SOAEFD flexible Fund Project RO 818

Report of Project Coordinator on data produced at the Rowett Research Institute (RRI)

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1. Introduction

In 1995 SOAEFD commissioned a 3-year multicentre project: *Genetic engineering of crop plants for resistance to insect and nematode pests: effects of transgene expression on animal nutrition and the environment*. The main objective of the programme was: "To identify genes encoding antinutritional factors which will be suitable for transfer into plants to enhance their resistance towards insect and nematode pests, but will have minimum impact on non-target, beneficial organisms, the environment, livestock fed on these plants, and which will present no health risks for humans either directly or indirectly through the food chain ".

Provision of genetically modified (GM) plants and measuring their environmental impact was the responsibility of the University of Durham (UD) and the Scottish Crop Research Institute (SCRI). The task of RRI was carry out thorough chemical analyses and establish whether the parent and transgene lines were compositionally equivalent or not and to determine in short-term (10 day) and long-term (3 months) rat feeding trials whether the effect of GM lines on the mammalian gut and metabolism was similar to that of parent lines or not. Our work has concentrated on tubers from GM-potato lines expressing the gene of snowdrop (*Galanthus nivalis*) bulb lectin, GNA, as this was the only GM crop plant which we received in time and in sufficient amounts to do nutritional trials with. No other lectin-expressing GM plants have been tested with rats although we did some analytical but no nutritional work on Con A-GM-potatoes which we received from SCRI well into the third year of the programme. This Report will therefore only describe the results of our analytical, nutritional and immunological work on GNA-GM-potatoes. For ease of reading, the main text will only give few data and only those necessary for understanding the significance of the findings. However, all the results will be given as Tables and Figures in a separate but linked Appendix allowing readers to check the accuracy and truthfulness of the conclusions drawn. All other non-essential and non-GM aspects of our work will be omitted from this Report as these can be found in the Audit Report produced by the Audit Commission.

2. Results & Discussion

2.1 Compositional analyses:

To facilitate the understanding of the relationship between the lines used in the study a lineage

chart is given in **Figure 1** (Appendix). Briefly, there were **two original GNA-transformed lines 71/1 and 7411** and their derivatives which were used in this study. Lines 71/1 and 7411 were grown at Rothamstead and passed on to UD and SCRI respectively where second generations were grown in either tunnels (71/2T and 74/2T) or glasshouses (71/2G and 74/2G) together with their respective parent lines. The data of tuber composition given in **Table 1** (Appendix) and the results of analyses of antinutrients in **Table 2** (Appendix) are as comprehensive as possible. However, as some lines were scarce, not all GNA-GM-potatoes have been used in feeding studies. The ones which have, are printed in bold in the Tables.

The results clearly show that the contents of some or all of the constituents of major nutritional importance in GM-potatoes are significantly different from those of their respective parent lines. Thus, although the true protein content of the tubers of 71/1 lines (used in D227) is similar, the GNA-GM 74/2T potato line used in 3 of the main feeding trials (D237, D242 & D249), regardless whether raw or baked, contained nearly 20% less protein than its respective parent. The starch and/or glucose contents of the parent and GNA-GM tubers were also different. Similar findings were made for antinutrient contents. Thus, the potato lectin (PL) content of most GM lines was significantly different from the appropriate parent. Indeed, in one instance, the PL content of 74/26 GNA-GM line was well over double of that of the parent line. Similarly and almost without exception, contents of trypsin inhibitors and chymotrypsin inhibitors of the GM- and appropriate parent lines were significantly different. The results of changes in protein, starch, sugar, lectin and trypsin/chymotrypsin inhibitor levels in potato tubers after GNA gene insertion taken in conjunction with the preliminary results by SCRI scientists (Interim SCRI Report - FF 818; April 1998) showing decreased foliar glycoalkaloid content in various lines of GM-potatoes clearly **"indicate possible gene silencing, suppression and/or somaclonal variation"** as a result of gene insertion. It is therefore clear that in contrast to the conclusions of the Audit Report the **GNA-GM-potato lines investigated as part of the Rowett's work programme in FF 818** were <u>not "substantially equivalent"</u> **to the appropriate parent tubers.**

