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Genetically modified plants and food hypersensitivity diseases: Usage and implications of experimental models for risk assessment

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Abstract

The recent advances in biotechnology in the plant industry have led to increasing crop production and yield that in turn has increased the usage of genetically modified (GM) food in the human food chain. The usage of GM foods for human consumption has raised a number of fundamental questions including the ability of GM foods to elicit potentially harmful immunological responses, including allergic hypersensitivity. To assess the safety of foods derived from GM plants including allergenic potential, the US FDA, Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO), and the EU have developed approaches for evaluation assessment. One assessment approach that has been a very active area of research and debate is the development and usage of animal models to assess the potential allergenicity of GM foods. A number of specific animal models employing rodents, pigs, and dogs have been developed for allergenicity assessment. However, validation of these models is needed and consideration of the criteria for an appropriate animal model for the assessment of allergenicity in GM plants is required. We have recently employed a BALB/c mouse model to assess the potential allergenicity of GM plants. We have been able to demonstrate that this model is able to detect differences in antigenicity and identify aspects of protein post-translational modifications that can alter antigenicity. Furthermore, this model has also enabled us to examine the usage of GM plants as a therapeutic approach for the treatment of allergic diseases. This review discusses the current approaches to assess the allergenic potential of GM food and particularly focusing on the usage of animal models to determine the potential allergenicity of GM foods and gives an overview of our recent findings and implications of these studies.

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Keywords: Genetically modified plants; Animal model; Food allergy; Decision tree; Regulatory T-cells; Assessment

Abbreviations: α AI, α -amylase inhibitor-1; Bean- α AI, purified bean-derived α AI; Chickpea- α AI, purified chickpea-derived α AI; Codex, Codex Alimentarius Commission; FAO, Food and Agriculture Organization of the United Nations; FHDs, food hypersensitivity disorders; GM, genetically modified; MALDI-TOF-MS, Matrix-assisted laser desorption/ionization, time of flight mass spectroscopy; OVA, ovalbumin; Pea- α AI, purified pea-derived α AI; SSA, sunflower seed albumin; Transgenic-Pea, Transgenic pea (*Pisum sativum* L.) expressing a common bean (*Phaseolus vulgaris* cv. Tendergreen)-derived α AI; WHO, World Health Organization.

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1. Introduction

The recent advances in biotechnology have led to the improvement of molecular techniques for plant genetic engineering. This technological advancement now allows recombinant proteins from prokaryotic and eukaryotic organisms to be expressed in genetically modified (GM) plants in a temporal and spatial manner for a variety of purposes including agronomic productivity, consumer benefits, and antibody and vaccine development (Mason et al., 2002; Stewart et al., 2000). Thus, the global cultivation area of GM crops has risen from 1.7 million hectares in 1996 (the first year GM crops were commercially grown) to 67.7 million hectares in 2003 (Engel et al., 2002). The majority of these GM crops are select crop varieties including maize, rape, potato, and soybean and are primarily genetically modified for agronomic productivity including herbicide tolerance or insect resistance (Brandt, 2003).

With the rapid development of GM crops, a number of fundamental issues regarding environmental and health concerns have been raised by the general public and the scientific community. Environmental risks including potential invasiveness of GM crops, vertical and horizontal gene transfer, and biodiversity and health concerns associated with GM crops such as toxicity and the potential of the introduced proteins to elicit a potentially harmful immunological response including allergic hypersensitivity have all been highlighted (Conner et al., 2003; Novak & Haslberger, 2000; Taylor & Hefle, 2002).

To address these concerns a number of agencies, US FDA, Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO), and the EU have developed protocols for stringent regulatory examination of GM plants using a weight of evidence approach to identify potential allergens in GM foods (Metcalf et al., 1996; Taylor, 2002).

1.1. The weight of evidence approach

To evaluate foods derived from biotechnology, the joint World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) program developed a decision tree approach in 2001 (Metcalf et al., 1996; Taylor, 2002). The Codex Alimentarius Commission (Codex) has since reviewed the decision

tree approach and other relevant material and concluded that one test could not accurately predict the allergenicity of a given protein (Alinorm 03/34: Joint FAO/WHO Food Standard Programme, 2003). The Codex principles and guidelines on foods derived from biotechnology calls for a weight of evidence approach. This approach involves assessment of allergenicity of the expressed protein using all available and scientifically relevant techniques and the weight of the evidence is then used to determine the likelihood of the protein being allergenic (Alinorm 03/34: Joint FAO/WHO Food Standard Programme, 2003; Goodman et al., 2005). Techniques used as part of the weight of evidence approach to assess the potential allergenicity of the newly expressed protein include consideration of the source of the gene, sequence similarity to known allergens, serum screens for IgE epitopes, ex vivo cell assays, and physico/chemical properties such as pepsin resistance and allergenicity in animal models (Alinorm 03/34: Joint FAO/WHO Food Standard Programme, 2003; Goodman et al., 2005; Metcalfe et al., 1996).

