Assessment of endogenous allergenicity of genetically modified plants exemplified by soybean – where do we stand?

Selb R[a], Wal JM[b], Moreno FJ[c], Lovik M[d], Mills C[e], Hoffmann-Sommergruber K[f] and Fernandez Dumont A[a]*

[a]European Food Safety Authority, Parma, Italy
[b]INRA–CEA, Gif sur Yvette Cedex, France
[c]Institute of Food Science Research, CIAL (CSIC-UAM), Madrid, Spain
[d]Norwegian Institute of Public Health, Oslo, Norway
[e]Institute of Inflammation and Repair, Manchester Academic Health Sciences Centre, Manchester Institute of Biotechnology, The University of Manchester, Manchester, United Kingdom
[f]Department of Pathophysiology and Allergy Research, Medical University of Vienna, Vienna, Austria

*Address correspondence:
Dr. Antonio Fernandez Dumont
European Food Safety Authority
Via Carlo Magno 1A
43126 Parma, Italy
antonio.fenandezdumont@efsa.europa.eu

Abstract:

According to EU regulation, genetically modified (GM) plants considered to be allergenic have to be assessed concerning their endogenous allergens before they can be placed on the European market, which is also in line with the international standards described in Codex Alimentarius. A quantitative relevant increase in allergens which might occur in GM plants as an unintended effect compared to conventionally consumed crops can pose a risk to consumers and has therefore to be monitored. Currently, data showing a connection between allergen dosage and allergic sensitisation are scarce since, generally, the pathophysiological mechanisms of sensitisation are only insufficiently understood. In contrast, data on dose-distribution relationships acquired by oral food challenge are available, showing a connection of allergenic protein consumed and the elicitation of allergic reactions. Soybean as the currently only recognised allergenic GM food by law for which EFSA has received applications for placement on the market was taken as an example for defining an assessment strategy. This approach for endogenous allergenicity assessment in soybean could, in the next step, be expanded to other allergenic foods in the future, whenever required. Identification of potential soybean allergens, the methodology to be used as well as risk assessment considerations

are discussed in this manuscript. A strategy is proposed for the identification, assessment and evaluation of potential hazards concerning endogenous allergenicity in food derived from plants developed by biotechnology.

Research Highlights:

- The history and the current state-of-the-art in endogenous allergenicity risk assessment of soybean plants developed by biotechnology is reviewed
- The current challenges of the endogenous allergenicity assessment are discussed
- A strategy is proposed for the identification and evaluation of potential risks concerning endogenous allergenicity in food derived from plants developed by biotechnology

Keywords: endogenous allergenicity, genetically modified plants, GMO, soybean, food allergy

Abbreviations:

LOAEL	lowest observed adverse effect level
DBPCFC	double blind placebo controlled food challenge
GM	genetically modified
LC	Liquid chromatography
MS	Mass spectrometry
MRM	Multiple reaction monitoring

Introduction:

Allergen dosage is a critical issue in the course of the allergic disease, and possible overexpression of allergenic proteins in plants used for food might pose a risk to allergic consumers' health. It is therefore important to monitor food derived from modern biotechnology regarding allergenicity and, if the food source is known to be allergenic, to evaluate the expression levels of endogenous allergens.

The allergic disease is a two-step process made up by sensitisation to the allergenic food and subsequent elicitation of the allergic reaction resulting in allergic symptoms when the allergen is administered in sufficient amounts (Taylor et al., 2009). The dose necessary for allergic sensitisation is only insufficiently studied and it is challenging to determine a threshold for sensitisation (Taylor et al., 2009). Also, it has been shown that encountering potential allergenic foods under specific circumstances at an early age might actually even lower the risk of food allergy (Du Toit et al., 2015). In contrast, the dose-distribution relationship for elicitation of an allergic reaction can be determined by oral food challenge of allergic individuals with increasing doses of the allergenic food and the minimum eliciting doses were determined for many allergenic food sources (Ballmer-Weber et al., 2015; Zhu et al., 2015). For soybean, the minimum amount of protein causing objective symptoms in allergic individuals was described to be 5.3 mg of soybean protein in one study (Ballmer-Weber et al., 2007), but others determined the lowest the dose resulting in observed adverse effect level (LOAEL) to be as low as 0.2 mg of total protein used in food challenge (Blom et al., 2013). However, depending on the individual allergenic protein contained within consumed food and especially depending on the single allergic individual and experimental procedures, this amount eliciting objective symptoms can vary on a large scale, which is undermined by a sometimes poor reproducibility of double blind placebo controlled food challenge (DBPCFC) (Glaumann et al., 2013).

Allergic subjects are generally advised to altogether avoid food containing the respective allergenic ingredients. However, unintentional ingestions of allergenic food happen on a regular basis and are mostly the reason for adverse reactions in allergic individuals who are aware of their condition. Several studies describing the prevalence of unintended exposure to food allergens have been reviewed (Boyce et al., 2010). On the other hand however, also intentional ingestions are not uncommon. Especially young adults are willing to take risks, as 54% of 13-21 year old food allergic allergic individuals admitted to eat small amounts of the food they are allergic to (Sampson, 2006). Other studies clearly indicate that around 20-30% of allergic subjects or their parents ignore their doctors dietary advice and food allergy warnings on products (Allen et al., 2014; Ben-Shoshan et al., 2012; Imamura et al., 2008; Noimark et al., 2009) and it was shown that the number of allergic consumers ignoring allergy advisory statements was increasing between 2003 and 2006 (Hefle et al.,

2007) Another reason why allergic individuals might consciously consume allergenic food is their experience that certain amounts of similar foods did not cause any allergic reactions previously (Sampson et al., 2006). It can therefore not be assumed that allergic subjects avoid the food they are allergic to at all times, and, while this can happen unintentionally, they might also consume the allergenic food consciously.

From a regulatory point of view, the assessment of endogenous allergenicity is a prerequisite before any genetically modified (GM) plant known to be allergenic can be placed on the European market (IR503/2013 EC, 2013). These guideline instructions are also in line with international standards described in Codex Alimentarius (Codex, 2009). Here, the major strategy of risk assessors is to determine whether GM food is as safe as the conventionally consumed non-GM comparator. To this end, any GM plant regarded to be allergenic has to be evaluated for potential changes in allergen composition by comparing the food source derived from modern biotechnology with appropriate non-GM comparators.

