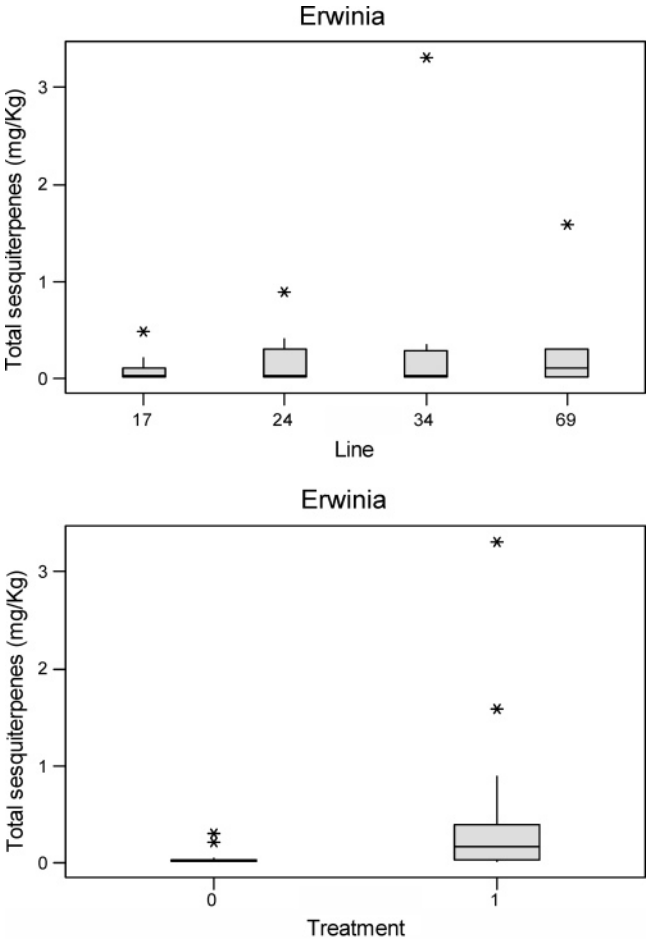

Figure 11. Box plots of sesquiterpene levels in the antisense invertase lines grown in 2001 and subjected to *Erwinia*: (lines 24, 34, and 69) cv. Record, antisense invertase; (line 17) cv. Record (NCC); (treatment 0) control; (treatment 1) infected.

