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The effect of genetic transformations for pest resistance on foliar solanidine-based glycoalkaloids of potato (*Solanum tuberosum*)

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Summary

Foliage of potato cv. Désirée was harvested from glasshouse-cultivated plants of five experimental transgenic lines expressing three different insecticidal proteins (snowdrop lectin, *Galanthus nivalis* agglutinin (GNA); jackbean lectin, Concanavalin A (Con A), cowpea trypsin inhibitor; (CpTi)), tissue-cultured control plants and standard control (non-tissue cultured) plants. The foliage was subdivided into stems, upper, middle and lower leaves and analysed separately by HPLC for the solanidine-based glycoalkaloids α-solanine and α-chaconine. The results demonstrate that one or more stages in the plant transformation process (i.e. insecticidal- and marker-gene insertions, gene expression and tissue culture) resulted in a lower level of leaf glycoalkaloids than that found in either the tissue-cultured controls or standard controls, based on the selected potato lines transformed for insecticidal protein expression. However, the distribution of glycoalkaloids throughout the plant foliage was unaffected by genetic transformation and tissue culture, with the highest glycoalkaloid levels being observed in the top third of the plant. The importance of investigating unexpected effects of genetic engineering on plant secondary metabolism is discussed from an ecological viewpoint.

Key words: Altered secondary metabolites, biosafety, foliar glycoalkaloids, genetic engineering, potato, insect resistance genes

Introduction

The predominant glycoalkaloids present in commercial potato cultivars are α-solanine and α-chaconine, which are differently glycosylated forms of the aglycone, solanidine (Friedman & McDonald, 1997). Both compounds are highly toxic to mammals, with LD_{50} values comparable to that of strychnine (Morris & Lee, 1984). Glycoalkaloids are synthesised in all parts of the potato plant, with the highest levels being associated with areas of greatest metabolic activity, such as young leaves, flowers and sprouts (Friedman & McDonald, 1997).

The ecological role of glycoalkaloids in the plant has not been fully elucidated but their toxicity and reported bitterness (Zitnak & Filadelfi, 1985) suggest that they may act as feeding deterrents to browsing mammals and to non-adapted insect herbivores. It has been shown that the level of foliar glycoalkaloids is highly correlated with resistance to the potato leafhopper (*Empoasca fabae* Harris) (Sanford *et al.,* 1990; Tingey & Sinden, 1982; Tingey *et al.,* 1978), but no such correlations were reported in studies with foxglove aphids (*Aulacorthum solani,* Kaltenbach), peach potato aphids (*Myzus persicae*, Sulzer) nor potato flea beetles (*Epitrix cucumeris* Harris) (Flanders *et al.,* 1992; Tingey & Sinden, 1982). Feeding purified solanidine-based glycoalkaloids in artificial diets to the potato aphid (*Macrosiphum euphorbiae* Thomas) revealed that neither α–solanine nor α-chaconine had strong lethal effects on this adapted potato-feeding species (Günter *et al.,* 1997) and indeed the latter compound stimulated aphid feeding at low levels. However, at high levels α-chaconine produced a significant decrease in aphid reproduction rates, whilst α-solanine increased reproduction rates at all concentrations tested. Similarly, low concentrations of α-chaconine and α-solanine marginally stimulated reproduction and diet acceptability by another potato aphid, *M. persicae,* but higher concentrations (80-160 mg 100 ml^{-1} diet) reduced aphid fecundity, feeding and survival (Fragoyiannis *et al.,* 1998).

Insecticidal properties of specific plant proteins, in particular the protease inhibitors and lectins, have been well documented (Birch *et al.,* 1999; Boulter, 1993). These insecticidal proteins have been expressed in transgenic plants, offering the potential to provide novel types of pest resistance lacking in many conventional cultivars. Lectins (particularly those from snowdrop and jackbean) have been evaluated recently against pest aphids because the current range of *Bacillus thuringiensis* toxins widely

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used in transgenic plants are not active against this important group of pests. Lectins have a broader spectrum of insecticidal activity than have *Bacillus thuringiensis* toxins and are therefore more likely to affect non-target organisms, including beneficial insect predators (Birch *et al.,* 1999).