2.2 Isolation of GNA from GNA-GM-potatoes and its identity with GNA from snowdrops.

Our work has clearly demonstrated that the GNA expressed in the potato tuber is similar (or identical) to the GNA in snowdrop bulbs. Using the same specific methodology of affinity chromatography as Van Damme et al. *(Febs Lett.* **215,** 140, 1987) originally employed for isolating GNA from snowdrop bulbs, we have purified sufficient amounts of "potato GNA" to allow us to establish by SDS-PAGE, haemagglutination, mannose-inhibition of haemagglutination and specific ELISA that the potato and the snowdrop GNA molecules were closely similar, perhaps identical. This similarity was further underlined by our findings that the behaviour and binding of "potato GNA" in the gut on feeding GNA-GM-potatoes (D227) was similar to that of "snowdrop GNA" which was fed as part of a diet containing parent potatoes spiked with GNA **(Table 3).** Interestingly, GNA levels in the tuber appeared to decrease on re-growing **(Table 2)** although further studies are needed to confirm this.

2.3 Rat feeding studies

a. <u>Background considerations:</u>

There were four feeding trials using GNA-GM-potatoes but none with Con A-GM-tubers. The protein content of potato tubers was low and the potato proteins were of relatively low quality to fully support the growth of young rats and to conform to Home Office requirements. Unfortunately this meant that according to the basic principles of nutritional science any potential differences in nutritional value between GM and parent potatoes may be diminished

and/or abolished by the presence of lactalbumin. An additional complication was due to major differences in protein content between some of the transgenic and parent lines. Therefore to formulate *iso*-nitrogenous diets all diets needed supplementation with different amounts of a high nutritional quality protein, lactalbumin, leading to further complications in the design of the experiments. However, this "protein effect" was expected to be less important in short-term (10 day) trials than in the long-term (110 day) experiment because the reserves of the animals could compensate for dietary imbalances. Therefore we concentrated on 10-day rat feeding trials and carried out three such experiments. Protein imbalance was a particularly acute problem in our long-term study (D237) because the GNA-GM-potato tuber, 71/2T, the line which was available in sufficient amounts (close to 100 kg) for feeding and also extensively used in the insect trials, contained almost 20% less protein than its appropriate parent line. Thus, to make the diets isoproteinic the GM-potato diets contained 26% more lactalbumin (LA) than the diet based on the parent line. Therefore, D237 should be regarded as a preliminary study from whose results we might be able to modify the diet in such a way that the next trial could be made under ideal conditions.

b. Experiment D227:

This was a preliminary 10-day study because tubers of the line 71/1 **GNA-GM** and its parent from Rothamstead were needed for re-growing at SCRI. No lymphocyte proliferation assays were done in this experiment. The total protein content of the raw potato diets was about 61 g/kg diet which was made up of 55 g potato protein/kg and just under 6 g LA/kg diet. With boiled potatoes the contribution of potato proteins was about 5 g less and therefore the total protein content of the boiled diet, including LA was only about 56-57 g/kg diet. These dietary protein concentrations were far less than the minimum of 10-12% thought to be required for proper growth. The following diets were tested: Raw parent, Raw parent + GNA, Raw transgenic, Boiled parent, Boiled parent + GNA, Boiled transgenic and LA control. All rats were pair-fed with the diets.

The growth curves and the wet weight data in Appendix 7 of the Audit Report are correct. Briefly, as expected, rat growth was significantly reduced on boiled potato diets and even more on diets containing raw potatoes. Therefore the presence of GNA, whether added to potato-based diets or expressed in the transgenic tuber line 71/1 had no significant effect on weight gain and weight change compared to parental controls although the difference between the final body weight and the empty body weight of rats (accounting for the food removed by washing) which were fed raw transgenic potato diets was more than that of rats given diets containing the raw parent line. Although the difference was not significant in this experiment (while it was in D242) the trend indicated that digestion and absorption of transgenic potato-based diets was retarded in comparison with ordinary potato diets.

