Brief communication

Endogenous allergens and compositional analysis in the allergenicity assessment of genetically modified plants

A. Fernandez a,⇑, E.N.C. Mills b, M. Lovik c, A. Spoek d, A. Germini a, A. Mikalsen e, J.M. Wal f

a European Food Safety Authority, Parma, Italy
b Institute of Inflammation and Repair, Manchester Academic Health Sciences Centre, Manchester Institute of Biotechnology, The University of Manchester, Manchester, United Kingdom
c Norwegian Institute of Public Health, Oslo, Norway
d Inter-University Research Centre for Technology, Work and Culture, Alpen-Adria Universität Klagenfurt, Graz, Austria
e Norwegian Scientific Committee for Food Safety, Oslo, Norway
f INRA–CEA, Gif sur Yvette Cedex, France

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A B S T R A C T

Allergenicity assessment of genetically modified (GM) plants is one of the key pillars in the safety assessment process of these products. As part of this evaluation, one of the concerns is to assess that unintended effects (e.g. over-expression of endogenous allergens) relevant for the food safety have not occurred due to the genetic modification. Novel technologies are now available and could be used as complementary and/or alternative methods to those based on human sera for the assessment of endogenous allergenicity. In view of these developments and as a step forward in the allergenicity assessment of GM plants, it is recommended that known endogenous allergens are included in the compositional analysis as additional parameters to be measured.

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1. Introduction

According to EU legislation (EC, 2001, 2003, 2013), genetically modified organisms (GMOs) for food and feed use can only be authorised for placing on the market following a scientific assessment of any risks that they may pose to human and animal health, which includes an allergenicity assessment. Allergenicity is the property of a substance to either induce allergy or elicit an immune-mediated adverse reaction in a susceptible individual. Allergic reactions can be triggered by a variety of environmental agents including foods, almost all the substances involved in driving allergic responses to food and feed being proteins. Food allergy is an important public health problem, which is caused by various immune mechanisms one of which is mediated by IgE. IgE-mediated food allergies pose particular risks since only small amounts of a food can trigger a reaction, which can be life threatening. In the absence of a cure, individuals with food allergies have to practice life-long food avoidance and those at risk of severe reactions are provided with rescue medication in event of accidental consumption of the incriminated food. As a consequence of concerns to protect food allergic consumers and the need to prevent introduction of new allergenic foods into the supply chain, allergenicity risk assessment has been the focus of different international guidance documents for the assessment of GMOs and Novel Foods (Codex Alimentarius 2009; EC, 1997; EFSA, 2010a, 2011a).

According to the EFSA guidance (EFSA, 2011a), the allergenicity assessment of GM plants includes two elements, the assessment of the newly expressed proteins and the assessment of the whole GM plant. One of the aspects in the assessment of the allergenicity of the whole GM plant is to ensure that the genetic modification does not affect the levels or characteristics of endogenous compounds that would adversely impact on the human and animal health (EFSA, 2011a; König et al., 2004; Metcalfe et al., 1996; Thomas et al., 2008). As a part of the assessment, EFSA (2011a) and Codex Alimentarius (2009) foresee the assessment of endogenous allergenicity when the plant receiving the new gene(s) is known to be allergenic. Thus, any potential change in the overall allergenicity of the whole GM plant compared with that of its non-GM comparator(s) is foreseen to be analysed.

EFSA has previously recommended the inclusion of endogenous allergens in the comparative compositional analysis as additional parameters to be measured (EFSA, 2010a, 2011a). Key allergens in allergenic food have been defined as allergens which are relevant for public health because of their allergenic potency and
abundance (Bjorksten et al., 2008). In this context, further discussion on this topic took place in a workshop entitled “Key allergens and compositional analysis in the allergenicity assessment of genetically modified plants” which was organised back-to-back with the OECD Task Force for the Safety of Novel Foods and Feed (EFSA, 2012). The objective of the workshop was to initiate an in-depth discussion on the possible inclusion of endogenous allergens in the compositional analysis of GM plants. Scientific matters relevant to this topic are further discussed in this publication.