It is incorrect to assume that the current methods of genetic engineering used to express single transgenes in plants are completely targeted and will have no, or minimal, effects on unrelated biosynthetic pathways in transformed plants. The issue of "substantial equivalence" to non-transgenic crop plants, particularly in terms of food safety, is a priority research topic within the European Union and elsewhere. However, so far there have been relatively few studies which have examined the ecological and biosafety aspects of any unintended changes in plant metabolism following genetic engineering for pest resistance.

Recent studies have shown that although the inhibition of amylose biosynthesis in potatoes by anti-sense RNA expression has no effect on tuber glycoalkaloid content, the insertion of an invertase gene from yeast into the same potato cultivar resulted in a 30% reduction in the level of α-chaconine in the tubers (Engel *et al.,* 1996). In contrast, potato tubers genetically modified to produce soybean glycinin have been reported to contain higher glycoalkaloid levels than the non-transgenic controls, but the levels detected were below the maximum recommended values for human consumption (Hashimoto *et al.,* 1999).

The objective of this study was to determine whether the transformation of potato resulted in unintended changes in the levels of foliar, solanidine-based, glycoalkaloids. A selection of genetically modified (GM) potato lines expressing different types of insecticidal proteins were used to provide baseline data on detectable variation in foliar glycoalkaloids. There is little information currently available on changes to foliar secondary metabolites in potato following genetic transformation. These data are likely to be important when considering ecological interactions between transgenically expressed insecticidal proteins and plant secondary metabolites known to influence insect-plant interactions in the agro-ecosystem.

Materials and Methods

Genetically modified plant material

Potato (*Solanum tuberosum* L.) cv. Désirée (virus-free, clonally propagated) was used as a recipient for the anti-insect transgenes under test and for producing clonal (asexually propagated) non-transgenic control and tissue-cultured plants for comparisons. Potato transformation was carried out as described by Gatehouse *et al*. (1999). The GNA and CpTi gene constructs were supplied under experimental licence from Pestax Ltd, Cambridge. The *Con A* gene was isolated and cloned from jackbean genomic DNA (Gatehouse *et al.,* 1999). All the inserted insecticidal genes were expressed constitutively under the CaMV 35S promoter, together with a selectable marker (*npt*II). Transgenic potato plantlets were propagated by standard tissue culturing techniques (Gatehouse *et al.,* 1997, 1999). For plants of the selected GM potato lines, leaf expression of the transgene products was found to be within the ranges of 0.01-0.05% (Con A), 0.1-0.53% (GNA), and 0.7-1.0% (CpTi) of the total leaf-soluble protein, after tissue culture and growth in a heated glasshouse at 18-20°C and 16-h photoperiod using high-intensity sodium lights (Birch *et al.,* 1999; Gatehouse *et al.,* 1997, 1999). The representative lines of each gene construct used in our experiments to investigate effects on plant glycoalkaloid synthesis were selected on the basis of those lines expressing the highest and most consistent levels of pest resistance in previously reported bioassays (Birch *et al.,* 1999; Gatehouse *et al.,* 1997, 1999).

Cultivation and sampling

All experimental plants were grown from tubers, pre-conditioned under standardised conditions to initiate sprouting. Tubers of similar size from both the non-tissue (standard) culture and tissue-culture controls and genetically modified lines were individually planted in 13-cm diameter pots containing Universal potting compost. The pots were randomly positioned and grown in an artificially lit glasshouse. Lighting was provided for 16 h per day, with the temperature maintained at 18-20°C during the day and 15-16°C during the 8 h night. The foliage was harvested after 48 days to standardise the growing period under identical environmental conditions, rather than attempting to standardise the physiological growth stage for each line at the time of sampling.

