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Effect of the insecticidal *Galanthus nivalis* agglutinin on metabolism and the activities of brush border enzymes in the rat small intestine

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With increasing use of lectin genes in crop plants to improve insect resistance, the dietary exposure of humans to lectins will rise and it is necessary to assess whether the presently most favored insecticidal lectin, Galanthus nivalis *agglutinin, would be harmful for mammals. Effects of* Galanthus nivalis *agglutinin on gut and brush border enzymes were studied in rats over a 10-day dietary exposure and compared with those of a known antinutrient, phytohaemagglutinin. At a level that provides insecticidal protection for plants but did not reduce the growth of young rats,* Galanthus nivalis *agglutinin had negligible effects on the weight and length of the small intestine even though there was a slight, but significant hypertrophy of this tissue. However, the activities of brush border enzymes were affected; sucrase-isomaltase activity was nearly halved and those of alkaline phosphatase and aminopeptidase were significantly increased. Although most of the changes in gut metabolism caused by the incorporation of* Galanthus nivalis *agglutinin in the diet were less extensive than those found with toxic phytohaemagglutinin, some of them may be potentially deleterious. Thus, further and longer animal studies are needed to establish whether it is safe to use* Galanthus nivalis *agglutinin in transgenic plants destined for human consumption.* © Elsevier Science Inc. 1996 (J. Nutr. Biochem. 7:677–682, 1996.)

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Introduction

Lectins are essential plant constituents and, thus, also found in the diet.[1,2] Unfortunately, at relatively high dietary intakes some lectins, such as phytohaemagglutinin (PHA), the lectin from kidney bean (*Phaseolus vulgaris*), wheat germ (*Triticum vulgare*) agglutinin, and many others can have harmful effects. However, they are not deleterious for germ-free rats[3] and in animals harboring a gut flora most of the harmful effects are due to the extensive *Escherichia coli* overgrowth.[3] Avidly binding lectins induce hyperplastic growth, increased turnover[4,5] and polymannosylation of epithelial membranes[6] of the small bowel and this facilitates its colonization by *E. coli* and other Type-1 fimbriated bacteria.

Some lectins are toxic for insects and other plant pests.[7] Moreover, with the availability of lectin genes and methods for their transfer into the genome of vulnerable plants, it is now possible to use transgenic technology to improve the pest-resistance and agronomic yield of crop plants. However, there are several essential requirements that a successful lectin-based transgenic strategy must satisfy. The most important of these are that an ideal transgene product must be maximally effective against harmful pests at as low a concentration as possible, but its deleterious effects on higher animals, including humans, must be negligible and it must not harm the environment and beneficial organisms. It is, therefore, imperative to explore which of the many lectins presently available satisfy the above conditions. For-

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tunately, the effects of lectins on the gut and its flora are not uniform. As the extent of changes induced is determined by the sugar specificity of the lectin, its binding and the number of gut receptors,[2] it is possible that differences in the glycosylation of the digestive system of target and non-target organisms are sufficiently large to allow some lectins to harm pests but be innocuous for humans and higher animals, raising expectations that lectin genes may be safely used in transgenic plants.

Insect feeding trials have revealed that the mannose-specific snowdrop (*Galanthus nivalis*) agglutinin, GNA, had a strong insecticidal effect[7] and that this was also manifest in transgenic plants expressing GNA.[7] Although several lectins showed similar insecticidal behavior, GNA was selected in preference to others because, in preliminary studies with rats, it had only a slight effect on growth at dietary levels likely to be encountered in practice.[8] Additionally, due to the scarcity of α-1,3 terminal mannose residues in brush border membranes (BBM) of the small bowel[3,6] GNA is non-mitogenic for the gut of higher animals in the short term and, therefore, its use in transgenic plants may be safe for humans. However, on longer exposure more extensive binding develops, partly because GNA replaces type-1 fimbriated bacteria, such as *E. coli*,[3,8] and partly because the emerging new mannosyl sites can bind more GNA.[6,8] It is, thus, possible that GNA becomes mitogenic on longer exposures and consequently harmful.