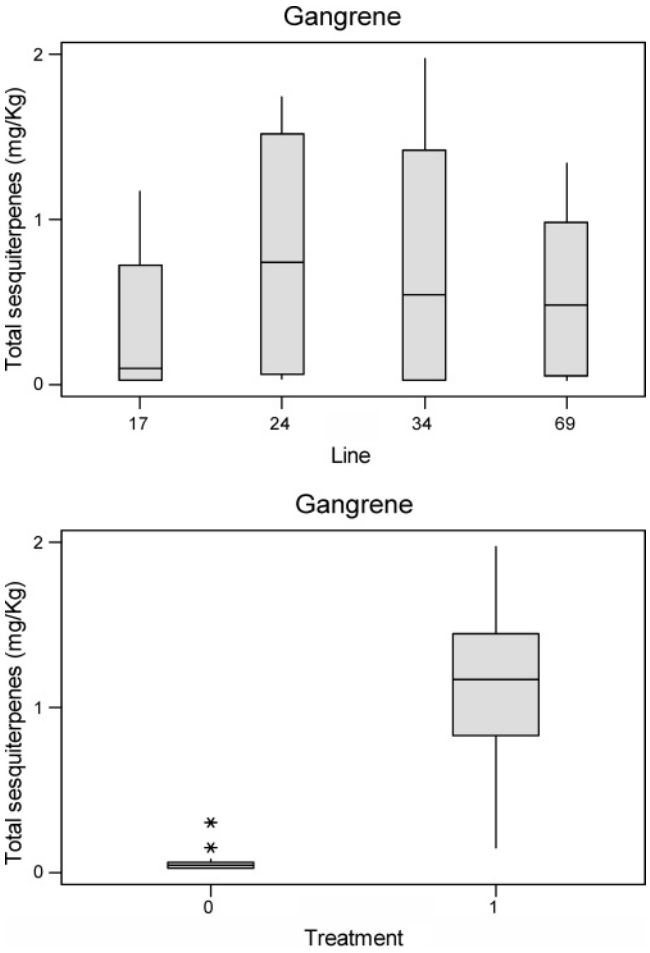

Figure 12. Box plots of sesquiterpene levels in the antisense invertase lines grown in 2001 and subjected to gangrene: (lines 24, 34, and 69) cv. Record, antisense invertase; (line 17) cv. Record (NCC); (treatments 0) control; (treatment 1) infected.

cant ($p < 0.001$) for material infected with tuber blight. The S−R−H test was not performed on these data due to missing values.

Tuber Levels of Sesquiterpene Phytoalexins: Antisense Invertase Lines Grown in 2001. In contrast to the trend observed for PGA levels, the transgenic lines tended to show elevated levels of sesquiterpenes compared to the non-coculti-vated controls (**Figures 10**−**13**). In all cases apart from damage due to slug infestation, infection resulted in elevated levels of sesquiterpenes as compared to uninfected controls. The ANOVA returned significant *p* values for the observed differences between infected and noninfected treatments in the case of gangrene ($p < 0.001$), *Erwinia* ($p = 0.046$), and tuber blight infection ($p < 0.001$). A significantly ($p < 0.001$) elevated level of sesquiterpenes in comparison with the control line was observed in lines 24 and 34. The S−R−H test returned highly significant *p* values for differences due to infection with *Erwinia*, tuber blight, gangrene, and physical damage (all $p < 0.001$).

Tuber Levels of Sesquiterpene Phytoalexins: Antisense Invertase Lines Grown in 2002. The box plots (**Figures 14** and **15**) show some small differences between the lines with line 24 having slightly elevated levels of sesquiterpenes in comparison with the other lines. Large differences, however, were observed between the lines due to PCN infestation, with the transgenic lines having lower levels of sesquiterpenes than the controls. The effect of gangrene infection was to increase sesquiterpene levels, whereas that of PCN was to lower them. ANOVA returned significant *p* values for differences among lines ($p < 0.001$) and treatments ($p = 0.03$) for gangrene infection. The S−R−H test returned significant values of *p* for gangrene treatment ($p < 0.001$) and among the lines for PCN infection ($p = 0.02$).

Tuber Levels of Potato Sesquiterpene Phytoalexins: Maize RIP and Stacked Lines Grown in 2002. Significant differences were observed between lines ($p < 0.001$), but with no consistent pattern with regard to the control, and between treatments, with infected material having significantly elevated levels with respect to uninfected material ($p = 0.033$). The S−R−H test was not carried out on this material due to missing values.

Summary of Results. Although in general the combined effect of GM construct and stress treatment was not identical for all combinations of treatments, a consistent pattern was observed in that antisense invertase material tended to have lower PGA levels than untransformed controls, a result that has been observed in other studies. The maize RIP lines were less predictable, some lines being unchanged while others had reduced and still others elevated levels of PGAs and sesquiterpenes. Few conclusions can be drawn about the behavior of the stacked lines due to the limited number that could be generated.

Tuber blight, gangrene, and *Erwinia* were the stresses that were mostly likely to lead to differences among the various lines.