The weight of evidence approach involves the assumption that if the protein's source is known to be allergenic, then the transferred protein is assumed to be an allergen unless scientifically proved otherwise. Proteins from a known allergenic source need to be assessed for serum IgE binding from patients allergic to the source material and other tests including skin prick tests and ex vivo T-cell assays (Alinorm 03/34: Joint FAO/WHO Food Standard Programme, 2003; Goodman et al., 2005). When the protein is not from an allergenic source, other assessment criteria including sequence homology analysis, IgE binding capacity and pepsin resistance are used. Animal models are also recommended to be used to screen proteins for allergenicity. Animal models are considered to be particularly useful when the GM plant expresses a protein that has not previously been consumed (Taylor, 2002). Recently, the development and validation of animal models to be used for the assessment of allergenicity in GM crops has become an area of intense research and development.

2. Genetically modified plants and allergy

Interestingly, only a small number of specific proteins are thought to induce food hypersensitivity disorders (FHDs). This is supported by the fact that 90% of food allergy in children is

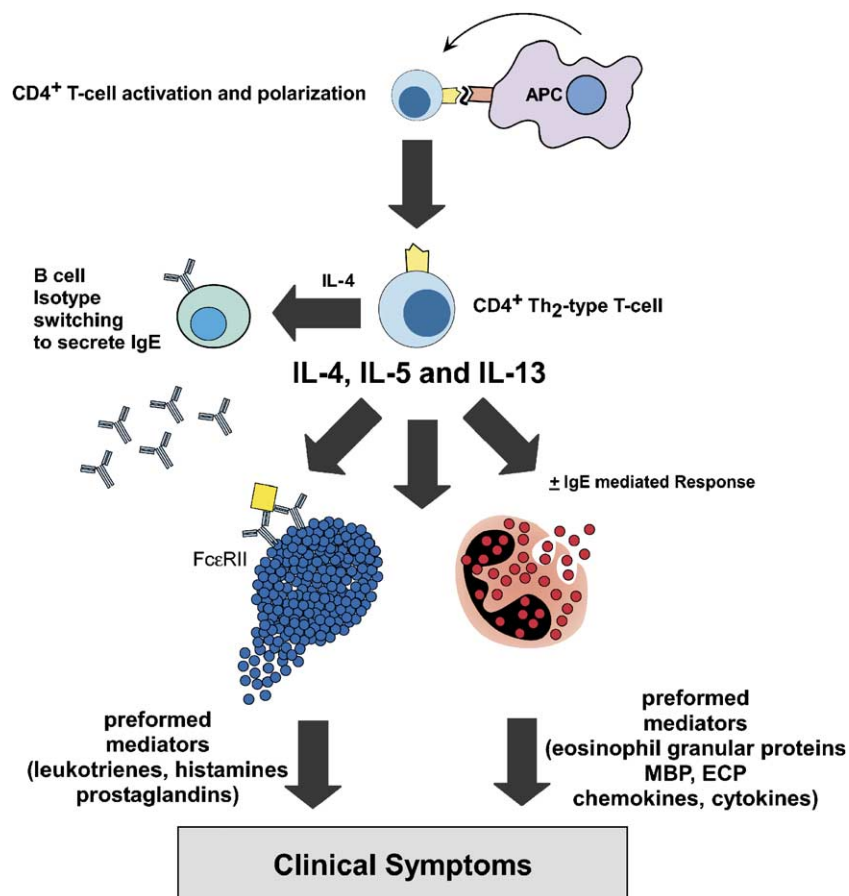


Fig. 1. CD4⁺ T-cell regulation of food hypersensitivity disorders. CD4⁺ Th₂ cells play a central role in orchestrating allergic inflammation. Following activation by antigen, Th₂ cells produce IL-4, IL-5, and IL-13. IL-4 is required for B-cell maturation and IgE synthesis; IL-3 and IL-5 promote eosinophil growth and maturation; and IL-4, IL-9, and IL-13 are required for mast cell function. IgE produced by B-cells binds to the Fcε receptor (FcεRI) present on mast cells and eosinophils. Cross linking of this receptor stimulates the release of a number of preformed mediators by mast cells and eosinophils including leukotrienes, histamines, prostaglandins, and eosinophilic granular proteins inducing the clinical symptoms.

