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Characteristics and safety assessment of intractable proteins in genetically modified crops

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ABSTRACT

Genetically modified (GM) crops may contain newly expressed proteins that are described as "intractable". Safety assessment of these proteins may require some adaptations to the current assessment procedures. Intractable proteins are defined here as those proteins with properties that make it extremely difficult or impossible with current methods to express in heterologous systems; isolate, purify, or concentrate; quantify (due to low levels); demonstrate biological activity; or prove equivalency with plant proteins. Five classes of intractable proteins are discussed here: (1) membrane proteins, (2) signaling proteins, (3) transcription factors, (4) N-glycosylated proteins, and (5) resistance proteins (R-proteins, plant pathogen recognition proteins that activate innate immune responses). While the basic tiered weight-of-evidence approach for assessing the safety of GM crops proposed by the International Life Sciences Institute (ILSI) in 2008 is applicable to intractable proteins, new or modified methods may be required. For example, the first two steps in Tier I (hazard identification) analysis, gathering of applicable history of safe use (HOSU) information and bioinformatics analysis, do not require protein isolation. The extremely low level of expression of most intractable proteins should be taken into account while assessing safety of the intractable protein in GM crops. If Tier II (hazard characterization) analyses requiring animal feeding are judged to be necessary, alternatives to feeding high doses of pure protein may be needed. These alternatives are discussed here.

Abbreviations: BW, body weight; CAH1, chloroplast-localized carbonic anhydrase; CC, coiled-coil; CMC, critical micelle concentration; CO, CONSTANS; CYP, cytochrome P450; D9DS, delta 9 desaturase; DGAT, diacylglycerol acyltransferase; EPSPS, 5-enol-pyruvylshikimate-3-phosphate synthase; ER, endoplasmic reticulum; ETI, effector-triggered immunity; FARRP, Food Allergy Research and Resource Program; GFP, green fluorescent protein; GI, gastrointestinal; GM, genetically modified; GPI, glucosyl phosphatidyl inositide; HLB, hydrophilic–lipophilic balance; HOSU, history of safe use; IFBiC, International Food Biotechnology Committee; ILSI, International Life Sciences Institute; IV, intravenous; LRR, leucine-rich repeat; NBS, nucleotide-binding site; NOAEL, no-observed-adverse-effect level; OECD, Organisation for Economic Co-operation and Development; PAMP, pathogen-associated molecular pattern; PAT, phosphinothricin acetyltransferase; PEPCK, phosphoenolpyruvate carboxylase kinase; PHA, phytohemagglutinin; PTI, PAMP-triggered immunity; R-proteins, plant pathogen recognition proteins that activate innate immune responses; RLK, receptor-like kinase; RLP, receptor-like protein; SDS, sodium dodecyl sulfate; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; STAND, signal transduction ATPase with numerous domains; TAG, triacylglycerol; TFAPs, transcription factor accessory proteins; TTC, threshold of toxicological concern; TIR, Toll and interleukin-1 receptor.

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1. Introduction

The safety of proteins expressed in genetically modified (GM) crops (hereafter "transgenic proteins") has been assessed ever since scientists first had the ability to introduce genes into crops. There are many opinions on what tests or studies should be required to scientifically document that transgenic proteins are safe, especially when the protein has a history of safe use (HOSU) or when the expression level is very low.

For the transgenic proteins that are expressed in GM crops today, a comparative safety assessment process was implemented in the 1990s in which scientific studies were carried out to identify the similarities and differences between a newly developed GM crop and its conventional (non-GM) counterpart that had a HOSU. This approach assesses (1) the agronomic/morphological characteristics of the GM crop; (2) macro- and micronutrient composition and content of important anti-nutrients and toxicants; (3) molecular characteristics, protein expression, and safety of the newly introduced protein(s) and their metabolites; and, if appropriate, (4) the nutritional characteristics of the novel product compared with that of its conventional counterpart, by testing wholesomeness in animal models (e.g., poultry feeding studies). Any identified biological differences are assessed further to determine whether safety issues or concerns exist and then to evaluate the associated risk. This comparative safety assessment process is also known as substantial equivalence. This approach has become standard for safety evaluation of GM crops and has been described in multiple publications (e.g., Delaney et al., 2008; Hammond, 2008). A more recent work examined whether the same approach was sufficient to demonstrate the safety of GM crops that have improved function by altering endogenous gene expression via RNAi technology or expression of transcription factors (Parrott et al., 2010). However, most of the transgenic proteins currently in GM crops are foreign to the target plant and either toxic to insects or afford tolerance to commercial herbicides. More importantly, they are amenable to production of significant amounts in heterologous systems, isolation, and subsequent testing. Some proteins from the next generation of transgenic crops are already proving to be much more difficult to study. In some cases the transgenic protein will be an integral part of the substructure of the plant cell, in others it may be closely related to a protein of the target plant, and in still others it may be present in the target plant, but be expressed ectopically in the GM crop. Some proteins may only exist at very low levels for a short time and be hard to detect and/or identify in the plant.