One of the best ways to establish whether orally administered lectins could have any negative effects on gut metabolism was to monitor possible changes in BBM enzyme activities. This was to see whether the digestive capacity of the gut is affected by the consumption of the lectin to be used in transgenic plants, particularly as there is some evidence that lectins included in the diet can affect the activities of BBM enzymes.[9] Therefore, to establish the effects of GNA on nutritional performance of rats and epithelial cells, activities of BBM enzymes were measured in the small intestine of rats fed for 10 days on either a control diet or a control diet including ~0.7% GNA or PHA as a toxic positive control lectin. As GNA has already been expressed at this level in transgenic plants, it is possible that similarly high levels will be used in practice. The effects of these two lectins were compared to each other and to those of control rats.

Methods and materials

Lectins

GNA was isolated from snowdrop bulbs by chromatography on immobilized mannose[10] and PHA from kidney bean seeds on fetuin-Sepharose-4B.[11] PHA-E_4 was from E-Y Laboratories, San Mateo, CA USA.

Animals and diets

All animal management and experimental procedures in this study were performed in strict accordance with the requirements of the UK Animals (Scientific procedures) Act 1986 by staff personally licensed under this act to perform such procedures.

Nine male, 30-day-old Hooded-Lister, specific pathogen-free rats of the Rowett colony, kept singly in metabolism cages, were prefed for three days (6 g rat^{-1} day^{-1}) on a semi-synthetic, good quality diet containing 100 g lactalbumin protein kg^{-1} (LA diet) as the sole protein.[6] The rats, weighing 79.6 g ± 1.7 g, were divided into three groups of three rats each and pair-fed on different experimental diets for 10 days. The control group was kept on the same LA-diet used for prefeeding. The GNA and PHA groups were fed diets based on the LA control diet in which 7% (w/w) of the lactalbumin was replaced by an equal amount of either GNA or PHA (93 g lactalbumin + 7 g GNA or PHA kg^{-1} diet). The intake of all three groups was restricted to the voluntary feed intake of the PHA-group (6 g diet rat^{-1} day^{-1}). This amount of diet contained 42 mg d^{-1} PHA or GNA and the results after feeding for 10 days were expected to be indicative of the cumulative effects of the ten day dietary exposure to these lectins. However, to standardize the conditions and relate the responses precisely to the time of the exposure and the dose of the respective lectins, in the evening of the tenth day the rats were fasted overnight, ensuring that they became hungry enough to eat very quickly the 1.5 g of the different diets offered to them in the next morning. The rats were then killed under anaesthesia 2 hr later, their small intestine was removed, rinsed with 20 mL PBS, pH 7.3 (90 mM NaCl, 9 mM KH_2PO_4, 34 mM Na_2HPO_4), cut into 4-cm pieces, weighed, and placed into liquid N_2 until further analysis.

Enzyme activities in small intestinal segments

Gut pieces (4 cm in length) were homogenized in ice-cold twice distilled water using a Virtis blender (The Virtis Company, Gardiner, NY USA) at full speed for 10 min at 0°C to give a final concentration of 5% (w/v). Subsequently, the homogenates were sonicated twice at 0°C for 15 sec, separated by a 30 sec interval, at an amplitude of 24 μm with an MSE Soniprep 150 (Beun de Ronde B.V., Abcoude, The Netherlands). The protein content of the resulting sonicates was determined,[12] adjusted to approximately 350 μg protein mL^{-1} and used to measure enzyme activities and DNA content.[13] Enzyme activities were first tested at three different dilutions to find the optimal range of enzyme concentration in the reaction mixture. The assays were then done in triplicate on each intestinal segment at this optimal enzyme concentration. The sum of the means of these values was taken to represent the total enzyme activity per whole small intestine. The activities of sucrase-isomaltase (EC 3.2.1.48) were measured with saccharose[14] as substrate (1 unit = 1 μmol disaccharide hydrolysed min^{-1}), alkaline phosphatase (EC 3.1.3.1) with *p*-nitrophenyl phosphate[15] (1 unit = mmol *p*-nitrophenol liberated h^{-1}), and aminopeptidase (EC 3.4.11.2) using L-alanine-*p*-nitroanilide[16] as substrate (1 unit = 1 nmol substrate hydrolysed min^{-1}) and expressed as enzyme units g^{-1} DNA.