The differences that were observed among the various GM and non-GM lines were in most cases exceeded by effects due

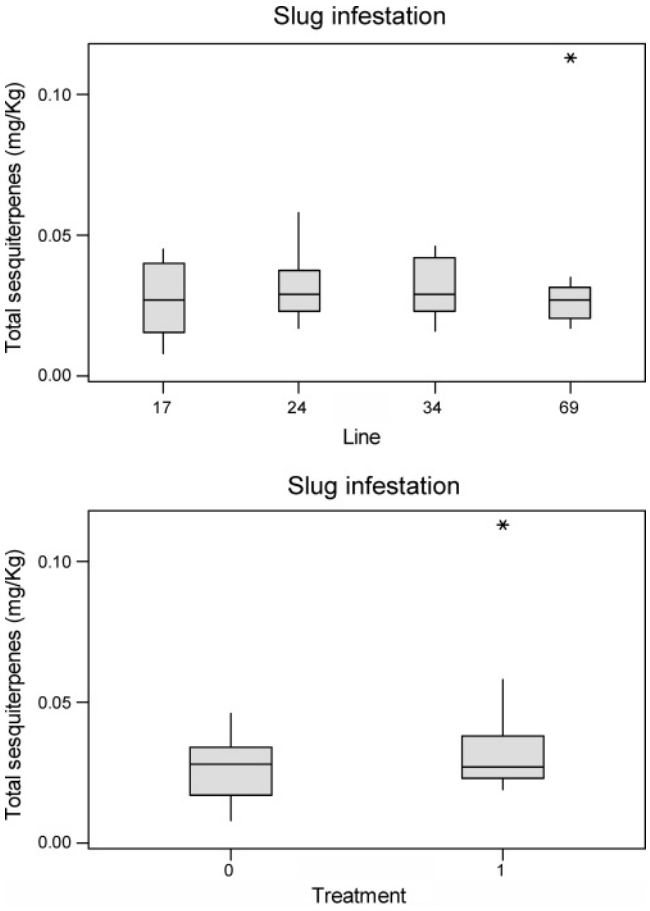

Figure 13. Box plots of sesquiterpene levels in the antisense invertase lines grown in 2001 and subjected to slug infestation: (lines 24, 34, and 69) cv. Record, antisense invertase; (line 17) cv. Record (NCC); (treatment 0) control; (treatment 1) infected.

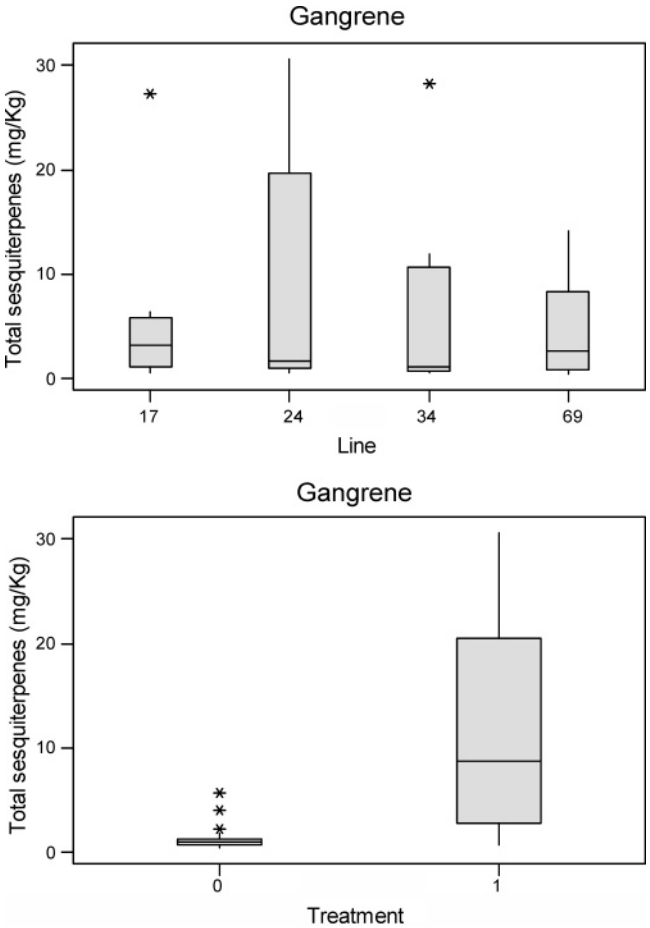

Figure 14. Box plots of sesquiterpene levels in the antisense invertase lines grown in 2002 and subjected to gangrene infection: (lines 24, 34, and 69) cv. Record, antisense invertase; (line 17) cv. Record (NCC); (treatment 0) control; (treatment 1) infected.

to the stresses alone; tuber blight, gangrene, and *Erwinia* infection generally led to elevated levels compared to the uninfected materials. For infected material, there were some instances when the observed levels of PGA were >3 times the accepted "safe" levels.