mediated by egg, milk, peanut, fish, and soy; whereas peanuts, fish, and shellfish mediate ~85% of hypersensitivity reactions in adults (Oehlschlager et al., 2001; Sampson, 1999). In general, the majority of dietary proteins consumed are proteolytically digested and degraded into small non-immunogenic peptides that evade immunosurveillance. The dietary proteins that are sampled by the GI immune system generally evoke a state of specific immunological unresponsiveness termed oral tolerance (Chehade & Mayer, 2005). However, for reasons that are not yet fully understood in patients with FHDs, some dietary antigens when presented by the GI immune system evoke an allergic-type inflammatory response and predispose to disease (Brandtzaeg, 2002; Burks, 2002). The primary mode of treatment for food hypersensitivity is avoidance of the affecting food types (Khakoo & Lack, 2004). For this reason, the introduction of GM plants into the human food chain that contain allergens or proteins of unknown allergic potential could be of risk. These concerns were highlighted in a study by Nordlee et al. (1996) who demonstrated that allergenicity is transferable by genetic modification. These investigators demonstrated that of the 8 out of 9 patients' serum IgE that bound the 2S albumin brazil nut protein, IgE of 7 patients bound to the 2S albumin from

extracts of soybeans that transgenically express the brazil nut 2S albumin but not to non-transgenic soybeans (Nordlee et al., 1996). Furthermore, they demonstrated positive skin prick test in patients with brazil nut hypersensitivity to brazil nut and the transgenic soybean expressing the brazil nut 2S albumin protein but not non-transgenic soybeans (Nordlee et al., 1996). Collectively, these studies demonstrated that IgE sensitivity could be transferred to the transgenic plants when an allergen is expressed in a non-native host through genetic modification.

2.1. Immunopathogenesis of food hypersensitivity disorders

The immunological mechanisms associated with the development of a food hypersensitivity reaction are not yet fully elucidated (Brandtzaeg, 2002; Sampson, 1999, 2003; Sicherer, 2002). Clinical and experimental investigations suggest that CD4⁺ T-lymphocytes through the secretion Th₂-type cytokines IL-4, IL-5, and IL-13 regulate the production of IgE and the effector function of mast cells and eosinophils in allergic disease. Indeed, peripheral blood and tissue from patients with FHDs contains elevated numbers of activated T-cells and their increased numbers correlate with elevated levels of Th₂-

cytokines, the degree of GI inflammation, and dysfunction (Eigenmann, 2002). Furthermore, in vitro stimulation of T-cells and T-cell clones from food allergic patients produce Th₂-cytokines (IL-4, IL-5, and IL-13) following antigen stimulation (Eigenmann, 2002; Turcanu et al., 2003).

IL-4, IL-5, IL-10, and IL-13 promote a number of allergic inflammatory processes. IL-4 and IL-13 are critical factors for the regulation of T-cell commitment to the CD4⁺ Th₂ phenotype, IgE production by B-cells, mast cell function, inflammatory responses to recall antigens, and the modulation of chemokine production. In conjunction with other Th₂-type cytokines, as well as IL-1 β and TNF- β , IL-4 may also regulate cell trafficking by activating adhesion systems in the vascular endothelium. IL-5 regulates the growth, differentiation, activation, and survival of eosinophils and provides the essential signals for the amplification and recruitment of this leukocyte to sites of allergic inflammation (Rothenberg, 1998).

Mast cells and eosinophils are thought to be the primary effector cell in FHDs (Sampson, 1999, 2003; Sicherer, 2002). Activation of mast cells by Antigen/IgE cross-linking to Fc ϵ R1 leads to the release of proinflammatory mediators including histamine, prostaglandin-D₂, and sulfated peptide leukotrienes (LTC₄, LTD₄, LTE₄), proteoglycans (heparin and chondroitin), proteases (tryptase and chymase), and lipid-derived mediators (prostaglandins and leukotrienes) (Lorentz et al., 1999; Santos et al., 1999; Siddiqui & Miner, 2004; Yu & Perdue, 2001) (Fig. 1). Eosinophils serve as major effector cells inducing tissue damage and dysfunction by releasing toxic granule proteins (major basic protein [MBP], eosinophilic cationic protein [ECP], eosinophil peroxidase [EPO], and eosinophil-derived neurotoxin [EDN]) and lipid mediators (Rothenberg, 1998). The mast cell and eosinophil-derived bioactive mediators are thought to exert the clinical symptoms associated with FHDs (Yu & Perdue, 2001).