2. The potential inclusion of endogenous allergens in the compositional analysis for the allergenicity assessment of GM plants – Scientific basis to be considered

2.1. Usefulness of human sera in the allergenicity assessment

IgE-mediated food allergy in humans impacts on the health and quality of life of the affected individuals. Prevention of allergic sensitisation and avoidance of the relevant allergens by allergic individuals are in principle the measures that can be undertaken to reduce the risk of allergy development and triggering of allergic reactions. However, little is known about the conditions and mechanisms of sensitisation and how it can be prevented. A major concern for the allergenicity assessment of GM plants is to evaluate whether the genetic modification introduces new allergens into the GM plant, and to verify that an increased expression of endogenous allergens in the GM plant has not taken place.

Historically and in applications for the placing of GM plants submitted under Regulation No. 1829/2003 (EC, 2003) on the market, the assessment of endogenous allergenicity has been mainly carried out using immunoassays based on the use of sera from humans with the relevant allergies, e.g. IgE binding capacity measured by immunoblotting and/or enzyme allergosorbent tests (EAST). Although it is recognised that specific serum screening using serum from food allergic individuals is the current reference method for in vitro detection and definition of an allergenic protein, there are several limitations associated with the use of human sera. At present, novel analytical methods and molecular profiling techniques, not based on human sera, are becoming available and could be considered as complementary and/or alternative methods for the comparative assessment of the endogenous allergenicity between the GM plant and its non-GM comparator(s).

Allergenicity risk assessment can involve the use of human sera for allergen identification and quantification although the use of such precious biological samples imposes limitations. An essential element in the triggering of an allergic reaction is the binding of the allergen to mast cell-bound IgE antibodies. It is well recognised that the presence of food specific IgE is not always linked with clinical disease. Some allergic individuals can have relatively low levels of circulating IgE antibodies, but still experience severe clinical reactions (Lieberman and Sicherer, 2011). Others may have high levels of specific IgE antibodies but no clinical reactions are triggered, or the clinical reactions may be mild. One of the reasons for these discrepancies is the fact that serum IgE does not always well reflect cell-bound IgE (Liang and Ganley-Leal, 2009; Dehlink et al., 2010). Thus, in any work utilising human sera it is essential that the sera come from well characterised allergic individuals who have preferably undergone oral food challenge to confirm their food allergy. IgE binding is usually highly specific and directed to particular proteins within an allergenic food or pollen. A single allergenic food may contain many different IgE-binding allergic proteins, that may differ in importance (Fonseca et al., 2012; L’Hocine and Boye 2007; Miller et al., 2012; Movérale et al., 2011), and the profile of IgE-binding proteins may be allergic individuals specific. The parts of the protein molecules to which IgE antibodies bind are known as epitopes and can comprise a combination of conformational epitopes, where IgE binding is determined by the three-dimensional conformation of the allergen, and linear epitopes comprising short contiguous stretches (e.g. 8–12 amino acids) of the polypeptide chain. The IgE antibody response of an allergic individual towards any given allergen will recognise multiple epitopes, and whilst certain epitopes may be immunodominant the pattern of recognised epitopes may also vary from an allergic individual to another. As a consequence, human allergic sera are very difficult to standardise – serum IgE from two individuals will, strictly, never be identical, even though they bind the same food allergens. An example of this variability in binding capacity between allergic individuals can be noticed in Fig. 1 (reprinted from Fonseca et al., 2012). It is also important to mention that in relation to antibody specificity, the use of sera does not allow usually isoform specific discrimination of allergens (Poms et al., 2004; L’Hocine and Boye, 2007). Therefore, before a serum can be reliably used for these purposes, it must be very well characterised and the clinical history and features of the donor allergic individual must be well known. This is demanding of resources and often difficult to achieve.