DISCUSSION

Although at the inception of this project there were no published reports of the responses of transgenic potatoes to environmental and biotic stresses to guide either the choice of transgenes or treatments, many of the trends observed in this study now have counterparts in the published literature.

Consistent with the results observed in this study, ref *11* showed that the introduction of an antisense invertase gene from yeast led to a reduction in the levels of steroidal glycoalkaloids in a number of potato cultivars. In contrast, however, suppression of amylase biosynthesis by antisense RNA expression did not affect PGA levels in the same cultivars. Similarly, ref *12* investigated the effect of genetic modifications of potatoes on the concentrations of glycoalkaloids. These authors found that the genetically modified lines had significantly reduced levels of total glycoalkaloids compared with the controls. These authors drew a correlation between the influence of genetic modification of carbohydrate metabolism (invertase and sucrose synthase) and the levels of glycoalkaloids. This is unlikely to be the sole cause, however, as transgenic potatoes without altered carbohydrate metabolism have shown similar reductions in PGA levels.

Two studies (*13*, *14*) examined a number of potato lines transformed with the insecticidal proteins *Galanthus nivalis* agglutinin, jackbean lectin, concanavalin A, and cowpea trypsin inhibitor for α-solanine and α-chaconine as in this study. In a manner similar to the tuber levels found in this study, these workers found lower levels of leaf glycoalkaloids in the transgenic lines than those found in either the tissue-cultured controls or standard controls. Although

Another study (*15*) suggests that the tissue culture process by itself may be sufficient to cause some of these changes. These workers investigated five clones of the cultivar Desiree transformed with the ech42 gene (encoding an endochitinase) for tuber glycoalkaloid levels and found no differences from the control material. In the same study, however, these authors noted variations in PGA levels among the somatic and interspecific hybrids they investigated and suggested that their results showed that, of the biochemical parameters they examined, glycoalkaloid content was the most sensitive to variation.

In this study, the blight and gangrene severity scores (data not shown) followed a pattern among the antisense invertase lines similar to that seen for glycoalkaloid levels, that is, transgenics had lower levels than controls. This is the converse of the situation observed for sesquiterpene levels, which were generally higher than the controls. This observation is consistent with the results of Yoshioka et al. (*16*), who showed that sesquiterpene biosynthesis via vetispiradiene synthase tends to compete with the synthesis of glycoalkaloids via squalene synthase. It is possible to speculate that the reduced sugar pool

