



Review

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



R. Selb^a, J.M. Wal^b, F.J. Moreno^c, M. Lovik^d, C. Mills^e, K. Hoffmann-Sommergruber^f,
A. Fernandez^{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 School of Biological Sciences, 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

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ABSTRACT

According to EU regulation, genetically modified (GM) plants considered to be allergenic have to be assessed concerning their endogenous allergens before placement on the EU market, in line with the international standards described in Codex Alimentarius. Under such premises, a quantitative relevant increase in allergens might occur in GM plants as an unintended effect compared with conventionally produced crops, which could pose a risk to consumers. Currently, data showing a connection between dose and allergic sensitisation are scarce since the pathophysiological mechanisms of sensitisation are insufficiently understood. In contrast, data on population dose-distribution relationships acquired by oral food challenge are available showing a connection between quantity of allergenic protein consumed and the population of allergic individuals experiencing reactions. Soybean is currently the only recognised allergenic GM food by law for which EFSA has received applications and was therefore taken as an example for defining an assessment strategy. Identification of potential allergens, methodology for quantification as well as risk assessment considerations, are discussed. A strategy is proposed for the identification, assessment and evaluation of potential hazards/risks concerning endogenous allergenicity in food derived from plants developed by biotechnology. This approach could be expanded to other allergenic foods in the future, whenever required.

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* Corresponding author. European Food Safety Authority, Via Carlo Magno 1A, 43126, Parma, Italy.

E-mail address: antonio.fenandezdumont@efsa.europa.eu (A. Fernandez).

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Abbreviations

LOAEL	lowest observed adverse effect level
DBPCFC	double blind placebo controlled food challenge
PAL	precautionary allergen labels
GM	genetically modified
2D-GE	Two dimensional gel electrophoresis
LC	Liquid chromatography
MS	Mass spectrometry
MRM	Multiple reaction monitoring

1. Introduction

Allergen dose is a critical issue in the course of the allergic disease, and possible over-expression of allergenic proteins in plants used for food might pose a risk to allergic consumers' health. In contrast to proteins newly introduced into plants which might be allergenic, endogenous allergens are proteins naturally occurring in plants. It is therefore not only important to monitor food derived from modern biotechnology regarding the allergenicity of newly expressed proteins, but moreover, if the food source is considered to be allergenic, to evaluate the expression levels of endogenous allergens.

Allergic disease develops in a two-step process comprising sensitisation to the allergenic food and subsequent elicitation of the allergic reaction resulting in symptoms on re-exposure to the allergen when administered in sufficient amounts (Taylor et al., 2009). The dose necessary for allergic sensitisation is little studied and it is challenging to determine a threshold for sensitisation (Taylor et al., 2009). Also, it has been shown that encountering potentially 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 population 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 have been determined for many allergenic food sources (Ballmer-Weber et al., 2015; Allen et al., 2014a). For soybean, the only food derived from modern biotechnology on the European market which is considered a common cause for food allergy by EU regulation (EC, 2003, 2011), the minimum amount of protein causing (subjective) symptoms in allergic individuals was calculated to be 5.3 mg of soybean protein in one study (Ballmer-Weber et al., 2007). The amount of total protein eliciting objective symptoms can vary to a large degree. This can depend on the individual allergenic proteins contributing to the total protein content contained within consumed food, the challenge protocol, and the attributes of the allergic individual. Furthermore the reproducibility of double blind placebo controlled food challenge (DBPCFC) can sometimes also be limited (Glaumann et al., 2013).

Allergic subjects are generally advised to avoid food containing the ingredients to which they are allergic. However, unintentional

ingestion of allergenic food happens on a regular basis and is the most common 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 intentional ingestion is not uncommon, especially in young adults who are willing to take risks, with 54% of 13–21 year old food allergic individuals admitting they eat small amounts of the food they are allergic to (Sampson et al., 2006). Other studies clearly indicate that around 20–30% of allergic subjects (or their parents, in the case of children) ignore precautionary allergen labels (PALs) on products or even dietary advice to avoid certain ingredients (Allen et al., 2014a,b; Ben-Shoshan et al., 2012; Cochrane et al., 2013; Imamura et al., 2008; Noimark et al., 2009). The number of allergic consumers ignoring allergy advisory statements increased 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). Therefore, it cannot 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 deliberately.