3. Animal models for the assessment of genetically modified plants

Animal models are important for testing proteins that are derived from sources with no previous history of ingestion or exposure to the GI tract. It is proposed that an animal model for the assessment of allergenicity of GM crops should preferentially meet the following criteria: (1) the model should not involve the use of adjuvants as adjuvants potentiate antigen-specific immunity and can also influence to type of immune response elicited; (2) the animal model should preferentially involve oral administration of novel food; however, low multiple doses of the GM plant should be used in animals given a balanced diet to prevent adverse effects unrelated to the material itself; (3) the test should detect an IgE response to the protein, as well as other Th₂-associated immune responses; (4) the clinical response should mimic that of humans with food allergy; (5) the model needs to be easy to conduct, reproducible over time, and the introduced protein should not be part of the diet prior to experimentation; and (6) the test animal should develop allergen-specific IgE antibodies to known allergens (Penninks & Knippels, 2001; Taylor, 2002;

Alinorm 03/34: Joint FAO/WHO Food Standard Programme 30 June–5 July, 2003).

Several models have been developed including models using the Brown Norway (BN) rat, BALB/c mouse, dogs, and pigs (Helm & Burks, 2002; Helm et al., 2003). It is unlikely that a single animal model will be able to definitively predict the potential allergenicity of the novel food antigens because food allergy in humans is a complex disorder that is dependant on a number of different factors to promote allergic sensitization (Atherton et al., 2002). It is predicted that the use of a number of appropriate animal models, in conjunction with other assessment criteria, will be able to identify potential hazards.

3.1. The Brown Norway rat model

The BN rat is a high-immunoglobulin (particularly IgE) responder strain, which resembles atopic humans in their (genetic) predisposition to preferentially produce antigen-specific immunoglobulins of the IgE isotype. In experimental investigations employing a model of feeding purified protein (1 mg/day for 42 days) in the absence of an adjuvant, the rats were shown to induce specific humoral (IgG and IgE) and cellular immune responses to the model antigen ovalbumin (OVA) (Knippels et al., 1998). Furthermore, studies have shown that when fed common allergenic whole foods (cows milk and egg white) the rats developed IgE-associated allergic responses to the same proteins that are commonly allergenic in humans (Knippels & Penninks, 2002). The rat is commonly used in routine toxicity testing, and because the oral sensitization properties of food proteins in the rat have already been tested, novel food products can be assessed in perspective to the total information of the potential health effects of a food on the health of the animal (Knippels et al., 1998; Penninks & Knippels, 2001).

The BN rat is therefore a very promising model for the assessment of allergenicity in GM foods. However, there is conflicting data concerning the variability of IgE production to OVA gavage treatment between experiments (Knippels et al., 1998). Further assessment and characterization of the responses of the BN rat to known allergenic proteins is therefore required.

3.2. Dog and pig models

Other animal models that have been employed to study IgE-associated disorders such as atopic dermatitis, nausea/vomiting, diarrhea, and anaphylaxis involved strains of dogs and pigs (Buchanan & Frick, 2002; Helm et al., 2003). Recent studies have demonstrated that priming the food allergy in these animals is comparable to food allergy in humans (Buchanan et al., 1997; Buchanan & Frick, 2002, 2002; Helm et al., 2003; Tryphonas et al., 2003).

3.3. The BALB/c mouse model

Another model under development is the BALB/c mouse model which, like the Brown Norway rat, has a propensity towards a Th₂-type phenotype and IgE production (Okano et al., 1999; Russo et

al., 2001). BALB/c mice have previously been used in animal models of food hypersensitivity where they have been shown to develop many of the clinical symptoms of food allergy such as diarrhea, anaphylaxis, antibody responses (IgE and IgG1), eosinophil and mast cell accumulation (Li et al., 2000; Hogan et al., 2001; Brandt et al., 2003; Untersmayr et al., 2003). BALB/c mouse models developed for the assessment of GM plants for allergenicity have primarily measured antibody responses to the protein and not other clinical symptoms associated with food allergy (Dearman et al., 2001; Atherton et al., 2002; Dearman & Kimber, 2001).

A BALB/c mouse model developed by Dearman et al. (2000, 2001) involves peritoneal administration of purified protein in the absence of an adjuvant and is useful because it circumvents the potential of oral tolerance. Moreover, this model can distinguish between the propensity of an allergenic or non-allergenic protein to induce IgE production (Dearman et al., 2000, 2001; Dearman & Kimber, 2001). However, Dearman et al. (2001) have also demonstrated that oral gavage of purified allergens induced an IgG response but only low variable amounts of IgE, indicating that this method may be limited by the level of sensitivity to allergenic protein.

4. Investigations using the BALB/c mouse model

Recently, we have utilized the BALB/c mouse model to assess the potential allergenicity of two GM plants. Firstly, we examined a transgenic pea (*Pisum sativum* L.) expressing a common bean (*Phaseolus vulgaris* cv. Tendergreen) (transgenic-Pea)-derived α -amylase inhibitor-1 (α AI). The transgenic peas were generated to protect the seeds from damage by inhibiting the α -amylase enzyme in old world bruchids (pea, cowpea, and azuki bean weevils) (Morton et al., 2000; Schroeder et al., 1995). The second plant was a narrow leaf lupin (*Lupinus angustifolius* L.) expressing the sunflower (*Helianthus annuus* L.) seed-derived sunflower seed albumin [SSA] (SSA-Lupin), which was generated to increase sulfur-rich amino acid content in livestock feed (Molvig et al., 1997).

