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A review of animal models used to evaluate potential allergenicity of genetically modified organisms (GMOs)

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Food safety regulators request prediction of allergenicity for newly expressed proteins in genetically modified (GM) crops and in novel foods. Some have suggested using animal models to assess potential allergenicity. A variety of animal models have been used in research to evaluate sensitisation or elicitation of allergic responses. However, protocols for sensitisation and challenge, animal species and strains, diets and other environmental factors differ widely. We present a comprehensive review of published, peer-reviewed experimental animal models used for the evaluation of allergenicity of genetically modified organisms (GMOs).

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Introduction

The prevalence of allergy and, in particular, food allergy with potentially life threatening reactions has increased in the last decades [1], without the identification of obvious environmental or genetic causal factors [2]. Food allergy is a complex disease resulting from primary (sensitisation) and secondary (elicitation) responses against food proteins. During sensitisation in susceptible individuals, food proteins may induce specific Th2-type allergic responses. T cells stimulate immunoglobulin class-switching in B cells to produce allergen-specific IgE, which binds to mast cells and basophils, and upon re-exposure to the allergen induces the release of mediators that elicit allergic symptoms. Although it is not clear why food allergies are more prevalent now, some authors suggest that this increase is due to widespread use of GM crops for food production ever since their introduction in 1996 ([3], http://www.globalresearch.ca/genetically-modified-foods-unsafe-evidence-that-links-gm-foods-to-allergic-responses-mounts/7277).

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Is there a need for animal models in GMO allergenicity assessment/evaluation?

An incomplete understanding of factors that affect allergic sensitisation has driven the search for predictive strategies in allergy risk assessment. International guidelines and regulations from various countries state that GMOs should be assessed for potential allergy risks based on the source of the gene, amino acid sequence identity matches to known allergens and stability to *in vitro* pepsin digestion (Box 1) [4,5]. In the European Union, however, the European Food Safety Authority (EFSA) has recommended using animal models to evaluate sensitising potential of novel proteins on a case-by-case basis [6], even though there are no validated animal models that are broadly predictive for allergenicity in humans [4,7]. Nevertheless, if a highly predictive animal model was developed, it would be useful for answering several critical questions about the basic mechanisms underlying food allergy (Box 2). Such models would likely improve the risk assessment process for GMOs that do not have a clear history of safe consumption by humans.

GMOs evaluated in animal models

To date, Cry1Ab and Cry1Ac proteins of *Bacillus thuringiensis* (Bt) and grain from the transformed maize host have been the most frequently tested materials in experimental animal models. Yet these proteins have not been found to induce allergy in animal models or in humans who have consumed food produced from the GM crops (see Bt-related references in Table 1). These crystal proteins are encoded by a non-allergenic source. They are relatively large proteins that are rapidly digested in pepsin and have a low abundance in the GM crop. In addition to Bt and its cloned Cry1 proteins, other GMOs have been tested *in vivo* including alpha amylase inhibitor (αAI) peas [8,9], PHA-E lectin in rice [10], sunflower seed albumin in narrow leaf lupin [11] and lactoferrin [12].

Box 1. Primary risks and overall focus for evaluating potential risks of allergy from GMOs [4]

- Is the protein from the transferred gene an existing allergen (food, airway or contact) as suggested by the allergenicity of the gene source or sequence comparison to known allergens? If indicated, perform serum IgE tests using samples from appropriately allergic donors.
- Is the protein encoded by the transgene likely to cause cross-reactions as suggested by even modest amino acid sequence identity matches to known allergens? If so perform serum IgE tests.
- Are the characteristics of the protein similar to known common food allergens; stable in pepsin at acidic pH and abundant in food grade materials suggesting potential risk?

Box 2. Questions potentially addressed using experimental animal models

- Is a protein without a history of safe human dietary exposure likely to sensitise and cause allergic reactions?
- Does the food matrix alter potential sensitisation, tolerance or elicitation?
- Are there 'threshold' doses for sensitisation or elicitation using various routes of exposure?
- How does proteolysis or heat processing alter the sensitising and eliciting properties of an allergen?

