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Detection of genetically modified material using immunoblot (Dot Blot)

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ABSTRACT

The production of genetically modified food using *Bacillus thuringiensis* (Bt) toxins (proteins) and *Galanthus nivalis agglutinin* (GNA) is not new; various food crops have been transformed to withstand biotic and abiotic stresses using the lectin (*agglutinin*) from *Galanthus nivalis*. The rice samples used in this work were analysed for the presence of the genetically modified material. Two of the rice samples used gave dot fluorescence of 223.513 and 150.032 with calculated GNA amount of 14.02 and 9.19 ng respectively. The samples contained GNA between 0.031 and 0.07 % per 1µg; the values are significant indicating that the samples express significant amount of the lectin, which implies that the rice analysed are genetically modified. Thus this study identified rice that has been genetically modified with the lectin from *Galanthus nivalis* using the immunoblot method.

Introduction

A major threat to agriculture is the issue of adapting both biotic and abiotic stresses that affects agricultural productivity. Various strategies are used to enhance crops' adaptability to the stresses. The introduction and application of biotechnology strategy which saw the development of genetically modified (GM) crops is one of the integrated approaches used to bring about agricultural sustainability. This has led to the development of GM foods using *Bacillus thuringiensis*, *Galanthus nivalis* among others (Atherton, 2002; Kier and Petrick, 2008). Consequently, a number of strategies to improve plant resistance to pests and diseases are being developed using genetic engineering. GM foods that can withstand stresses such as those transformed using *Bacillus thuringiensis* and *Galanthus nivalis* (snowdrop) are being developed. GM foods have become part of the food chain; the adoption and commercialization of GM food is due to their numerous roles in tackling biotic and abiotic stresses. In Europe and some developed countries, regulations guiding the sale, use and / or consumption of GM derived foods require the labelling of foods that have been genetically modified in order to keep buyers or consumers informed of the food they are purchasing. *Galanthus nivalis agglutinin* (GNA) is widely used to produce GM foods (McCafferty et al., 2008). The lectins found in snowdrop are naturally occurring proteins that possess at least one non-catalytic domain that binds reversibly to a specific mono- or oligosaccharide. These lectins are found in plants, but also in animals, bacteria and fungi. Various explanations have been made on the mode of action of lectins; Some lectins are toxic to insects and other herbivores. This may be facilitated by binding of lectins to chitin in the peritrophic matrix or by interacting with glycoproteins on the epithelial cells of the insect midgut. A study on cowpea bruchid (*Callosobruchus maculatus*) demonstrated that the binding of a legume lectin to cell surface receptors in the gut of the insect was essential for its anti-feeding activity (Zhu-Salzman *et al*., 1998).

GNA, a lectin isolated from bulbs of the snowdrop plant has the ability of recognizing only mannose residues. It agglutinates rabbit erythrocytes but is inactive against human red blood cells. Mannose-recognizing lectins have also been isolated from flowering monocots but their toxicity to higher animals limits their use in crop plants for human consumption. The low binding capacity of GNA in the jejunum is responsible for its non-toxicity to mammals (Poulsen *et al*., 2007; Foissac *et al*., 2000; Gatehouse *et al*., 1993).

The detection, identification and quantification of the presence of genetically modified (GM) material in foods or crops that are genetically engineered are necessary due to biosafety guidelines and requirements. The present work is aimed at detecting and quantifying the presence of *Galanthus nivalis agglutinin* (GNA) in rice extracts or has GNA transgene embedded in its genetic material and has expressed the protein (Kier and Petrick, 2008).

Materials and Methods

Protein Extraction and quantification

Rice seeds were obtained from Gatehouse research laboratory in the School of Biology Newcastle University. Total soluble protein (TSP) was extracted from rice seeds as described by Sambrook and Russel (2001). In brief, the outer husks of the seeds were removed and the seeds were put in a micro-centrifuge tube and 100µl of extraction buffer was added. The tubes were placed in the 50ºC water bath for 15 minutes. The seeds were homogenised using the micro pestle. 200µl of extraction buffer was added to each tube after the seeds were fully homogenised. The seed homogenates were incubated for 30 minutes at room temperature with the sample being vortex every five minutes to extract the soluble protein (TSP). After the 30 minutes incubation, the extracts were centrifuged at

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maximum revolutions per minutes for 10 minutes to extract the insoluble material from the TSP. The TSP extract was transferred into a fresh micro-centrifuge tube for quantification and kept on ice.

10µl and 100µl of rice seeds TSP extract were added to 1.0ml Bradford reagent in duplicates and were incubated at room temperature for five minutes; the absorbance was measured at 595nm. The equation from the standard curve was used to calculate the amount of protein in each Rice TSP sample.

Construction of a protein standard Curve

Five cm^3 of a 10mg/cm^3 BSA stock solution was prepared in extraction buffer; 0.05g was weighed and mixed in 5ml of the buffer. Dilutions were made from the stock. A quantity (10µl) of each standard was added to 0.01 cm^3 Bradford reagents and mixed (Bradford, 1976); this was repeated in duplicates for each standard. It was incubated for 5 minutes at room temperature and the absorbance was measured at 595nm. Standard curve of concentration of BSA against absorbance at 595nm was prepared in excel.