The bean-derived α -amylase inhibitor-1 is a member of the α -trypsin inhibitor family, and unlike the cereal α -amylase inhibitors (Baur & Czuppon, 1995; Baur & Posch, 1998; Kanny & Moneret-Vautrin, 1995) there is no report of the legume α AI inhibitor inducing allergic hypersensitivity responses. In contrast, sunflower seed albumin (SSA), a member of the 2S albumin protein family, has been identified as a candidate allergenic protein in sunflower seed hypersensitivity (Kelly et al., 2000; Kelly, 2000). SSA has been shown to contain IgE binding epitopes and possesses amino acid sequence similarity (34%) with the known allergenic 2S albumin protein from the brazil nut (Kelly et al., 2000; Kelly, 2000).

4.1. Immunological responsiveness assessment of genetically modified pea expressing α -amylase inhibitor-1 in a BALB/c mouse model

Initially we utilized the BALB/c mouse model to examine whether oral consumption of transgenic plants predisposed to

immunological responsiveness. We employed a protocol whereby mice were oral gavage challenged with homogenized GM (transgenic-pea) or native plant (non-transgenic pea) flour twice a week for 4 weeks and then subsequently challenged subcutaneously (s.c.) and intratracheally (i.t.) with purified α AI and assessed for delayed type hypersensitivity (DTH) and Th₂-type inflammatory responses (Prescott et al., 2005). Firstly, we examined the production of α AI-specific atopy-associated immunoglobulin production (IgG₁ and IgE) and demonstrated that oral consumption of the transgenic peas elicited an immunological response. α AI-specific IgG₁ was detected at 2 weeks and at significant levels after 4 weeks of oral exposure in mice that were fed transgenic-pea, but not non-transgenic pea (Prescott et al., 2005). Furthermore, these mice exhibited a significant DTH response compared to the non-transgenic pea exposed group when purified pea-derived α AI (pea- α AI) was injected into the footpad (Prescott et al., 2005). Thereby, suggesting that the consumption of transgenic-pea promoted α AI-specific immunological responsiveness. To characterize the type of immune response elicited against pea- α AI following oral consumption of transgenic-pea, we employed a well characterized murine model of CD4⁺ Th₂ cell-mediated inflammation (Wills-Karp, 2000). We show instillation of purified pea-derived α AI (pea- α AI) into the lungs of mice fed with transgenic pea induced key features of Th₂-type inflammation including pulmonary eosinophilia, mucus hypersecretion, and AHR (Prescott et al., 2005). Pulmonary eosinophilia, mucus hypersecretion, and AHR are critically linked to the effector function of the Th₂ cytokines interleukins (IL)-4, -5, and -13 (Wills-Karp, 1999). In vitro stimulation of T-cells from transgenic pea-fed mice revealed that oral exposure of mice to transgenic-pea, but not non-transgenic seed meal, predisposed to systemic immunological responsiveness characterized by a Th₂-type immune profile (Prescott et al., 2005).

BALB/c mice are known to have a propensity towards Th₂ immunity. To test if the response seen in these BALB/c mice was a strain-specific response, we fed C57BL/6 mice the transgenic-pea and non-transgenic pea. We found that the C57BL/6 mice also developed α AI-specific IgG₁ antibody responses to α AI when fed the transgenic-pea and not the non-transgenic pea (unpublished data).

To clarify that natively expressed α AI does not predispose to immunological responsiveness and Th₂-type inflammation, mice were fed common bean (Pinto bean), which express a native form of α AI and subsequently received purified bean-derived α AI (bean- α AI) in the skin and lung. These studies demonstrated that oral consumption followed by respiratory exposure to native bean- α AI did not promote immunological responsiveness or inflammation (Prescott et al., 2005).

