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Editorial

A Special Issue of the Journal of Regulatory Science on Genetically Modified Organisms

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Ever since Herbert Boyer and Stanley Cohen [1] developed the first genetically modified organism (GMO) in 1973, advances in biotechnology – or genetic engineering as the science of gene manipulation has come to be known throughout the world – have made it possible to develop numerous plant crops, microorganisms, and more recently animals, whose genetic makeup has been modified to include one or more genes that confer desirable traits belonging to other organisms. After extensive research in the 1980s, the 1982 approval of the first GMO product developed commercially through genetic engineering: human insulin synthesized in genetically engineered bacteria [2], and the publication of the Coordinated Framework for the Regulation of Biotechnology, which involves the U.S. Department of Agriculture (USDA), the Food and Drug Administration (FDA), and the Environmental Protection Agency (EPA), by the White House Office of Science and Technology Policy (OSTP) in 1986 [3], the world saw the introduction of the first commercial GMOs in the 1990s. The OSTP updated the Coordinated Framework in 1992 and again in 2017. Excellent short and extensive reviews of the history of genetic engineering can be found in a blog by Gabriel Rangel [4] and Colwell [5], respectively.

More than 25 years later, GMO corn is approved for planting in 38 countries and GM soybeans are the most widely planted crop in the whole world. Many other GM plants are being grown today, including alfalfa, papaya pineapple, apples, potatoes, sugar cane, eggplant, etc. After years of mishandling, approval for raising GM salmon was granted by the FDA in 2015, but a court recently ordered the FDA to conduct an environmental risk assessment of the salmon [6], which is not yet available to consumers.

The development and expansion of approvals for growing GM crops has been the subject of stringent regulations in many countries. Many of these regulations are openly or surreptitiously based on the precautionary principle, and there is no global agreement on the requirements GM organisms must comply with to be approved for planting, growing, raising or importation. Some

countries rely on "substantial equivalence" in their regulations on genetic engineering, whereas others use a "case-by-case" approach. Because of the enormous implications of biotechnology on human and animal health, nutrition, and the environment, it would seem timely to pursue a standardization and globalization of such regulations. In 2000, the Cartagena Protocol on Biosafety – an international accord on regulating the transfer, use and handling of GMOs – was adopted by 172 countries (now 173) and went into effect in 2003 [7], and in 2003 the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Food Standards Program, through its Codex Alimentarius Commission, published a set of Principles and Guidelines on Foods Derived from Biotechnology [8]. Therefore, the basis for international harmonization of regulations on GMOs does exist, but many regulatory agencies around the world, for political or technical reasons, fail to update their requirements and corresponding methodologies, with the resulting delays and unnecessary costs associated with various elements of GMO regulations.

The Journal of Regulatory Science, now in the first year in continuous publication mode, is proud to publish its first Special Issue, which examines some proposals from industry to facilitate the structuring of a more widely accepted, science based, standardized future international regulatory framework for approval of genetically modified organisms.

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Regulatory Assessment of Off-Target Changes and Spurious DNA Insertions in Gene-Edited Organisms for Agri-Food Use

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Abstract

Worldwide, an increasing number of regulatory systems have begun to consider applications for the authorization of activities involving gene-edited organisms for agri-food use. Although a handful of countries have made advances in establishing regulatory criteria and gathering practical experience in this regard, there is still a general need for regulatory cooperation concerning capacity building and development of harmonized criteria. Consequently, many biotechnology regulators need to quickly become more acquainted with the numerous technological possibilities enclosed under the concept of "gene editing", and to incorporate criteria for their regulatory assessment. This article contains a simplified introduction to the state of the art in genome editing, described from a regulatory perspective. In particular, two issues of higher practical importance are covered in detail, namely, off-target effects and unintended DNA insertions. The detailed review of current evidence regarding those issues serves as the basis for proposing concrete regulatory criteria to address them.

Keywords: gene editing, genome editing, off-target, CRISPR-Cas, biotechnology regulation, new breeding techniques

Abbreviations: ACR, anti-Crispr proteins; CRISPR, clustered, regularly interspaced, short palindromic repeat; crRNA, CRISPR RNA; Cas, CRISPR-associated; GE, genetically engineered; GMO, genetically modified organism; LMO, living modified organism; NBT, new breeding techniques; ODM, oligonucleotide-directed mutagenesis; PAL, potentially affected locus/loci; PAM, protospacer adjacent motif; nt, nucleotide/s; r-DNA, recombinant-DNA; sgRNA, single-guide RNA; SDN, site-directed nuclease; TALEN, transcription activator-like effector nucleases; WGS, whole-genome sequencing; ZFNs, zinc-finger nucleases

1. Introduction

1.1. The Established Regulation of "Modern Biotechnology"

With the advent of recombinant-DNA (r-DNA) techniques applied to the genetic modification of organisms for agri-food uses, governments developed *ad hoc* regulatory frameworks for the so-called "modern biotechnology" [16, 7, 79]. Typically, such *ad hoc* regulation does not replace but supplement other regulations of broad applicability to organisms of agricultural use and the foodstuffs derived from them.

Concurrently, intergovernmental organizations developed international standards for "modern biotechnology". The main corpus of regulatory guidance in this regard includes several Guidelines from *Codex Alimentarius* [132], the text of the Cartagena Protocol on Biosafety [14] and many reference documents from the Organization for Economic Co-operation and Development [84].

These national regulations and intergovernmental documents were crafted from the early 1990s to the late 2000s. In that period, the only kind of products developed for agri-food use with the aid of recombinant-DNA technology consisted of transgenic organisms. Other agricultural "biotechnologies" also emerged in that period, such as plant micropropagation, marker-assisted breeding, animal cloning, microbial bioinputs, and mutagenesis from somaclonal variation. However, these other innovative biotechnologies do not use r-DNA, and for that reason, they were not included in the scope of the new regulations intended for transgenic organisms.

In general, national regulations and international guidelines are nowadays quite developed and harmonized in terms of their scientific basis and the principles to conduct a safety assessment of transgenic organisms. Basically, these frameworks begin with a comprehensive characterization of the artificial genetic construct inserted in a host organism and the resulting novel (or modified) traits. Such characterization constitutes a foundation for subsequently performing risk analyses based on a comparative approach with "conventional counterparts" (typically, wild-type organisms with a similar genetic background). The core of those analyses is the safety assessment of novel substances - in most cases meaning new proteins - and changes

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in composition (food/feed safety assessment), or biosafety assessment of changes in phenotype and the interactions with the environment.

In contrast to a high level of harmonization regarding the underlying scientific criteria, international guidance and national regulations are very diversified regarding the use of terms and definitions mandating which organisms and products are under their scope. Examples of this diversity include "genetically modified organism" (GMO), "living modified organism" (LMO), "modern biotechnology products", "genetically engineered (GE) organism", "organisms/products derived from recombinant-DNA", "organisms with novel traits", and "organisms containing pest components". Moreover, the term most frequently used by governmental regulations, which is "GMO", can have diverse operational definitions in different territories.

Nevertheless, these discrepancies were of little relevance when only transgenic organisms were presented to the regulators because, in most cases, they would be encompassed under any of the definitions for these terms. However, the lack of harmonization in this respect became very relevant during the last half of the past decade with the advent of gene (or genome) editing applied to species of agricultural use.

1.2. The Evolution in The Regulation of Gene-Edited Products

In the early 2010s, some regulators in the field of agricultural biotechnology became aware of innovations in breeding techniques using r-DNA technology [70]. These innovations, in many cases, can be used to generate changes in the host genome that do not result in transgenic organisms. They were called "new breeding techniques" (NBTs), and gene editing is their most prominent member.

Eventually, it became necessary to clarify the status of organisms and products obtained using these techniques as subjected, or not, to governmental regulations for agricultural biotechnology [51, 109]. During the first half of the decade, regulators in Canada, the United States, and some European countries took some case-by-case decisions in this regard [19, 45]. However, their decisions were based on very particular scoping definitions used only in their territory; therefore, these early decisions cannot be readily replicated in third countries having different definitions.

In 2015, Argentina issued a regulation establishing general criteria and a mechanism to define the regulatory status of these products [124, 126]. The Argentine regulation is based on definitions taken from the Cartagena Protocol on Biosafety, which are the more widely recognized. Therefore, the Argentine approach was the first that could be replicated by other countries also abiding by the Cartagena Protocol definitions. To date, another seven countries in Latin America have enacted similar regulatory approaches. At the same time, other countries in Asia and Africa have also endorsed approaches that may be compatible for most products or are officially considering to do so [22, 33, 98, 118, 120].

Finally, in 2018 the European Court of Justice issued a legal interpretation that contradicted earlier regulatory decisions of European Member States as well as official scientific reports [111]. Currently, many governments are still analyzing if (and eventually how) to regulate gene-edited agricultural organisms and the products derived from them [32, 45, 59, 88, 24].

1.3. Scope of this Article

1.3.1. Checking for off-target activity and DNA insertions in SDN products

Argentina has now accumulated significant practice in the regulatory analysis of gene-edited organisms [66, 127], and also has cooperated intensely on this subject with various governments and intergovernmental organizations. From this experience, it has become clear now that two practical issues of high regulatory relevance require harmonized approaches. These are the assessment of, (a) the so-called "off-target" activity, and (b) spurious DNA insertions. Therefore, this article proposes model criteria in this regard. These criteria have not been crafted merely on theoretical considerations; they are a result of several refinement rounds from practical experience on the regulatory assessment of different gene edited products.

To begin, we may consider the simpler scenario where gene editing is applied to generate a small number of point mutations and/or a short *indel* (insertion and/or deletion of a few nucleotides) in a specific locus. In this scenario, changes in the DNA sequence are due only to random spontaneous errors in the DNA repair process following the cut performed by a site-directed nuclease (SDN). Therefore, for this scenario the expected result should be a short stretch of nucleotide base substitutions and/or deletions and/or additions (due to DNA polymerase error), but not in the insertion of any pre-existing DNA fragment. This result/scenario is named "Site-Directed Nuclease, Type 1 (SDN1)" according to the current regulatory jargon [20]. Typically, the intent of such interventions is to knock out an endogenous gene.

1.3.2. Relevance and timeliness

The criteria presented here would be useful under any of the diverging options for regulatory approaches that are being considered worldwide. On one side, they would be applicable in situations where it may be necessary to assess if a product is (or is not) a GMO or the analogous category of regulated products. On the other hand, the criteria would be useful also in situations where it has already been decided that a product will be regulated as GMO (or analogous category). In the latter case, the criteria would be the first step in identifying those genetic alterations that would be further scrutinized by established GMO risk analysis practices.

Moreover, since many cases correspond to agri-food products that are traded internationally, these regulatory criteria, if widely adopted, could help in facilitating an unavoidable crosstalk between similar as well as dissimilar regulatory frameworks across national borders.

The specific issues and criteria discussed in this article are thus quite independent of the regulatory environment where they may be applied. In addition, they are based only on scientific considerations. They are not technically trivial, since they require considering a state of the art that is quite diversified and

continuously evolving. They tackle a regulatory challenge consisting of balancing the case-by-case approach with the principle of avoiding arbitrariness and providing equal treatment to all applicants under laws and regulations.

1.3.3. What this article is not about

This article is not intended to address any of the topics under debate with regard to the governance of genome editing in agriculture [6, 99], nor neglect their importance. These topics include whether the gene-edited products are GMOs or not [110], ethical aspects [21] and socioeconomic considerations [53, 125, 127], consumer acceptance [44] or the applicable risk assessment criteria [19, 29]. Instead, this article is anticipating that whatever the outcome of such debates, the regulatory criteria presented here would be useful for regulators when implementing any of the policy options that are being considered worldwide.

This article suggests how to harmonize the approach for searching every possible change in genetic sequences derived from a gene-editing intervention, considering that it would be a common need of regulators in different countries and for different purposes. However, there will be subsequent regulatory steps after such a search that are beyond the scope of this article and would be different for each case.

After searching, there would be a need for *characterizing* each genetic change. Characterization needs a case-by-case approach. For instance, it would be different if assessing a mutation that knocks out an endogenous gene vs. another that "resurrects" a pseudogene [74]. Also, a specific characterization would be warranted, for instance, if novel polypeptides are possibly expressed after the random insertion of foreign DNA. Finally, characterization is dependent on specific regulatory endpoints, such as analyzing if the resulting organism should be regulated as GMO or not or for the safety assessment of each change.

2. Further Considerations

2.1. What Else Can Happen at or Around a Cleaved Site?

As explained above, the activity of SDNs can lead to point mutations and indels in the cleaved site, generated during the subsequent DNA repair process. In contrast, ordinary SDNs are reported not to cause epigenetic changes [65]. Nevertheless, other kinds of changes in the host genomic sequence caused by SDN cleavage are possible, and they are briefly reviewed next.

2.1.1. Unintended DNA insertion in cleaved sites

Genome editing methods based on genomic DNA cleavage can result in unintended DNA insertion at the cut loci. Such inserted DNA may come from the host cell as well as from foreign sources [2, 46, 67, 37]. In the case of foreign DNA, the possibilities are not limited to the more obvious source of plasmids purposely introduced in the cell for expressing the SDN. The insertion of foreign DNA has been reported even in case of allegedly "DNA-free" techniques based on the intracellular delivery of nuclease proteins. In one case, for instance, the foreign DNA was identified as the *in vitro* transcription template of a Cas nuclease's RNA component; such an outcome could not be avoided even after DNAse I treatment [3]. Besides, more remote sources of minute DNA contamination have also led to foreign DNA insertions in cleaved loci. For instance, *E. coli* DNA from bacteria used to multiply plasmids, or mammalian DNA from fetal serum added to culture media [85]. For this reason, in the current state of the art no SDN technique can be claimed to be absolutely "DNA free". Developers should minimize the presence of foreign DNA and genomic DNA breakage as much as possible.

The latest improvements in sequencing and bioinformatic tools allow for a more exhaustive search of foreign DNA insertions compared with the early days of GMO safety assessment when that search could be based only on Southern Blot techniques. These improved techniques, however, given their level of detail, raise the issue of how to distinguish foreign (artificial) from endogenous (spontaneous) DNA insertions, especially for very short sequences.

2.1.2. Mutations from refilling

It has been reported [96] that the gap-refilling activity of the DNA repair mechanisms acting after SDN cleavage can lead to mutations at a short distance (up to 24 nt up/downstream) of the cleavage site. Such "satellite" mutations can happen even if there is no mutation in the cleavage site itself.

2.1.3. Local chromosomal rearrangements

It has also been reported recently [61] that large deletions and complex rearrangements (translocations, inversions, or large insertions) can result from SDN activity. Such chromosomal rearrangements can be quite distal to the cut site. Therefore, they can be missed by conventional short-range sequencing but they would still be in reach of long-range PCR or long-read sequencing. Such rearrangements also seem to be possible even if the sequence of the cut site is not modified.

2.2. CRISPR-Cas Nucleases as a Case Study

Various site-directed nucleases have been used for gene editing over the past two decades [90]. However, the advent of the CRISPR-Cas technology in 2012 [50, 92] generated an enormous increase in the number of projects and developers of genome-edited organisms [141]. CRISPR-Cas is considered easier to use by many researchers, particularly in regard to the programmability of the target sequence.

Clustered, regularly interspaced, short palindromic repeat (CRISPR) RNA-guided nucleases are highly efficient genome editing tools. These CRISPR-associated (Cas) ribonucleoprotein complexes with endonuclease activity generate a double-strand break in those DNA molecules having sequence complementarity to a specific section of its RNA component (CRISPR RNA "crRNA" or single-guide RNA "sgRNA").

2.2.1. Sequence specificity

The specificity-relevant section of the Cas RNA component consists of a "protospacer adjacent motif" (PAM), followed upstream by a so-called "spacer" sequence. A perfect pairing of the PAM with DNA is required for cleavage, while mismatches may be tolerated in the spacer.

The first nucleotides within the spacer sequence that are located immediately upstream of PAM are called the "seed region"; mismatches in this region greatly impair nuclease activity. Conversely, the middle of the spacer sequence appears to have a higher tolerance for mismatches [31, 91, 50, 52, 71, 82, 115].

Significant knowledge on the molecular basis of mismatch tolerance comes from studies of the ribonucleoprotein/DNA interaction; these studies combine molecular modeling, statistical thermodynamics, and kinetics [25, 48, 52, 129, 135]. The first critical stage of the enzymatic mechanism is PAM site recognition. The second one is the formation of a DNA/RNA bound structure (R-loop), leading to a conformational gating mechanism driven by the 14th-17th nt region of the spacer.

It has been shown that the specificity of Cas nucleases can be modified by protein mutation, leading to alterations in mismatch tolerance and the PAM canonical sequence [8, 12, 57, 58, 106, 140]. Specificity has also been modulated by creating homo- or hetero-dimeric fusion proteins [91, 119, 36].

2.2.2. Variants of Cas proteins

There are many types of Cas nucleases in nature, and just a handful have been employed so far for genome editing. The more widely used and perhaps best characterized Cas nuclease was obtained from *Streptococcus pyogenes* (SpCas9). SpCas9 PAM has the canonical sequence "NGG". The possible length of its spacer sequence ranges from 17 to 24 nt; and its seed region is reported to measure from 8 to 12 nt.

In addition to SpCas9, other natural Cas9 homologs include those obtained from *Neisseria meningitides* (NmCas9), *Treponema denticola* (TdCas9), *Staphylococcus aureus* (SaCas9), and *Streptococcus thermophilus* (StCas9) [23, 54, 112, 128].

Cas12a (a.k.a. Cpf1) is a different group claimed to have higher specificity than Cas9 nucleases [114]. The group includes the Cas12a nuclease of *Francisella novicida* (FnCas12a), *Acidaminococcus sp. BV3L6* (AsCas12a), and *Lachnospiraceae* bacterium (LbCas12a) [56, 116]. Cas12b is a related group, including representatives from *Alicyclobacillus acidophilus* (AaCas12b), *Alicyclobacillus acidoterrestris* (AacCas12b), *Bacillus thermoamylovorans* (BthCas12b), and *Bacillus hisashii* (BhCas12b) [75]. These other Cas nucleases have PAMs that are different from SpCas9 in canonical sequence and length (from 2 to 7 nt). The length of the spacer region and the relative span of the seed subregion within it can also vary.

2.2.3. Other factors affecting specificity

The standard design of Cas9 spacer sequences has a length of 20 nt. Fu et al. [31] showed that using a truncated RNA molecule having a spacer of 17-18 nt can decrease the generation of mutations in loci harboring mismatches. Conversely, spacer sequences with a high GC content (50-70 percent) might favor tolerance towards mismatches [71, 119, 134, 47].

It has been suggested that the delivery of purified Cas ribonucleoprotein complex may result in lesser cleavage of loci having mismatches compared to *in vivo* expression of the protein from a Cas gene delivered to the cell. This difference is explained using kinetic speculations based on the fast degradation rate of the protein [55]. There seems to be enough evidence from several studies that increases in cleavage efficacy (e.g., by using a more processive nuclease, increasing GC content, generating a high concentration or a longer-term presence of the nuclease) likely lead to a trade-off regarding specificity.

Finally, the current state of the art also includes techniques for modulating "tissue specificity". They include the application of anti-CRISPR (Acr) proteins [42] and photoactivatable systems [81]. However, such techniques are not expected to modify "off-target specificity" and would be applied mostly in health-related applications and basic research.

In summary, many studies show that gene editing specificity displays variability from one intervention to another. However, such variability has boundaries that can be assumed for predicting the broadest scenario with regard to loci that could be affected. Moreover, these studies also illustrate that it is possible to find and characterize every significant change generated by a particular gene editing procedure.

3. "Off-Target" Analysis

3.1. From "Off-Target" to "Potentially Affected Loci"

Numerous reports have shown that Cas nucleases are capable of performing their DNA cleavage activity in loci having some mismatches with the spacer sequence [46, 117, 134, 140, 62, 121, 101]. As a result, one of the main concerns related to technologies based in Cas and other SDNs is the possibility of "off-target" cleavage [139].

A simplistic portrayal of the "off-target" issue would be a situation where: (a) the sequence of the locus that is intended to be modified is present only once in the genome and has perfect sequence identity with the spacer sequence (i.e., one "intended" "target" loci with zero mismatches); and concurrently (b) there are other loci with imperfect sequence identity scattered along the rest of the genome that are not intended to be modified (i.e., "unintended" "off-target" loci with mismatches).

However, real-world situations are usually more complex. On one side, the developer's intention could be to modify more than one related sequence, perhaps not having a 100 percent sequence identity among them (e.g., when attempting to modify different alleles or homolog genes all at once). In such cases, the design of the spacer sequence would contemplate mismatch tolerance to reach every locus of interest. On the other hand, in addition to a locus of interest, there could be other loci with 100 percent identity with the PAM + spacer sequences (e.g., genes with repetitive sequences or random coincidences throughout the genome). In such situations, the meaning of terms like intended/unintended and target/off-target can be blurry (and ultimately irrelevant) from a regulator's perspective. Moreover, such "target/off-target" labels usually lead the

developer to study potentially affected loci using different tools and depth, on a subjective basis. Subsequently, this can lead to a situation where the developer/applicant presents the case to regulators in a way that may mislead them towards paying different attention to "loci of interests" compared with "off-target loci". Nevertheless, all potentially affected loci should receive the same treatment in regulatory issues like deciding if a product is a GMO or not, and for the safety assessment of genetic modifications.

Therefore, the term "potentially affected locus/loci" (PAL) is proposed to encircle all possibilities under a homogeneous umbrella of regulatory oversight, leaving aside the developer's subjective point of view. PAL would include every sequence of the host genome that has a significant probability of being altered by the activity of the SDN.

3.2. Ex-ante Assessment of PAL

Whole-genome studies have confirmed that Cas proteins do not affect loci lacking homology with the spacer sequence [117, 27]. Therefore, the identification of PAL should be based exclusively on a bioinformatics search of loci having a certain degree of homology with the PAM and spacer sequences.

With so many different Cas proteins and protocol options available that can affect specificity, it becomes a difficult task to establish standard consensus rules for identifying PAL. Moreover, caution should be taken when generalizing results across different reaction milieus. *In vitro* environments vs. bacterial cells vs. eukaryotic cells can display differences affecting protein function and kinetic and thermodynamic factors such as redox potential, pH, water activity, DNA repair machinery, as well as the concentration of key molecules and their degradation routes.

Developers usually base their searches for PAL on specificity rules taken from a reduced number of publications on the NnCasX protein that they are using, or a closely related one. Furthermore, in some cases developers just rely on one of the online tools available for this purpose [4, 17, 38, 39, 43]. However, these online tools do not always agree on their results, given that their algorithms were devised separately by different experts, based on different sets of publications.

This situation is particularly problematic for regulators, who have the three following options:

(i) Rely blindly on the applicant's (or the server's) criteria. This option would not be a preferred choice, since an important part of the regulator's oversight duties would be discharged to the interested party (or even worse, a third party that takes no legal responsibility on the reliability of the prediction, in the case of online tools). The applicant may select rules with bias or incomplete knowledge on the state of the art, thus missing and underreporting PAL. Besides, this would lead to different applicants arbitrarily receiving tailored regulatory stringency. In summary, there would be unacceptable regulatory slips regarding both safety assessment and fairness.

(ii) Wait until the state-of-the-art leads to repeated validation of distinctive specificity rules for each protocol (i.e., each particular combination of a NnCasX protein, host organism, and delivery method). This option could appear as the more appropriate one in theory. However, in practical terms, it would lead to a never-ending moratorium on the use of the technology, or at best to the forced use of just a couple of older and best characterized Cas proteins, thus halting innovation.

(iii) Apply a set of canonical rules based on the more characterized Cas proteins, including how to adapt these rules cautiously in case of novel Cas versions and protocols. This option seems to be the more reasonable one, after considering that there is already a significant amount of knowledge about these proteins; thus, it is further developed below.

3.2.1. General considerations

The rules for predicting PAL should be used by default in most cases, for the sake of fair treatment to all applicants and to seek harmonization between regulatory offices. They should incorporate evidence from a broad base of literature sources. The rules should be clearly made available to the public in advance, so that any potential developer can incorporate (and consult with regulators about) them during the design stage of a gene-editing procedure.

Regulators should exert a conservative criterion regarding how to identify all the genome locations potentially affected by the gene-editing procedure. Conservative in this context means that less available information on specificity may lead to more stringent criteria. Such criteria would be prone to identify more "false" PAL as a trade-off in avoiding to miss any "real" PAL (i.e., type II error over type I error) [11].

Being conservative, the rules should initially treat the latest innovations (for instance, using mutant Cas9 proteins) with the same or increased stringency. Lowering stringency shall be contemplated as an infrequent case-by-case possibility, only after strong evidence of increased selectivity is gathered and where the evidence also allows extracting clear alternative rules. "Strong evidence" in this context means a significant number of studies, from different authors, under similar conditions (NnCasX protein, host organism, delivery method) as the case presented by the applicant.

Comprehensive *in vivo* and *in vitro* empirical studies searching for effectively affected (mutated) sites have been performed in diverse species [27, 28, 30, 91, 64, 65, 75, 80, 86, 89, 95, 117, 130, 142]. These studies on specificity usually report the number of loci that have been affected (mutated) despite having mismatches against the sequence of the spacer region. Studies are diverse, but most of them report a tolerance of typically one (occasionally two) mismatch in the seed region and up to three (occasionally up to five) mismatches in the whole spacer region. In contrast, some studies report no other affected loci than those with perfect matches.

There could be many explanations for such varied results, ranging from differences in the Cas protein and the protocol used, up to the strategy used to identify affected loci. Another relevant factor could be the trueness of the genomic sequences

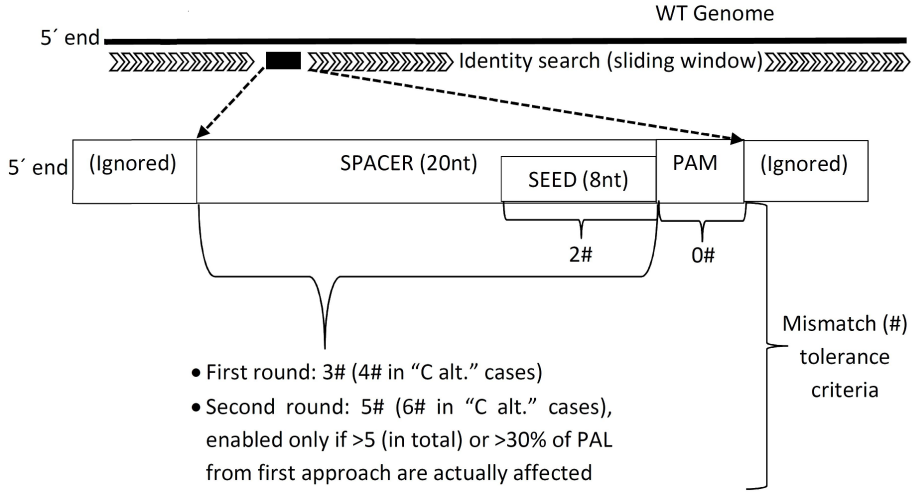

Figure 1: Proposed rules for identifying PAL

used for the *ex-ante* bioinformatics analysis compared to the actual genome of the specific strain/breed/variety to be gene-edited; for instance, when only a generic reference sequence for the species is available.

Off-target mutations appear to be reported more frequently in mammals than in plants. However, it is not clear if such difference is factual or just an artifact from more intense search in mammalian studies (given serious inferences for human health applications), vs. lesser studies in plants (where removal of mutations is feasible through backcrossing) [38].

3.2.2. Proposed rules

Considering the current state of the art, for regulatory purposes in agricultural applications, a locus should be assumed to be a PAL when **all the following** apply (see also Figure 1):

(i) It has perfect complementarity to the PAM. This assumes that the regulator acknowledges the PAM region to be sufficiently characterized for the nuclease used (such as the "NGG" motif in SpCas9). If this is not the case, only the first two nucleotides of the alleged PAM region would be assumed to be acting as PAM (i.e., not allowing mismatches), and the remainder would be considered to be part of the seed region. This approach is conservative because, in any case, it would increase the number of PAL and thus would lean towards type II errors while avoiding type I errors.

(ii) It has up to two mismatches in the seed region, assuming an 8 nt seed region for any NnCasX protein. Although the seed region has been postulated to be longer in some cases, this shorter assumption would, in any case, increase the number of PAL.

(iii) It has up to three mismatches in the whole spacer region, including any mismatch in the seed region.

Most designs use a 20 nt spacer region. However, the same limit of three mismatches should be used for other lengths, as follows:

(a) If a truncated RNA molecule is used to shorten the spacer (17-18 nt), the same limit of three mismatches would, in any case, increase the number of PAL.

(b) If the applicant claims to be using a "shorter" spacer but without truncating the RNA molecule (i.e., there is still a short stretch of 2-3 contiguous nucleotides at the tail of the molecule that could be considered part of a 20 nt spacer).

(c) If the use of a spacer *longer* than 20 nt is claimed. For regulatory analysis, the extra nucleotides should be ignored and still apply the same limit of three mismatches over a spacer region of a 20 nt. To accommodate different types of uncertainties, the limit of three mismatches in the whole spacer should be increased to four in any of the following cases:

- The sequences used for the bioinformatics analysis are not from the very same strain/breed/variety employed later in the gene editing procedure.
- Usage of a Cas nuclease that has very few studies on their specificity (regarding homologs from other microorganisms and mutant versions as different proteins). This criterion would apply especially in cases where the nuclease or the protocol is postulated to have "increased" cleavage activity (which has been shown to antagonize specificity).
- The absence of any practice that may reduce the number of loci effectively affected. This includes, where possible, designing a spacer with a low GC content (<45 percent), performing ribonucleoprotein delivery, and doing repeated backcrossing (>3 times) to bred-out undetected affected sites.

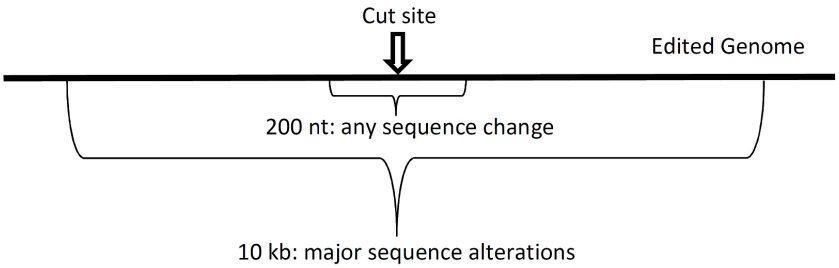

Figure 2: Bioinformatic analysis of PAL after application of the gene editing technique

The 3-4 mismatch limit is based on the assumption that very few PAL are actually found to be changed later. This assumption allows for avoiding an initial characterization of unlikely affected sites having a higher number of mismatches. Nevertheless, affected loci with up to six mismatches have been reported; this seems to be the limit imposed by the ribonucleoprotein/DNA binding process thermodynamics. Therefore, in cases where a significant number of PAL end up being affected (>5 in total or >30 percent of all PAL) using a 3-4 mismatch limit, and if the applicant is still interested in continuing, the analysis shall be repeated and expanded to loci with 5-6 mismatches.

These rules are devised on the assumption that there is a complete set of reference genome sequences available for the organism before the technique is applied, thus allowing the prior bioinformatics analysis. Nevertheless, this does not mean that deregulation shall be impossible in cases where such a reference genome is not available. A speculative possibility in this regard is the use of unassembled whole-genome sequences, which would be cheaper (S. Feingold, personal communication, November 28, 2019). Another possibility consists in the use of physicochemical methods for isolating genomic DNA fragments containing PALs; for instance, affinity chromatography using modified SDNs [131]. Finally, an alternative solution could be the "inverse approach", based on identifying affected loci after the gene editing procedure has been performed (as described later).

3.3. Ex-post Analysis of PAL

After executing a gene editing technique, in order to find which PAL have actually been affected by the SDN, developers may resort to preliminary analysis such as PCR/restriction enzyme assay [26, 48, 130, 68, 102] or T7E1 assay [134, 100]. However, for regulatory purposes these tests provide insufficient information and could miss some kinds of genomic sequence alterations. Therefore, the following should be required for regulatory purposes:

3.3.1. Whole-genome sequencing

Whole-genome sequencing (WGS), with at least 20 passes is the most straightforward way to assess if and how a PAL has been affected by the activity of the SDN. It allows exploring any PAL with any number of mismatches. It also allows any DNA insertion or satellite mutations or chromosomal rearrangements to be discarded. Moreover, this option is less prone to raise debates between applicants and regulators regarding experimental design and interpretation of results. Besides, in case of controversy regarding the parameters of the sequence analysis, it is relatively simple to perform them again.

The use of WGS does not mean that a whole-genome comparison with the reference sequence should be made (except when the "inverse approach" is used, see below). Such a comparison will always report differences in other locations not related to the PAL, which are expected due to spontaneous mutation, somaclonal variation, or pre-existing differences between the genome of the strain/variety/breed used and the reference genome [27, 117]. These kinds of changes are not a consequence of SDN activity and are not under the regulatory frameworks usually applied to agricultural biotechnology products. However, a few countries have a very particular criterion in this regard, including France (according to the latest court interpretations, see [63]), or perhaps Canada, if one of those other spurious mutations generates a novel trait [103, 93]. Therefore, only the WGS data corresponding to the PAL identified earlier should be used. The suggested approach for the bioinformatics analysis applied to every PAL should involve (see also Figure 2):

(i) Sequence alignment of a region spanning 200 nucleotides centered in the predicted cut site (100 upstream to 100 downstream). This would enable analyzing for (a) indels or change of bases at the cut site, (b) DNA insertion in the PAL, and (c) adjacent mutations from repair/refilling.

(ii) Sequence alignment of a region spanning 10 kb centered in the predicted cut site (5000 nt upstream to 5000 nt downstream). Parameters of the comparison should be adjusted for finding and reporting large deletions and complex rearrangements.