Currently, provision of information to the consumers concerning food containing allergens is mandatory under EU regulation No 1169/2011 (EC, 2011). However, it can be anticipated that a reference level will be in place in the future, whereby the content of an allergic food source in the overall product will be decisive if precautionary labelling is necessary. In products where the overall amount of an allergic food source is too low and which will therefore not be labelled, the endogenous allergen content gains crucial importance. A single allergen enhanced in the original crop would then mean that even though the food product would still contain the same (low) amount of protein from the allergen source, it might contain a multiple of the single allergenic protein. This might pose a risk to particular allergic consumers who would be unaware of the content of the food product.

Because of these facts and also because the impact of allergen dosage on sensitisation to potentially allergenic proteins is only insufficiently understood, it is crucially important to know whether or not the levels of endogenous allergens of a GM crop, in relation to appropriate comparators, have been modified to a level that may cause concerns. To this end, a thorough investigation of endogenous allergenicity of GM food and comparison with appropriate comparators is essential in order to inform risk managers of (absence of) increased risks.

Out of foods recognised to be allergenic and therefore pose a risk to a given population (EC 2003, 2011), EFSA so far only received applications for genetically modified (GM) soybean to be placed on the European market where an endogenous allergenicity assessment was performed (EFSA GMO

Panel 2007, 2011, 2015a). Therefore, this article will concentrate on endogenous allergenicity assessment of soybean.

Soybean, a crop of the *Fabaceae* family, is recognised as an allergenic food source by European Regulation (EC 2003, 2011). Soybean products are traditionally consumed in Asia and in the US, however, the use of soybean has increased in Europe during the last years. Several studies reported varying prevalence data of allergy to soybean in Europe, while the highest prevalence of self-reported soybean allergy was 8% in a birth cohort of 4 year old Swedish children (Ostblom et al., 2008). However, prevalence assessments applying measurements of objective clinical symptoms by DBPCFC are rare. Studies on the worldwide prevalence of soybean allergy have been summarized in a systematic review, concluding that 0.27% of the general population and 2.7% of atopic children are soybean allergic if assessed by oral food challenge. However, sensitisation of atopic children is significantly higher, as 12.9% of these children reacted to soybean in skin prick testing and 27.1% had specific IgE antibodies to soybean (Katz et al., 2014). Soybean allergy is more common in children than in adults and by the age of 4 years already 25% of allergic children outgrow their allergy (Savage et al., 2010). While sensitisation to soybean occurs mostly in young children, birch-pollen or peanut cross-reactivity can lead to an onset of soybean allergy in older children, peaking at the age of 8 years (Savage et al., 2010). Even though reactions to soybean are usually mild, also severe reactions including anaphylactic shock after the ingestion of soybean products have been reported (reviewed in EFSA 2014).

Soybean is currently the most relevant GM food commodity concerning endogenous allergenicity risk assessment for EFSA. However, considering the rapid developments in the research field of GM plants, it is, on the one hand, likely that in the near future applications dealing with other potential allergenic GM foods (e.g. apples, kiwis, nuts) might be received for authorisation. Interestingly, several examples are available where genetic modifications of plants were actually applied to reduce or silence the expression of endogenous allergens (Chu et al., 2008; Dodo et al., 2008; Dubois et al., 2015; Gilissen et al., 2005; Knoll et al., 2011) and these new varieties were consequently also able to cause less allergic symptoms upon challenge of allergic individuals. This might become an interesting task for risk assessors dealing with future applications. On the other hand, it also cannot be excluded that other crops apart from soybean used for food might be considered to have allergenic potential of public health importance in the future, and therefore pose a risk to consumers. Endogenous allergenicity and its assessment is currently discussed intensively and the topic has been the subject of several other recent publications (Graf et al. 2014, Fernandez et al. 2013, Ladics et al. 2014, Panda et al. 2013).

Background: History of assessment of endogenous allergenicity in soybean

Presently, the Implementing Regulation EU No. 503/2013 (EC, 2013) asks for the assessment of relevant allergens as part of the comparative analysis and provides a reference to the OECD consensus documents on compositional considerations for new plant varieties. The OECD consensus document on compositional considerations for new varieties of soybean contains a table listing potential soybean allergens (Table 20 in OECD, 2012), which was adapted from a review publication (L'Hocine and Boye, 2007) and complemented with data from the WHO/IUIS database. However, the significance and relevance of some single potential allergen molecules listed in the table was questioned previously (Ladics et al., 2014). While this was on the one hand assigned to the fact that sequences are not available for all potential allergens, also the published scientific evidence of clinical relevance was questioned for others. Currently, an EFSA guidance document is developed which provides assistance for applicants and risk assessors regarding these issues (EFSA, public consultation document on allergenicity guidelines 2016).

Historically, applications received by EFSA for the placing of a GM soybean on the market included the assessment of endogenous allergenicity. The first EFSA opinion containing such an assessment was published in 2007 (EFSA, 2007). Examples of different methodologies used in applications can be found in previous EFSA opinions, and constant further development of these methodologies is taking place. Previously, the standard methodologies applicants used for endogenous allergenicity assessment were based on human serum IgE (EFSA, 2003, 2013). As a further development, 2-dimensional gel electrophoresis (2D-GE) in combination with spot quantification was employed in other applications (EFSA, 2015abc) and, lately, also absolute quantification methods were used by industry and these applications are currently under assessment. The different methodologies are discussed in detail below. Up to this date, the EFSA GMO Panel did not find evidence that the genetic modification might significantly change the overall allergenicity of GM soybeans when compared with that of its comparator(s) in the context of the applications assessed.

In the first studies, semi-quantitative analyses using IgE containing sera of allergic individuals were used to assess the allergen content of a plant, and most applications received for placing a GM soybean on the market under Regulation No. 1829/2003 (EC, 2003) used this methodology.

On the one hand, IgE- immunoblotting methods are useful to determine the presence of allergy-causing antibodies against a certain allergen source in human serum, and it is also a valuable tool to assess whether a certain allergen in food is still a potential hazard after processing (Verhoeckx et al., 2015). On the other hand however, the use of serum IgE in measuring the quantitative allergen content in plant materials has several disadvantages. Usually, the antibody repertoire of a single allergic subject is highly unique. The quantity and quality of IgE antibodies may vary greatly between individuals and most do not react to all known allergens of one source. IgE antibodies against certain

single allergen molecules might therefore be quantitatively underrepresented compared to others in human sera or might occur in only few subjects altogether. To this end, some allergen molecules might be overseen by immunoblotting. To solve these problems, well characterized sera of several allergic individuals would be necessary; assuring that IgE antibodies against all known allergens in a certain allergen source are present and recognise these allergen proteins sufficiently. However, serum samples from allergic subjects are precious and not easy to come by and this is especially true for food allergies with a relatively low prevalence like allergy to soybean. Due to the restrictions of the human serum IgE-based techniques, standardisation is not feasible and the possibility to compare data from different assessments is limited. The potential allergenic proteins which are quantified in the endogenous allergenicity assessment have usually been described by IgE binding assays previously and they are measured in their native form (i.e. in the raw plant material or protein extracts thereof). The recognition of these potential allergens by human IgE can therefore be considered a pre-requisite. Within the comparative approach, more robust and reliable methods with a better readout are available, which can be standardised more easily and do not depend on limiting human material.