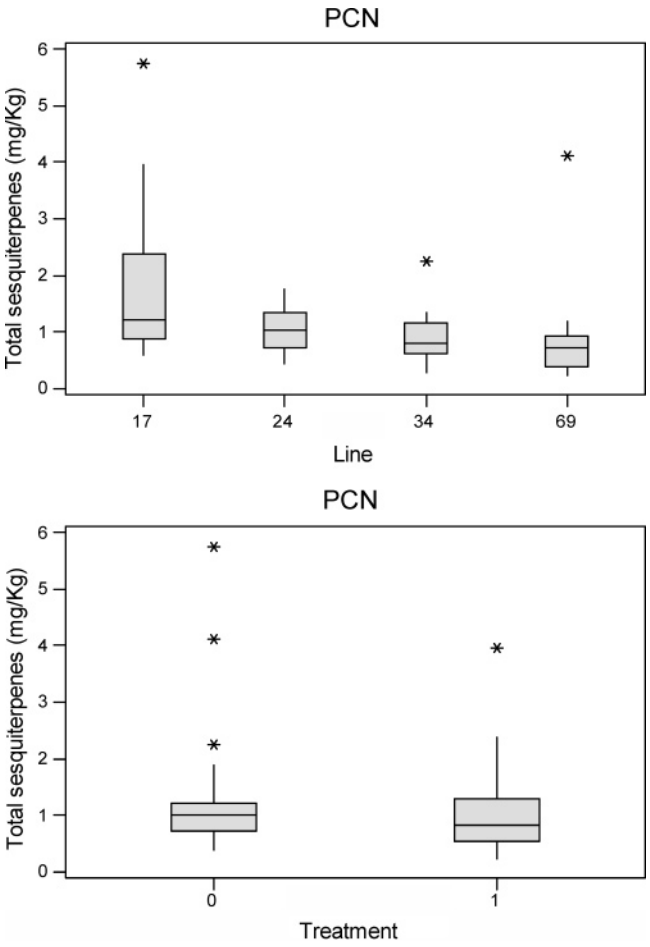

Figure 15. Box plots of sesquiterpene levels in the antisense invertase lines grown in 2002 and subjected to potato cyst nematode infection: (lines 24, 34, and 69) cv. Record, antisense invertase; (line 17) cv. Record (NCC); (treatment 0) control; (treatment 1) infected.

in this material may reduce the conversion of solanidine to γ-chaconine and γ-solonidine via UDP-glucose:solanidine glucosyltransferase and UDP-galactose:solanidine galactosyltransferase, respectively. This in turn might reduce the pool of α-solanidine and α-chaconine measured in this study. Further work will be needed to determine whether this could favor an equilibrium shift in favor of sesquiterpene synthesis or whether these changes are elicited as a result of increased predation due to reduced glycoalkaloid levels.

The maize RIP and stacked lines produced for the second year showed no consistent trend of glycoalkaloid or sesquiterpene compared to the controls

From the results of this study and the limited literature on the subject, it is clear that genetic manipulation of carbohydrate metabolism and pathogen resistance often leads to changes in the profile of plant defense compounds present in the organs of potato plants including the tubers. This is most frequently characterized by a reduction in the level of the main glycoalkaloids α-solanine and α-chaconine. Whether the elevated levels of sesquiterpene phytoalexins observed in this study are also as common is not clear, because these metabolites have not previously been examined in the context of genetic manipulation. At the present time, the mechanisms behind the changes observed in this study are not clear but may include direct effects due to changes in the hexose pool and/or indirect effects due to changes in the susceptibility of the plants to infection and infestation.

An observation from this study, which may be of some concern, is the high levels of total glycoalkaloids found in infected material (both GM and non-GM). Mean levels frequently reached twice the accepted threshold of 20 mg/100 g, with some individual samples exceeding 72 mg/100 g; clearly, tubers destined for human consumption containing these levels of glycoalkaloids could represent a significant health concern. This must raise the question of whether similar levels would be observed in potatoes grown under low input or organic regimes, when higher levels of infection might be expected.

In conclusion, the results of this study clearly demonstrate that the introduction of anti-invertase and maize RIP genes into potato can result in significant changes in the levels of glycoalkeloids and sesquiterpenes in tuber tissue. Given the relatively small number of transgenic lines produced during this work, it may be concluded that the levels of these metabolites are particularly sensitive to genetic manipulation. As this study included material that had been through the tissue culture procedures associated with the transformation process in addition to unmanipulated controls, it may be concluded that the effects observed in this study were due either to the placement or activity of the transgenes rather than the process of inserting the genes per se.

As a general trend it was found that manipulation of carbohydrate levels reduced glycoalkaloid levels and increased sesquiterpene levels, whereas the introduction of the maize RIP gene had a more unpredictable effect on the levels of these two metabolites. Although these changes were small in comparison with the effects of stress and predation, we conclude that it is important that transgenic potato lines be assayed for these metabolites under realistic field conditions before they are approved for human consumption.

ACKNOWLEDGMENT

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