3.3.2. Alternatives to whole-genome sequencing

As mentioned earlier, WGS would be the preferred option for obtaining regulatory data on genetic changes derived from a gene editing procedure. However, this option may have a prohibitive cost in some cases. Therefore, alternative methods based on other techniques but still generating information of similar regulatory value may be used in some cases. The cost and hustle of these alternative approaches increase with

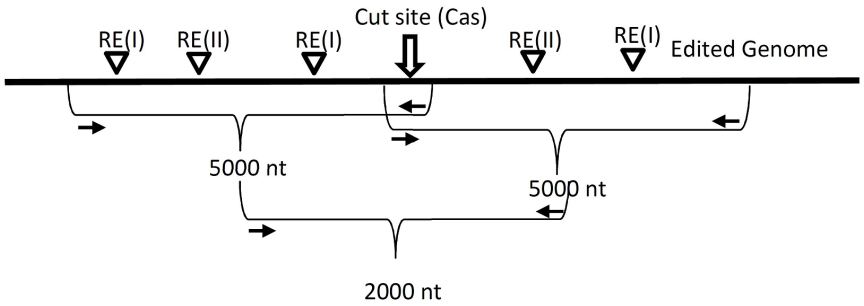

Figure 3: Alternative PCR/restriction enzyme analysis

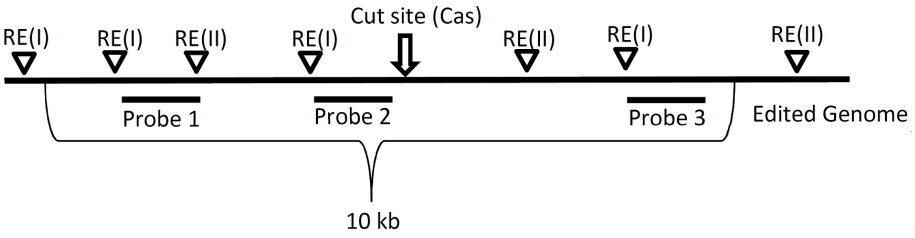

Figure 4: Alternative Southern blot analysis

the number of loci to be studied. Therefore, its relative convenience relies on the developers' capacity to find spacer sequences that minimize the number of PAL. Besides, their relative convenience will decrease over time as WGS cost gets cheaper. The suggested approach, regarding every PAL, would involve:

(i) Standard PCR, followed by sequencing and alignment, of a region spanning at least 200 nucleotides centered in the predicted cut site (100 upstream to 100 downstream). This approach would enable analyzing for (a) indels or change of bases at the cut site, (b) DNA insertion in the PAL, and (c) adjacent mutations from repair/refilling, and

(ii) a single long-range PCR spanning a 10 kb region centered in the predicted cut site (5000 bp upstream to 5000 bp downstream), followed by sequencing of the PCR fragment and alignment, where parameters of the bioinformatics comparison should be adjusted for finding and reporting large deletions and complex rearrangements, or

(iii) alternative A: Three separate long-range PCRs covering a region spanning approximately, (a) 5000 bp upstream to 50 bp upstream of the predicted cut site; (b) 2000 bp upstream to 2000 bp downstream of the predicted cut site; (c) 50 bp downstream to 5000 bp downstream of the predicted cut site. The exact location of the primers and PCR parameters should be adjusted (i.e., appropriate GC content, avoiding repetitive sequences, etc.) to avoid artifacts or misleading results. In addition, an RFLP analysis with two different restriction enzymes should be applied to assess the presence/absence and size of the amplicons. The restriction enzymes used should cut at least once on each amplicon according to the reference sequence and be used in combination (see also Figure 3), or

(iv) alternative B: Southern blot analysis of a DNA fragment spanning 10 kb centered in the predicted cut site, by using at least three probes spanning that fragment in combination with separate treatments using a different restriction enzyme each, chosen to cut in different sites spanning the fragment (see also Figure 4).

With regard to (ii), (iii Alternative A), or (iv Alternative B) above, it is relevant to note that all of them would add a significant regulatory cost and are based on currently scarce evidence (only [61]) concerning large deletions and complex rearrangements. Therefore, as more scientific evidence accumulates, this requirement might be left out in cases where the regulator has confidence that local chromosomal rearrangements are not likely to have occurred.

3.4. "Inverse Approach": Ex-post Detection of Affected Loci

An alternative approach that does not require presenting an *ex-ante* assessment of PAL begins with a comprehensive whole-genome comparison between the gene-edited organism and a closely related, wild type reference genomic sequence. As mentioned, such whole-genome comparisons will report numerous differences, most of which are not likely to be caused by the action of the SDN. Therefore, after all differences are found, the corresponding local sequences in the wild type genome should be tested with the PAL rules described previously.

This alternative approach has pros and cons. The reference (wild type) genomic sequence should be exactly the same strain/breed/variety used; if not, the parameters of the bioinformatics comparison may become quite subjective and therefore

debatable. The quantity of PAL is not examined *ex-ante*; therefore it might make more sense for techniques where the ability to redesign the tool to reduce the amount of PAL is more limited (e.g., TALEN vs. Cas). It may lead to a higher cost (two WGS determinations, on the wild type and edited organisms); however, that cost is put off until a gene-edited organism of a satisfactory phenotype is obtained.

4. Spurious DNA Insertions (Outside of PAL)

It has been shown that spurious insertions of foreign DNA in random locations (different from PAL) can occur under certain circumstances. This outcome seems to have a significant probability for techniques that introduce high quantities of DNA in the cell (e.g., those expressing the Cas and its RNA component *in vivo*). The latter is especially valid when combined with biolistic or other physical introduction methods that may increase the rate of random nuclear DNA breakage. In contrast, no random DNA insertion has been reported in protocols that use modified *Agrobacterium*-mediated methods to deliver the nuclease gene [5]. A recent article by FDA regulators [83, 137, 10] is enlightening about the possibility of spurious foreign DNA insertions, as well as insertions in PAL, to stay unnoticed by the developers because of assumptions about the "cleanliness" of an SDN technique.

Conversely, the probability of having spurious foreign DNA insertions can be dismissed as highly unlikely in cases where the presence of DNA in the milieu introduced in the cell has been greatly reduced. This is applicable for techniques where the nuclease protein is delivered into the cell instead of its gene, the Cas RNA component has been synthesized chemically, and the milieu to be inserted in the cell has been treated extensively with DNAse I, fulfilling the three conditions altogether.

In any case, further breeding, including backcrossing, as well as phenotype studies showing no changes in polygenic traits can increase regulatory confidence in many aspects, including the absence of DNA insertions in the final product that could have safety relevance.

4.1. Whole-Genome Sequencing

A bioinformatics search for foreign DNA insertion should be required in every case where the developer has performed a WGS. It should be based on the alignment of sliding windows of 100 nt having 100 percent identity. This comparison, when applicable, should be made against the following:

(a) every DNA known to have been purposely introduced in the cell; in the case of restriction fragments or PCR amplicons from a larger DNA molecule, the study should be made against the whole larger molecule;

(b) every DNA molecule used as a template in the process of producing the ribonucleoprotein complex in separate expression systems, including both the templates for the Cas protein and the RNA component;

(c) an appropriate whole reference genome of the system used for producing plasmids and/or as a separate expression system for Cas protein, typically *E. coli*; and

(d) an appropriate whole-genome reference sequence of organisms contributing with components of culture media that are presumed to contain considerable amounts of DNA (typically *Bos Taurus* in an animal cell culture medium, because of fetal calf serum).

Perhaps (c) and (d) would be an over-exaggeration for those cases described previously as having minimal possibilities of spurious DNA insertions. However, if the applicant has already invested in a WGS determination and the associated capabilities of bioinformatics analysis for assessing PAL, this additional request would not be significantly costly. Besides, the empirical experience gathered this way will enhance the regulators' ability to address different cases where other applicants base their analysis in one of the approaches described next.

4.2. Alternatives to Whole-Genome Sequencing

When the applicant is not able to provide WGS data, regulators should decide if there is a need for demanding proof of absence regarding spurious insertions of foreign DNA. This decision can be taken based on counting "flags", corresponding to those factors mentioned before that affect the possibility of having such insertions.

"Red flags" would be raised for protocols that are prone to generate random breaks in the genome and introduce significant amounts of DNA in the cell. Conversely, "green flags" can be acknowledged when repeated backcrossing has been performed and extensive phenotype information about polygenic traits is available.

If, after counting flags, such proof is required, in the absence of WGS it could be based on PCR and Southern blot analysis. For this purpose, it can be acknowledged that biotech regulators have plenty of experience with the analysis of transgenic organisms having transformation events. In that analogous situation, the applicant has to prove how many independent foreign DNA insertions from the construct and its molecular vector may be present. For years, this has been done with a combination of PCR and Southern blot analysis. Adhering to a strict scientific base as well as the principle of fairness towards all applicants, the experimental design required for the search of random DNA insertions in gene-edited and transgenic organisms should be the same. As further guidance, a suggestion of a strict design that could be equally applied to both situations is:

(i) Southern blot analysis using probes of 700 nt corresponding to at least five different, roughly equally spaced sections across the whole of each foreign DNA molecule, preferably on coding regions, or

(ii) PCR using primers targeting gross sources of foreign DNA (e.g., DNA purposely introduced in the cell, plasmids from *in vitro* transcription if the milieu was not treated with DNAse I). They should be selected to produce fragments not longer than 700 kb nor shorter than

200 kb, taking into account both the efficiency of ordinary PCR detection methods and seeking short insertions. Amplicons should span at least five different, roughly equally spaced sections across the whole target molecule, preferably on coding regions because of their higher importance on safety considerations.

Once again, regulators should make sure that whatever their criteria may be, either they are equivalent to their previously established criteria for finding the number of inserts in a transgenic organism, or they should update those earlier requirements to match the ones used for this purpose.

5. Other Gene-Editing Techniques and Site-Directed Effectors

5.1. Other Nucleases

Nowadays, dossiers reaching the regulator's desks include similar proportions of products obtained using Cas nucleases vs. products obtained using other genome editing techniques. These other techniques resort to the use of Transcription Activator-like Effector Nucleases (TALEN) [9, 78, 133, 138], zinc-finger nucleases (ZFNs) [122], and meganucleases [104, 108]. In addition, some products are obtained with oligonucleotide-directed mutagenesis (ODM) [97], a set of gene-editing techniques that do not use SDNs.

In most cases, these other products originated in projects that started before CRISPR-Cas tools were widely available, and they will likely become a minority in the near future. However, they may not disappear entirely because of intellectual property aspects that could balance their alleged technical disadvantages, and some companies that have been investing in developing proprietary technology may stick to them. In the long term, older technologies becoming off-patent sooner may be preferred by low-budget developers such as the public research sector and SMEs because they are free after entering the public domain. In principle, the overall approach presented here can be adapted to these other techniques, provided that enough information on specificity is available. For instance, it has been shown that TALEN pairs can tolerate up to 3-4 mismatches in their recognition sites [13]. However, studies characterizing the possibility of unintended genetic changes in these other techniques [15, 35, 18, 40, 73, 77, 87, 94, 107, 136] are far less in number compared with the literature on Cas nucleases. This asymmetry represents a challenge for regulators, who should adopt the same level of stringency toward different applicants, regardless of the technique used.

5.2. SDN2 and SDN3

The criteria proposed here were explained based on the simpler SDN1 scenario. However, as mentioned, there are other classes of gene editing techniques [20, 126]. One class employs an additional short DNA molecule as a template for repairing the cleaved locus (SDN2, a.k.a. "allelic repair" in some cases). Another class uses a bigger DNA molecule for its intended insertion in or replacement of the target locus (SDN3, a.k.a. "allelic replacement" in some cases).

In principle, the criteria presented here are also applicable to SDN2 and SDN3, with due consideration to the fact that these techniques always involve the deliberate introduction of specific foreign DNA in the cell in significant quantities. Hence, the additional DNA molecule has a high probability of ending up inserted, perhaps in tandem insertions, inversions, etc., in any of the PAL or elsewhere in random locations of the genome [96]. Therefore, this molecule's sequence should be taken into account in the search for spurious foreign DNA insertions, as described.

In addition, for SDN3, the short-range sequence identity analysis of those PALs where there has been an insertion should be extended. Sequence information should reach at least from 200 nt downstream of the first junction, between the inserted DNA molecule and the host genome, until 200 nt upstream of the second junction. This approach would be effective in finding unintended effects such as multiple head-to-tail insertions that can be misidentified as single insertions when only conventional PCR analysis is performed [105].

5.3. Other Site-Directed Activity

The criteria presented here perhaps could also be used as inspiration for developing regulatory criteria for SDNs-related proteins that have a different function. For instance, "prime editors" that edit single bases without double-strand breaks [34, 69], epigenome editing [41, 113, 123], transcriptional activation [60, 72], or the use of Cas13a (a.k.a. C2c2) for knocking down endogenous mRNA [1]. It has been shown that it is possible to have off-target activity in some of these techniques [49, 143].

6. Discussion and Conclusions

This article proposes a pragmatic and proportionate approach for addressing the possible existence of off-target editions and spurious DNA insertions caused by gene-editing techniques in agricultural applications. If widely adopted, there would be a harmonized approach for this important regulatory issue. Moreover, its explicit availability would help developers improve a safety aspect of their experimental design and protocols *ab initio*, lowering costs and complications during the subsequent regulatory assessment.

The value of counting with a standardized regulatory approach of the kind presented here can be compared with the allergenicity analysis of novel proteins expressed in GMOs. In 2003, the Codex Alimentarius generated guidance in this regard based on a simple bioinformatic analysis [132]. At that time, different scientific opinions were proposing alternate rules, which were similar albeit not entirely coincident. The Codex guidance was ultimately a compromise solution, agreed on the understanding that it may be a little bit prone to type II errors, but not as much as to hinder the use of GMOs for food.

The Codex guidance on allergenicity assessment was widely adopted and applied during the past two decades, and such a harmonized approach greatly facilitated that developers receive a fair and similar regulatory response in different countries. This guidance has never failed nor changed, despite updating proposals inspired by theoretical advances in the field,

since the robustness and efficacy of its original criteria are remarkable.

Similarly, the current body of knowledge on off-target effects and DNA insertions from SDN is enough for adopting criteria like the ones proposed here. Nevertheless, as more information continues to be made available, such criteria can be updated and extended to other techniques. Ongoing work on systematic reviews and information maps (such as [76]) may be very useful in this regard.

It could be argued that establishing a set of standard rules based on the specificity displayed by SDNs used nowadays would discourage the development of enhanced alternatives. Regulators may not easily relax the rules, unless an increased selectivity is considered to be fully proven, case by case; therefore, the quantity of PAL to be verified might not be affected much by innovation. However, the development of more specific SDNs or techniques will remain appealing, since they would reduce the number of loci that are actually affected. Therefore, such innovations may still reduce the overall regulatory burden.

Finally, it is important to highlight the relevance of examining phenotype to reinforce the molecular genetics approach presented here. In particular, the absence of unexpected changes in polygenic traits would act as an additional reassurance regarding the lack of genetic changes that could have gone undetected. For this purpose, polygenic traits could include agrophenotypic characterization in the case of crops, overall health indicators in the case of animals, and compositional analysis of derived foodstuff in both cases. In addition, assessing the predicted phenotypic change caused by the gene editing intervention is highly recommended. This assessment would serve as a reassurance that the biological function(s) of the target gene and the possible consequences of the intervention are understood well enough to assess their safety.

7. Declaration of Conflicting Interest

The author declares no conflicts of interest.

8. Disclaimer

The information and views are those of the author, and do not necessarily represent those of the organizations where he works.

9. Article Information

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Recommendations for Science-Based Safety Assessment of Genetically Modified (GM) Plants for Food and Feed Uses

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Abstract

Since the commercial introduction of genetically modified (GM) plants in agriculture over two decades ago, technology developers and regulatory authorities have gained significant experience in evaluating their safety based on assessing potential impact to humans, animals and the environment. Over 3,500 independent regulatory agency reviews have positively concluded on the safety of GM plants for food and feed. Yet, divergent and increased regulatory requirements have led to delayed and asynchronous approvals and have restricted access to innovative products for farmers and consumers. With accumulated knowledge from safety assessments conducted so far, an enhanced understanding of plant genomes, and a history of safe use, it is time to re-evaluate the current approaches to the regulation of GM plants used for food and feed. A stepwise approach using weight-of-evidence should be sufficient for the safety assessment of newly expressed proteins in GM plants. A set of core studies including molecular characterization, expression and characterization of the newly expressed proteins (or other expression product), and safety assessment of the introduced protein, are appropriate to characterize the product and assess safety. Using data from core studies and employing a "problem formulation" approach, the need for supplementary hypothesis-driven or case-by-case studies can be determined. Employing this approach for the evaluation of GM plants will remove regulatory data requirements that do not provide value to the safety assessment, and provide a consistent framework for global regulation.

Keywords: genetically modified plant, food and feed, safety assessment, core studies, supplementary studies, risk, problem formulation, regulations

1. Introduction

The first genetically modified (GM) plants used as a source of food were commercialized in 1994 [17], and in 2018 GM plants were grown on over 190 million hectares across 26 countries [16]. Over the past 25+ years, technology developers and regulatory agencies have gained knowledge and experience from studying and assessing the safety of GM plant products. To date, more than 4,000 independent regulatory agency reviews issued by 70 countries have concluded on the safety of GM plants, of which 3,524 reviews have been for food and feed use [16]. The approvals have unanimously found in each case that the GM plant in question was as safe as its conventional counterpart. Moreover, global economic gain of 186 billion USD over 21 years, and savings of 27.1 billion kilograms of CO^2 emissions in 2016 [15], have been realized as a result of the commercialization of GM plants.

While the 1,000+ years of safe use of conventionally-bred agricultural plants demonstrate that plants developed in this manner are generally safe for human and animal consumption, the introduction of GM plants generated questions about their safety despite the similarities in the development of both conventional and GM plants. In a typical commercial breeding program, hundreds of thousands of plants are produced and tested in hundreds of environments over many years to select

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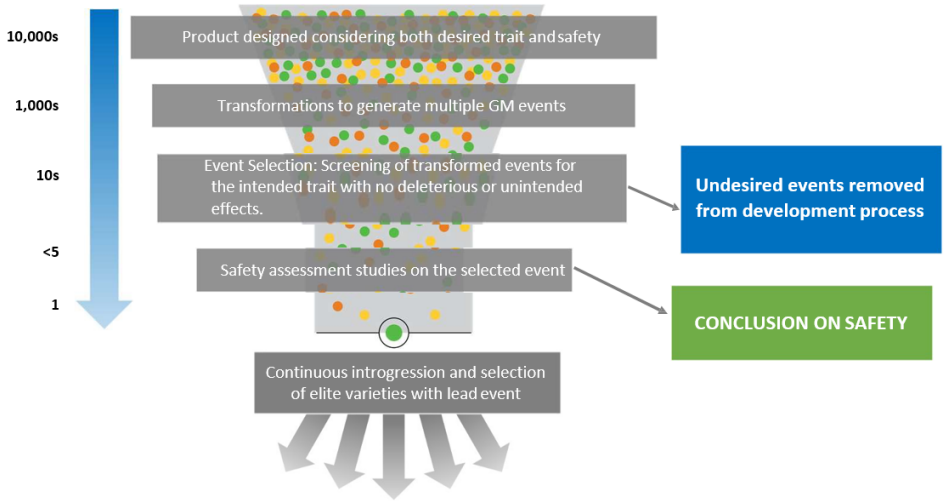

Figure 1: Screening process for the selection of the lead GM event for further safety assessment and introgression into elite varieties

a commercially viable, new variety. The screening out of undesired and unintended agronomic effects is an integral part of the breeding process for both conventional and GM varieties, and acts as a mechanism to reduce or eliminate undesirable plants and events from the development process. The extensive screening and selection process during variety development has led to a general recognition that conventional breeding of food crops does not present a risk to human or animal health.

During the development of new GM varieties, transgene(s) are typically introduced into an easily transformable host plant to produce thousands of GM events [21, 22]. Following an initial safety screen that is performed during the design phase, the GM events themselves are subject to an extensive screening process that includes molecular profiling, assessments of trait efficacy, and observations for unintended agronomic phenotypes [21]. At this point, for sexually propagated crops, one or more lead events are selected for introgression into elite germplasms. Introgression typically involves multiple backcrosses with locally-adapted germplasm. Following these backcrosses, more than 99 percent of the DNA in the GM variety is derived from the local germplasm [22]. The trait introgression process, along with the lead event selection process, substantially reduce any possibility of unintended effects in commercial GM varieties [22]. For vegetatively-propagated crops (e.g., sugarcane, potatoes, perennials), alternative selection and breeding strategies may be required [1]. An overview of the commercial development process for new sexually propagated GM varieties is shown in Figure 1.

Despite the rigorous breeding and selection process, record of safety, environmental benefit, and increasing familiarity with GM plants, their development and commercialization has, in some cases, been under increasingly stringent regulatory oversight and new safety data requirements. Many of these new regulatory requirements are not scientifically justified and do not add value to a safety assessment. Advances in science and accumulated experience should be considered during the safety assessment process. As discussed below, some existing data requirements and/or data that do not add value should be removed from regulatory oversight. This would reduce and provide consistency to product development timelines, greatly benefiting industry, including small and public sector developers. Another pragmatic approach to the regulation of GM plants employs the recognition of safety assessments completed in other regions through a significantly streamlined approach to the safety assessment process or through the mutual recognition of safety assessments. This approach maintains a high level of safety for human/animal health and the environment, while reducing regulatory timelines and enabling timely access to technology.

This paper presents the aligned view of the authors and recommends study designs and scientific data appropriate for the initial safety assessment of GM plants for food and feed use. These recommendations are modified from earlier guidelines and recommendations for the safety assessment of GM plants containing newly expressed proteins (e.g., [5, 9]).

1.1. Current food and feed safety assessment for GM plants

With the commercialization of GM plants, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) held consultations on biotechnology and food safety in 1990 and 1996, and the Codex Alimentarius Commission (CAC) published guidelines for the conduct of food safety assessments in 2003 [3, 4]. The Codex Alimentarius (Codex) recognized the need for a focused approach for the safety assessment of foods produced from GM plants, differing from the classical safety assessment approaches for discrete hazards that may be present in foods, such as food additives or pesticide residues. Codex also recognized the need for a comparative approach, and the concept of "substantial equivalence" was emphasized as an important first step in the safety assessment process to identify differences and sim-

ilarities between the new food (GM plant) and its conventional counterpart. Codex recommended that the safety assessment include an evaluation of both intended effects (consideration of the safety of any newly expressed proteins, NEPs, or intended metabolic changes) and unintended effects (identifying any new or altered hazards). These comparisons were to be made relative to an appropriate conventional counterpart with a history of safe use (HOSU).

Codex Alimentarius principles and guidelines have served as a valuable and consistent standard for the development of national and regional safety assessment guidelines and regulations since their introduction. However, those guidelines should be supplemented by the familiarity and established history of safety of GM plants over the past 25 years. Additionally, the divergent implementation of these guidelines by regulatory authorities has, in some cases, led to excessive data requirements prior to regulatory approval. The unique requirements of different agencies have resulted in significant delays in regulatory approvals, leading to asynchronous approvals globally. Notable areas of divergence from Codex include requirements for animal feeding studies without a testable risk hypothesis; expanding compositional analysis requirements, statistical approaches and appropriate comparators; extensive allergenicity assessments of introduced proteins and endogenous allergens in whole foods; and requests for excessive molecular and protein characterization data. Regulatory authorities in some countries where GM plants or their products are imported, but not cultivated, also require submission of agronomic and environmental data that are not relevant to the assessment of safety of the GM plant and its products for animal and human consumption. Additionally, some countries require specific studies that have already been conducted in another country or region to be repeated locally, adding further time, complexity, and cost to the approval process, when data from existing studies in other countries are fully applicable to these countries.

Although all countries agree that the primary purpose of regulation is the protection of human and animal health and the environment, divergent approaches to the regulation of GM plants globally have had major impacts in other policy areas. For example, asynchronous approvals have resulted in delays in commercial launches of innovations [18], despite the benefits of the cultivation of GM plants being well documented. The divergent approaches to current global regulation of GM plants hinder innovation as well as the wider adoption of the technology, resulting in loss of significant economic and environmental benefits. In fact, a recent report estimated that the value of corn production and soybean production in major export countries would increase by 4.3 billion USD and 4.9 billion USD, respectively, between 2018 and 2022 if GM plant approvals were achieved in a more timely manner [14].

Some regulatory authorities have revised their oversight of GM plants as a result of their familiarity with GM traits and plants and extensive experience with their regulation. In Japan, some previously approved agronomic traits (for example, traits conferring herbicide tolerance) stacked through conventional plant breeding are now subject to a simplified risk assessment [25]. Japan also excluded GM crops that do not have wild relatives in Japan from mandatory field trial requirements if they contain familiar traits [26]. Canada has reduced the requirement of certain agronomic data needed for the approval of a GM trait which has 'sufficient similarity' to a previously approved GM trait [7]. The USDA recently proposed modernization of its biotechnology regulations to exempt GM plants obtained through certain genetic engineering techniques and some previously approved traits from regulation [24].

In 2017, the U.S. National Academy of Sciences recommended an updated approach to the regulation of future products of biotechnology to address the needs of, "*supporting innovation, protecting health and the environment, promoting public confidence in the regulatory process, increasing transparency and predictability, and reducing unnecessary costs and burdens*". The recommendations also suggested an expedited, simplified process for products containing previously assessed traits (i.e., familiar products) [19].

1.2. Recommendations for future food and feed safety assessments for GM plants

Despite recent developments in the regulatory approaches followed in some countries, complex and unnecessarily burdensome regulatory requirements continue to underscore the importance of a science-based testing paradigm for safety assessments. With the vastly enhanced understanding of plant genomes since the publication of the Codex principles and guidelines over 15 years ago, as well as more than two decades of experience in the development, commercialization and safety assessment of GM plants, it is time to re-examine approaches for the safety assessment of GM plants used as food or feed.

A weight-of-evidence (WOE), stepwise, and science-based approach that uses a set of core studies to evaluate the safety assessment of GM plants is recommended. Depending on the nature of the introduced trait, intended use, and data obtained from core studies, supplementary (hypothesis-driven or case-by-case) studies may be required to fully evaluate the safety of the GM plant. Figure 2 provides a schematic overview of the studies required to ascertain the safety of GM plants as proposed in this article, distinguishing between core studies and supplementary studies.

1.3. Core studies for food and feed safety assessment

Safety assessment of GM plants used as sources of human and/or animal nutrition requires a collection of information about the host plant and donor organism from which the GM trait is derived, history of food and feed use (if applicable), and detailed knowledge of the GM trait [4]. In this article, the following core sets of studies are recommended to characterize the product and assess safety, namely: (1) molecular characterization of the GM event; (2) expression levels and characterization of the NEP or other expression product (e.g., double-stranded RNA); and (3) safety assessment of the introduced protein (or expression product). These studies are discussed in more detail elsewhere [2].

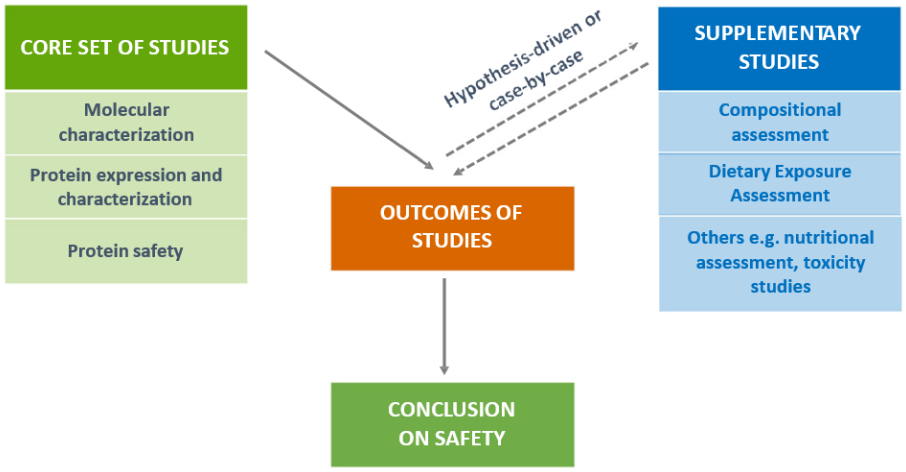

Figure 2: Schematic overview of the studies necessary to assess the safety of GM plants for food and feed uses. Core studies are a set of studies necessary for a science-based risk assessment of a GM plant. These are suggested core studies for typical GM plants. There may also be alternative newly expressed substances (e.g. RNAi). Supplementary studies are studies to be conducted upon identification of information and/or hypothesis that indicates increased risk to human or animal health. The conduct of these studies depends on the nature of the introduced trait, intended use and data obtained from core studies.

1.4. Supplementary hypothesis-driven or case-by-case studies and study design

Data obtained from core safety assessment studies can be used to determine which additional studies may need to be conducted before fully characterizing the product and evaluating the safety of a GM plant for food and feed. A "problem formulation" approach should be employed [23, 28] to address specific safety questions relevant to the nature of the GM product. The problem formulation approach generates risk hypotheses arising from the nature of the trait and the genes introduced to confer the new trait. Evaluation of the potential risks is then conducted according to the science-based process established by Codex. Risk analysis is a stepwise process requiring hazard identification, hazard characterization, exposure assessment, and risk characterization [11]. In simpler terms, risk is a function of hazard and exposure (Figure 3). Understanding the mode of action (MOA) of the expression product also provides meaningful information on potential hazards. To employ problem formulation, the study design must be testable and specifically address the questions or concerns raised to enable developers and regulators to efficiently assess risk and evaluate the safety of the product.

Extensive compositional analysis has historically been considered a core study. However, over 25 years of safety assessments evaluating composition data have demonstrated a lack of notable difference between GM plants and conventional comparators, especially in the context of the natural variability that already exists between plant varieties [8, 27]. As discussed by Herman and co-authors, there is enough scientific evidence today to merit a shift to conducting compositional analysis as a supplementary hypothesis-driven study [13]. In some cases, nutritional and dietary exposure assessments are performed to fulfill regulatory requirements. These studies should also be supplementary and performed if required upon hazard or exposure identification. Similarly, for traits intended to improve the nutritional profile of grains (e.g., increased oleic acid), changes in the levels of other grain components (e.g., other fatty acids) should be assessed through supplementary studies.

1.5. Risk evaluation

Risk evaluation requires consideration of potential "hazards", as well as an evaluation of likely "exposure" to the evaluated substance [20]. Even in commonly-consumed foods, there are hazardous substances. Some foods, for example kidney beans, tomatoes, and potatoes, contain naturally-occurring toxins that could be "hazardous" to our health (e.g. phytohemagglutinin, tomatine, solanine/glycoalkaloids). However, various means (e.g. plant breeding and variety selection, proper storage and/or preparation, cooking, etc.) have been used to mitigate "exposure" to these toxins and thereby reduce the risk to an acceptable level. Safety assessments of new foods produced from GM plants should focus on new or altered hazards as they pertain to the NEP, rather than trying to identify every potential hazard associated with that food [3]. The safety of food substances *per se* is regulated through other food regulation mechanisms [12, 6]. A WOE and stepwise approach should be followed for assessing the safety of newly expressed substances (protein or DNA) in GM plants, delineating the safety/risk assessment into its components of hazard and exposure in alignment with Codex principles [3, 5]. In cases where no new hazards are identified, or where there is no human or animal exposure to identified hazards, there would be no new risk or need for a full safety assessment. For example, in the case of a highly purified food ingredient, such as sugar or oil produced from a GM plant where there would be no exposure to a newly expressed substance, a hazard assessment is not scientifically justified. When a full safety assessment is necessary, both hazard and exposure must be evaluated to understand risk. Figure

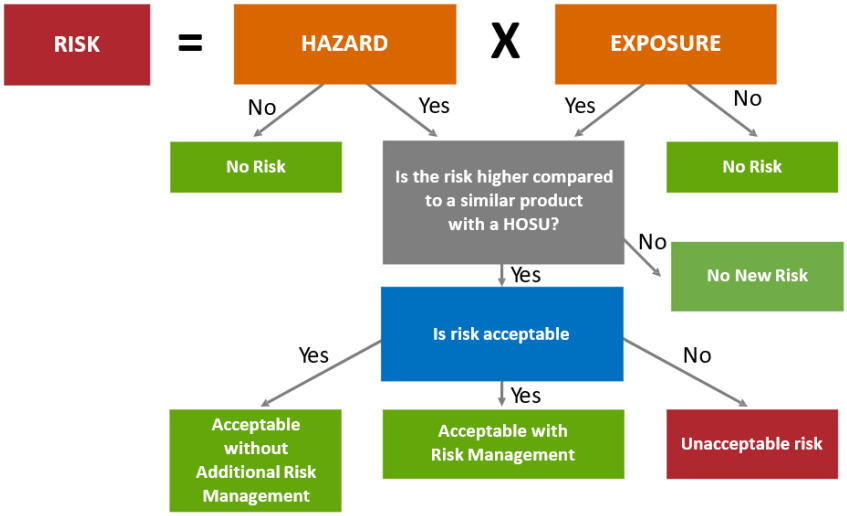

Figure 3: Risk evaluation process and possible outcomes [10]. Risk is a function of the probability of an adverse health effect and the severity of that effect, consequential to hazard(s) in food. Hazard is a biological, chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect. Exposure is the likely intake of biological, chemical, and physical agents via food as well as exposures from other sources if relevant.

3 highlights the risk evaluation process and the three possible outcomes of risk evaluation:

- No risk/no new risk
- Acceptable risk with or without risk management
- Unacceptable

2. Conclusions

The development of GM technology in agriculture was rightly accompanied by the development of regulatory safety guidance at the international level through the Codex Alimentarius. With the knowledge, experience, and HOSU gained from over 25 years of developing, commercializing, and consuming GM plants, it is time to re-evaluate current approaches to their regulation. A stepwise and science-based method using a set of core studies and a problem formulation approach to determine the necessity for supplementary studies for the safety assessment of GM plants used for food and feed is proposed for all GM plants. These studies, outlined in Figure 2, are described in further detail by Brune et al. [2].