Furthermore, and more importantly, there were highly significant differences in the wet and dry weights, both absolute and relative weights, of many essential body organs **(Table 4),** indicating that the effects of GNA-GM-potatoes on body and organ metabolism were significantly different from those of control potatoes. Although the Audit Committee apparently had these very data in their possession, they were not included in their Report even though that some of the effects, such as the partial liver atrophy observed on feeding boiled transgenic potato-based diets may have major repercussions on liver function. Other effects which included the enlargement of the pancreas, jejunum and testes on raw GNA-GM-potato diets suggested that the lack of compositional equivalence might also be extended to a lack of equivalence in the metabolic consequences between feeding of GM and parent potatoes. This is of particular importance because as shown before **(Table 3)** this occurs despite that the behaviour in the gut lumen of

"potato GNA" after GNA-GM-potato diets was closely similar to that of "snowdrop GNA" in parent potato diets spiked with GNA **(Table 3).**

c. Experiment D242:

This was 10-day trial using second generation tubers of GNA-GM-potatoes 71/2T line and a mixed control consisting 19% 71/2T and 81% 74/2G control lines due to the short supply of 71/2T control potatoes. In addition to observing the nutritional and metabolic effects of feeding rats with transgenic potatoes for 10 days, in this experiment we also carried out mitogenic lymphocyte responsiveness measurements to test the effects of these diets on immune function using the well-established lymphocyte proliferation assay.

The total protein content of the 71/2T GNA-GM potato tubers was less than that of the 71/1 lines and therefore in the transgenic diets potato proteins contributed less than 43 g protein to the total of 55 g/kg. The difference was made up by supplementing the diet with 12 g LA/kg diet (twice of that in D227). The mixture of the 71/2T and 71/2G control potatoes contained more protein **(Table 1)** and these diets therefore contained 51.8 g potato proteins and 12 g LA, making a total of over 63 g protein/kg diet. The following diets were tested: Raw parent, Raw parent + GNA, Raw transgenic, Boiled parent, Boiled parent + GNA, Boiled transgenic and LA control. All rats were pair-fed.

The results were similar to those in D227 and the growth curves and the wet weight data in Appendix 9 of the Audit Report are correct. Briefly, as expected, rat growth was significantly reduced on boiled potato diets and even more on diets containing raw potatoes compared with LA diet. Therefore the presence of GNA, whether added to potato-based diets or expressed in the transgenic tuber line 71/1 had no significant effect on weight gain and weight change compared to parental potato lines. However, in this instance the difference between the final body weight and empty body weight of rats (accounting for food in the gut lumen) which were fed raw transgenic potato diets was significantly higher than that of rats given diets containing the raw parent line. This again indicated that digestion and absorption of nutrients of transgenic potato diets was retarded in comparison with ordinary potato diets.

Feeding rats with diets containing raw GNA-GM-potato tubers 71/2T for 10 days induced significantly large changes in the absolute and relative wet weights of most major organs in comparison with parent line diets **(Table 5).** Most of the changes were leading to reduction in organ weights of transgenic potato-fed rats and this may have been the result of the significantly reduced rate of digestion and absorption of nutrients in the digestive tract of these animals. This effect was reversed with the thymus and gastrocnemius muscle. Feeding rats with baked transgenic potatoes also significantly affected some of their vital organs including the kidneys, thymus and gastrocnemius muscle **(Table 5).** Similar to findings in D227 feeding rats diets containing the baked transgenic line reduced their liver weight but the results were not significant in this case.

No dry weight data have been obtained in this study.

Results of our immune assays clearly indicated that incorporation of raw transgenic potatoes in the diet highly significantly depressed the responsiveness of rat peripheral lymphocytes to both Concanavalin A (Con A) and *Phaseolus vulgaris* agglutinin (PHA) as mitogens. As the results were the same as in Appendix 9 of the Audit Report these are not reprinted. However, as on re-evaluation some of the significances became slightly different, the T-test results are tabulated in **Table 6** and **Table 7.** Although the depression of lymphocyte proliferation was less with baked

transgenic potato diets vs baked parent diets, at 6 μ g Con A/well mitogen concentration the difference was significant ($p < 0.05$). Moreover, even in those cases where the differences were not statistically significant the trend was unmistakeable: lymphocytes from rats given transgenic potato diets were almost always less responsive to mitogenic stimuli than those from rats fed parent line diets. It was particularly interesting that in contrast to the depression with GNA-GM-potato diets, GNA added to parent potatoes, regardless whether raw or baked, had in some instance stimulated the responsiveness of the lymphocytes, thus increasing the differences between transgenic and parent lines.