1.1. Regulatory aspects

The safety assessment of GM plants in general is strictly regulated by international guidelines (Codex, 2009) and EU regulations (EC, 2013). These thorough regulations have helped to maintain a high safety standard of GM food, and a recent review of the scientific literature has suggested that GM plants currently on the market are as safe as their parental species, even though long term studies are scarce (Domingo, 2016). From a regulatory point of view, possible allergenicity of a newly expressed protein in food derived from modern biotechnology has to be assessed for all crops. In contrast, regarding endogenous allergens, after genetic modification of plants considered to be allergenic and considered common causes of food allergy, such as soybean, any potential changes in endogenous allergenicity have to be assessed before placement on the European market according to specific regulations (EC, 2013). The main principles of 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 genetically modified (GM) food is as safe as the conventionally consumed non-GM comparator. To this end, any plant derived from a source considered to be allergenic which was GM has to be evaluated for potential changes in allergen composition by comparing the food source derived from modern biotechnology with appropriate non-GM comparators.

1.2. Risk assessment considerations

Currently, provision of information to consumers concerning the deliberate presence of regulated food 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 amount of an allergenic food source in the overall

product forms the basis of a decision to use or not use precautionary labelling. In products where the overall amount of an allergic food source falls below such a reference level and a risk assessment indicates no PAL needs to be applied, the endogenous allergen content gains crucial importance. A single allergen enhanced in the original crop would mean that even though the food product could still contain the same (low) amount of total 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 dose on sensitisation to potentially allergenic proteins is not sufficiently understood, it is important to know whether or not the levels of endogenous allergens of a GM crop, in relation to appropriate comparators, have been enhanced and may cause concern. 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.

1.3. Soybean allergenicity

Out of foods that are recognised to be common allergenic sources by European Regulation (EC, 2003, 2011) and therefore pose a risk to a given population, EFSA has 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 the 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 the USA, although 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 being 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, severe reactions including anaphylactic shock after the ingestion of soybean products have also 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 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

consequently also caused less allergic symptoms upon challenge of allergic individuals. It also cannot be excluded that other crops apart from soybean might be considered to have allergenic potential of public health importance in the future, and therefore be considered as common allergenic sources. Endogenous allergenicity and its assessment is currently discussed intensively and the topic has been the subject of several other publications (Graf et al., 2014; Fernandez et al., 2013; Ladics et al., 2014; Panda et al., 2013).

2. Background: history of assessment of endogenous allergenicity in soybean

Codex Alimentarius (2009) set the pillars for the safety assessment of “Foods derived from modern biotechnology”, which are grounded on the assessment of the “new” food relative to that of the appropriate conventional counterpart that can be considered as safe. This approach is rooted in the concept of substantial equivalence, initially developed by OECD (1993). Therefore, in the current risk assessment context of foods derived from modern biotechnology including GMOs, absolute safety is not pursued but the safety of the “new” food is relative to those widely consumed and considered to have a history of safe use. In this context, it is therefore the final goal of the GM plant safety assessment to ensure that the genetic modification does not affect the levels of key components of a given GM plant to a level raising concern, when compared to the appropriate conventional counterpart plant. The relevance of such assessment for plants produced by biotechnology in general (Ladics et al., 2015; NAS, 2016) and for endogenous allergenicity in particular has been previously debated (Graf et al., 2014; Fernandez et al., 2013; Panda et al., 2013).

Presently in Europe, the Implementing Regulation EU No. 503/2013 (EC, 2013) asks for the assessment of relevant allergens as part of the comparative analysis as a mandatory requirement and provides a reference to the OECD consensus documents on compositional considerations for new plant varieties. This requirement has been a subject of debate (Ladics et al., 2014). 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 of the potential allergens listed in the table was questioned previously (Ladics et al., 2014), since either their sequences are not available or the published scientific evidence of clinical relevance is disputed. Currently, an EFSA guidance document is being developed which provides assistance for applicants and risk assessors regarding measurement of allergens under the most recent European Regulation requirements (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, under Regulation No. 1829/2003 (EC, 2003) the standard methodologies applicants used for endogenous allergenicity assessment were based on human serum containing allergen-specific IgE (EFSA, 2007, 2013). As a further development, 2-dimensional gel electrophoresis (2D-GE) in combination with spot quantification was employed in other applications (EFSA, 2015a, b, c). Lately, also absolute quantification methods were used by industry and these applications are currently under assessment. The different methodologies are discussed in detail below.