A further complication is that only small volumes of serum can usually be obtained from the same allergic individual, and because serum characteristics may change over time, sera from blood drawn from the same allergic individual at different times cannot be assumed to be identical. In addition, suitable allergic individuals with the relevant allergy may be difficult to find in sufficient numbers. Finally, there are ethical/legal constraints regarding the collection, storage and use of human material, and in particular regarding sera from children who constitute a considerable part of the food allergic population.

Thus, each human allergic serum sample is more or less unique and available only in very limited amounts. To achieve better standardisation of investigations into the allergen content of foods and to ensure reproducibility of studies across different laboratories, there would be a need for sera that can be well standardised and that are available in more or less unlimited quantities. This might only be possible with animal sera or monoclonal antibodies. Human sera will continue to be important for the detection of possible new allergens and the confirmation that a protein is an allergen but there is a need to find alternative approaches to profile allergens in foods, especially for those cases where the plant allergen(s) of clinical relevance are well-known and – characterised (i.e. have been identified and reported in allergen databases). Providing that human sera will still be available in sufficient quantity and quality, they should be spared and used for the detection and identification of new allergens rather than for routine risk assessment purposes.

2.2. Methods available for allergen identification and quantification without the use of human sera

An alternative to using human allergic serum for assessing the profile of known allergens in GM plants would be to rely on the structure and physico-chemical characteristics of the allergenic proteins. Profiling techniques and analytical methods for protein identification and quantification have evolved in the past few years and have become additional tools for the characterisation of the protein profile of plants, including allergenic proteins. Advances in mass spectrometry (MS) techniques in combination with separation steps can allow nowadays for an accurate and reliable identification and quantification of known allergens. In fact the combination of ionization techniques, such as electrospray ionization (ESI), nanospray ionization (NSI) and matrix assisted laser desorption ionization (MALDI), and mass analyzers, such as quadrupole (Q), ion-trap (IT) and time-of-flight (TOF), has dramatically
increased the possibility of analysing biomolecules such as allergens. In particular Single Reaction Monitoring (SRM) and Multiple Reaction Monitoring (MRM) MS approaches have emerged as reliable tools for the absolute quantification of proteins in complex samples (Picariello et al., 2011). The recent developments of these techniques have highlighted their potential to become valuable tools to also support the risk assessment of allergenic proteins (Sancho and Mills, 2010).

MS-based methodologies may represent a reliable alternative for the identification and quantification of known allergens in the comparative assessment of the endogenous allergenicity. In this regard, examples of these applications have recently appeared in the literature for the two most cultivated GM plants worldwide: soybean and maize.

Natarajan et al. (2009) used two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry, and liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) to separate, identify and quantify different classes of soybean seed proteins in different genotypes of wild Glicyne soja and cultivated Glicyne max. The authors concluded that the overall distribution pattern of allergen proteins identified in the study were similar in both wild and cultivated genotypes; however, major variation was observed in terms of the number of spots and their intensities between genotypes.

In a comparative analysis of GM and non-GM soybeans, Rouquié et al. (2010) used 2D-PAGE in combination with MALDI-TOF to identify soybean seed allergens. The authors were able to qualitatively identify 37 IgE reactive protein spots and, by performing a quantitative comparison, concluded that the values observed in the GM soybean fell within the range of biological variation observed in conventional soybean varieties.

In a paper addressing the natural variability of soybean allergens, Houston et al. (2011) quantified 10 soybean allergens from 20 non-GM commercial soybean varieties using mass spectrometry approaches. Relative quantification was performed by spectral counting on trypsin-digested protein samples analysed with LC–MS/MS. Absolute quantification of allergen concentrations in seeds was carried out using an LC-triple quadrupole MS system using MRM analysis with synthetic, isotope-labelled peptides as internal standards. The authors concluded that their method could effectively profile the natural variation of soybean allergens and that in the future, quantification of each allergen using MS may provide accurate information for soy product labels.