d. Experiment D237

This was the only long-term experiment carried out with GNA-GM-potatoes, line 71/2T. However, only baked transgenic and parent potatoes could be used in the diets as in preliminary studies we found that weight gains with raw potatoes were so much depressed that they would have breached Home Office rules. In addition to observing the nutritional and metabolic effects of feeding rats with transgenic potatoes for 110 days, in this experiment we also carried out mitogenic lymphocyte responsiveness measurements to test the effects of these diets on immune function using the well-established lymphocyte proliferation assay.

As the protein content of the 71/2T GNA-GM potato tubers was lower than that of the parent lines it was nearly impossible to formulate isoproteinic diets with them. Moreover, to overcome the likely low growth rates when rats are long-term fed on diets of inadequate protein content, it was decided to increase the amounts of the high-quality LA protein used for diet supplementation even though that feeding such diets might diminish (abolish) possible differences between the effects of transgenic and parent potato diets. Thus, as in the transgenic diets potato proteins contributed less than 41 g protein to the total of 88 g/kg, this diet contained more high-quality LA protein, 47 g LA/kg diet, than potato protein. The mixture of the 71/2T and 71/2G control potatoes used parent control contained more protein **(Table 1)** and these diets therefore contained 49 g potato proteins and 35 g high-quality LA, making a total of 84 g protein/kg diet. The following diets were tested: Baked parent, Baked parent + GNA and Baked transgenic. In a separate study groups of rats were fed LA control diet (94 g LA/kg diet) or LA diet + GNA. All rats were pair-fed.

The growth curves and the wet weight data in Appendix 8 of the Audit Report are correct and therefore are not duplicated here. Clearly, rats fed all of the potato diets grew slightly but significantly less well than pair-fed LA controls. However, to maintain such an equal growth rate with the potato diets the transgenic diet not only had to contain more total protein (8.8%) than the parent line diets (8.4%) but also it had to be supplemented with over a quarter more LA than the control. LA is a nutritionally better quality protein than potato proteins and therefore its high dietary inclusion may have contributed to the generally similar organ weights of rats whether fed transgenic or parent line diets. The only significant difference found was in the liver weight. However, in contrast to all previous studies (D227 and D242) in which there was a partial liver atrophy in rats given transgenic potatoes, in this experiment both the absolute and relative weights of the liver of the animals on the transgenic diet were significantly ($p < 0.05$) increased compared to the parent control. This suggested that it was possibly the higher dietary level of the high quality LA in the transgenic diet that might have caused a general increase in the organ weight of the rats on this diet. This was further supported by the findings that when the effects of the transgenic diet were compared with those of the parent + GNA spike, the weights of other organs, such as the caecum, kidneys and thymus also became significantly different.

The scale of the lymphocyte responsiveness of rats fed potato diets was so much compressed

that no significant differences were found between transgenic and parent lines. The results given in Appendix 8 of the Audit Report are therefore not duplicated here. Clearly, under the conditions of long-term feeding with potatoes many factors contributed to the overall highly depressed lymphocyte responsiveness. Thus, one of the possible major contributors could have been the same that caused the depression seen in transgenic-fed rats in the 10 day trial, D242.

In addition, it is also known that immune responses are also diminished by feeding animals on diets of low protein content particularly when the proteins in the diet are of poor nutritional quality such as potato proteins. Moreover, the cumulative depressing effect on long term feeding of some possibly harmful component(s) (lectins, protease inhibitors, glycoalkaloids etc) in potatoes could have also contributed to the depression of some of the functions of the immune system and for these reasons any potential effects of the transgene on the immune system may have been hidden. To clarify the role of these factors further studies are needed at time points intermediate between the 10 and 110 days used in this study.

e. Experiment D249

To overcome the problems and the immune depression caused by feeding potato diets of low protein content and quality an experiment was designed in which the protein content of the diet was increased well above the daily requirement of the rats. This 10-day feeding experiment was in progress during the sitting of the Audit Committee and therefore its results could not be evaluated by them. However, as the results of the feeding studies and the lymphocyte responsiveness assays make a very significant contribution to our understanding, they will be described here.