The recommendations are based on extensive global experience and an enhanced understanding of plant genomes and genetic diversity. Further, the extensive screening process for new GM events and the plant breeding/trait introgression process together significantly reduce the possibility of unintended effects in commercial varieties. Removal of regulatory requirements that do not provide value to the safety assessment would reduce product development timelines, which would enable smaller and public sector developers to bring diverse agricultural innovations to the marketplace. It would also lower the cost barriers to working on non-traditional crops and traits and make product launch timelines more predictable. With the increasing repertoire of GM plant products anticipated in the future, a science-based regulatory paradigm will enable innovation and delivery of products that will have a positive impact on the global economy, the environment, and food security sustainability.

3. Declaration of Conflicting Interest

All the authors of this paper are currently employed by, or have been employed by, the agricultural biotechnology industry.

4. Disclaimer

The findings and conclusions in this publication are those of the author(s) and should not be construed to represent any official USDA or U.S. government determination or policy.

5. Article Information

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Stacked Trait Products Are As Safe As Non-Genetically Modified (GM) Products Developed By Conventional Breeding Practices

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Abstract

International safety assessments and independent publications conclude that stacking genetically modified (GM) traits (events) through conventional breeding poses no greater risk to food or feed safety than stacking multiple non-GM traits by conventional breeding. Stacked trait products are not substantially different from their conventional comparator or their GM parent plants. Additional safety assessment of a stacked trait product produced by conventional breeding should not be required unless there is a plausible and testable hypothesis for interaction of the traits. However, the different approaches employed for the regulation of stacked trait products between countries results in asynchronous approvals, increasing the potential for trade flow disruptions, and adds to the regulatory burden for product developers. Considering their proven safety and benefit over the past 20+ years, regulatory authorities in some countries do not regulate stacked trait products, while others have simplified the approval process based on experience and sound science, reducing or eliminating the need for additional regulatory oversight. Countries that choose to regulate stacked trait products should consider integrating the more than 20 years of safety assessment experience, history of safe use, and global regulatory experience, in their approach to reduce redundancy, simplify regulations, and minimize the likelihood for trade disruption.

Keywords: stacked trait product, breeding stack, genetically-modified plant, GM event, GM trait, single event

1. Introduction

Genetic engineering has been used for more than 25 years to incorporate novel traits into plants. This tool has provided innovative and beneficial products to farmers around the world since genetically modified (GM) plants were first commercialized in 1994. Originally, individual traits such as herbicide tolerance and insect resistance were introduced into plants. These were, and continue to be, subject to regulatory review before being authorized for commercial use [9]. Over the years, as the safety and benefits of genetically modified (GM) plants were realized, a logical progression in the evolution of product development was to introduce multiple GM traits in the same plant, resulting in a "stack" or "stacked trait product" [15] that exhibits the phenotype of each of the GM traits.

Stacking of traits is accomplished through two methods: 1) by conventional plant breeding, where parents with single GM events are crossed to produce progeny that contain two or more GM events, commonly referred to as stacked trait products (also known as "breeding stacks"), or 2) by using molecular methods, where two or more traits are simultaneously or sequentially introduced into a host plant. The difference between the two stacking methods is that stacked trait products produced via conventional breeding do not contain a new event(s) that has not been assessed and approved by regulatory authorities [11, 12]. In this rapid communication we focus on the scientific rationale that additional regulatory oversight and further safety assessment of stacked trait products produced through conventional breeding, where the individual traits have already been assessed and approved, is unnecessary.

1.1. Global importance of stacked products

Stacked trait products offer multiple solutions for the farmer in one plant, allowing for expanded and enhanced management practices to maximize productivity and realize environ-

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mental benefits through improved agronomic practices (e.g., reduced/minimum tillage). For example, stacking of insect resistant traits can result in crops that are protected from damage by multiple pests and/or provide multiple modes of action to protect against similar pests, thereby delaying the development of insecticidal resistance among the target pests. Similarly, the stacking of herbicide tolerant traits allows farmers to utilize diverse modes of action for weed management and improve product durability specific to more prevalent or problematic local environments, while providing flexibility to combat difficult-to-control and resistant weeds. In both cases, this results in options for farmers to employ best management practices to improve farming productivity and expand the use of integrated pest management systems [14]. The value of stacked trait products to agriculture is highlighted by their rapid adoption by farmers. Over 80 percent of corn acres planted in 2018 in the United States were stacked trait products, a 70 percent increase in the last 15 years [16]. This same trend has been observed globally, with a market adoption rate increase of over 115 percent in just 10 years, reflecting the rapid and widespread adoption of these products and their importance to advancing agriculture [9].

1.2. Conclusions of international regulatory bodies and independent studies

Conventional plant breeding has a long, established history of safe use (HOSU), predictably providing safe food and feed products throughout history [22]. The World Health Organization (WHO) issued food safety evaluation guidelines in 1995 recognizing that when two GM plants that are substantially equivalent to conventional varieties are crossed by conventional breeding techniques, the resulting stacked trait product is expected to be substantially equivalent to the individual events [21]. Since stacked trait products do not contain a new GM trait (event) or additional introduced DNA, they are not considered new genetically modified organisms (GMO) or new living modified organisms (LMO), as defined by the Cartagena Protocol on Biosafety [6, 12].

Today, there are significant differences in the approach to regulation of stacked trait products between countries, frequently resulting in asynchronous approvals. For example, some jurisdictions do not require pre-market authorizations or only require a notification of commercialization if stacked trait products will be introduced to the marketplace, while others require additional data to be submitted for a safety assessment. The Codex Alimentarius principles and guidelines have been broadly applied to the evaluation of the safety of single events. Once these single events have been assessed and approved for use, conventional breeding can be utilized to incorporate these events into the commercial cultivars without the need for additional safety assessment [5]. Numerous publications support the conclusion that stacking GM traits through conventional breeding poses no greater risk to food or feed safety than combining multiple non-GM traits by conventional breeding [12, 13, 20, 21], and several recent reports have demonstrated that stacked trait products are not substantially different from their conventional comparator or the GM parent plants [3, 11, 23].

Given that single GM trait products are approved after a rigorous regulatory safety review, and that this process far exceeds the review employed for non-GM crops produced through conventional breeding, it is reasonable to conclude that stacked trait products do not pose additional risk and are as safe as the parental single events, unless there is a potential for the stacked traits to interact [15].

1.3. Current trends in the regulation of stacked trait products

As stated above, international guidelines and standards state that safety assessments performed on the single GM event are sufficient to assess the safety of stacked trait products and the associated intermediate stacks (sub-stacks) when these products are developed using conventional breeding [8]. Regulatory authorities in many countries do not require additional regulatory data to approve stacked trait products, as long as the traits are not predicted to interact [4, 19, 2]. Additional studies would only be warranted if two or more of the events present in the stacked trait product can potentially interact in a manner that would in some way change the conclusions of prior safety assessments of the single events. Interactions between traits are plausible only if they are predicted based on their mechanisms of action. Usually, such an interaction can be tested directly (such as through bioassays of insect-active proteins) and, only when necessary, by studies on the safety of the stacked trait product. The potential for interaction is rare but predictable, and can typically be evaluated within the context of the single event and the mode of action of the individual traits [13, 15]. To date, there has never been a documented occurrence of trait interaction as a result of stacking that has caused a safety concern.

With experience and familiarity gained through the evaluation and adoption of stacked trait products internationally, many countries have simplified or are currently in the process of simplifying their regulations for stacked trait products, including Japan, Brazil, and Argentina. For example, based on 20+ years of experience and familiarity with stacked trait products in which no safety concerns were observed, Japan streamlined its regulations. The Ministry of Health, Labor and Welfare (MHLW) of Japan has authorized stacked trait products for food and feed import with previously approved agronomic traits that are considered category 1 traits without the need for additional data [17]. Argentina's Ministry of Production and Labor recently published a new normative regarding stacked trait products with a "low probability of synergism" (i.e., interaction) between previously assessed single events, indicating that these products will not require any further assessment before commercial release and marketing [1, 18]. Additionally, the European Food Safety Authority (EFSA) has extensively reviewed more than 30 stacked trait products without finding any safety concerns [7, 10].

2. Conclusion

Since conventional breeding and selection does not by itself introduce novel hazards, and the process of stacking GM

traits has been shown to be as safe as stacking non-GM traits, the safety assessment of stacked trait products is unnecessary unless there is a plausible and testable hypothesis of trait interaction [15].

Despite a HOSU of GM plants with single traits, and extensive regulatory and commercial experience with stacked trait products, regulatory policies and data requirements for their approval differ globally. While some countries have eliminated or streamlined their stacked trait data requirements in recent years based on that experience, others continue to increase their requirements. Additional regulatory oversight and further safety assessment of stacked trait products where the individual traits are approved is unnecessary and duplicative. Simplification and streamlining of existing stacked trait product regulations would reduce regulatory burden and asynchronous approvals, while continuing to deliver innovations with a history of safe use to farmers globally.

3. Declaration of Conflicting Interest

All the authors of this paper are currently employed by, or have been employed by, the agricultural biotechnology industry.

4. Disclaimer

The findings and conclusions in this publication are those of the author(s) and should not be construed to represent any official USDA or U.S. government determination or policy.

5. Article Information

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Streamlining Data Requirements for the Environmental Risk Assessment of Genetically Modified (GM) Crops for Cultivation Approvals

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Abstract

Genetically modified (GM) crops have been cultivated safely for more than 25 years, and the framework for conducting an environmental risk assessment (ERA) is well-established. Currently, there is alignment of broadly-stated protection goals across global jurisdictions; however, there is a lack of consistency for the data that are required by regulators. Risk assessors have over 25 years of collective experience assessing the environmental safety of GM crops and have conducted hundreds of ERAs to evaluate GM crop safety. This experience provides a scientific basis to help determine which data informs the ERA, and which data does not inform the safety assessment. The goals of this paper are to: 1) define the process for identifying potential pathways to harm based on robust problem formulation; 2) provide an overview of data that inform the science-based ERA for cultivation approval; 3) provide examples of data that are routinely or occasionally required but do not inform the ERA; and 4) make recommendations for harmonization of global ERA data requirements. Refinement and harmonization of data requirements across global regulatory authorities will add transparency and predictability to the ERA of GM crops globally, while ensuring that each country's protection goals are respected.

Keywords: environmental risk assessment, genetically modified plant, problem formulation, data requirements, cultivation

1. Introduction

Genetically modified (GM) crops are cultivated on over 191.7 million hectares worldwide [30]. Prior to commercial approval, GM crops undergo thorough safety assessments to characterize food and feed safety in countries that cultivate the crops and those that import GM grain [13, 21, 59]. Additionally, in countries cultivating GM crops, environmental risk assessments (ERA) are conducted as part of the regulatory approval process to assess impacts on the agricultural and surrounding environments where the crop is intended to be grown. ERA investigates the potential types and magnitude of harm to valued elements of the environment that could arise from the crops' environmental release.

To date, the majority of commercialized GM crops has been limited to commodity row crops (e.g., corn, soybean, cotton, canola) containing herbicide tolerance and/or insect protection traits. As a result, there is a large body of knowledge (i.e., familiarity) surrounding the potential environmental risks associated with cultivation of these crops and traits. New insect-protection traits, tolerance to new herbicidal active ingredients, disease protection traits, and traits that improve agronomic performance, shelf-life and nutritional profiles are also being developed in corn, soybean, cotton, canola, sugar beet, as well as in new crops (apples, potatoes, banana, eggplant, etc.) [16, 15, 3, 48, 57, 52, 54]. As new crop and trait combinations are developed, additional considerations (potential pathways to environmental harm) may become relevant to consider as part of the ERA. However, a science-based ERA framework should be robust and flexible enough to be applied to any crop or trait combination to enable regulatory decision making (for example, [55]).

While most countries have similar broadly-stated protection goals (e.g., protection of biodiversity), there is a lack of global

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alignment and consistency in the data that are required for ERA of GM crops, and not all data that are required globally inform science-based decision making in the ERA. The goals of this paper are to: 1) define the process for identifying potential pathways to harm based on robust problem formulation; 2) provide an overview of data that informs the science-based ERA for cultivation approval; 3) provide examples of data that are routinely or occasionally required but do not inform the ERA; and 4) make recommendations for harmonization of global data requirements for ERA. Refinement of data requirements to those that inform the ERA and harmonization of data requirements across global regulatory authorities will add transparency and consistency to the ERA of GM crops globally while ensuring that countries' protection goals are respected.

1.1. Problem Formulation to Identify Potential Pathways to Harm

Protection goals are established by local legislation or by regulatory authorities to describe the species, habitats, and/or ecosystem services that are to be protected. These protection goals are typically broadly stated and are often translated into operational protection goals with clear relevance to the ERA [19, 24]. Understanding the operational protection goals of each regulatory authority is important for understanding what is to be protected and determining relevant risk assessment endpoints. In the context of a GM crop, a broad protection goal (e.g., protection of biodiversity) could be translated into an operational protection goal (e.g., protection of beneficial or charismatic species). While impacts on protection goals can be difficult or impossible to measure, information is available or can be developed on relevant assessment endpoints (e.g., non-target organism abundance or diversity), and studies may be performed to measure specific relevant effects (e.g., honeybee mortality). Protection goals must be accompanied by standards to judge adverse effects in the agricultural context (for example, 50 percent reduction in population abundance; [2]).

For cultivation approval, a hypothesis-based approach should be used to determine potential environmental risks, and the data that are required should inform the ERA by providing reliable scientific evidence that addresses a plausible, testable hypothesis. Problem formulation is used to develop hypotheses of potential harm, based on knowledge of the receiving environment, the biology of the crop, and the characteristics of the introduced trait [47, 58, 43, 40]. Often, a testable hypothesis can be addressed with the use of existing knowledge/studies, and no additional data need to be generated. Because risk is a function of both hazard and exposure, if exposure can be shown to be low or negligible, additional hazard characterization may not be needed to inform the risk assessment. For example, if it can be demonstrated that there is low or negligible exposure of a non-targeted organism (NTO) to an insecticidal trait in a GM crop, additional hazard data are not needed to conclude low or no risk to that specific NTO. This is one of the fundamental reasons why hazard data on NTOs should not be necessary for import approvals [42]. Due to low-level exposure scenarios associated with import of GM grain (e.g., grain spillage at port or processing facility), there are rarely plausible hypotheses for harm to the protected elements of the environment [42, 29, 41]. Most potential harms would arise in the country of cultivation due to higher potential exposure, so the ERA conclusions in a cultivation country should be sufficient to inform potential risk in an importing country. In other instances, if the potential for harm cannot reasonably be ruled out based on existing information on the environment, crop, or trait, further examination of potential exposure or hazard may be warranted to assess risk. For instance, if the importing country has wild relatives of the crop that are not present in the cultivating country, there may be a potential for gene flow, which could trigger assessment of the environmental risk that could result from transgene introgression into populations of the wild relatives in that country. The problem formulation approach to ERA is a robust way to structure the risk assessment to consider plausible hypotheses of harm, assess available information that addresses those hypotheses, generate additional data that reduce uncertainty in the identified risks, and enable decision making that is relevant to the protection goals.

1.2. Overview of Data that Inform the Science-Based ERA for Cultivation Approval

ERA of a GM crop evaluates the likelihood of harm arising from the interaction of the GM crop with the environment, compared with non-GM counterparts. Therefore, the data that universally inform the ERA for all crops and traits include: 1) an understanding of the receiving environment and the basic biology of the unmodified plant; 2) an assessment of the agronomic similarity of the GM crop to its conventional counterparts; and 3) an understanding of the intended trait of the GM plant and assessment of how the intended trait may lead to environmental harm (Table 1).

1) Understanding of the receiving environment and the basic biology of the unmodified plant

By definition, the agroecosystem is ecologically disturbed, unstable, and dynamic, and the potential effects of cultivating a GM crop must be considered relative to the effects of cultivating the non-modified crop. Agricultural fields generally do not support high biodiversity, but instead are dominated by one or a few cultivated plant species and are managed to maximize yield (e.g., tillage, weed, insect, and pathogen management). Therefore, if a general protection goal to protect biodiversity is to be observed, the ERA should consider if the GM plant adversely affects biodiversity relative to the non-modified plants growing in the same agroecosystem and managed under standard agronomic practices.

An understanding of the basic biology of the crop is also a key component of problem formulation. For example, understanding if the unmodified crop has any weediness characteristics [5], if it survives outside of managed cultivation, or if it outcompetes other plants, are important considerations in the context of ERA. Most agricultural crops are highly domesticated, and the agronomic traits that make them efficient at meeting human needs under cultivation have been selected for by breeding over hundreds or thousands of years. Many of these characteristics selected for in the domestication process also make them

Table 1: Data that universally inform the environmental risk assessment for cultivation of a genetically modified (GM) crop

Data relevant for ERA of all crops and traits	**How data informs the ERA**
Understanding of the receiving environment and the basic biology of the unmodified plant	Understanding of the receiving environment allows for relevant pathways to harm related to the receiving environment to be considered. For example, does the receiving environment contain any wild or weedy relatives? Do wild relatives grow near or adjacent to the GM plant? Understanding the basic biology of the unmodified plant allows for relevant pathways to harm related to survival, weediness, reproduction, gene flow, etc., to be considered. For example, does the non-modified plant have weediness characteristics [6]? Can the non-modified plant survive outside of cultivation? Does the non-modified plant outcross with wild relatives?
Comparative assessment of the agronomic similarity of the GM crop to its conventional counterparts	Assessment of the agronomic similarity of the GM crop to its conventional counterparts allows for relevant pathways to harm related to survival, weediness, reproduction, gene flow, etc., to be considered. For example, is the GM crop similar to the non-modified crop in terms of the standard agronomic endpoints? Does the GM plant have traits that may increase weediness (seed shattering, dropped ears, etc.)?
Understanding of the intended trait of the GM plant and assessment of how the intended trait may lead to environmental harm	Understanding of the intended phenotype of the GM plant allows for relevant pathways to harm related to the trait to be considered. For example, does the intended trait confer insect protection? Herbicide tolerance? Drought tolerance? Understanding the intended trait(s) and a basic understanding of its mode of action will inform problem formulation and may indicate additional relevant data requirements for the ERA (as described in Table 2).

poor competitors with natural vegetation in the absence of human intervention. Maize, for example, is highly domesticated, and populations of maize do not survive outside of cultivation [36]. There is extensive information and knowledge about basic weediness and reproductive and survival characteristics for all major row crops like maize, soybean, cotton, and canola, which can be leveraged to inform the ERA [38, 37, 36, 35].

2) Assessment of the agronomic similarity of the GM crop to its conventional counterparts

Like conventional breeding programs, GM plants are assessed and screened through many rounds of event selection to ensure the agronomic and phenotypic characteristics of the commercialized event meets farmer needs. If the agronomic and phenotypic characteristics of a GM plant have deleterious effects or are not desired by the developer or customer, the event is eliminated from further development. This basic process of selection that is used for both conventional breeding programs and GM plant development programs is important for developing robust, commercially viable products, while it also ensures that plants with undesirable phenotypes are not advanced [25].

Standard agronomic endpoints are collected throughout event selection as well as from large multi-site field trials, and these agronomic data can be used to assess the similarity of the GM plant to its conventional counterparts. Conventional crops can have an extensive range of agronomic properties, enabling them to be grown across diverse environments or to meet various societal needs. This range of agronomic properties is accepted by society because it does not present an unreasonable environmental risk and there is an established history of safe use of domesticated crops. If a GM plant is shown to be agronomically similar to non-modified plants with a history of safety, it would have no novel risks outside the range of the conventional crop other than the introduced GM trait. Therefore, the ERA can focus on the intended traits, and additional data would only be needed to inform the ERA if plausible risk hypotheses can be developed for potential environmental harm caused by that trait. For example, the basic biology of maize is well established and accepted [36]. As previously mentioned, maize does not survive outside managed agricultural environments [11], and its survival and reproduction is limited by environmental conditions (heat stress, frost, drought, excessive rainfall, etc.) [51]. If the GM plant is shown to be agronomically similar to non-modified maize, which has no weediness characteristics, and the intended phenotype is not related to a weediness characteristic, then there is no plausible hypothesis for how the GM plant could increase weediness potential. In this case, generating data that are related to plant weediness would not further inform the risk assessment. Risk can be assessed based on the biology of the unmodified plant, understanding of the intended phenotype, and similarity of agronomic characteristics.

3) Understanding of the intended trait of the GM plant and assessment of how the intended trait may lead to environmental harm

An understanding of the intended trait of the GM plant

helps inform problem formulation. A basic understanding of the mode of action of a newly expressed protein in a GM plant, which is often investigated as part of the food and feed risk assessment, can support the understanding of the intended trait. If plausible risk hypotheses can be developed for how a novel trait could lead to environmental harm, they can guide the risk assessment and help determine which data are relevant for assessing risk. For example, if the intended phenotype of the GM plant is to protect against insect pests, this information helps guide the ERA towards assessing hazards to non-target insects in the agroecosystem. In this example, a basic understanding of the mode of action of the insecticidal protein (e.g., receptor binding, pore forming, enzymatic catabolism, etc.), may support the understanding of the trait and problem formulation. A full understanding of how the insecticidal protein works at the molecular, cellular, or anatomical level should only be required if plausible hypotheses for harm could be developed and addressed with mode of action information. For a second example, if the intended phenotype of the GM plant is to confer drought tolerance, there may be no plausible hypothesis for hazard to non-target insects, but there may be a plausible hypothesis for increased survival of the GM plant. Understanding the intended phenotype is therefore important because it informs the science-based ERA for cultivation approval; however, the data requirements that are relevant for characterizing the intended phenotype and the need for extensive mode of action data should be assessed on a case-by-case basis, and driven by the development of relevant pathways to harm related to the intended trait (as described in Table 2).

1.3. Overview of Data that may be Relevant in the Science-Based ERA for Cultivation Approval and Should be Considered on a Case-by-Case Basis

Data that may be considered relevant for some crops and traits, in addition to the core data described above, are determined by problem formulation and the development of plausible hypotheses for harm (Table 2). The relevance of these data depends on the crop, trait and receiving environment, and therefore should be considered on a case-by-case basis. These data are related to characterization of the GM crop, which includes: 1) assessment of potential changes in agricultural practices; 2) generation of additional agronomic data based on relevant pathways to harm related to increased survival, weediness, reproduction, gene flow, etc.; and 3) generation of additional data based on relevant pathways to harm related to the intended trait. Depending on the intended trait (e.g., insect protection, herbicide tolerance, or stress tolerance), additional data that may be relevant to consider for ERA include: (a) characterization of potential hazard to NTOs; (b) characterization of trait expression; (c) characterization of environmental fate in soil, sediment or surface water; and (d) characterization of potential effects on soil microbial communities and other plants.

1) Characterization of the GM crop: Assessment of potential changes in agricultural practices

In some cases it may be relevant to consider if the introduced GM trait is likely or intended to alter the standard agricultural practices in ways that could cause adverse effects on the environment. Consider for example a GM plant that contains an herbicide tolerance trait (HT). In this case, there could be a change in management practices relative to the non-modified crop (herbicide application, tillage, etc.) that is associated with the HT trait. In most cases, changes in agricultural practices will remain within the normal accepted practices for that crop (for example, even with an HT trait, herbicides would still be applied per the labeled rates), and the potential for change may not result in harm. If there is a plausible hypothesis for how the GM crop could result in a change in agricultural practices (for example, if a GM trait allows the crop to be cultivated in new environments) that could lead to a new or heightened adverse effect on the environment, additional data may be required to assess that risk.

2) Characterization of the GM crop: generation of additional agronomic data based on relevant pathways to harm related to increased survival, weediness, reproduction, gene flow, etc.

As described above, standard agronomic endpoints are collected during event selection and from large multi-site field trials for any new crop variety (GM or conventional), and these agronomic data can be used to identify any phenotypic or agronomic differences from the conventional crop that could result in a relevant pathway to harm. If these agronomic data show that a GM crop is similar to its conventional counterpart, the ERA for the GM crop can focus on the intended traits, and additional data would only be needed to inform the ERA if plausible risk hypotheses can be developed for potential environmental harm. Therefore, the requirement for generating additional agronomic data related to survival, weediness, reproduction, gene flow, etc., should be based on the development of a plausible pathway to harm and testable hypothesis. For example, if the non-modified plant does not outcross to wild relatives or no wild relatives grow in the vicinity of pollen deposition, and the GM plant is agronomically similar to the non-modified plant, then there is no plausible hypothesis for harm arising from gene-flow, and no additional data are needed to assess the gene flow potential of the GM plant. Similarly, if the non-modified plant does not have weedy characteristics, and the GM plant is agronomically similar to the non-modified plant, there is no plausible hypothesis for the GM crop becoming more weedy or invasive than its non-modified counterpart. On the other hand, if the non-modified plant does have weediness or invasiveness characteristics and/or the GM plant is not agronomically similar to the non-modified plant for relevant endpoints (for example, if the GM plant has increased seed dormancy or dispersal compared to the non-modified plant), there may be a plausible hypothesis for increased weediness or invasiveness potential of the GM plant, and additional information may be needed to fully assess the likelihood and magnitude of this risk. Similarly, if the GM trait introduces increased weediness potential and there are sexually compatible wild relatives in the areas of intended cultivation, an assessment of the likelihood and consequences of trait introgression into the wild relative population may be warranted.

Table 2: Data that may be relevant in the science-based ERA for cultivation approval and should be considered on a case-by-case basis

Data that may be relevant for the ERA	**Cases when data may inform the ERA**
Characterization of the GM crop	
Assessment of potential changes in agricultural practices	The agricultural practices associated with the GM crop need to be considered within the context of the agricultural practices that are typical for the non-modified crop. If there is a plausible hypothesis for how the GM crop could change an agricultural practice, additional data may inform the ERA. For example, if a GM crop that confers tolerance to an herbicide could result in a change on herbicide application, tillage, etc., an assessment of the effects of this change in management practice should be considered. Alternatively, a GM crop that confers protection against an insect pest may not result in any relevant changes in management practices, and additional data may not inform the risk assessment.
Generation of additional agronomic data based on relevant pathways to harm related to increased survival, weediness, reproduction, gene flow, etc.	Standard agronomic data is collected as part of event selection and multi-location field trials. Generation of additional data related to weediness, invasiveness, survival, and gene flow should only be considered if a plausible hypothesis can be generated for environmental harm. If no plausible hypothesis can be generated, understanding of the basic biology of the non-modified crop, the intended phenotype of the plant, and agronomic similarity to non-modified plants should be sufficient to assess risk (Table 1). For weediness, invasiveness, survival: agronomic endpoints related to weediness, invasiveness, and survival from the standard agronomic assessment can be used to assess risk (for example, seed dormancy, dropped ears, etc.). In some cases, additional data beyond the standard agronomic endpoints may be required to inform the risk assessment (e.g., an overwintering study may be deemed appropriate for a GM plant where the intended phenotype is a cold tolerance trait). For gene flow: agronomic endpoints related to reproductive endpoints from the standard agronomic assessment can be used to assess risk (for example, days to flowering, time to silking). In some cases, additional data beyond the standard agronomic endpoints may be required to inform the risk assessment (e.g., if the intended phenotype is related to a reproductive trait). The occurrence of sexually compatible wild relatives (SCWR) in the cultivation area is also relevant to consider; if there are SCWR, additional data beyond the standard agronomic assessment may be required to inform the risk assessment (for example, an outcrossing study). In all cases, the trigger for generating additional agronomic data should be based on problem formulation and a generation of plausible risk hypothesis and pathways to harm.
Generation of additional data based on relevant pathways to harm related to the intended trait. Depending on the intended trait, additional data that may be relevant to consider for ERA include:	
Characterization of potential hazard to non-target organisms	Understanding the spectrum of activity (specificity) of the newly introduced trait is only relevant for traits with a toxic mode of action (e.g., insecticidal traits). Spectrum of activity studies provide a foundation for NTO testing strategy for a newly expressed trait that confers insect protection. Similarly, NTO insect bioassays are only relevant for traits with a toxic mode of action. For traits that do not have a toxic mode of action, or where there is no plausible hypothesis for harm, understanding the specificity of the protein and/or conducting insect bioassays to assess NTO hazard has limited value for ERA (for example, EPSPS protein that confers tolerance to glyphosate).

Table 2: Continued

Characterization of trait expression	Characterization of trait expression in plant tissues is only relevant for traits with a toxic mode of action (e.g., insecticidal traits) or that otherwise directly harm valued entities. Risk is a function of both hazard and exposure. The concentration of a newly expressed trait in a GM plant is relevant for a trait that confers insect protection because this information is used to characterize the magnitude of an NTO potential exposure. If the newly expressed trait is not insecticidal or there is no toxic mode of action, trait expression in plant tissues does not inform the ERA, unless there is a plausible hypothesis (e.g., pathway to harm).
Characterization of environmental fate in soil, sediment, or surface water	Characterization of trait concentration in soil, sediment, or surface water is only relevant for traits with a toxic mode of action (e.g., insecticidal traits) and if there is a plausible hypothesis for why the newly introduced trait would persist in the environment. If the newly expressed trait does not have a toxic mode of action, environmental fate studies do not inform the ERA because there is no hazard. Generation of data on a new trait should only be needed if there is no existing data on closely-related traits to inform the risk assessment or if there is a plausible hypothesis for why the newly introduced trait would persist in soil, sediment, or surface water differently than other traits (i.e., proteins or dsRNA).
Characterization of potential effects on soil microbial communities and other plants	The potential effects of a newly expressed trait on soil microbial communities should only be considered if there is a specific hypothesis for how the trait could negatively affect the soil microbial community or specific microbes (for example, a trait that confers antimicrobial or antifungal properties). Consideration of the potential for allelopathic effects on other plants should be assessed if there is a specific hypothesis of changes in germination or growth inhibition based on biochemical properties of the introduced trait.

3) Characterization of the GM crop: generation of additional data based on relevant pathways to harm related to the intended trait

As discussed above, during problem formulation an understanding of the intended phenotype of the GM plant allows for relevant pathways to harm related to the trait to be considered. The ERA should focus on the known or expected effects of the trait on valued components of the biotic and abiotic environments. For instance, a trait conferring insect protection will generate different potential pathways to harm and require different data compared with a trait conferring drought tolerance. The data that are required for an ERA should be driven by problem formulation, assessment of the core data (Table 1), assessment of the intended trait, formulation of potential pathways to harm, and development of plausible hypotheses, which is why these data are considered on a case-by-case basis.

Assessment of the intended trait: (a) Characterization of potential hazard to non-target organisms (NTOs)

The need to understand and characterize the spectrum of activity (specificity) of a newly introduced trait and to assess potential hazard to NTOs is limited to traits that confer insect protection (insecticidal traits), have a toxic mode of action, or that otherwise could directly harm a valued entity. The spectrum of activity of the active ingredient will inform the risk assessment for insecticidal traits. For example, Cry1 protein activity is limited to the order Lepidoptera and Cry3 protein activity is limited to the order Coleoptera [56, 49]. Understanding the specificity of the insecticidal trait can be used to determine what non-target orders or species make sense to assess for potential hazard. Typically, for insecticidal traits, several non-target surrogate species from different orders are selected for testing (for example, honeybee, lady bird beetle, non-target lepidopteran [46, 44, 45]). Surrogate species are selected based on their relatedness to the target pest, relevance to beneficial NTOs of interest, and ability to be reared and tested in the laboratory using standardized methods. As described by Bachman et al. [4], laboratory hazard studies on surrogate species conducted in one country can be used in problem formulation for the ERA in countries. The spectrum of activity of the trait, as well as the potential for exposure and hazard to NTOs, can be used to develop potential pathways to harm and plausible hypotheses, which can direct if additional non-target organism laboratory assessment will inform the risk assessment. Likewise, the spectrum of activity of the trait, combined with information generated in laboratory assessments (Tier I and/or Tier

II testing), should be used to determine if additional non-target hazard assessment in a greenhouse (Tier III) or in the field (Tier IV) are required for risk assessment. A tiered testing approach to non-target hazard assessment should always be leveraged to avoid unnecessary higher-tier greenhouse or field studies when they are not informative for the risk assessment [7].

Assessment of the intended trait: (b) Characterization of trait expression

Data characterizing the expression of the newly introduced trait in plant tissues should only be required for the ERA on a case-by-case basis. For example, for an insecticidal trait, understanding the concentration of the insect-active substance (e.g., protein or dsRNA) in appropriate plant tissues is relevant to consider, as it helps inform problem formulation (i.e., potential exposure to NTOs). In the case of a lepidopteran active trait that is expressed in maize pollen, there is a plausible hypothesis for risk to a non-target lepidopteran that could incidentally ingest maize pollen while feeding on leaves on which pollen has deposited. On the other hand, if the lepidopteran active trait is not expressed in maize pollen, there is no plausible hypothesis for exposure to non-target lepidopterans. A lepidopteran that feeds on other maize tissues is not considered in this scenario, as it would be viewed as a pest. The concentration of the insecticidal trait in pollen can also inform potential exposure to other non-target insects in agroecosystems that also may consume pollen (for example, ladybird beetles). Similarly, understanding the concentration of the insect-active trait in other crop tissues is also informative for potential exposure to other NTOs. For example, predatory insects that feed on herbivorous prey may be exposed to the insect active traits through prey feeding [7]. There is a large body of knowledge about the lack of bioaccumulation and persistence of *Bacillus thuringiensis* (Bt) Cry proteins in prey [45]. Therefore, a predator is unlikely to be exposed to a higher concentration of a Cry protein via prey, relative to the concentration of the Cry protein that is in the crop tissue. Understanding the insecticidal trait concentration in crop tissues can help inform the potential exposure to predatory insects and can be used to develop plausible hypotheses for harm, since risk is a function of both hazard and exposure.