Julka et al. (2012) developed a two-dimensional liquid chromatography assay with ultraviolet and mass spectrometric detection (2DLC–UV/MS) for the quantification of the soybean allergen Gly m 4 in seeds from 10 commercial non-GM soybean varieties. The assay allowed for the separation and detection of Gly m 4 and isoforms, thereby enabling successful quantification. The authors concluded that this approach could be applied to other soybean allergens.

In a study addressing the effect of the environment on the allergen profile of soybean, Stevenson et al. (2012) used triple quadrupole MS to analyse four commercial varieties grown in six different locations. The authors used MRM to perform the quantification of eight known allergens using synthetic heavy-labelled internal standard.

Kuppannan et al. (2011) developed a LC–UV/MS method for the quantification of the maize allergen lipid transfer protein (LTP) in seeds from 14 commercial non-GM maize varieties. The method allowed for the differentiation between LTP and its variants, enabling for their quantification.

In an additional study on maize allergens, Fonseca et al. (2012) used 2D-PAGE in combination with MALDI-TOF/TOF to perform a comparative analysis of GM and non-GM maize varieties.

The examples provided above demonstrate how these novel methods could represent valuable tools to be used as complementary and/or alternative methods to human serum-based methods for the identification and quantification of known allergens. Regardless of the method of choice for the identification and quantification of known endogenous allergens in the allergenicity assessment, there are challenges to any comparative measurement of endogenous allergens which require more detailed considerations.

2.3. Challenges to the allergenicity assessment of endogenous allergens

2.3.1. Natural variability and the relevance of the lack of baseline data from databases

It is recognised that when the endogenous allergenicity of a GM plant is compared with that of its non-GM comparator(s), natural variability must be taken into account. As for any other plant component assessed in the comparative compositional analysis, the levels of food allergens can differ across commercial lines due to genetic background differences and can also be influenced by environmental conditions, agronomic practices, etc. Therefore, the data obtained from the comparative assessment must be interpreted taking into account the natural variation of food allergen levels. The natural variability of allergens in plants has been recently investigated using novel technologies not based on human sera (e.g. Natarajan et al., 2009; Natarajan, 2010; Houston et al., 2011; Stevenson et al., 2010, 2012; etc.). Data on allergen levels in crops are therefore becoming available.

Nevertheless, another approach has been recommended by EFSA that has developed a new field trial design for the comparative compositional analysis which does not rely on baseline data from databases for the estimation of the natural variability of the endpoints of interest (EFSA, 2010b, 2011a). The purpose of this design is to allow the direct comparison of the GM plant, its non-GM
comparator and a number of non-GM reference varieties with a history of use. These reference varieties are included in the same field trials where the GM and its non-GM comparator are grown and are used to provide an estimation of the ranges of natural variation (baseline data) for each endpoint, under the same environmental conditions. The analysis of the data is then performed using a combination of two statistical tests, i.e. difference tests with the conventional counterpart and equivalence tests with the reference varieties (EFSA, 2010b, 2011a). This EFSA’s experimental set up has become mandatory for the assessment of GM plants for applications submitted under the new EFSA Guidance Document (EFSA, 2011a; EC, 2013).

Integration of such trial designs with new profiling methods could also be used to analyse endogenous allergen levels in plants without the need for baseline data from databases for the estimation of the natural variability.

2.3.2. Thresholds values for sensitisation and elicitation

Food allergy consists of two separate phases: (i) sensitisation, a phase where the immune system develops hyperreactivity to the allergen but no symptoms occur; and (ii) elicitation, a phase where clinical manifestations are triggered.

There is a general consensus that thresholds probably apply to both sensitisation and elicitation phases (Crevel et al., 2008), although data are lacking regarding any potential threshold for the sensitisation phase for food allergens. The balance between sensitisation and tolerance development is likely to be influenced not only by the amount of allergen, but also by time distribution, age of the individual, genetic and environmental factors, other food components and microbiota (Berin and Sampson, 2013). In relation to this, an important aspect to be considered is that the extent to which sensitisation to a protein occurs is also influenced by its abundance in the food product and by the amount of protein that will reach the immune system in an immunogenic form. It is generally believed that an increased exposure to an allergen in a given population will raise the number of people reacting to that allergen in that specific population, although it has been reported that it might not be the case in all circumstances (Du Toit et al., 2008). An example of this general assumption is that the prevalence of allergy to a particular food varies with the country/geographic conditions and it is directly linked with dietary habits.