In the first studies, semi-quantitative analyses using IgE containing sera of allergic individuals were used to assess the allergen content of a plant. 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 with others in human sera or might occur in only few subjects. To this end, some allergen molecules might be overlooked 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 also that these antibodies recognise these allergen proteins sufficiently well. However, serum samples from allergic subjects are precious and accessible only in limited amounts, 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). Within the comparative approach, the focus lies on quantification rather than characterisation. For this, more robust and reliable methods with a better readout are available, which can be standardised more easily and do not depend on limited human material.

Because of the limitations described above for 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). To identify and quantify soybean proteins, including potential allergens, another group used a 2D-GE and mass spectrometry based approach, also using spot intensity quantification (Natarajan et al., 2009). However, the focus of Natarajan and colleagues was on identification of proteins rather than their quantification. While these spot quantification methods might be an improvement compared with 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.

Up to this date, the EFSA GMO Panel has not found 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.

3. Identification of relevant soybean allergens for assessment of GM plants

According to IR 503/2013, 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 to select potentially relevant soybean allergens. This can be further complemented by searching the databases listed in the EFSA scientific opinion on allergenicity (EFSA, 2010), especially the WHO/IUIS database (www.allergen.org), the FARRP 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. 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, in the context of the issues addressed in this publication, it should be highlighted that one major drawback of current clinical practice is the fact that data on clinical reactivity are obtained by DBPCFC to (the composite) soybean extract or soybean formulas only because information is required on the allergenicity of foods as they are consumed (Ballmer-Weber et al., 2007; Blom et al., 2013). While DBPCFC is considered the gold standard for assessment and diagnosis of food allergy (Sampson et al., 2012; 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 have not yet been performed. Reactivity with the individual proteins is therefore mainly expressed by sensitisation data using IgE binding assays, or, in few cases, basophil activation or histamine release experiments (Gly m 4, Gly m 5, Kleine-Tebbe et al., 2002; Zheng et al., 2012; Gly m 7, Riascos et al., 2016) 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 (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, 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 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.

Acknowledging the challenges mentioned above, the criteria for selection of allergens like those suggested by international institutions like WHO/IUIS can be used as a tool to select allergens. WHO/IUIS prescribes the IgE-reactivity of at least 5 allergic individual's sera, or at least 5% of all sera tested, 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 WHO/IUIS primary criteria were applied to the list of potential allergens as a starting point. Considerations for including molecules into current

Table 1
Information on (potential) soybean allergens 2016.