Because of the described limitations of quantifying endogenous allergens on the basis of human serum IgE binding, the 2D-GE methodology was developed and used as an alternative. 2D-GE and protein spot quantification by densitometry has been used previously to quantitatively assess five potential soybean allergens in a GM soybean variety and non-GM comparators (Rouquie et al., 2010). In order to identify and quantify soybean proteins, including potential allergens, another group used an 2D-GE and mass spectrometry based approach, also using spot intensity quantification (Natarajan et al., 2009). However, the focus of this publication lies on identification of proteins rather than their quantification. While these spot quantification methods might be an improvement compared to those using human serum IgE, analyses not done simultaneously still cannot be compared. Therefore, the methodology for the endogenous allergenicity assessment has recently moved towards absolute quantification by mass spectrometry, as discussed previously (Fernandez et al., 2013), and quantitative ELISA based on IgG antibodies raised in animals. These appropriate methods for the quantitative assessment of endogenous allergens will be described and discussed in more detail below.

Identification of potential soybean allergens for assessment

The table provided in the OECD consensus document on soybean compositional considerations (Table 20 in OECD, 2012), as well as the supporting review publication by L'Hocine and Boye (L'Hocine and Boye, 2007) can be used as the basis for the research on potentially relevant soybean allergens. The search can be further complemented using the databases listed in the EFSA scientific

opinion on allergenicity (EFSA, 2010), especially the IUIS/WHO database (www.allergen.org), the FAARP database of the University of Nebraska-Lincoln (www.allergenonline.org) and Allergome (www.allergome.org). Table 1 lists the information collected for all potential soybean allergens after applying this search (Table 1). A more detailed version of the outcome can be found in the EFSA public consultation document on the guidance on allergenicity assessment of GM plants (Table C1, EFSA public consultation document 2016). Data on single potential soybean allergens as well as on the allergic individuals reported were retrieved, as well as data on possible clinical reactions to the single, purified or recombinantly produced allergen molecules. However, it should be highlighted that one major drawback of the current clinical practice is the fact that data on clinical reactivity is obtained by DBPCFC to (the composite) soybean extract or soybean formulas only (Ballmer-Weber et al., 2007; Blom et al., 2013). While this is the gold standard for assessment and diagnosis of food allergy (Hoffmann-Sommergruber et al., 2015), it does not provide data on reactions to single soybean proteins unless the individual tested is mono-sensitised. Currently, data on allergic individuals reacting to single allergens is mainly restricted to component resolved diagnostics (Tuano and Davis, 2015), which allows an analysis of the correlation between levels of serum IgE to single allergens with clinical reactivity to the composite food or food extract. However, DBPCFC with single soybean allergen molecules are not yet performed. Reactivity with the actual proteins is therefore mainly expressed by sensitisation data using IgE binding assays, or, in few cases, basophil histamine release experiments (Gly m 4, Gly m 5, Kleine-Tebbe et al., 2002, Zheng et al., 2012) and in one case possible clinical reactivity after inhalation (KTI, Quirce et al., 2002). In one study, IgE reactivity of allergic individual's sera with the single soybean allergens Gly m 5 and Gly m 6 was found to be related to clinical symptoms of soybean allergy and this has therefore been connected to clinical reactivity (Holzhauser et al., 2009). Moreover, IgE antibodies to Gly m 8 were shown to have a predictive value for soybean allergy in another study (Klemans et al., 2013). However, it is well known that the presence of IgE antibodies specific for a certain molecule or food does not necessarily imply clinical reactivity to the allergen source (Sicherer and Wood, 2012). Here, apart from quantity (Sampson, 2001), also quality of IgE antibodies and the number and proximity of IgE epitopes on the allergen molecule might play a role (Christensen et al., 2008; Gieras et al., 2007; Gieras et al., 2015; Mari et al., 2008), but also other factors like blocking IgG or IgA antibodies might have to be taken into account (van Neerven et al., 2006, Wright and Kulis et al 2016). Taken together, reactions in DBPCFC to soybean extracts in combination with IgE reactivity profiles might be strongly indicative, but they cannot unequivocally imply clinical reactivity to single allergen molecules.

Given the aforementioned mentioned challenges, the definitions of renowned, international institutions like WHO can be used as a tool to define allergens. IUIS/WHO describes the IgE-reactivity of at least 5 patient's sera with a specific molecule as a primary criterion for this purpose. In the next

step, an expert peer-review process is conducted before a protein is included among allergens in the database. In table 1, the IUIS/WHO primary criteria were applied to the list of potential allergens as a starting point. Considerations for including molecules into current endogenous allergenicity assessments are described on the basis of the further strategy applied by IUIS/WHO, suggesting a peer-review process, as well as on possible methodological limitations (Table 1). A more detailed description of this strategy can be found in the EFSA guidance document on allergenicity assessment (EFSA public consultation document, 2016). It should be noted that, due to the constant progress in science, these considerations might be outdated in the future, making adaptions necessary.

Methodology for the assessment of endogenous allergens

Well established and suitable methods to assess endogenous allergens are mass spectrometry and quantitative ELISA. In ELISA, specific IgG antibodies well characterized for their specificity and affinity should be used, which were raised in animals after immunisation with purified allergens. Examples for endogenous allergenicity assessment of soybean from the current literature using these methods are elaborated below.

Helpful mass spectrometry based protocols to quantify endogenous allergens have been established recently. Houston et al. (2011) and Stevenson et al. (2012) developed similar analytical methods based on the LC-MS/MS analysis of trypsin-digested allergens to characterize their natural variation in different non-GM soybean varieties. The quantification approach relied on the use of “signature” peptides as surrogates for the endogenous allergens using synthetic stable isotope-labelled peptide spiked into the sample of interest. These peptides are selected based mainly on their ionization properties and uniqueness, and, then, analysed by the most sensitive triple-quadrupole MS-based data acquisition mode (that is, targeted multiple reaction monitoring (MRM)), to be further validated by MS^2 spectrum acquisition.