Protein detection and quantification via immunoblot (immuno-Dot Blot)

A strip (Invitrogen) was used according to manufacturer's information. It contained 8 dots that corresponded to the following standards and samples; standard 0ng GNA, standard 5ng GNA, standard 10ng GNA, standard 20ng, sample 1 of 20µg control rice, sample 2 of 20µg unknown potentially GM rice, sample 3 of 20µg unknown potentially GM rice and sample 4-20µg unknown potentially GM rice. The quantification was carried out as described by McCafferty *et al*., 2008. Membrane incubations were performed with 8ml volume of reagent in an approximately sized container as was designed. The membranes were contained within the 15ml centrifuge tube. The incubations were performed on a rocking machine under continuous motion. Membrane was washed in 8ml TBS-T for 5 minutes with gentle shaking. The membrane was incubated in 8ml blocking reagent for 10 minutes at room temperature with continuous rocking. The blocking reagent was removed and was washed twice for 5 minutes each in anti sera buffer. 8ml of anti sera buffer was added and 4 µl of primary antibody was added to the tube. The membrane was incubated at room temperature for 30 minutes with constant gentle rocking. Membrane was washed in 8ml TBS-T twice for 5 minutes each with gentle shaking. 8ml of anti sera buffer was added to the membrane and tube. 4 µl of Qdot® secondary antibody conjugate of 0.5nM final concentration was added. The membrane was incubated at room temperature for 1.5 hour with constant gentle rocking. The membrane was washed in 8ml TBS-T two times for five minutes each with gentle rocking. The membrane was transferred to TBS and was washed twice for three minutes each. It was stored in TBS at room temperature until it imaged. The blot was imaged under UV illumination and image and fluorescence of each 'Dot' was recorded. The GNA standards were used to construct standard curve. The amount of GNA in each rice sample was calculated using the standard curve. Percentage GNA per µg TSP was also calculated.

Results

In order to quantify the amount of protein in the test samples, the amount of protein in the dilutions of the standards were used to calculate the standard curve as shown in Table 1. The standard curve was used to calculate the amount of proteins in the rice samples (Table 1). The amount of total soluble proteins in rice extracts ranges from 0.396µg – 0.6050µg per 1µl as in Table 1. GNA was quantified using the fluorescence intensity; the amount of GNA in the rice extracts was between 6.274 – 14.05ng (Table 2). The percentage of GNA in the total soluble proteins was calculated and shown in Table 2.

Table 1: Protein quantification

Sample	Amount of Protein (BSA or TSP) (µg)
Standard 1	0
Standard 2	10
Standard 3	20
Standard 4	30
Rice Extract (Control)	0.398
Rice Extract (GNA 1)	0.478
Rice Extract (GNA 2)	0.510
Rice Extract (GNA 3)	0.605

Table 2: Determination of GNA concentration in samples

Sample	Fluorescence of dot	Amount of GNA (ng)	% GNA per 1µgTSP
Standard 0ng	0	0	
Standard 5ng	91.302	5	
Standard 10ng	175.637	10	
Standard 20ng	306.425	20	
Control Rice (20µg TSP)	117.315	7.037	0.035
GNA rice 1 (20µg TSP)	223.510	14.025	0.071
GNA rice 2 (20µg TSP)	105.708	6.274	0.031
GNA rice 3 (20µg TSP)	150.032	9.190	0.045

Discussion

Maximum total soluble protein values of 0.517 and 0.605 µg/µl were obtained from two rice samples. Two samples gave dot fluorescence of 223.513 and 150.032 (Table 2) with calculated GNA amount of 14.02 and 9.19 ng respectively, others gave lower fluorescence but fluorescence was shown by the standard (0 ng) and control which may have occurred due to cross reaction or sensitivity of the method (Nahalkova *et al*., 2001; Howarth *et al*., 2005). The percentage of GNA per 1µg was between 0.031 and 0.07 %; earlier workers have reported varying percentages of 0.02 to 0.05, and 0.03 to 0.7 up to 1% of GNA per total soluble proteins depending on the method used and the level of expression of the protein driven by the promoters used. Thus two rice samples (1 and 3-Table 2) that gave significant amounts of GNA are likely to contain *Galanthus nivalis* agglutinin (McCafferty *et al*., 2008). The maximum percentage GNA expression in 1µg total soluble protein of 0.07% will have no significant effect on non-target organisms; endoparasitoids like *Meteorus gyrator* (Wakefield *et al*., 2006).

Galanthus nivalis agglutinin (GNA) gene that encodes snowdrop lectin has been introduced into variety of GM crops and has been shown to integrate into the genome of these plants and is expressed. Transgenic tobacco plants expressing *GNA* demonstrated strong inhibition to aphids (Zhang *et al*., 2007). Thus, the rice used in the present study is safe as it will not affect the non-target organisms since unintended effects are not likely to occur.

As part of biosafety regulations there is requirements that GM crops or food containing a transgene be labelled (Atherton, 2002; Wakefield *et al*., 2006). Thus, it is highly important to use an accurate and reliable technique to detect and quantify GM materials in genetically modified foods to satisfy these biosafety

regulations. As has been demonstrated in this work, the immublot technique is a good method of the identification of proteins.

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