However, for gene products that do not have a toxic mode of action, and gene products that do not otherwise directly harm valued entities in the environment, understanding the concentration of the newly-expressed trait in plant tissues has limited value for the ERA. In these cases, since there is no a plausible hypothesis for hazard to NTOs, it is not informative to characterize the concentration of the newly introduced trait. Therefore, trait expression should only be required to assess environmental risk on a case-by-case basis, which is limited to traits that confer insect protection or otherwise have a toxic mode of action.

Assessment of the intended trait: (c) Characterization of environmental fate in soil, sediment, or surface water

Similar to trait expression in plant tissues, data characterizing the persistence of a newly expressed trait in environmental compartments such as soil, sediment, or surface water should only be required on a case-by-case basis. As described above, risk is a function of both exposure and hazard. The duration of exposure of a newly expressed trait to an NTO is only relevant for insect protection traits or traits with a toxic mode of action or that otherwise could directly harm a valued entity. For example, for a non-insecticidal trait where there is no plausible hypothesis for hazard to an NTO, understanding the persistence of a newly-expressed protein in environmental compartments has limited value for the ERA. Therefore, soil or water dissipation data should only be required to assess environmental risk on a case-by-case basis, which is limited to traits that confer insect protection or have a toxic mode of action.

In cases where there are existing data about the persistence of an insect protection trait in soil, additional studies may not be necessary to characterize risk. For example, from over 20 years of commercial use and risk assessment, there is a large body of evidence that Bt Cry proteins do not accumulate or persist in soil [28, 12, 53]. Cry proteins, in general, dissipate rapidly in soil [50, 33, 27]. The lack of persistence in soil can be used to understand persistence of Bt Cry proteins in sediment, surface water or other environmental matrices. The ERA for a GM crop that expresses a Bt Cry protein may be able to use existing data, and additional soil dissipation data may not further inform the risk assessment. Similarly, the existing soil fate data on Bt proteins can inform the risk assessment of non-Bt proteins. Characterizing the soil dissipation of a non-Bt protein may not be needed to inform the risk assessment, unless there is a specific hypothesis for why the source of the non-Bt protein would alter a proteins dissipation and degradation in soil. Additionally, for GM plants using RNA interference, there is strong evidence that dsRNA does not persist in soil [39, 20], sediment, or surface water [1, 23]. The degradation kinetics and persistence of dsRNA is not sequence dependent [22], and additional soil dissipation studies for GM plants that contain different sequences of dsRNA may not be necessary to characterize exposure or risk.

Assessment of the intended trait: (d) characterization of potential effects on soil microbial communities and other plant

The potential effects of a newly expressed trait on soil microbial communities should only be considered if there is a specific hypothesis for how the trait could adversely affect the soil microbial community or specific microbes. For example, a plausible hypothesis could be developed for how an antimicrobial trait or an antifungal trait could affect soil microbial communities. In these cases, if the concentration and persistence of the trait in the environment is deemed meaningful (see *(c)*, above), assessing the soil microbial community to ensure there are no unreasonable adverse effects on microbial-mediated soil processes may inform the risk assessment. Similarly, consideration of the potential for allelopathic effects on other plants should be assessed if there is a specific hypothesis of changes in germination or growth inhibition based on biochemical properties of the introduced trait. However, for most GM traits commercialized to date (HT traits, insect protection traits, etc.),

there is no plausible hypothesis for harm to microbial communities in soil or allelopathy, and studies assessing the number, abundance, or community structure of soil microbial communities, or on production of neighboring or following crops, do not inform the risk assessment. To date, there are no indications that GM plants negatively affect soil microbial communities [28]. Soils are inherently dynamic, and soil microbial communities are known to be impacted to some degree by crop rotation, management practices, and other environmental variables. Changes in soil microbial communities does not necessarily indicate harm, and there is evidence to suggest that the magnitude of change in microbial abundance due to GM crops is small relative to the overall variability in the soil [28]. In any event, if evaluation of the soil microbial community is relevant on the basis of problem formulation, functionally-focused studies of relevant soil microbial processes will likely be more meaningful than community-wide studies.

1.4. Data Requirements that Can Add to the Weight of Evidence but Are Not Required to Inform the ERA for Cultivation

As part of the overall cultivation application, some data are collected for product characterization. While these data may not directly inform the ERA, it can add to the weight of evidence (WOE) of safety. This data should not be required specifically to conduct an ERA but could be used on a case-by-case basis to help add information and context to the risk assessment (Table 3). For example, understanding and characterizing the source of the donor genes is commonly included in cultivation dossiers as part of the molecular characterization of the event. The source of the gene is also considered in the context of the food and feed safety assessment for import approvals, because if the source of the gene can be shown to have a history of safe use (HOSU), it adds to the WOE that the gene products are safe for food and feed [18]. For ERA, genes that come from a donor that has a HOSU may also add to the WOE of safety (i.e., familiarity). For instance, the greater than 20 years of knowledge and experience gained during the cultivation of GM crops expressing Bt Cry proteins helps inform the ERA for new crops expressing Bt Cry proteins. In these cases, there may be less need for additional data to be generated, because published literature can be used to assess the risk of a new Cry protein based on familiarity with Cry proteins in general. Similarly, if a novel source of proteins is utilized, but the source is widely dispersed in agricultural or natural habitats, familiarity with the source can add to the WOE to support the ERA. For a GM crop that expresses a novel protein from a novel source, prior information about the environmental effects of that source may be useful as part of the problem formulation for the risk assessment. However, in the absence of such prior knowledge, establishing the safety of the inserted gene is more informative for the risk assessment than establishing the safety of the source of the gene.

Similarly, the mode of action of a newly expressed protein in a GM plant is often investigated as part of the food and feed risk assessment, and can also be leveraged in the ERA, but should not be required to assess risk. Some understanding of the mode of action of a protein informs problem formulation and helps determine and develop plausible hypotheses for harm. For example, for a GM crop expressing a Bt Cry protein, it is helpful to understand that this protein is insecticidal, binds to specific receptors in the midgut of certain insects, and is ingested in the diet: knowledge of the receptor specificity can be used to guide the NTO testing scheme. However, a detailed understanding of how the insecticidal protein works at the molecular, cellular, or anatomical level is not required to assess environmental risk if the effects of the GM trait, as expressed in the crop, on valued components of the environment are understood sufficiently well to address plausible risk hypotheses. Similarly, for an HT trait, understanding the basic mechanism for tolerance to the herbicidal active ingredient may add to the WOE, but is not needed to assess the environmental safety of the GM plant. In many cases, herbicide tolerance is conferred by an enzyme that can detoxify the herbicidal active ingredient when expressed in the plant. In the case of an enzyme, confirmation of substrate specificity and the affinity of the enzyme for the herbicide (inhibitor) may inform the safety assessment. In both of these examples, if the GM crop is shown to have substantially equivalent agronomics to the non-modified plant, and there is an understanding of the intended function of the trait, additional refinement of the specific mode of action of the trait would only be required if plausible hypotheses for harm could be developed and addressed with mode of action information.

1.5. Data Requirements that Do Not Inform the ERA for Cultivation

As part of the overall cultivation application, additional molecular, protein, and event characterization data are collected, but these data do not directly inform the ERA. For example, as part of product characterization, molecular studies are conducted to confirm that the insert is an intact single copy, stable across generations, and that there is no plasmid backbone DNA [8, 10, 26, 32]. Southern blots, and more recently, next generation sequencing (NGS) data are submitted as part of the cultivation application, but these data are not a requirement for an ERA (Table 4). Similarly, characterization studies are conducted to confirm that the surrogate test material (e.g., microbially produced protein) is equivalent to the plant-expressed trait. Because plant-expressed traits are typically difficult to extract in high enough amounts to support safety hazard testing, surrogate test materials are generated for laboratory safety testing. In the case of a proteinaceous trait, characterization of the microbial-produced protein (e.g., amino acid analysis, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot, N-terminal sequencing, mass spectrometry for intact mass determination and peptide mapping, glycosylation staining) are conducted to demonstrate equivalence, but these protein characterization data are not directly relevant for the ERA. It is the effects of the plant-expressed protein, rather than its sequence, that inform the risk assessment.

For food and feed safety assessment, the composition of the grain and forage of the GM plant has historically been compared to the composition of the non-modified plant. However, there is strong evidence, based on more than two decades of experience, that compositional assessment beyond assessment of the intended change(s) is unwarranted for food and feed safety

Table 3: Data that can add to the weight of evidence but are not required to inform the ERA for cultivation

Data that are not necessary for ERA	Adds to weight of evidence
Characterizing the source of the donor gene	Prior knowledge of the safety of the source of the inserted gene in a GM plant may add to the WOE (for instance, if the source can be shown to have a history of safe use). However, in the absence of such prior knowledge, establishing the safety of the inserted gene is more informative for the risk assessment than establishing the safety of the source of the gene.
Characterizing the mode of action/mechanism of action	An understanding of the mode of action (MOA) of an introduced protein may inform problem formulation and be used to help develop pathways to harm. However, a full understanding of how the insecticidal trait works at the molecular, cellular, or anatomical level is not required to assess environmental risk if the effects of the GM trait, as expressed in the crop, on valued components of the environment are understood sufficiently well to address plausible risk hypotheses.

Table 4: Data requirements that do not inform the ERA for cultivation

Data that are not necessary for ERA	Not required for ERA for cultivation
Molecular characterization	Southern blots, and more recently, next generation sequencing (NGS) data, are submitted as part of the cultivation application. This information is needed to support the molecular characterization of the inserted gene, but it does not directly inform the ERA.
Trait characterization	If a surrogate test material is used in acute toxicology studies or NTO laboratory hazard studies, characterization data are typically generated to demonstrate equivalence to the plant-expressed protein. This information is needed to support use of surrogate test material for use in hazard studies, and is therefore relevant in the context of protein safety, but it does not directly inform the ERA.
Composition	The composition of the grain and forage of the GM plant is compared to the composition of the non-modified plant. These data have historically been required for food and feed safety assessment. For ERA, compositional assessment should only be considered if there can be a plausible pathway to harm to the environment.
Product efficacy	The overall efficacy of the trait is relevant when considering the commercial value and benefits of the product, but this information is not directly relevant to the ERA. In the context of the overall assessment of the product, benefits should be factored into the decision-making process, because in some cases the benefits of a product or trait may outweigh the risks. Nevertheless, assessment of benefits does not directly inform the ERA.
Horizontal gene transfer (HGT)	There is no evidence that HGT occurs under natural conditions at rates that have an environmental impact. Therefore, while HGT may be considered as part of the ERA, generating data specific to the inserted gene is not needed to assess risk.

assessment. There is, in fact, more natural variability in composition among conventional varieties, which all have a history of safe use, than there is between a GM crop and its genetically-close conventional comparator [14]. For food and feed safety, composition of a new GM plant should only be assessed if the data, as determined using a hypothesis-driven, stepwise approach, will inform the safety assessment [9]. Likewise, for ERA, compositional assessment should only be considered if there is a plausible pathway to harm to the environment. For GM products, crops, and traits commercialized to date, composition data has not been scientifically relevant for the ERA. Likewise, while the efficacy of the product is considered in the overall product submission, data on product efficacy is not a relevant consideration for ERA. Finally, the requirement to assess the potential for horizontal gene transfer (HGT) of the inserted DNA into microbes in soil or digestive tracts does not inform the ERA because there is extensive evidence that HGT does not occur under natural conditions [31, 17, 34].

1.6. Recommendations for Harmonization of Global Data Requirements for ERA

The ERA framework is robust and flexible. This framework uses problem formulation to generate plausible risk hypotheses and allows risk to be assessed using a science-based approach. There are only a few key pieces of data that should be universally required to inform the ERA for all crop and trait combinations. These include an understanding of the receiving environment and the basic biology of the unmodified plant; an understanding of the intended phenotype of the GM plant and assessment of how the intended phenotype may lead to environmental harm; and an assessment of the agronomic similarity of the GM crop to its conventional counterparts. These key pieces of information serve as the foundation of any ERA, and most global regulatory data requirements assess these key pieces of information for cultivation approvals; however, there remains a lack of global alignment and scientific consistency for when additional data are required for the ERA of GM crops.

Problem formulation and the development of plausible risk hypotheses are both important steps in the ERA, which help identify additional data that are needed to inform the ERA. The concepts of familiarity (i.e., using the existing body of knowledge for GM crops and traits with a history of safety) and data transportability (for example, using an agronomic study conducted in one country can be used to assess the need for additional agronomic data to be generated in another cultivation country) are both key components of problem formulation that are used to structure the risk assessment. These key pieces of data serve as a foundation for the risk assessment, and the need for additional data is assessed on a case-by-case basis, depending on the crop, trait, receiving environment, and protection goals. Refinement of data requirements to those that inform the ERA and harmonization of data requirements across global regulatory authorities would add transparency and consistency to the ERA of GM crops globally.

2. Declaration of Conflicting Interest

All the authors of this paper are currently employed by, or have been employed by, the agricultural biotechnology industry.

3. Disclaimer

The findings and conclusions in this publication are those of the author(s) and should not be construed to represent any official USDA or U.S. government determination or policy.

4. Article Information

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Data Transportability for Studies Performed to Support an Environmental Risk Assessment for Genetically Modified (GM) Crops

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Abstract

Laboratory and field data generated on genetically modified (GM) plants in one country can inform the environmental risk assessment and support regulatory decision-making for GM plants being cultivated in another country. Well-designed studies that test clear risk hypotheses and that follow well-established methods allow for conclusions to be made about potential environmental effects from cultivation of a GM plant relative to its conventional counterparts. Following the principle of data transportability, if no biologically relevant differences between a GM plant and its conventional counterparts are observed in one country or region, data from these studies can be used to inform the risk assessment in another country, regardless of agroclimatic zone. Similarly, if biologically relevant differences are observed in studies conducted in one country, these data can be used to assess potential environmental harm in another country. Gathering additional data for the ERA in a different country or in expanded regions may increase the weight of evidence of environmental safety, but additional field study data are only warranted if specific hypotheses of risk remain after assessing risk based on the existing data, and if they would affect the outcome of decision-making. Transportation of product data across regions has been successfully used by multiple countries to eliminate redundancy, create regulatory efficiencies and enable timely realization of the benefits of GM plants.

Keywords: data transportability, environmental risk assessment, genetically modified plants, agroclimate

1. Introduction

Laboratory and/or field studies on genetically modified (GM) plants are conducted as part of an environmental risk assessment (ERA) to determine whether cultivation or incidental release of the GM plants could cause unreasonable environmental harm. Data that are developed as part of a science-based ERA for cultivation of GM plants should be driven by problem formulation and the identification of plausible pathways to harm [2, 30]. Problem formulation in the ERA for the cultivation of GM plants is based on information related to the receiving environment, the biology of the plant, the phenotypic similarity of the GM plant relative to its conventional counterparts, and the characteristics of the introduced trait [2]. These represent the core data for ERA and can be used to establish plausible relevant pathways to harm related to plant persistence, weediness or invasiveness, and gene flow. Any need for additional data for the ERA should be considered on a case-by-case basis, guided by problem formulation and development of risk hypotheses based on the core data and trait interactions with the environment [2]. For example, for a trait that has insect resistance properties, concentration data for the introduced gene product and non-target organism (NTO) laboratory hazard data may be necessary to understand potential effects beyond the target pest.

Sometimes, despite a lack of country-specific hypotheses of unique risks, regulatory agencies require local laboratory and/or field studies in a country intending to cultivate the GM plant. Some agencies also require local agronomic studies when the GM plant products (e.g., grain) is intended for import and will not be cultivated. For example, regulatory agencies in Japan have required local field studies for import approvals for some GM events, depending on the crop and trait (GM soybean re-

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quires a local field study to be conducted in Japan, but corn and cotton containing familiar traits do not). The regulatory agency in China (Ministry of Agriculture and Rural Affairs, MARA) accepts global data as part of the import permit application, and local field studies as well as other laboratory-based studies are then commissioned by MARA to be conducted by a local institution in China. The requirements to repeat studies in different countries lead to duplication of data and adds time and complexity to the regulatory process of GM plant approvals without providing additional information essential to the ERA [8]. The time added to the regulatory process delays accrual of benefits of new products to growers and consumers in both cultivation and import countries. Additionally, taxpayers bear the cost of unnecessary regulatory review of duplicative data. A more efficient approach for conducting a science-based ERA in a new location, known as data transportability (DT), consists of leveraging existing data generated in other places. This Policy Commentary explores the concept of DT and provides scientific justification for its use with both laboratory and field data employed in the ERA of GM plants.

2. Overview of Environmental Risks Assessed for GM Plants

Protection goals are derived by each country according to local laws and legislation; however, protection goals related to the environment tend to be broadly similar, such as protecting sustainable food production and biodiversity. Three core areas are typically considered as part of the ERA of GM plants [5, 12, 14]: assessment of weediness/invasiveness potential; assessment of the potential for and effects of transgene flow; and, for insect resistance traits, assessment of potential adverse effects on beneficial NTO populations. Problem formulation, which is based on knowledge of the receiving environment, biology of the plant, agronomic comparison of the GM plant to conventional counterparts, and the characteristics of the introduced trait, is used to assess whether sufficient information and data already exist to address these elements of the ERA and to develop specific hypotheses of harm relating to specific protection goals.

2.1. Weediness and invasiveness

Assessment of a plant's weediness potential considers whether the GM plant has increased weediness characteristics compared with the non-modified crop. An example of a specific hypothesis for harm related to weediness is that the GM plant has introduced traits that increase its ability to outcompete and reduce the abundance of a valued plant species in the environment, including other crops in the agroecosystem. Highly domesticated crops such as maize, cotton, and soybean have a long history of cultivation, and selective breeding has reduced or removed their weediness traits [24]. Many weedy traits, such as seed dormancy or shattering, are agronomically unfavourable and have been selected against in modern crop varieties. As discussed by [2], information about the receiving environment and the biology of the crop, understanding of the intended trait and how it may lead to increased weediness potential, and agronomic field data assessing the similarity of the GM plant to its conventional counterparts, allows for relevant pathways to harm related to weediness to be considered.

2.2. Effects of transgene flow to sexually compatible wild relatives

Gene flow can occur naturally among plants that are sexually compatible and sympatric. For the ERA of a GM plant, it is important to assess whether the introduced gene, if successfully introgressed into a wild relative population, could provide a selective advantage to that population to a greater level than other native genes in the cultivated species. As with weediness assessments, information about the receiving environment and the biology of the plant, an understanding of how the intended trait may lead to increased weediness potential of wild relatives, data on relative agronomic performance, coupled with an understanding of the potential for successful outcrossing and transgene introgression into a wild relative population, allows for relevant pathways to harm related to transgene flow to be considered.

A specific hypothesis for harm related to gene flow for GM soybeans that are to be cultivated in an area where wild soybeans are present would consider if the introduced trait in the GM plant become introgressed into the wild soybean population and confers a selective advantage. Cultivated soybean (*Glycine max*) is sexually compatible with wild soybean (*Glycine soja*), and genes from cultivated soybean can be found in wild soybean populations. An understanding of GM trait in cultivated soybean can be used to assess potential for selective advantage if the trait is introgressed in the wild soybean population. In many cases, introgression of a trait does not result in harm. For example, yield genes and abiotic stress tolerance genes that have been selected for through traditional breeding for generations have not been observed to provide a selectable advantage to wild soybean in North Asian countries such as Japan, China, Korea, and Taiwan, as evidenced by the lack of adverse effects on wild soybean populations in these countries after years of import of non-GM domestic soybeans with improved yield and stress phenotypes [9, 15, 16, 17, 18, 19, 20, 21, 22]. Therefore, it is reasonable to expect that GM traits that increase yield or abiotic stress would likewise not provide a selectable advantage to wild soybean. Similarly, it has been demonstrated that outcrossing by a GM soybean modified with an insect resistance gene from *Bacillus thuringiensis* (*Bt*) does not provide a selective advantage to wild soybean, based on the outcome of insect feeding damage surveys in wild soybean populations [10].

Agronomic endpoints related to reproduction (pollen viability, pollination rates, etc.), can be used to inform whether the trait has increased the potential for outcrossing, and information on the crop biology and the receiving environment can be used to inform the likelihood of outcrossing with sexually compatible wild relatives. There is rarely a plausible hypothesis that the trait in a GM plant has altered the outcrossing rates relative to the outcrossing rates of the non-modified crop. The only hypothetical exception to this would be if the GM trait alters

pollen dispersal or pollination rates, perhaps to increase crop yield. However, no such traits have been developed to date.

2.3. Effects on beneficial non-target organisms

Efficient crop production depends on valuable interactions between the crop and its biotic environment. For example, arthropod populations can be beneficial to agricultural production; many crops rely on insects for pollination; insect predators and parasitoids provide important ecosystem services by reducing the populations of insect pests that feed on the crop; some soil arthropods are important in processing decaying vegetable matter and maintaining soil function. Therefore, it is important to understand the potential for a particular GM trait to reduce the abundance of beneficial taxa representing core ecological functions (e.g., pollinators, predators, parasitoids, and decomposers). The need to generate data to assess the effects on NTOs typically only informs an ERA for GM plants with traits that confer insect protection (insecticidal trait), such as Cry proteins from *Bt* and traits based on RNAinterference (RNAi). The ERA of insect-protected crops includes an assessment of the effect of the trait on beneficial non-target arthropods (NTAs) that may be exposed to the trait and may be sensitive to it. As *Bt* proteins and RNAi are highly specific in their spectrums of activity, focused NTO testing under laboratory conditions is generally sufficient to detect meaningful effects (e.g., >50 percent mortality [29]) and higher tier studies (greenhouse or field study) are not conducted unless triggered by uncertainty in the tier 1 studies.

Beneficial microbial components of the agricultural environment, such as soil bacteria and fungi, could be considered within the ERA for GM plants if the introduced trait has antimicrobial activity, for example, through studies of soil microbial activity. However, no such traits have been developed to date and such studies are not warranted for existing GM traits.

3. Data Transportability (DT)

DT for the ERA of a GM plant can be defined as the use of data generated in one region or country to inform the ERA of the GM plant in another region or country. DT requires proper scientific justification to demonstrate that the data are suitable to inform the risk assessment. For example, studies should have clearly defined and relevant environmental risk hypotheses, follow well-established methods, have a suitable study design (e.g., adequate replication, randomization, and sampling), and use appropriate statistical analyses that are suitable for the environmental risk assessment.

3.1. Field studies

Agronomic field studies are typically conducted across multiple locations that are representative of the growing region in the country where the GM plant was developed. The purpose of these field studies is to assess the phenotypic and agronomic similarity between the GM plant and the conventional counterpart and to determine the concentration of an introduced gene product in different plant tissues and across multiple growth stages. If a GM plant and its conventional counterpart are observed across a range of environmental conditions in one country or region, these agronomic and concentration datasets can be used to support the risk assessment in another country, regardless of agroclimatic zone. Gathering additional data for the ERA in a different country or in expanded regions may increase the weight of evidence of environmental safety, but additional field study data are only warranted if specific hypotheses of risk remain after assessing risk based on the existing data, and if the additional field data would affect the outcome of decision-making.

3.1.1. Agronomic and phenotypic observation

Field studies for phenotypic/agronomic observations should not be designed or expected to characterize the agronomy of the GM plant in as much detail as possible in a given region or climate. Instead, field studies are used to identify any biologically relevant adverse changes to the GM plant as a result of the GM trait and compare these changes against a range of conventional counterparts grown in the same area and under the same conditions [26]. Agronomic field studies that follow current guidance (for example, [7]) are conducted in multiple locations that represent a diversity of the commercial crop growing areas, measure a standard suite of agronomic endpoints (some of which are relevant for the ERA), and are analyzed with appropriate statistical analyses to detect biologically-meaningful differences between GM plants and near-isoline control and/or representative reference lines. Environmental conditions, including climate, weather, and soil type, can influence how both GM and conventional crops grow. Changes in growth patterns of crops due to local environmental conditions are inherent and expected for both conventional and GM plants systems, but this natural variability is not indicative of environmental risk. There is a large range of agronomic properties that enable crops to grow in different environments with an established history of safety. Without a plausible mechanism based on the characteristics of the introduced trait, the potential for unintended or unanticipated harmful differences to occur in one environment and not in other environments is remote. In most cases, data from confined field trials can be transported across regions, regardless of agroclimatic conditions. When a plausible hypothesis can be developed for how the GM plant could result in harm in a different region, studies designed specifically to investigate the likelihood and magnitude of potential harm can be conducted [2]. For example, a cold hardiness trait may have an impact in temperate zones that may not be apparent in tropical areas, and additional testing in a temperate zone may be warranted. Other scenarios may exist where similarity of environments may be useful to justify the transportability of data, such as when there is an expectation that the expression of intended phenotype is heavily dependent on environment (e.g., drought tolerance) [8].

Selection against weedy traits during crop domestication is desired in modern crops, and agronomic endpoints that are related to weediness characteristics (for example, seed shattering, dropped ears) are typically measured in agronomic field studies. If the host plant (conventional crop) exhibits no weed-

iness characteristics, the GM trait is not related to weediness characteristics, and agronomic endpoints related to weediness characteristics are shown to be comparable to the non-modified plant, then no plausible hypothesis exists to support increased weediness potential of the GM plant, and the agronomic field study data collected in one country can and should be transported and used to inform the ERA in another country. On the other hand, if the host plant does exhibit weedy characteristics [23], and/or if the GM trait is observed to affect the agronomic endpoints that are related to weediness characteristics, the agronomic field study data collected in one country still can and should be transported and used to inform the ERA in another country. As discussed above, the comparative nature of well-designed field studies examines the GM plant in comparison to its conventional counterpart in a range of environments, and agronomic endpoints that are related to weediness characteristics should be able to be transported to inform the ERA in another region. If there remain additional plausible hypotheses for environmental harm related to weediness after considering the available transported agronomic field study data, additional studies to address those hypotheses may be warranted in another country, but the decision to request additional in-country data should be on a case-by-case basis to inform a hypothesis for harm that cannot be addressed with the available data from other countries.

As with weediness risk assessments, environmental risks associated with transgene flow to sexually compatible wild relatives can be assessed in one country using field study data from another country. Data and conclusions from field studies that demonstrate lack of biologically relevant differences in agronomic performance of the GM plant and its conventional counterpart across a range of environments can be used to inform the transgene flow risk assessment in another country.

i. Environmental exposure

For certain aspects of risk assessment, measures of exposure to environmental stressors can be necessary. These measures are warranted for assessment of risks to potentially sensitive NTOs like beneficial arthropods, on a case-by-case basis only when a potential hazard of a GM trait is identified by problem formulation, such as one that confers insect protection [2]. Potential exposure of NTOs are typically informed by measuring tissue specific concentrations of a gene product (newly expressed protein, dsRNA, etc.,) collected from field studies conducted under a range of field conditions. As with agronomic field study data, the concentration data for the gene products are collected from plants grown in multiple locations that represent the major growing areas for the crop, typically in the country of development. Protein or dsRNA concentration is measured in different plant tissues and different growth stages, and it can be used to estimate potential for exposure to NTOs (for example, a honey bee may be exposed to a protein/dsRNA expressed in maize pollen). Expression product concentration data and conclusions from studies conducted in one country are transportable to other countries for the purpose of assessing potential exposure to NTOs, and generating new expression data in one country should not need to be repeated to inform the ERA in another country.

ii. Laboratory data

When an NTO risk assessment for GM plants with traits that confer insect protection is needed, NTO testing should follow a tiered approach whereby laboratory studies are first conducted at high concentration of the GM gene product in the laboratory. Higher tier testing using GM plant tissue, greenhouse trials, or field studies to assess potential effects on NTOs are only warranted when they are triggered by effects seen in the lower tier laboratory assessment.

The transportability of laboratory data has been widely accepted by regulatory agencies globally for both GM plants [27, 28] and traditional chemistry testing [13, 25] because laboratory conditions are not intended to represent realistic environmental conditions. When laboratory studies are considered for use in a risk assessment or regulatory decision making, they should be evaluated for relevancy and reliability. Methods should be reconstructable, interpretable, reliable, and include appropriate statistical analysis. Test systems and study design should follow standardized and internationally accepted guidelines (e.g., Organisation for Economic Co-operation and Development, U.S. Environmental Protection Agency) or peer-reviewed published methodologies, when available. Finally, laboratory studies should be conducted under widely accepted quality criteria (e.g., good laboratory practices, International Organization for Standardization) to ensure reproducibility of the data. Numerous authors have set forth recommendations of laboratory testing to support the ERA of GM plants [3, 27]. Surrogate species are often used in laboratory testing, and surrogate species are selected based on relatedness to target pest and beneficial NTOs, amenability to testing under laboratory conditions, availability of standardized methods, etc. [4, 28]. The surrogate species concept is well-accepted, and testing at high concentration in the laboratory in the early tier hazard assessment (for example, 10X the concentration an NTO could be exposed to in the environment) provides a high margin of exposure and protection for other species that may be in the environment but not directly assessed in the laboratory. The data and conclusion from the laboratory hazard studies are transportable across regions due to the controlled nature of laboratory studies, validated, robust, and reproducible methods, and use of surrogate species. When triggered by the tiered testing approach, a field study may be conducted to assess the consequences of the hazard to NTOs under environmentally relevant conditions. As with other field study data, field NTO study data can be transported across regions if they are designed to detect meaningful differences in NTO abundance or function between the GM plant and its non-GM counterparts. Additional field testing should only be conducted in another country if there is a specific hypothesis for harm that cannot be addressed using all of the existing data, for example, if there is an NTO taxon of particular concern in one region that is not present in the original one, and familiarity with the GM trait and lower tier laboratory data are insufficient to assess risk.

For import-only scenarios (e.g., for food, feed, and processing) the potential exposure of individual NTOs to a GM plant is

low and the potential for population level exposure is negligible (i.e., seed spillage during transportation and/or Low Level Presence in conventional planting seeds) relative to cultivation scenarios, and therefore the risk to NTOs from import of GM grain is negligible when compared with the risk from cultivation of GM plants. In the case of import countries, the data and conclusions from the cultivation country should be considered, and additional data are not warranted to assess risk.

4. Data Transportability Case Studies

Garcia Alonso et al. [8] presented a case for the transportability of field study data for ERA along with a conceptual framework and process for both regulators and the regulatory community. This approach to DT relied on the similarity of agroclimatic zones as the foundation to enable the transportability of field study data by encouraging the comparison of physical characteristics of the field study environment to the region where the data could be used. Identification of analogous agronomic climates in a given country could then allow for easy acceptance of data generated in the same agronomic climate in another country. This approach is intended to provide very explicit evidence to justify a regulatory agency's decision to accept data generated in another location.

In recent years, however, more studies examining field data from different environments have revealed that agroclimatic similarity is in fact not necessary for DT to be employed as part of the ERA of GM plants. Horak et al. [11] demonstrated that data collected to evaluate the weediness potential of soybean is transportable between cultivation countries. In this example, comparative studies were conducted in diverse locations across the U.S. and Argentina over three years, evaluating two GM soybean products and their conventional control. Data collected from distinct geographic and environmental conditions yielded similar results and conclusions regarding a lack of environmental risk. Where statistically significant differences were observed, no consistent trends across years and regions for these weediness characteristics were observed, and these differences were within the range of natural variability for soybean, thus providing additional evidence that these differences were not associated with the genetic modification process or the locations where the field study data were generated.

Nakai et al. [23] demonstrated that confined field trial data for GM plants are also transportable between cultivation and import countries. While GM plants are not cultivated in Japan and China, these countries require that in-country (local) confined field trials be performed for GM grain imported as food/feed or for processing. Currently, Japanese authorities will accept data derived in a cultivation country for GM maize with familiar traits (e.g., already registered). By examining the parameters under which the confined field trials are conducted, demonstrating that the endpoints assessed are relevant for the protection goals in import countries, and highlighting the low exposure scenario inherent in import scenarios, Nakai et al. [23] concluded that field study data, regardless of the characteristics of the inserted gene(s), are transportable from cultivation countries to importing countries (e.g., from the U.S. to Japan). Japan has accepted confined field trial data from other countries for ERA of GM maize for which the inserted gene(s) had already been assessed in other GM maize events to grant cultivation and import approval since December, 2014 [23]. As of March, 2018, confined field data collected in the U.S. for three GM maize products (MON87416, MZHG0JG, MZIR098) have already been accepted for implementing ERA in Japan.

In Mexico and other countries, cultivation approval requires in-country field assessment of the potential effects of the GM plants on NTAs. Corrales Madrid et al. [6] demonstrated that NTA data is transportable within diverse ecoregions in Mexico. Relevant NTA data from three types of GM maize were shown to be transportable across four ecoregions in Mexico. Importantly, the sites of the field studies represented high geographic and environmental diversity. No statistically significant differences in NTA taxa abundance between tests and controls were observed, and likewise, no adverse effects on NTAs were reported. As reported elsewhere [1], within the maize agroecosystem, a high conservation of taxa exists that link to core ecological functions (e.g., herbivores, predators, parasitoids, etc.). These conserved ecosystem services are present irrespective of the regional biodiversity and across temperate and tropical agroclimatic zones. This repetition of taxa facilitates their use as representative taxa for maize systems, thus enabling the transportability of data collected from one region to another [1]. The breadth of sites and high conservation of taxa shown in Corrales Madrid et al. [6], further demonstrates that NTA data and the associated conclusions regarding risk are transportable even in mega-diverse countries.

As described in Corrales Madrid et al. [6], the above studies provide empirical support that data from well-designed, comparative assessments and the associated conclusions on potential environmental risk are independent of the local environments and are transportable to other regions to inform the risk assessment.

5. Transportability of ERA Conclusions

This paper has focused on transporting data from one region to another to form the basis of an ERA in the recipient one. This principle can often be extended so that not just the data, but also the ERA conclusions, can be transported across regions based on problem formulation. Countries tend to have broadly similar protection goals for their agricultural environments, such as protecting sustainable food production and biodiversity, and therefore, similar risk hypotheses for the same crop. The risk assessments conducted based on those risk hypotheses are, therefore, similar and the conclusions are the same.