To establish the effect of free or bound PHA-E_4 on the activity of sucrase-isomaltase, the sonicates of gut pieces 14, 15, and 16 of all groups were incubated for 60 min in the absence or presence of 2 μg PHA-E_4 mL^{-1}, 2 μg fetuin mL^{-1} or the combination of PHA-E_4 and fetuin. To exclude the possibility that PHA-E_4 or fetuin act on glucose-oxidase (EC 1.1.3.4) and peroxidase (EC 1.11.1.7) in the sucrase-isomaltase assay, different lectin concentrations (0, 2, 10, 50, 100, 1000 μg mL^{-1}) were tested with glucose as substrate. Similar experiments were performed using 2 μg GNA in the presence or absence of 10 μmol mannose to test its possible interference with sucrase-isomaltase.

Statistical analysis

Statistical significance between control and PHA or GNA-fed rats or between PHA and GNA-fed rats was assessed by one-way analysis of variance (ANOVA) plus comparison of means. Differences were considered significant at $P < 0.05$ level using the Student's *t*-test.

Results

Nutritional data

The inclusion of GNA at 42 mg rat^{-1} day^{-1} had no significant effect on the growth of rats or accumulation of body nitrogen (*Table 1*) in comparison with the pair-fed control group. The significance of this finding was highlighted by the finding that the toxic positive control PHA at the same dietary concentration significantly depressed the weight gain and the final dry body weight of rats and reduced the nitrogen balance.

Small intestinal tissue weight and composition

The total weight and length of the small bowel of the GNA group were not significantly different from the control values although the protein and DNA contents were slightly, but significantly, higher than in control rats (*Table 2*). The contrasting significant increases in the wet weight, length and DNA and protein contents of the small intestine induced by PHA-feeding further underlined the relative inertness of GNA (*Table 2*).

When the small intestine was subdivided, the consecutive 4-cm pieces of the GNA group were generally lighter than the lactalbumin controls (*Figure 1a*) although differences in weight were rarely significant. Tissue sections from GNA-fed rats in most instances contained more DNA and protein than corresponding controls (*Figure 1*). Differences in protein content of the gut pieces between GNA-fed and lactalbumin control rats were significant throughout the small bowel, whereas differences in DNA content were seldom significant (*Figure 1b,c*). In contrast, in rats given toxic PHA diet for 10 days, the weight of the gut pieces (*Figure 1a*) and their DNA and protein contents were significantly elevated over the corresponding lactalbumin control values (*Figure 1b,c*).

Content and distribution of enzymes in the small intestine

Total alkaline phosphatase activity of the entire small intestine of both GNA and PHA groups was significantly higher than that of lactalbumin control rats (*Table 2*). Although the increase was slightly higher in GNA-fed rats than in the PHA group, the difference was not significant.

Table 1 Nutritional effects of PHA and GNA on rats

	Diet			
	Control	PHA	GNA	Pooled SD
Weight gain (g)	9.3[a]	3.1[b]	8.4[a]	1.6
Dry body weight (g)	26.7[a]	21.9[b]	26.5[a]	0.7
Nitrogen balance (mg)	721[a]	368[b]	718[a]	20

Rats were fed (6 g diet rat^{-1} day^{-1}) for 10 days on control and test diets containing either PHA or GNA. The rats were weighed regularly. Feces and urine were collected daily during the experiment and their nitrogen content was determined. For details, see text. Values are means of three rats per group with pooled SD values. Different superscripts in a row denote significantly different values ($P < 0.05$).