Animal models for testing potential allergenicity of GMOs

Several animal species have been fed material from GM plant varieties, near isogenic or non-GM (nGM) varieties. The evaluated animal responses included weight gain and overall health, toxic effects or development of allergy. In particular, Bt-maize expressing Cry1Ab has been studied in pigs [13,14], salmon [15], sheep [16], cattle [17], zebrafish (cross generational feeding) [18], rats [19,20], and mice [21]. Although a scientific rationale could be argued for using one or more of these species to evaluate nutritional or ecological impacts of agricultural plant varieties, each species differs markedly from humans in some physiological and immunological responses. Rodents are the most frequently used model for food allergy, even though there is little evidence that their responses are highly predictive for ranking the allergenicity of diverse proteins in humans [22]. Thus, we sought to review data from studies in rodents and other animal models looking for evidence that they are useful for the evaluation of allergic sensitisation, elicitation and adjuvant activity (Table 1). We have excluded studies designed to evaluate nutritional or toxic properties without evaluating potential immunogenicity or allergenicity.

Allergic sensitisation

Most of the studies identified in this review used rats or mice of a single genetic strain. Sensitisation was accomplished with either purified protein or extracts or feed containing whole GM crop materials. The antigens were administered orally by feeding or gavage, by dermal application, intraperitoneal (i.p.) injection or intranasal (i.n.) dosing. In most studies, the materials were provided repeatedly over time and in some cases with added adjuvants, such as alum or cholera toxin. For oral sensitisation, the length of daily exposure varied from 30 days to more than 90 days [21], and in a few cases multigenerational exposure was evaluated [23,24]. Finamore *et al.* [21] fed mice with diets incorporating MON810 maize or control maize for 30 or 90 days, evaluated CD4+ T cell counts in peripheral blood and measured differences in levels of IL-6, and IL-13 in the serum of both newly weaned and old mice. They reported that the parameters varied little between groups [21]. Although differences were found for non-antigen-specific immunological markers, clarification for these findings is

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Table 1. Summary of animal models used for assessing allergenicity and/or immunogenicity of GMOs.