There are a few cases where ERA conclusions may differ in one region from another. First, if the agronomic data show meaningful differences between a GM plant and its conventional counterparts under certain environmental conditions, and those conditions are more prevalent in one region than another, the risk assessments may reach different conclusions. If there is a plausible hypothesis for how that agronomic difference could lead to environmental harm, additional assessment

may be warranted. Second, if there are sexually compatible wild relatives in one country that are absent in another country, the risk assessment conclusions may differ. Third, if there are specific (usually protected due to being endangered) NTOs in one country that may be affected by an insecticidal trait, the conclusion of the NTO risk assessment may differ. However, even in these situations where the risk assessment conclusions cannot be transported, the risk assessment data upon which they are based can still be transported.

6. Discussion and Conclusions

The transportability and acceptance of ERA data and/or conclusions from well-designed laboratory and field studies can facilitate the efficiency of regulatory approvals of GM plants across countries and regions. Acceptance of such data leads to more rapid access to benefits for farmers, reduces duplicative requirements, and ensures consistent science-based testing, data, and conclusions. This approach is similar to the standard practice of mutual acceptance of data that has broad acceptance within the chemical industry and is supported by international organizations such as the Organisation for Economic Cooperation and Development (OECD). The controlled nature of laboratory studies and comparative nature of field studies conducted across diverse environmental conditions allow for data to be viewed and conclusions to be made independent of an agroclimate or region, unless a specific risk hypothesis exists to oppose that consideration. Numerous peer-reviewed publications have demonstrated the scientific rationale for DT, and both cultivation and import countries are beginning to adopt and benefit from this practice.

7. Declaration of Conflicting Interest

All the authors of this paper are currently employed by, or have been employed by, the agricultural biotechnology industry.

8. Disclaimer

The findings and conclusions in this publication are those of the author(s) and should not be construed to represent any official USDA or U.S. government determination or policy.

9. Article Information

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Core and Supplementary Studies to Assess the Safety of Genetically Modified (GM) Plants Used for Food and Feed

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Abstract

Genetically modified (GM) plants used for food and feed have an established history of safe use over more than 25 years of their commercialization. Developers and regulatory authorities have accumulated extensive experience in evaluating their safety over time. The studies required for the safety assessment of GM plants used for food and feed should now be re-defined to leverage this experience and increased scientific knowledge. This paper, a companion paper for Waters et al. also published in this issue, presents a systematic approach for the safety assessment of newly expressed proteins (NEPs) in GM plants by evaluating the two components of risk: hazard and exposure. Although the paper focuses on NEPs, the principles presented could also apply to other expression products that do not result in a NEP. A set of core studies is recommended, along with supplementary studies, if needed, to evaluate whether the GM plant poses risk. Core studies include molecular and protein characterization and hazard identification encompassing toxicity and allergenicity. In the absence of hazard, core studies are sufficient to conclude that GM plants are as safe as their conventional counterparts. Depending on the GM trait and intended use, supplementary studies should be performed to characterize hazard and exposure when a hazard is identified. Problem formulation should be used to identify hypothesis-driven supplementary studies. Acute toxicity studies, compositional assessment, and dietary exposure assessment are recommended to be hypothesis-driven supplementary studies. Further discussion on the current food and feed safety assessment landscape for GM plants and the use of problem formulation as a tool for identifying supplementary studies can be found in the companion paper [62].

Keywords: genetically modified, safety assessment, food and feed, hazard, exposure, risk, core studies, supplementary studies

Abbreviations: Codex, Codex Alimentarius Commission; FAO, Food and Agriculture Organization of the United Nations; GM, genetically modified; HPP, hydroxyphenylpyruvate; NEP, newly expressed protein; OECD, Organization for Economic Co-operation and Development; ORFs, open reading frames; RNAi, RNA interference; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SNPs, single nucleotide polymorphisms; WHO, World Health Organization; WOE, weight-of-evidence

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1. Introduction

Since the commercial introduction of genetically modified (GM) plants in 1994, regulatory decisions have been made internationally to authorize their use for food and feed and for cultivation [34, 35]. In 2003, the Codex Alimentarius Commission (Codex) published guidelines for conducting safety assessments of GM plants [8] that have constituted the basis for developers and global regulatory authorities to evaluate their safety. To date, regulatory agencies have issued over 3,500 approvals for the use of GM plants for food and feed [34]. Although experience and scientific knowledge about GM plants has expanded, regulatory requirements for scientific data have been increasing disproportionately with the observed potential for risk [33, 71]. Even with the continued relevance of the Codex guidelines, there is an opportunity to leverage both the familiarity and established history of safety of GM plants to revise the safety assessment approach, given the expanded experience of product developers, regulatory authorities, and researchers. As further discussed in Waters et al., current scientific understanding and experience warrants redefining the studies that are sufficient to evaluate whether a GM plant is as safe as its conventional counterpart [71].

Safety assessment is part of an overall risk analysis [8]. Risk is a function of hazard and exposure [14]:

(Risk = Hazard x Exposure)

Lack of either hazard or exposure would imply that there is no risk. If it is determined that the newly expressed protein (NEP) presents both a potential hazard and potential exposure risk, a problem formulation approach considering both familiarity and the history of safe use should be used to identify specific questions relevant to the safety of the GM plant [5]. Evaluation of these identified risks should then be conducted according to the Codex science-based process employing a stepwise approach to hazard identification, hazard characterization, exposure assessment. and risk characterization [74]. It is recommended to evaluate hazard and exposure systematically during the safety assessment process using a problem formulation approach [71], where the broad 'problem' (i.e., food and feed safety of the GM plant) must be addressed based on the specific trait introduced, host plant, and intended use. Hypotheses that address specific safety questions must be framed, and study designs developed, to address these questions. Although a hazard-led approach has typically been followed for safety assessment of GM plants [14], exposure-based approaches for risk assessment have also been discussed recently [41, 52]. It is important to perform hazard identification studies as a basis for safety assessment of GM plants, although the approach for assessing potential hazards for these products is reconsidered in this manuscript based on knowledge and experience gained to date. Exposure-led studies, which are performed for small molecules [17], can also be helpful if relevant to the NEP and its expression in the GM plant when hazards are present.

In this paper, a core set of studies is recommended that is focused on characterization and safety assessment of the introduced trait. These recommendations are modified from earlier guidelines and recommendations for the safety assessment of GM plants (e.g., Codex, 2009 [9]; Delaney et al., 2008 [14]). A schematic overview of the recommended core and supplementary studies is available in Figure 1. Using the data resulting from the recommended core studies, and employing a "problem formulation" approach, the need for supplementary hypothesis-driven or case-by-case studies can be determined.

Depending on the nature of the introduced GM trait and intended use, supplementary hypothesis-driven or case-by-case studies may be further needed to complete the safety assessment. As outlined in Waters et al. [71], when the weight-of-evidence from core studies is not sufficient to determine the absence of hazard, supplementary studies may provide additional hazard characterization and/or exposure characterization to better understand the hazard presented by the NEP. As an example, one of the studies proposed to be supplementary is dietary exposure assessment, which is unnecessary if the weight-of-evidence [18] supports a conclusion of low or negligible hazard associated with consumption of a GM plant [41]. However, if the weight-of-evidence failed to provide support for a low or negligible hazard conclusion, a supplemental dietary exposure assessment and other supplemental data may be necessary to conclude on risk.

As previously discussed, thousands of safety assessments conducted globally have been consistent in their outcomes. Consequently, some jurisdictions have chosen to implement a streamlined and pragmatic approach to regulate GM plants for food or feed use by empowering the appropriate governmental body to authorize products based on the safety determinations of authorities in one or more other countries. This allows for efficient use of regulatory resources while maintaining a high level of safety for human/animal health and the environment. This approach to regulation is also embedded in the Codex guidelines which clearly state that "*where appropriate, the results of a risk assessment undertaken by other regulatory authorities may be used to assist in the risk analysis and avoid duplication of work*" [8].

The recommendations presented in this paper build on earlier guidelines and recommendations for the safety assessments of GM plants, and also incorporate the history of safety and familiarity that can be employed after 25 years of commercial use (e.g., Codex, 2009 [9]; Delaney et al., 2008 [14]) towards standardizing those assessments.

Core Studies: Characterization and Safety Assessment

It is noted that there may be alternative newly expressed substances that are not addressed in this manuscript; however, the principles presented could also apply to other expression products that do not result in a NEP.

The suggested core studies for typical (i.e., sexually propagated) GM plants producing a NEP are:

2.1 *Molecular Characterization*

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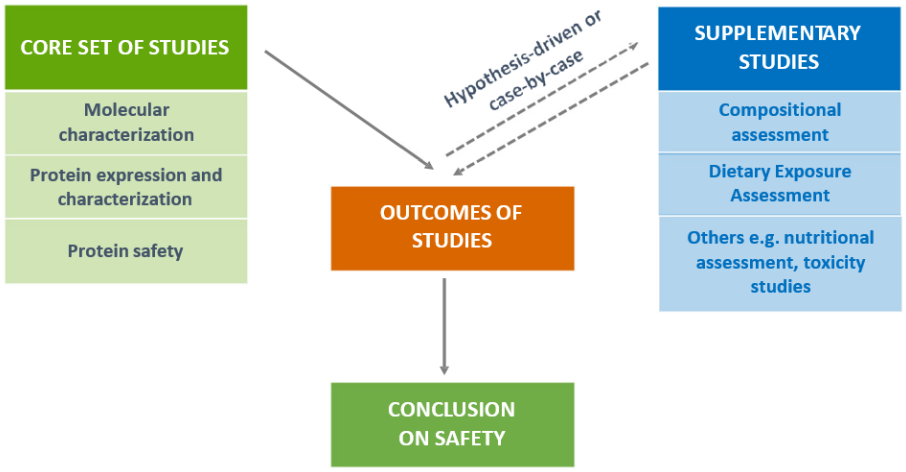

Figure 1: Schematic representation of core and supplementary studies for typical GM plants (reprinted from *Recommendations for science-based safety assessment of genetically modified (GM) plants for food and feed uses* [62]. Core studies are a set of studies necessary for a science-based risk assessment of a GM plant. These are suggested core studies for typical GM plants. There may also be alternative newly expressed substances (e.g. RNAi). Supplementary studies are studies to be conducted upon identification of information and/or hypothesis that indicates increased risk to human or animal health. The conduct of these studies depends on the nature of the introduced trait, intended use and data obtained from core studies.

2.1.1 *Number of insertion loci and inserts per locus*

2.1.2 *Presence or absence of unintended sequences (e.g., plasmid backbone)*

2.1.3 *Sequence of the inserted DNA*

2.1.4 *Stability of inserted DNA across multiple generations*

2.2 *Protein Expression and Characterization*

2.2.1 *Core characterization of the NEP isolated from the GM plant*

2.2.2 *Determining that the surrogate protein test substance and the plant-produced protein are sufficiently similar: Core comparative studies*

2.2.3 *Quantification of NEP expression levels in planta*

2.3 *Protein Safety: Hazard Identification Encompassing Toxicity and Allergenicity*

2.3.1 *Toxicological Assessment*

2.2.2 *Allergenicity Assessment*

Supplementary Studies

Supplementary studies should be conducted when core studies identify a hazard or are not sufficient to conclude a negligible risk, or when certain GM traits require additional analyses for complete characterization. Problem formulation can be used to design hypothesis-driven studies to answer specific safety questions. Depending on the nature of the NEP, case-by-case studies may be required for complete characterization. Examples of supplementary studies include:

3.1 *Protein abundance*

3.2 *Processing*

3.3 *Resistance to digestion*

3.4 *Toxicity studies*

3.5 *Compositional assessment*

3.6 *Dietary exposure assessment*

3.7 *Case-by-case protein characterization studies*

3.8 *Nutritional assessment*

3.9 *Immunoglobulin E binding*

GM traits could be the outcome of the expression of NEPs, double-stranded RNA to target silencing of a target pest gene, or altered expression of endogenous proteins. Studies described in this paper focus on traits derived from NEPs, and although the other types of modifications are not discussed in detail here, they may be mentioned or referred to, with the principles discussed in this paper still being applicable.

2. Core Studies: Characterization and Safety Assessment

The integrity and genetic stability of the introduced DNA and expression of the trait should be evaluated for all GM plants. Molecular and protein characterization are core characterization studies. Some data obtained from these studies also inform certain aspects of the protein safety assessment.

2.1. Molecular Characterization

Molecular characterization contributes important data and information that underlies the safety assessment of GM plants according to both Codex and FAO/WHO principles and guidelines. While the molecular characterization of GM plants is not a safety assessment in and of itself (nucleic acids are generally regarded as safe), it helps confirm the novel gene product(s) [74]. Molecular characterization elucidates the molecular changes that have been introduced into the plant during the transformation process. There are four primary endpoints for molecular characterization of a GM plant: (1) Number of insertion loci and inserts per locus; (2) Presence or absence of unintended sequences (e.g., plasmid backbone); (3) Sequence of the inserted DNA; and (4) Stability of the inserted DNA across multiple generations.

2.1.1. Number of insertion loci and inserts per locus

Some current established techniques employed to transfer genes into plant cells, including *Agrobacterium*-mediated transformation and particle bombardment, could result in random integration of insert(s) into the recipient genome [2]. Furthermore, using these transformation methods there is no control over the number of integrations (inserts) or whether the DNA transferred is complete, truncated, or rearranged. Determining the number of inserts integrated in the GM plant genome is a necessary molecular characterization endpoint to support risk assessments described in subsequent sections.

Transgene copy number can be positively or negatively associated with transgene expression and associated with inheritance/segregation patterns from generation to generation. Therefore, determining the number of insertion locations (loci) and number of inserts per location (locus) in the GM plant genome is a useful molecular characterization endpoint. For example, confirmation of a single locus containing a single transgene can help ensure that there are no unexpected anomalies in transgene expression levels that could impact expressed trait protein levels. In some GM plants, such a confirmation could provide assurance of heritable product efficacy and quality.

2.1.2. Presence or absence of unintended sequences (e.g., plasmid backbone)

A plant transformation plasmid is usually composed of the DNA that is intended to be transferred to the recipient plant genome for the intended trait, and a plasmid backbone. The plasmid backbone contains origin(s) of replication and selectable marker(s), as well as sequences that allow for the propagation and maintenance of the plasmid in bacteria, including *Agrobacterium*. The microbe-derived origin of replication and selectable marker genes in the plasmid backbone, however, are unnecessary for trait gene expression in the plant cell. Although the presence of the plasmid backbone fragment in an event has not resulted in any safety concerns [56], confirmation that no plasmid backbone DNA or any other plasmids used in transformation process have been inserted into the genome of the transgenic plant remains an important characterization endpoint.

2.1.3. Sequence of the inserted DNA

It is important to sequence the inserted DNA, with special emphasis on the transgene(s) to ensure that the predicted protein(s) sequence would be produced. Through translation of the observed transgene nucleotide sequence, protein-based bioinformatics that address potential allergenicity or toxicity can be performed. To fully characterize the insert DNA, obtaining the genomic flanking sequence is also necessary to confirm the termini of the insertion.

Mutations such as single nucleotide polymorphisms (SNPs), truncations, and re-arrangements such as inversions, insertions/deletions, duplications, and translocations within the DNA insert, and between the insert and the integration site of the recipient genome (i.e., flanking genomic DNA or flanking site), have been reported in transgenic plants [6, 38, 62]. However, the genomes of plant species are dynamic and possess natural variability arising from events like single-nucleotide changes, transposon insertions, and horizontal gene transfer [37]. Moreover, conventional breeding techniques have a much larger impact on the plant genome compared with plant transformation [62]. Therefore, while sequencing of the inserted transgene DNA provides the most accurate information of integrated sequence(s) and variations that may have occurred, the studies assist in characterization of the GM event rather than providing data to inform the safety assessment.

2.1.4. Stability of inserted DNA across multiple generations

Trait stability is part of any successful breeding program regardless of the technique used, be it GM or conventional. Molecular stability of the inserted DNA from generation to generation can be affected by multiple factors (e.g., genetic recombination) [47]. Testing across a minimum of three generations should provide sufficient data to demonstrate generational stability of the introduced trait [55].

A transgenic insert located in the nuclear genome is expected to follow Mendelian segregation principles [44]. Although not unique to GM plant insertions, there are some instances when non-Mendelian inheritance of transgenes occurs in a variety of crops due to transgene deletion, duplication, or rearrangement [70]. Structural variations are also observed in conventional diploid and polyploid crops [76]. In this situation, the developer may choose to discard the transgenic event if it exhibits instability of the desired phenotype [76]. Transgenic inserts that are in organellar genomes, such as plastids, are expected to be inherited maternally, in which case Mendelian segregation principles would not apply [24]. Plants that are propagated through asexual reproduction (e.g., vegetatively) would also not follow Mendelian inheritance patterns. Non-Mendelian inheritance in these instances are not considered to be instability of the trait and demonstration of molecular stability is not necessary.

Further studies, routinely required to complete the molecular characterization requirements established by certain regulatory authorities, should not be considered core or supplementary studies, because they do not inform the safety assessment. These studies are discussed in Box 1.

Box 1: Bioinformatic assessments enabled by molecular characterization that do not provide additional value to the safety assessment

The assessments below are typically performed to meet current registration requirements in specific jurisdictions, but are not universally required and do not inform the safety assessment for GM plants.

Open Reading Frames (ORF) bioinformatic analysis: The bioinformatic assessment of ORFs in the insert and adjacent flanking genomic sequence is performed to determine if non-canonical transcription and/or translation can yield a novel protein sequence that is allergenic, toxic, or displays some other undesirable characteristic such as inhibition of proteases or nucleases found in animal digestive systems. Unlike the bioinformatic evaluation of the actual transgene-encoded protein itself (which is confirmed through protein characterization studies), the analysis of potential ORFs created due to transgenic insertion is theoretical and disregards basic biological processes. Theoretical ORF analysis provides no additional value to a safety assessment, unless the ORF contains a contextually correct initiation codon and is appropriately located relative to promoter and terminator (or gene expression) elements. Such detail would be uncovered through inspection of the organization of genetic elements in the transgenic insert sequence, which is included within the core molecular characterization studies. Furthermore, the potential for an unintended ORF generated through transgenic insertion is no greater than that for conventional breeding, and a safety issue arising from such random events is statistically negligible, consistent with the history of safety for new crop varieties (both conventional and GM) [62].

***Repeated bioinformatic analyses for endogenous genes*:** Although obtaining genomic flanking sequences for determining whether an endogenous gene was disrupted is required by some regulatory authorities, this analysis is not necessary to support the safety assessment of GM crops, as the risks associated with disrupting a gene by insertion of transgenic DNA is the same or less than that for conventional breeding [62]. When bioinformatic analyses are performed with well-annotated genome assemblies, definitive conclusions on the interruption or deletion of endogenous genes can be drawn. The database update of genomic assemblies including new information on gene annotation, function, etc., should not alter the conclusions made initially if the region of the recipient genome is not changed in the updates. Since gene disruption is not a unique risk of transgenesis, repeated bioinformatic analyses do not add value to safety assessments.

2.2. Protein Expression and Characterization

Protein characterization and *in planta* expression studies are part of the core characterization of GM plants in which NEPs are introduced (Figure 2). The objective of protein characterization studies is to confirm the identity of the NEP and to verify that the protein is expressed in the plant as intended. The objective of *in planta* protein expression studies is to quantify the levels of the NEP under representative growing conditions, to enable protein exposure assessments for humans and animals if there is uncertainty about protein hazard.

Since it is generally not feasible to isolate large amounts of NEP from the plant due to low concentrations, heterologous production of a surrogate protein test substance in another expression system is often necessary and aids to provide sufficient protein for both characterization and safety studies [57]. In this latter case, a further objective of protein characterization studies is to ensure that the protein test substance produced exogenously is a suitable surrogate and is sufficiently similar to the plant-produced protein for the purposes of safety assessment studies.

The sections below outline the core studies that are essential to characterize the NEP and describe the studies that are essential to establish that a surrogate protein test substance is sufficiently similar to the plant-produced protein for the purposes of safety assessment studies. To date, many of the proteins expressed in GM plants have been isolated and purified from either the plant or heterologous systems. However, intractable proteins - those that can be difficult to express or challenging to isolate in a functional form (e.g., membrane proteins, transcription-factors) - may require alternative approaches to establish protein safety [3, 39].

2.2.1. Core characterization of the NEP isolated from the GM plant

a) Molecular Weight: Determining the molecular weight of the protein expressed by the plant and comparing it with the theoretical mass calculated using the inserted DNA sequence and any known or intended proteolytic processing sites provide a key indication that the NEP is being expressed in the GM plant as intended. Knowledge of the molecular weight of the NEP also provides indications of any post-translational modifications (e.g., glycosylation, proteolytic processing, etc.) and may allow further insights into relevant characteristics of the protein, such as the formation of quaternary structures.

b) Amino Acid Sequence: The amino acid sequence of the NEP provides information about any protein processing that may occur in the plant, such as N-terminal methionine cleavage. While it is often not feasible to obtain com-

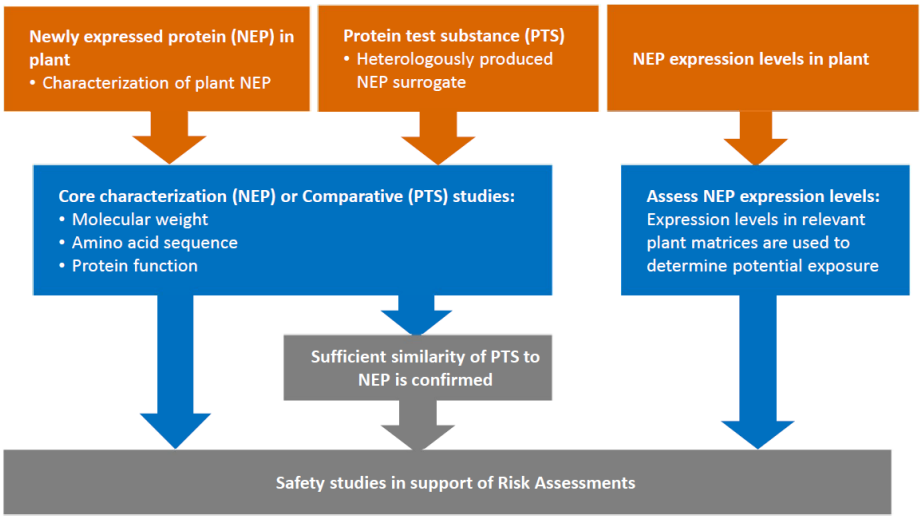

Figure 2: Schematic overview of the expression and characterization of newly expressed proteins (NEPs) in genetically modified (GM) plants

plete amino acid sequence coverage for the NEP isolated from the plant, determining a partial amino acid sequence and ensuring that it matches the complete inserted DNA sequencing results and molecular weight data will further determine protein identity. The adequacy of the level of amino acid sequence coverage should be assessed on a case-by-case basis, depending on the type of protein.

c) Protein Function: It is important to confirm that the NEP functions as expected. For NEPs that function as enzymes, functional activity analysis verifies that the NEP has the intended activity. For insecticidal proteins, an insect bioassay is usually conducted on a target organism to determine potency against target organisms. When the functional activity of a protein cannot be measured *in vitro* or in laboratory bioassays, the characterization and safety assessment must rely on alternative weight-of-evidence (WOE) information (e.g., field or trait performance data can provide important indirect evidence for the functional expression of the NEP in the newly designed GM plant).

2.2.2. *Determining that the surrogate protein test substance and the plant-produced protein are sufficiently similar: Core comparative studies*

A surrogate protein test substance of appropriate purity can often be produced in microbial organisms such as Gram-negative bacteria (e.g., *Escherichia coli*, *Pseudomonas fluorescens*), Gram-positive bacteria (e.g., *Bacillus* sp.), yeast, fungi, or in other cell culture systems such as insect cells or plant cells, as described in detail by Raybould et al. [57]. When an adequate amount of protein test substance is obtained, it is necessary to confirm that it is suitable as a surrogate for the plant-produced protein in subsequent characterization or safety assessment studies. The protein test substance and plant-produced protein need not be 100 percent identical in their characteristics if any observed differences do not impact functional or biochemical properties of the test protein, as described previously [57].

The determination of sufficient similarity is based on a weight-of-evidence approach, following comparisons of properties of the protein test substance and plant-produced protein, to confirm suitability for use in protein safety assessment studies [57]. The comparative studies are discussed below. Similarity in these pertinent attributes of the proteins derived from both sources allows them to be used interchangeably in protein characterization and safety studies.

a) Molecular Weight: Molecular weight of the protein test substance and plant-produced protein should be compared to assess similarity. If differences exist, it will be necessary to understand whether the differences arise because of changes in the amino acid sequence or post-translational modifications.

b) Amino Acid Sequence: Amino acid sequence comparison of the protein test substance and the plant-produced protein is important in establishing sufficient similarity. The amino acid sequences do not necessarily need to be complete, nor do the sequences need to be identical, for the protein test substance to be considered suitable for use in safety studies, as described in detail by Raybould at al. [57]. For example, minor changes to the plant-produced protein (e.g., single amino acid substitutions, N-terminal modification, affinity tags added to aid purification, or differential cleavage of N-terminal targeting peptides) may be considered acceptable when there is evidence indicating that the changes do not impact biochemical and functional properties relevant to the safety assessment.

c) Protein Function: If the NEP has a measurable functional activity (e.g., enzyme, receptor, insect-toxin, etc.), determination of the functional activity of the protein test

substance and the plant-produced protein contributes to the weight-of-evidence assessment of sufficient similarity, even when the activity levels are not quantitatively equivalent [57]. In instances where the functional activity of a protein cannot be measured (e.g., protein cannot be isolated in a functional form) or an *in vitro* assay does not exist, the comparative studies must rely on alternative weight-of-evidence information to confirm the suitability of the protein test substance as a surrogate, as suggested, for example, by Bushey et al. and Delaney et al. [3, 14]. For proteins with no activity, or where activity is not readily measurable (e.g., transcription factors, storage proteins, or plant resistance proteins [R-proteins]), a protein functionality comparison is not applicable.

2.2.3. Quantification of NEP expression levels in planta

When an exogenous protein is being expressed in the GM plant, or expression of an endogenous protein has been intentionally altered, quantitative information about protein expression levels in GM plants provides important information in support of the risk assessment. Protein expression data enable an accurate assessment of human and animal exposure and would form the basis for certain safety studies and an exposure-led safety assessment. For example, if abundance or dietary exposure assessment studies are deemed necessary for the food safety assessment (Section 3), protein expression levels enable assessment of exposure. In cases when a hazard is identified and hazard characterization is necessary, determination of NEP expression levels in relevant plant matrices is important. Expression data are also needed for calculating safety margins in certain toxicology studies performed for hazard characterization [60]. In cases where novel plant traits are enabled without NEPs, e.g., by the silencing or over-expression of an endogenous plant protein, the expression level of the impacted endogenous protein (or an appropriate surrogate endpoint) should still be measured to understand the potential impact on safety.

The levels of NEP in plants can be influenced by environmental factors. Therefore, analyzing plants grown in field trials is desirable for determining expression levels under commercially relevant conditions.

2.3. Protein Safety

Following molecular and protein characterization, hazard identification encompassing toxicity and allergenicity should be conducted, and the outcome of this step and other core studies will determine the need for additional supplementary studies. A brief background discussion of toxicity and allergenicity assessments that may supplement the core studies on a case-by-case basis is provided below. Further detail on these assessments can be found in Roper et al. [60] and McClain et al. [42].

2.3.1. Toxicological Assessment

As a result of the acidic conditions and digestive enzymes of the gastrointestinal tract, dietary proteins are typically rapidly degraded into small peptides and individual amino acids before absorption and metabolic use by the body. Some biological barriers may restrict the oral bioavailability of intact proteins after dietary consumption. Several factors may affect protein such as ionic charge and lipophilicity. Additionally, protein size may be a consideration as systemic absorption of any orally consumed substance is typically inversely proportional to its molecule size [21]. Proteins resistant to degradation by digestive enzymes may have limited systemic uptake due to their large molecular weight (e.g., lectin proteins). The effectiveness of these biological barriers has been demonstrated through the unsuccessful attempts to orally administer proteins for therapeutic purposes [23, 25, 46, 63]. Therefore, as also concluded in Roper et al., consumption of proteins is not normally associated with adverse effects, and additional studies to confirm the dietary safety of a protein should only be conducted on a case-by-case basis where there is an identified hazard (see Section 3.4) [60].

Applying a weight-of-evidence approach, key hazard identification studies for toxicity are required to assess the safety of all NEPs [14]. Hazard identification can be built by evaluating the four elements described below.

(1) History of safe use of the NEP: Probable dietary safety of the NEP can be established through a history of safe consumption of closely related proteins (considering both structure and function) by humans and/or animals [1, 9, 14]. To demonstrate history of safe use, evidence of structural and/or functional similarity and exposure to other endogenous proteins found in foods or other species expressing these proteins or similar proteins is necessary [26, 42, 60]. However, the absence of a clear history of safe use does not automatically indicate a hazard, only that some further evidence and analysis is needed for the safety assessment.

(2) History of safe use of the source organism: The history of safe use in the food or feed chain of the source organism for the gene encoding the NEP provides additional evidence about the safety of the protein. The safe consumption of the source organism indicates that the NEP should also have limited potential for allergenicity, toxicity or other anti-nutrient for animals or humans [13, 42, 60].

(3) Bioinformatics for sequence comparison: Bioinformatic screens are an excellent tool for placing a protein within the context of related proteins based on recognizing localized homologies, common domains, and larger protein families or super-families. This screen should be done early in the hazard identification phase and can be useful in providing the preliminary protein and protein family context that will help determine the scientific rationale for conducting supplementary toxicology studies. However, bioinformatics results should not be regarded as necessarily indicative of toxicity, and any hazard prediction based upon bioinformatic results must subsequently be examined in conjunction with other data from core studies when assessing risk.

(4) Mode of action and functional specificity: The potential of the NEP as an allergen, toxin, or anti-nutrient can also be established by understanding the mode of action and

functional specificity of the protein. If the mode of action and functional specificity of the NEP are well understood and have been shown to have low relevance to humans or animals, this provides confidence that it is unlikely to cause harm when consumed.

2.3.2. Allergenicity Assessment

Since the initial Codex guidance documents for allergenicity assessment of GM plants were published, improved tools have been developed to more accurately and precisely identify allergens [42]. With current knowledge of molecular biology, genomics and bioinformatic techniques, a revised approach for assessing the allergenic potential of NEPs is warranted, hinging on the standard risk equation (Risk = Hazard x Exposure). Since there is no single test or predictive assessment to establish whether a protein will act as an allergen, hazard identification and exposure characterization require measurement of several physiochemical properties. In *Allergy risk assessment for newly expressed proteins (NEPs) in genetically modified (GM) plants*, a stepwise approach is recommended where hazard identification is first performed for all NEPs [42]. If a hazard is identified, exposure characterization should be done (supplementary study). Fundamental to this allergenicity assessment is the degree of similarity of the NEP to known allergens.

a) History of safe use of the NEP and familiarity with the source organism: These concepts are one of the fundamental and initial elements in the overall safety assessment and are used to evaluate potential for allergenicity in a manner similar to the evaluation of toxicity (see Section 2.3.1).

b) Amino Acid Sequence Similarity and Bioinformatics: The best use of bioinformatics for protein safety assessments is the combination of a thorough understanding of existing allergens with a coordinated review of putative allergens and their placement into a qualified database [11]. To enhance the accuracy and reliability of bioinformatic assessments for allergenic potential of NEPs, a stepwise approach is recommended as below; conclusions from step 1 would determine the necessity for further analyses described in steps 2 and 3:

   1. *Sequence level consideration*: Bioinformatic algorithms evaluate sequence identity and similarity, and the probability that two sequences share structure and common evolutionary origin. Such relationships also provide a measure of likely physicochemical similarity among proteins that might reflect immunoglobulinE (IgE) cross-reactivity between a NEP and known allergens. Conventional linear sequence-based algorithms Fast All (FASTA) and Basic Local Alignment Search Tool (BLAST) are used for these analyses and expectation value (E-value or E-score) is the typical statistical measure of relatedness.
   2. *Structural relatedness*: The potential of cross reactivity can be assessed by determining if a NEP shares structural features with known allergens. The degree to which structure is compared can include determination as to whether the NEP is in the same protein family as known allergens, if it shares a domain with known allergens or, at the finest level of granularity, if the NEP contains known IgE-binding epitopes. Such structural comparisons then contribute to a weight-of-evidence conclusion.
   3. *Structural considerations*: Three-dimensional modelling offers a more sophisticated measure of similarity between a NEP and an allergen, but it would need to be performed based on the results of sequence level analyses. The knowledge of any specific allergens and their associated epitopes and other clinically relevant sequence mapping is a key to understanding similarity with the NEP.

2.4. Outcome of Core Studies

If no hazard is identified after conducting core studies, further hazard and exposure characterization for GM plants should not be required according to established principles for risk analysis. In this case, core studies alone would be sufficient to conclude that the GM plant has negligible risk and is as safe as its conventional counterpart. It is noteworthy that food and feed safety assessments of many diverse GM plants over the past 25 years have not identified unique hazards associated with GM plants [19, 33, 45].

3. Supplementary Studies

If the weight-of-evidence from core studies is not sufficient to determine negligible hazard, further hazard and exposure characterization are needed to support the safety assessment. Alternately, depending on the nature of the NEP, case-by-case studies may be required for complete characterization even when hazard is absent. As mentioned in the introduction, the choice of supplementary study or studies would depend on the introduced GM trait and intended use.