In this experiment the GNA-GM-potato line 71/1 and its corresponding control line was used. Potato proteins contributed 47 g protein to the total protein concentration of 147 g/kg in the diets; the rest was high-quality LA (100 g/kg diet). The high dietary level of LA was chosen deliberately to establish whether the differences in organ weights and lymphocyte responsiveness found in previous short-term experiments with diets containing low levels of LA could be overridden by greatly increasing the concentration of the high-quality LA in the diet, so that the protein concentration in the diets was superoptimal. Four major experimental groups (12 rats of each group) were set up: Raw parent, Raw parent + GNA, Raw transgenic and LA control. All rats were pair-fed.

At these high dietary protein concentration no significant differences were found in the growth rate of the rats **(Figure 2**). Even the LA control rats grew at the same rate. However, the empty body weight of the transgene-fed rats was significantly higher than that of the parent control. Moreover, despite the high LA concentration in all diets, there were still significant differences in organ weights between the rats fed transgene *vs* parent diets **(Table 8).** In addition to other organs, both the spleen and thymus appeared to be stimulated by feeding the rats on transgenic potato diets. It was also quite worrying that the previously observed partial liver atrophy and also a reduction in the size of the brain was still observed despite the expected levelling effect of the high concentration of the high-quality LA in the diet.

The lymphocyte proliferation assay carried out with spleenocytes showed the same trend as in D242 with significant differences in lymphocyte responsiveness between raw transgenic and parent lines. Apparently, even the high level of LA in the diet could not reverse the depressing effect of the transgene previously found at low dietary inclusion levels of the high-quality LA protein.

Summary conclusions:

After **GNA** gene insertion into potatoes changes in protein, starch, sugar, lectin and trypsin/chymotrypsin inhibitor levels were observed in the tubers of two generations of **two GNA-GM lines** suggesting **"possible gene silencing, suppression and/or somaclonal variation"** in the potato genome. **The GNA-GM-potato lines investigated as part of the Rowett's work programme in FF 818 were therefore not "substantially equivalent" to the appropriate parent tubers.**

Four feeding trials were carried out with two lines of GNA-GM-potatoes. In all four experiments feeding transgenic potatoes to rats induced major and in most instances highly significant changes in the weights of some or most of their vital organs. This was not abolished even when high-quality lactalbumin supplied two-third of the protein in the diet (D249). Particularly worrying was the partial liver atrophy observed with cooked transgenic potatoes in all short-time (10 day) studies. Immune organs, such as the spleen and thymus were also frequently affected. These results therefore indicated that **similar to the lack of equivalence in composition there is also a lack of equivalence in the metabolic consequences between feeding of GM and parent potatoes** even though that "potato GNA" in GNA-GM-potato diets appears to show functional equivalence to "snowdrop GNA" in parent potato diets spiked with GNA.

The growth rate of rats fed potato diets was slightly but significantly less than that of rats fed a high-quality control diet but the presence of GNA, whether added to potato-based diets or expressed in the transgenic tuber line 71/1, had no significant effect on weight gain and weight change compared to parent potato lines. However, in most instances the presence of GNA-GM-potatoes in the diet caused some slowing down the digestion and absorption of nutrients in the gut in comparison with parent line diets. This was only observed with diets in which potatoes supplied the major part of dietary protein (D227 and D242) and the effect reached full significance in experiment D242.

Feeding rats with GNA-GM-potatoes significantly reduced their lymphocyte responsiveness to mitogenic stimuli after 10 days compared to parent controls that was not abolished by raising the high-quality protein (lactalbumin) concentration to superoptimal nutritional levels. However, as in long-term feeding the lymphocyte proliferative response of all rats fed potato-based diets was reduced to non-stimulated levels, **no significant differences were observed in lymphocyte responsiveness between GNA-GM-potatoes and their parent counterparts in long-term (110 day) feeding experiments.**

Accordingly, the Coordinator of FF 818 SOAEFD commissioned programme is of the opinion that the existing data fully support our suggestion that the consumption by rats of transgenic potatoes expressing GNA has significant effects on organ development, body metabolism and immune function that is fully in line with the significant compositional differences between transgenic and corresponding parent lines of potatoes.