Five plants from each of seven lines were harvested individually. Leaves from the top, middle and base positions of each stem were bulked from each individual plant for fresh weight (fr. wt) measurements, taken immediately after each harvest to ensure uniformity. The samples were then immediately frozen in liquid nitrogen and then lyophilised in an industrial freeze-drier (Millitor Engineering Ltd, Manchester, UK). The freeze-dried samples were then ground in a laboratory mill (Glen Creston Ltd, Stanmore, UK) fitted with a 1 mm screen. The resulting material was stored at -20°C until taken for dry weight determinations (freeze-dried matter; FDM) and for chemical analysis.

Chemical analysis

The glycoalkaloids were extracted by suspending

100 mg freeze-dried leaf or 250 mg freeze-dried stem samples in 10 ml 5% aqueous acetic acid (Dao & Friedman, 1996). After shaking for 30 min, the resulting suspension was centrifuged and the levels of the individual glycoalkaloids were determined using a high performance liquid chromatographic method, based on that of Hellenäs (1986). Total glycoalkaloid (TGA) content was calculated as the sum of the individual values obtained for α-solanine and α-chaconine and expressed as mg total glycoalkaloids per 100 g fr. wt (mg TGA 100 g^{-1} fr. wt).

The dry matter content of the samples was determined by calculating the weight loss during lyophilisation and expressed as g freeze-dried matter per 100 g fr. wt (g FDM 100 g^{-1} fr. wt).

Statistical analysis

To overcome the problem of base, middle and top data being correlated, as they were observations from the same plants, a Principal Component Analysis was carried out treating base, middle and top as separate characters. Analysis of variance (all data) and Principal Component Analysis (leaf position data) were carried out using Genstat 5 (Payne, 2000). Where, on the basis of the residual plots, transformation was deemed necessary, both the transformed (log_e) and raw data are shown in the Tables.

Results

The total dry-matter yield of whole shoots (leaves plus stems) that were harvested after 48 days growth from the tissue culture control line was significantly lower than that for the control plants (Table 1). This was primarily due to the lower amount of leaf material produced by the former. Similarly, all the genetically modified lines, with the exception of that expressing the *Con A* gene, produced significantly less total foliage than the control plants (Table 1). In three of the genetically modified lines (CpTi, GNA 74-1 and GNA 71-1) and the tissue culture control plants, the yields of leaf material were significantly lower than in the standard control plants and did not differ significantly from each other. Stem production was also significantly reduced, relative to both controls, in three of the genetically modified lines (CpTi, GNA 71-1 and GNA 2#28), but was significantly higher in the Con A 4-1 line, reflecting the bushy growth characteristics of the latter transgenic plants. Although above ground growth rates differed slightly between the experimental lines, all were pre-flowering at the time of harvest.

The dry-matter content of leaves (Table 2) from the control, tissue-culture control and in the genetically modified line GNA 2#28 was significantly higher than in the other lines studied.

Table 1. *The yield (g FDM $plant^{-1}$) of foliage harvested from normal and genetically modified plants derived from the potato cv. Désirée*

Line	Leaf	Stem	Whole Shoot
Control	4.5	2.6	7.2
TC Control	2.8	2.9	5.7
Con A 4-1	4.3	4.5	8.8
CpTi	2.5	1.1	3.6
GNA 74-1	2.9	2.3	5.1
GNA 2#28	4.0	1.7	5.7
GNA 71-1	2.4	1.8	4.2
LSD ($P < 0.05$)	0.53	0.59	1.03

Table 2. *The freeze dried matter concentration (g FDM 100 g^{-1}fr. wt) of foliage harvested from normal and genetically modified plants derived from the potato cv. Désirée*

Line	Leaf	Stem
Control	11.2	9.0
TC Control	10.6	8.8
Con A 4-1	9.3	7.0
CpTi	7.9	5.2
GNA 74-1	8.9	8.1
GNA 2#28	11.6	9.0
GNA 71-1	7.8	7.6
LSD ($P < 0.05$)	0.80	0.94

Similar results were found for dry matter contents of the stems of the experimental lines except for GNA 74-1, which in addition to line GNA 2#28, were not significantly different from either type of control.