Protein	Animal strain and sex	Sensitisation route	# of immunisations	Conventional adjuvant	Challenge	Measured parameters	Notes	Year/citation
Soluble Cry1Ac protoxin from E. coli JM103, crystalline from BTHD-73, or BSA	Female BALB/c mice	i.g. or i.p. with Mg-Al hydroxide and i.p. in PBS as well as	3 times on weekly intervals	Either no adjuvant, cholera toxin, or Alum	No	Specific IgM, IgG, and IgA	Cry 1Ab produced from *E. coli* JM103 (pOS9300) obtained from D. Dean of Ohio State University. Immunogenicity (IgA, IgG and IgM) measured by i.g. or i.p. crystalline or soluble Cry1 administration	1999 Vazquez-Padron [47]
Cry1Ac protoxin	Female BALB/c mice	i.p., rectally, or i.n.	3 times on weekly intervals	No	No	Specific IgA, IgM, IgG in sera, BAL, vaginal, small intestine, and large intestine wash fluids	Observed mucosal immunogenicity of Cry1Ac, but no control protein used. The negative control was PBS	2000 Moreno-Fierros [34]
Cry1A protoxins Cry1A (Cry1Aa, Cry1Ab, and Cry1Ac) (130–133 kDa), Cry3A protoxin, devoid of C-terminal half	Male BALB/c mice	i.p. or i.n.	3 times on weekly interval	No	No	Specific IgG, IgM, and IgA	Aimed to define immunogenic regions of Cry proteins using i.p. and i.n. route with $n = 5$ mice per treatment measuring IgG, IgM and IgA to suggest N-terminal region more immunogenic, no treatment replicates	2004 Guerrero [36]
Seed meal from GM αAI peas, nGM peas; αAI purified from beans; OVA; SSA-Lupin; GM αAI chickpeas or nGM pinto beans	BALB/c mice (sex was not indicated)	i.g., or i.p.	Twice per week for 4 weeks	No (positive control mice received alum, but treatment mice did not)	By airway (asthma) and in foot pad (subcutaneously for delayed type hypersensitivity responses)	Footpad thickness measured, specific IgG1; mucus secreting cells, eosinophils, and Th2 cell number	Multiple sensitisation and challenge schemes. Indicates i.g. αAI peas increased OVA responses. Compare to Lee *et al.* [8]	2005 Prescott [9]
Cry1A toxins (Cry1Aa, Cry1Aa8 and Cry1Ab2)	Male BALB/c mice	i.n.	1	Various compounds: LPS, DT, ConA, GalNAc.	Re-stimulation of immune cells *in vitro* with Cry 1 proteins	IgG1, IgG2a, cytokines: Th1 (IFNγ, IL-12p70); Th2 (IL-10, IL-4)	Wild-type Cry1A induced Th1 responses, but not Th2 responses	2007 Guerrero [48]
Bt-MON810 maize diet, Cry1Ab	Male BALB/c mice	Inclusion diet: 50% MON810 or parental control maize flour	Diet given to recently weaned or old mice for 30 and 90 days (old only 90 days)	No	No	IELs, spleen lymphocytes, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IFN γ, TNF-R, MCP-1 (CCL2), and mMCP-1	Evaluated potential immunotoxicology of GM, parental and commercial maize lines. deoxynivalenol mycotoxin was higher in GM compared to non-GM, and immune markers of inflammation in mice fed GM were reportedly higher in number and statistics, but no clear associations were found to suggest harm	2008 Finamore [21]
Cry1Ab, peanut protein extract	Female BALB/c mice	i.g.	5 times on days 1, 7, 13, 19 and 25	Cholera toxin in comparison to adjuvanticity of Cry1Ab	Yes (intra-tracheal)	Specific IgE, IgG1 and IgG2a and Th1/Th2/Th17 cytokine, bronchoalveolar lavage fluid (BAL), and splenocytes analysed	Sensitisation to peanut proteins only observed in mice sensitised with PE and CT, as measured by T cell responses. No Cry1Ab adjuvant activity. Conclusion: Cry1Ab did not demonstrate adjuvant activity compared to cholera toxin	2008 Guimaraes [49]
PHA-E transgenic rice or Cry1Ab, with or without added purified Cry1Ab or PHA-E	Male and female Wistar rats	Dietary and Inhalation	28 day and 90 day feeding	No	No	Specific IgM, IgG1, IgG2a, IgA antibody to PHA-E and Cry1Ab and total IgM, IgG, and IgA	PHA-E lectin had an immune modulatory effect, but Cry1 did not	2008 Kroghsbo [10]
Recombinant Cry1Ac protoxin with Naegleria fowleri lysate	Male BALB/c STAT6++ and STAT6−/− mice	i.n.	4 times on weekly interval	No	Challenged with lethal doses of *N. fowleri* trophozoites	Th2, IgG1, IL-4, IFNγ, IL-12, IgG2a, IgA, IgM	Assessed adjuvanticity of Cry1Ac and conferred protection to lysate	2010 Carrasco-Yepez [50]

Table 1 (*Continued*)