Hypothesis-driven studies identified by problem formulation can be used for the characterization of hazard and exposure [61]. Hazard characterization expands beyond the hazard identification step to more fully understand the conditions under which the hazard may be present [68]. The appropriate supplementary hazard characterization studies needed should be determined based on the results of the core studies and an understanding of the nature of the identified hazard, and may include toxicological studies with the NEP or IgE binding studies, as examples. Expression levels, likely protein degradation during processing (e.g., heat stability), resistance to digestion, and dietary exposure assessments are some studies that can be considered that are relevant to exposure characterization.

Examples of hypothesis-driven and case-by-case supplementary data studies are discussed below.

3.1. Protein abundance in food and feed

While protein expression data in plant tissues may be helpful as part of the environmental risk assessment for specific traits, exposure estimates related to consumption by humans and animals are less relevant for proteins for which no hazard has been identified [1]. The abundance of a protein has historically been recognized as supportive information for allergy safety assessment. However, if the NEP is not allergenic or cross reactive, abundance is not relevant to safety [7, 9, 42]. While low abundance does suggest a lower probability of allergy relevant exposure, if there is not an identified hazard, greater or lower abundance is not a contributing factor in an allergy risk assessment for an NEP. This topic is further discussed in *Allergy risk assessment for newly expressed proteins (NEPs) in genetically modified (GM) plants* [42].

3.2. Processing

Processing is another factor that can be considered in exposure characterization when a hazard has been identified. Processing has typically referred to the assessment of how stable a NEP may be when the grain in which it is contained is processed, using methods that would be typical for turning grains into food and feed fractions. Measuring NEP functional intactness after heat treatment(s) that mimics food processing conditions could contribute to an exposure assessment but does not otherwise characterize allergy or toxin hazard for NEPs. Although exposure assessments are required by some regulatory agencies, they provide no quantitative value for risk assessment if negligible hazard has been determined [54].

3.3. Resistance to digestion

The *in vitro* degradation of a protein using simulated gastric fluid (SGF) and/or simulated intestinal fluid (SIF) assays can also be used as part of the WOE safety assessment [42, 60]. SGF/SIF studies aid in the understanding about the potential digestive fate of a NEP in food and feed and also inform about potential human and animal exposure to NEPs. When there is a known hazard, SGF/SIF assays help to understand and assess internal exposure.

Traditionally, stability of a NEP in SGF was used as a distinguishing feature of food allergens, resulting in the wide adoption of this criterion as part of the WOE approach supporting the allergenic risk assessment of NEPs [73]. However, follow-up studies showed the SGF assay to be an inconsistent predictor of impact on the immune system (allergenicity), and modifications of digestion studies to include more physiological gastric conditions and SIF were explored, without any notable improvement in the contribution to the WOE for assessing the allergenic risk of NEPs [28]. As discussed recently, there is poor correlation between digestion results and the allergenic status of proteins [29, 31]. SGF stability provides value only when there is a known hazard, as digestion characteristics would contribute to exposure considerations in the risk assessment.

3.4. Toxicity studies

The toxicological evaluation of all NEPs as a default assessment is not hypothesis-driven, nor supported by the current weight-of-evidence. As discussed in Roper et al., "*defaulting to in vivo toxicology studies, as is often required for regulatory approvals, does not reflect ethical use of animals in scientific research and testing as outlined by the 3R's of responsible animal use (Replacement, Reduction and Refinement) that have been increasingly incorporated into regulatory in vivo studies*" [60, 66].

Acute oral toxicology studies with proteins should only be conducted if deemed necessary to address specific hazard hypotheses arrived at through problem formulation [60]. When toxicity studies are deemed necessary, acute toxicity studies are generally sufficient given the observation that, while most proteins do not present a hazard, most protein toxins elicit their toxicity through acute mechanisms of action [64].

Evidence to date for NEPs in GM crops indicates that when no hazard is identified, no evidence of adverse effects is observed in acute oral toxicology studies [4, 14, 36, 40, 65, 75]. Nevertheless, acute toxicology studies are still required by many regulatory authorities regardless of the nature of the protein [43].

The routine requirement for repeated dose toxicity studies with proteins in the safety assessment of GM plants is also not scientifically justified, as discussed in Box 2. No evidence exists to suggest that protein digestion is altered as a result of repeated exposure or consumption of proteins [14]. Furthermore, most protein toxins act acutely, and therefore, do not have repeated dose or cumulative toxicity [50].

3.5. Compositional assessment

Currently, extensive assessment of the nutritional composition of a new GM crop is a requirement by many government regulatory authorities around the world. The main purpose of these compositional assessments has been to determine whether introduction of the GM trait(s) has altered the nutritional profile in a way that would have a meaningful impact on the food or feed use of the GM crop. These compositional studies do not attempt to show that the GM crop and the conventional crop are identical, but merely that one crop could be substituted for the other in the diet without any meaningful impact. Any noted changes in nutritional component levels in the GM crop are evaluated against the breadth of component variability in the conventional crop.

The risk assessment of GM crops according to Codex guidelines includes, "*an assessment of a whole food or a component thereof relative to the appropriate conventional counterpart: A. taking into account both intended and unintended effects; B. identifying new or altered hazards; C. identifying changes relevant to human health and key nutrients*" [7]. Although the assessment has included intended effects due to the GM trait(s) of interest, much of the assessment continues to be focused on uncovering possible unintended effects due to the trait insertion process. However, Codex recognized that, "*many unintended effects are largely predictable based on knowledge of*

Box 2: Routine 28-day and 90-day repeated dose toxicity studies are scientifically unjustified

If the NEP is related to a family of proteins that has a history of safe use based on bioinformatics and literature review, and is not homologous to known protein toxins, then any supplementary toxicology study is not necessary. Furthermore, if an acute oral toxicology study has been performed with no observed adverse effects, then a 28-day repeated-dose toxicity study with the protein is unlikely to contribute any additional valuable information to the protein safety assessment [3,14,15,26].

The routine requirement for a 90-day toxicity study with whole foods (e.g., grains) in the safety assessment of GM plants is also not scientifically justified. However, these studies are required in some countries to supplement the molecular and compositional data included in the risk assessment, even in the absence of a plausible risk hypothesis [10]. Studies to date have shown that unintended effects on the composition of GM plants occur less frequently and are of a lower magnitude as a result of the process of genetic modification or transformation compared with their occurrence in traditionally-bred crop varieties [30].

When a 90-day study is performed to meet individual country requirements, it is performed by feeding whole food/feed material, ostensibly to identify potential adverse effects of consumption of edible fractions from GM plants. The methods are principally based on existing guidance for the identification and characterization of potential hazards of chemicals from repeated oral administration during a critical period of animal growth and development, with necessary adaptations for evaluation of whole food and feed [49]. Although the study design provides satisfactory estimates of no-effect and no-adverse-effect levels, the incorporation of whole food or feed fractions into these studies has inherent limitations for exposure due to nutritional and satiety concerns that impact animal performance irrespective of the GM crop fraction included in the diet. Additionally, feeding studies conducted in the absence of a risk hypothesis are generally considered to lack sufficient sensitivity to yield meaningful results relevant to the safety assessment of GM plants, create challenges for study design due to difficulties in determination of adequate sample size for appropriate statistical power, place undue emphasis on feeding study results in comparison with other components of the safety assessment, and are inconsistent with the principles of reduction, refinement, and replacement of animals in research [16]. These conclusions have been reinforced by the recent completion of two European Commission work programs, GMO Risk Assessment and Communication of Evidence and GM Plants Two Year Safety Testing [20]. The findings of these European projects are consistent with those presented above that 90-day feeding studies with GM food/feed materials are scientifically superfluous to the overall risk assessment.

the inserted trait and its metabolic connections or of the site of insertion", and that unintended effects also result from use of conventional breeding. Likewise, more than ten years ago, the European Commission [19] stated that, "*The main conclusion to be drawn from the efforts of more than 130 research projects, covering a period of more than 25 years of research, and involving more than 500 independent research groups, is that biotechnology, and in particular GMOs, are not per se more risky than e.g., conventional plant breeding technologies*".

Variability in nutritional components occurs naturally in conventionally bred crops due to the influence of both genotype and growing environment [12, 59]. Genotype differences arise spontaneously in plants (e.g., transposon movement, mutation, chromosome crossing-over, etc.), and transgenic modification leads to molecular changes in the genome similar to insertions and disruptions that occur naturally in plant genomes [37]. Researchers have demonstrated that conventional breeding methods contribute more to compositional variability than the process of transgene insertion [62, 69, 72]. Human and livestock animal populations have been exposed to the full breadth of variability of components within crop commodities (within recommended dietary intake levels) without evidence of harm. Therefore, variability in levels of nutritional components does not in itself indicate an impact on safe consumption.

Within the context of this inherent variability in composition, the accumulated experience in evaluation of compositional data has revealed the lack of biologically meaningful differences between GM crops and their conventional comparators [22, 27]. To date, compositional studies have not documented evidence of notable consequences attributed to the process of developing a new GM plant [30]. Just as it is done for conventional breeding, extensive evaluation prior to selection of the GM line for commercial development greatly reduces chances of unintended impacts of the GM process on the commercialized crop variety [22]. When biologically relevant compositional changes have been observed, these changes can be predicted from the mode of action of the introduced trait. Additionally, in a 2010 review of transgenic safety assessments, Parrott et al. noted that, "*results emphasize that the GM and non-GM comparators are of similar composition*" and that, when considering other expression products (such as RNAi or transcription factors), "*there is no scientific rationale to justify new or more*

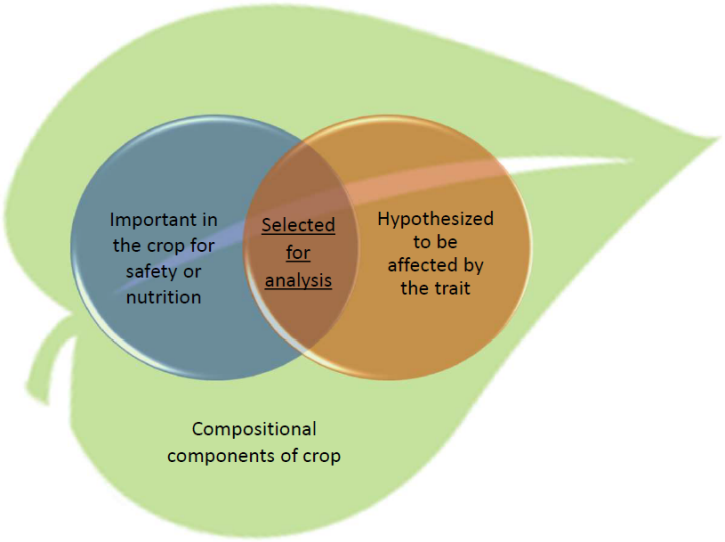

Figure 3: Component selection for genetically modified (GM) plant composition analyses in support of food and feed safety assessment

complex safety assessments" [51].

Based on the compelling body of evidence collected since Codex guidelines were developed [8], it is recommended that a compositional assessment of a new GM plant should follow a stepwise approach to determine if further data generation is necessary, and if so, what data should be collected. The goal of this approach is to focus the compositional assessment on the key components that are critical to the nutritional and/or safety considerations for the crop and also have potential to be altered by the introduced trait(s) (Figure 3), since not all compositional changes are an indicator of a hazard [51].

Our proposal is to first formulate sound hypotheses, based on the trait mode of action, to further refine the list of components to be targeted for analyses, and then use a stepwise approach to evaluate known information and decide what additional information is necessary to inform the safety assessment as detailed in Box 3.

As an example of implementation of the proposed approach, consider the tyrosine catabolic pathway and a trait that affects levels of hydroxyphenylpyruvate (HPP). HPP is dehydrogenated into homogentisate, which is upstream from the tocochromanols (tocopherols and tocotrienols). (α-Tocopherol is the most biologically active form of vitamin E in the diet). All tocopherols and tocotrienols act as antioxidants when present in vegetable oils, preventing development of rancidity. The null hypothesis to test is that there are no differences in levels of tocopherols and tocotrienols between the GM crop and its non-GM comparator. There is no reasonable expectation, and thus no sound hypothesis, that the trait, based on its mode of action, would affect other crop components outside of this pathway (e.g., levels of minerals, crude protein, dietary fibers) any more than is possible with conventional breeding. Therefore, the components to be measured and compared should only be those hypothesized to be impacted as a result of the trait mode of action and impacting health or safety. Generation of other data for unrelated components is superfluous and would be detracting from the safety assessment of the novel GM crop. This hypothesis-driven approach is also in line with the problem formulation approach described by Raybould and MacDonald [58] for environmental risk assessment of GM crops, who emphasized that there should be movement, "*toward hypotheses that help decision-making and realization of policy objectives*".

The automatic requirement of in-depth, multi-component compositional studies within the set of safety evaluations of a new GM crop has been called into question by the increasing body of knowledge regarding the extent of natural variation in crop composition, the innate variability and plasticity in plant genomes, and the empirical evidence supporting a negligible impact of the transgenesis on composition [30]. The hypothesis-driven approach to compositional studies described here serves to characterize the impact of the trait(s) on the levels of the targeted components. This focused approach is consistent with the established practices of conventional variety registration and meets food and feed product standards.

3.6. Dietary exposure assessment

Dietary exposure assessments are recommended to be hypothesis-driven studies. If, during core safety assessment, the weight-of-evidence points to negligible hazard, a formal dietary exposure assessment is unnecessary for the overall risk assessment [41]. Conversely, problem formulation may demonstrate that there is minimal or no exposure to a NEP, precluding the necessity for additional hazard assessment. This holds true for NEPs and also for other expression products such as RNA-based mechanisms for gene regulation [53].

In the case where a dietary exposure assessment is needed, e.g., if hazard is not negligible or is uncertain, a stepwise approach should be taken, using the most straight-forward di-

Box 3: Recommended stepwise approach for compositional assessment

At each step, a decision is made whether the available information is sufficient to assess possible risk or whether additional information may be needed (e.g., further information concerning mode of action, generation of appropriate data to address the hypothesized risk).

Step 1: Based on knowledge of the* mode of action *or function of the introduced GM trait, determine whether a supplementary compositional study will be useful for informing the overall risk assessment

GM plants possess specific phenotypic traits determined by the mode of action of the introduced genetic material. The expected functional or biological activity of the intended genetic modification is studied prior to commercialization of the new GM plant (information gained from core studies). A compositional study is not necessary if there is no scientifically reasonable hypothesis that the GM trait introduction will compromise crop composition in a manner that could lead to a safety or nutritional concern. For some GM traits, there is a reasonable hypothesis, based on the mode of action or function of the introduced trait, to justify a compositional assessment [32]. If the outcome of Step 1 concludes that a targeted compositional assessment is necessary to address hypothesized changes in composition, then the assessment proceeds to Step 2.

Step 2: Determine which components are relevant to include in a composition study

In cases where a composition study will provide informative data that are meaningful to the safety assessment, the decision on which components to include are limited to those components that are predicted to be both affected by the introduction of the trait and relevant to the safety or nutritional properties of the crop (Figure 3). If levels of the selected components are within what is considered typical for the crop, no further assessment is necessary. If the introduced trait is predicted to potentially result in the production of a metabolite novel to the crop, then levels of this metabolite are to be evaluated as well. If the assessed components are present at levels outside the natural range for the crop commodity, or if further evaluation of a novel metabolite is deemed necessary, then Step 3 is performed.

Step 3: Evaluate the safety and nutritional relevance of altered component levels

The focused compositional analysis may indicate that the level of one or more components falls outside the range of values previously observed for the crop commodity. However, such a result does not necessarily signify that the new GM plant is less safe, but that further assessment of the implications of the change may be necessary. The scope of the additional assessment would depend on the nature of the change and on the intended use(s) of the crop. Particular component changes could mandate a change in the use of, or the level of inclusion in, downstream products (e.g., processed food/feed). For example, the level of inclusion of cottonseed meal in livestock diets can be influenced by the level of the anti-nutrient gossypol, and the functionality of soybean oil used in food service or processed food could be impacted by intentional alterations in its fatty acid profile. Novel metabolites would be similarly assessed for possible impacts to safety and nutrition: history of safe use of the metabolite, levels of exposure from other food sources, etc.

etary exposure assessment method, starting with an unrefined, conservative assessment. Strengths and limitations of available databases should be considered, and the exposure duration selected should be relevant to the NEP. In cases where the unrefined assessment does not allow for acceptable risk, a refined dietary exposure assessment may then be leveraged to provide more realistic quantitative exposure estimates. Refinement factors include market share, food processing effects, variety-specific NEP data, NEP digestibility, and probabilistic modelling. Assessment of human dietary exposure to NEPs in GM plants have been described recently [41].

The Codex guidance on biotechnology-derived plants does not address the safety assessment for animals fed with feed produced from GM plants. Dietary exposure assessments for animal species should only be performed for a NEP expressed in GM plants if deemed necessary during the risk assessment process. Such an assessment can follow a similar stepwise approach as proposed for a human dietary exposure assessment, but should consider both the relevant animal species and the crop fractions that they consume. The major livestock species should be sufficient, as crop products are traditionally the main ingredient sources for livestock feed and animals are fed at high inclusion levels. The Organization for Economic Cooperation and Development (OECD) provides a single, international source of body weight, feed intake, and dietary feed inclusion data for livestock species [48]. Feed consumption databases are lacking for other animal species, in particular companion animal species, where animal protein sources are becoming more common ingredients. Crop fractions should be relevant and justified for the application; for example, seed im-

port applications should not require a dietary exposure assessment for forage.

3.7. Case-by-case protein characterization studies

In addition to the core studies performed for protein characterization (see Section 2.2), studies may be needed on a case-by-case basis for complete characterization of certain NEPs.

3.7.1. Case-by-case studies with the plant-produced protein or protein test substance

a) Post-Translational Modifications: Post-translational modifications can affect the activity, tertiary structure, and biophysical properties of the NEP. If there are indications that the plant-derived NEP is post-translationally modified, this should be confirmed through analytical methods specific to the potential modification. One common post-translational modification of plant proteins is glycosylation, which can change physicochemical properties of the protein [57].

b) Mode of Action: The mode of action is a mechanistic understanding of how the NEP functions to produce the desired trait. An understanding of the mode of action can support establishing the design of safety studies for a particular trait product. However, the requirement for more complex or detailed understanding of the mode of action, in addition to what is done for hazard identification (Section 2.3.1), is supplementary and only required in cases in which an impact on safety is identified through, for example, the problem formulation process.

c) Substrate Specificity: For a NEP which is an enzyme that adds a new capability to the plant, assessment of the substrate specificity of the NEP may be necessary. Knowledge of how an enzyme acts on a substrate can help identify the range of substrates on which it might act. This may provide information about potential impact on existing metabolic pathways or on the potential to produce newly formed metabolites. The safety implications of such changes would need to be addressed, possibly through a compositional assessment.

3.7.2. Comparative studies of the protein test substance and plant-produced protein

In addition to the core comparative studies to demonstrate sufficient similarity between the protein test substance and the plant-produced protein, additional studies may be required in some cases to demonstrate the suitability of the protein test substance for use in a safety assessment.

Post-Translational Modifications: If a NEP isolated from the GM plant is found to be modified, the impact of that modification on safety should be assessed. If this modification impacts the function or biochemical properties of the protein, it will be necessary to produce a protein test substance modified in a similar manner for conducting safety assessment studies.

3.8. Nutritional assessment

Nutritional assessments of GM plants are based on a comparative assessment of the composition of food and feed derived from the GM plant. Extensive nutritional analysis should only be performed on a case-by-case basis when compositional assessment demonstrates that analytes critical to the nutritional value of the diet are altered, i.e., when Step 3 of Compositional assessment (see Section 3.5) is performed. In fact, in studies where compositional analyses demonstrated no meaningful differences between the GM plant and comparator or commercial varieties, no differences in intake, digestibility or other parameters have been found [67].

However, numerous nutritional studies with fast growing animal species such as broiler chickens have historically been required by regulatory authorities to assess the nutritional value (or "wholesomeness") of GM plant products compared with those from conventional plants, even in cases where compositional equivalence had already been established. These historical data do not support the standard requirement of more extensive nutritional analysis without a hypothesis for nutritional change.

3.9. Immunoglobulin E binding

Traditionally [7], the need to perform IgE binding studies for an assessment of the allergenic potential of a NEP was conducted only in the case of significant similarity identified through bioinformatics. With the advent of more sophisticated bioinformatic techniques and in using the proposed problem formulation approach described herein, the application of IgE binding would be considered a case-by-case study performed to evaluate the potential allergy risk identified through bioinformatics [42].

4. Summary and Conclusion

Earlier guidelines and recommendations for the safety assessment of GM plants containing NEPs still provide a valid resource for the risk assessment of GM plants. However, given the history of safety and familiarity after many years of experience with these products, it is time to reconsider the approach to safety assessments for GM plants.

Despite the accumulated knowledge and familiarity of developers, academic scientists, and regulators with GM plants, regulatory reviews of their safety for food and feed use continue to be inconsistent internationally. In some cases, the safety assessment data required has continued to increase without adding value to the risk assessment. In this paper, a systematic approach for the safety review of GM plants used as food or feed is presented. A set of core studies is recommended, including characterization and protein safety assessment. It is important to perform hazard identification in core studies, and if hazard is determined to be negligible, then core studies should be sufficient to conclude that the GM plant is as safe as its conventional comparator. Rather than making additional assessments a routine requirement, these additional assessments would only be needed if, given the trait mode of action, the hazard and

exposure assessments from the core studies were not conclusive. Only when the information from the core studies is clearly not adequate to conclude on risk may supplementary studies be necessary.

Waters et al. [71] present a compelling rationale and concepts for the adoption of science-based approaches to GM plant safety assessment, and the present paper details a systematic approach to evaluate the safety of GM plants. The approach for safety assessment discussed in these papers, if implemented, could provide a first step towards standardizing requirements across regulatory systems based on current scientific knowledge and 25+ years of experience in the development and food/feed safety assessment of GM plants. Examples of case studies that use problem formulation and hypothesis-driven studies will be explored in future articles.

5. Declaration of Conflicting Interest

All the authors of this paper are currently employed by, or have been employed by, the agricultural biotechnology industry.

6. Disclaimer

The findings and conclusions in this publication are those of the author(s) and should not be construed to represent any official USDA or U.S. government determination or policy.

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Toxicological Assessment of Newly Expressed Proteins (NEPs) in Genetically Modified (GM) Plants

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Abstract

This paper details the weight of evidence (WOE) and stepwise approaches used to assess the food and feed safety of newly expressed proteins (NEPs) in genetically modified (GM) plants, based on previously reported principles. The WOE approach is critical, as in a vast majority of cases no single assay or biochemical characteristic can identify a protein as a hazard. A stepwise approach is recommended to evaluate the safety of NEPs taking the totality of information into account. Potential triggers for the need for supplementary toxicology studies are discussed, and an alternative *in vitro* method for the acute toxicology study is proposed.

Keywords: genetically modified, toxicological assessment, food and feed, hazard, exposure, risk, core studies, supplementary studies

Abbreviations: GM, genetically modified; GRAS, generally recognized as safe; HOSU, history of safe use; MOA, mode of action; MOE, margin of exposure; NEP, newly expressed protein; NOAEL, no observable adverse effect level; WOE, weight-of-evidence

1. Introduction

Proteins are a natural part of human and animal diets, and when subjected to rapid degradation by digestive enzymes and acidic conditions in the gastrointestinal tract, are catabolized into individual amino acids and small peptides that can be absorbed by the body. There are many biological barriers in mammals and livestock that restrict the oral bioavailability of intact proteins after dietary consumption [24, 26] and there are many factors, including size, charge (e.g., many proteins are charged, which restricts permeation), and lipophilicity (logP, diffusion across lipid membranes) that affect their absorption. In general, systemic absorption of any orally consumed substance is inversely proportional to the size of the molecule, with smaller molecules more readily absorbed in comparison to larger ones [12]. Thus, even for proteins with the unusual property of resistance to degradation by digestive enzymes (for example, lectin proteins), systemic uptake is limited by their large molecular weight. Unsuccessful attempts to use orally administered proteins for therapeutic purposes exemplify the effectiveness of these natural barriers [13, 15, 31, 36].

Consumption of proteins as a general class of macronutrients is not normally associated with adverse effects. While some proteins have shown toxicity via parenteral routes (non-oral exposure to venoms), very few are known to exhibit evidence of adverse effects following oral exposure. Most of the proteins that are toxic via oral exposure are lectins and tend to exhibit effects at the intestinal epithelium, although in some cases, such as with ricin, systemic effects can also occur [6].

A stepwise assessment approach is recommended to evaluate the hazard of newly expressed proteins (NEPs) taking the totality of information into account [7]:

- **NEP Hazard Identification (Core Studies)**: Key hazard identification studies are required to assess the safety of all NEPs.
- **NEP supplementary toxicology studies (Supplementary studies)**: If the above studies are unable to conclude on the absence of hazard of the NEP with reasonable cer-

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tainty, then additional supplementary studies need to be conducted (Supplementary studies).

- **Exposure assessment (Supplementary studies)**

2. NEP Hazard Identification (Core Studies)

Evidence from initial hazard identification can be built by considering the following elements: (a) history of safe use (HOSU, consumption) of the protein of interest; (b) HOSU of the source organism; (c) protein mode of action (MOA); functional specificity; and (d) bioinformatics for sequence comparison (e.g., primary amino acid sequence homology and overall structural similarity [30] to proteins with a known HOSU and evaluation for similarity to known toxins or other biologically active proteins that produce adverse effects in humans and animals. If a hazard has been identified, exposure can be determined by performing studies (e.g., dietary exposure assessments) as necessary, depending on the NEP.

2.1. History of Safe Use of the NEP

History of safe use (HOSU) is one of the initial analyses in the safety assessment of NEPs in genetically modified (GM) plants. Demonstration of prior human and/or animal consumption of the NEP or closely related proteins, structurally and/or functionally, provides familiarity with respect to probable safety of the NEP.

The concept of HOSU is similar to the GRAS (generally recognized as safe) concept employed by the U.S. Food and Drug Administration (FDA) [41]. GRAS classification indicates that a food ingredient is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use, either through scientific procedures or through common use in food. FDA extended the GRAS concept to proteins used in biotechnology (genetically modified) plants in 1992. The concept of HOSU was also included in a recent European Food Safety Authority (EFSA) guideline [9], suggesting no need for any specific toxicity or allergenicity testing in cases where both the plant and proteins expressed in the GM plant have a history of safe consumption by humans and animals. The concept of protein HOSU has also been emphasized in peer reviewed publications and other guidance documents related to safety assessment of genetically modified plants [3, 7].

It is important to note that absence of HOSU does not automatically indicate that the protein presents a hazard; it only indicates that further analysis of other lines of evidence is required. In order to demonstrate HOSU, evidence of structural and/or functional similarity and exposure to other endogenous proteins found in foods or other species expressing these proteins or similar proteins is necessary [16]. Protein similarity can be determined by either primary amino acid sequence alignment or structural/functional similarity, depending on the class of the protein. Protein phylogenetic analysis also helps determine protein similarity (with well characterized proteins) in the absence of higher primary sequence identity. Regarding exposure to similar proteins or species expressing these proteins, the appropriate methods for establishing this similarity need to be determined on a case-by-case basis.

2.2. HOSU of the Source Organism

HOSU of the source organism of the protein plays a supportive role in the weight-of-evidence (WOE) approach for determining the safety of the NEP. The HOSU of the source organism as a food ingredient, supplement, pharmaceutical, source of pest resistance (e.g., *Bacillus thuringiensis, B.t.*), or through environmental exposure can provide additional evidence about safety of the NEP. Use of a safe source organism can be used to demonstrate the limited potential for the NEP to be a toxin or anti-nutrient (or allergen) that could be relevant to humans or animals [4]. On the other hand, knowledge of the source organism does not, in and of itself, directly answer the question of whether the NEP presents a likely hazard. Safe proteins can be sourced from "unsafe" organisms because it is very likely that only a small number of an organism's genes are responsible for causing pathogenicity, toxicity or allergenicity.

Tools to characterize the hazard of NEPs derived from organisms known to cause any pathogenicity or toxicity (or allergenicity) include comparison of the amino acid sequence with fully curated protein toxin databases, and mathematical modeling of higher levels of structural similarity (if primary sequence information shows similarity between the protein and a putative toxin and there is information available on conformational epitopes or other key structural features).

Where there is clear identification of those genes in the source organism that produce a toxin or an anti-nutrient, other proteins would be presumed to be non-toxic unless empirical evidence indicates otherwise. We can use this information to demonstrate that the gene encoding the NEP does not have the potential for toxicity, thereby providing supportive evidence in a WOE approach that the protein is not hazardous.

2.3. Mode of Action/Functional Specificity

Knowledge of MOA and functional specificity of the NEP are important elements in the WOE for hazard identification, and may be helpful in determining the NEP's potential for causing toxicity to humans or animals. If the MOA and functional specificity of a NEP are well understood and are shown to have low relevance to humans, it lowers the concern about the safety of the NEP. For example, enzymes generally do not have a toxic MOA, and knowing that a NEP has an enzymatic MOA, for example herbicide metabolism in plants, suggests that the NEP is unlikely to present a dietary hazard. Alternatively, a pesticidal (insect resistant) MOA triggers further investigation into putative hazards and potential risks that can be further understood considering a more detailed mechanism of action. In the case of B.t. insect resistance proteins, the proteins bind to a receptor not present in mammals, which reduces concerns about the protein's potential for human harm.

2.4. Bioinformatics for Toxin Screening

Bioinformatic screens are an excellent tool for placing a protein within the context of related proteins, based on recog-

nizing localized similarity, common domains, and larger protein families or protein super-families. Consequently, bioinformatics plays an important role in the hazard assessment of toxins. This *in silico* screen is typically applied early in the hazard assessment phase and can be useful in providing the preliminary protein and protein family context, which will help determine the need and scientific rationale to conduct any supplementary toxicology (hazard characterization) studies. Bioinformatics results should be regarded as guiding rather than predictive. They allow for a more holistic understanding of a protein or protein family but are not a predictive tool for hazard identification.

The analyses most apt to provide this contextual information are traditional primary sequence alignment algorithms such as Fast All (FASTA) or Basic Local Alignment Search Tool (BLAST), which return localized protein alignments. These alignments can then be reviewed to establish the contextual information, which will serve as the driving reason behind determining the necessity for supplementary studies. Ultimately, as the understanding of domain architecture and function continues to develop, the observed linear alignments - when analyzed in tandem with domain information - will play the greatest role in reconciling protein function and identifying a potential for toxic hazard. For example, use of a domain-based approach has recently been used to help put sequence homology data into context for protein safety evaluation [11, 30]. This analysis demonstrated that simply having a domain or region with homology to a toxin does not necessarily signal potential toxicity.

Bioinformatics will only serve as an identifier of proteins with a "hazard potential" based on some level of similarity. This defined potential, as established by the contextual information gathered by the bioinformatics assessment, will then guide the decision as to whether supplementary toxicology studies are necessary or warranted to enable the classification of a protein as hazardous.

While bioinformatics is an excellent tool for rapid screening and protein identification during the discovery or product development phases, any hazard characterization based upon bioinformatic results must ultimately be examined in conjunction with other hazard and exposure assessment data when generating a risk hypothesis (e.g., HOSU, heat lability, digestibility, MOA, functional specificity, etc.). If a risk is hypothesized, it can be further validated through supplementary toxicology studies. Although bioinformatic analysis may be of limited value for directly demonstrating protein safety, it is an important component of the WOE for hazard identification of the NEP.

3. NEP Supplementary Toxicology Studies (Supplementary Studies)

The weight of the scientific evidence derived from hazard identification can be used to evaluate the necessity for further evaluation, i.e., if the WOE following hazard identification is not sufficient to determine absence of hazard. The toxicological evaluation of all NEPs as a default assessment is not hypothesis driven and is not supported by the WOE established from the history of protein hazard assessments conducted with NEPs in GM plants. Defaulting to *in vivo* toxicology studies, as is often required for regulatory approvals, does not reflect ethical use of animals in scientific research and testing as outlined by the 3R's of responsible animal use (Replacement, Reduction and Refinement) that have been increasingly incorporated into regulatory *in vivo* studies [39]. Such a default approach is, therefore, not science based and is inconsistent with the tiered approach outlined for the safety assessment of NEPs [7]. The initial protein hazard identification should be conducted to build a WOE that can serve as a guide to determine the necessity for supplementary protein hazard characterization.

3.1. Acute Oral Toxicology Study

Evidence to date for NEPs in GM plants indicates that, when no hazard is identified based on the WOE, no evidence of adverse effects is observed in acute oral toxicology studies [2, 7, 23, 28, 38, 44]. Nevertheless, acute toxicology studies are still required by some regulatory authorities regardless of the nature of the protein [29]. These studies have been conducted largely due to the observation that, while most proteins do not present a hazard, most protein toxins elicit their toxicity through acute mechanisms of action [37]. A notable exception to this is the lectins, a group of proteins characterized as anti-nutrients that can cause injury through cell agglutination from binding cell surface carbohydrate moieties.

It is well recognized that the vast majority of dietary proteins are degraded into individual amino acids and small peptides, and absorbed by the intestine for nutritive purposes. This degradation results in a loss of biological activity. Furthermore, most dietary proteins are too large to be absorbed intact, which further minimizes their potential for systemic effects [10, 35]. Lectins have been demonstrated to be highly resistant to proteolytic degradation, and their ability to cause adverse effects is dependent on this property [42].

Given these factors, it is perhaps not surprising that the small number of proteins known to be hazardous when ingested, including ricin and the kidney bean lectin phytohaemagglutinin E (PHA-E), often exert effects on the intestinal epithelium [22, 25, 33, 43, 45]. Lectins can also act systemically [42]. The common features of 'protein toxins' is they typically are cytotoxic, act acutely, and cause damage to an epithelial surface (i.e., non-systemically).