For the elicitation phase, there is experimental evidence from studies with quantitative dose-distribution data from double-blind placebo-control food challenge (DBPCCF) indicating that for a given allergic individual at a given time, threshold values for elicitation exist (Ballmer-Weber et al., 2007; Crevel et al., 2008; Hourihane and Knulst, 2005; Madsen et al., 2009, 2010, 2011; Taylor et al., 2009a,b, 2010; Threshold Working Group 2008; et al.). There are different reasons why threshold values for food allergens have not been defined yet for regulatory purposes. Although thresholds for individuals can be established at a specific time point, they cannot be estimated in the same way for a given population. The sensitivity to an allergenic food in individuals can differ by orders of magnitude, e.g. from 0.5 up to 10,000 mg of peanut (Taylor et al., 2009b). Some allergic individuals react so strongly that they are not tested by DBPCCF, and some of these individuals are sensitive to so minute levels of allergen that it may in reality be impossible or without practical value to set a threshold to protect everybody. Furthermore, thresholds for individuals may vary over the time due to stress, exercise, medications, etc. Probabilistic modelling has been proposed as the most promising approach for population risk assessment (Bindslev-Jensen et al., 2002; Crevel et al., 2007; Kruizinga et al., 2008; Madsen et al., 2009, 2011; Spanjersberg et al., 2007). Taylor et al. (2009b, 2010) showed that data on peanut are available for this model to be used. Using this model, the management or regulatory threshold value is related to a percentage of the allergic population which is unlikely to react to a given dose (e.g. 90% or 95%). This approach is associated with a cut off value and the definition of an “acceptable” risk which is under the remit of risk managers and policy makers.

Even in the case that threshold values were readily available for regulatory purposes, their usefulness for the allergenicity assessment of GM plants would need to be discussed. The allergenicity assessment of GM plants is not meant to address the adventitious presence of an allergen in a given food but rather to understand whether a GM plant might be more allergenic than its non-GM comparator(s) to such an extent to be of concern for human and animal health. Therefore, although threshold values could provide crucial information for labelling purposes, their applicability to the allergenicity assessment of GM plants might need further considerations.

2.3.3. Avoidance of the offending food

Avoidance of the offending food is an obvious strategy for allergic individuals to prevent an allergic reaction. In reality, in everyday life strict avoidance of specific foods is difficult to achieve (Crevel et al., 2008) and not effective enough (Madsen et al., 2010). Allergic individuals might be intentionally or accidentally exposed to the offending food due to its presence as an ingredient deliberately introduced in food products and/or through cross-contamination of foods with an allergenic source (Madsen et al., 2009). Since the manufacturing processes are complex (Crevel, 2005), the adventitious presence of small amounts of allergens is a reality in many food products. In fact, most of the severe reactions, including anaphylaxis, are due to accidental ingestion of the allergenic food (Decker et al., 2008; Lieberman et al., 2010). For these reasons, precautionary labelling of allergens in food was proposed. However, a significant proportion of allergic consumers ignore such warnings (Hefle et al., 2007). In addition, allergic individuals with a moderate allergy usually know from experience the amount of offending food that they can tolerate and that they need to avoid potential exposure to only certain amounts of the allergen (Hourihane and Knulst, 2005). They self-regulate the intake of offending food that they can tolerate since they know the composition and recipe of the food they consume. Therefore, a substantial increase in allergen levels in a GM plant or in food products derived from this plant above those levels found in similar products derived from conventional plants (tolerated by an allergic individual) might have unpredictable consequences (e.g. elicitation of severe allergic reactions). Different views on this topic have been previously presented (Goodman et al., 2008; Panda et al., 2013).