Protein	Animal strain and sex	Sensitisation route	# of immunisations	Conventional adjuvant	Challenge	Measured parameters	Notes	Year/citation
Soluble Cry1Ac from E. coli JM103	Male BALB/c mice	i.n.	4 times on weekly intervals	No (controls received cholera toxin)	No	Specific IgA and IgG, phenotypic and activation analysis, IL-4, IL-5, and IL-10	*E. coli* JM103 (pOS9300) with Cry1Ac insert was obtained from D. Dean of Ohio State University. Endotoxin in Cry1Ac quantified.	2010 Rodriguez-Monroy [51]
Cry1Ab, BLG, Ara h 1, KLH	Female BALB/cJ mice	i.g. or i.p.	5 for i.g. or 2 for i.p.	Incomplete Freund's adjuvant or cholera toxin	Yes	Specific IgE, IgG1, IgG2a, cytokines, murine metabolic biomarkers	Observed Cry1Ab is immunogenic, but does not have allergenic potential. Endotoxin quantified in test proteins.	2011 Adel-Patient [52]
Bt-MON810 (Cry1Ab) maize diet and non-GM maize	Female swine	Diet	Sows fed daily for 143 days during gestation, then lactation	No	No	Cry1Ab-specific antibody, leukocyte phenotyping, hematology	Sows fed MON810 maize (Cry1Ab) or non-GM through gestation and lactation. Immune function evaluated including tests for Ab to Cry 1Ab in sows and piglets, which were negative	2012 Buzoianu [23]
Native human milk lactoferrin (LF) and recombinant (rLF) in Aspergillus or rice	Female BALB/c mice	i.p.	2 or 3 times on weekly intervals	No	No	Specific IgE, IgG1, IgG2a, Th1 and Th2 cytokines	Endotoxin quantified. LF was more immunogenic then rLF. Mannose- and fucose (Le^x)-containing ligands have adjuvant properties depending on glycan profile	2013 Almond [12]
Bt-MON810 maize diet, Cry1Ab	Atlantic salmon	Diet	33 day or 97 day feeding trial	No	No	Histomorphology of main organs, mRNA expression levels of genes in distal intestine, IgM	No specific anti-Cry1Ab IgM detected	2013 Gu [15]
OVA and transgenic αAI from peas, chickpeas and cowpeas compared to non-transgenic controls	Female BALB/c mice	i.p., i.g., or i.n.	2 for i.p., 6 for i.n., i.g. twice weekly for 4 weeks	No	Challenged with 1% OVA by an ultrasonic nebulizer	Specific IgG1, IgG2a, IgE in sera, lung and airway inflammation and mucus hypersecretion	No major differences were found between the immune and inflammatory responses between extracted proteins from GMs. The isogenic pea induced immune responses to pea lectin that were cross-reactive with αAI	2013 Lee [8]
Bt-MON810 maize diet, Cry1Ab	Atlantic salmon	Diet	99 day feeding trial	No	No	Histological changes, mRNA expression levels, and inflammation scored in distal intestine	Cry1Ab protein or other compositional differences in GM Bt-maize may cause minor alterations in intestinal responses in juvenile salmon, while not affecting overall survival, growth performance, development or health of the animal	2014 Gu [53]
Bt-maize, nGM maize, and OVA	Female BALB/c mice	Diet for Bt-maize, i.p. or i.n. for OVA	Bt-maize: diet; OVA i.p. 2 times	No	Yes (aerosol challenge twice daily on 4 days)	Specific IgG1, IgG2a, IgE, lung inflammation and mucus hypersecretion	No adjuvant effect on allergic response to non-cross-reactive OVA after diet containing Bt-maize (Mon810)	2014 Reiner [43]
Bt-MON810 pollen or leaves extract, Cry1Ab from Bt spores, OVA, and trypsinized Cry1Ab from E. coli	Female BALB/c mice	i.n.	6 times on days 0, 1, 2, 21, 22 and 23	Cholera toxin	Yes	OVA specific: IgE, IgG1, IgG2a. MCP-1, BAL cytokines	No adjuvant effect of pollen grains as Allakhverdi *et al.* observed [54]. No treatment replicates	2015 Andreassen [3]
Bt-MON810 pollen or leaves extract, Cry1Ab from Bt spores, and trypsinized Cry1Ab from E. coli	Female BALB/c mice	i.n.	6 times on days 0, 1, 2, 21, 22 and 23	No	Yes	IgE, IgG1, IgG2a, MCP-1, BALF cytokines	Claim that Bt spores are not a good source and that E. coli trypCry1Ab may be more relevant, but their results indicate that trypsinised protein is more immunogenic then Mon810. There are no cutoff values for IgE. There are no treatment replicates	2015 Andreassen [35]

necessary [21]. Studies using i.p. injection for sensitisation typically used three doses separated by seven days. Allergen was used for clinical challenges. Blood was usually collected between the third and seventh day after the last injection and was used for measuring specific IgE concentrations.

Measured disease parameters

The readouts measured in animals are similar to those used or observed to evaluate allergy in humans. For example, a primary marker of sensitisation in humans is antigen-specific IgE. Antigen-specific IgE or IgG1 levels were frequently measured in exposed mice as useful markers of a Th2 response and potential allergy. Additional markers including a differential measure of cytokines (IL-4, IL-5, IL-13 vs. IFN-γ, IL-2, IL-10) were sometimes measured from direct protein assays or mRNA detection. While antibody binding demonstrates immune recognition of a specific antigen, clinical manifestations of allergic responses require activation and degranulation of mast cells and basophils as a result of IgE binding (or possibly IgG1 in mice) to two or more epitopes on a single allergen. Additional tests in rodents and other species include protein-specific dermal mast cell degranulation with either active- or passive-cutaneous anaphylaxis, which mimics skin prick tests (SPT) with allergenic extracts to diagnose humans [25]. Allergen-specific production of Th2 cytokines, release of histamine and mast cell protease usually correlate with *in vivo* signs including anaphylaxis, hypothermia, hypotension or reduced pulmonary function in various animal models [26–29]. Allergic sensitisation and elicitation are complex processes that manifest differently in allergic individuals depending on genetic and environmental factors. Thus, it is not surprising that animal models may not mimic all clinical responses in humans.