In all the lines investigated (genetically modified and control lines), the level of glycoalkaloids in the leaves was considerably higher than that present in the stems, with the leaves containing on average over nine times the TGA level of the stems. The levels present in both plant tissues were significantly correlated ($r = 0.8858$, $P < 0.01$). The TGA level of the leaves and stems of the control and tissue culture control plants were almost identical (Table 3).

All the genetically modified lines had statistically significant lower levels of leaf-TGA than the control plants. The lowest level was found in the CpTi line, at 30% that of the control plants. The insertion of the two selected plant lectin genes (GNA, Con A) produced similar but generally less pronounced effects on foliar TGA levels. The insertion of the Con A gene resulted in a 24% reduction in foliar TGA levels, whilst the three lines containing GNA had, on average, leaf-TGA levels 44% lower than that in the control plants. The TGA levels found in the stems of the genetically modified plants were

also lower than that for the controls, with the observed decrease being statistically significant for all but the GNA 74-1 line.

A study of the effects of leaf position on TGA level (Table 4) revealed that the highest levels were consistently found in the top third of the plants, where metabolic activity would be expected to be highest. In the non-modified control plants, leaf TGA level was approximately 50% lower in the mid-plant region as compared to the top third, and was reduced by a further 50% in leaf samples taken from the bottom third of the plant.

TGA levels in the top, middle and base leaves of the tissue culture controls were not significantly different from those of leaf samples taken from same parts of the non-modified control. The glycoalkaloid levels in leaves from the base and middle areas of plants from the genetically modified lines were all significantly lower than those of leaves taken from the equivalent areas of plants from the non-modified and tissue-culture controls. Similarly, with the exception of line Con A 4-1, leaves harvested from the tops of plants from all the genetically modified lines had significantly lower glycoalkaloid levels than leaves from the tops of plants from either the non-modified or tissue culture control.

The first Principal Component accounted for 86% of the variation and described a simple average profile of base, middle and top. Analysis of variance of the scores of this Principal Component generated the same conclusions as the analysis of variance for total plant leaf glycoalkaloid level (Table 3). Plots of the scores of the second and third Principal Components revealed no patterns or clusters among the cultivars. Therefore, on the basis of the Principal Components Analysis performed it was concluded that the plant profiles of glycoalkaloid levels (base, middle and top leaves) were the same in all the lines studied.

The levels of the individual glycoalkaloids, α-solanine and α-chaconine were determined for both leaf and stem samples (Table 5). The process of transformation reduced the levels of both compounds

Table 3. *The concentration (mg TGA 100 g^{-1} fr. wt)* of total solanidine-based glycoalkaloids in foliage harvested from normal and genetically modified plants derived from the potato cv. Désirée*

Line	Leaf	Stem	Whole Shoot
Control	160 (5.06)	16	99
TC Control	158 (5.05)	14	78
Con A 4-1	120 (4.77)	10	55
CpTi	47 (3.84)	5	31
GNA 74-1	116 (4.75)	13	69
GNA 2#28	73 (4.27)	10	52
GNA 71-1	78 (4.35)	11	50
LSD ($P < 0.05$)	(0.236)	3.2	14.3

* Log_e transformed data in parentheses

Table 4. *The effect of leaf position on the concentration (mg TGA 100 g^{-1} fr. wt)* of total solanidine-based glycoalkaloids in normal and genetically modified plants derived from the potato cv. Désirée*

Line	Base	Middle	Top
Control	79	163 (5.07)	312
TC Control	87	179 (5.18)	244
Con A 4-1	58	99 (4.65)	248
CpTi	35	37 (3.60)	103
GNA 74-1	51	127 (4.83)	210
GNA 2#28	36	64 (4.14)	173
GNA 71-1	36	65 (4.16)	157
LSD ($P < 0.05$)	17.8	(0.283)	77.3