Houston et al. (2011) studied the natural variability of five soybean allergens and their subunits in 20 commercial soybean varieties. The authors concluded that some of the assessed proteins were highly variable among the tested soybean crops. These variations were mainly found in two of the five studied Gly m 6 subunits (i.e. G3 and G4) and in the Gly m 5 α-subunit. In another publication by the same group, high endogenous allergen variability between varieties was concluded to be rather connected to environmental conditions than to the variety itself. However, also the crop variety played a role for some allergens (Stevenson et al., 2012).

Although the above mentioned studies have developed a high-throughput and multiplexed LC/MS^2/MRM method for quantification of 8-10 allergens (including Gly m 6 subunits) in a single

297 analysis, data on other allergens and subunits are not available. As an example, for the major seed
298 storage protein Gly m 5, consisting of α- , α'- and β-subunits, only the α-subunit was measured.

299 Julka et al. (2012) studied the natural variability of the Bet v 1 homologue allergen Gly m 4 in 10
300 soybean varieties using 2D liquid chromatography followed by ultraviolet (UV) and MS detection of
301 the intact protein. Gly m 4 quantification in soybean seeds was based on the UV response factor
302 calculated from the purified reference standard of Gly m4, whereas the MS detection confirmed the
303 absence of any co-elution with the Gly m 4 peak during the separation of protein extracts. The
304 authors concluded that Gly m 4 was successfully quantified in the 10 commercially available soybean
305 varieties; and the difference between the lowest and highest allergen content only differed by less
306 than a factor of 2 (approx. 360-600 ug/g). However, possible isoforms of the allergen were not
307 assessed.

308 Using the same methodology and soybean varieties, in another work by the same group the Gly m 1
309 and Gly m 4 allergens were simultaneously quantified, resulting in 7.5 and 3-fold differences,
310 respectively (Kuppannan et al., 2014).

311 There are several examples available describing quantification of soybean allergens by ELISA. The
312 ELISA method is a valuable and helpful tool to quantitatively assess endogenous allergens, and is able
313 to provide accurate readouts. However, it has to be assured that defined antibodies raised in animals
314 against single allergen molecules are used. Previously, also antibodies raised against whole soybean
315 protein extract were applied to test for the occurrence of single allergens in ELISA (Cucu et al. 2012).
316 This method is however not recommended for endogenous allergenicity assessment, and some
317 problems are comparable to the limitations described above, occurring when human serum IgE is
318 used. Importantly, the animal immune response is highly variable and single allergenic proteins might
319 display only limited immunogenicity compared to others. Therefore, the variety of antibodies present
320 in these sera might be restricted concerning specificity, affinity and concentration. As a consequence,
321 some allergic proteins might be over- or underrepresented, respectively, and some allergens might
322 even be overlooked entirely.

323 In the publication by Geng et al. (2015), polyclonal antibodies as well as a monoclonal antibody
324 raised against Gly m 4 in animals were used to quantify the allergen in 24 natural soybean varieties
325 grown in eight different locations. The authors describe a high variability of Gly m 4 between these
326 varieties, differing by a factor of approximately 13. Considering the MS characterisations of Gly m 4
327 described above (Julka et al., 2012, Kuppanan et al. 2014), this is an interesting discrepancy.

328 Hei et al. (2012) developed a robust and reliable double antibody sandwich ELISA to quantify Gly m 5
329 in 469 soybean seed samples of different origin and breed. Levels varied from 40 to 148 mg of Gly m

5 per g of dry matter (3.7-fold difference) with more than 60% of the assessed samples falling in the range of 80-110 mg/g. Using a rather similar methodological approach, Chen et al. (2014) quantified Gly m 6 in 469 soybean samples from different origins and cultivars. Results revealed higher variability than in Gly m 5, since Gly m 6 concentrations ranged from 27 to 196 mg per g of dry matter, equivalent to a 7.3-fold difference, with more than 20% of the tested samples falling in the range of 120-140 mg/g. These data are in agreement with those observed by Houston et al. (2011) using a LC/MS^2/MRM approach, where moderate to high variability of Gly m 5 α-subunit and Gly m 6 subunits was concluded .

Wilson et al. (2008) used a monoclonal antibody raised in animals to assess the endogenous allergen content of Gly m Bd 30K in 138 soybean germplasm collection samples. The authors concluded that there is a high natural variability between the samples, ranging from approximately 2.5 to 30 mg/g of extracted protein.

The content of the profilin Gly m 3 was assessed in different soybean products (Amnuaycheewa et al., 2010). The group concluded that the concentration of allergen might depend on the country of origin as well as on processing of the food products.

Currently, publications regarding quantification are not available for all soybean allergens mentioned in the WHO/IUIS database or for all potential allergens included in the OECD consensus document. As described, there are also discrepancies between publications concerning variability of certain allergenic molecules. However, while this might be a technical issue and therefore depend on the method used, it could also be due to different soybean varieties investigated, and also depend on growth sites of the crops and, therefore, environmental factors. Moreover, possible isoforms in the case of Gly m 4 or subunits of Gly m 5 or Gly m 6 might react with the same antibodies due to structural similarities in ELISA, while these varieties are not detected by MS because of sequence restrictions. To this end, the development of further protocols as well as fine tuning of current approaches should be highly encouraged. In order to be able to compare the allergen content of different soybean varieties, also between research groups, it would be helpful to optimize and standardise ELISA and MS methods. To this end, standardised and harmonised protocols using defined antibodies in ELISA or defined allergen peptides in MS could be developed and applied, providing a basis for optimum quantification and comparison.

Data interpretation

Determination of endogenous allergen levels should be carried out as a part of the comparative analysis of composition (EC, 2013). Therefore, apart from comparing the GM food to its conventional counterpart, a suitable set of non-GM reference varieties should be assessed in parallel. These

varieties can present a set of data on the natural variation of the protein or allergen in question and ideally can provide an upper (and lower) limit of the natural allergen protein content. Any possible changes observed between the GM crop and its conventional counterpart can then, in the next step, be evaluated statistically by taking these varieties and the natural range of endogenous allergenic protein into account.

There is a need for better understanding the natural variability of allergens. Industry and research institutes should be strongly encouraged to collect as many data as possible on allergen natural variability of soybean varieties representative of the ones consumed by the at-risk population. In order for these data to be readily usable and comparable, the methodology used should be standardised and harmonised.