Protein	Family/ Similarities	Protein sequence availability	Allergic individuals			evidence for clinical reactivity to soy corresponding with reaction to the specific allergen	contra evidence	References	Endogenous allergenicity assessment - considerations
			Individuals tested and positives in IgE binding (examples)	Allergenic activity described by	Patients' group				
Gly m 1	Lipid transfer protein	yes	⁶ 20 patients: 95% positive; ⁷ 18 patients: 83% positive	IgE binding, ELISA	adults	^{5,6} SPT to soy	^{8,10} no sensitisation to Gly m 1 in occupational asthma of soy allergic individuals (bakers/ workers)	1-10	included in IUIS/WHO, underwent peer-review process
Gly m 2	Storage protein	20AA N- terminal sequence	³ 18 patients: 83% positive	IgE binding, ELISA	adults		⁸ no sensitisation to Gly m 2 in occupational asthma of soy allergic individuals (workers)	2,3,8,11,12	included in IUIS/WHO, sequence is currently not available
Gly m 3	Profilin	yes	¹² 22 patients: 25% positive; ¹³ 13 patients: 69% positive	IgE binding	adults (children)	¹² DBPCFC to soy		12-14	included in IUIS/WHO, underwent peer-review process
Gly m 4	Bet v 1 family	yes	¹⁵ 21/30 soy allergic patients; ¹⁸ 8 birch allergic children reacting to Gly m 4; ²¹ 22 birch allergic patients: 96% positive	IgE binding (ELISA, CAP, microarray) ¹⁹ basophil histamine release	adults, children	^{12,15,21} DBPCFC; ¹⁸ open challenge; ^{12,15,17,18} SPT, ^{12,19,20} oral allergy syndrome; ^{17,19,20} strong link to severe systemic reactions to soy products		12, 15-21	included in IUIS/WHO, underwent peer-review process
Gly m 5	Cupin, vicilin- like proteins	yes, 3 subunits	²⁶ 13/30 patients (43%), 5/5 children (100%)	IgE binding, ³² dose- dependent histamine release in basophil testing	adults, children	^{24,26} DBPCFC, ²⁶ history of anaphylaxis to soy, ^{26,27} strong link to allergic symptoms, ²⁴ AD patients, ²² exercise induced anaphylaxis, ^{8,23} possible also respiratory sensitisation		8, 22-32	included in IUIS/WHO, underwent peer-review process
Gly m 6	Cupin	yes, several subunits	²⁶ 11/30 patients (36%), 3/5 children (60%);	IgE binding, RBL assay (but no patient's IgE used)	adults, children	²⁶ DBPCFC, ²⁶ history of anaphylaxis to soy, ^{26,27} strong link to allergic symptoms, ^{8,23} possible also respiratory sensitisation		8, 23, 26, 27, 33-39	included in IUIS/WHO, underwent peer-review process
Gly m 7	seed-specific biotinylated proteins (SBP)	yes	18/23 tested patients positive	IgE binding, basophil activation	adults, children	⁴⁰ self-reported allergy to peanut and soybean (adults), positive oral food challenge to peanut		40	included in IUIS/WHO, underwent peer-review process
Gly m 8	prolamin, plant LTP	yes	³¹ 4/8 anaphylactic patients; ⁴¹ 17/19 symptomatic children	IgE binding (ELISA, microarray)	adults, children	⁴² IgE to 2S A with strong link to severe reactions	⁴³ 23 european soy allergic patients show no reactivity with Gly m 8	9, 31, 41- 43	included in IUIS/WHO, underwent peer-review process
Gly m BD 28K	vicilin-like glycoprotein, Cupin	yes	⁴⁵ 10/10 patients (100%); ⁴⁸ 5/8 patients (62.5%)	IgE binding	adults, children			44-48	not included in IUIS/ WHO, but fulfills primary criteria of IUIS/WHO, considerable peer- reviewed literature available, measurement suggested by other scientists (Ladics et al., 2014)
Gly m BD 30K	thiol proteinases of papain family, Peptidase C1	yes	⁵⁰ serum pool 4 patients; ⁵³ 9/31; ⁵⁴ 69 patients, 65% bind to 30 kD protein in 7S fraction,	IgE binding	adults, children	^{50,51} DBPCFC to soy, ^{50,51} positive SPT, ⁵³ significant correlation between soy IgE and IgE to P34		49-56	not included in IUIS/ WHO, but fulfills primary criteria of IUIS/WHO, considerable peer- reviewed literature available, measurement suggested by other

(continued on next page)

Table 1 (continued)

Protein	Family/ Similarities	Protein sequence availability	Allergic individuals			evidence for clinical reactivity to soy corresponding with reaction to the specific allergen	contra evidence	References	Endogenous allergenicity assessment - considerations
			Individuals tested and positives in IgE binding (examples)	Allergenic activity described by	Patients' group				
Soybean lectin	Agglutinin, Lectin_legB	yes	⁴³ 5/16 positive; ⁵⁷ commercially obtained SBA: 3/ 14 soybean sensitized bakers; ⁵⁸ peanut allergic patients weakly cross-react	IgE binding	adults, children	⁵⁹ SPT and oral provocation to peanut	⁶⁰ investigation of different lectins, soy lectin not reactive	43, 57–61	scientists (Ladics et al., 2014) fulfills primary criteria of IUIS/WHO, but evidence from peer- reviewed scientific literature is limited/non- existent. A peer-review process including clinicians is recommended.
Lipoxygenase		no	⁵⁷ 6/14 soybean sensitized bakers	IgE binding	adults			57	fulfills primary criteria of IUIS/WHO, but evidence from peer- reviewed scientific literature is limited/non- existent. A peer-review process including clinicians is recommended.
Kunitz trypsin inhibitor	trypsin inhibitors	yes	⁹¹ 6 patients; ⁵⁷ 12/14 patients; ⁶² 1/5 patients; ⁶⁵ 6/45 patients	IgE binding, inhalation testing (uncontrolled)	adults, children	⁶² DBPCFC and SPT to soybean; ⁶³ possible anaphylaxis linked to KTI; ⁶⁴ symptoms after inhalation (uncontrolled)		9, 57, 62– 65	not included in IUIS/ WHO, but fulfills primary criteria of IUIS/WHO, considerable peer- reviewed literature available, measurement suggested by other scientists (Ladics et al., 2014)
Gly m 39KD		12AA N- terminal sequence	⁹² 6 patients positive	IgE binding	adults, children			9, 66	does not fulfill primary criteria of WHO/IUIS
Gly m 50KD		17AA non terminal residue	15/29 patients positive	IgE binding	adults, children	⁶⁷ SPT to soybean hulls		67	fulfills primary criteria of WHO/IUIS, but evidence from peer-reviewed scientific literature is limited/non-existent. sequence is currently not available
P22-25		no	?	IgE binding (humans?)				68	does not fulfill primary criteria of WHO/IUIS
Gly m CPI	squash aspartic acid proteinase inhibitor	yes	⁶¹ 1/6 patients positive	IgE binding				61	does not fulfill primary criteria of WHO/IUIS
Gly m EAP	Late embryogenesis abundant protein	yes	⁶¹ 3/6 patients positive	IgE binding				61	does not fulfill primary criteria of WHO/IUIS
Unknown possible allergen	Protease inhibitor/seed storage/LTP family	yes	none	homology prediction				69	does not fulfill primary criteria of WHO/IUIS