Factors that may limit predictability of animal models

Animal models are useful for mechanistic insights in allergy and may be useful for assessing allergenicity of GMOs. However, the lack of a complete understanding of the factors that impact sensitisation in humans creates obstacles for the development of a predictable animal model.

A major problem in this field is the lack of standardised models for testing novel foods and GMO allergenicity. The models are designed with different sensitisation protocols, species or genetic strains, routes of allergen exposure (e.g., oral, inhalation, gastro-intestinal, dermal and intraperitoneal), added adjuvants (e.g., cholera toxin, alum, lectins, and lipids) and GMO test materials (e.g., purified proteins in their native conformation or denatured, whole food matrix, contaminants like endotoxin), which markedly influence sensitisation and elicitation responses [30–33]. It is possible that certain genetic differences between animal strains will result in disparate responses to specific proteins, which implies that similar experiments with different strains in the same lab might be necessary to fully assess materials. There is also the possibility that the GM materials contain cross-reactive proteins. Notably, both nGM and αAI peas upon consumption in mice induced allergic responses upon re-challenge that were caused by the cross-reactive pea lectin [8]. Different protocols, animal strains, and materials as well as lab-to-lab variation often lead to disparate experimental outcomes and low predictability. It is important to consider whether conflicting results from different models will alter risk assessment for human food safety.

Another challenge for establishing predictable models is the inclusion of appropriate negative and positive controls. When testing food grade material from a GMO, ideally a near isogenic line with overall minimum genetic diversity compared to the GMO and one or more genetically diverse commercial lines with similar intended use would be included in separate treatment test groups. In many of the studies (Table 1), the authors have not included a positive control, that is, crop materials that will induce a strong allergic response as a comparator because, in many cases, the perfect positive control does not exist. This is also the case when using a GM protein, which should be compared with (1) a protein inducing no allergic response, (2) an allergenic protein and (3) vehicle alone. For example, in one mouse study, the protein Cry1Ac from Bt induced immune responses in mice, but was only compared to the vehicle [34].

An important consideration for experimental animal models is that there is often high variation in the induced immune response and thus, it is important to test an appropriate number of animals and perform a sufficient number of experimental replicates to assess biological variance. For the inclusion of animal experiments into a risk assessment, it is essential to perform intra- as well as inter-lab comparisons, using the same test materials because there may be external or internal (e.g., gastrointestinal microbiota) environmental variables that could influence the outcome. Nevertheless, there are published animal studies assessing GMO allergenicity with only one experiment [3,24,35,36] that may be the result of strict animal ethics rules which may prohibit the replication of experiments. However, biological repeats are necessary to ensure that results are not biased by undefined variables. The 'one experiment approach' may lead to unsubstantiated results that are unsuitable for risk assessment.

Experimental models used for risk assessment must be reproducible. Inter-experiment and inter-lab variations are expected, but experimental protocols should be designed in a way that the test results are related to controls, thereby allowing comparisons of results between laboratories. When results in an experimental animal model are contradictory, as they were for αAI GM peas [8,9], it is impossible to conclude whether the GMO is allergenic. Notably, these two labs used the same materials, protocol, and mouse strain and yet, the

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results were contradictory, emphasising the importance of repeated experiments in independent laboratories [8,9].