A solid database containing a broad range of measurements concerning the natural variation of endogenous allergens is the main basis for interpreting data acquired for a new GM variety. The endogenous allergen content of a new GM variety is less likely to raise an uncertainty the more the natural variation of these proteins is understood. In the case a potential allergenic protein differs statistically significant from its non-GM counterpart regarding the concentration of one or several endogenous allergens and additionally falls outside the range of natural variation, the consequences are uncertain and the biological relevance as well as the impact on human health has to be evaluated in the next step. Not only the possible enhanced risk of eliciting an allergic reaction in already allergic individuals has to be assessed, but also the likelihood that new sensitisations might occur to a greater extent due to the enhanced allergen content. To this end, a case-by case approach evaluating additional considerations and/or experimental data might be applied, depending on the specific potential allergen(s) in question, the number of allergens involved, their clinical relevance and the magnitude of the change. While it is currently challenging to evaluate the impact of an enhanced allergen content on sensitisation, the use of dose-distribution curves obtained by DBPCFC are a relevant tool and could be taken into consideration to assess the risk for elicitation of an allergic reaction. However, as mentioned above, dose-distribution curves are not readily available for single allergens, and they are usually obtained by challenging allergic individuals which are not at high risk to encounter an anaphylactic reaction.

Dose-distribution curves with a readout for single allergen molecules are in this context especially important for soybean allergy, since, due to several “major” allergens, the allergic individuals are highly heterogeneous. While the ones “truly” sensitised by soybean allergens Gly m 5 or Gly m 6 might be at risk if these molecules are enhanced in a GM crop compared to natural varieties, allergic individuals sensitised to the major allergen of birch, Bet v 1, may be highly sensitive to the cross-reactive allergen Gly m 4 and, for them, the Gly m 4 content of soybean may be crucially important.

Dose-distribution curves obtained by using total soybean protein might therefore be critically skewed depending on the allergic individuals assessed in DBPCFC, and also the quality and allergen content of the challenge material can play a role. Indeed, if the challenge material is well characterised and the allergen-content is known, dose-distribution curves corresponding to a single allergen may be acquired if allergic individuals reacting to only this single allergen are assessed.

Taking these considerations into account, the allergenicity assessment would certainly take advantage from future scientific research on component-resolved matters and from obtaining and strengthen the data on single potential allergen molecules in the light of causing clinical symptoms. Indeed, oral challenge tests with recombinant Mal d 1, the major food allergen in apple, have been performed recently (Kinaciyan et al., 2016).

If an enhanced endogenous allergen content in a GM crop is detected, raises concerns and the consequences for soybean allergic individuals cannot be estimated theoretically, DBPCFC comparing challenge material from the GM and a closely related non-GM counterpart is a powerful tool which could be applied, if considered necessary. However, several critical aspects have to be taken into account, including the choice of the non-GM comparator and the allergic individuals to be challenged. The conventional soybean variety used for comparison would have to be chosen carefully, since it should not significantly differ from the new GM variety in any other aspect than in the content of the enhanced allergen in question. Regarding the allergic individuals used in the DBPCFC, they should be well characterized by molecular diagnosis to react with the allergen in question. A comparative assessment should be done, whereby the same allergic individuals are challenged with both, material from the GM crop and the non-GM crop, respectively.

Upon an enhanced endogenous allergen content detected, also exposure considerations might be taken into account as a last step in the risk assessment process. Therefore, the GM food should be further characterized concerning its anticipated intake (EC, 2013) and efforts should concentrate on most at-risk groups. In the case of airborne allergens, the likelihood of exposure to the airways has to be considered. A potentially enhanced allergen content should even strengthen the anticipations of strictly fulfilling safety measures (Pont et al., 1997).

Conclusion:

Connecting single protein molecules to clinical reactivity of IgE-mediated food allergy remains challenging. For soybean, several potential allergens have been described, but a connection to clinical reactions in soybean allergic individuals has not been unequivocally shown for all potential allergens. Since the assessment of endogenous allergenicity in soybean as a part of the compositional analysis started to be mandatory with the Implementing Regulation EU No. 503/2013 (EC, 2013), a

need arose to define relevant allergens to be measured. International definitions and parameters, like the strategy applied by WHO/IUIS can be taken as a basis for these considerations. However, due to the constant scientific progress these definitions have to be evaluated on a regular basis and, if necessary, adapted. It is important to pro-actively include clinicians into the risk assessment process, including into defining appropriate molecules to be measured as well as into the final strategies to be applied for the safety assessment to protect consumers, in particular those at higher risk. The natural variability of endogenous allergens in soybeans traditionally consumed by the population is an invaluable tool to interpret data acquired for new varieties generated by biotechnology. There is therefore a need to generate comprehensive and robust databases for natural levels of endogenous allergens. To this end, the methodology of assessment should be standardised, allowing a harmonisation of the risk assessment process.

References

Allen, K.J., Turner, P.J., Pawankar, R., Taylor, S., Sicherer, S., Lack, G., Rosario, N., Ebisawa, M., Wong, G., Mills, E.N., Beyer, K., Fiocchi, A., Sampson, H.A., 2014. Precautionary labelling of foods for allergen content: are we ready for a global framework? World Allergy Organ J 7, 10.

Amnuaycheewa P., Gonzalez de Mejia E., 2010. Purification, characterisation, and quantification of the soy allergen profilin (Gly m 3) in soy products. Food Chemistry 119, 1671-1680.

Ballmer-Weber, B.K., Fernandez-Rivas, M., Beyer, K., Defernez, M., Sperrin, M., Mackie, A.R., Salt, L.J., Hourihane, J.O., Asero, R., Belohlavkova, S., Kowalski, M., de Blay, F., Papadopoulos, N.G., Clausen, M., Knulst, A.C., Roberts, G., Popov, T., Sprikkelman, A.B., Dubakiene, R., Vieths, S., van Ree, R., Crevel, R., Mills, E.N., 2015. How much is too much? Threshold dose distributions for 5 food allergens. J Allergy Clin Immunol 135, 964-971.

Ballmer-Weber, B.K., Holzhauser, T., Scibilia, J., Mittag, D., Zisa, G., Ortolani, C., Oesterballe, M., Poulsen, L.K., Vieths, S., Bindslev-Jensen, C., 2007. Clinical characteristics of soybean allergy in Europe: a double-blind, placebo-controlled food challenge study. J Allergy Clin Immunol 119, 1489-1496.