References can be found in the supplementary information.

endogenous allergenicity assessments are described on the basis of the further strategy applied by WHO/IUIS, 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 become outdated in the future, making adaptations necessary.

4. Methodology for the assessment of endogenous allergens

2D-GE in combination with spot quantification can be used for direct comparative analysis of endogenous allergens between crops

developed by biotechnology and their comparators ([Rouquie et al., 2010](#)). However, for a more accurate assessment, mass spectrometry and quantitative ELISA are well established and suitable methods. 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 assessment of endogenous allergens in soybean from the current literature using these methods are elaborated below.

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 subsequently analysed by the most sensitive triple-quadrupole MS-based data acquisition mode (that is, targeted multiple reaction monitoring (MRM)), to be further validated by MS² 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, it was concluded that high endogenous allergen variability between varieties was rather a function of environmental conditions than of the variety itself. However, the crop variety also played a role for some allergens (Stevenson et al., 2012).

Although the above mentioned studies have developed a high-throughput and multiplexed LC/MS²/MRM method for quantification of 8–10 allergens (including Gly m 6 subunits) in a single analysis, data on other allergens and subunits are not available. For example, for the major seed storage protein Gly m 5, which consists of α -, α' - and β -subunits, only the α -subunit was measured.

Julka et al. (2012) studied the natural variability of the Bet v 1 homologue allergen Gly m 4 in 10 soybean varieties using 2D liquid chromatography followed by ultraviolet (UV) and MS detection of the intact protein. Gly m 4 quantification in soybean seeds was based on the UV response factor calculated from the purified reference standard of Gly m 4, whereas the MS detection confirmed the absence of any co-elution of other proteins together with Gly m 4 during the separation of protein extracts. The authors concluded that Gly m 4 was successfully quantified in the 10 commercially available soybean varieties; and the difference between the lowest and highest allergen content (approx. 360–600 $\mu\text{g/g}$) differed by less than a factor of 2. However, possible isoforms of the allergen were not assessed.

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

There are several examples available describing quantification of soybean allergens by ELISA. The ELISA method is a valuable and helpful tool to quantitatively assess endogenous allergens, and is able to provide accurate readouts. However, it has to be assured that defined antibodies raised in animals against single allergen molecules are used since only these antibodies make the quantification of single allergens in ELISA possible.

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

Hei et al. (2012) developed a robust and reliable double antibody sandwich ELISA to quantify Gly m 5 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/MS²/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 and Gonzalez de Mejia, 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 nor for all potential allergens included in the OECD consensus document. As described, there are also discrepancies between publications reporting on the variability of certain soybean allergens. 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. Environmental factors, like available nutrients or weather conditions, were shown to influence protein levels (Trtikova et al., 2015; Nguyen and Jehle, 2007). Here, epigenetic factors might play a role like promoter methylation influencing gene expression (Meyer et al., 1992) as well as post-translational mechanisms are able to alter the protein content of plants due to environmental stress (Guerra et al., 2015). Enhanced protein degradation due to heat stress has also been described (Chen et al., 2005). Another reason why discrepancies between publications seem to arise could be the presence of possible isoforms, in the case of Gly m 1 or Gly m 4, or subunits of Gly m 5 or Gly m 6. Sequence and structural similarities of isoforms or subunits might lead to recognition by the same antibodies in ELISA and sequence similarity might also lead to detection by the same signature peptide in MS. However, other antibodies might be specific for only certain isoforms or subunits and for some sequence deviations additional signature peptides might be needed in MS. To this end, the development of further protocols as well as fine tuning of current approaches should be highly encouraged. To be able to compare the allergen content of different soybean varieties, also between research groups, it would be helpful to optimise and standardise ELISA (including methods of protein purification and antibody generation) and MS methods. To this end, standardised and harmonised protocols using defined antibodies in ELISA or defined allergen peptides in MS, also considering possible sequence variability, could be developed and applied, providing a basis for optimal quantification and comparison.