A margin of exposure (MOE) calculation compares the estimated daily exposure that might occur in a given set of circumstances, such as for a specific country/region or sub-population to the No Observable Adverse Effect Level (NOAEL) determined in experimental animals. In the case of NEPs, the NOAEL typically comes from the acute oral toxicity study where the limit dose of 2,000 mg/kg bw is often utilized based on OECD guidelines [40, 32] for testing at high levels when there is no reason to suspect toxicity at lower dose levels.

The MOE is the magnitude by which the NOAEL of the critical toxic effects exceeds the estimated daily exposure, in this case through oral consumption, and is calculated as follows:

MOE = 2,000 mg/kg ÷ estimated consumption (acute consumption values x NEP concentration)

Another method of calculating the MOE is to set dose levels based on multiples of the maximum theoretical human exposure. There may be cases where the test substance solubility is limited or the production of the test substance in large quantities is extremely challenging or virtually impossible, and therefore, using an MOE approach based on exposure estimates, rather than defaulting to testing at a limit dose, would be appropriate. In these cases, one would consider the population and country/region of interest (or highest consumers globally if considering worldwide consumption), and the NEP concentration in a relevant plant commodity or by-product to calculate the MOE.

3.2. Potential Future Approaches to Supplementary Toxicology Studies: In Vitro Evaluations

Conducting an acute toxicology study with a NEP requires the production and isolation of multiple grams of protein from plant or microbial sources. This can be technically difficult for some proteins and virtually impossible for others [1]. Proteins in the latter category include integral membrane proteins and some transcription factors [5, 18, 34]. Proteins such as these have been referred to as intractable proteins, to indicate that it may not be possible to isolate them in quantities required to conduct acute toxicology studies [1].

In view of these protein production challenges, as well as animal welfare consideration, it would be desirable, in the future, to be able to employ *in vitro* methods as a substitute for *in vivo* toxicology studies, as described previously [1]. A feature of toxic proteins is their impact on the intestinal epithelium and/or cytotoxic mechanisms of action. In the unlikely event that a NEP was to be hazardous, it is likely that it would cause damage to the intestinal epithelium. On this basis, intestinal epithelial cell line monolayers from rodents and humans have been investigated to evaluate the effects of known hazardous proteins, including ricin [22] and PHA-E [19]. A number of recently published experiments demonstrate the utility of immortalized [20, 21] and primary [8] human epithelial cell culture models for differentiating proteins with associated hazards from those considered to be innocuous, in both the presence and absence of simulated gastric and intestinal digestive enzymes [6, 27].

4. Exposure Assessment (Supplementary Studies)

As mentioned above, when a hazard is identified by the WOE approach, it is necessary to determine exposure to the NEP. Various factors such as stability of the NEP under different conditions and resistance to digestion influence exposure. Evaluation of these considerations will impact the overall safety assessment. Under conditions where there is no exposure to the NEP, such as in highly-processed foods like oil or sugar, a safety assessment may not be necessary, since there is no apparent risk (Risk = Hazard x Exposure).

4.1. Stability (Heat/pH/Processing)

Demonstration of a lack of biological activity or function following exposure to heat, pH extremes, or processing conditions common in milling, cooking or other processing methods will contribute to the safety assessment of the NEP. This is because these conditions reduce exposure to the functional protein, thereby reducing the hazard potential [17].

4.2. Resistance to Digestion

Proteins, in general, are a natural and necessary part of human and animal diets, and are subjected to rapid degradation by digestive enzymes in the gastrointestinal tract into individual amino acids and small peptides that can be absorbed by the body to support nutritional needs. Large proteins are not known to be absorbed by the intestinal epithelium. As part of the WOE approach, a protein's ability to resist degradation *in vitro*, in the presence of digestive enzymes (pepsin and pancreatin) is tested, and aids in the understanding about the potential digestive fate of a NEP in food. This, in turn, provides information about any potential for systemic absorption of intact active proteins, since proteins that are rapidly and thoroughly degraded by digestive enzymes present no opportunity to be absorbed intact. If the NEP is rapidly degraded in pepsin and pancreatin, it can be inferred that it has limited or no biological activity and is less likely to impart toxic effects upon consumption, and thus less of a concern for safety to humans and animals. However, if proteins are resistant to degradation by digestive enzymes, it does not necessarily indicate that the protein presents a potential hazard, as stability does not, in and of itself, answer the question about whether the NEP is a likely hazard.

5. Conclusion

Toxicological assessment of NEPs in GM plants is performed to inform the overall safety assessment process. In conjunction with allergenicity assessment, the results of toxicity evaluation enable risk characterization and the evaluation of safety of GM plants for food and feed use. A stepwise approach is proposed here to evaluate toxicity that uses WOE gathered from different attributes of the NEP. Key hazard identification studies should first be performed for all NEPs (core studies) and, if a hazard is identified, further toxicity studies and exposure characterization should be done (supplementary studies). An excellent example of the application of this proposed stepwise approach to the safety assessment of a NEP is described in Habig et al., wherein the WOE for safety of the intractable protein VNT1 was successfully concluded using only those approaches described as "core" studies [14]. Acute oral toxicology studies are not informative in the absence of hazard attained from the WOE assessment, and *in vitro* toxicology studies are proposed for intractable proteins. *In vitro* studies are also beneficial for animal welfare. Exposure considerations such as stability and resistance to digestion contribute to the WOE for overall safety assessment and should be done when a hazard is identified. When there is no hazard identified, there would be no risk, and therefore, further hazard and exposure characterization is unnecessary.

6. Declaration of Conflicting Interest

All the authors of this paper are currently employed by, or have been employed by, the agricultural biotechnology industry.

7. Disclaimer

Portions of this policy commentary were used to inform policy commentaries, *Core and supplementary studies to assess the safety of genetically modified (GM) plants used for food and feed* and *Allergy risk assessment for newly expressed proteins (NEPs) in genetically modified (GM) plants*. These portions were written by the same authors and the commentaries are published in this journal issue.

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9. Article Information

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Allergy Risk Assessment for Newly Expressed Proteins (NEPs) in Genetically Modified (GM) Plants

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Abstract

Based on experience and scientific advancements over the past two decades, a revised approach for the assessment of the allergenic potential of newly expressed proteins (NEPs) in genetically modified (GM) plants is warranted. NEPs are most often not native to the crop genome, and thus regulatory reviews of the safety of GM plants include an assessment of the allergenic potential of NEPs. International standards for the assessment of allergenicity first developed in the mid-1990s required a series of characterization studies to be conducted that are, to some extent, still applicable today to the risk assessment of GM plants, with most modern versions represented in the Codex Alimentarius. This standardized guidance on allergenicity assessments, including the required characterization studies, presented two primary challenges. First, there was (and still is) no defined and accepted model (animal or *in vitro*) for directly testing allergy potential. Second, bioinformatic analyses were prescribed using thresholds for hazard identification that were neither universal for all allergens nor tested prior to the implementation of requirements into guidance documents. Herein, risk assessment principles are applied to structure the assessment of the allergenic potential of NEPs. This allergy risk assessment is built on a foundation of: 1) identifying hazard by assessing similarity to known allergens, and 2) assessing exposure when a hazard is identified. Supplementary studies such as IgE binding may need to be performed in special cases. These recommended revisions to current approaches to the assessment of allergy potential are designed to ensure a realistic, case-by-case approach, and consider updated molecular biology, genomics, and bioinformatic techniques that were unavailable when earlier allergy risk assessment approaches were established.

Keywords: genetically modified, allergenicity assessment, food and feed, hazard, exposure, risk, core studies, supplementary studies

Abbreviations: GM, genetically modified; GRAS, generally recognized as safe; HOSU, history of safe use; NEP, newly expressed protein; SGF, simulated gastric fluid; WOE, weight-of-evidence

1. Introduction

There is no single measure or combined set of measures that are universally common to allergens. For example, a high level of stability (e.g., peanut Ara h 1 protein) in the presence of pepsin enzyme (simulated gastric digestion), is common to many proteins, only some of which are allergens, and is not a characteristic of all known allergens. Therefore, by itself, stability across the many structural groups of allergens is too inconsistent to be a predictive indicator of allergenic potential. As no validated animal testing system is available and there is no single criterion that sufficiently predicts allergenic potential, the hazard characterization of potential allergens must use a weight-of-evidence (WOE) approach, combining several biophysical characterizations of the newly expressed protein (NEP) [4]. Fundamental to this hazard assessment approach is the degree of structural similarity to known allergens. A denoted high level of similarity to a known allergen is a hazard threshold that, in most situations, either triggers further studies to more clearly define sensitization/allergy risk or is the basis to discontinue commercial development of the NEP. As described by Nordlee et al. [23], the discovery of similarity between the NEP and the Brazil nut storage protein allergen did not happen until after transformation of the intended soybean crop. In this

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case, appropriate sequence screening tools were not available to allow for early hazard identification; there were no comprehensive protein allergen databases at the time and access to comprehensive sequence databases was limited. Advances in bioinformatics and molecular characterization techniques, along with a better understanding of protein allergens in general, offers the opportunity to revise the approach to allergy characterization to more effectively and accurately inform allergy risk assessment.

2. Scientific Guidance Documents for Allergy Assessment

Experiences early in the history of genetically modified (GM) plant development encouraged the adoption of scientifically reviewed guidelines to assess the allergy safety of proteins introduced into GM plants [21]. The Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) revised guidance for food allergy assessments [33], included a flow diagram of characterizations that concluded with the introduced protein being considered as either having, "*no clear risk of being allergenic*" or having, "*evidence for a clear potential allergy risk*". The Codex Alimentarius [3] further refined food allergy assessments with updates in 2009 by providing an integrated, case-by-case "tiered" approach that uses a combination of criteria to assess where, on a continuum of allergenicity potential, the introduced protein fits. Codex uses a WOE approach, recognizing that no single criterion is sufficiently predictive of allergenic potential. The first tier in the Codex process characterizes the source organism of the introduced protein and determines whether that organism is a known source of allergenic proteins. This tier employs bioinformatics to assess whether significant sequence homology and/or structural similarity exists between the introduced protein and known allergens. Other criteria assessed following the Codex process characterize whether the protein is susceptible to degradation by pepsin (many, but not all known allergens are pepsin resistant and many non-allergens are also pepsin resistant), and, if appropriate based on information from the first tier, conducting specific serum screening studies. In the Codex process, if the first tier determines that there is no substantial similarity with known allergens, then risk is characterized as low or negligible. If there is similarity to a known allergen, then an appropriate human serum IgE binding study should be conducted to address potential shared epitope binding between the introduced protein and the known allergen. The Codex process is considered tiered, since it recognizes that none of the individual characterization assessments can be fully predictive of allergenic risk and that potential risk can be more clearly defined by serum-based screening. The guidance does not prescriptively indicate specific protocols but does include relatively specific endpoints about cross-reactivity and shared sequence identity. For example, sequence similarity methods are defined no more specifically than "bioinformatics", but do include a reference for more than 35 percent identity match over 80 or more amino acids when comparing a NEP to allergens.

The guidelines for allergy assessment were built to address two aspects of food allergies, since clinical response occurs in a two-step process: 1) the initial exposure to the protein that sensitizes the individual, and 2) elicitation of a clinically manifested response upon re-exposure to the same or similar protein [22]. Given that proteins are grouped into families that share homology (similarity in structure and function), assessing the elicitation potential of an introduced protein is a key element included in the updated guidance language. Characterization of several distinct physiochemical properties of the introduced protein form the basis for this "elicitation response" assessment. As an example, bioinformatics that assess the sequence similarity between an allergen and a NEP is essentially assessing potential cross-reactivity, or the likelihood that elicitation in sensitive patients to the NEP can occur.

Guidance documents also address the potential risk that the introduced protein may newly sensitize individuals consuming the protein in a food derived from a GM plant that has not previously included the NEP or a related homolog (*de novo* sensitization potential). This second type of risk assessment uses the same physicochemical properties as the elicitation response assessment, but differentially applies them to the WOE conclusion. In other words, without a defined test or criteria that can predict likelihood of allergy, characterization parameters such as sequence similarity are used as a surrogate to assess the likelihood of an allergy risk in an undefined segment of the population.

More recently, proteins introduced into GM plants have been referred to as "novel proteins" in some guidance documents, since the protein is "new" to that plant. However, it should be recognized that, to date, all introduced proteins in GM plants share structural and/or sequence homology with known dietary proteins and are therefore not '"novel" in terms of available data on human dietary exposure to the protein or its homologs [13].

In summary, when guidance was first proposed by Metcalfe et al. [21], later adopted by the World Health Organization in the late 1990s, and expanded in 2001 [33], there were methodological limitations and assumptions made because of limited knowledge about allergens, compared with today. Further along, in 2003 Codex built upon the previous FAO/WHO guidance but stepped away from a decision-tree approach towards a tiered and cumulative WOE approach.

All these earlier guidance documents were effectively based on identifying allergens using approaches that were not empirically tested to distinguish allergens from non-allergens, especially in the case of the bioinformatics thresholds. Since those guidance documents were published, better tools have become available that more accurately and precisely identify allergens (Figure 1). A brief breakdown of the state-of-the-science in 1995 versus the current status of each type of assessment is the following:

i. *1995:* Genome sequencing and databases. As there was no prior knowledge of whole organism genomes, any protein from an organism causing allergy was assumed hazardous. *Currently:* Source organisms and specific genes can be sequenced, identified, and characterized. Allergen databases enable identification of NEPs belonging to an allergen group and allow identification of an al-

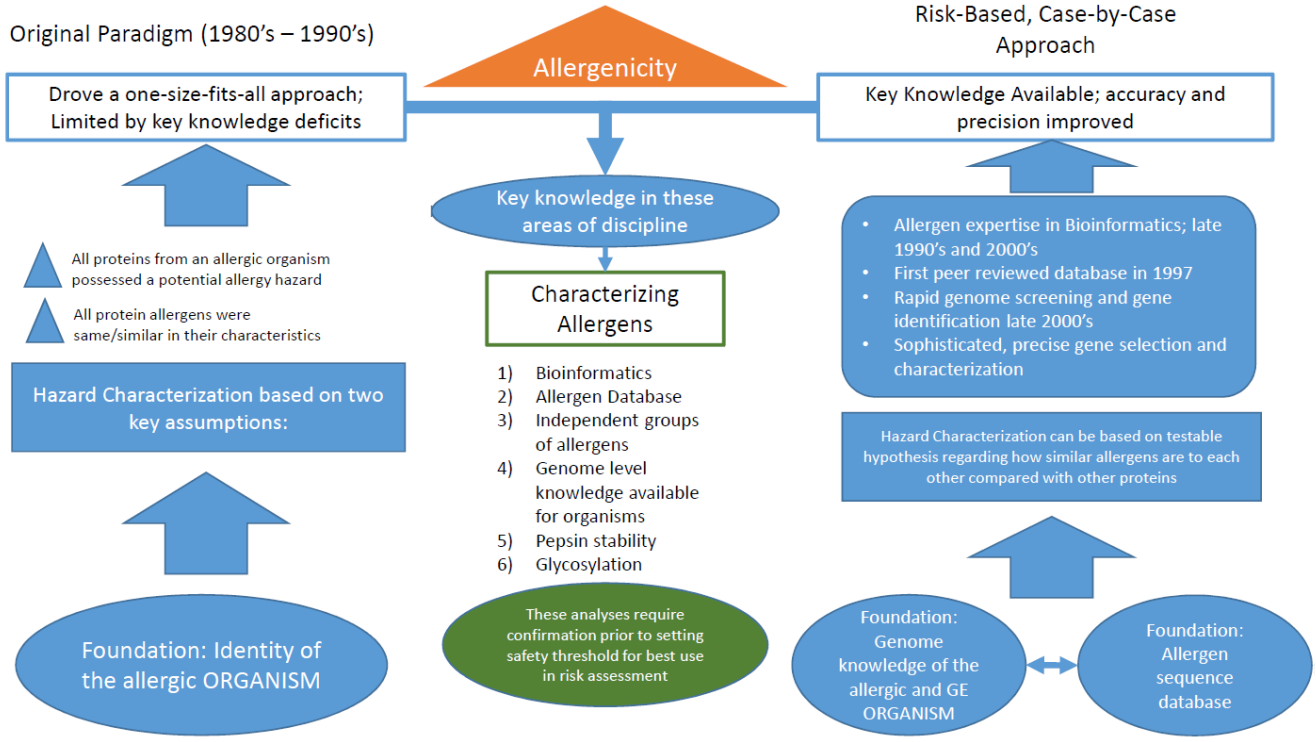

Figure 1: The evaluation of allergenicity of newly expressed proteins in genetically modified plants according to the original paradigm developed twenty-five years ago, and modern approaches using bioinformatics and hypothesis testing

lergen defined by serology (elicitation) risk with known exposure and response by sensitive patients.

ii. *1995:* Gastric fluid simulation (pepsin enzyme stability) was used to assess stability to digestion and exposure of the lower gut immune system. *Currently:* Pepsin is now understood to not be a predictive indicator of a hazard as not all allergens are stable and not all non-allergens are unstable to digestion.

iii. *1995:* Glycosylation of NEPs used as an indicator of hazard. *Currently:* Recognized that glycosylation may support IgE binding, but that it is not a causative factor in the initiation or elicitation of clinical allergy.

iv. *1995:* Serology testing with human serum and IgE-binding readouts were used to identify cross-reactivity. Serology provided the only available method to identify allergens. However, it was only implemented if there was some other hazard identifier for the NEP being similar to an allergen, not as a "test" for allergy potential. *Currently:* Serology testing is still recognized as a valuable method to study patient response to an allergen. However, it does not provide utility in routine screening NEPs for allergenicity without a prior "trigger" being identified, which is typically a concern raised by relevant bioinformatic similarity.

v. *1995:* Abundance of a NEP in the plant was used as a hazard identifier, but without specific quantified guidance thresholds. This approach was based solely on knowing that some allergens, such as plant storage proteins, represent a high percentage of total consumed protein. *Currently:* Abundance has no predictive capacity for allergenic potential (see Exposure section); threshold levels for clinically relevant exposure levels have not been identified for most allergens.

In view of methodological developments and a modern understanding of allergenicity, a revised approach for assessing the allergenic potential of NEPs is warranted. This revised approach supports an overall risk assessment to ensure that foods derived from GM plants are safe for consumption. It assigns various WOE characteristics into respective risk assessment categories of "hazard" and "exposure", and is based on well-recognized risk paradigms that, in the absence of hazard, there is no risk, and therefore no need to assess exposure. Similarly, in cases where there is no exposure there is no need to assess hazard. From a technical standpoint, there is a much better understanding in the last 15-20 years regarding molecular characterization, the bioinformatic assessments of NEPs, and recognition of qualified allergen database(s). A key concept to the revisions discussed herein is to place the biophysical assessments of NEPs into their respective risk-use domains.

A suggested approach for allergenicity evaluation, separating assessment of hazard from assessment of exposure is the following:

- **Allergen-Specific Hazard Assessment (Core Studies)**:

These should be performed for all NEPs.

- **Exposure Characterization (Supplementary Studies)**: If a hazard is identified, exposure characterization should be performed. In the absence of hazard, there would be no risk, and therefore, no need to characterize exposure.
- **Hazard Characterization: Allergenic Potential and IgE Binding (Supplementary Studies)**: These may need to be performed on a case-by-case basis.

3. Allergen-Specific Hazard Assessment (Core Studies)

A critical question to address in the allergy hazard assessment for NEPs is whether the protein is similar to a known allergen. This addresses the potential for cross-reactivity between the NEP and a known allergen (i.e., elicitation), and the potential for a novel NEP to present a hazard as a *de novo* allergen (i.e., sensitization followed by elicitation). The assessment of the latter focuses on primary or first exposure of a *de novo* protein allergen to a person who has potential for sensitization and the consideration of whether that person, upon subsequent exposure, would develop an elicited, clinically-relevant response.

The paradigm of risk assessment for allergens, hinging on the standard risk equation (Risk = Hazard x Exposure), has caveats to distinguish allergens from other toxicants. Specifically, there is no single test or predictive assessment for whether a protein will act as an allergen. Consequently, hazard identification involves measures of several of the physiochemical properties of the NEP. The approach is based on identifying relevant properties that are considered "common" to allergens. The key characterization parameters and revised approaches to assess allergenic potential of NEPs are detailed below.

3.1. History of Safe Use of the NEP

History of safe use (HOSU) of the NEP is one of the fundamental and initial endpoints in the safety assessment. Demonstration of prior human and/or animal consumption provides familiarity with respect to the probable safety of a given protein. This is similar to the GRAS (Generally Recognized as Safe) concept employed by the U.S Food and Drug Administration (FDA) [28]. GRAS classification indicates that a food ingredient is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use, either through scientific procedures or through common use in food. FDA extended the GRAS concept to proteins used in biotechnology plants in 1992. The concept of HOSU was also included in a recent European Food Safety Agency (EFSA) assessment guideline document suggesting no need for any specific toxicity or allergenicity testing in cases where both the plant and proteins expressed in the GM plant have a history of safe consumption by humans and animals. The concept of protein HOSU has also been emphasized in peer reviewed publications and other guidance documents related to safety assessment of GM plants [4, 8]. In order to demonstrate HOSU, evidence of similarity and exposure to the other consumed proteins or species expressing these proteins or similar proteins is needed [13]. Protein similarity can be determined, on a case-by-case basis, by primary sequence alignment and by structural or functional similarity, depending on the class of the protein. Protein phylogenetic analysis also helps in determining protein similarity with well-characterized proteins known to be safe. Similarly, familiarity with the mode of action and the specificity of protein activity also contributes to an evaluation of the HOSU of proteins. Depending on similarities, it may not be necessary in all cases to model exposures, as sufficient history of exposure may be demonstrated semi-quantitatively (e.g., by comparing concentrations of the similar protein in foods and relative consumption levels). While epidemiological and experimental evidence should also be considered when available, an extended history of use in the diet with no reported adverse outcomes can suffice for a safety assessment. It is important to note that absence of a clear HOSU for a protein does not represent a hazard but only indicates that further analysis of other lines of evidence may be needed in the assessment of protein safety.

3.2. Familiarity of the Source Organism

Familiarity with the source organism of the protein can also play a vital role in the WOE approach for determining safety of the NEP. The absence of any biosafety risk associated with the source organism provides strong evidence about the safety of the NEP. If the source of the protein has a HOSU then any protein from the source is also likely to be safe [6]. Animal toxicology and nutrition studies, as well as human exposure, with the source organism of the NEP can also support a safety assessment of the NEP. Use of an organism with a HOSU can demonstrate the limited potential for the NEP to be a toxin, allergen, or anti-nutrient [6]. On the other hand, if a source organism does have some pathogenicity, toxicity, or allergenicity, established knowledge of that organism can also support a safety assessment of the NEP. Typically, only a few proteins or a small fraction of an organism's genes are responsible for these properties. With modern molecular characterization of source organisms, fully curated allergen sequence databases, vast knowledge of toxic protein sequences and modes of action, and capability in modeling higher levels of structural similarity, the source organism as such does not necessarily describe a hazard for an NEP. In other words, there is usually clear identification of the genes within a genome that produce an allergen, an allergen homologue, a toxin or an anti-nutrient. Other proteins encoded in the genome would be expected to be non-allergenic and non-toxic, and the organism itself does not define a hazard. It is more important to establish what degree a NEP (not already known to science as an allergen) is significantly similar to a known allergen early in the NEP development process; thus, it becomes a foundation in describing whether a NEP is in any way similar to allergens or more similar to other safely consumed proteins.

3.3. Amino Acid Sequence Similarity and Bioinformatics

Traditionally, assessing protein similarity at the amino acid sequence level was considered as "bioinformatics", and hazard identification involved a binary condition considering two

measures of sequence alignment. The amino acid sequence of the NEP was compared with that of known allergens using algorithms to assess sequence similarity with a focus on locally aligned "domains". Those two measures were: 1) shared percent *identity* > 35 percent, and 2) *overlap* length of alignment ≥ 80 amino acids.

This dual criterion and binary bioinformatic approach has since been demonstrated to limit two key understandings of protein allergens [19, 26]. First, an untested set of identity and overlap criteria do not describe a minimum understanding of similarity between two proteins. Allergens (i.e., an allergen database) were not originally modeled bioinformatically whereby only identity and overlap length were determinately known to predict biological relevance. Therefore, identity was used early on in allergen similarity assessments, but now is not enough to uniformly apply as an indicator of relatedness across the many groups of allergens [20]. A much better understanding of applying bioinformatic when comparing a NEP with allergens has come into place since the initial bioinformatics guidance [32] was published.

Although bioinformatics is a wide-ranging discipline combing informatics and biology, it is a special-case application when using local alignment algorithms or other methods to determine similarity among two or more sequences for the purposes of allergy risk assessments. The best use of bioinformatics for protein safety assessments in this context is the combination of a thorough understanding of existing allergens with a coordinated review of allergens and their placement into a qualified database that has more statistical power to detect structural relationship [5].

The use of bioinformatic tools should be such that the results are both accurate and precise without reliance on arbitrary endpoints (as discussed earlier). This can be enabled by application of structural biochemistry to support structural classifications of all proteins so that individual structural classes of protein allergens are recognized. The structural analyses of allergens then become "case-specific", because it is the combination of the clinical phenomenon of some proteins being allergen sensitizers/elicitors along with their unique biochemistry that allows placing them into an allergen database. It remains to be defined whether there is common structural biochemistry across all allergens; in the meantime, a case-by-case analysis when addressing the similarity of a NEP with known allergens is required.

To date, there is no evidence that a single sequence alignment feature such as percent identity (or percent identity and sequence length) is both conservative (from a safety standpoint) and accurate in describing allergy potential and/or allergy cross-reactivity. In fact, inspection of the Comprehensive Protein Allergen Resource (COMPARE) database reveals some allergen families are composed of highly similar sequences from a highly diverse group of hosts, while other allergen families consist of highly diverse sequences from closely related hosts.

Recent test cases and exploration of allergen similarity using common algorithmic approaches highlight the extent to which accurate measures of similarity, which extend past the use of percent identity and alignment length, can be applied. To enhance the accuracy and reliability of bioinformatics assessments for allergenic potential of NEPs, a step-wise approach is recommended:

Step 1: Sequence level consideration – Does a protein share relevant similarity with any proteins in a qualified allergen database?

Bioinformatic algorithms have been designed to highlight and measure the probability that two sequences share a substantial portion of their structure and otherwise share a common evolutionary origin. The conventional local sequence alignment-based algorithms Fast All (FASTA) and Basic Local Alignment Search Tool (BLAST) produce several output measures that demonstrate shared identity, domain-specific overlaps, and similarity with the summary statistic, expectation value (E-value) typically being the culmination.

The recommended endpoint measure is E-value, the most reliable and sensitive indicator of likely sequence homology. E-value depends on the database size and simply reports the number of times a similarity score is expected by chance, or the number of expected false positives (non-homologs) per search. Generally, an alignment of two sequences with E-value < 0.01 are homologous, but to be certainly homologous for two sequences, E-value needs to be less than 10^{-6} [24]. E-value determinations for specific cases (i.e., specific NEP comparison to allergen databases) should be evaluated depending on the following:

a) Is the E-value biased by amino acid composition, i.e., is the normal distribution of the 20 amino acids expected for a typical protein, or is there reduced distribution? This is critical to understanding alignment scoring due to a bias in "significance" if reduced distribution (i.e., low complexity) is present. This can be assessed by shuffling the sequence of the NEP and repeating the search. If shuffled, and if these sequences yield E-values of ~1.0 or greater, the corresponding alignments demonstrate that alignment results are reliable (i.e., indicate false positivity) because the random shuffling abolished the unique sequence structure only present in the intact, native, and original sequence.

b) Do alignments with significant similarity identify two or more different families of allergen proteins? In such cases, sequence masking should be employed to remove so-called "low complexity" sequences from the search. If masking eliminates apparent significantly similar alignments, the significance of low complexity sequence alignment should be assessed.

Step 2: Structural relatedness – Does the NEP belong to a structurally defined group of allergens?

Allergens can be grouped taxonomically to some degree, and more often structurally, to help determine similarity (when they are well characterized proteins) with the goal of assessing

the potential for cross-reactivity. Regardless of whether a protein possesses relatively low or high allergenic potential, identifying relationships among groups of allergens can be informative.

Step 3: Further structural considerations – Does a protein that is similar (either significantly or borderline) in sequence level consideration share direct measures of similarity using modeling of three-dimensional structures?

Dimensional modeling offers a more sophisticated measure of similarity between a NEP and an allergen, but it would only be performed if necessary, based on the results of sequence level analyses. Because dimensional modeling would be expected to extend beyond the limits of the linear sequence similarity typically performed during sequence level consideration, an additional assessment with modeling may offer clarity. Specifically, modelling may reveal dramatically different 3D structures despite the observed primary sequence similarity.

Specific metrics from modeling would be addressed on a case-by-case basis with the knowledge of any specific allergens and their associated epitopes, and other clinically relevant sequence mapping being a key to understanding similarity with the NEP (i.e., IgE binding epitopes).

4. Exposure Characterization (Supplementary Studies)

If a hazard is identified, exposure should be characterized to obtain an understanding of risk. Digestion, processing, and abundance are studies that address exposure to the NEP.

4.1. Digestion

Stability of proteins in simulated gastric fluid (SGF) was first suggested as a distinguishing feature of food allergens in 1996 [1]. While subsequent studies largely found this correlation to be weak or non-existent [2, 10, 17], this initial study and the intuitive appeal of reduced exposure in the intestine, where sensitization and elicitation were believed to predominantly occur, resulted in the wide adoption of this criterion as part of the WOE approach supporting the allergenic risk assessment of NEPs [33]. The SGF method [29] was adopted as a surrogate for human digestion in the stomach because this is the method for which results were initially reported to correlate with the allergenic status of proteins [1], and because this method has been used commonly to assess the digestion of pharmaceuticals [11].

As follow-up studies began to show that the SGF assay was a poor predictor of the allergenic status of proteins, modifications and expansion of digestion studies to include more physiological gastric conditions and simulated intestinal fluid were explored, without any notable improvement in the contribution to the WOE for assessing the allergenic risk of novel food proteins [16]. This is not surprising, as a robust body of literature exists in the animal science arena showing that a better correlation between *in vitro* and *in vivo* digestion often occurs when non-physiological *in vitro* digestion conditions are employed [9]. As layers of complexity are added through the inclusion of "physiological conditions", it becomes increasingly difficult to draw meaningful conclusions due to the introduction of additional variables. It is important to note that SGF and simulated intestinal fluid are not designed to mimic the highly complex and variable conditions of human digestion, but rather to allow relative rates of digestion among substances (in this case proteins) to be compared [16].

While SGF is often considered to measure pepsin susceptibility, this is typically not the case. Rather, it is the combination of acid denaturation and pepsin digestion. Pepsin is a relatively promiscuous enzyme and will digest most proteins very rapidly when the proteins are linearized by denaturation [14]. It is the folding of proteins, resulting in the shielding of these pepsin cleavage sites, that slows digestion. The acidic conditions in the stomach and those specified for SGF (pH 1.2) denature many proteins, and results of the SGF assay are largely dependent on the kinetics of this denaturation [14]. Furthermore, pepsin describes a family of highly similar enzymes with different amino acid sequences. Multiple pepsins exist in humans, and the porcine pepsin used for SGF assays is even known to cleave proteins into different peptides compared with human pepsin [30]. Thus, the SGF assay is a surrogate for human gastric conditions but is only designed to give relative information among proteins. It is not designed to mimic physiological conditions that are highly complex and variable. Surrogate digestion assays such as SGF have been developed recognizing this reality. Clearly, increased digestion of a protein reduces exposure in the intestines. For sensitized individuals, reduced exposure to the offending allergen reduces allergenic symptoms [27]. With the poor correlation between digestion results and the allergenic status of proteins, these recent findings do not support the use of digestion results in the WOE for assessing sensitization risk for novel food proteins [15, 31] .

The characteristics of hazard and the triggering level of elicitation need to be established for a risk evaluation [7]. Under the risk-based approach recommended, SGF stability provides value only when there is a known hazard, as digestion characteristics would contribute to exposure considerations in the risk = hazard x exposure equation.

4.2. Processing

Processing has typically referred to the assessment of how apparent the stability of a NEP may be when the grain in which it is contained is processed using processes that would be typical for turning grains into food and feed fractions. The premise is that the application of heat during processing can alter protein structure, which is key to both enzymatic/biological function and IgE-binding site access (i.e., allergenic potential). From a safety perspective, the goal is presumably to understand whether some form of unique "stability" is apparent that is not otherwise identified by the SGF pepsin assay. Discussed below is the clarification of the limited hazard characterization utility in performing this type of study on NEPs.

To address the limited use of a "processing stability" assessment, Privalle et al. [25] reviewed this type of characterization of NEPs. The basis of the premise stated above is that several

allergens maintain functional intactness and allergenicity after exposure to various heat conditions during food processing like cooking [25]. As a result, heat stability, also known as thermal stability, is required in Tier I of the Codex WOE approach [4]. Typically, *in vitro* heat stability is measured by two methods, enzymatic/biological function assays and non-human immunodetection assays. These methods were reviewed extensively [25]. Measuring functional intactness after food processing or heat treatment(s) could contribute to an exposure assessment. In the non-human immunodetection assays, animal IgG serum is used to show loss of the immune-binding to the introduced protein after varying levels of heat treatment. The IgG serum is produced by exposing an animal to the protein of interest and serves as a "surrogate" for human IgE serum, which is unavailable due to lack of human exposure to the NEP. However, the animal IgG serum is not a suitable surrogate because its production relies on the animal's immune systems, and there is no way to predict or ensure the human IgE and animal IgG binding sites are the same. As a result, it was concluded that the non-human immunodetection assay measures immunogenicity, not allergenicity, and cannot contribute in any meaningful way to assessments of allergenicity [25]. In the function assays, enzymatic/biological activity of the introduced protein is measured before and after heating; a loss of function suggests a low risk of allergenic potential. However, functional stability is not consistently correlated with allergenic potential because heat conditions have been shown to increase, reduce, or unalter allergenicity in foods such as roasted peanuts, hazelnut, and soybean meal, respectively [25]. Measuring functional intactness after food processing or heat treatment(s) could contribute to an exposure assessment but does not otherwise identify allergy hazard for NEPs if the NEP is not otherwise determined to possess allergen similarity or is clearly an allergen. There is no presumptive endpoint for processing stability that would contribute to allergy risk assessment for the currently approved NEPs that lack allergen similarity and are known to be safe. Exposure assessments may be an expectation for some regulatory agencies but provide no quantitative value for risk assessment in the absence of hazard.