In addition, evaluating the history of safe use of introduced genes and proteins as well as consideration of any cooking or processing that would normally be used to prepare food from the GM source, should be evaluated for impacts on the specific GM protein levels, structure and immune reactivity. The αAI gene was transferred from common beans (*Phaseolus vulgaris*) into peas, chickpeas and cowpeas to inhibit damage caused by the bruchid seed storage beetles. The αAI pea is notable because (1) there are no reports of bean αAI allergy in humans thus, there is a history of safe use of αAI; (2) αAI is produced at high levels in the GMO (1–2% of protein) [37]; (3) there is differential post-translational modifications depending on the host plant [38], which may lead to new conformational allergenic epitopes leading to potential allergenicity; and (4) food processing is an important factor in food safety because we consume beans and other legumes including peas after cooking, which inactivates a number of protease inhibitors or lectins [39]. The αAI pea raises several important points for food safety evaluation: (1) when there is low GM protein expression, the probability of associated allergenicity is low (e.g., Cry 1Ab in Mon810 represents approximately 0.01% of the total crude protein in wholegrain maize [40]), (2) it is necessary to consider post-translational modifications of the proteins, (3) it is essential to evaluate such GM materials upon heat-treatment [8], and (4) it is necessary to measure protein levels in processed food and feed products.

While some animal models test whole GMO materials (including the food matrix), there are also experiments in which the isolated or recombinant GM proteins (purified, isolated or recombinant GM proteins (proteins derived from *Escherichia coli* or other GM microbes)) were tested [8,12,34,36,41,42]. When GM proteins are derived from different sources and processes, there might be a response in the animal that is unrelated to the GM protein. For instance, lipopolysaccharide (LPS) skews the immune response to proteins [31] and might be a contaminant along with targeted recombinant proteins such as Cry1, which might explain disparate results between studies [3,43]. Lectins and carbohydrate binding proteins are present in plants and antigen presenting cells have receptors that bind different classes of lectins. Some lectins stimulate antigen uptake, which has the potential to influence immune responses to unrelated proteins [33,44]. Careful characterisation of diet is essential as many factors can influence the immune response. Improper storage of the GM-food can also have drastic consequences if fungal growth occurs, resulting in significant levels of aflatoxin or other mycotoxins, potent toxins that may directly or indirectly affect immune responses, as well as the fungal structural carbohydrates such as chitin, an immune stimulating adjuvant [45].

With a better understanding of the factors that impact sensitisation in humans, known obstacles can be avoided when developing a predictive animal model. Some authors have attempted to evaluate potential adjuvanticity of specific GM proteins. For instance, Lee *et al.* found that neither αAI peas nor Bt maize had adjuvant effects in mice [8,42], whereas Prescott *et al.* found that consumption of peas together with ovalbumin (OVA) increased OVA responses [9]. In one report, the effect of cholera toxin as an adjuvant was confirmed in the positive control group, but Cry1Ab's adjuvant activity was not assessed at a dose that is relevant to expression in the plant [3,42]. Furthermore, there is little evidence that pure proteins or proteins in the context of commonly consumed food matrices act as adjuvants. It is important for researchers to characterise the proteins and GMO raw materials used in tests to prove identity and appropriate biochemical structure and function if the tests are to be useful. For example, as mentioned above, many plant proteins are modified post-translationally by proteolysis or covalent addition of lipids or carbohydrates (e.g., asparagine-linked glycosylation) [9,46].

While a predictive animal model of allergenicity would be of great value, it is worth considering the possibility that a perfect model may not come to fruition. Without a single validated animal model, scientists and regulators will need to carefully consider the positives and negatives of a given model and determine the relevance of the results based on careful analysis of the controls, immune markers, protein characterisation, and animals used on a case-by-case basis. Further developments such as *in vitro* and *ex vivo* models (discussed elsewhere in this section) that take into consideration high genetic variation in the human population and environmental factors, for example, microbial skewing, might also lead to improved risk assessment of GMOs and novel foods.

Conclusions

The major risks of food allergy are minimised by evaluating the source, amino acid sequence similarity to allergens and when indicated, testing for specific serum IgE. Nevertheless, further risk reduction by identifying the allergenic potential of novel foods including GMOs using *in vitro* and *in vivo* assays would be valuable. Experimental animal models are particularly useful for understanding the mechanisms underlying the allergic response to food. However, there are many potential limitations that hinder the development of standardised and validated animal models used for predicting GMO allergenicity. There is a pressing need to validate experimental models with whole food materials and known allergenic, as well as non-allergenic, food proteins in carefully controlled experiments using the best-suited species and strains and ensuring statistical power. For the successful use of animal models in allergenicity risk assessment, a consensus approach must be identified with sufficient predictive power to mimic human allergic risks.

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