There are important questions associated with these "intractable" proteins. Do these proteins pose a safety issue? Do they need to be regulated? If necessary, how does one perform a safety assessment on intractable proteins? While it is unlikely that a protein that is unstable outside of its normal plant environment can be toxic to an animal or human, there will be intractable proteins for which a safety assessment is appropriate, or requested by regulators. In these cases, there are studies that can contribute to the safety assessment of intractable proteins should it be necessary, and those studies are the focus of this paper.

Intractable proteins are defined here as those proteins with properties that make it impossible or extremely difficult to (1) express in a heterologous system; (2) quantify (due to low levels); (3) isolate, concentrate, or purify from either heterologous expression systems or the GM plant; (4) demonstrate functionality of the isolated protein; or (5) prove equivalency of the heterologously produced protein with the plant-expressed protein. These limitations are important, because in 2008, a document jointly published by the International Life Sciences Institute (ILSI) and the International Food Biotechnology Committee (IFBiC) recommended a systematic weight-of-evidence tiered approach to assess the safety of novel proteins expressed in GM crops (Delaney et al., 2008). Safety evaluation of the candidate novel protein begins with a Tier I potential hazard identification, which includes HOSU, bioinformatics analysis, mode of action, *in vitro* digestibility and stability in the presence of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), expression level, and dietary intake evaluation. If these elements of the safety evaluation are satisfactory, then it can be concluded that the protein is safe to express in GM crops. If, however, the safety of the protein cannot be confirmed in the Tier I analyses, then Tier II hazard characterization studies should be considered. These Tier II studies could include acute and possibly repeated-dose toxicity studies in mice or rats and, if warranted, hypothesis-based evaluations. Some of the Tier I tests and all of the Tier II hazard tests require grams of protein. However, production, isolation, or concentration of sufficient quantities of functionally active proteins for use in safety studies may not be possible. For example, integral membrane proteins are not only difficult to express in heterologous systems, they (due to their hydrophobicity) have very limited solubility in the types and levels of vehicles that would be appropriate for toxicity testing. Many proteins form suspensions at high concentrations, but membrane proteins oligomerize into uncharacterizable forms. The question then becomes, how does one provide appropriate scientific data to support the safety assessment of a GM crop that contains an intractable protein?

Classes of proteins in which all or at least some of the proteins might be intractable include (1) membrane proteins, (2) signaling proteins, (3) transcription factors, (4) N-glycosylated proteins, and (5) resistance (R)-proteins. The characteristics that could render each type of protein intractable are discussed along with tools and science-based solutions for safety assessment of intractable proteins. The scope of this paper focuses only on the safety assessment and functionality of the intractable protein itself and not the safety of the crop containing the protein.

2. Classes of intractable proteins

Table 1 summarizes the classes of intractable proteins discussed in this section.

2.1. Membrane proteins

2.1.1. Definition of membrane proteins

"Membrane protein" is a biochemical term used to describe polypeptides that associate with lipid membranes, either stably

Table 1
Overview of issues associated with different intractable protein classes.

Issue	Protein class				
	Membrane proteins	Signaling proteins	Transcription factors	N-glycosylated proteins	R-proteins
Absence of suitable heterologous expression system	✓		✓	✓	
Low level of expression in GM crop	✓	✓	✓		✓
Inability to test the functionality of isolated protein	✓				✓
Inability to determine equivalence of heterologously produced protein and plant-expressed protein	✓	✓	✓	✓	✓