To investigate if transgenic expression of α AI in a plant unrelated to the pea would still have altered immunogenicity to the native bean α AI when fed to mice, mice were fed a transgenic chickpea (*Cicer arietinum* L.) expressing α AI [transgenic chickpea] or non-transgenic chickpea homogenate (250 μ L/mouse twice a week as previously described; Prescott et al., 2005) and challenged in the lung with purified chickpea-derived α AI (chickpea- α AI). Unlike transgenic

pea, mice fed with transgenic chickpea did not develop a α AI-specific IgG₁ antibody responses, eosinophilia, mucus hypersecretion, or AHR following intratracheal challenge with α AI (V. Prescott and S. Hogan, unpublished data). The level of α AI-specific IgG₁, mucus hypersecretion, eosinophilia, and AHR in transgenic chickpea-fed mice was comparable to that observed in non-transgenic chickpea-fed mice challenged in the lung with α AI. Concurrently, the responses were significantly less than observed in mice with antigen/adjuvant-induced Th₂-type inflammation. Chickpea- α AI were also assessed for cell-mediated immune responses to α AI by challenge in the footpad with purified chickpea- α AI and measuring footpad oedema. Mice fed with the transgenic or non-transgenic chickpea homogenate did not develop a footpad swelling response 24 hr following footpad challenge with chickpea- α AI when compared to the positive control (α AI/Alum primed and α AI footpad-challenged mice) (V. Prescott and S. Hogan, unpublished data). These studies demonstrate that oral consumption of a transgenic chickpea expressing α AI does not promote Th₂-type inflammation or cell-mediated footpad hypersensitivity responses in mice. Furthermore, these studies show that transgenic expression of α AI inhibitor in the chickpea does not induce immunogenicity of α AI in mice.

The contrasting differences between the chickpea and pea α AI studies prompted us to investigate the potential mechanism of altered immunogenicity of α AI. The immunological responsiveness of food proteins is regulated by multiple factors including peptide sequence, post-translational modification, and structure. Since the nucleotide sequences were identical between pea- α AI and bean- α AI, the demonstration of immunological differences between the transgenic α AI in pea and the natively expressed bean forms prompted us to assess potential structural differences between these proteins by matrix-assisted laser desorption/ionization, time of flight mass spectroscopy (MALDI-TOF-MS) analysis (Prescott et al., 2005).

Previous studies have shown that bean- α AI is synthesized as a pre-pro- α AI polypeptide that is cleaved following Asn⁷⁷ to form 2 peptide chains (α and β), both of which are glycosylated and have 1 or more amino acid residue(s) removed from their C-termini. This post-translational processing results in major forms of the α - and β -chains and minor forms containing alternative glycans (Yamaguchi et al., 1992; Ishimoto et al., 1995; Martin Young et al., 1999; Sawada et al., 2002). MALDI-TOF-MS analysis of purified bean- α AI (*P. vulgaris* L. cv. Tendergreen) closely matched a previously published spectrum of another bean- α AI (*P. vulgaris* L. cv. Greensleeves) confirming that our bean- α AI possessed similar well-characterized post-translational modifications and very similar relative abundance of minor processing variants (Yamaguchi et al., 1992; Ishimoto et al., 1995; Martin Young et al., 1999; Sawada et al., 2002). Interestingly, MALDI-TOF-MS analysis of transgenic pea- and bean- α AI revealed differences in both the α - and β -chain (Hoffman et al., 1982). We identified modifications in the ratios of Man₆GlcNAc₂–Man₉GlcNAc₂ glycan variants in α AI between transgenically expressed pea- α AI and bean-derived α AI (Prescott et al., 2005). Since then,

DNA sequencing of the transgene in pea and comparison with the published sequence (Hoffman et al., 1982) confirmed that the nucleotide sequences were identical; these data suggest that the observed further forms of the α -chain are related by variations in post-translational modifications including glycosylation.

Collectively, these studies suggest that transgenic expression of a bean- α AI in pea leads to the formation of a modified form of α AI with altered glycan variants and enhanced antigenicity. The glycan variants of the chickpea-derived α AI has not yet been extensively analyzed; however, Western blot analysis has revealed banding different to bean- and pea-derived α AI (results not shown). By analyzing and comparing the glycan variants of the pea, bean, and chickpea-derived α AI, we may be able to identify the glycan variants mediating allergic sensitization.

Previous investigations have also demonstrated that carbohydrate moieties of glycoprotein allergens are important for IgE reactivity. Studies characterizing the cereal α -amylase-inhibitor family (unrelated to legume α AI) demonstrated that differential glycosylation of subunit enhances IgE-binding capacity (Sanchez-Monge et al., 1992). Moreover, glycosylated cereal α AI subunits have been shown to possess significantly enhanced IgE-binding affinity when compared to the unglycosylated forms (Sanchez-Monge et al., 1992). These proteins possess identical amino acid sequences and only differ in their carbohydrate moieties, indicating that glycosylation can confer IgE-binding capacity and Th₂-inflammation. Interestingly, recent investigations have also demonstrated that glycan side chains linked to high mannose-type N-glycans on plant-derived glycoproteins can confer immunogenicity and are IgE binding determinants (Faye et al., 1993; Van Ree et al., 2000). Furthermore, α (1,3)-fucose and β (1,2)-xylose linkage to high mannose-type N-glycans (Man₅GlcNAc₂–Man₉GlcNAc₂) have been shown to promote immunogenicity and IgE binding. The β -chain of pea- α AI possesses β (1,2)-xylose linked high mannose-type N-glycans and other complex glycoforms and the α -chain may possess an as yet undefined glycoform variant; however, it remains to be determined how these modifications alter pea- α AI immunogenicity.