Ben-Shoshan, M., Sheth, S., Harrington, D., Soller, L., Fragapane, J., Joseph, L., St Pierre, Y., La Vieille, S., Elliott, S., Waserman, S., Alizadehfar, R., Harada, L., Allen, M., Allen, M.H., Clarke, A.E., 2012. Effect of precautionary statements on the purchasing practices of Canadians directly and indirectly affected by food allergies. J Allergy Clin Immunol 129, 1401-1404.

Blom, W.M., Vlieg-Boerstra, B.J., Kruizinga, A.G., van der Heide, S., Houben, G.F., Dubois, A.E., 2013. Threshold dose distributions for 5 major allergenic foods in children. J Allergy Clin Immunol 131, 172-179.

Boyce, J.A., Assa'ad, A., Burks, A.W., Jones, S.M., Sampson, H.A., Wood, R.A., Plaut, M., Cooper, S.F., Fenton, M.J., Arshad, S.H., Bahna, S.L., Beck, L.A., Byrd-Bredbenner, C., Camargo, C.A., Jr., Eichenfield, L., Furuta, G.T., Hanifin, J.M., Jones, C., Kraft, M., Levy, B.D., Lieberman, P., Luccioli, S., McCall, K.M., Schneider, L.C., Simon, R.A., Simons, F.E., Teach, S.J., Yawn, B.P., Schwaninger, J.M., 2010. Guidelines for the Diagnosis and Management of Food Allergy in the United States: Summary of the NIAID-Sponsored Expert Panel Report. J Allergy Clin Immunol 126, 1105-1118.

Chen, J., Wang, J., Song, P., Ma, X., 2014. Determination of glycinin in soybean and soybean products using a sandwich enzyme-linked immunosorbent assay. Food Chem 162, 27-33.

Christensen, L.H., Holm, J., Lund, G., Riise, E., Lund, K., 2008. Several distinct properties of the IgE repertoire determine effector cell degranulation in response to allergen challenge. J Allergy Clin Immunol 122, 298-304.

Chu, Y., Faustinelli, P., Ramos, M.L., Hajduch, M., Stevenson, S., Thelen, J.J., Maleki, S.J., Cheng, H., Ozias-Akins, P., 2008. Reduction of IgE binding and nonpromotion of Aspergillus flavus fungal growth by simultaneously silencing Ara h 2 and Ara h 6 in peanut. J Agric Food Chem 56, 11225-11233.

Cucu, T., Devreese, B., Kerkaert, B., Rogge, M., Vercruysse, L., De Meulenaer, B., 2012. ELISA-Based Detection of Soybean Proteins: A Comparative Study Using Antibodies Against Modified and Native Proteins. Food Anal Methods 5, 1121-1130.

Codex Alimentarius, 2009. Foods Derived from Modern Biotechnology. Codex Alimentarius Commission, Joint FAO/WHO Food Standards Programme, Rome.

Dodo, H.W., Konan, K.N., Chen, F.C., Egnin, M., Viquez, O.M., 2008. Alleviating peanut allergy using genetic engineering: the silencing of the immunodominant allergen Ara h 2 leads to its significant reduction and a decrease in peanut allergenicity. Plant Biotechnol J 6, 135-145.

Du Toit, G., Roberts, G., Sayre, P.H., Bahnson, H.T., Radulovic, S., Santos, A.F., Brough, H.A., Phippard, D., Basting, M., Feeney, M., Turcanu, V., Sever, M.L., Gomez Lorenzo, M., Plaut, M., Lack, G., 2015. Randomized trial of peanut consumption in infants at risk for peanut allergy. N Engl J Med 372, 803-813.

Dubois, A.E., Pagliarani, G., Brouwer, R.M., Kollen, B.J., Dragsted, L.O., Eriksen, F.D., Callesen, O., Gilissen, L.J., Krens, F.A., Visser, R.G., Smulders, M.J., Vlieg-Boerstra, B.J., Flokstra-de Blok, B.J., van de Weg, W.E., 2015. First successful reduction of clinical allergenicity of food by genetic modification: Mal d 1-silenced apples cause fewer allergy symptoms than the wild-type cultivar. Allergy 70, 1406-1412.

EC, 2003. Directive 2003/89/EC of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs.

EC, 2011. Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004.

EC, 2013. Commission Implementing Regulation (EU) No. 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No. 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No. 641/2004 and (EC) No. 1981/2006. Off. J. Eur. Union L157, 1–48.

EFSA, 2010. EFSA panel on genetically modified organisms (GMO). Scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed. EFSA J. 8 (7), 1700.

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms) 2003. Opinion of the Scientific Panel on Genetically Modified Organisms on an application (Reference EFSA-GMO-NL-2005-18) for the placing on the market of the glufosinate tolerant soybean A2704-12, for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Bayer CropScience, The EFSA Journal (2007) 524, 1-22.

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms) 2007. Opinion of the Scientific Panel on genetically modified organisms (GMO) on an application (Reference EFSA-GMO-NL-2005-18) for the placing on the market of the glufosinate tolerant soybean A2704-12, for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Bayer CropScience. doi:10.2903/j.efsa.2007.524.

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms) 2013. Scientific Opinion on application EFSA-GMO-NL-2011-93 for the placing on the market of the herbicide-tolerant genetically modified soybean MON 87708 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Monsanto. EFSA Journal 2013;11(10):3355, 30 pp. doi:10.2903/j.efsa.2013.3355.

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms) 2011. Scientific Opinion on application (EFSA-GMO-UK-2007-43) for the placing on the market of herbicide tolerant genetically modified soybean 356043 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Pioneer. EFSA Journal 2011;9(7):2310, 40 pp. doi:10.2903/j.efsa.2011.2310.EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms) 2015a. Scientific Opinion on application (EFSA-GMO-NL-2012-108) for the placing on the market of the herbicide-tolerant genetically modified soybean MON 87708 × MON 89788 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from. EFSA Journal 2015;13(6):4136, 26 pp. doi:10.2903/j.efsa.2015.4136.

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2015b. Scientific Opinion on an application (EFSA-GMO-NL-2010-85) for the placing on the market of MON 87769 × MON 89788 soybean, genetically modified to contain stearidonic acid and be tolerant to glyphosphate for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Monsanto. EFSA Journal 2015;13(10):4256, 25 pp. doi:10.2903/j.efsa.2015.4256.

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2015c. Scientific Opinion on an application (Reference EFSA-GMO-NL-2011-100) for the placing on the market of the herbicide-tolerant, increased oleic acid genetically modified soybean MON 87705 × MON 89788 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Monsanto. EFSA Journal 2015;13(7):4178, 30 pp. doi:10.2903/j.efsa.2015.4178

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2016. Draft guidance on allergenicity assessment of genetically modified plants. EFSA Journal 20YY;volume(issue):NNNN, 49 pp. doi:10.2903/j.efsa.20YY.NNNN.