Table 2 Changes in growth, composition and digestive enzyme activities of the rat small intestine

	LA-fed	GNA-fed	PHA-fed	Pooled SD
Length (cm)	88.0	90.8[b]	106.7[a]	2.4
Wet weight (g)	4.71	4.50[b]	6.76[a]	0.4
Protein (mg)	322	401[a,b]	563[a]	31.1
DNA (mg)	23.3	29.0[a,b]	63.3[a]	4.8
Sucrase-isomaltase (U)	12.50	7.43[a]	6.93[a]	1.8
Alkaline phosphatase (U)	36.01	46.82[a]	43.36[a]	7.9
Aminopeptidase (U)	6.59	9.50[a,b]	12.18[a]	1.9

The results are mean values of length, wet weight, protein and DNA contents and mean activities of sucrase-isomaltase, alkaline phosphatase and aminopeptidase per small intestine of three rats. Pooled SD values were obtained by ANOVA.
[a]Significantly different from LA-fed ($P < 0.01$) and [b]significantly different from PHA-fed ($P < 0.01$).

Similarly, treatment with both GNA and PHA significantly elevated the total amino peptidase activity of the rat small intestine. In this instance, the increase was more substantial with the PHA group and the difference between the two groups was significant. In contrast, the total mean sucrase-isomaltase activity of the small intestine of both GNA and PHA-fed rats was almost halved in comparison with controls (*Table 2*).

The distribution of the activities of the three digestive enzymes expressed as units of respective enzymes g^{-1} DNA along the length of the rat small intestine (*Figure 2*) revealed an uneven distribution of these enzymes in the consecutive 4-cm pieces. Thus, sucrase-isomaltase activity gave two peaks; one in the duodenum/proximal jejunum and a second one mid-jejunum. Aminopeptidase activity was highest at mid-jejunum, but slowly decreased toward the ileum, whereas alkaline phosphatase activity was very high in the duodenum and slowly, but evenly, decreased towards the ileum. The shape of the distribution patterns generally remained the same in the lectin-exposed rats, but there were significant changes in the activities of the individual enzymes. Thus, in comparison with lactalbumin controls, there was a major decrease in sucrase-isomaltase activity of rats given GNA or PHA (*Figure 2*) throughout the small intestine although this was significantly less with GNA than PHA. GNA had only marginal and non-significant effect on the activities of both alkaline phosphatase and aminopeptidase in proximal small intestinal segments. However, the activities of both enzymes were markedly increased in the distal half of the small intestine and the elevation in some of these segments was, in fact, significant. In contrast to these relatively slight changes, exposure to PHA caused a major reduction of alkaline phosphatase activity in all gut segments. Although PHA also induced a significant reduction in aminopeptidase activity of the proximal small bowel, this effect was gradually reduced in distal parts where the differences became non-significant (*Figure 2*).

To exclude the possibility that the PHA effect was simply due to inhibition of the enzymes by binding to the sugar side chain, the activity of sucrase-isomaltase was measured in the presence or absence of PHA and/or fetuin. It was shown that neither the activity of sucrase-isomaltase (*Table*