Studies with α AI support the usage of animal models to assist in the assessment of GM plants for allergenicity. These studies highlight the need for the weight of evidence approach to not only consider the source of the gene but also to take into consideration the potential for post-translational modifications to alter the structure and antigenicity of a protein when expressed in a new host. Full characterization of the protein of interest expressed in the native and transgenic state might also be warranted for assessment of allergenicity. Furthermore, these studies suggest that sequence comparison analysis with known allergens may have limited predictive ability.

4.2. Immunological responsiveness assessment of genetically modified lupin expressing sunflower seed albumin in a BALB/c mouse model

To assess the antigenicity of SSA-Lupin, we employed a protocol whereby mice were oral gavage challenged with

homogenized GM (SSA-Lupin) or native plant (lupin) flour twice a week for 4 weeks and then subsequently challenged subcutaneously and intratracheally with purified SSA and assessed DTH and Th₂-type immune responses (Smart et al., 2003). Firstly, we examined the production of SSA-specific atopy-associated immunoglobulin production (IgG₁ and IgE). We found that consumption of SSA-Lupin did not induce an SSA-specific IgG₁ or IgE response (Smart et al., 2003). Furthermore, we also examined whether consumption of SSA-Lupin in mice predisposed to hypersensitivity responses or Th₂-type inflammation and showed that consumption of SSA-Lupin does not systemically sensitize animals and predispose to atopy (Smart et al., 2003). We concluded that oral consumption of the GM plant (SSA-Lupin) did not promote a Th₂-type immune response and predispose to allergic hypersensitivity of skin or lung in mice.

4.3. Experimental analysis of the role of genetically modified plants promoting a Th₁-type immune response and suppressing allergic inflammation

Clinical and experimental studies have provided corroborative evidence demonstrating that allergic diseases are regulated by allergen-specific CD4⁺ Th₂ cells in the respiratory tract. Allergen-specific CD4⁺ Th₂ cells through the release of cytokines enhance the growth and differentiation of eosinophils, basophils, mast cells, and B-cells producing IgE (2). The resulting inflammatory response is associated with the development of symptoms associated with various allergic diseases including asthma, eczema, and food allergy (3, 4, 5). Recently, there has been considerable focus on the role of allergen-specific CD4⁺ Th₁ cells, producing high levels of IFN γ , as regulators of allergic inflammation. IFN γ down-modulates Th₂-biased immune responses in the respiratory tract, thereby protecting against the symptoms of allergy and asthma. Experimental investigations have demonstrated that induction of CD4⁺ Th₁ cells and IFN γ limits the development of Th₂-biased immune responses and prevents the development of allergic disease.

We found interestingly that oral consumption of SSA-Lupin in fact promoted an SSA-specific IgG_{2a} antibody response, suggesting that oral administration of SSA promoted an antigen-specific Th₁-type as opposed to a Th₂-type immune response. Further analysis revealed elevated levels of IFN γ in PBLN-derived T-cell culture supernatants from SSA-Lupin-fed but not in lupin-fed SSA sensitized and challenged WT mice following SSA stimulation (Smart et al., 2003). These studies support the concept that consumption of SSA-Lupin promoted the development of a CD4⁺ Th₁-type immune response.

We were therefore interested in examining whether oral consumption of SSA-Lupin could protect against the development of SSA-induced experimental asthma. To assess this, mice were fed lupin- and SSA-Lupin seed meal and were intraperitoneally and intratracheally challenged with purified SSA to induce experimental asthma (Smart et al., 2003). We showed that intraperitoneal and intratracheal challenge of

lupin-fed mice promoted experimental asthma as characterized by mucus hypersecretion, pulmonary eosinophilia, and AHR. Notably, experimental asthma was suppressed in SSA-challenged mice fed with SSA-Lupin. These data suggest that oral consumption of a genetically modified plant expressing SSA promoted an antigen-specific Th₁-type response and suppressed the development of experimental asthma.

To examine whether this protective response was mediated by an IFN γ -dependent process, we employed IFN γ -deficient mice. We demonstrated that in the absence of IFN γ , there was a protective effect of SSA-Lupin on the induction of allergic airways disease (AAD). Consistent with this observation, previous studies have shown that IFN γ is associated with the protection against allergic diseases (Cohn et al., 1999; Hogan et al., 1998).