4.3. Abundance

Abundance has been recognized as additional information that can support an allergy safety assessment [3, 4]. The premise is that generally, protein allergens tend to be abundant on a per weight basis and that this is part of the characterization that separates allergens from non-allergens. Several allergens, like Ara h 1 in peanut and glycinin in soybean, are abundant proteins and represent at least one percent of the total protein from the source organism [12, 18]. Due to the high concentration, abundant proteins are more likely to endure digestion in humans and animals and crop processing (although stability is a factor) and may increase the risk of allergenic response in sensitized people. In this regard, abundance has been treated as an associative factor in considering exposure; i.e., more of the protein equates to more exposure and a higher probability of allergy risk. For this reason, abundance of the introduced protein could be considered as a contributing factor to the exposure assessment for known allergens. However, abundance is not understood in a way that endpoints can be applied to proteins that are not otherwise allergens or cross-reactive proteins. And, there are many allergens expressed at low concentrations because they are not seed storage proteins or other proteins expressed at high levels relative to others in plant and animal tissues. Therefore, low abundance suggests a low probability of allergy relevant exposure, but independent of an identified hazard, greater or lower abundance cannot be a contributing factor in describing allergy risk for a NEP.

5. Hazard Characterization: Allergenic Potential and IgE Binding (Supplementary Studies)

Traditionally (Codex), the need to perform human serum IgE binding to a purified NEP was considered as part of a tiered approach. In this approach, the expectation was that either the source organism of the NEP being an allergen-containing source or a bioinformatic similarity trigger, was a requirement. With the advent of more sophisticated bioinformatic techniques and using the proposed refined approach described herein, the necessity of adding IgE binding data to an allergy risk assessment would be considered a "special case". The WOE across all the characterization metrics for the NEP and the details of the bioinformatic analyses will be unique for each NEP. To support rapid and routine safety screening of introduced proteins, bioinformatics is the primary way in which introduced proteins are screened. When determined to be necessary, IgE binding data could help confirm elicitation potential, but the expectation is that this would only be performed in rare cases, if at all. Therefore, it is recommended that IgE binding as an allergy assessment strategy be delegated as "case-by-case".

6. Conclusion

The assessment of a NEP for allergic potential is based on a characterization of its overall biophysical similarity with known allergens. In turn, advancements in allergen discovery and characterization support an increasingly robust characterization of NEPs. A key advancement is an established database of known allergens as well as transparent and well-documented processes for maintaining this as an accessible resource for safety assessments. In addition, molecular characterization of genomes has redefined the safety focus to be on individual genes rather than whole organisms for those that are the source of a NEP and those of allergen-containing organisms. While identifying a source organism for a NEP as a source of allergy requires additional investigation, a greater knowledge of the genes and proteins within organisms allows those genes related to allergy to be separated from those that are not. As such, the vast majority of safe genes and proteins within the source organism are not falsely implicated as contributing to allergenic risk. Overall, addressing NEP health concerns by assessing whether it is an allergen or similar enough to cross-react with a known allergen is the hallmark of the allergenicity assessment. Predicting whether a NEP can "become" an allergen remains the

most difficult health concern to address, as there is no single test for this potential. Yet, improvements in allergen databases (e.g., COMPARE) [5], genome-level gene identification, and the recognition that NEPs are rarely "novel" and are expected to be proteins that can be fully characterized, alleviate most of this concern. In the modern era, allergenicity assessment relies on the newest allergy and molecular science to maintain a low level of risk for the consumer.

7. Declaration of Conflicting Interest

All the authors of this paper are currently employed by, or have been employed by, the agricultural biotechnology industry.

8. Disclaimer

Portions of this policy commentary were used to inform policy commentaries, *Core and supplementary studies to assess the safety of genetically modified (GM) plants used for food and feed* and *Toxicological assessment of newly expressed proteins (NEPs) in genetically modified (GM) plants*. These portions were written by the same authors and the commentaries are published in this journal issue.

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Can Mass Spectrometry Analysis of *In Vitro* Digestion Products Improve the Assessment of Allergenic Potential of a Newly Expressed Protein?

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Abstract

The rigorous safety assessment conducted on genetically modified crops includes an evaluation of allergenic potential for an associated newly expressed protein (NEP). Since no single method is recognized as a predictor for protein allergenicity, a weight of evidence approach (WOE) has been adopted. *In vitro* digestion is a part of the WOE approach and is used to evaluate the susceptibility of a NEP to digestion by gastrointestinal proteases. In 2017, the European Food Safety Authority outlined additional digestion conditions and suggested liquid chromatography tandem mass spectrometry (LC-MS/MS) as an analytical method to detect small post-digestion peptides. This technical review paper focuses on the question of whether LC-MS/MS can aid in assessing allergenic potential of *in vitro* digestion products generated under the newly proposed conditions. After an extensive review, it was determined that LC-MS/MS can detect very small digestion products. However, the method cannot provide relevant information to differentiate whether these products are allergenic or non-allergenic. Therefore, the use of LC-MS/MS for a standard *in vitro* digestibility assessment provides no improvement in allergenicity prediction.

Keywords: genetically modified crops, allergenicity assessment, *in vitro* digestion, mass spectrometry

1. Introduction

Genetically modified (GM) crops undergo an extensive safety assessment prior to commercialization. A key component of the food safety assessment for a GM crop is an assessment of allergenic potential of the newly expressed protein (NEP). Due to the lack of clear understanding of mechanisms of allergenic sensitization and elicitation, there is no single test or characteristic that can predict the allergenic potential of a protein or peptide [6, 27]. As a result, an overall weight-of-evidence (WOE) approach with a cumulative body of evidence was adopted to assess the allergenic potential of NEPs in GM crops [6, 9, 43]. The registration requirements to address the allergenicity potential of a GM crop include the source of the NEP, a review of the history of safe use for the NEP and its homologs, a bioinformatics analysis of amino acid sequences, the stability of the protein when heated, and the susceptibility of the protein to gastrointestinal enzyme digestion (e.g., pepsin). Resistance to pepsin digestion was initially linked to allergenicity by one research publication and an opinion paper [2, 25] under the premise that more stable proteins tended to be allergens via the potential for increased exposure to the gut immune system, one route for sensitization and elicitation of allergy. However, subsequent investigations have revealed that there is no direct correlation between pepsin resistance and allergenicity [3, 13, 17, 32]. Despite the lack of a clear correlation, the *in vitro* pepsin digestion test remains as one component in the WOE approach.

The current standardized *in vitro* digestion assay has been adopted and accepted globally for almost two decades; this assay consists of a pepsin resistance time course of the NEP followed by a qualitative assessment for the presence or absence of intact protein or degradation fragments (>3 kDa) on a stained sodium dodecyl sulfate-polyacrylamide gel and/or western blot [38]. In 2017, the European Food Safety Authority (EFSA) Panel on Genetically Modified Organisms outlined new *in vitro* digestion conditions and proposed an additional detection method, liquid chromatography tandem mass spectrometry

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(LC-MS/MS) [26]. The hypothesis is that the combination of newly outlined *in vitro* digestion conditions and detection of small peptides by LC-MS/MS could enhance the identification of the allergen potential of a NEP [11, 26]. The newly proposed *in vitro* digestion conditions consist of pepsin digestion to represent gastric digestion followed by trypsin and chymotrypsin digestion to represent intestinal digestion. For pepsin digestion, the EFSA GMO Panel recommended the use of classic (low pH and high enzyme-to-test protein ratio) and suboptimal (high pH and low enzyme-to-test protein ratio) conditions to consider populations with impaired or underdeveloped digestive systems [38]. Recent observations suggested that pepsin digestion tests under suboptimal conditions would not provide useful information because some proteins that are readily digested by pepsin could show resistance to degradation and lead to the inability to distinguish between pepsin labile and pepsin resistant proteins [1, 41].

For the proposed detection of smaller digestion peptide fragments, the EFSA GMO Panel recommended LC-MS/MS to identify and track abundance of small peptides ($\geq$ 9 amino acids in length) in conjunction with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for intact protein and large peptide observations. The premise is that an LC-MS/MS detection method would aid in identification of a hazard and its exposure by providing valuable information through distinguishing allergens from non-allergens and tracking their abundance. At a first glance, the recommendation to use LC-MS/MS as an alternative detection method seems reasonable considering it has been used for peptide identification since the 1990s and applications have advanced with continued improvements in modern separation, detection, and computation technologies [33]. For example, LC-MS/MS has been used to detect and identify peptide-based biomarkers involved in disease, like the beta-amyloid fragments associated with Alzheimer's disease [7, 18, 22]. Researchers have used this technology to understand protein degradation in a variety of matrices, such as the digestive fluid, milk, urine, and stool [4, 19, 28]. This technology has been used to detect and identify *in vitro* digestion products of several allergenic and non-allergenic proteins [14, 39]; and theoretically, LC-MS/MS methods can be optimized to detect peptides from *in vitro* digestion of NEPs expressed in GM crops. However, prior to implementing a new detection method to the evaluation of the *in vitro* digestion, the LC-MS/MS method should be evaluated as to whether it adds improved utility with respect to the assessment of allergenic potential. This paper examines the technical capability of LC-MS/MS analysis for detection, identification and tracking of *in vitro* digestion products under the proposed conditions, and whether LC-MS/MS provides improved utility to differentiate allergens from non-allergens [11, 26]. Although a discussion of the proposed *in vitro* digestion conditions is important, the focus of this communication is the proposal to use LC-MS/MS for identification and its ability to assess allergenic potential.

2. Applicability of LC-MS/MS to Evaluate *In Vitro* Digestion

Mass spectrometry (MS) is an instrumental technique for separation of electrically-charged molecules as ions in a gas phase. When paired with liquid chromatography (LC) separation capabilities followed by powerful software tools, mass spectrometry can give detailed information on both the identity and abundance of peptides. This technology has demonstrated great analytical potential for purified proteins and complex matrices; it is crucial to apply this technology properly to understand the unique aspects of mass spectrometry data generation and analysis. The LC-MS/MS method requires technical establishment for GM crop *in vitro* digestion analyses with respect to sample preparation (digestion and desalting), LC-MS/MS analysis (ionization and detection), and data analysis. Ultimately, the resulting data are critically linked to the efficiency of each step and the peptides' physiochemical properties, which have significant variations in size, structure, and abundance. Some peptide fragments from the digestion could be lost during sample preparation or become essentially undetectable to the LC-MS/MS [35]. For instance, there can be high variability in the ranges of peptide abundance, which could lead to masking of less abundant peptides [36]. Also, some high-abundance peptides may not ionize with commonly used positive-charged ions mode proteomics due to the presence of acidic amino acids or cysteine residues [10, 12]. For these reasons and others outlined below, some peptides may not be detected by LC-MS/MS and others may appear to have an artificially enhanced relative abundance. To evaluate LC-MS/MS in perspective for *in vitro* digestion analysis of NEPs, we provide a technical review of mass spectrometry methods based on sample preparation, MS analysis, and data analysis, as well as a discussion of its utility for *in vitro* digestibility assessment.

2.1. Sample Preparation

Many factors are considered when designing MS sample preparation strategies, including sample source, type, physical properties, abundance, and complexity. As a result, it is important to describe how the *in vitro* digestion samples are prepared. The *in vitro* digestion sample preparation steps that the EFSA GMO Panel recommended are outlined in Figure 1. Briefly, a NEP hydrolysis time course is performed with a gastric enzyme (i.e., pepsin) under classical or suboptimal conditions. Pepsin has relatively low specificity, with preferential hydrolysis of the peptide bonds for aromatic amino acids, and it can cleave other peptide bonds with hydrophobic amino acids. The lack of specificity could result in variability between repeated digestion assays. Next, the enzyme is deactivated, and the sample is further hydrolyzed by intestinal enzymes (i.e., trypsin and chymotrypsin) with bile salts. Trypsin and chymotrypsin have relatively high specificity to basic amino acids and aromatic amino acids, respectively. In addition, bile salts are considered an important component for intestinal digestion; therefore, they may be included during trypsin and chymotrypsin digestion [26]. After hydrolysis, trypsin and chymotrypsin are deactivated. The precise timing

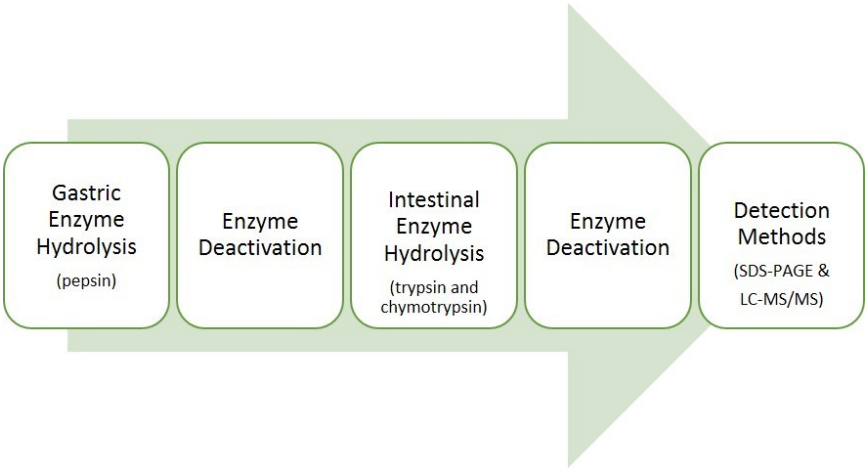

Figure 1: The proposed *in vitro* digestion steps include the following steps sequentially: gastric enzyme hydrolysis (e.g., pepsin) 60 min time course of NEP at low pH/high enzyme (classic) or high pH/low enzyme (suboptimal) with bile salts; deactivation of gastric enzyme by pH neutralization; intestinal enzyme (e.g., trypsin and chymotrypsin) hydrolysis 60 min time course with bile salts; deactivation of intestinal enzymes by acid or inhibitors; and application of SDS-PAGE and LC-MS/MS detection methods.

of enzyme activity deactivation is important for reproducible observations of stable peptides. The inhibition of pepsin can be achieved by neutralizing the pH, since pepsin is irreversibly deactivated at pH 7 [29], while inhibition of trypsin and chymotrypsin can be achieved by addition of acids or protease inhibitors such as 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), Tosyl phenylalanyl chloromethyl ketone (TPCK), and Tosyl-L-lysine chloromethyl ketone (TLCK). Finally, the digestion products are analyzed by SDS-PAGE and LC MS/MS.

Considering the steps outlined above, sample preparation and the removal of unwanted components are essential for MS analysis. For the proposed *in vitro* digestion of a NEP, purified protein (1-5 mg/ml) is generally used. The absence of other biological matrix components (lipids, starch, etc.) make the three-enzyme (pepsin, trypsin, and chymotrypsin) *in vitro* digestion system less complex compared to biological samples from tissues or fluids [26]. However, the purified protein sample may be at a pH required for purification or stable storage that is unsuitable for LC-MS/MS or contain incompatible buffer components, such as non-volatile salts, detergents and bile salts. The final quenched digestion mixture may include components that are incompatible with the electrospray ionization step, interfering with the MS detection process (see Mass Spectrometry Ionization Methods section). Surfactants, such as bile salts, could be detrimental and suppress ionization of peptides. Therefore, the removal of bile salts may require a desalting step. Optimization of desalting is necessary to prevent any significant loss of peptides.

Top-down proteomic methodology analyzes intact protein while bottom-up proteomic methodology analyzes enzyme hydrolyzed peptides. In traditional bottom-up proteomics, there is a common workflow to prepare proteins for enzyme digestion [15]. Concurrently or after treatment with a chaotropic reagent, disulfide bonds within the proteins and peptides are cleaved to allow further processing. Dithiothreitol is commonly used for this purpose and would modify any cysteine amino acids involved in forming disulfide bridges at these specific sites within the protein. Once the bonds are disrupted, an alkylating reagent is typically used to "cap" the residues capable of forming disulfide bonds and thus prevent any bond reformation. When assessing *in vitro* digestion of NEPs, typical pre-MS proteomic sample preparation, such as reduction and alkylation, would be eliminated to avoid breaking disulfide bonds and artificially impacting NEP stability. The absence of these steps allows detection of disulfide-bond linked protein/peptides but the resulting peptides with multiple disulfide bonds may be difficult to characterize due to incomplete fragmentation [20].

After digestion, sample enrichment and/or clean-up is a critical step. The enrichment can be accomplished in several ways, including, but not limited to, acid precipitation, solvent extraction, and/or molecular weight-based filtration (e.g., 10 kDa cut-off selects for peptides less than 10 kDa). Peptides may be lost with enrichment steps due to non-specific binding or inability to resolubilize. Samples could be cleaned-up by solid phase extractions (e.g., C18 cartridges or filter plates) to remove intact protein and bile salts for improved peptide detection; however, it is important to note that very hydrophobic peptides may never be recovered. These steps need to be carefully evaluated and the sample preparation process must be controlled to achieve reproducible results.

2.2. Liquid Chromatography Separation

High-performance liquid chromatography (HPLC) has often been included as a physical separation technique prior to MS analysis to improve detection. This process involves incorporating the analytes (peptides and proteins) into a mobile phase that is pumped across a stationary phase contained in a column, and allows separation of the analytes by their relative affinity for either phase. Based on their properties, peptides

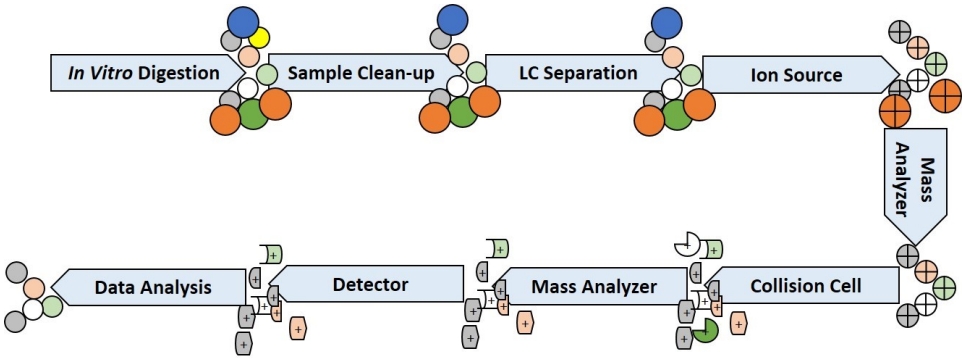

Figure 2: Peptides (circles) are generated during in vitro digestion. Sample clean-up removes incompatible components but may cause peptide loss (yellow circle is lost). Peptides are then separated during liquid chromatography prior to ionizations in the ion source. Ionization efficiency may lead to loss of peptide detected (blue circle is lost). Ions are separated in the mass analyzer and fragmented (large white and orange circle are lost). The resulting ions are separated and detected (partial circles are lost). Finally, peptides are identified during data analysis. As illustrated, peptide (ion) loss may occur at multiple steps.

present within a digestion mixture can be separated; this separation allows more peptides to be efficiently ionized and generate mass information, as there is less competition for the ionization potential at a given time. A variety of LC flow rates can be employed from high flow rates (>0.2 mL/min) to microflow (1-200 μL/min) or nanoflow (<1 μL/min), and there are compatible columns and systems used specifically for these conditions. The low flow rate in microflow and nanoflow allows ionization of a sample to last for minutes and enables orders of magnitude more observations compared with the observations from a regular flow of electrospray ionization (ESI) [24]. Nanoflow LC has unique advantages in supporting detection of more peptides than other techniques due to increased ionization efficiency and reduced ion suppression that leads to higher MS sensitivity. Likewise, microflow would also yield a nearly ideal platform for high sensitivity analysis by LC-MS/MS [42]. As a result, the technique exhibits high sensitivity and helps identify very minor populations of peptides at as low as femtomole concentrations.

2.3. Mass Spectrometry Ionization Methods

After sample preparation and LC separation, peptides would be analyzed on a mass spectrometer, which is an analytical instrument having multiple components, including an ion source, mass analyzer, collision cell and detector (Figure 2). The peptides in the liquid phase from the LC instrument go into the ion source and are converted to gas phase ions (ionization) that can be separated by the mass analyzer. There are many ionization techniques used in mass spectrometry. ESI is the most popular ionization technique due to its advantage of compatibility with LC. ESI creates an aerosol by applying a high voltage to a flow of liquid at atmospheric pressure. This aerosol is dried using gases and heat so that the protein or peptide of interest in the droplet retains a charge. ESI is very useful for biomolecules such as proteins and peptides because it is a "soft ionization" technique, which causes minimal fragmentation [5, 24].

Efficient ionization of peptides within the digested samples is critical for accurate LC-MS/MS detection and analysis. Each peptide from the *in vitro* digestion has unique physiochemical properties and capabilities for ionization. Ionization can theoretically occur in either the solution phase or the gas phase and is strongly affected by all components of the sample, including other peptides. Differential suppression or enhancement of specific ions can occur due to the differences in amino acid composition, mass and/or charges of individual peptides. In some cases, one peptide could be a source of ion suppression or enhancement for another. For example, acidic peptides do not readily accept a positive charge, which makes them difficult to ionize using positive mode methods, and therefore, less observable [10]. Ion suppression often occurs due to changes in the spray droplet solution properties depending on the presence of less volatile or non-volatile solutes such as salts, ion-pairing agents, or endogenous compounds like bile salts. These non-volatile materials can change the efficiency of droplet formation or evaporation and alter the amount of charged ions in the gas phase that ultimately reach the detector. When analyzing *in vitro* digestion samples, bile salts, phosphatidyl choline, and other lipids, if present in the assay, will reduce ionization efficiency; therefore, desalting, enrichment and/or clean-up post sample preparation and LC separation are needed to significantly reduce or remove incompatible compounds.

2.4. Mass Spectrometry Analyzers and Fragmentation

Once ionized, the mass analyzer (or filters) measures the mass-to-charge ratios (*m/z*) of the ions in a sample, and *m/z* detected from a sample provides an atomic signature. Currently, there are several types of mass analyzers suitable for a variety of applications. For example, low-resolution mass spectrometers have analyzers (e.g., triple quadrupole MS) that measure nominal mass and are often used for targeted quantification of peptides. On the other hand, high-resolution mass spectrometers have analyzers (e.g., Orbitrap) that measure exact mass and are used for identification and non-targeted analyses. The high-resolution Orbitrap MS analyzers can achieve *m/z* ratio up to 280,000 for mass range at 50-8,000 Da. As a result, MS instruments with different mass analyzers will likely yield results

that are very similar but not identical.

One key step to identifying peptides is to use tandem mass spectrometry (MS/MS). In MS/MS ions are separated and fragmented, and the resulting fragments are further separated and detected to yield spectra with characteristic peptide fragmentation patterns. Fragmentation of the peptide backbone generally produces characteristic ions, termed *a*, *b*, *c* and *x*, *y*, *z* ions, and allows reliable interpretation of spectra to predict amino acid sequences [31]. Collision-induced dissociation (CID) is the most widely applied fragmentation method for peptide identification with MS/MS. CID provided the largest contribution to the identified peptides from human blood plasma compared with high-energy collision dissociation (HCD) and electron transfer dissociation (ETD) [34], while ETD outperformed CID and HCD in the analysis of ubiquitylated proteome [30]. CID is suitable for identification of small peptides in digestion products, since it is most effective for small and low-charged peptides. Understanding fragmentation methods can help improve identification rates from digestion products. Like fragmentation, for reproducible peptide identification from *in vitro* digestion of NEPs, assay parameters, such as spray voltage, collision energy, dynamic range, limit of detection, and other parameters such as total injection amount, need to be harmonized among technology developers, contract research organizations (CROs), and research institutes.

2.5. Data Analysis

In traditional bottom-up proteomics the resulting spectra are searched against a known *in silico* spectra database generated from known protein sequences to compare observed and predicted masses of peptides. A query software with search algorithms, such as Mascot (Matrix Science, Boston, MA), XTandem, or Sequest, is needed to analyze peptides from *in vitro* digestion studies by predicting theoretical peptides based on potential pepsin, trypsin, and chymotrypsin cleavage sites [21]. The interpretation of amino acid sequences and their assignment to the spectra relies heavily on accurately predicted masses generated from the protein amino acid sequence of the protein, including potential modifications. This is especially true for a database generated with less specific enzymes such as pepsin. However, allowing for one or two amino acid mismatches during spectra analysis would minimize the impact. Accordingly, for the proposed *in vitro* digestion of a single NEP, protein analysis and the reliance on a database are not a factor, especially since the NEP sequence is known. The major limitation to data analysis is linked to the data input; any missing peptides or ions cannot be identified during data analysis.

Besides identification, the EFSA GMO Panel proposed using LC-MS/MS to evaluate the stability or persistence of a peptide by its temporal occurrence or presence throughout a digestion time course. Although there are several quantitative or semi-quantitative MS paradigms to choose, label-free methods would be most amenable to the *in vitro* digestion outlined above. Label-free methods do not require modification of the protein or peptides. In the absolute quantitative methods, protein abundance is calculated based on a linear correlation with MS collected data for each peptide [8]. Relative quantitative techniques compare peak areas intensities or spectral counts. Such relative techniques are simple and easy to perform but do not provide absolute concentrations, which can be used to compare multiple peptides. Relative parameters may be reported, such as the total number of unique peptides or the number of unique peptides derived from a specific segment of the protein during a time course. It is important to be aware that total peptide number does not directly correspond to protein stability.

The presence or number of peptides is directly related to digestion conditions, protein properties, LC MS/MS instrumentation, etc. Therefore, the same peptide from a protein may have a different occurrence profile under classic versus the sub-optimal pepsin digestion conditions. At pH 1.2, the optimal pH for pepsin, pepsin is fully active, yet the test protein may or may not be fully digested if it is not completely acid denatured and peptide bonds are not exposed for cleavage. For example, alpha lactalbumin is fully denatured at pH 1.2 while beta-lactoglobulin is still in its native structure at this pH [37]. As a result, there might be decreased observations of unique peptides from alpha-lactalbumin due to near completion digestion by pepsin at the end of time course. In contrast, the subsequent digestion by trypsin/chymotrypsin could result in increased observations of peptides from beta-lactoglobulin due to more effective degradation by the two enzymes. Such data can be easily generated by LC-MS/MS using the outline described above. Evidence suggests that peptides can be observed from digestion products of allergens or non-allergens at different digestion conditions [39, 42]. However, the connection between the presence of peptides and their allergenicity potential is unclear, indicating that the additional data from the LC-MS/MS analysis of the *in vitro* digestion assay does not add any information to inform the allergenic potential of the NEP.

3. Discussion

Recently, the EFSA GMO Panel outlined a suggested *in vitro* digestion protocol as a part of WOE of the allergenicity assessment for the GM crop regulatory submissions with a hope the new methods will have improved predictability of allergenicity potential of NEPs [26]. It described sequential digestions with additional sub-optimal digestion conditions and proposed the use of LC-MS/MS as a method to detect the digestion products. *In vivo* physiological digestion is highly complex, from oral to gastrointestinal digestions, and therefore it is impossible to mimic the physiological digestion *in vitro*. Currently, the *in vitro* digestion designed using purified protein at high concentration (1 to 5 mg/mL) along with the proposed three-enzyme system allows better forecasting for smaller numbers of potential peptides relative to the large numbers of potential peptides from more complex biological samples or food substances. Resistance to pepsin digestion was initially hypothesized to be linked to allergenicity by limited historical reports, but subsequent investigations have revealed that there is weak correlation between pepsin resistance and allergenicity [3, 32, 17, 13].

A few key points need to be addressed before answering the question posed in this manuscript, whether LC-MS/MS can

improve the risk assessment of NEPs. As part of WOE for allergenic assessment of an NEP, knowledge of the protein concentration, heat stability, and digestive stability of the protein are exposure criteria, while structural similarity with known allergens and history of safe use are hazard criteria [16]. Regardless of SDS-PAGE results that show the presence of an intact protein, LC-MS/MS methods are so sensitive that lead to observation of peptides from the protein; therefore, exposure assessment and allergenicity determination of that protein from LC-MS/MS analysis remain uncertain. It was reported that non-allergen soybean lipoxygenase has more resistant peptides than allergen beta-casein, from optimal or suboptimal digestion condition [39]. Similar observation was reported between non-allergen phosphofructokinase and allergen beta-lactoglobulin [42]. Evidence suggests that unique and stable peptides are present in digestion products from both allergen and non-allergens Also, no clear patterns in the persistence or abundance of peptides exist between allergen and non-allergen digestion products; therefore, the presence of peptides gives no information on their allergenic potential [39, 42]. Fundamentally, a simple correlation between protein digestibility and allergenicity has not been established, and therefore the presence of the protein or specific peptides may only be used in relation to the exposure assessment. It should be noted that most risk assessment bodies would consider that information on exposure is only informative for the risk assessment if a potential hazard has been identified. Therefore, it can be questioned if the mere presence of a peptide that is otherwise considered safe provides useful information for a risk assessment.

Although the proposed *in vitro* digestion conditions contain extra steps and enzymes compared with the classic pepsin resistance assay, they can be made amenable for LC-MS/MS analysis of the digested samples. LC-MS/MS technology can detect and identify unique peptides from *in vitro* digestion with some important technical challenges that could significantly impact the interpretation of the results and their utility in the WOE for allergenicity assessments. As discussed in previous sections, an inability to detect a peptide does not mean the peptide is absent from the digestion samples. The occurrence of a peptide over a time course may be directly related to properties of the parent protein and the digestion conditions, but may also result from the sensitivity of the instrument or other experimental conditions. A specific peptide may be present, but detection may not be guaranteed due to several factors, including sample preparation, low ionization, efficiency, and sensitivity of the mass analyzer. Despite this limitation, LC-MS/MS is still very sensitive and capable of detecting peptides missed in other technologies (e.g., SDS-PAGE). A peptide could be lost during the enrichment and desalting step due to its hydrophobicity, low abundance, or size; it could also be too small to be ionized or identifed because of repetitive amino acids within the sequence. Therefore, the ability to detect all small peptides in an in vitro digestion can be challenging (if not impossible).

The EFSA GMO Panel has not recommended a standard protocol for peptide identification or quantification using LC-MS/MS analysis. Criteria to achieve reproducible and consistent peptide identification from *in vitro* digestions are challenging to harmonize among different labs. There are many more parameters and settings, such as spray voltage, collision energy, and survey scan, required for a mass spectrometry instrument that are not relevant to SDS-PAGE. Different labs may have different models of mass spectrometers and parameters from one model do not perform the same way on another model. As a result, the lack of standardization seems likely considering the variability described previously. Nevertheless, modern LC-MS/MS exhibits sensitivity down to fmol concentrations, so it is technically possible for a peptide to be observed throughout the entire digestion. The LC-MS/MS methods for peptide identification from *in vitro* digestion cannot quantify the mass or concentration of the peptides. Moreover, observation of peptides is not necessarily linked directly to exposure.

Can mass spectrometry analysis of *in vitro* digestion products improve the assessment of allergenic potential of newly expressed proteins? Based on the technical review above, the answer is "no" or "not at this time". Like SDS-PAGE, LC-MS/MS can identify and track the abundance of digestion products albeit with technical challenges that may skew results. Results from an SDS-PAGE assay do not provide helpful information to assess whether a digestion product is an allergen or not. Unfortunately, results on digestion products using LC-MS/MS do not improve this shortcoming. Neither method, whether used in a tiered process or used alone, provides the capability of distinguishing between allergen and non-allergen.

A determining factor that could aid in the identification of an allergen from a non-allergen is sequence homology to known allergens. NEPs have been successfully screened and excluded from allergenicity concern using sequence homology and epitope searching that are done *in silico* with bioinformatics analysis on the intact protein, without conducting *in vitro* digestion and LC-MS/MS analysis. Use of LC-MS/MS can be informative if peptide presence from *in vitro* digestion is an indication of allergenicity of a NEP. The approach to monitor the peptide presence and abundance with a demonstration of the lasting presence of a peptide and counting the number of unique peptides throughout a digestion has a drawback because the number of peptides from a digestion of the protein is protein dependent rather than allergenicity dependent. In addition, the LC-MS/MS identification method is not for quantification, and therefore cannot provide concentration information. The value of LC-MS/MS analysis of digestions for allergenicity prediction needs to be demonstrated prior to adoption for allergenicity assessment of NEPs.

4. Conclusion

State-of-the-art, LC-MS/MS has become a familiar technology for the characterization of food proteins and peptides [23]. Detection of some stable peptides from *in vitro* digestion can be achieved through this technique. Nanoflow or microflow LC along with ESI are highly sensitive and can detect and identify a large number of peptides, including trace amounts of unique peptides. The relatively simple *in vitro* digestion design (purified test protein and three-enzyme system) and high concentration of test protein may be compatible with LC-MS/MS pep-

tide identification. However, LC-MS/MS methods need to be carefully developed and evaluated taking several factors into consideration, such as buffer compatibility, enrichment, signal suppression, etc., prior to data interpretation. Resistance to digestion has no direct correlation with allergenicity potential. The peptides observed from *in vitro* digestion may not reflect what occurs *in vivo*, and therefore, detection of peptides is unlikely to provide a good prediction of potential for allergenicity. More work needs be done for a better understanding of allergenic sensitization and elicitation before adding complexity to the *in vitro* digestion assay that does not translate into improved assessment of allergenic potential for NEPs from GM crop products, and which may only add to more confusion in evaluating assay results [40].

5. Declaration of Conflicting Interest

The authors are employed by the agricultural biotechnology industry. Employment affiliation of the authors is given on the first page of this paper.

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7. Article Information

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