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2014. Scientific Opinion on the evaluation of allergenic foods and food ingredients for labelling purposes. EFSA Journal 2014;12(11):3894, 286 pp. doi:10.2903/j.efsa.2014.3894.

Fernandez, A., Mills, E.N., Lovik, M., Spoek, A., Germini, A., Mikalsen, A., Wal, J.M., 2013. Endogenous allergens and compositional analysis in the allergenicity assessment of genetically modified plants. Food Chem Toxicol 62, 1-6.

Geng, T., Liu, K., Frazier, R., Shi, L., Bell, E., Glenn, K., Ward, J.M., 2015. Development of a Sandwich ELISA for Quantification of Gly m 4, a Soybean Allergen. J Agric Food Chem 63, 4947-4953.

Gieras, A., Focke-Tejkl, M., Ball, T., Verdino, P., Hartl, A., Thalhamer, J., Valenta, R., 2007. Molecular determinants of allergen-induced effector cell degranulation. J Allergy Clin Immunol 119, 384-390.

Gieras, A., Linhart, B., Roux, K.H., Dutta, M., Khodoun, M., Zafred, D., Cabauatan, C.R., Lupinek, C., Weber, M., Focke-Tejkl, M., Keller, W., Finkelman, F.D., Valenta, R., 2015. IgE epitope proximity determines immune complex shape and effector cell activation capacity. J Allergy Clin Immunol 9, 01422-01420.

Gilissen, L.J., Bolhaar, S.T., Matos, C.I., Rouwendal, G.J., Boone, M.J., Krens, F.A., Zuidmeer, L., Van Leeuwen, A., Akkerdaas, J., Hoffmann-Sommergruber, K., Knulst, A.C., Bosch, D., Van de Weg, W.E., Van Ree, R., 2005. Silencing the major apple allergen Mal d 1 by using the RNA interference approach. J Allergy Clin Immunol 115, 364-369.

Glaumann, S., Nopp, A., Johansson, S.G., Borres, M.P., Nilsson, C., 2013. Oral peanut challenge identifies and allergy but the peanut allergen threshold sensitivity is not reproducible. Plos One 8:e53465.

Graf, L., Hayder, H., Mueller, U., 2014. Endogenous allergens in the regulatory assessment of genetically engineered crops. Food Chem Toxicol 73, 17-20.

Hefle, S.L., Furlong, T.J., Niemann, L., Lemon-Mule, H., Sicherer, S., Taylor, S.L., 2007. Consumer attitudes and risks associated with packaged foods having advisory labeling regarding the presence of peanuts. J Allergy Clin Immunol 120, 171-176.

Hei, W., Li, Z., Ma, X., He, P., 2012. Determination of beta-conglycinin in soybean and soybean products using a sandwich enzyme-linked immunosorbent assay. Anal Chim Acta 734, 62-68.

Hoffmann-Sommergruber, K., Pfeifer, S., Bublin, M., 2015. Applications of Molecular Diagnostic Testing in Food Allergy. Curr Allergy Asthma Rep 15, 56.

Holzhauser, T., Wackermann, O., Ballmer-Weber, B.K., Bindslev-Jensen, C., Scibilia, J., Perono-Garoffo, L., Utsumi, S., Poulsen, L.K., Vieths, S., 2009. Soybean (Glycine max) allergy in Europe: Gly m

578 5 (beta-conglycinin) and Gly m 6 (glycinin) are potential diagnostic markers for severe allergic
579 reactions to soy. J Allergy Clin Immunol 123, 452-458.

580 Houston, N.L., Lee, D., Stevenson, S.E., Ladics, G.S., Bannon, G.A., McClain, S., Privalle,L., Stagg, N.,
581 Herouet-Guicheney, C., MacIntosh, S.D., Thelen, J.J., 2011. Quantitation of soybean allergens using
582 tandem mass spectrometry. J.Proteome Res. 10, 763–773.

583 Imamura, T., Kanagawa, Y., Ebisawa, M., 2008. A survey of patients with self-reported severe food
584 allergies in Japan. Pediatr Allergy Immunol 19, 270-274.

585 Julka, S., Kuppannan, K., Karnoup, A., Dielman, D., Schafer, B., Young, S.A., 2012. Quantification of Gly
586 m 4 protein, a major soybean allergen, by two-dimensional liquid chromatography with ultraviolet
587 and mass spectrometry detection. Anal Chem 84, 10019-10030.

588 Katz, Y., Gutierrez-Castrellon, P., Gonzalez, M.G., Rivas, R., Lee, B.W., Alarcon, P., 2014. A
589 comprehensive review of sensitization and allergy to soy-based products. Clin Rev Allergy Immunol
590 46, 272-281.

591 Kinaciyan, T., Nagl, B., Faustmann, S., Kopp, S., Wolkersdorfer, M., Bohle, B., 2016. Recombinant Mal
592 d 1 facilitates sublingual challenge tests of birch pollen-allergic patients with apple allergy. Allergy 71,
593 272-274.

594 Kleine-Tebbe, J., Vogel, L., Crowell, D.N., Haustein, U.F., Vieths, S., 2002. Severe oral allergy
595 syndrome and anaphylactic reactions caused by a Bet v 1- related PR-10 protein in soybean, SAM22. J
596 Allergy Clin Immunol 110, 797-804.

597 Klemans, R.J., Knol, E.F., Michelsen-Huisman, A., Pasmans, S.G., de Kruijf-Broekman, W., Bruijnzeel-
598 Koomen, C.A., van Hoffen, E., Knulst, A.C., 2013. Components in soy allergy diagnostics: Gly m 2S
599 albumin has the best diagnostic value in adults. Allergy 68, 1396-1402.

600 Knoll, J.E., Ramos, M.L., Zeng, Y., Holbrook, C.C., Chow, M., Chen, S., Maleki, S., Bhattacharya, A.,
601 Ozias-Akins, P., 2011. TILLING for allergen reduction and improvement of quality traits in peanut
602 (Arachis hypogaea L.). BMC Plant Biol 11, 81.

603 Kuppannan, K., Julka, S., Karnoup, A., Dielman, D., Schafer, B., 2014. 2DLC-UV/MS assay for the
604 simultaneous quantification of intact soybean allergens Gly m 4 and hydrophobic protein from
605 soybean (HPS). J Agric Food Chem 62, 4884-4892.