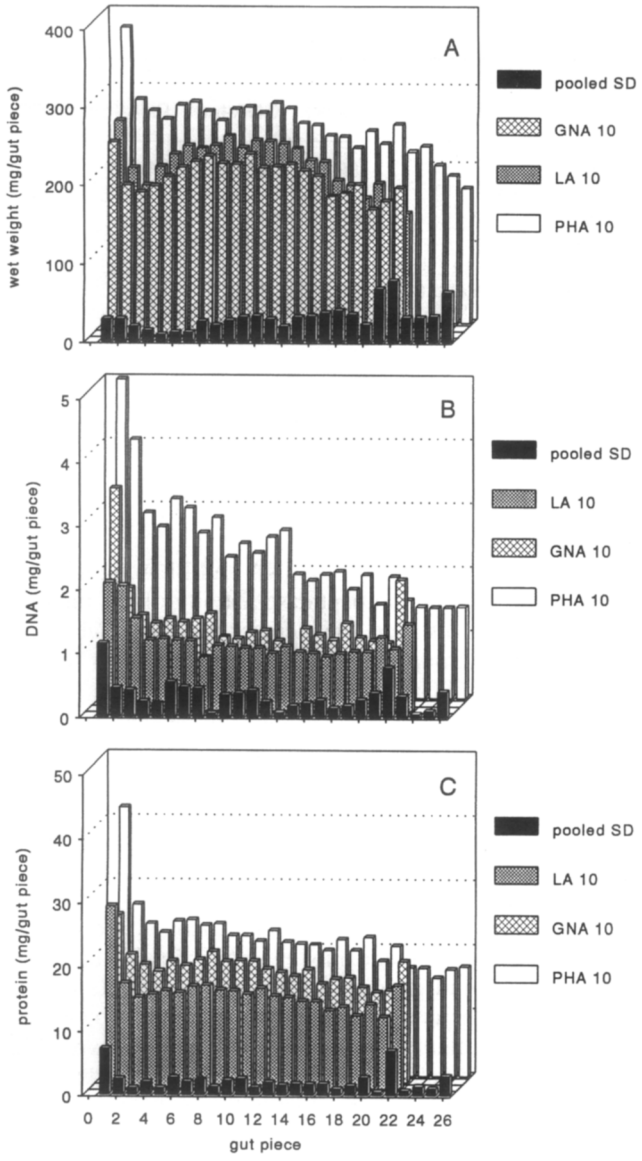

Figure 1 Growth characteristics of the small intestine of rats fed on diets containing PHA, or GNA, or control diet without lectins. Rats (groups of three) were pair-fed for 10 days on control or test diets containing PHA or GNA whose overall protein content was 10% (w/w). For details see text. Wet weight (A), DNA (B), and protein contents (C) were determined. The results are expressed as mg wet weight/gut piece ($n = 3$), mg DNA/gut piece ($n = 3$) and mg protein/gut piece ($n = 3$). The first row of these three-dimensional graphs represents the pooled SD values obtained by ANOVA.