* Log_e transformed data in parentheses

Table 5. *The concentrations (mg 100 g^{-1} fr. wt)* of individual solanidine-based glycoalkaloids in foliage harvested from normal and genetically modified plants derived from the potato cv. Désirée*

	Leaf			Stem		
Line	α-Solanine	α-Chaconine	Ratio†	α-Solanine	α-Chaconine	Ratio†
Control	69 (4.21)	91 (4.49)	1.32	6.9	9.6	1.42
TC Control	67 (4.19)	91 (4.51)	1.37	5.4	8.2	1.52
Con A 4-1	48 (3.86)	71 (4.26)	1.49	3.9	5.9	1.52
CpTi	20 (2.99)	27 (3.29)	1.35	2.4	2.9	1.21
GNA 74-1	50 (3.91)	66 (4.18)	1.31	5.5	7.9	1.44
GNA 2#28	33 (3.47)	40 (3.67)	1.22	4.9	5.1	1.06
GNA 71-1	33 (3.49)	45 (3.80)	1.36	4.6	6.2	1.38
LSD ($3 < 0.05$)	(0.265)	(0.218)	0.092	1.49	1.77	0.110

† Ratio of α-Chaconine to α-Solanine

* Log_e transformed data in parentheses

equally in the leaves of most genetically modified lines, i.e. no significant change in the ratios of α-chaconine : α-solanine. With the exceptions of Con A 4 and GNA 2#28 transgenic lines, the chaconine to solanine ratios in the leaves of the other genetically modified lines did not differ significantly from that found in the control plants. In the Con A line, the reduction in α-chaconine level was 22%, as compared with 34% in α-solanine level. However, in GNA 2#28 the converse was found, with reductions of 56% and 52% in α-chaconine and α-solanine levels respectively. In the stems, as with the leaves, only minor differences in the chaconine to solanine ratios were observed, with only the values for the CpTi and GNA 2#28 lines differing significantly from the controls. In the former, the levels of α-chaconine and α-solanine were reduced by 70% and 65%, whilst in line GNA 2#28 the reductions were 47% and 29% respectively.

Discussion

The levels of glycoalkaloids detected in potato foliage during this study were comparable with previously reported results (Dao & Friedman, 1996; Friedman & McDonald, 1997). However, direct comparisons with previously published results are difficult, since levels depend not only on stage of plant development (Dao & Friedman, 1996), but also on growth conditions and on the sampling regime adopted (Brown *et al.,* 1999).

The process of genetic transformation for the insecticidal proteins tested, which included tissue culture, resulted in a lower level of foliar TGA at the time of sampling than standard control plants of the same cultivar. This was generally due to a similar fall in level for both constituent leaf glycoalkaloids rather than just in α-chaconine, as was reported in tubers of cv. Désirée containing different invertase genes (Engel *et al.,* 1996). There appears to be only limited information about changes in foliar glycoalkaloid levels during plant development and growth. Consequently, the lower levels in the genetically modified lines could be due to the effects of the processes of transformation and tissue culture on the rate of plant development, rather than a direct effect on the ability of the genetically modified plants to synthesise glycoalkaloids *per se*. The lower glycoalkaloid levels detected in the genetically modified lines were, however, discernible in old, middle-aged and young leaves harvested from the base, middle and top of the plants respectively (Table 4). It would also appear unlikely that these differences in foliar TGA level could be attributed to the early onset of tuber initiation in the genetically modified lines, as it has been previously demonstrated (Roddick, 1982) that tuber glycoalkaloids are synthesised *in situ* and not transported from foliage to tubers. A more likely explanation is that transformation and tissue culture affected some basic physiological process(es). This is indicated by the altered yields of foliage and respective % FDM measurements in some of the transformed lines, relative to the untransformed control plants.