5. Data interpretation

Determination of endogenous allergen levels should be carried out as a part of the comparative analysis of composition in the frame of GMO applications for market approval in the EU, as a mandatory requirement specified under the most recent European Regulation (EC, 2013). In general terms, for the compositional analysis, EFSA has developed a field trial design for the comparative compositional analysis which requires that apart from comparing the GM food to its conventional counterpart, a suitable set of non-GM reference varieties should also be considered in the assessment (EFSA, 2011). These varieties are used to provide an estimation of the ranges of natural variation of the allergen in question for the

same environmental conditions. There is a need for better understanding the natural variability in the content of allergens, and for collecting as many data as possible on natural variability of allergens in soybean varieties representative of the ones consumed by the at-risk population. For these data to be readily usable and comparable, the analytical methodology used as well as protein extraction procedures 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. In the case a GM plant differs statistically significant from its non-GM counterpart, displaying a higher concentration of one or several endogenous allergens which additionally falls outside the range of natural variation, the biological relevance as well as the impact on human health has to be evaluated in the next step.

Depending on the allergen in question for which an enhanced content was determined, specific isoforms of the allergen might be considered. Variants of allergens in soybean have been described for Gly m 1 and Gly m 4 and it can be speculated that the isoform proportion might play a role in allergenicity. Bet v 1, the major birch pollen allergen, which is very similar to Gly m 4 in soybean, is found in pollen as several isoforms which display different properties concerning their ability to cause allergic reactions (Ferreira et al., 1996).

Not only does the possible enhanced risk of eliciting an allergic reaction in already allergic individuals have to be assessed, but also the likelihood that a higher frequency of sensitisations might occur 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. It is currently challenging to evaluate the impact of an increased allergen content on sensitisation. However, the use of population dose-distribution curves obtained by DBPCFC is a relevant tool and could be taken into consideration to assess the risk for elicitation of an allergic reaction. However, as mentioned above, population dose-distribution curves are not readily available for single allergens, and they are not always obtained by challenging allergic individuals who are not at high risk of experiencing an anaphylactic reaction. Population dose-distribution curves with a readout for single allergen molecules are, in this context, especially important for soybean allergy, since soybean contains several “major” allergens and soybean 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 were enhanced in a GM crop compared with 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. For such individuals, the Gly m 4 content of soybean may be crucially important. Population 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 characterized and the allergen-content is known, population 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 benefit from future scientific research on component-resolved diagnosis and obtaining and strengthening the data on the capacity of single potential allergen molecules to elicit clinical symptoms of food allergy in allergic patients. 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 that 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.

Should an enhanced endogenous allergen content be detected, exposure considerations might also 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. For Gly m 1, safety measures are in place reducing the exposure to the allergen at storage facilities. A potentially enhanced allergen content in soybean should even strengthen the efforts of strictly fulfilling such precautionary standards. (Pont et al., 1997).

6. Conclusion

The assessment of endogenous allergenicity is required as part of the overall assessment of food derived from biotechnology in line with agreed international standards. The purpose of the assessment is to ensure that no overexpression of endogenous allergens has taken place which may result in greater risk to consumers. In general, connecting single protein molecules to clinical reactivity of IgE-mediated food allergy remains challenging. In particular 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 allergens in soybean as a part of the compositional analysis started to be required with the Implementing Regulation EU No. 503/2013 (EC, 2013), a need arose to identify relevant allergens to be measured. International criteria 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 proactively involve clinical scientists in the risk assessment process, when defining appropriate molecules to be measured and when finalising 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 a valuable basis 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.

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Appendix A. Supplementary data

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Transparency document

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Supplementary information:

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