The dietary proteins that are sampled by the GI immune system generally evoke a state of specific immunological unresponsiveness termed oral tolerance (Brandtzaeg, 2002; Mowat, 2003). Oral tolerance is mediated by 3 mechanisms including deletion, anergy, or suppression of antigen-reactive CD4⁺ T-cells. Experimental evidence exists for an active suppressor mechanism to play a role in oral tolerance (Chehade & Mayer, 2005). Early investigations provided circumstantial evidence that specific suppressor T-cells are responsible for oral tolerance involving both humoral and cell-mediated immunity (Ngan & Kind, 1978; Miller & Hanson, 1979; Mowat et al., 1982; Strobel et al., 1983). Since these investigations, various T-cell subsets including CD4⁺ CD25⁺, CD4⁺ CD45RB^{low}, and IL-10 producing T regulatory 1 (Treg1) T-cells have been identified to possess suppressive activity (Whitacre et al., 1991; Weiner, 2000; Read & Powrie, 2001; Brandtzaeg, 2002). These CD4⁺ T-cell subsets are thought to suppress activated antigen-specific T-cells via a number of mechanisms including cell contact-mediated and cytokine (TGF- β , IL-10, and IFN- γ)-mediated suppression (Chehade & Mayer, 2005).

Using FACS analysis we examined the CD4⁺ T-cell population in the draining mesenteric lymph nodes of SSA-Lupin and lupin-fed mice in an attempt to identify the phenotypic characteristics of the suppressor T-cell subpopulation. FACS analysis on PBLN of Lupin and SSA-Lupin-fed mice following intratracheal challenge revealed an increase in the percentage of CD4⁺ CD45RB^{low} T-cells present in PBLN of SSA-Lupin-fed mice sensitized and challenged with SSA as compared to that observed in lupin-fed mice, suggesting that CD4⁺ CD45RB^{low} regulatory T-cells may be involved in the suppressive effects. To examine the contribution of this cell population in the GM-plant-mediated suppression of experimental asthma, we adoptively transferred CD4⁺ CD45RB^{low} regulatory T-cells from SSA-Lupin- and lupin-fed mice to syngeneic naive mice and induced experimental asthma protected against the development of AAD. CD4⁺ CD45RB^{low} regulatory T-cells from SSA-Lupin-fed mice were protected against the development of experimental asthma, suggesting that CD4⁺ CD45RB^{low} regulatory T-cells were involved in GM plant mediated suppression of AAD (Smart et al., 2003).

These investigations are consistent with previous experimental clinical studies demonstrating a role for IFN γ in oral tolerance (Lee et al., 2000; Nagata et al., 2000; Turcanu et al., 2003). Examining the cytokine profile of PBMC-derived T-cells from peanut-allergic (PA), non-allergic patients (NA), and also from donors that have outgrown their peanut allergy (OG), investigators demonstrated that T-cells from NA and OG produced Th₁-type cytokine profile (IFN γ ^{high}, IL-4^{low}, IL-5^{low}, and IL-13^{low}) following antigen stimulation (Turcanu et al., 2003). Similarly, other clinical studies have shown that PBMCs from non-allergic patients secrete high levels of IFN γ following in vitro stimulation (Nagata et al., 2000). Employing a TCR Tg⁺ mouse model system, investigators have demonstrated that IFN γ may regulate oral tolerance through modulating T-cell migration to peripheral sites of inflammation (Lee et al., 2000). We currently do not know what involvement IFN γ plays in the development and regulation of CD4⁺ CD45RB^{low} regulatory T-cell activity.

These experimental investigations revealed that oral consumption of a GM plant expressing a known allergen does not necessarily predispose to allergy but in fact may stimulate the development of a protective regulatory T-cell response and suppress the development of AAD.

5. Conclusion

The development, characterization, and production of GM plants is a rapidly evolving industry. To address potential health concerns particularly regarding the ability of GM crops to elicit a potentially harmful immunological response including allergic hypersensitivity responses, the Codex has developed stringent regulatory assessment criteria (Taylor, 2002, p. 98; Metcalfe et al., 1996). One mode of assessing the potential immunological consequence of consumption of GM plants is the development and employment of animal models. We describe here a murine model to assess the potential allergenicity of 2 GM plants. We show that consumption of a GM plant expressing an allergen does not necessarily predispose to allergic hypersensitivity responses. In fact, we demonstrate that consumption of GM plants can promote a protective regulatory T-cell response and protect against the subsequent development of allergic disease specific to that protein. However, we also show that diversity in translational and post-translational modification pathways between species can influence the molecular architecture of the expressed protein and subsequent cellular function and antigenicity. These findings highlight the need for further investigation into the usage of animal models for the assessment of antigenicity of GM plants and suggest that post-translational mechanisms may contribute to altered antigenicity.

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