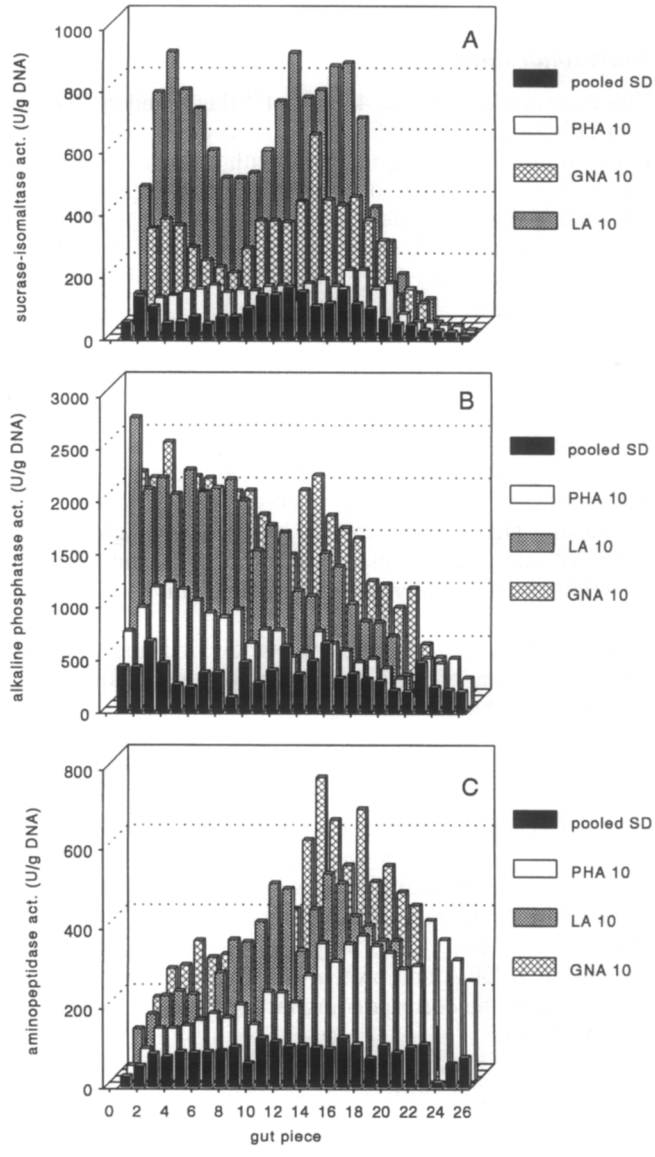

Figure 2 Activities of digestive enzymes of the small intestine of rats fed on diets containing lactalbumin, PHA, or GNA. Details of the test as in *Figure 1* with a daily lectin intake of 42 mg of PHA or GNA. The specific activities of sucrase-isomaltase (A), alkaline phosphatase (B), and aminopeptidase (C) was determined. The results are expressed as U/g DNA ($n = 3$). The first row of the three-dimensional graphs gives the pooled SD values obtained by ANOVA.

3) nor that of the assay-specific intrinsic enzymes, glucose oxidase and peroxidase (data not shown) were changed by incorporating PHA in the reaction mixture alone or together with fetuin, its specific haptenic inhibitor. This was equally true for the small intestinal homogenates obtained from PHA-fed or control rats (*Table 3*). GNA-fed animals were not tested because previous results showed that exposure to GNA does not induce significant changes in brush border membrane glycosylation.[6] In similar tests, PHA appeared to have no effects on the activity of the other two brush border enzymes either. Additionally, the presence of extra GNA in the reaction mixtures was also without effect on the activity of these enzymes (data not shown).

Discussion

In this study the number of rats per groups had to be kept to a minimum (three rats per group) mainly because the isolation of nearly 1.5 g of pure GNA was a major and expensive task and also because the large number of enzyme assays that had to be carried out. Despite this, as the individual results were highly consistent as shown by the small pooled SD values, the conclusions are not invalidated by the small number of rats.

Table 3 Effect of PHA and/or fetuin on the specific activity of sucrase-isomaltase in sections of the rat small intestine

Sonicate	Sucrase-isomaltase activity	
	LA-fed	PHA-fed
No addition	59 ± 3	25 ± 3
2 μg PHA-E_4/mL	59 ± 6[a]	26 ± 3[b]
2 μg fetuin/mL	59 ± 3[a]	25 ± 2[b]
PHA-E_4 + fetuin	59 ± 6[a]	24 ± 1[b]

Sucrase-isomaltase activity was measured in representative sections of control or PHA-fed rat small intestine in the absence or presence PHA-E_4 lectin, fetuin and PHA-E_4 + fetuin. The results are expressed as the mean sucrase-isomaltase activity ± SD in mU mg^{-1} protein (n = 8).
[a,b]Not significantly different from the corresponding control group (no addition) based on Student's t-test.

As expected from the poor initial binding of the strictly mannose-specific lectin GNA to the brush border epithelium,[6] incorporation of this lectin in the diet had little effect on the growth rate, nitrogen balance and final dry body weight of rats (*Table 1*). Moreover, GNA was also a poor growth factor for the small intestine and the slight growth it induced was mainly by hypertrophy (*Table 2*). This virtual lack of negative effects on gross body metabolism was highlighted by the fact that at the same dietary level, PHA used as a toxic control lectin induced substantial hyperplastic growth of the small intestine and seriously depressed the growth of the rats (*Table 1*). A likely explanation for this difference in the effects of the two lectins is that, in contrast to the PHA-induced major changes in membrane glycosylation[6] and consequent bacterial overgrowth,[3,17] GNA does not affect glycosylation[6] or the microbial flora[3] of the small intestine and is therefore non-toxic.