606 L'Hocine, L., Boye, J.I., 2007. Allergenicity of soybean: new developments in identification of
607 allergenic proteins, cross-reactivities and hypoallergenization technologies. Crit Rev Food Sci Nutr 47,
608 127-143.

609 Ladics, G.S., Budziszewski, G.J., Herman, R.A., Herouet-Guicheney, C., Joshi, S., Lipscomb, E.A.,
610 McClain, S., Ward, J.M., 2014. Measurement of endogenous allergens in genetically modified
611 soybeans--short communication. Regul Toxicol Pharmacol 70, 75-79.

612 Mari, A., Ooievaar-de Heer, P., Scala, E., Giani, M., Pirrotta, L., Zuidmeer, L., Bethell, D., van Ree, R.,
613 2008. Evaluation by double-blind placebo-controlled oral challenge of the clinical relevance of IgE
614 antibodies against plant glycans. Allergy 63, 891-896.

615 Natarajan, S.S., Xu, C., Cregan, P., Caperna, T.J., Garrett, W.M., Luthria, D., 2009. Utility of proteomics
616 techniques for assessing protein expression. Regul Toxicol Pharmacol 54, S32-36.

617 Noimark, L., Gardner, J., Warner, J.O., 2009. Parents' attitudes when purchasing products for children
618 with nut allergy: a UK perspective. Pediatr Allergy Immunol 20, 500-504.

619 OECD, 2012. Revised consensus document on compositional considerations for new 452 varieties of
620 soybean [Glycine max (L.) Merr.]: key food and feed nutrients, 453 antinutrients, toxicants and
621 allergens. Series on the Safety of Novel Foods and 454 Feeds No. 25.

622 Ostblom, E., Wickman, M., van Hage, M., Lilja, G., 2008. Reported symptoms of food hypersensitivity
623 and sensitization to common foods in 4-year-old children. Acta Paediatr 97, 85-90.

624 Panda, R., Ariyarathna, H., Amnuaycheewa, P., Tetteh, A., Pramod, S.N., Taylor, S.L., Ballmer-Weber,
625 B.K., Goodman, R.E., 2013. Challenges in testing genetically modified crops for potential increases in
626 endogenous allergen expression for safety. Allergy 68, 142-151.

627 Pont, F., Gispert, X., Canete, C., Pinto, E., Dot, D., Monteis, J., 1997. [An epidemic of asthma caused
628 by soybean in L'Hospitalet de Llobregat (Barcelona)]. Arch Bronconeumol 33, 453-456.

629 Quirce, S., Fernandez-Nieto, M., Polo, F., Sastre, J., 2002. Soybean trypsin inhibitor is an occupational
630 inhalant allergen. J Allergy Clin Immunol 109, 178.

631 Rouquie, D., Capt, A., Eby, W.H., Sekar, V., Herouet-Guicheney, C., 2010. Investigation of endogenous
632 soybean food allergens by using a 2-dimensional gel electrophoresis approach. Regul Toxicol
633 Pharmacol 58, S47-53.

634 Sampson, H.A., 2001. Utility of food-specific IgE concentrations in predicting symptomatic food
635 allergy. J Allergy Clin Immunol 107, 891-896.

636 Sampson, M.A., Munoz-Furlong, A., Sicherer, S.H., 2006. Risk-taking and coping strategies of
637 adolescents and young adults with food allergy. J Allergy Clin Immunol 117, 1440-1445.

638 Savage, J.H., Kaeding, A.J., Matsui, E.C., Wood, R.A., 2010. The natural history of soy allergy. J Allergy
639 Clin Immunol 125, 683-686.

640 Sicherer, S.H., Wood, R.A., 2012. Allergy testing in childhood: using allergen-specific IgE tests.
641 Pediatrics 129, 193-197.

642 Stevenson, S.E., Woods, C.A., Hong, B., Kong, X., Thelen, J.J., Ladics, G.S., 2012. Environmental effects
643 on allergen levels in commercially grown non-genetically modified soybeans> assessing variation
644 across North America. Front. Plant Sci. 3, 1-13.

645 Taylor, S.L., Gendel, S.M., Houben, G.F., Julien, E., 2009. The Key Events Dose-Response Framework:
646 a foundation for examining variability in elicitation thresholds for food allergens. Crit Rev Food Sci
647 Nutr 49, 729-739.

648 Tuano, K.S., Davis, C.M., 2015. Utility of Component-Resolved Diagnostics in Food Allergy. Curr
649 Allergy Asthma Rep 15, 32.

650 van Neerven, R.J., Knol, E.F., Ejrnaes, A., Wurtzen, P.A., 2006. IgE-mediated allergen presentation and
651 blocking antibodies: regulation of T-cell activation in allergy. Int Arch Allergy Immunol 141, 119-129.

652 Verhoeckx, K.C., Vissers, Y.M., Baumert, J.L., Faludi, R., Feys, M., Flanagan, S., Herouet-Guicheney, C.,
653 Holzhauser, T., Shimojo, R., van der Bolt, N., Wichers, H., Kimber, I., 2015. Food processing and
654 allergenicity. Food Chem Toxicol 80, 223-240.

655 Wilson, S., Martinez-Villaluenga, C., De Mejia, E.G., 2008. Purification, thermal stability, and
656 antigenicity of the immunodominant soybean allergen P34 in soy cultivars, ingredients, and products.
657 J Food Sci 73, T106-114.

658 Wright, B.L., Kulis, M., Orgel, K.A., Burks, A.W., Dawson, P., Henning, A.K., Jones, S.M., Wood, R.A.,
659 Sicherer, S.H., Lindblad, R.W., Stablein, D., Leung, D.Y., Vickery, B.P., Sampson, H.A., 2016.
660 Component-resolved analysis of IgA, IgE, and IgG4 during egg OIT identifies markers associated with
661 sustained unresponsiveness. Allergy. doi: 10.1111/all.12895.

662 Zheng, S., Tian, H., Ma, N., Qin, G., Sun, Z., Yu, C., 2012. Purification and IgE-binding properties of
663 soybean beta-conglycinin subunits. Process Biochemistry 47, 2531–37.

664 Zhu, J., Pouillot, R., Kwegyir-Afful, E.K., Luccioli, S., Gendel, S.M., 2015. A retrospective analysis of
665 allergic reaction severities and minimal eliciting doses for peanut, milk, egg, and soy oral food
666 challenges. Food Chem Toxicol 80, 92-100.