From the results presented here, it is not possible to determine the molecular and genetic mechanisms causing these effects, which could be due to target gene insertion, marker gene insertion, chromosomal re-arrangements, altered gene expression and/or tissue culture. The fact that similar TGA levels were found in the foliage of both the standard control and tissue-cultured control plants indicates that, for the GM potato lines tested, transformation events combined with tissue culture caused more pronounced alteration of glycoalkaloid metabolism than did the events involved in the process of tissue culture on their own. This finding has been confirmed in two other separate studies on the same transformed plant lines, each with independent tissue cultured control plants (A N E Birch and D W Griffiths, unpublished). Further studies, examining the extent and molecular biological causes of somaclonal and transformation-induced variation in the glycoalkaloid level of potato foliage of a larger range of transgenic lines, are required before broader conclusions can be drawn. As in all risk:benefit and environmental impact assessments of GM crop lines, each is handled as a "case-by-case" study. Broad conclusions about the biosafety of a particular GM crop, GM trait (e.g. pest resistance) or interactions of specific GM lines with the environment should be avoided because each GM plant line is likely to be genetically unique and could therefore interact differently within a dynamic ecosystem and changing growing environment.

Potato foliage is not normally consumed by domesticated animals or man, although it has been reported that potato leaves do form part of the diet of the UK Bangladeshi community (Phillips *et al.,* 1996). With this exception, levels of glycoalkaloids in foliage are unlikely to be a major mammalian toxicity problem and indeed their reduction in this sense (food safety) could be considered beneficial.

Studies with artificial diets have demonstrated that for the two potato-feeding aphid species, *M. euphorbiae* and *M. persicae* (Fragoyiannis *et al.,* 1998; Günter *et al.,* 1997), glycoalkaloids at low to medium levels (e.g. 10-40 mg TGA 100 ml^{-1} in artificial diets) can stimulate feeding and reproduction. Nevertheless, like many naturally occurring secondary plant compounds involved in defence against partially adapted insects, their action can be reversed to that of feeding deterrents or toxins if they are present at levels above a critical threshold value in the relevant plant tissue upon which the

insect feeds. Thus, any inadvertent lowering of foliar glycoalkaloids in transgenic potato plants could cause an undesired increase in susceptibility to those pests which are sensitive to threshold concentrations of glycoalkaloids for insect deterrence or toxicity, potentially reducing the benefits of expressing anti-insect transgenes in potato.

The results presented here demonstrate that the processes of transformation and tissue culture can result in an unintended and unexpected modification in the level of bioactive secondary plant metabolites of potato leaves. The nutritional status and ecological fitness (e.g. susceptibility to specific pests and pathogens) of the transformed plants may be adversely affected by such changes in levels of endogenous plant defence compounds, but this remains to be investigated under field conditions. Therefore, we think that it is as important to monitor unintended changes in the levels of such secondary plant compounds as it is to evaluate the potential risks and benefits of the intended transgene product (anti-insect gene products) in the agro-ecosystem. It is possible that in some GM plant lines expressing higher levels of insecticidal proteins there could be a "trade-off" between maintaining ecologically functional levels of natural defence compounds and expressing effective amounts of insecticidal proteins through genetic engineering. However, to test this hypothesis rigorously and to determine ecological effects on specific target pests, it would also be necessary, in future experiments, to measure amounts of expressed insecticidal proteins and natural plant defence toxins within the specific plant tissues consumed by the herbivore (e.g. plant phloem for aphids). Measuring only the contents of insecticidal GM protein and natural defence compounds in leaf or stem tissues as a whole may provide information on metabolic "trade-offs" to the plant, but will not necessarily provide accurate information on the ecological effects of such "trade-offs" to specific target pests and their natural enemies. We also consider it important to consider potential toxicological interactions between the transgene products (e.g. insecticidal proteins) and the plant's primary and secondary metabolites *in planta*; since such biochemical interactions are difficult or impossible to simulate using artificial diets containing simplified components, when testing toxicity to target and non-target insects.

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