Because of its nutritional toxicity, it is not unexpected that exposure to PHA resulted in major changes in the level of expression and/or activity of BBM enzymes. However, it was somewhat surprising to find that despite its slight effects on nutritional performance, GNA also had major effects on BBM enzyme activities. Moreover, changes in BBM enzyme activities were apparently not due to the direct inhibition of these enzymes by PHA (*Table 3*) or GNA (unpublished). Thus, the total mean sucrase-isomaltase activity of the small intestine of rats given GNA was reduced after 10 days almost to the same extent as with toxic PHA (*Table 2*). However, reduction in the activity expressed as units g^{-1} DNA was less extensive (*Figure 2a*) mainly because initially, GNA was non-mitogenic and therefore, the increase in small intestinal DNA was slight. Its effect on the activities of alkaline phosphatase and aminopeptidase expressed in units g^{-1} DNA, was distinct from that on sucrase-isomaltase; they were slightly increased in the distal small intestine (*Figure 2b,c*). In comparison, the strongly mitogenic toxic PHA depressed the activities of all three enzymes, particularly sucrase-isomaltase and alkaline phosphatase.

The major changes in the expression and activities of BBM enzymes on GNA exposure for 10 days appear to correlate poorly with its initial weak binding to the brush border epithelium.[6,8] However, as the binding becomes progressively more extensive on continuous exposure,[8] possibly because the competitive displacement by GNA of endogenous ligands from the gut surface and its preferential binding to the mannosyl receptors of newly emerging cells generated by the epithelial turnover, its effects on brush border cellular metabolism can become more extensive with time. This, in turn, can induce more binding and this self-amplification process may eventually lead to a progressive interference with the expression and activities of BBM enzymes. Although in nutritional terms, this interference was not significant after 10 days with GNA (*Table 1*); on longer exposures, more anti-nutritional effects of GNA may emerge.

The present study was not designed to establish the mechanism of the effects of lectins in the diet on the brush border epithelium and enzymes associated with it, nonetheless it highlighted some of the potential nutritional hazards of the use of lectin genes to improve the pest resistance of plants. Obviously, lectin genes such as that of PHA are not likely to be acceptable because PHA is a major antinutrient at high dietary concentrations and, by inducing bacterial overgrowth, has a negative impact on growth and health. However, the results of the present work indicated that caution should be exercised even with apparently non-toxic lectins such as GNA. Thus, although in initial feeding tests it showed no significant antinutritive effects, by reducing sucrase-isomaltase activity, GNA is likely to have some deleterious effects for the digestive capacity of the gut. This finding, therefore, underlines the general need to test any potential transgene product at dietary concentrations likely to be encountered in the transgenic plant not only for its effects on nutritional performance, but also for its effects on BBM enzymes, glycosylation, gut-associated lymphoid tissue and the bacterial flora before costly genetic manipulations and the transfer of insecticidal lectin genes into crop plants is undertaken and they are released for human consumption. A further warning that changes in the activity of sucrase-isomaltase in the small intestine of rats exposed to GNA may have a negative effect on health was suggested by the finding that similar changes in enzyme activity were also observed in rats exposed to PHA, which is known to have potent toxic effects at this dietary level.[1–5,8,18] Although the connection between reduced intestinal function and potential toxicity for the consumer is not yet understood, this problem needs to be urgently addressed because the GNA gene has already been transferred into several major crops including potatoes, rice, rape seeds, etc., based on the general presumption that GNA has no oral toxicity for humans and higher animals.[7]

In conclusion, the growth of young rats was not significantly affected by feeding them diets containing GNA for 10 days, whereas diets containing the toxic positive control PHA strongly depressed growth. However, despite its slight effect on nutritional value, GNA interfered with the activities of small intestinal BBM enzymes, particularly with sucrase-isomaltase. Thus, the possibility that the consumption of transgenic plants expressing GNA may in the long-term reduce the digestive capacity of the small intestine and have other negative effects on the health of humans and higher

animals, highlights the potential hazards implicit in the use of transgenic crop plants containing lectin/antinutrient genes and the need for further testing before their general release.

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