Avoidance of the offending food by allergic individuals can therefore not be assumed in all cases. In order to avoid unexpected high exposures to the allergen, it is important to ascertain that the allergenic food derived from a GM plant does not contain relevant higher levels of allergens than food derived from conventional plants.

2.3.4. Interpretation of data obtained from the inclusion of allergens in the compositional analysis

According to EFSA guidance (2011a), the risk assessment strategy for GM plants is to compare a GM plant with their appropriate comparator(s). The reasoning behind this concept is that traditionally cultivated plants have a history of use in humans and animals, and therefore can serve as comparators in the food and feed safety assessment. As previously mentioned, for the comparative compositional analysis, EFSA (2011a) and Codex Alimentarius (2009) acknowledge the need to consider the range of natural variation of the endpoints analysed in the assessment.

Based on the information gathered from the inclusion of allergens in the compositional analysis, the starting point in the assessment would be the identification of statistically significant
differences between the GM and its non-GM comparator. A further evaluation should investigate whether or not the differences observed fall within or outside the range of natural variation (i.e. equivalent or not to the non-GM reference varieties). In the hypothetical case that the amount of a specific allergen of a GM plant might be statistically significantly different to its non-GM comparator(s) and/or falls outside the range of natural variation, the biological relevance in relation to its impact on human and animal health of allergic individuals needs to be determined. Biological relevance and statistical significance are not necessarily linked since a statistically significant effect may exist but still be biologically irrelevant (EFSA, 2011b). In general terms, EFSA (2011b) has highlighted the importance of defining a priori the size of an effect that would be considered biologically relevant in order to design a proper experiment leading to reliable results upon which the decision making process can be based. However, it was also recognised that the identification of a biologically relevant size of an effect that can be predefined in every situation is not always easy or even possible.

For allergens as for other compounds already included in the compositional analysis, the size of an effect that would be considered biologically relevant cannot be defined a priori. In order to further characterise those changes with a potential to be of biological relevance, additional considerations and/or experimental data, other than those obtained from the inclusion of allergens in the comparative analysis, might be needed on a case-by-case basis. As for other compounds included in the compositional analysis, the nature of these additional considerations and/or experimental data needed in the assessment might depend on the number and magnitude of the changes identified as well as on the clinical/safety relevance of the specific allergen(s)/compound involved.

Changes found to be of biological relevance need to be communicated to the risk managers for a broader consideration in the decision making process, such as for the establishment of acceptable risk levels and the need for management measures.

3. Conclusions/discussion

According to international guidelines (EFSA, 2010a, 2011a; Codex Alimentarius, 2009), endogenous allergenicity is an important component in the risk assessment of allergenic GM plants since unintended effects may lead to over-expression of endogenous allergens. For this purpose, the overall allergen content of the GM plant is tested against that of its non-GM comparator(s). Historically, it has been performed using techniques based on human sera. Currently, novel analytical approaches not depending on human sera are now available for the identification and quantification of known allergens in plants. Studies have been published providing information not only on the variability of allergen levels in plants but also on the applicability, specificity, sensitivity and accuracy of these novel techniques (e.g. Houston et al., 2011; Julka et al., 2012; Kuppannan et al., 2011; Natarajan et al., 2009, Natarajan, 2010; Rouqué et al., 2010; Stevenson et al., 2010, 2012).

In order to achieve better standardisation and avoid the use of human sera on a routine basis for the allergenicity assessment of GM plants, complementary and/or alternative methods based on recent technological advances that do not require human sera need to be considered. Furthermore, the inclusion of endogenous allergens in the comparative compositional analysis, as additional parameters to be measured by these novel technologies, would provide robust, quantifiable, reproducible and reliable information for the risk assessment of endogenous allergenicity of GM plants. Scientific arguments of different nature have been described in this document to support this proposal.

4. Disclaimer

The authors Fernandez A. and Germini A. are employed by the European Food Safety Authority (EFSA). The positions and opinions presented in this article are those of the authors alone and do not necessarily represent the views or scientific works of EFSA.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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